Isolation of endophytic bacteria from *Gynura pseudochina* (L.) DC.growing in Zn/Cd contaminated soil and the effect of endophytic bacteria to phenolic compounds

Ruttanakorn Munjit

A Thesis Submitted in Partial Fulfillment of Requirements for

degree of Doctor of Philosophy in Biology

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The examining committee has unanimously approved this Thesis, submitted by Miss Ruttanakorn Munjit , as a partial fulfillment of the requirements for the Doctor of Philosophy Biology at Mahasarakham University

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TITLE Isolation of endophytic bacteria from *Gynura pseudochina* (L.) DC.growing in Zn/Cd contaminated soil and the effect of endophytic bacteria to phenolic compounds

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ABSTRACT

This research aims to isolate zinc (Zn) and cadmium (Cd) tolerant endophytic bacteria from *Gynura pseudochina* (L.) DC., a Zn/Cd hyperaccumulative plant. In addition, the effects of the endophytes inoculation on Zn and Cd accumulation and phenolic contents in the plants were investigated in both tissue culture and pot systems including the metal stress. Seventy-three entophytic bacteria were isolated from the plant samples (tuber, stem, and leaves), which grew in a reserved forest area of a closed Zn mine, in Mae Sot district, Tak province, Thailand. All isolates were selected by streaking on trypticase soya agar (TSA) plate containing various concentrations of Zn (25, 20, 100 and 200 mg/l) and Cd (5, 10, 20 and 50 mg/l). The results showed that there were 17 isolates tolerating the high concentrations of Zn and Cd at 200 mg/l and 50 mg/l, respectively. The 16S rDNA gene identifications indicated that the bacteria isolates belonged to the genus of *Acinetobacter*, *Curtobacterium*, *Yokenella*, *Cupriavidus*, *Cellulomonas*, *Rhizobium*, *Enterobacter*, *Stenotrophomonas*, *Methylobacterium*, *Beijerinckia*, *Chryseobacterium*, *Klenkia* and *Marmoricola*. However, nine bacterial strains that tolerated the dual stress with Zn (200 mg/l) plus Cd (50 mg/l) were selected for study their plant-growth promoting properties under the metal stress. These results indicated that the Zn plus Cd stress induced the production of siderophore and indole-3-acetic acid by *S. maltophilia* GSD10 and *Chryseobacterium* sp. GTID13. In addition, *Curtobacterium* sp. GLD03, *S. maltophilia* GSD10 and *Chryseobacterium* sp. GTID13, which have had no reports of pathogenicity, were selected for monitoring the bacterial inoculation affecting on *G. pseudochina* plants.

In tissue culture system, *Curtobacterium* sp. GLD03, *S. maltophilia* GSD10 and *Chryseobacterium* sp. GTID13 were separately inoculated into the plants, then cultivated for one-month before dual treatment with Zn (100 mg/l) plus Cd (15 mg/l) for the next two weeks. In pot system, the three endophytes were separated inoculated into the tubers before growing in a fertile soil supplemented with Zn 1,000 mg/kg soil plus Cd 50 mg/kg soil for 2 months. The plant growth, accumulation of Cd and Zn in roots and shoots, and phenolic compounds in leaves were investigated. The results

obtained from the tissue culture system were not significant difference in the effects of the bacterial inoculation, because the high humidity might decrease the plants' stress. In focusing, the results from the pot system indicated that *S. maltophilia* GSD10 increased Zn and Cd accumulation in roots, whereas *Curtobacterium* sp. GLD03 and *Chryseobacterium* sp. GTID13 increased amounts of Zn and Cd in the shoot. The three strains could colonize in the stem and root tissues. The picture of GFP-labelled *S. maltophilia* GSD10 colonizing on the surfaces of the primary root tissue under the confocal microscope represented the colonization. In comparison with the phenolic compounds of the leaf extracts from the plants treated with Zn plus Cd, the inoculation of *Curtobacterium* sp. GLD03 and *S. maltophilia* GSD10 resulted to decrease total phenolic content (TPC) and total flavonoid content (TFC) in the leaf extracts. Whereas, TPC and TFC of the leaf extracts from the plants inoculated with *Chryseobacterium* sp. GTID13 were similar to the extracts from the control plants, without bacterial inoculation. In addition, the leaf extracts of the plant are inoculated with *Chryseobacterium* sp. GTID13 had the highest radical scavenging activity. High Performance Liquid Chromatography (HPLC) showed that chromatogram of the leaf extracts had similarly with four main peaks of chlorogenic acid (CGA), caffeic acid (CA), rutin (RUT) and unknown compound. The inoculation of *Curtobacterium* sp. GLD03 and *S. maltophilia* GSD10 into the plans affected the decrease of all four main peaks. Whereas, the inoculation by *Chryseobacterium* sp. GTID13 might modulate the increase of CGA, RUT and unknown compound. Consequently, the results demonstrated that *Chryseobacterium* sp. GTID13 could be applied in a bioaugmentation process to decrease Zn/Cd phytotoxicity and support phytoextraction of the metals by growing *G. pseudochina*.

Keyword : Cadmium, endophytic bacteria, phenolic, phytoremediation, zinc

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CHAPTER 1
INTRODUCTION

1.1 Background

Doi Padaeng, Phatat Padaeng sub-district, Mae Sot district, Tak Province, Thailand, is a rich source of zinc mineralization (Simmons *et al.*, 2005). High concentrations of zinc (Zn) and cadmium (Cd) have been reported in vegetable crops and crops in the region. The metals have established a health risk to the local population living near the mining activity (M. N. V. Prasad & Nakbanpote, 2015; Swaddiwudhipong et al., 2010; Teeyakasem et al., 2007) After the Padaeng Zn mine closed its operations in 2016 (Nakbanpote et al., 2018) The mine has relied on phytomanagement processes to recover the ecosystem, in which native flora has been chosen as to accumulate high concentrations of Zn and Cd from the contaminated land. An ideal process for sustainable development, plant biomass could then be harvested and converted into ash in an incinerator before metal extraction through the mining process. Furthermore, medicinal plants are a model for sustainable agriculture by the phenolic plant containing antioxidant properties, which could be extracted from the harvesting part, in particular the growing part (Ghosh & Singh, 2005; Nakbanpote et al., 2010; Prasad et al., 2015)

Gynura pseudochina (L.) DC., a tuberous plant from the Asteraceae Family, was discovered in a zinc mine and could be used for phytoremediation of Zn and Cd (Panitlertumpai et al., 2003). Phaenark et al. (2009) reported that *G. pseudochina* was a Cd/Zn hyperaccumulator, the concentrations of Cd and Zn accumulated in the shoot at 457 mg Cd kg^{-1} dry mass and 6171 mg Zn kg^{-1} dry mass. Panitlertumpai et al. (2013) reported that dual treatment with Cd and Zn decreased the toxicity of metals and demonstrated the properties of the plant to accumulate Cd. In addition, *G. pseudochina* is usually used as a medicinal herb, have a certain pharmacological effect, especially anti-inflammatory (Plant Genetic Conservation Project, 2009). Nakbanpote et al. (2010) reported that extraction of *G. pseudochina* leaves had polyphenolic compounds, particularly rutin and caffeine. Extract of *G. pseudochina* leaves contain saponin, steroids, monoterpene, sesquiterpene and numerous phenolic compounds such as flavonoids, tannins, rutin, caffeic acid derivatives and polyphenols (Batubara et al.,

2012; Moektiwardoyo et al., 2014). Freeze drying and microwave drying resulted in *G. pseudochina* retention in leaf extract showing high levels of total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activity (Sukadeetad et al., 2018)

Under stress conditions, plants induced the accumulation of production of phenolic compounds (Lavola et al., 2000; Sakihama & Yamasaki, 2002) Heavy metals are an abiotic stress, one of their effects on plants is the generation of harmful reactive oxygen species (ROS), resulting in oxidative stress. The by-products of ROS are toxic to destroy cell (Díaz et al., 2001). Phenolic compounds are one of the plant's defense systems for metal stress, in which phenolic compounds have been reported to play an important role as antioxidants and metal detoxification (Chaudiere and Ferrari-iliou, 1999) such as *Capsicum annum* L. (Díaz et al., 2001), *Nympheae* (Lavid et al., 2001) and *Lupinus albus* L. (Jung et al., 2003). *Phaseolus vulgaris* exposed to Cd^{2+} accumulated soluble and insoluble phenolics, and leaves of *Phyllantus tenellus* had more phenolic content than control plants after being sprayed with copper sulphate (Díaz et al., 2001). Kitisin et al. (2013) investigated crude rice oil extracted from cadmium-contaminated rice, and they found an alternative to the use of cadmium-contaminated rice without risk to public health. *G. pseudochina* leaf extract increased the content of caffeic acid and rutin under Zn and Cd contamination (Mongkhonsin et al., 2016).

Endophytic bacteria are defined as those that colonize the internal tissues of healthy plants without causing symptoms of disease or harmful effects on their host (Schulz & Boyle, 2007). Recently, the advantage of combining heavy metal-resistant-endophytic bacteria with metal hyperaccumulation plants is to increase contaminant remediation. In addition, they can uptake and accumulate heavy metals in plants (Chen et al., 2010; Shin et al., 2012). Cd-resistant endophytic bacteria isolated from seeds of *Nicotiana tabacum* were able to increase the accumulation of Cd in shoots (Mastretta et al., 2009). Endophytic bacteria have the abilities to promote plant growth in order to improve of plant growth in metal contaminated soils, which various mechanisms such as production of indole acetic acid (IAA), 1-aminocyclopropane-1-carboxylate (ACC) deaminase, nitrogen fixation, solubilization of mineral phosphate, and siderophore (Verma et al., 2001; Chen et al., 2010; Ma et al., 2011; Zhang et al., 2011). Endophytic bacteria can be isolated from medicinal plants. Bhore et al. (2010) isolated

Pseudomonas resinovorans, *Paenibacillus polymaxa*, and *Acenitobacter calcoaceticus* from leaves of *Gynura procumbens* (Lour.) Merr. Yan et al. (2014) isolated the endophytic bacteria from the roots of *Salvia miltiorrhiza*, and they discovered that *Pseudomonas brassicacearum* sub sp. *neoaurantiaca*, *Rhizobium radiobacter*, *Pseudomonas thivervalensis* and *Pseudomonas frederiksbergensis* significantly improved the activity tensions.

Therefore, this research aims to study the isolation and characterization of Zn/Cd resistant endophytic bacteria from *G. pseudochina* growing in the Padaeng zinc mine. The isolates were selected for non-pathogenic with host plant and plant growth promoting properties. Then, the effects of endophytic bacterial inoculation on phenolics, growth and accumulation of Zn and Cd of *G. pseudochina* were investigated in both plant tissue culture and pot system. The results obtained could be applied to a field system in the Zn/Cd contaminated area for phytoremediation, phytomining, phytomanagement and sustainable development.

1.2 Objectives

This research aims to:

1. Isolate and characterize endophytic bacteria from tubers, stems and leaves of *G. pseudochina*, a Zn/Cd hyperaccumulator.

2. Screen the endophytic bacterial isolates base on non-pathogenic with the host plant, Zn/Cd tolerance properties and plant growth promotion.

3. Study effects of the selected endophytic bacterial inoculation on *G. pseudochina* growing in plant tissue culture and pot system by focusing on phenolic compounds, plant growth and Zn/Cd accumulation.

1.3 Hypothesis of the study

1. The diversity of endophytic bacteria is different for tubers, stems and leaves of *G. pseudochina*.

2. The endophytic bacteria have different Zn/Cd resistance and plant growth promoting properties.

3. Inoculation of selected endophytic bacteria from *G. pseudochina* affects phenolic compounds, plant growth and accumulation of Zn and/or Cd.

1.4 Advantages of the study

1. The results of isolation and characterization will be a database of endophytic bacteria associated with *G. pseudochina*, a Zn/Cd hyperacumulative tuber plant.

2. Endophytic bacteria screening by Zn/Cd resistance and plant growth promoting properties could be applied to promote the growth of *G. pseudochina* in the Zn/Cd contaminated area.

3. Effect of endophytic bacteria on phenolic compounds and Zn/Cd accumulation in *G. pseudochina* could promote plant cultivation and phytomanagement in the contaminated Zn/Cd area.

CHAPTER 2
LITERATURE REVIEWS

2.1 *Gynura pseudochina* (L.) DC.

Gynura pseudochina spread from eastern Sierra Leone through the Central African Republic and Ethiopia to Somalia and southward to Malawi, Zambia and Angola. It also appears in Australia, India, Sri Lanka, China, Bhutan, Myanmar, Vietnam and Thailand. The taxonomy of *G. pseudochina* is indicated as follows.

Kingdom: Plantae
Division: Spermatophyta
Class: Dicotyledoneae
Order: Asterales
Family: Asteraceae
Genus: *Gynura*
Species: *Gynura pseudochina*

G. pseudochina (L.) DC. is showed in Figure 1. The botany of the plants is a perennial herb 0.4–1 m high, with an unpleasant musky smell; tuberous rootstock, the tubers can be 10 cm long and up to 5 cm across. Leaves slightly fleshy, green or purplish, the basal leaves ovate or spatulate, 4–22 cm long, 2.5–11 cm wide, margins entire, apex obtuse; middle and upper leaves narrower, elliptic, obovate or narrowly obovate, lobed to pinnatisect, 6–25 cm long, 2–7 cm wide, base clasping the stem in the uppermost leaves, margins 1–6-lobed, the lobes toothed or lobed; all leaves pubescent or glabrous, glandular. Florets orange or yellow, corolla 9–12.5 mm long, expanded in the upper part, lobes 0.9–1.7 mm long. Achenes 3 mm long, pubescent or glabrous; pappus 7–11 mm long (Vanijajiva, 2009).

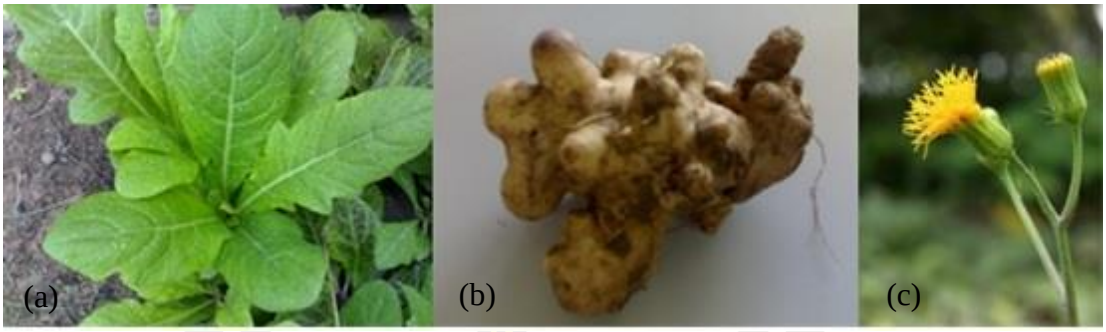


Figure 1 *Gynura pseudochina* (L.) DC. (a) leaves, (b) tuber, and (c) flower. (Photograph by Nakbanpote, W.)

2.2 Benefits of genus *Gynura* plants

Benefits of a plant in *Gynura* (such as *G. bicolor*, *G. divaricate*, *G. procumbens*, *G. segetum* and *G. pseudochina*) are generally used as a traditional medicine to treat of inflammation, herpes simplex virus, fever, skin rash, rheumatism, kidney disease, cancer, migraines, constipation, diabetes and high blood pressure (Li et al., 2009). In Thailand, *G. pseudochina* leaves have been used for the treatment of herpetic infections, burning pains and poultice against abscesses, all uses are associated with with potential anti-inflammatory effects (Plant Genetic Conservation Project, 2009). The petroleum ether, ethyl acetate, and methanol extracts from the fresh leaves of *G. pseudochina* L. (DC) and *G. pseudochina* L. (DC) var. *hispida* as Thai plant species anti-inflammatory showed the most promising inhibitory effect of the nuclear factor-αB (NF-αB) (Siriwatanametanon et al., 2010). The water extract of the leaves has been prescribed to treat AIDS (Woradulayapinij et al., 2005). *G. pseudochina* leaf extract could kill *Aedes aegypti* instar IV mosquito larvae (dengue) in a significant way, hence the extract used for controlling the *A. aegypti* population in its larval form (Panghiyangani et al., 2009). In addition, the alcoholic extract or water extract from fresh leaves and rhizome of *G. pseudochina* L. (DC) var. *hispida* have been used externally against inflammation and viral infections (herpes). Root extract can be used internally to treat pain and fever (Vanijajiva, 2009). The underground of *G. pseudochina* L. (DC) has been used as an anti-inflammatory, relieving symptoms of hot pain, fevers, and treatment of herpes infections (Plant Genetic Conservation Project, 2009). In addition, root part has been

used against bruises, and poultice from the leaf part has been applied against pimples. Both roots and leaves are used as a hemostasis and against breast tumors. In Vietnam, the root has been used as a tonic, the leaves have been used as an emollient to treat sore throat (Vanijajiva, 2009). Moreover, *G. pseudochina* has potential use for Zn and Cd phytoremediation in the Zn and Cd contaminated area. This plant was defined as a Cd/Zn hyperaccumulator based on high concentrations of Cd and Zn accumulated in the shoot to 457 mg Cd kg^{-1} dry mass and 6171 mg Zn kg^{-1} dry mass, respectively (Phaenark et al., 2009).

Secondary metabolites have been extensively investigated, and some compounds have been identified. Purwanto et al. (2010) compared secondary metabolites profiles from the extracts of *in vitro* culture and external *G. pseudochina* by Thin-layer chromatography (TLC). Terpenoid content was found similar, but there was some evidence on differences of flavonoids and alkaloid content in both conditions. On the other hand, *in vitro* cultures can produce a higher amount and variety of secondary metabolites than the external plant. Total phenolic and flavonoid contents in the leaf extract of *G. pseudochina* in Indonesia were 68.1 µg gallic acid equivalent/mg dry extract and 13.31 µg quercetin equivalent/mg dry extract, respectively (Batubara et al., 2012). Moektiwardoyo et al. (2014) reported that *G. pseudochina* leaf extract contained flavonoids, tannins, steroids, monoterpene, sesquiterpene, and polyphenol. The methanol extract from the *G. pseudochina* leaves had four compounds of quercetin 3- rutinoside, 3,5-di-caffeoylquinic acid, 4,5-di-caffeoylquinic acid, and 5-monocaffeoylquinic acid (Siriwatanametanon & Heinricha, 2011).

Although *G. pseudochina* has medicinal value, pyrrolizidine alkaloids (PA) are reported to be present in the leaves of this plant and other *Gynura* species. The presence of PAs in medicinal plants is of concern due to their toxicity to humans and animals. PAs are reported to be hepatotoxic, pneumotoxic, genotoxic, neurotoxic, and cytotoxic. Windono et al. (2012) found that two PAs, senecionine and senkirkine were isolated from the *G. pseudochina* tuber.

2.3 Heavy metal toxicity in plants

Environmental pollution involving high concentrations of heavy metals is a major challenge for plants and other organisms worldwide. In places such as current or

former mining areas, metal ion levels will be caused by human activity. Heavy metals are defined as that group of elements that have specific weights greater than about 5g/cm^3. A number of them (Co, Fe, Mn, Mo, Ni, Zn, Cu) are essential micronutrients and are necessary for normal growth and participation in electron transfers and other significant metabolic processes in plants. Non-essential metals (Pb, Cd, Cr, Hg) are potentially highly toxic to plants (Michalak, 2006). Excessive concentrations of trace elements (Cd, Co, Cr, Hg, Mn, Ni, Pb, and Zn) are toxic, inhibiting growth, reducing biomass and killing the plant. Furthermore, they can inhibit physiological processes such as respiration, photosynthesis, cellular elongation, plant-water relationship, N-metabolism, and mineral nutrition (Zenk, 1996).

The presence of Zn in the environment is usually related to the presence of Cd due to its association with nature and their chemical similarity (Nriagu & Pacyna, 1988; Smith & Brennan, 1983). However, it is known that higher concentrations of both essential metals such as Zn and non-essential metals such as Cd result in oxidative stress and inhibition of plant growth. In order to adapt to the excess of heavy metals in the environment, plants have developed various detoxification mechanisms mainly based on exclusion, chelation, and subcellular compartmentalization (Hall, 2002; Ovečka & Takáč, 2014). Panitlertumpai et al. (2013) reported that *G. pseudochina* treated with higher concentrations of Zn and Cd increased chlorosis and metal accumulation in roots and shoots. The low concentration of Cd induced growth and a high translocation factor, and the dual treatment with Zn and Cd decreased metal toxicity and accumulation of Cd.

Some external mechanisms that restrict the uptake of metals by roots can help plants to tolerate a certain amount of toxic metals in the soil. One of them is the formation of non-toxic chelates of metallic ligand in the rhizosphere involving organic acids and other exudates from the roots. Metals can be transported via an apoplastic system and immobilized in cell walls (Zornoza et al., 2002). In many cases, plants that are resistant to heavy metal stress have lower nutritional needs and specific minerals (cadmium, potassium, and phosphorus) and a water limit with this stress. Remarkably resistant plants are involved in the phytoremediation of metallic contaminated sites (Ali et al., 2013). A mechanism of high concentrations of heavy metals, which can damage plant tissues, is to stimulate the potential of free radical by imposing oxidative (Díaz et

al., 2001). The accumulation of reactive oxygen species (ROS) activates the antioxidant defence mechanisms of plants. ROSs are known to damage cell membranes by causing lipid peroxidation. They also cause damage DNA, proteins, lipids, and chlorophyll (Devi & Prasad, 1998; Breusegem et al., 2006). The most popular free radicals of ROS in plants are singlet oxygen ($^1O_2$), superoxide radicals ($^{\cdot}O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\cdot}OH$). However, the plant has a capacity to produce phenolic compounds, which have been suggested as playing key roles as antioxidants in stress plants, metal chelators and metal detoxification (Chaudière & Ferrari-Iliou, 1999).

Plants produce an extremely diverse range of low molecular mass compounds, secondary metabolites, or natural products that have an essential metabolic plasticity to anticipate and respond to biotic and abiotic stresses. Damage to plants occurs when the capacity of antioxidant processes and detoxification mechanisms is below the amount of ROS production. Aerobic organisms have developed complex systems that protect them from ROS, consisting of several enzymes and antioxidants (Michalak, 2006).

2.4 Actions of phenol on heavy metal stress

Plants are potential sources of naturally occurring bioactive compounds such as secondary metabolites and antioxidants. Phenolic compounds are secondary plant metabolites, which are electron donors. Hence, they can act as antioxidants that are one of the most common and widespread groups of substances in the plants. They act as reducing agents, hydrogen donors, and singlet oxygen quenchers and prevent the evolution of free oxidizing radicals and reactive species derived from metallic catalysis (Jung et al., 2003; Michalak, 2006). Phenolic has at least one aromatic ring (C6) with one or more hydroxyl groups. They are mainly synthesized from cinnamic acid, which consists of phenylalanine through the action of L-phenylalanine ammonia-lyase PAL, the branch point enzyme between primary (shikimate pathway) and secondary (phenylpropanoid) metabolism (Dixon & Paiva, 1995). A summary of the highly complex phenylpropanoid pathway is represented and important steps for the formation of some phenolic compounds as shown in Figure 2 (Dias et al., 2016). Furthermore, the most important pathway in the biosynthesis of phenolic compounds is through shikimic

acid pathway. Shikimate formation is an essential branch for forming phenolic compounds (Cohen & Kennedy, 2010; Karakaya, 2004).

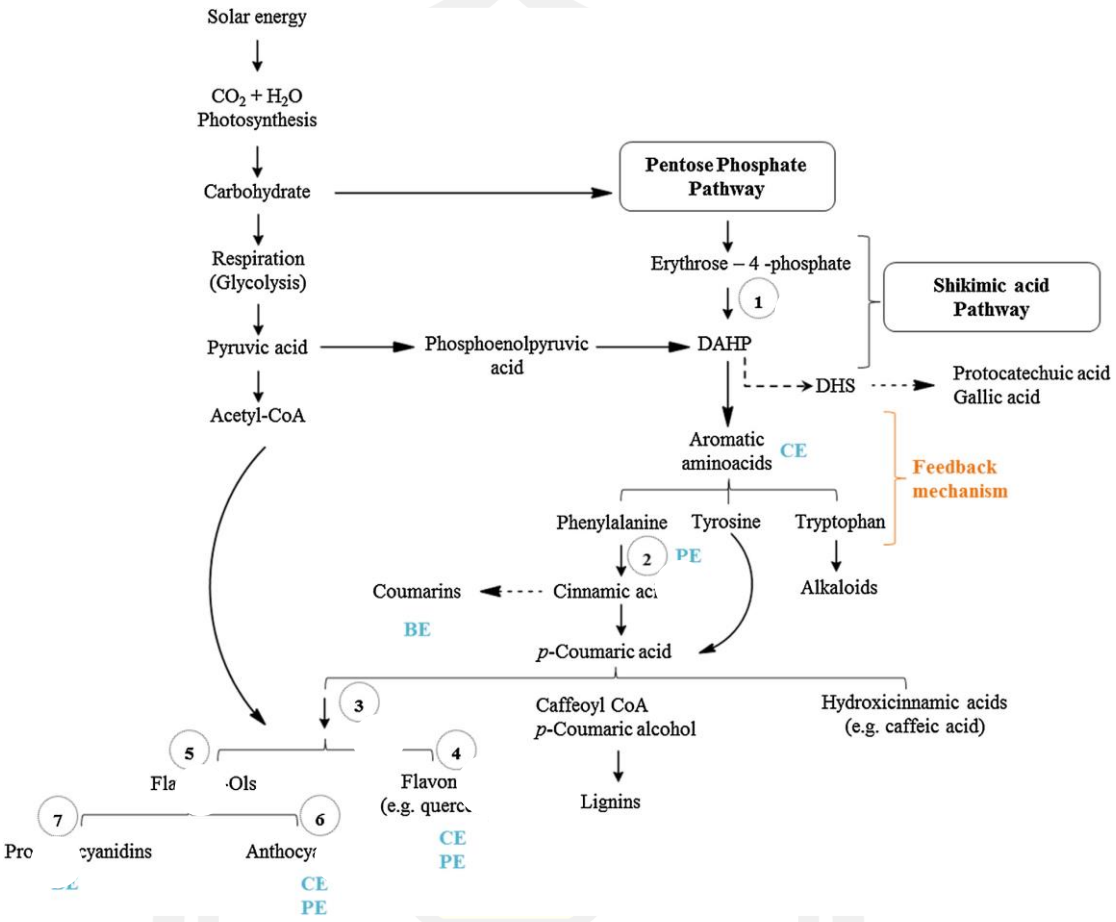


Figure 2 Biosynthetic pathways of some phenolic compounds (Dias et al., 2016).

The antioxidant action of phenolic compounds has many reports of induced accumulation of phenolic compounds and peroxidase activity in plants treated with high concentrations of metals (Figure 3). If the concentration of phenolics and ascorbate can be present at the level of mM in vacuoles, the ascorbate/phenolic/POX (peroxidase) system enables the effective reduction of H_2O_2 without accumulating oxidized phenolic products. Vacuoles and apoplast are proposed to function as sinks of H_2O_2 in plant cells, which allow the delocalized detoxification mechanism against H_2O_2 produced in other compartments under stress and development (Michalak, 2006).

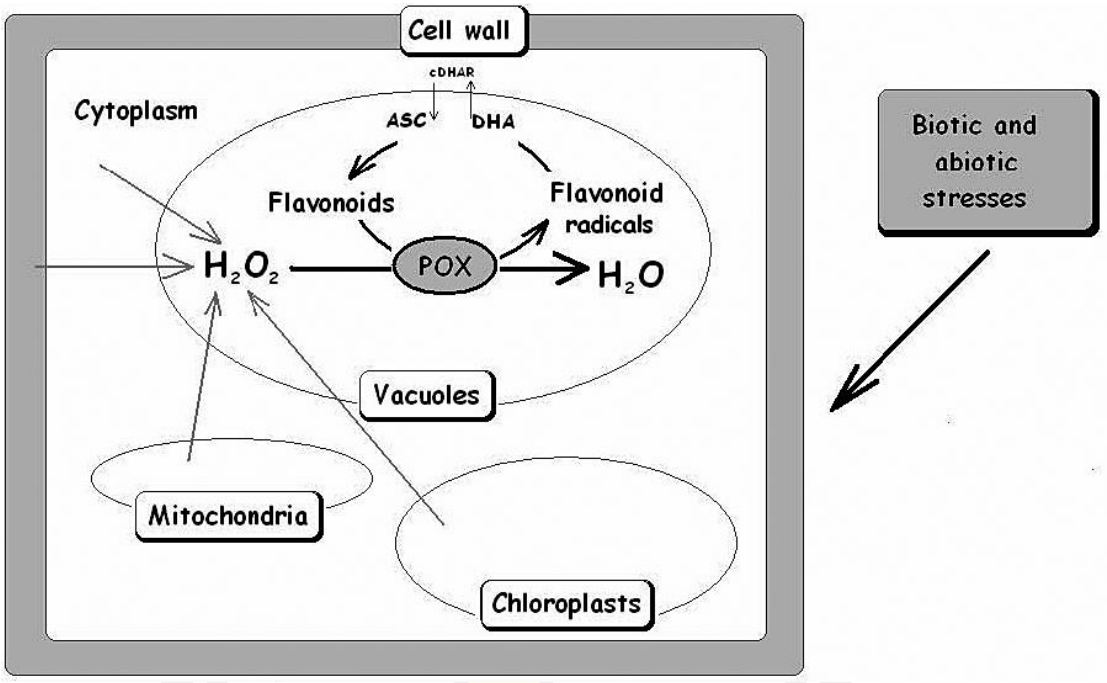


Figure 3 Phenolic activity for the reduction of H_2O_2 toxicity (Michalak, 2006).

Flavonoids are a group of phenolic compounds, a larger class of secondary plant metabolites, with a broad range of biological functions, for example: signaling of interactions between plants and microorganisms, defense against herbivores and pathogens, protection against UV radiation, pollen pigmentation, and flowers for attracting pollinators, or stimulating the pollen germination and pollen tube growth. Many of these biological characteristics and functions seem to be associated with the high antioxidant activity of flavonoids (Winkel-Shirley, 2001; Ylstra et al., 1992). The antioxidant activity of phenol is due to its strong tendency to chelate metals. Phenolic have hydroxyl and carboxyl groups that can bind iron and copper in particular (Jung et al., 2003). The roots of many plants exposed to heavy metals exude high levels of phenolic (Winkel-Shirley, 2002). Direct chelation, or polyphenol binding, was observed with methanol extracts of rhizome polyphenols from *Nympheae* for Cr, Pb, and Hg (Lavid et al., 2001). The increase of soluble phenolic such as intermediates in lignin biosynthesis can reflect the typical anatomical change induced by stress factors, which increase the endurance of the cell wall and the creation of physical barriers to the harmful effects of heavy metals. *Phaseolus vulgaris* exposed to Cd^{2+} accumulates soluble and insoluble phenolics, and leaves of *Phyllantus tenellus* contain more

phenolics than control plants after being sprayed with copper sulfate (Dias et al., 2016). Cd induced the total levels of phenolics and flavonoids and the total antioxidant capacity of *Erica andevalensis* growing in mine soils. Cinnamic acid derivatives, epicatechin, and rutin were increased in the presence of Cd, and levels of chlorophylls did not change the ratio. Phenolic compounds play a major role in *E. andevalensis* metabolism to survive in soils polluted by heavy metal (Márquez-García et al., 2012). Consequently, phenolic compounds have a role in a plant's mechanisms for surviving in the presence of toxic metals in its natural habitat.

2.5 Plant-microbe interaction in phytoremediation

Phytoremediation is the use of green plants to eliminate environmental pollutants (Cunningham & Berti, 1993; Raskin et al., 1994; Wenzel, 2009). The success of the process, in which metals are effectively removed from the soil, depends on an adequate plant yield and efficient transfer of metals from plant roots to their shoots. Plants are termed as "hyperaccumulators" (Ghosh & Singh, 2005). Overall the microbial activities in the root/rhizospheric soils improve the efficiency of phytoremediation processes in metal-contaminated soils in two complementary ways (Figure 4): (1) direct promotion of phytoremediation in which plant-associated microbes enhance metal translocation (facilitate phytoextraction) or reduce mobility and availability of metallic contaminants in the rhizosphere (phytostabilization), and (2) indirect promotion of phytoremediation in which microbes confer tolerance to plant metals and/or enhance the production of plant biomass in order to remove/arrest the pollutants (Rajkumar et al., 2012).

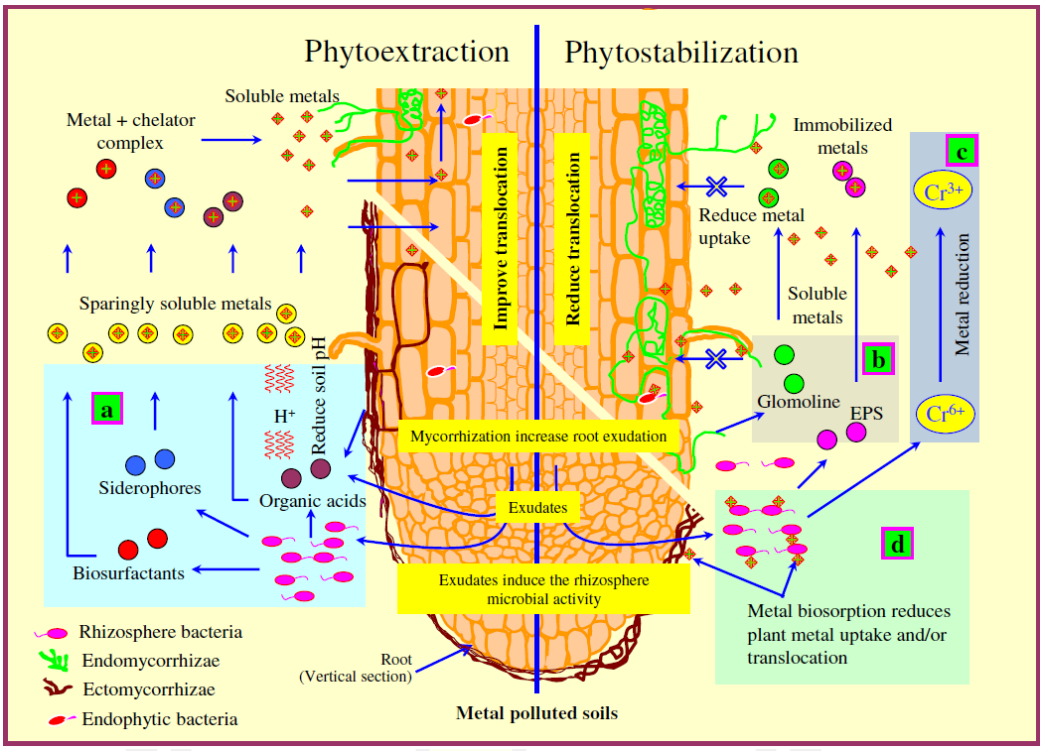


Figure 4 Plant-associated microbes accelerate the phytoremediation process in metal contaminated soils by enhancing metal mobilization/immobilization.

(a) Plant-associated microbes improve plant metal uptake by producing metal mobilizing chelators. Plant associated microbes reduce plant metal uptake and/or translocation through.

(b) Producing metal immobilizing metabolites.

(c) Metal reduction.

(d) *M*etal biosorption.

Abbreviations: extracellular polymeric substances (EPS) (Rajkumar et al., 2012).

2.6 Application of Endophytic bacteria

Endophytic bacteria are defined as those that colonize the innernal tissues of healthy plants without causing symptoms of disease or harmful effects on their host. They may complement certain metabolic properties, such as promoting plant growth, controlling soil-borne pathogens (Schulz & Boyle, 2007). Endophytic bacteria can be either facultative or obligate to the host, depending on the genotype of the host plant

and the life strategy. Facultative endophytic bacteria can survive and colonize outside the plant during a period of their life cycle. However, obligate endophytic bacteria depend on the host plant for survival and metabolic activities and can be transmitted from one generation to another by seeds or vegetative plant tissue (Su et al., 2010; Hamilton et al., 2012; Afzal et al., 2014). The host plant also benefits from the endophytes by its natural resistance to soil contamination, their ability to degrade xenobiotics, or their action as vectors to introduce degrading traits in plants, which is a significant contribution to phytoremediation (Ryan et al., 2008). Mechanisms of endophytic bacteria beneficial to their host plants can include the production of phytohormones, enzymes involved in regulatory growth metabolisms, such as ethylene, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, indole-3-acetic acid (IAA), and production of a siderophore (Glick et al., 1998; Hardoim et al., 2008; Rajkumar et al., 2009). In addition, they can also improve plant growth via the fixation of nitrogen and improve the availability of phosphate during initial colonization (Kuklinsky-Sobral et al., 2004; Luo et al., 2011). In addition, some endophytes improve plant growth and health by improving mineral nutrition or increasing resistance/tolerance to biotic and abiotic stresses (Ryan et al., 2008).

2.6.1 Diversity of endophytic bacteria in Zn and/or Cd hyperaccumulative plants

At present, the interactions between endophytic bacteria and hyperaccumulative plants have attracted the attention of several researchers because of biotechnological applications for bioremediation and to investigate the composition of bacterial communities living in a naturally contaminated environment (Rajkumar et al., 2009). For example, endophytic bacteria resistant to Zn and/or Cd have been isolated from various hyperaccumulative plants, as shown in Table 1. The hyperaccumulator can be colonized simultaneously by a high number of divisions, genera and species of metal-resistant endophytic bacteria. Distinct microbial communities colonize various plant compartments, and they have been found in different parts of the plant such as roots, stem, leaves, flowers as well as fruits and seeds.

Table 1 Overview of Zn and/or Cd hyperaccumulative plants and their associated endophytic bacteria.

Hyperaccumulators Plant	Metal	Portion	Endophytes	References
Thlaspi caerulescens	Zn	Stem	*Sphingomonas* sp., *Methylobacterium* sp., and *Sphingobacterium multivorum*	Lodewyckx et al. (2002)
		Root	Phyllobacterium sp., *Devosia* sp., *Afibia* sp., *Sphingomonas* sp., and *Rhodococcus* sp.	
Nicotiana tabacum	Cd	Seed	*Enterobacter* sp., *Xanthomonadaceae*, *Pseudomonas* sp., *Pseudomonas fulva*, *Stenotrophomonas* sp., *Clostridium aminovalericum*, and *Sanguibacter* sp.	Mastretta et al. (2009)
Sedum alfredii Hance	Zn/Cd	Stem	*Stenotrophomonas maltophilia, Bacillus cereus, Pseudomonas synxantha*	Xinxian et al. (2011)
		Leaves	*Pseudomonas fluorescens, Bacillus pumilus, Bacillus subtilis*	
Solanum nigrum L.	Cd	Root Stem	*α-, β-, γ- Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria*	Chen et al. (2012)

Table 1 (Cont.)

Hyperaccumulators Plant	Metal	Portion	Endophytes	References
S. plumbizincicola	Zn/Cd	Stems, leaves	*Bacillus pumilus* E2S2, *Bacillus sp.* E1S2, *Bacillus sp.* E4S1, *Achromobacter sp.* E4L5, *Stenotrophomonas sp.* E1L	Ma et al. (2015)
Murdannia spectabilis (Kurz) Faden.	Zn/Cd	Tubers, leaves, peduncles, storage root	*Bacillus, Pantoea, Microbacterium, Curtobacterium, Chryseobacterium, Cupriavidus, Siphonobacter, Pseudomonas*	Rattanapolsan et al. (2021)

2.6.2 The role of endophytic bacteria in phytoaccumulation

Indole-3-acetic acid (IAA), a phytohormone, is a central role in cell division, cell enlargement, and root initiation (Vessey, 2003). Some endophytes can produce auxins to improve the growth of host plants in polluted soils. For example, endophytic bacteria *Serratia nematodiphila* LRE07, *Enterobacter aerogenes* LRE17, *Enterobacter* sp. LSE04, and *Acinetobacter* sp. LSE06 from *Solanum nigrum*, *Enterobacter* sp. 12J1 from *Allium macrostemon*, and *Acinetobacter*, *Agrobacterium tumefaciens*, *Bacillus* sp., *B. subtilis*, and *B. megaterium* from *Commelina communis* was able to produce IAA to stimulate plant growth and enhance phytoremediation (Sheng et al., 2008; Chen et al., 2010; Zhang et al., 2011).

Ethylene (C_2H_4) is an important phytohormone to regulate plant growth and cellular metabolism (Ping & Boland, 2004), but the overproduction of ethylene promoted by stresses can inhibit plant development processes, such as root elongation, lateral root growth, root hair development from seed germination to shoot growth and

leaf abscission (Mayak et al., 2004). The role of ACC deaminase in decreases stress ethylene levels by the enzymatic hydrolysis of ACC into α-ketobutyric acid and ammonia, as one of the major mechanisms of plant growth-promoting endophyte (PGPE) to promote root and plant growth (Hardoim et al., 2008). The production of ACC deaminase is thus probably an important and efficient way for endophytes to manipulate their host plants. Madhaiyan et al. (2007) reported the greater potential of the methylotrophic bacteria, *Methylobacterium oryzae* and *Burkholderia* sp. (isolated from rice tissue) to protect tomato (*Lycopersicon esculentum*) seeds from the toxicity of high concentrations of Ni and Cd grown under biotic conditions.

Nitrogen fixation, nitrogen is an essential component of many critical plant compounds. It is an important component of all amino acids, nucleic acids, and chlorophyll. Xinxian et al. (2011) found that *Bacillus pumilus*, which was an endophytic bacterium isolated from the roots and leaves of Zn/Cd hyperaccumulator *Sedum alfredii*, had nitrogen fixation capability.

Phosphorus (P) is another essential macronutrient for biological growth and development. Soils may have large reserves of total P, but the amount available for plants is usually a small fraction of that total. Some endophytic bacteria also showed the solubilization of mineral phosphates (Verma et al., 2001), suggesting that during initial colonization, endophytic bacteria could increase phosphate availability for the host plant. Results from Kuklinsky-Sobral et al. (2004) supported this suggestion, demonstrating that 52% of the endophytic bacteria isolated from soybean could solubilize mineral phosphate.

The various mechanisms of interaction of microorganisms with plants in relation to micronutrient accumulation or resistance are summarized in Figure 5 (Pilon-Smits, 2005; Sessitsch et al., 2013). Plant-associated bacteria could potentially improve phytoaccumulation by altering the solubility, availability, and transport of heavy metal and nutrients through the production of organic acids, the release of chelators, siderophores, or redox changes and/or metal mobilization (Saravanan et al., 2007; Sheng et al., 2008). Siderophores are organic molecules that have a strong affinity to ferric ions, but they can also form complexes with other bivalent heavy metal ions such as Al, Cd, Cu, Ga, In, Pb, and Zn. The binding of the siderophore to a metal increases the concentration of soluble metal (Rajkumar et al., 2010). Many endophytes

that produce siderophores from a variety of plants have been reported, and they improved plant growth in low nutrition environments (Idris et al., 2004; Barzanti et al., 2007; Chen et al., 2010; Sheng et al., 2008; Ma et al., 2011; Zhang et al., 2011; Shin et al., 2012).

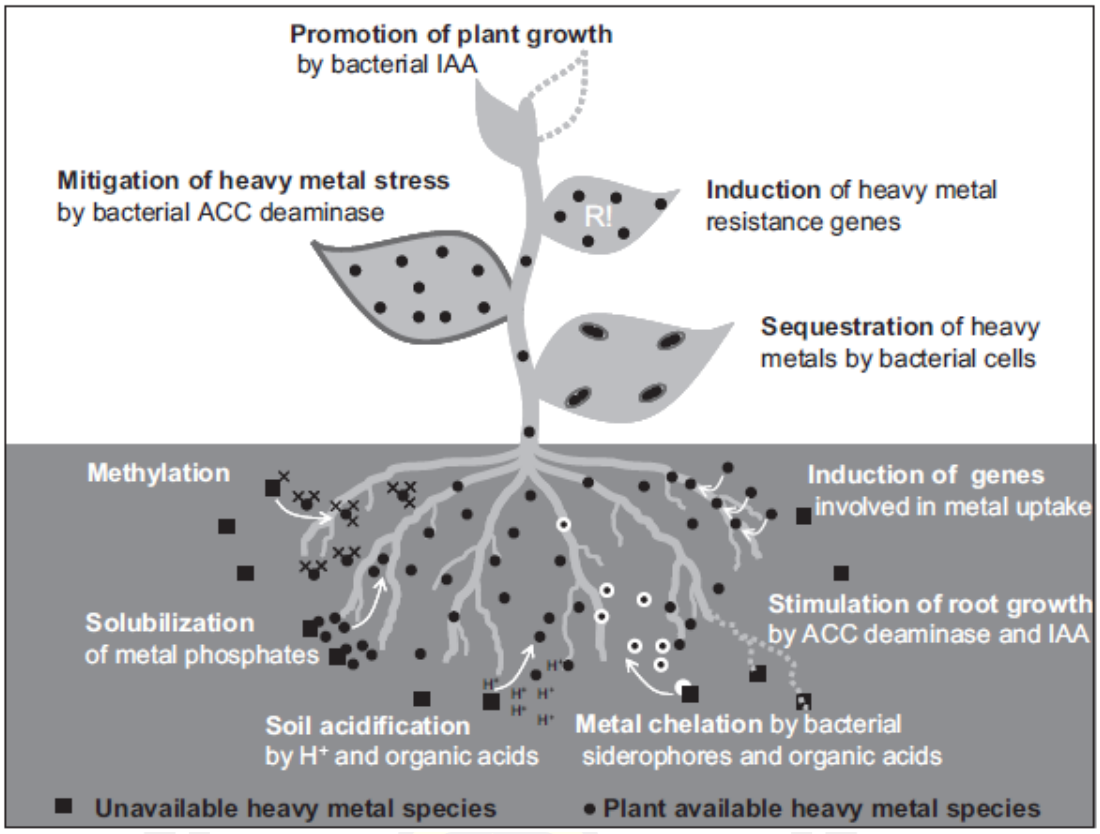


Figure 5 Putative plant-microbe interactions influencing trace element accumulation in plants (Pilon-Smits, 2005; Sessitsch et al., 2013).

Chen et al. (2010) found that four heavy-metal-resistant endophytic bacteria improved the accumulation of Cd in the roots, stems, and leaf tissues of *Solanum nigrum* L., which grew in three levels of soil contaminated with Cd. In addition, they also found that the accumulating capacity changed with the concentration of Cd in the soil. Mastretta et al. (2009) reported that the inoculation of *Nicotiana tabacum* with *Sanguibacter* sp., a Cd-resistant endophyte, increased the concentration of Cd in shoot tissues by approximately three-fold compared to the respective un-inoculated control. In addition, Sheng et al. (2008) found that inoculation of *Brassica*

napus with Pb-resistant endophytic bacteria increased the uptake of Pb in the shoot. Lodewyckx et al. (2002) showed the inoculated plant with endophytes, which was isolated from a hyperaccumulative Zn, accumulated a higher concentration of Zn in the root of the inoculated plant than the control plant (non-inoculated).

2.6.3 Endophytic bacteria in medicinal plants

Medicinal components are stored in plant leaves. Most secondary metabolites of herbs and spices are commercially important and used in a variety of pharmaceutical compounds. The identified determinants of this competitive ability include the production of antimicrobial compounds, detoxification of reactive oxygen species (ROS), and secondary plant metabolites by antioxidant enzymes, ring-cleaving by dioxygenases, presence of efflux pumps (Barret et al., 2011). Most of the plant species, which have already been studied, had on or more endophytic microbes (Ryan et al., 2008). Endophytic microbes have been recognized as potential sources for bioactive secondary metabolites (Strobel et al., 2004) as medicinally therapeutic important agents (Firáková et al., 2007; Huang et al., 2008), which have significance in medicine, agriculture and industry (Joseph & Mini, 2011; Nair & Padmavathy, 2014). Endophytes may therefore produce the same bioactive compounds as their host plants. In addition, it is recognized that a microbial source for a valued product can be easier and more cost-effective to produce, effectively reducing its market price. Some medicinal plants and their associated endophytic bacteria are shown in Table 2.

Table 2 Medicinal plants and their associated endophytic bacteria.

Medicinal plants	Portion	Endophytic bacteria	References
Gynura procumbens (Lour.) Merr.	Leaves	*Psuedomonas resinovorans*, *Paenibacillus polymaxa*, *Acenitobacter calcoaceticus*	Bhore et al. (2010)

Table 2 (Cont.)

Medicinal plants	Portion	Endophytic bacteria	References
Plectranthus tenuiflorus	Root, stem, leaves	*Bacillus* sp., *Bacillus megaterium, Bacillus pumilus, Bacillus licheniformis, Micrococcus luteus, Paenibacillus* sp., *Pseudomonas* sp., *Acinetobacter calcoaceticus*	El-Deeb et al. (2012)
Tridax procumbens Linn	Leaves, stems	*Bacillus* sp., *Cronobacter sakazakii, Enterobacter* sp., *Lysinibacillus sphaericus, Pantoea* sp., *Pseudomonas* sp., *Terribacillus saccharophilus*	Preveena & Bhore (2013)
Aquilaria species	Leaves, stems	*Acinetobacter radioresistens, B. altitudinis, B. anthracis, B. arbutinivorans, B. arsenicus, B. aryabhattai, B. cereus, B. licheniformis, B. megaterium, B. methylotrophicus, B. pumilus B. stratosphericus, B. subtilis B. tequilensis, Pantoea agglomeransniv, Rahnella aquatilis, Roseomonas mucosa, Vibrio cholera*	Bhore et al. (2013)
Zingiber officinale	Rhizome	*Bacillus* sp., *Pseudomonas* sp., *Stenotrophomonas sp, Staphylococcus* sp.	Jasim et al. (2014)
Salvia miltiorrhiza	Root	*Pseudomonas brassicacearum* sub sp. *neoaurantiaca, Rhizobium radiobacter, Pseudomonas thivervalensis, Pseudomonas frederiksbergensis*	Yan et al. (2014)

Table 2 (Cont.)

Medicinal plants	Portion	Endophytic bacteria	References
Capparis sinaica	Aerial parts	*Bacillus* sp. *B. subtilis, B. amyloliquefaciens subsp. Plantarum, Bacillus benzoevorans, Staphylococcus* sp.	Bahgat et al. (2014)

2.6.4 Colonization of endophytic bacteria

Although previous studies have reported that inoculation of plants with endophytic bacteria has a promising potential to improve plant health in an environment stressed by metals, only a few bacterial endophytes demonstrated beneficial effects on plants when inoculated on crops under field conditions. These results may be due to poor survival and colonization potential of the inoculated strains (Ma et al., 2011). These are specific characteristics required for endophytic competence i.e., the potential to successfully colonize the host plant (Hardoim et al., 2008). Passive penetration can occur in cracks, such as those occurring at root emergence sites or created by deleterious microorganisms, as well as by root tips. Under natural conditions, some plant pathogenic bacteria can also disrupt the endodermis, allowing endophytic bacteria to pass simultaneously into the central cylinder (Figure 6). After crossing the endodermis barrier, the endophytic bacteria must penetrate the pericycle to further reach the root xylem vessels of their hosts.

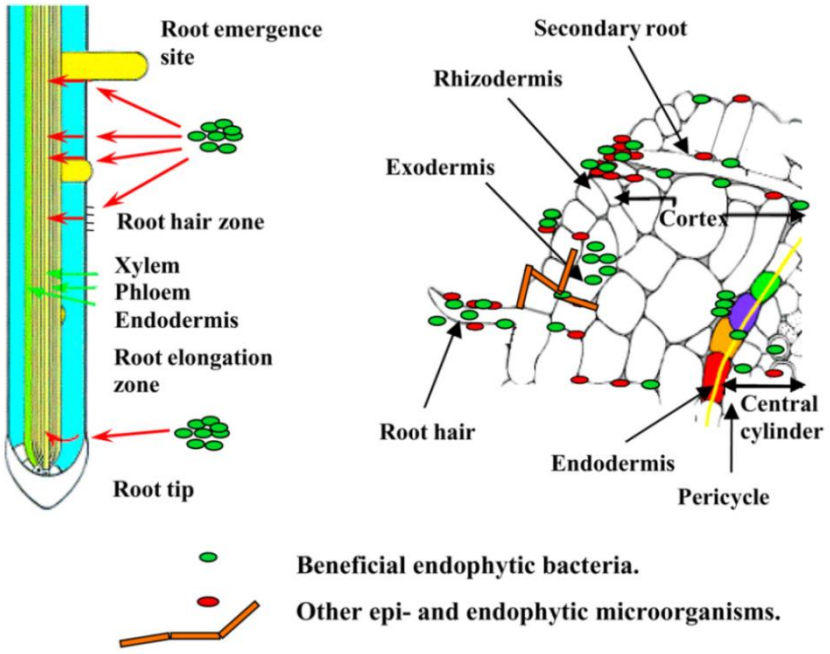


Figure 6 Sites of plant colonization by endophytic bacteria (Reinhold-Hurek & Hurek, 1998).

Since a high rate of survival and colonization ability is essential for achieving beneficial effects, it is important to analyze the possibility of survival and colonization of the strains inoculated in the host cells. Therefore, various methods have been developed for detecting inoculated endophytes in host plant tissues. For example, cultivation methods are commonly used to isolate and monitor their survival and colonization within the plant tissue. However, the disadvantage of using these methods is that it can be difficult to distinguish between inoculated strains and indigenous endophytic populations (Brehm-stecher & Johnson, 2004). Similarly, spontaneous mutants of parental endophytic strains are resistant to specific antibiotics such as chloramphenicol and rifampicin have been used in a number of studies to differentiate them from native endophytic populations (Sheng et al., 2008; Ma et al., 2015). However, antibiotic resistance in indigenous populations should first be identified prior to using this method. Once an appropriate method has been developed, a quantitative analysis of bacterial survival and colonization based on the number of colony-forming unit should be conducted using suitable media amended with appropriate antibiotics.

Moreover, autofluorescent protein (AFP) methods are now a key tool for investigating processes such as microbial–plant interactions and biofilm formation (Larrainzar et al., 2005). These techniques are used for the detection and enumeration of microorganisms *in situ* on plant surfaces and in plants (Gage et al., 1996). One of these AFP strategies employs a marker system, which encodes for green fluorescent protein (GFP). GFP is a useful AFP biomarker because it requires neither a substrate nor a cofactor for fluorescence. Bacterial cells with *gfp* chromosome integration can be identified by epifluorescent microscopy or confocal laser scanning microscopy (Tombolini et al., 1997; Villacieros et al., 2003; Germaine et al., 2009), as shown in Figure 7.

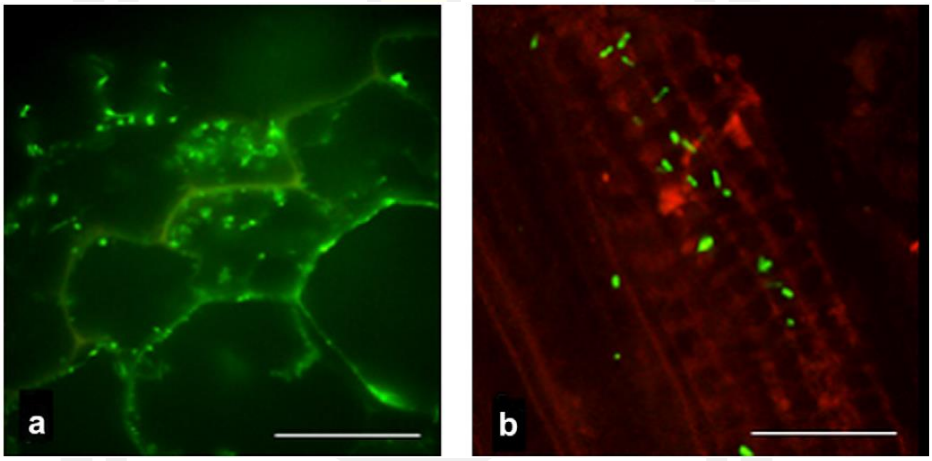


Figure 7 Endophytic colonization of a plant growth-promoting bacterium observed via fluorescence in situ hybridization with green fluorescent protein tagging.

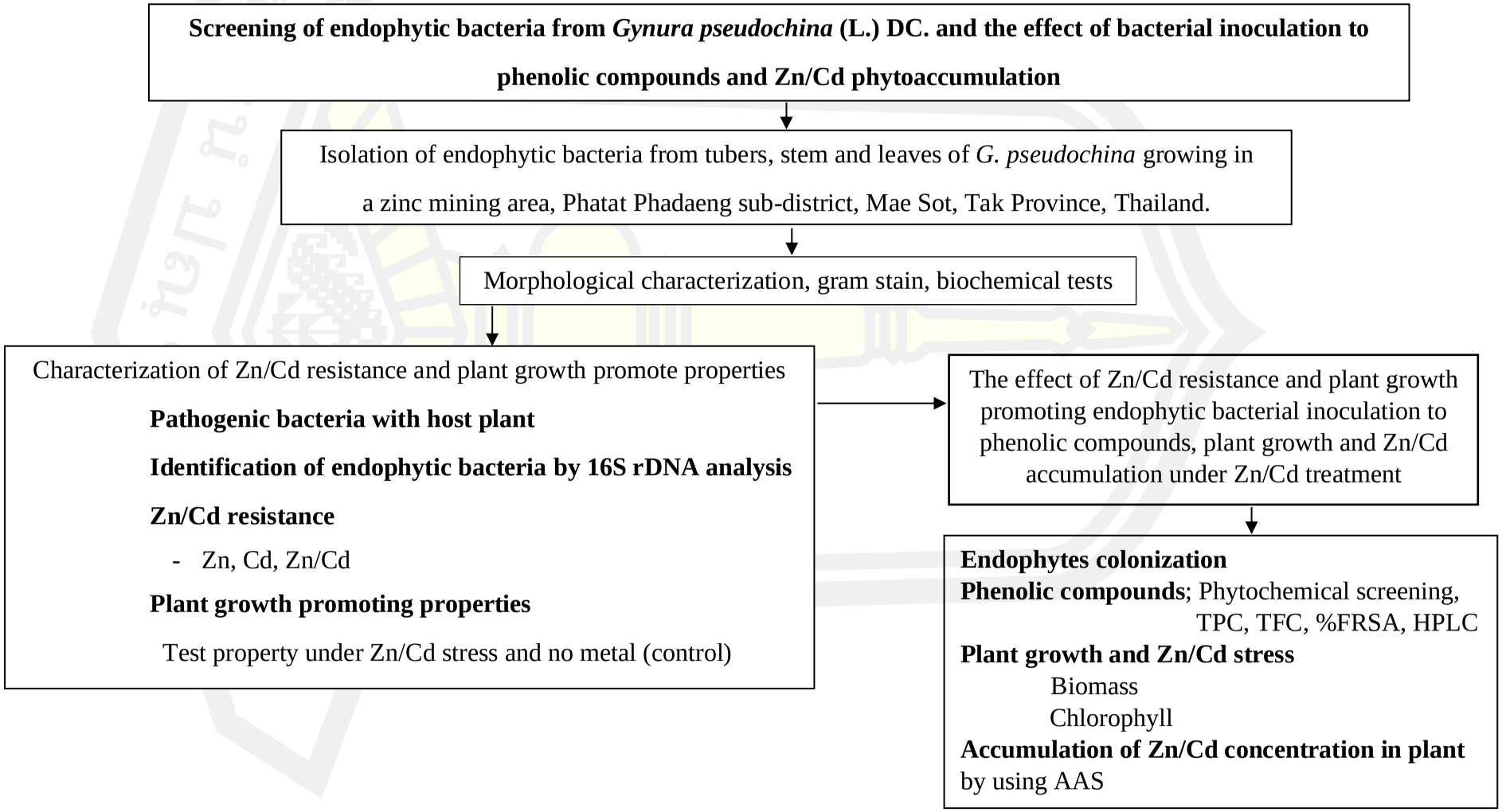
(a) Bacterial cells as green rods inside cortex. Scale bars: 25 mm.

(b) *Burkholderia phytofirmans* PsJN cells as green rods inside xylem vessels. Scale bars: 10 mm (Compant et al., 2008).

CHAPTER 3
METHEOLOGY

This research aims to isolate and characterize the endophytic bacteria from *Gynura pseudochina* that grew in the Padaeng Zn mine, Phatat Padaeng sub-district, Mae Sot district, Tak Province, Thailand. Endophytic bacteria were screened by Zn/Cd resistance and their ability to promote plant growth, including the production of indole-3-acetic acid (IAA) and siderophore, nitrogen fixation, and phosphate solubilization. The purpose of this study is also to investigate the effect of endophytic bacterial inoculation on phenolic compounds, plant growth, and accumulation of Zn/Cd. Additionally, Zn and Cd distribution and inoculation of endophytic bacteria were performed in the plants.

3.1 Research diagram



3.2 Materials and Methods

3.2.1 Plant materials

The plants were collected from a forest area of in the Padaeng Industry Public Company Limited (PDI) Mae Sot mine, DistrictPhatat Phadaeng sub-district, Mae Sot, Tak Province, Thailand (N 16° 39′ 6.6″, E 98° 39′ 41.2″) on 19 August 2016 in the rainy season.

*Soil analyze*s: Soil samples were collected from plant roots. Then the soil samples were oven-dried at 80 ºC for 24 hours in a hot air oven (Redline-Binder Germany), and then ground and sieved through a 2-mm nylon sieve. Soil temperature was examined with a digital thermometer (Hanna HI98501, Romania). Soil pH was determined in a 1:5 soil: deionized water suspension and measured using a pH meter (Denver Model 215, USA) (Estefan et al., 2013). Total Zn and Cd concentrations in the soil sample were determined by digestion of 0.5 g dry weight soil digestion with 15 ml of aqua regia (a mixture of 35% (w/v) HCl and 70% HNO_3 as 3:1) following a modified method of Miller (198). The extractable Zn and Cd concentrations in soil were determined by shaking the soil at 150 rpm for 2 hours in 0.005 M diethylene triaminepenta acetic acid (DTPA) using a soil: extractant ratio of 1:5 (Lindsay & Norvell, 1978). Total and extractable concentrations of Zn and Cd were determined by atomic absorption spectroscopy (AAS) (AA-680 Shimadzu, Japan).

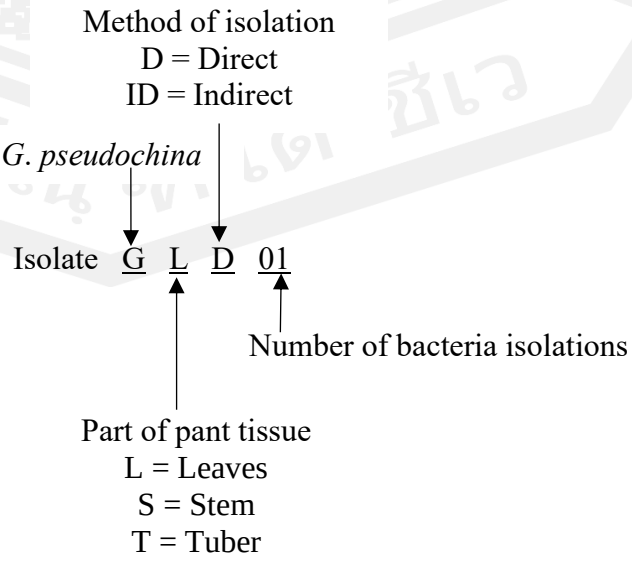
Accumulation of Zn and/or Cd in the plant: A plant sample was collected and divided into two parts of the root and the shoot. The sample was rinsed with excess tap water, washed three times with deionized water, and oven-dried at 80 ºC. Each dried sample was digested with HNO_3 conc. (70% v/v) and $HClO_4$ (70% v/v) according to a modified method of Miller (1998). The digestion was analyzed for Zn and Cd concentration by using an Atomic Absorption Spectrophotometer (AAS) (Shimadzu AA-680, Japan).

3.2.2 Isolation of endophytic bacteria

The plant samples were washed with excess tap water, then rinsed three times with deionized water before being separated into the tubers, stems, and leaves. The plant tissue samples were surface-sterilized following a modified method of Chen

et al. (2010) by immersion in 70% (v/v) ethanol for 40 s, then 15% (v/v) Clorox (0.9% NaOCl) for 30 min for tuber tissue, 70% (v/v) ethanol for 40 s, then 15% (v/v) Clorox (0.9% NaOCl) for 15 min for stem and leaf tissue, and finally 3 rinsings in sterile distilled water. To confirm the success of the surface disinfection process, a 100 µl sample of the final wash water was applied to tryptone soya agar (TSA) containing Nystatin (50 mg/ml of each) to inhibit fungal growth (Williams & Davies, 1965), and then the plate was incubated at 30 ± 5 °C for 3-7 days.

Endophytic bacteria were isolated from the surface-sterilized samples by direct and indirect methods (Rattanapolsan et al., 2021). In the case of the direct method, the tuber, stem, and leaf tissue of the whole plant were sliced into 1 to 2 cm × 1 to 2 cm pieces. Fragments of tubers, stems, and leaves were aseptically transferred into Petri dishes containing TSA medium supplied with 50 mg/ml Nystatin. For the indirect method, 1 g of each tuber, stem and leaf tissue were ground and macerated with a sterile mortar and pestle in 9 ml of sterile phosphate-buffered saline (PBS, g/L: 1.44 Na_2HPO_4, 0.24 KH_2PO_4, 0.20 KCl, 8.00 NaCl, pH 7.4). The tissue extracts were prepared for 10^{-4}, 10^{-5}, and 10^{-6} series dilution with PBS. Then 0.1 ml of each dilution was spread on a TSA medium supplied with 50 mg/ml Nystatin. After incubation at the 30 ± 5 °C for 7 days, colonies with different morphologies were picked up and re-streaked repeatedly on TSA media until the pure bacterial isolates were obtained. Cell morphology was studied by microscopy and Gram staining. The bacterial isolates were stored at -20 °C in TSA with 15 % (v/v) glycerol added (Pereira & Castro, 2014). Pathogenic in host plants was determined before studying other properties. The endophytic bacterial isolates were given code named as follows.



3.2.3 Characterization of Zn/Cd resistance and plant growth promotes traits of endophytic bacteria

3.2.3.1 Determination of bacterial pathogenesis on host plant

To prepare the inoculation, an endophytic bacterial strain was grown overnight in tryptone soya broth (TSB), before being adjusted to an optical density at 600 nm (OD_{600}) of 0.1. Pathogenesis were studied on hot plant by two techniques; (1) bacterial suspension was injected into the abaxial side of the leaf and the stem of a 4-week-old plant in plant tissue culture system, using a blunt end syringe with 1.0 ml of bacterial suspension or PSB as a control, and (2) bacterial suspension or PSB as a control was swapped on the leaves of plants growing in pots, which was made wound by sterilized needle. The treated plants were recovered in plastic boxes containing tissue and distilled water sterilized based on a modified method of Blakney & Patten (2011). After inoculation for 10 days, the plants were visually assessed for symptoms of the disease, as described by Ryu et al. (2003). The symptoms on the leaf was scored from 0 to 5; 0=no symptoms, 1=mild chlorosis, 2=chlorosis only, 3=chlorosis and mild necrosis, 4=necrosis, and 5=severe necrosis of the inoculated area.

3.2.3.2 Identification of endophytic bacteria

The endophytic bacteria were identified by 16S rDNA sequencing analysis. The genomic DNA of the test bacterial strains cultured on TSB broth was extracted by a modified phenol: chloroform procedure of Sambrook and Russel (2001). Partial 16S rDNA gene was PCR amplified by using 100 ng genomic DNA as a template with 1pM of each universal primers fD1 (5’ AGAGTTTGATCCTGGCTCAG 3’) and rP2 (5’ ACGGCTACCTTGTTACGACTT 3’) (Invitrogen, São Paulo, Brazil) according to a modified method of Weisburg et al. (1991). The PCR mixture (50 µl) contained 1 µl template, 5 µl of 10×Tap DNA polymerase buffer (Mg^{2+} plus), 4 µl dNTP at 2.5 mM, 0.25 µl of 5 unites Taq polymerase. The PCR was performed in a DNA Engine Thermal Cycler (TaKaRa TP 600, Germany) which conditions consisted of 94 ^{o}C (3 min), followed by 30 cycles of 95 ^{o}C (3 min), 55 ^{o}C (30 s), and 72 ^{o}C (1 min), and a final extension performed at 72 ^{o}C for 5 min. The amplified DNA was purified with TaKaRa Agarose Gel DNA

Purification Kit (TaKaRa, China), and sequencing was performed at the Macrogen sequencing service (Seoul, Korea). The sequence of 16S rDNA was compared to the GenBank database of the NCBI Blast program (Xinxian et al., 2011), by using the BLAST options of exclude uncultured/environmental sample sequences. All sequences were aligned following ClustalW multiple alignment by BioEdit. The MEGA 7 (version 7.0.26) software with the neighbor-joining statistical method and Kimura-2-parameter model with 1000 bootstraps was applied to construct phylogenetic trees (Kumar et al., 2016).

3.2.3.3 Zn/Cd resistance

The endophytic bacteria were refreshed by aerobic cultivation and shaking at 150 rpm, 30 ± 5 ^{o}C for 18-24 hours. Then, the bacterial suspensions were adjusted to a 0.5 at OD_{600}. To determine metal tolerance, 5 µl of bacterial suspension was dropped onto a TSA plate containing Zn concentrations (25, 20, 100 and 200 mg/l) and Cd concentrations (5, 10, 20 and 50 mg/l), and 20, 40 and 50 mg/l Cd plus 100, 150 and 200 mg/l Zn. The bacterial inoculation was incubated at 30 ± 5 ^{o}C for 24-48 hours. The Zn and Cd stock solutions were prepared from $ZnSO_4.7H_2O$ (Ajax Finechem, Australia) and $3CdSO_4.8H_2O$ (Ajax Finechem, Australia), respectively.

3.2.3.4 Plant growth-promoting properties

Indole-3-acetic acid (IAA) production

The bacteria were cultured in TSB containing 0.2% (w/v) tryptophan as a control (no metals) and Cd plus Zn treatments. After cultivation in the dark in an incubator shaker at 150 rpm at 30 ± 5 ^{o}C for 48 hours, the bacterial cultures were centrifuged and the supernatants were mixed with the Salkowski's reagent at a ratio of 2:1. The reaction mixtures were incubated for 20 min in the dark and at room temperature, and then the light absorbance was measured immediately at 530 nm (Glickmann & Dessaux, 1995). Concentration of IAA in supernatants was calculated from a standard curve prepared from the IAA analytical standard (Sigma-Aldrich, St. Louis, MO).

Nitrogen fixation

Each bacterial isolate was cultured in the TSB prior to collecting the cells through centrifugation. They were washed and re-suspended with 0.85% (w/v) NaCl and the bacterial suspensions were adjusted to a 0.5 at OD_{600}. Then 5 µl of each bacterial suspension was dropped onto a nitrogen-free medium plate containing 0.0025 % (w/v) bromothymol blue (Watanabe et al., 1979). A N-free medium was a control, and the medium supplied with Cd plus Zn was the treatment. After 1 week of incubation at 30 ± 5 ^{o}C, the appearance of a blue-green color change from a green color around the colony indicated that the isolate had nitrogen-fixing activity.

Phosphate solubilization

A 5 µl sample of each bacterial suspension (OD_{600} of 0.5) was dropped onto the National Botanical Research Institute's phosphate growth (NBRIP) agar containing of 0.5% (w/v) $Ca_3(PO_4)_2$ (Nautiyal, 1999). The NBRIP agar was the control, and the agar contaminated with Cd plus Zn was the treatment. After incubation for 7 days at 30 ± 5 ^{o}C, the clear zone around the growing area was calculated for a solubilization index (SI), which was the diameter of the clear zone (mm) divided by the diameter of the colony (mm).

Siderophore production

The bacterial isolates were streaked on NA plates supplied with Zn and Cd and incubated for 24 hours at 30 ± 5 ^{o}C. Siderophore detection was detected by an overlaid Chrome azurol S (O-CAS) assay (Pérez-Miranda et al., 2007). The medium for a liter of an overlay was as follows: Chrome azurol S (CAS) 60.5 mg, hexaecyltrimetyl ammonium bromide (HDTMA) 72.9 mg, Piperazine-1,4-bis (2-ethane sulfonic acid) (PIPES) 30.24 g, and 1 mM $FeCl_3 \cdot 6H_2O$ in 10 mM HCl 10 ml. Agarose (0.9% w/v) served as a gelling agent. Establishing a clear yellow zone around the bacterial colony was considered to be positive for siderophore production.

3.2.4 Effects of endophytic bacterial inoculation on phenolic compounds, plant growth, and Zn/Cd accumulation

3.2.4.1 Determination of endophytes colonization

Method (1): electroporation of a selected bacterial isolate was based on the protocol for *E. coli* (Sambrook & and Russel, 2001). One milliliter of overnight cultures of bacterial was inoculated into a 250 ml Erlenmeyer flask containing 50 ml of pre-warmed LB (Luria-Bertani) medium (30 °C for 10 min) and cultivated in a shaking incubator at 30 °C. To obtain the optimal electrocompetent cells, 5 ml of the cell cultures was successively harvested at different OD_{600} of the early exponential phase at 30 °C until 0.5 at OD_{600}. The flask was placed on ice for 15 min and then poured 25 ml of culture into 2 chilled 50 ml Corning tubes. The bacterial culture was centrifuged at 2,500 rpm and 4 °C for 15 min. The supernatant was discarded and the cell pellets were re-suspended in 25 ml of ice-cold 10% glycerol. The cell suspension was centrifuged at 4000 rpm and 4 °C for 5 min. The washing step was repeated twice re-suspending in 10 ml and lastly 5 ml of ice-cold 10% glycerol. After the final washing step, cell pellets were re-suspended in 100 µl of ice-cold 10% glycerol, and then aliquot 50 µl of electrocompetent cells were transferred into chilled 1.5-ml microcentrifuge tubes and store at −80 °C before using in the next step. An aliquot of 50 µl of electrocompetent cells was mixed thoroughly with 6 µl of *E. coli* JM105 AKN100 containing gfp pBK-mini-Tn7-Ω-Gm, Cm (79.3 ng/µl) (plasmids from UWE) according to a modified method of Ye et al. (2014). The bacteria mixture was placed on ice for 5 min. Then all the mixture was transferred to a chilled 0.2-cm cuvette and electroporated by a single pulse with the Gene Pulser (Bio-Rad) setting at 1.8 kV, 25 µF, 200 Ω, and 4.6 Ms. Immediately after the pulse, the sample was suspended in 0.95 ml of LB medium and incubated at 30 °C for 1 hour. The bacteria suspensions were serially diluted with sterile ¼ Ringer solution (g/L: 2.25 NaCl, 0.105 KCl, 0.12 $CaCl_2$, 0.05 $NaHCO_3$, pH 7.4). The cells were plated on TSA agar containing 34 µg/ml of chloramphenicol and incubated at 30 °C for 30 hours before counting the colonies. The transformed cells were confirmed expressing green fluorescent protein (GFP) by using a fluorescence microscope (Leica TCS4D Microsystems Wetzlar

GmbH, Germany) and confocal laser scanning microscope (Leica Microsystems Wetzlar GmbH, Germany).

Method (2): Colonization of endophytic bacteria was isolated from the inoculated plants. The plantlets after cultured for 4 weeks were cut and immersed in endophytic bacterial suspension at OD_{600} of 0.1 and ¼ Ringer solution as a control for 1 hour. The plantlets were placed in a sterile petri dish filled with sterilized soft paper. Then, the plants were cultured in 20 ml of half-strength Murashige and Skoog (½ MS) nutrient agar. Roots, stems, and leaves were separated and sterilized surface. Then, each part of the plant was grounded by a sterilized mortar and pestle. Serial dilutions of the plant tissue solution were spread on TSA agar supplemented with Zn 200 mg/l and Cd 50 mg/l base on the metal tolerance of the isolates. The bacteria were incubated for 3-5 days at 30 °C. The metal-resistant properties of bacterial strains were verified by their morphological characteristics.

3.2.4.2 Plant tissue culture

A selected bacterial strain was cultured overnight in LB broth at 30 ºC on a rotary shaker. A bacterial pellet was collected by centrifugation at 13,000 rpm for 1 min and washed twice with a ¼ Ringer solution (Merck Millipore, Germany). The ¼ Ringers solution is a solution that maintains the osmotic balance of bacteria (g/L: 0.12 $CaCl_2$, 0.105 KCl, 0.05 $NaHCO_3$, 2.25 NaCl). The pellet was re-suspended in ¼ Ringer solution to obtain a final inoculum density of 10^8 CFU/ml. The 2 cm plant shoots (explants) from four weeks-old tissue plants were cut. Shoots were soaked in bacterial suspension or ¼ Ringer solution (control) for 1 hour before being placed in a sterile petri dish filled with sterilized soft paper. Then, the plant shoots were cultured in 20 ml of ½ MS nutrient agar. The plants were grown in a light plant growth chamber at 25 °C with 16 hours of light per day and 80% humidity (Fitotron SGC 120). Four-weeks old plants were separately treated with (1) *Curtobacterium* sp. GLD03 with metals, (2) *Stenotrophomonas* sp. GSD10 with metals, (3) *Chryseobacterium* sp. GTID13 with metals, (4) ¼ Ringer solution with metals, (5) Control (¼ Ringer solution). Each treatment was performed in five replicates. The dual-metal treatment was 100 mg/l of Zn plus 15 mg/l of Cd for two weeks, according to the research of Mongkhonsin et al. (2016). The plants were carefully removed from the plant tissue culture bottle and

cleaned several times with sterilized deionized water. Root and shoot length, fresh weight, and dry weight (oven at 80 ºC for 24 h) were measured for the growth of plants. The phytotoxicity of the metals was associated with the appearance of any adverse effects on the leaves. The percentage of phytotoxicity was derived from the number of replicates of plants with chlorosis or necrosis spots on the leaves. The colonization of *Curtobacterium* sp. GLD03, *Stenotrophomonas* sp. GSD10 and *Chryseobacterium* sp. GTID13 in plant tissues (roots, stems, and leaves) was followed by the method (2) of the determination of endophytes colonization.

3.2.4.3 Pot experiment

The M2-professional growing medium (levintonR pot and bedding compost 75 lines every) was used as soil in pot experiment. The soil was air-dried and sieved to be smaller than 4 mm. The soil artificially contaminated with the solutions of $ZnSO_4.7H_2O$ and $3CdSO_4.8H_2O$ to obtain the concentration of Zn 1,000 mg/kg soil and Cd 50 mg/kg soil. The extractable Zn and Cd concentration was carried out by shaking the soil with DTPA. Before use, the soil was left in the greenhouse for 7 days to stabilize the metals before being autoclaved at 121 °C, 15 min over three consecutive days. Bacterial culture was cultivated overnight in LB broth at 30 °C. The cell pellet was collected by centrifugation at 13,000 rpm for 1 min and washed twice with the ¼ Ringer solution. Then, the pellet was re-suspended in ¼ Ringer solution and the OD_{600} was adjusted to 0.1 (10^8 CFU/ml).

The 5-6 g of tubers were surface sterilized by sequentially immersing in 70% (v/v) ethanol for 1 min, and 3% NaOCl for 3 min and washed several times with sterilized deionized water. Then, the tubers were separately soaked for 2 hours in bacterial suspension and ¼ Ringer solution (control). The treated tubers were transplanted into plastic pots containing 250 g of soil contaminated metals. The treatments were (1) *Curtobacterium* sp. GLD03, (2) *Stenotrophomonas* sp. GSD10, (3) *Chryseobacterium* sp. GTID13, (4) Control (uninoculated), (5) Control (no metals and uninoculated). The tubers were grown in a greenhouse at 25 ºC and a 16:8 h day/night regime. Plants were watered by sterilized deionized water for three days per week. Each treatment was performed in six replicates. After 8 weeks, the whole plants were carefully removed from the pots and the soil was removed from the roots. The roots

were immersed in 10 mM EDTA for 30 min and then rinsed thoroughly with deionized water to remove surface adsorbed metal. The leaves were cut from the plant to determine chlorophyll. The percentage of phytotoxicity was calculated from the number of plant replica showing chlorosis or necrosis spots on the leaves. Accumulation of Zn and Cd in roots and shoots, Zn/Cd stress, and phenolic compounds in leaves were investigated.

3.2.4.4 Extraction of phenolic compounds by percolation method

The leaf samples from the tissue culture and the pot experiment were harvested and washed with deionized water. The leaves were separated from the clean plants, then freeze-dried and ground into a powder. A powder sample was packed into a column, which was a 1-ml syringe. The conditions of extraction following the method of Mongkhonsin et al. (2016) and Liu (2010) were 1:10 (sample: solvent). The effluent of 10 ml loading was collected as a fraction. 95% (v/v) Hexane, 99.5% (v/v) ethanol, 99.9% (v/v) methanol, and 50% (v/v) methanol were used as solvent extraction in order of low to high polarity. Flow rate of the solvents was controlled at 0.1-0.2 ml/min by vacuum manifold. Every fraction was filtered through Whatman no. 4 and adjusted the volume to 10 ml. The extract was kept in an amber glass bottle with a tight stopper at −20 °C until further analysis. The extraction material samples were assayed for phytochemical screening**,** TPC, TFC, FRSA and HPLC.

3.2.4.5 Phytochemical screening

Leaf extracts from ethanol, methanol, and 50% (v/v) methanol fractions were determined by preliminary phytochemical testing using the modified methods of Tripathi & Mishra (2015) and Sadat et al. (2017).

Carbohydrates (Molish's test): A 100 µl of the extract was added 1 drop of Molish's reagent (5% (v/v) α-napthol in absolute alcohol). Two drops of concentrated H_2SO_4 were then added into the tube. The formation of the violet ring in the interface indicated the presence of carbohydrates.

Alkaloids: Two drops of 1.5% (v/v) HCl were added in 100 µl of extract. Then, two drops of Wagner's reagent (dissolve 2g of iodine and 6g of KI in 100

ml of water) were added. The appearance of yellow/ brown precipitation was indicative of alkaloids.

Proteins: For the Biuret assay, two drops of 1% (w/v) NaOH were added to 100 µl of extract, followed by two drops of 1% (w/v) $CuSO_4$. Blue/ purple or violet/ pinkish color indicated the presence of proteins.

Resins: A 100 µl of the extract was dissolved in acetone, and then 100 µl of distilled water was added. Turbidity was a sign of rasins.

Tannins: A 100 µl of 5% (w/v) $FeCl_3$ was added to 100 µl of extract. The dark blue or greenish black color was a positive tannin assay.

Steroids: A 100 µl of chloroform was added to 100 µl of extract. Two drops of concentrated H_2SO_4 were then added. The formation of Bluish red to cherry color in the chloroform layer showed the presence of steroids.

Saponins: A 100 µl of extract was mixed with 500 µl of water before shaking, and observed for foam as saponins.

Anthocyanin and betacynin: A 100 µl of the extract was treated with 100 µl of 2 N NaOH. The bluish green formation indicated the presence of anthocynin, while the yellow color showed the presence of betacynin.

Starch: A 100 µl of iodine solution was mixed with 100 µl of extract. The formation of a blue color indicated starch in the extract.

Glycosides: A 100 µl of 5% (v/v) $FeCl_3$ and two drops of acetic acid was mixed with 100 µl of extract. Then, one drop of concentrated H_2SO_4 was carefully added to the mixture. A greenish blue color on the interface indicated the presence of glycosides.

Terpinoids: A 200 µl of chloroform was added to 100 µl of extract. Then, 400 µl of concentrated H_2SO_4 was carefully added to form a layer. The appearance of terpinoids was indicated by the reddish-brown color of the interface.

Anthraquinone glycosides: A 100 µl of the extract was added to 50 µl of 10% (v/v) NH_3 solution and stirred. A combination of light rose color with more green color indicates a positive result of anthraquinone glycosides.

Reducing sugars (Fehling's test): The 100 µl solutions of Fehling A and Fehling B were added to 200 µl of extract. The mixture was shaken and

incubated at 100 ºC for 10-15 minutes. A rust brown or brick red-colored precipitate confirmed that reducing sugars were present in the sample.

3.2.4.6 Phenolic compounds determination

The leaf extracts obtained from ethanol, methanol, and 50% (v/v) methanol fractions were compared in terms of TPC, TFC, FRSA and HPLC chromatogram.

Total phenolic content (TPC): The TPC was measured using the modified Folin Ciocalteu method (Cicco et al., 2009). Briefly, a test used a 100 µl of samples or a standard solution of Gallic acid (GA). A 500 µl of 10% (v/v) Folin–Ciocalteu phenol reagent, 100 µl of 7.5% (w/v) Na_2CO_3 and 300 µl of distilled water were added to the sample and mixed thoroughly. Then, the mixture was incubated in the dark for 90 min. The total phenolic contents were measured at 731 nm using a UV-Vis spectrophotometer. The standard curve was prepared with 0, 5, 10, 20, 40, 60, 80 and 100 mg/l of GA solutions. The TPC value was expressed in terms of a GA equivalents (mg GA/g dry weight plant).

Total flavonoids content (TFC): The TFC was measured using a modified colorimetric method (Marinova et al., 2005; Yoo et al., 2008; Khamis Al-Jadidi & Hossain, 2015). A 500 µl of deionized water and 100 µl of sample or standard solution of epicatechin (EC) were added to a 1.5-ml Eppendorf. Then, 30 µl of 5% (w/v) $NaNO_2$ was added and mixed. The mixture was kept in the dark for 5 min before addition of 60 µl of 10% (w/v) $AlCl_3$. After mixing and incubation for 6 min, 200 µl of 1 M NaOH and 110 µl of deionized water were added to the mixture and mixed. After incubation for 5 min in the dark, the absorbance was measured at 510 nm. The measurement was compared to a standard curve prepared with 5, 10, 20, 40, 80, 100 and 200 mg/l of EC solutions. The TFC values were expressed in terms of an EC equivalent (µmol ECE/ g dry weight).

Free radical scavenging activity (FRSA): Antioxidant activity was measured based on the 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) free radical method (Cotelle et al., 1996). Briefly, 20 µl of different concentrations of each sample and ascorbic acid were mixed with 180 µl of 0.08 mM DPPH solution on 96 wells of the microplate. A microplate was mixed, covered and incubated in the dark at room

temperature for 30 min, and then the absorbance was measured at 515 nm using a microplate reader. The DPPH assay method was reported as free radical scavenging activity (FRSA) using the following equation:

$$FRSA\ (\%) = ((A_0 - A_1) / A_0) * 100$$

Where A_0 was the absorbance of the control, and A_1 was the absorbance of the test sample. The DPPH solution with methanol was used as the control. IC_{50} value corresponded to the concentration of the sample needed to inhibit 50% of the DPPH radical. An IC_{50} of the sample was calculated from the inhibition curve and compared with the IC_{50} of ascorbic acid (Musa et al., 2016).

High-performance liquid chromatography (HPLC): Phenolic compounds in the extracts were investigated by HPLC (Shimadzu SIL-10AD, Japan) with a C-18 guard column (4.6 mm x 10 mm x 5 µm) (VetiSepTM UPS C-18, Thailand) and a C-18 reversed-phase column (4.6 mm x 25 mm x 5 µm) (GL Science Lab InsertSustain C-18, Japan). The mobile phase consisted of a gradient elution between 3% (v/v) acetic acid in water (solvent A) and 99.9% (v/v) methanol (solvent B) (Zuo et al., 2002; Sukadeetad et al., 2018), with a flow rate of 1 ml/min and a column temperature of 40 °C. The gradient profile was shown in Table 3. Each extract was filtered through a 0.22-µm nylon filter (Whatman, GE Healthcare, UK), before injecting 20 µl of the sample. The HPLC chromatogram was detected by UV-diode array detector (SPD-M20A, Shimadzu, Japan) at 280 nm for both phenolic acids and flavonoids. The identification of each compound was based on a combination of retention time between sample solution and standard solution with the reference standards; gallic acid (GA), chlorogenic acid (CGA), caffeic acid (CA), catechin (CAT), vanillin (VAN), *p*- cumaric acid (PCA), epicatechin (EC), and rutin (RUT).

Table 3 The gradient profile condition of mobile phase for HPLC analysis

Time (min)	Solvent A	Solvent B
0	100	0
5	90	10
10	80	20
15	70	30
20	60	40
35-40	0	100

3.2.5 Data analysis

The data were analyzed by a one-way analysis of variance (ANOVA) in a randomized complete block design (RCBD). The variance and separation of means were performed using Duncan's new multiple range test (DMRT) at $p < 0.01$. The statistical analysis was performed with SPSS statistical software version 13.0 (SPSS Inc., IL, USA).

CHAPTER 4
RESULTES

4.1 Isolation and characterization of endophytic bacteria from *G. pseudochina*

4.1.1 Plant collection

G. pseudochina plants were collected from the conserved forest area of the Padaeng zinc mine during the rainy season of August 2019. Figure 8 shows the environment and samples of plants growing at the site before sample collection. The temperature, pH and moisture content of soil at the sampling time were 25.5±0.3 ºC, 7.07±0.06 and 30.0±8.1%, respectively. The concentrations of DTPA extractable Zn and Cd from bulk soil were 1,254.97±120.70 and 49.23±26.77 mg/kg dry wt., respectively. Table 4 shows Zn and Cd contents accumulating in the plant organs. The highest Cd and Zn contents were found in the leaves and stem, respectively.

(a)

(b)

Figure 8 The environment of the conserved forest area of the Padaeng zinc mine (a), and (b) *G. pseudochina* plants growing at the site of N 16° 39′ 6.6″, E 98° 39′ 41.2″ before sample collection.

Table 4 Zn and Cd contents and total endophytic bacteria in the plant organs of *G. pseudochina* growing in Zn-/Cd-contaminated soil of the zinc mine.

Plant organ	Metal accumulation (mg/kg dry wt.)		Total endophytic bacteria (CFU/g fresh wt.)	Endophytic bacterial isolate
	Zn	Cd		
Leaves	999.41 ± 100.66	281.96 ± 21.91	$2.9x10^3$	20
Stem	4,665.25 ± 47.83	90.81 ± 4.41	$5.9x10^3$	27
Tuber	520.66 ± 49.93	36.98 ± 5.29	$6.3x10^4$	26

4.1.2 Isolation and screening for Zn and Cd tolerant endophytic bacteria

The total culturable endophytic-bacterial counts in the tuber were greater than in the stems and leaves (Table 4). Endophytic bacteria were isolated from surface-sterile healthy tissues (leaf, stem, and tuber) of *G. pseudochina* by both direct and indirect methods (Appendixes A1-A3). The endophytic bacteria were primary isolated by bacterial colony morphology and Gram's stain (Appendixes A4-A6). The numbers of bacteria isolated from the leaf, stem and tuber tissues were 20, 27 and 26, respectively (Table 4). All 73 isolates were separately streaked on TSA plate containing different concentrations of Zn (25, 20, 100 and 200 mg/l) and Cd (5, 10, 20 and 50 mg/l). The bacterial growth on the TSA plates with and without Zn or Cd was rated as shown in Appendixes A7. There were 17 isolates tolerating both high concentrations of Zn (200 mg/l) and Cd (50 mg/l) (Appendixes A8-A10). The 4 isolates from the leaves were GLD02, GLD03, GLD06 and GLID08. The 8 isolates from the stem were GSD01, GSD02, GSD04, GSD06, GSD07, GSD10, GSID03 and GSID04. The 5 isolates from tubers were GTID01, GTID06, GTID13, GTID18 and GTID20.

The bacterial suspension of the 17 isolates were separately dropped on the TSA plates containing Zn (100, 150 and 200 mg/l) plus Cd (20, 40 and 50 mg/l). The colony diameter of each isolate was evaluated to assess the bacteria's tolerance to the metals. Examples of the colonial growth on the Zn plus Cd contaminated media are presented in Appendix A11. The 7 isolates tolerating to every Zn plus Cd concentrations and received the high growth symbol were GLD03, GSD06, GSD10, GTID01, GTID06, GTID13 and GTID20 (Table 5).

Table 5 The growth of 17 endophytic bacterial isolates on the TSA plates containing various concentrations of Zn plus Cd.

Isolates	TSA	Concentrations of Zn plus Cd in TSA medium[a] (Zn + Cd, mg/l)								
	(control)	100+20	100+40	100+50	150+20	150+40	150+50	200+20	200+40	200+50
GLD02	+++	++	-	-	-	-	-	-	-	-
GLD03	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
GLD06	+++	++	++	++	++	++	++	++	++	++
GLID08	+++	++	++	++	++	++	++	++	++	++
GSD01	+++	++	++	++	++	++	++	++	++	++
GSD02	+++	++	++	++	++	++	++	++	++	++
GSD04	+++	++	++	++	++	++	++	++	++	++
GSD06	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
GSD07	+++	++	++	++	++	++	++	++	++	++
GSD10	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
GSID03	+++	+	+	+	+	+	+	+	+	+
GSID04	+++	+/w	+/w	+/w	+/w	+/w	+/w	+/w	+/w	+/w
GTID01	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
GTID06	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
GTID13	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
GTID18	+++	++	++	++	++	++	++	++	++	++
GTID20	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

[a]The symbol of growth: +++ high growth, ++ moderate growth, + low growth, +/w growth/week, - no growth.

The partial 16s rRNA gene sequences of the 17 isolates were analysed for bacterial identification. More than 98.8% or higher 16S sequence similarity and well separated intra- and interspecific genetic distances between the reference strain were the criterion for identifying the strain at the species level. The nucleotide sequence data were deposited in the NCBI database under accession numbers MZ314060 to MZ314071 and MZ314073 to MZ314077. They were phylogenetically related to 13 genera of *Acinetobacter*, *Curtobacterium*, *Yokenella*, *Cupriavidus*, *Cellulomonas*, *Rhizobium*, *Enterobacter*, *Stenotrophomonas*, *Methylobacterium*, *Beijerinckia*, *Chryseobacterium*, *Klenkia* and *Marmoricola* (Figure 9).

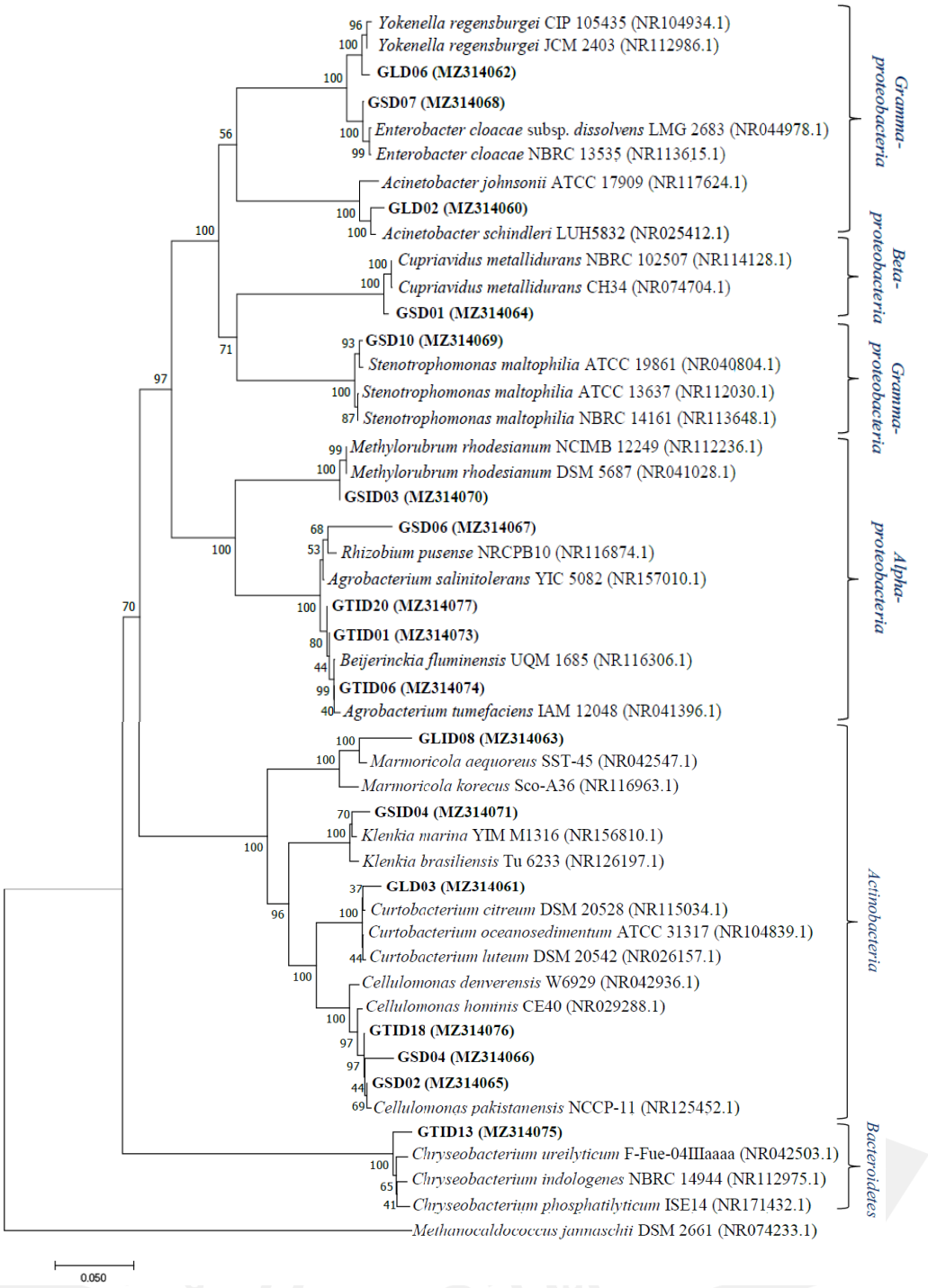


Figure 9 Phylogenetic analysis of 16S rDNA sequencing of the 17 endophytic bacteria isolated from *G. pseudochina* and sequences from the NCBI databases using the neighbour-joining statistical method with 1,000 bootstrap replicates.

There was a total of 1,633 positions in the final dataset. Evolutionary analyses were conducted with MEGA 7. Bootstrap values are indicated at the node. The bar indicates 0.05 substitutions per nucleotide position.

4.2 Plant growth promoting properties of the Zn and Cd tolerant endophytic bacteria

4.2.1 Plant growth promoting properties

The 17 bacterial strains were studied for their plant growth-promoting properties of IAA production, N_2-fixation, siderophore secretion and phosphate solubilization. Table 6 shows that all strains were able to produce IAA. In which, *Rhizobium sp*. GSD06, *B. fluminensis* GTID01, *B. fluminensis* GTID06 and *B. fluminensis* GTID20 produced high IAA concentrations at 100, 70, 50 and 50 mg/l, respectively. There were 12 strains had N_2-fixation properties, and 10 strains could secrete siderophore. In addition, *Y. regensburgei* GLD06, *Rhizobium* sp. GSD06, and the 3 strains of *B. fluminensis* GTID01, GTID06 and GTID20 were able to solubilize phosphate. From both the high Zn/Cd tolerant properties (Table 5) and the plant growth promoting properties (Table 6), the 9 endophytic bacteria were selected for further study plant growth-promoting properties under the Zn plus Cd stress.

Table 6 Characteristics of plant growth promoting properties of 17 endophytic strains isolated from *G. pseudochina*.

Bacterial identification	Similarity (%)	Strain	Plant growth promoting property			
			IAA production[a] (mg/l) at 48 hours	N_2-fixation[b]	Phosphate solubilization[b]	Siderophore production[b]
Acinetobacter sp.	97.57	GLD02	25.43±0.29	+	-	-
Curtobacterium sp.	98.04	GLD03	26.09±2.08	+, slowly weak	-	-
Yokenella regensburgei	98.81	GLD06	48.13±12.76	+	+	-
Marmoricola sp.	96.17	GLID08	0.86±0.43	-	-	-
Cupriavidus sp.	98.66	GSD01	8.82±0.58	+	-	-
Cellulomonas pakistanensis	99.43	GSD02	3.82±0.27	-	-	+
Cellulomonas sp.	97.73	GSD04	4.00±0.19	-	-	+
Rhizobium sp.	95.52	GSD06	103.12±1.54	+	-	-
Enterobacter sp.	98.23	GSD07	32.99±1.95	+	+	-
Stenotrophomonas maltophilia	98.89	GSD10	10.16±0.43	+, slowly weak	-	-
Methylorubrum rhodesianum	99.42	GSID03	9.49±0.68	+	-	-
Klenkia sp.	98.64	GSID04	0.97±0.622	+, slowly weak	-	-
Beijerinckia fluminensis	99.42	GTID01	71.24±9.85	+	+, slowly weak	+
Beijerinckia fluminensis	99.13	GTID06	53.47±17.74	+	+, slowly weak	+
Chryseobacterium sp.	98.01	GTID13	10.89±0.56	-	-/-	+
Cellulomonas pakistanensis	99.71	GTID18	2.79±0.28	-	-/-	+
Beijerinckia fluminensis	99.63	GTID20	54.57±11.39	+	+, slowly weak	+

Note: [a] IAA is expressed as mean±SD (n=3), [b] Nitrogen fixation, phosphate solubilization and siderophore production: -, not able to produce; +, able to produce; +, slowly weak, able to produce at 48-72 hours.

4.2.2 Plant growth promoting properties under Zn plus Cd stress

The 9 select Zn/Cd tolerant endophytic bacteria were studied for their plant growth-promoting properties of IAA production, N_2-fixation, siderophore secretion and phosphate solubilization under Zn plus Cd treatment. Examples of tested agar plates are shown in Appendixes A12-A14. Table 7 shows that *Enterobacter* sp. GSD07 and *S. maltophilia* GSD10 increased the IAA production under the Zn plus Cd stress. While, the production of IAA by the other 7 strains decreased under metal stress by comparing without metal contamination (control). Almost the select strains had the N_2-fixation property under metal and metal-free stresses, except *Curtobacterium* sp. GLD03. The phosphate solubilization property under metallic stresses was retained in *Y. regensburgei* GLD06 and *Enterobacter* sp. GSD07. The property of siderophore production by *S. maltophilia* GSD10 and *Y. regensburgei* GLD06 were more pronounced under metal contamination.

4.2.3 Determination of pathogenicity test on *G. pseudochina*

The select endophytic bacteria were studied for pathogenicity on *G. pseudochina*, which is their host plant, under both moisture box for leaves and tissue culture systems for stem part. *B. fluminensis* strains of GTID01, GTID06 and GTID20 caused crown-gall disease in both leaves and stem (Table 7). The occurring of crown-gall disease was shown in Appendix 15A-16A. In addition, the possibility of pathogenic bacteria in plants and humans was investigated. Consequently, endophytic bacteria of *Curtobacterium* sp. GLD03, *Stenotrophomonas* sp. GSD10, and *Chryseobacterium* sp. GTID13, which still have no reported of plant and human pathogen, were selected for study plant colonization and their effects on phytochemicals and Zn/Cd phytoaccumulation.

Table 7 Plant growth promoting properties of Zn/Cd endophytic bacteria under control (metal-free) and Zn plus Cd contamination (200+50 mg/l) and pathogenicity test on *G. pseudochina* plant.

Bacterial identification	Strain	Plant growth promoting properties [b]								Pathogenicity
		IAA production (mg/l) [a]		N_2-fixation		P-solubilization		Siderophore production		
		Control	Zn+Cd	Control	Zn+Cd	Control	Zn+Cd	Control	Zn+Cd	
Curtobacterium sp.	GLD03	17.52±0.69	7.58±1.19	+, slowly weak*	-	-	-	-	-	No symptoms found
Y. regensburgei	GLD06	19.89±2.56	15.93±2.13	+++	+	++	++	+	+++	No symptoms found
Rhizobium sp.	GSD06	84.40±5.45	17.4±1.32	+++	++	-	-	+	-	No symptoms found
Enterobacter sp.	GSD07	34.61±2.54	56.71±2.00	+++	++	++	++	+	-	No symptoms found
S. maltophilia	GSD10	13.03±0.04	21.16±2.38	++, slowly weak*	+, slowly weak	-	-	-	++	No symptoms found
B. fluminensis	GTID01	139.78±12.32	87.29±9.08	+++	++	+, slowly weak*	-	+	+	Crown gall
B. fluminensis	GTID06	126.37±4.99	65.30±5.68	+++	++	+, slowly weak*	-	+	+	Crown gall
Chryseobacterium sp.	GTID13	12.34±0.15	14.74±0.87	-	-	-	-	++	++	No symptoms found
B. fluminensis	GTID20	129.28±8.44	80.01±14.09	+++	++	+, slowly weak*	-	+	+	Crown gall

Note: [a] IAA is expressed as mean±SD (n=3), [b] Nitrogen fixation, phosphate solubilization and siderophore production: -, negative; +, positive/weak; ++, intermediate; +++, strong and slowly weak, able to produce at 48-72 hours.

4.3 Effects of endophytic bacterial inoculation on *G. pseudomonas* under Zn/Cd stress

The effects of endophytic bacterial inoculation of *Curtobacterium* sp. GLD03, *Stenotrophomonas* sp. GSD10 and *Chryseobacterium* sp. GTID13 on *G. pseudomonas* under Zn (100 mg/l) plus Cd (15 mg/l) stress, which was the Zn plus Cd threshold concentration of the plant, were studied in both plant tissue culture and pot systems. Plant growth, phytotoxicity, bacterial colonization, accumulation of Zn and Cd, and transformation of phenolic compounds were monitored.

4.3.1 Plant tissue culture system

4.3.1.1 Plant growth under the Zn plus Cd stress

The appearance of plants, both controls and treatments growing in the tissue culture system are shown in Figure 10. The wet weight and dry weight of leaves, stems and roots indicated that *G. pseudomonas* plants separately inoculated with *Curtobacterium* sp. GLD03, *Stenotrophomonas* sp. GSD10 and *Chryseobacterium* sp. GTID13 and treated with Zn plus Cd did not affect plant growth differently from the control (Figure 11).

Figure 10 The appearance of *G. pseudochina* plants growing in ½ MS, (a) control (without bacterial inoculation and metal treatment, (b) Zn 100 mg/l plus Cd 15 mg/l treatment, (c) *Curtobacterium* sp. GLD03 and Zn plus Cd treatment (d) *Stenotrophomonas* sp. GSD10 and Zn plus Cd treatment, and (e) *Chryseobacterium* sp. GTID13 and Zn plus Cd treatment.

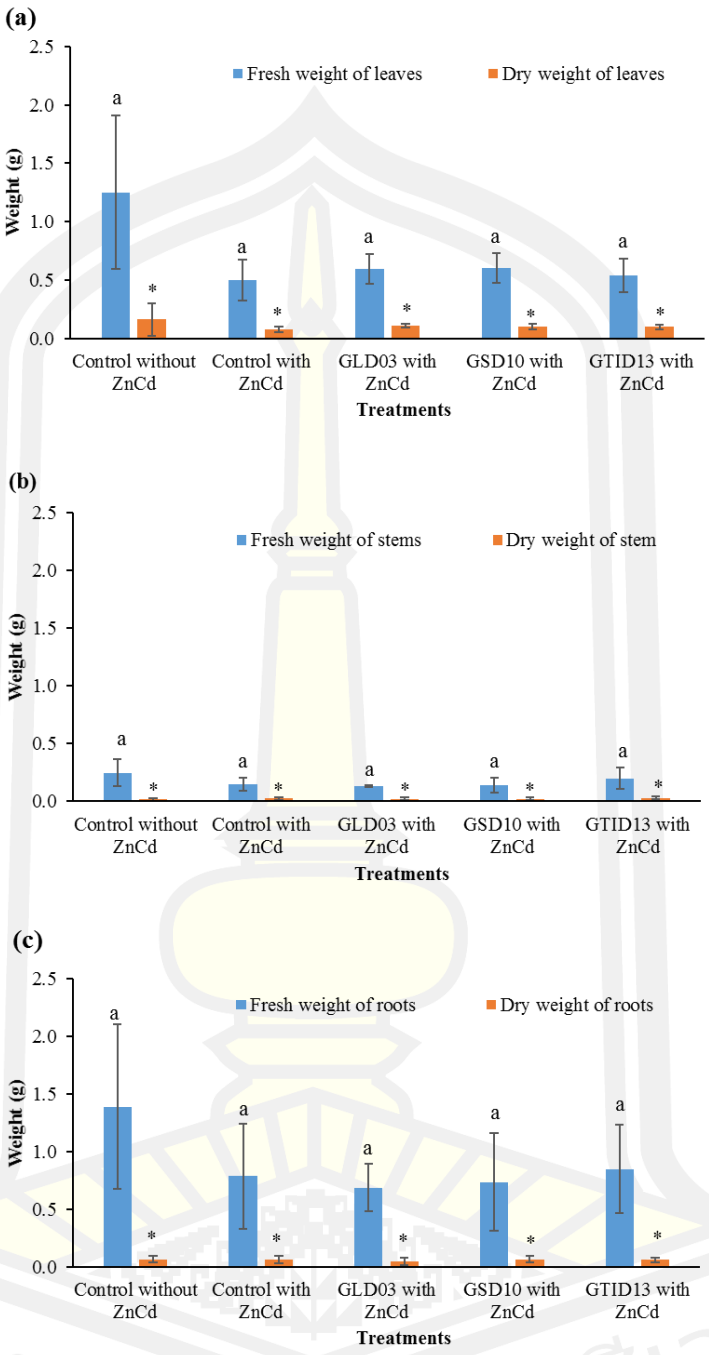


Figure 11 Fresh and dry weight of *G. pseudochina* plants; (a) leaves, (b) stems and (c) roots after inoculated with *Curtobacterium* sp. GLD03, *Stenotrophomonas* sp. GSD10 and *Chryseobacterium* sp. GTID13 and treated with Zn 100 mg/l plus Cd 15 mg/l in tissue culture system. Results shown with different letters and symbols on error bar are significantly different ($p < 0.01$, Duncan's new multiple range test). Data are given as means ± SD ($n = 3$).

4.3.1.2 Percentage of phytotoxicity in *G. pseudochina*

Although Figure 10 did not clearly show the difference, the number of chlorotic and necrotic leaves were investigated to evaluate possible phytotoxicity from the bacterial inoculation and/or the metallic stresses. Figure 12 indicated that the percentages of chlorotic and necrotic leaves were found to increase in the plants treated with Zn plus Cd combined with *Curtobacterium* sp. GLD03. The inoculation with *S. maltophilia* GSD10, and *Chryseobacterium* sp. GTID13 seem to decrease the percentages of chlorosis and necrotic leaves from the metal toxicity.

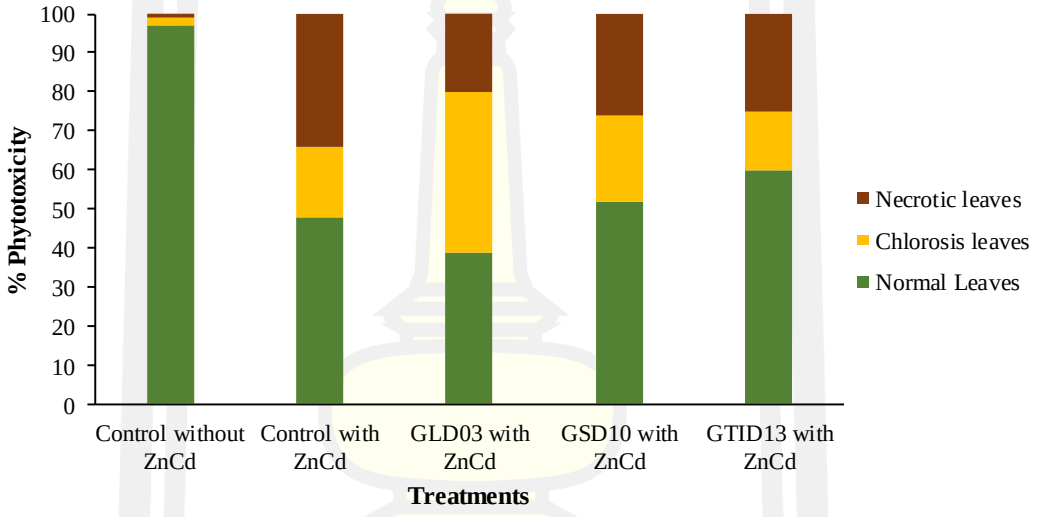


Figure 12 The percentages of chlorotic and necrotic leaves found in *G. pseudochina* plants after treated with Zn 100 mg/l plus Cd 15 mg/l, and inoculated with *Curtobacterium* sp. GLD03, *S. maltophilia* GSD10 and *Chryseobacterium* sp. GTID13 in tissue culture system. Data are given as means (n = 6).

4.3.1.3 Colonization of endophytic bacteria

The colonization and maintenance of the inoculated endophytic bacteria in the plant tissues (leaves, stems, roots) after inoculation for 4 weeks and further treated with Zn plus Cd for 2 weeks were observed. Figures 13-15 clearly show that *Curtobacterium* sp. GLD03, *Stenotrophomonas* sp. GSD10 and *Chryseobacterium* sp. GTID13 could colonize in the internal tissue of plants, especially in the parts of root and stem. In addition, the maintenance of inoculated endophytes in plants was monitored weekly for another 4 weeks and 2 weeks after the Zn plus Cd exposure. The

persistence of the endophytic bacteria along the experiment are shown in Appendixes A17-A19).

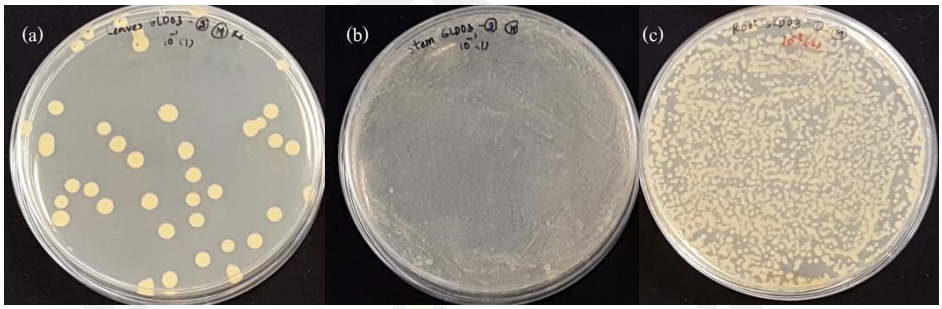


Figure 13 Colonization of *Curtobacterium* sp. GLD03 in (a) leaves, (b) stems and (c) roots of *G. pseudochina* plants after 4 weeks inoculation and further treated with Zn 100 mg/l plus Cd 15 mg/l for 2 weeks.

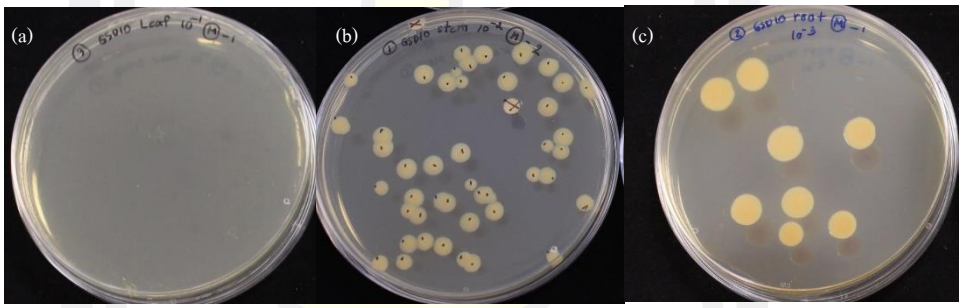


Figure 14 Colonization of *Stenotrophomonas* sp. GSD10 in (a) leaves, (b) stems and (c) roots of *G. pseudochina* plants after 4 weeks inoculation and further treated with Zn 100 mg/l plus Cd 15 mg/l for 2 weeks.

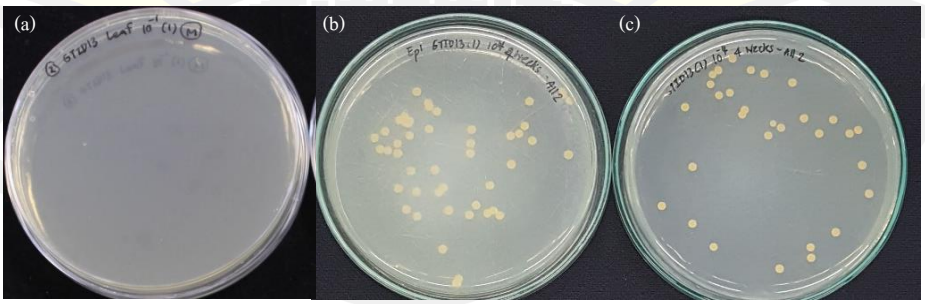


Figure 15 Colonization of *Chryseobacterium* sp. GTID13 in (a) leaves, (b) stems and (c) roots of *G. pseudochina* plants after 4 weeks inoculation and further treated with Zn 100 mg/l plus Cd 15 mg/l for 2 weeks.

Stenotrophomonas sp. GSD10 cells transform with AKN100 plasmid was able to grow on TSA with chloramphenicol. Colony morphology of the transformant GSD10 were not different from the wild types. In addition, the transformed cells showed an expression of green fluorescence protein (GFP) under a fluorescence microscope and confocal microscope (Appendixes A20-A21). 24 hours after the roots were exposed to the transformant GSD10, the root samples were sectioned and examined under a confocal laser microscope. Figure 16 clearly showed that the endophytic bacterium colonized the surface of the primary root in the root tissues. On the contrary, no GFP-labelled *Stenotrophomonas* sp. GSD10 cells were not found in the control plants.

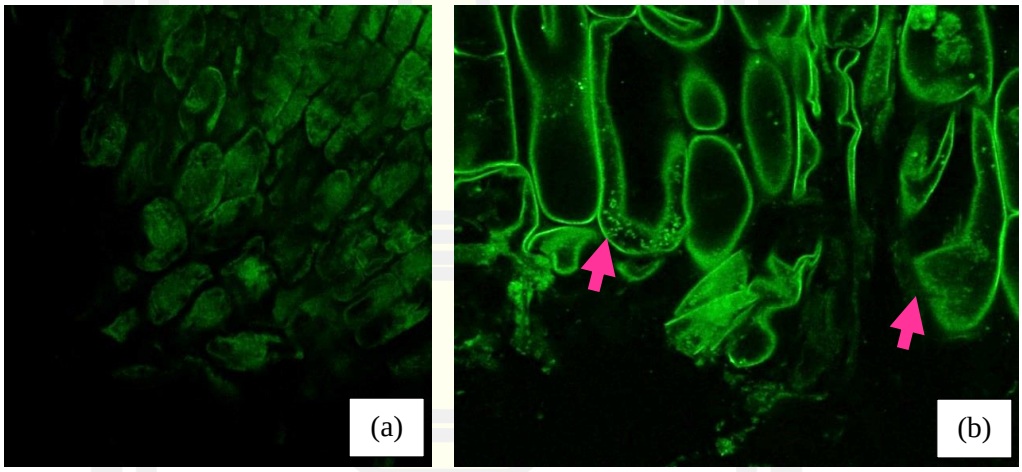


Figure 16 Confocal image of GFP-labelled *Stenotrophomonas* sp. GSD10 cells in root tissue of *G. pseudochina* under a confocal laser scanning microscope (60X) at 524-594 nm. (a) Control, (b) GFP-labelled *Stenotrophomonas* sp. GSD10 cells colonized the surfaces of the primary root (arrows).

4.3.1.4 Zn and Cd accumulation

The effects of Zn plus Cd treatment and the endophytic inoculation on Zn and Cd accumulation in leaves, stems and roots are shown in Figure 17. In the tissue culture system, the amounts of Zn and Cd in the inoculated plants did not differ significantly different from the metal content of the un-inoculated plants (control).

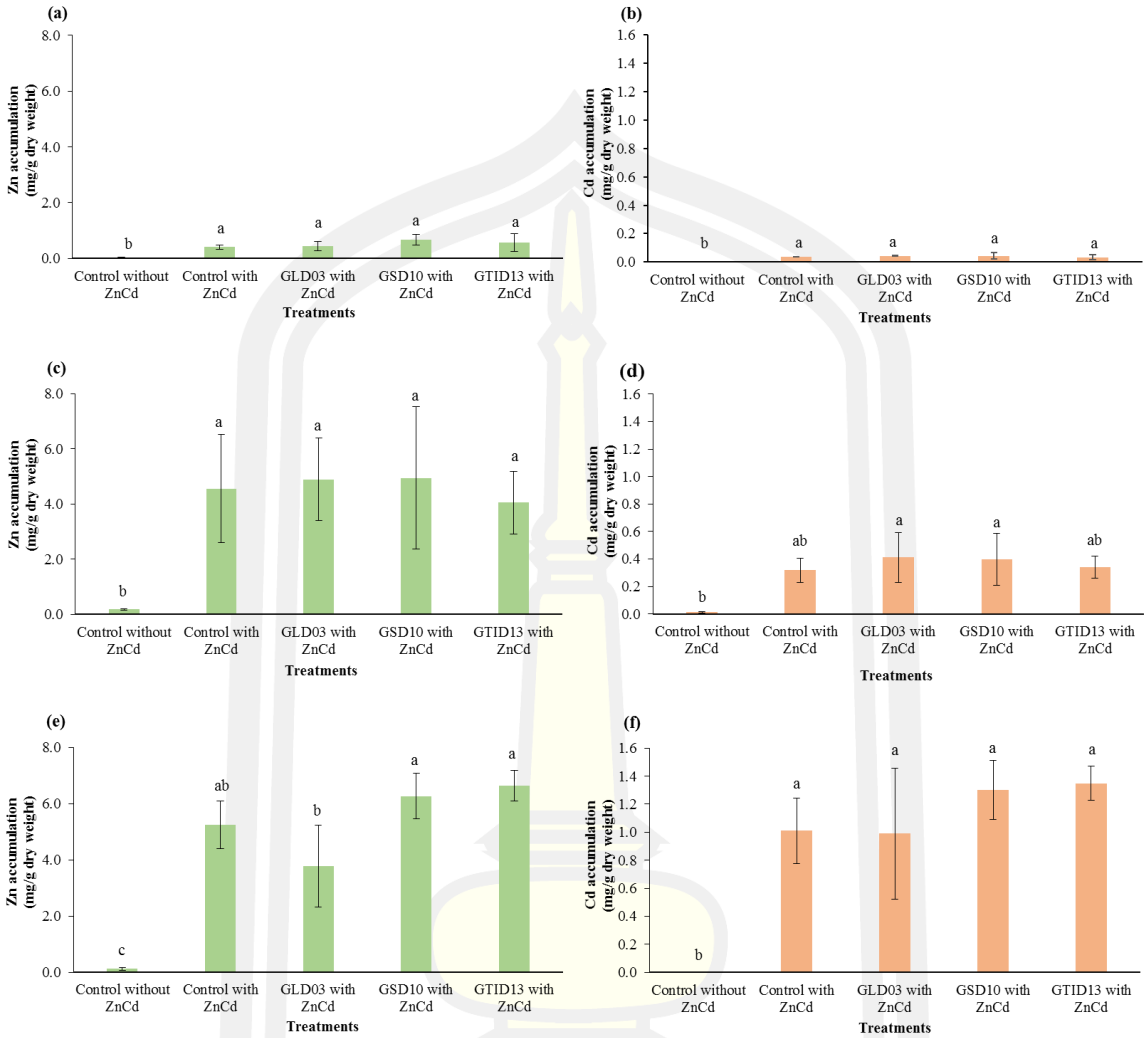


Figure 17 Zn and Cd accumulation in *G. pseudochina* after Zn plus Cd treatment for two weeks in tissue culture system. (a), (c) and (e) are Zn content in leaves, stems and roots, respectively. (b), (d) and (f) are Cd content in leaves, stems and roots.

Results shown with different letters on error bars are significantly different ($p < 0.01$, Duncan's new multiple range test). Data are given as means ± SD ($n = 3$).

4.3.1.5 Effects of endophytic bacterial inoculation to phenolic compounds

(1) Phytochemical screening

The *G. pseudochina* leaf extracts were obtained from the serial extraction from low to high polarity solvents of 99.9% (v/v) ethanol, 99.9% (v/v) methanol and 50% (v/v) methanol, respectively. Phytochemical screening showed that tannins, sponins, betacyanins, terpenoids, glycosides and carbohydrates were mainly presented in the extracts (Table 8). Sponins and terpenoids were found in all solvent extracts of all treatments. Only control (without metal stress) showed negative test of glycosides and carbohydrates in 50% (v/v) methanol fraction. In addition, tannins showed positive test in 99.9% (v/v) ethanol of the metals stress condition, and 99.9% (v/v) ethanol, 99.9% (v/v) methanol in the control. After the preliminary phytochemical screening, the extracts were analyzed for antioxidant activity, TPC, TFC and characterization by HPLC.

Table 8 Phytochemical screening of the 1st fraction of the leaf extracts obtained from Zn plus Cd and inoculation treated *G. pseudochina* plants.

Test	Control			Control with Zn+Cd			GLD03 with Zn+Cd			GSD10 with Zn+Cd			GTID13 with Zn+Cd		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Alkaloids	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Tannins	+	+	–	+	–	–	+	–	–	+	–	–	+	–	–
Sponins	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Anthocyanins	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
Betacyanins	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+
Terpenoids	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Starch	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Glycosides	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+
Steroids	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Carbohydrates	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+
Reducing sugars	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Anthoqinones	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Resins	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Proteins	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Note: The 1st fraction of serial extraction; 1, 99.9% (v/v) ethanol; 2, 99.9% (v/v) methanol; 3, 50% (v/v) methanol

(2) Total phenolic content (TPC) and total flavonoids content (TFC)

The leaves from the Zn plus Cd treatment and the endophytic inoculated plants were extracted by low to high polarity solvent in each fraction (Appendixes B1 and B2). Figure 18 showed TPC and TFC of the leaf extracts in each solvent. The results indicated that phenolic contents of the leaf extracts from the plants inoculated with *Curtobacterium* sp. GLD03 and *Chryseobacterium* sp. GTID13 were similar to the extracts from the control plants. Whereas, *Stenotrophomonas* sp. GSD10 decreased the phenolic content as shown in Table 9.

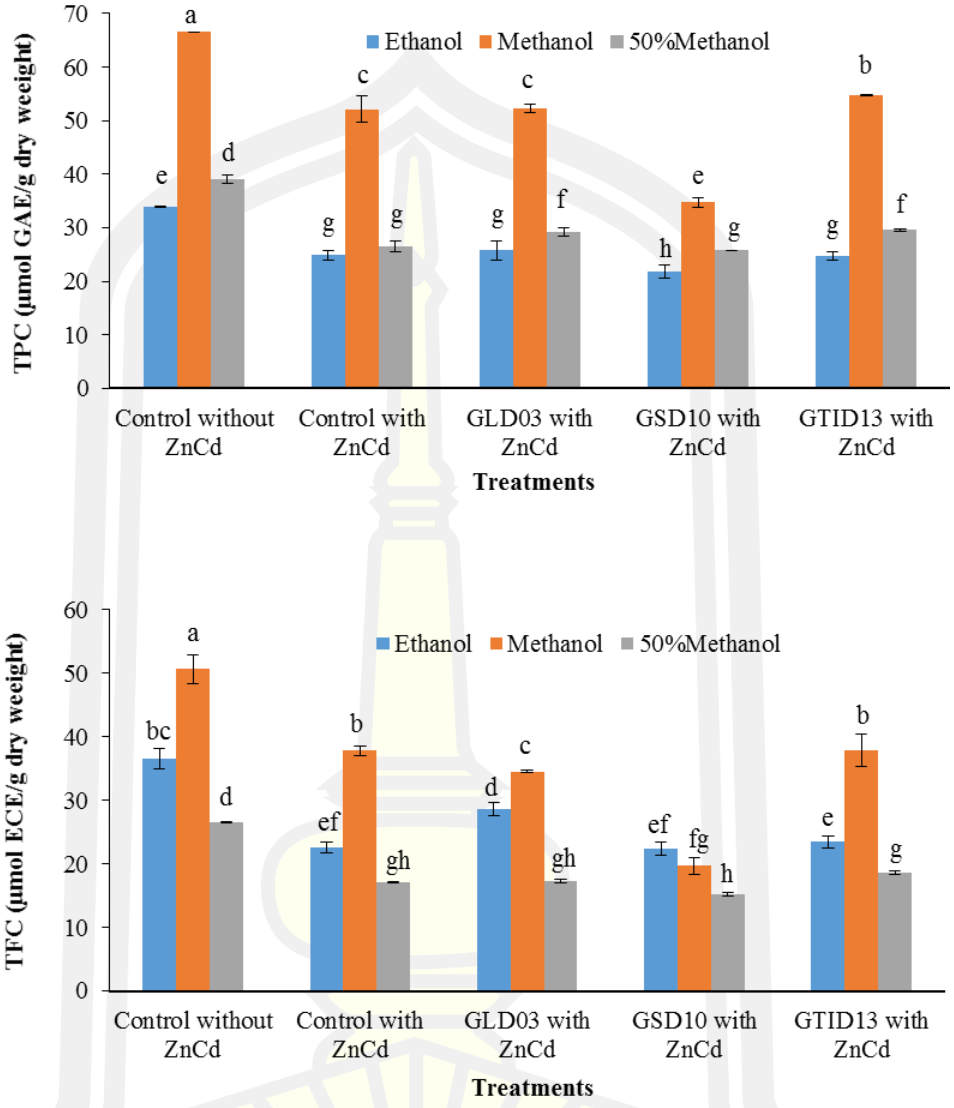


Figure 18 TPC and TFC in sum of all fractions (1, 2 and 3) from the leaf extracts of the treated *G. pseudochina* plants in tissue culture system. The percolation and partition methods were performed by 99.9% (v/v) ethanol, 99.9% (v/v) methanol and 50% (v/v) methanol. Results shown with different letters on error bars are significantly ($p < 0.01$, Duncan's new multiple range test). Data are given as means ± SD ($n = 3$).

Table 9 Summary of TPC and TFC in *G. pseudochina* leaf extract affected by different treatments in tissue culture system.

Treatments	TPC (μmol GAE/ g dry weight)	TFC (μmol ECE/ g dry weight)
Control	139.43 ± 0.64[a]	113.78 ± 3.73[a]
Control with Zn+Cd	103.63 ± 4.01[b]	77.47 ± 1.50[b]
GLD03 with Zn+Cd	107.28 ± 3.56[b]	80.37 ± 3.62[b]
GSD10 with Zn+Cd	82.32 ± 2.12[c]	57.28 ± 2.46[c]
GTID13 with Zn+Cd	109.06 ± 1.12[b]	79.97 ± 3.43[b]

Note: Difference the letters (a-c) in the same column are significant differences according to Duncan's test (p<0.01). Data are given as means ± SD (n=3).

(3) Antioxidant activity

The antioxidant activities of the leaf extracts are shown in Figure 19. A low IC_{50} value indicates a high antioxidant activity. Table 10 shows that the IC_{50} of leaf extract from the plants inoculated with *Chryseobacterium* sp. GTID13 in methanol fraction had the highest DPPH radical scavenging activity. In addition, this treatment was similar to the extracts obtained from the control plants in the same solvent fraction.

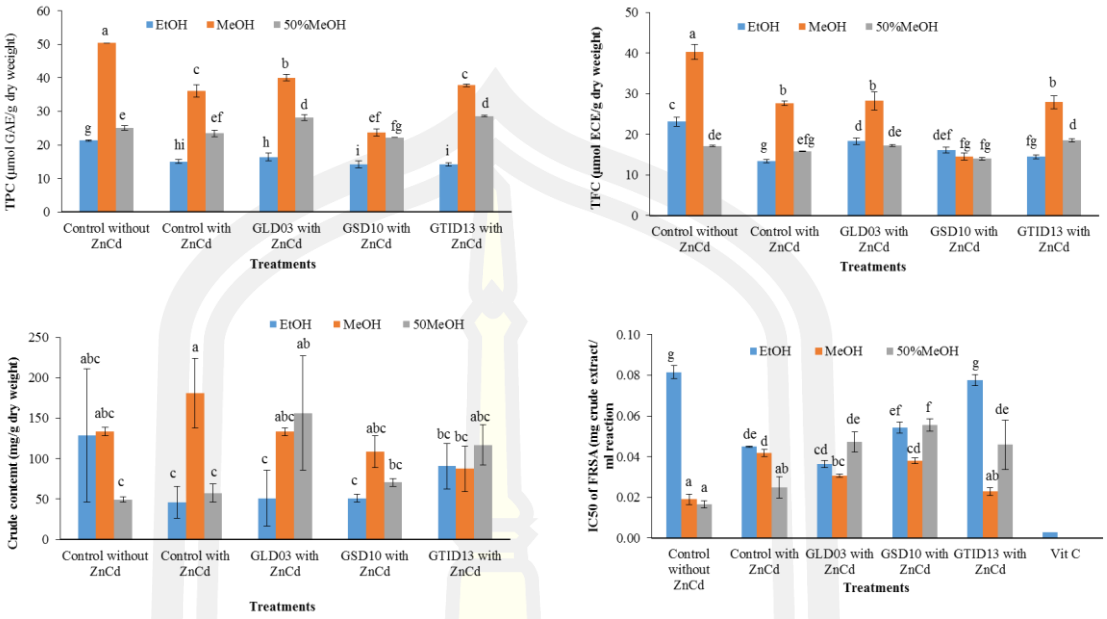


Figure 19 TPC, TFC, crude content and IC_{50} of FRSA in the 1st fraction from the leaf extracts of the treated *G. pseudochina* plants in tissue culture system.

The percolation and partition methods were performed by 99.9% (v/v) ethanol, 99.9% (v/v) methanol and 50% (v/v) methanol.Results shown with different letters on error bars are significantly different ($p < 0.01$, Duncan's new multiple range test). Data are given as means ± SD ($n = 3$).

Table 10 Sum totals of TPC, TFC, crude content and IC_{50} in *G. pseudochina* leaf extracts of the 1st fraction in tissue culture system.

Treatments	TPC (µmol GAE/ g dry weight)	TFC (µmol ECE/ g dry weight)	Crude content (mg/g dry weight)	IC_{50} (mg crude extract/ ml reaction)
Control	96.76 ± 0.66^{a}	80.52 ± 2.88^{a}	311.75 ± 80.82^{a}	0.12 ± 0.00^{a}
Control with Zn+Cd	74.46 ± 3.23^{c}	56.89 ± 0.79^{c}	284.66 ± 51.01^{a}	0.11 ± 0.01^{a}
GLD03 with Zn+Cd	84.64 ± 2.71^{b}	63.81 ± 3.02^{b}	340.32 ± 100.29^{a}	0.11 ± 0.00^{a}
GSD10 with Zn+Cd	60.10 ± 2.05^{d}	44.69 ± 1.65^{d}	230.26 ± 19.74^{a}	0.15 ± 0.00^{b}
GTID13 with Zn+Cd	80.57 ± 0.39^{b}	60.94 ± 2.00^{bc}	295.14 ± 24.73^{a}	0.15 ± 0.01^{b}

Note: Difference the letters (a-d) in the same column are significant differences according to Duncan's test (p<0.01). Data are given as means ± SD (n=3).

(4) Phenolic compounds analysis by HPLC

HPLC chromatogram of the extracts exhibited similar patterns and main 4 peak positions at 280 nm (Figure 20). Identification of the peaks was performed based on comparison with retention time (RT) of phenolic compound standards. Under the Zn plus Cd treatment, the plants inoculated with *Chryseobacterium* sp. GTID13 increased level of Chlorogenic acid (CCA), Caffeic acid (CA), Rutin and unknown (Table 11).

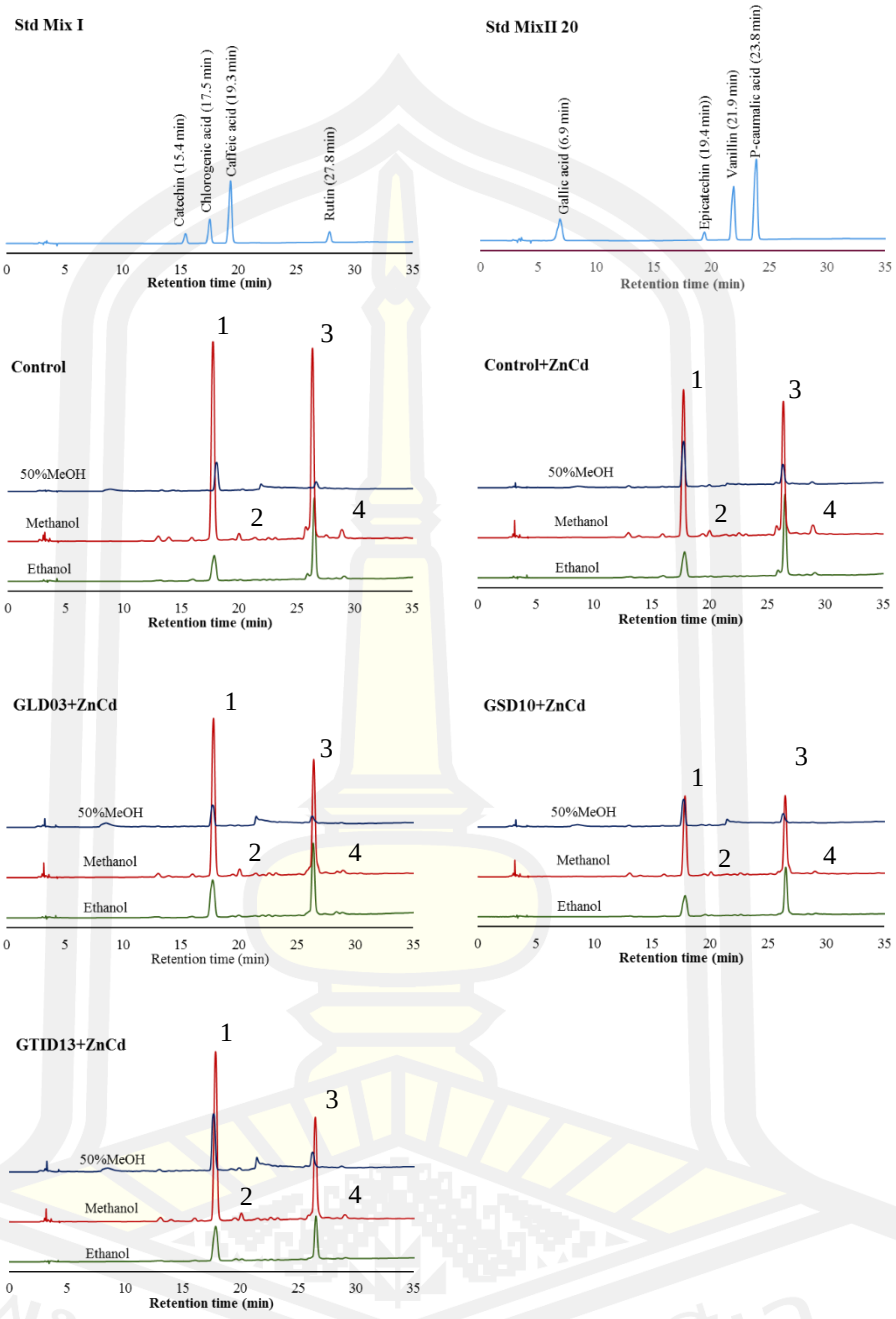


Figure 20 HPLC chromatograms of solvent extracts obtained from *G. pseudochina* inoculated with endophytic bacteria and treated with Zn plus Cd in tissue culture system. 1= Chlorogenic acid (CCA), 2=Caffeic acid (CA), 3=Unknown and 4 = Rutin (RUT).

Table 11 Concentration of Chlorogenic acid (CCA), Caffeic acid (CA) and Rutin (RUT) and peak area of unknown from HPLC chromatogram of *G. pseudochina* leaves extract at 280 nm in tissue culture system.

Treatments	CGA (mg/g dry weight)	CA (mg/g dry weight)	RUT (mg/g dry weight)	Unknow (Area)
Control	7.47 ± 0.01[a]	0.25 ± 0.01[b]	1.60 ± 0.16[a]	8,477,914 ± 1,393,862[a]
Control with Zn+Cd	5.88 ± 0.43[b]	0.27 ± 0.00[a]	1.16 ± 0.04[b]	5,851,183 ± 30,1583[b]
GLD03 with Zn+Cd	5.30 ± 0.05[b]	0.17 ± 0.00[d]	0.71 ± 0.02[c]	5,175,238 ± 124,865[b]
GSD10 with Zn+Cd	3.82 ± 0.45[c]	0.20 ± 0.00[c]	0.71 ± 0.00[c]	5,199,736 ± 872,957[b]
GTID13 with Zn+Cd	7.47 ± 0.03[a]	0.28 ± 0.00[a]	1.69 ± 0.03[a]	6,261,833 ± 1,299,761[b]

Note: Difference the letters (a-d) in the same column are significant differences according to Duncan's test (p<0.01). Data are given as means ± SD (n=3).

4.3.1 Pot system

4.3.1.1 Plant growth and phytotoxicity under the Zn plus Cd stress

The morphological changes in *G. pseudochina* plants inoculated with *Curtobacterium* sp. GLD03, *Stenotrophomonas* sp. GSD10 and *Chryseobacterium* sp. GTID13 growing in Zn (1,000 mg/kg) plus Cd (50 mg/kg) contaminated soil for 2 months are shown in Figure 21. The results showed the presenting of leaf chlorosis and growth inhibition when compared with control. The chlorophyll concentrations (Figure 22) indicated that the chlorophyll contents of the plants inoculated with endophytic bacteria and growing under the metal stress were decreased. The number of yellow and die leaves was investigated percentage of phytotoxicity. The highest percentages of chlorotic and necrotic leaves were found in the plants inoculated with *Stenotrophomonas* sp. GSD10 under the metal stress (Figure 23).

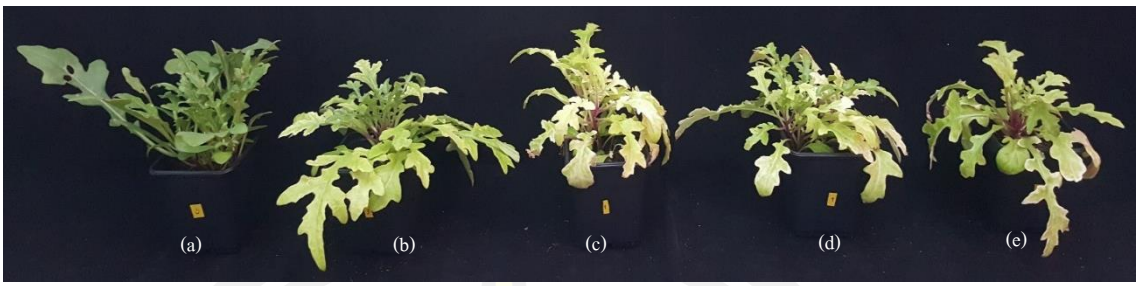


Figure 22 The morphological changes in *G. pseudochina* plants inoculated with endophytic bacteria and Zn plus Cd treatment for two months in pot system. (a) Control (without bacterial inoculation and metal treatment), (b) Zn 1,000 mg/kg soil and Cd 50 mg/kg soil treatment, (c) *Curtobacterium* sp. GLD03 and Zn plus Cd treatment, (b) *Stenotrophomonas* sp. GSD10 and Zn plus Cd treatment, and (e) *Chryseobacterium* sp. GTID13 and Zn plus Cd treatment.

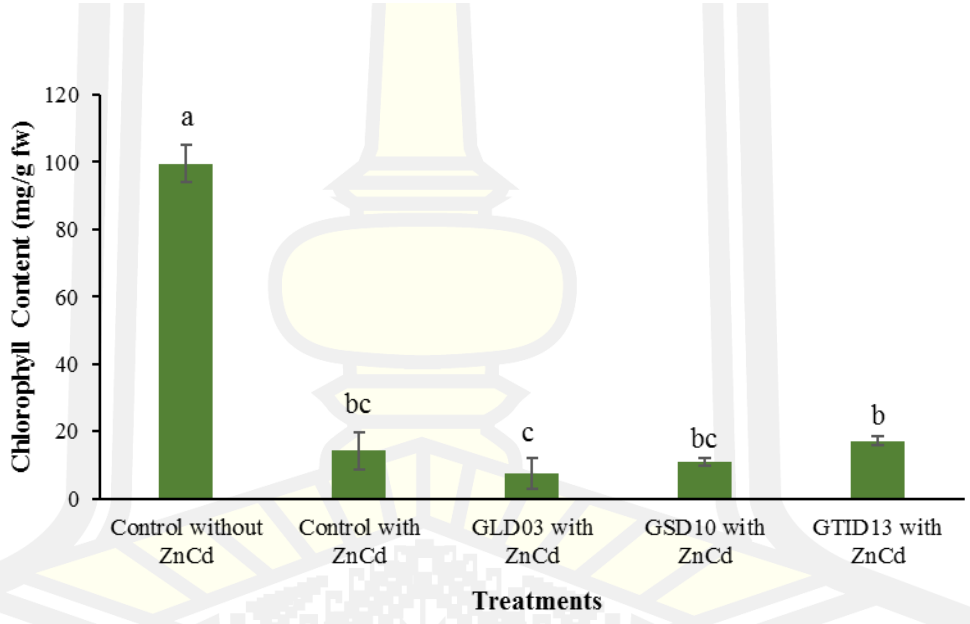


Figure 21 Total chlorophyll content of *G. pseudochina* plants treated with Zn 1,000 mg/kg soil and Cd 50 mg/kg soil, and inoculated with *Curtobacterium* sp. GLD03, *S. maltophilia* GSD10 and *Chryseobacterium* sp. GTID13 for two months in pot system. Results shown with different letters and symbols on error bars are significantly different ($p < 0.01$, Duncan's new multiple range test). Data are given as means ± SD ($n = 4$).

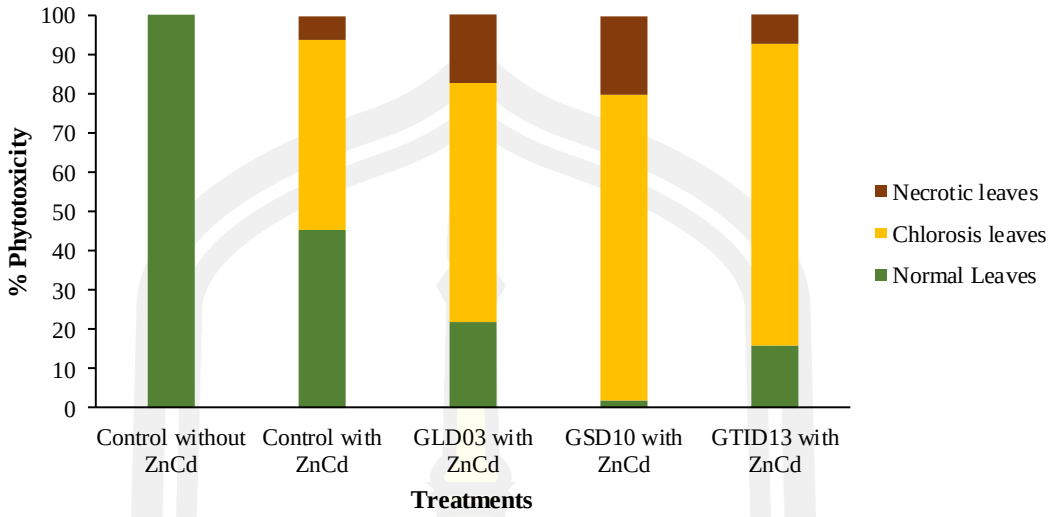


Figure 23 The percentage of chlorotic and necrotic leaves found in *G. pseudochina* plants treated with Zn 1,000 mg/kg soil and Cd 50 mg/kg soil and inoculated with *Curtobacterium* sp. GLD03, *S. maltophilia* GSD10 and *Chryseobacterium* sp. GTID13 for two months in pot system.

4.3.1.2 Zn and Cd accumulation

The effects of endophytic bacterial inoculation on Zn and Cd accumulation are shown in Figure 24. The plants inoculated with *Stenotrophomonas* sp. GSD10 increased amounts of Zn and Cd in roots. Whereas, *Curtobacterium* sp. GLD03 and *Chryseobacterium* sp. GTID13 increased amounts of Zn in the shoot when compared with the control plants.

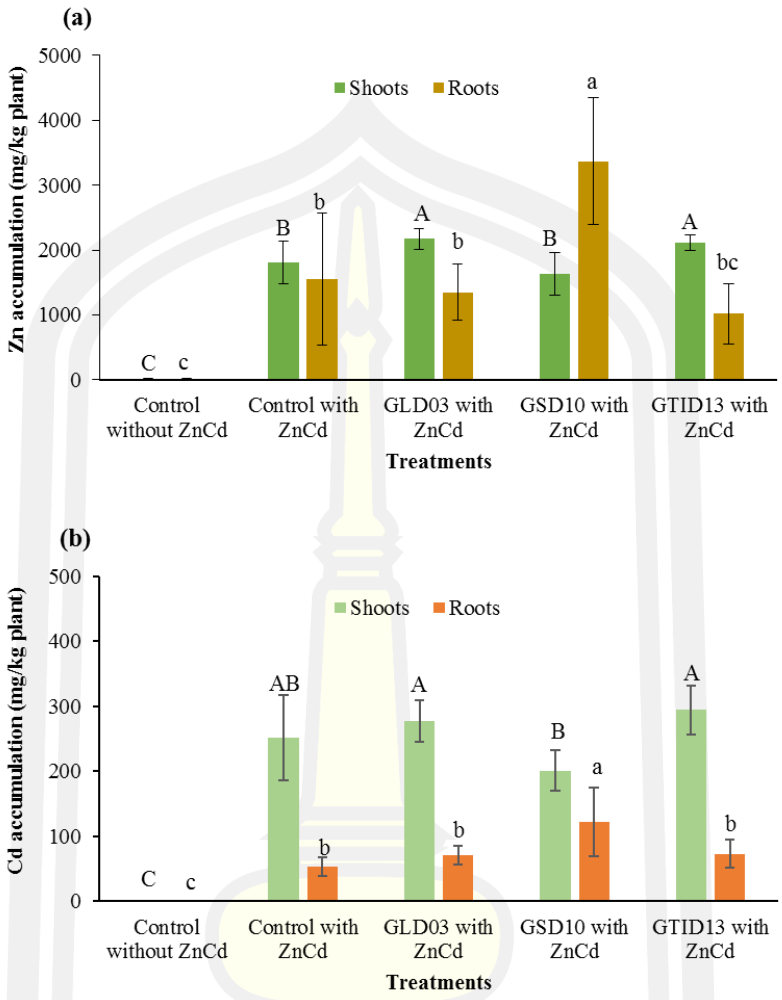


Figure 24 Zn and Cd accumulation of *G. pseudochina* shoot and root after Zn plus Cd treatment for two months in pot system. (a) and (b) are Zn and Cd content in plants. Results shown with different letters on error bars are significantly different ($p < 0.05$, Duncan's new multiple range test). Data are given as means ± SD ($n = 5$).

4.3.1.3 Endophytic bacterial inoculation to phenolic compounds

(1) Phytochemical screening

The leaf extracts of *G. pseudochina* in 99.9% (v/v) ethanol, 99.9% (v/v) methanol and 50% (v/v) methanol fraction from all treatments were detected for phytochemical. For phytochemical screening, carbohydrates and betacyanins were presented in all solvent fraction extracts of all treatments (Table 12). The extract from the plants inoculated with *Chryseobacterium* sp. GTID13 and the control with Zn plus Cd showed positive test of tannins in all solvent fractions and 99.9% (v/v) ethanol from the control without metal stress. The plants inoculated with endophytic bacteria showed positive test of saponins only in 50% (v/v) methanol fractions. 99.9% (v/v) methanol and 50% (v/v) methanol fraction in the plants inoculated with *Curtobacterium* sp. GLD03 and *Stenotrophomonas* sp. GSD10 showed negative test of terpenoids. The extracts from the plants inoculated with *Stenotrophomonas* sp. GSD10 absented glycosides. Steroids were found in leaf extracts on pot system. The leaf extracts from the plants inoculated with *Chryseobacterium* sp. GTID13 and control showed positive test of steroids in all solvent fractions under metal stress.

Table 12 Phytochemical screening of different treatments in 1st fraction *G. pseudochina* leaf extracts on pot system.

Test	Control			Control with Zn+Cd			GLD03 with Zn+Cd			GSD10 with Zn+Cd			GTID13 with Zn+Cd		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Alkaloids	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Tannins	+	–	–	+	+	+	–	–	–	–	–	–	+	+	+
Sponins	+	+	+	–	+	+	–	–	+	–	–	+	–	–	+
Anthocyanins	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Betacyanins	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Terpenoids	+	+	+	+	+	+	+	–	–	+	–	–	+	+	+
Starch	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Glycosides	+	–	+	+	–	–	+	–	+	–	–	–	+	–	+
Steroids	–	–	+	+	+	+	+	–	+	–	–	–	+	+	+
Carbohydrates	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Reducing sugars	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Anthoqinones	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Resins	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Proteins	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Note: The 1st fraction of serial extraction; 1, 99.9% (v/v) ethanol; 2, 99.9% (v/v) methanol; 3, 50% (v/v) methanol

(2) Total phenolic content (TPC) and Total flavonoid content (TFC)

The leaves from the Zn plus Cd treatment and the endophytic inoculated plants in pot system were extracted by low to high polarity solvent in each fraction (Appendix B3 and B4). Figure 25 shows a summary of TPC and TFC in the leaf extracts. Table 13 indicated that all endophytic inoculations decreased TPC and TFC, when compared with control plants. However, *Chryseobacterium* sp. GTID13 caused a higher level more than *Curtobacterium* sp. GLD03 and *Stenotrophomonas* sp. GSD10, and similar to the extracts from the control plants.

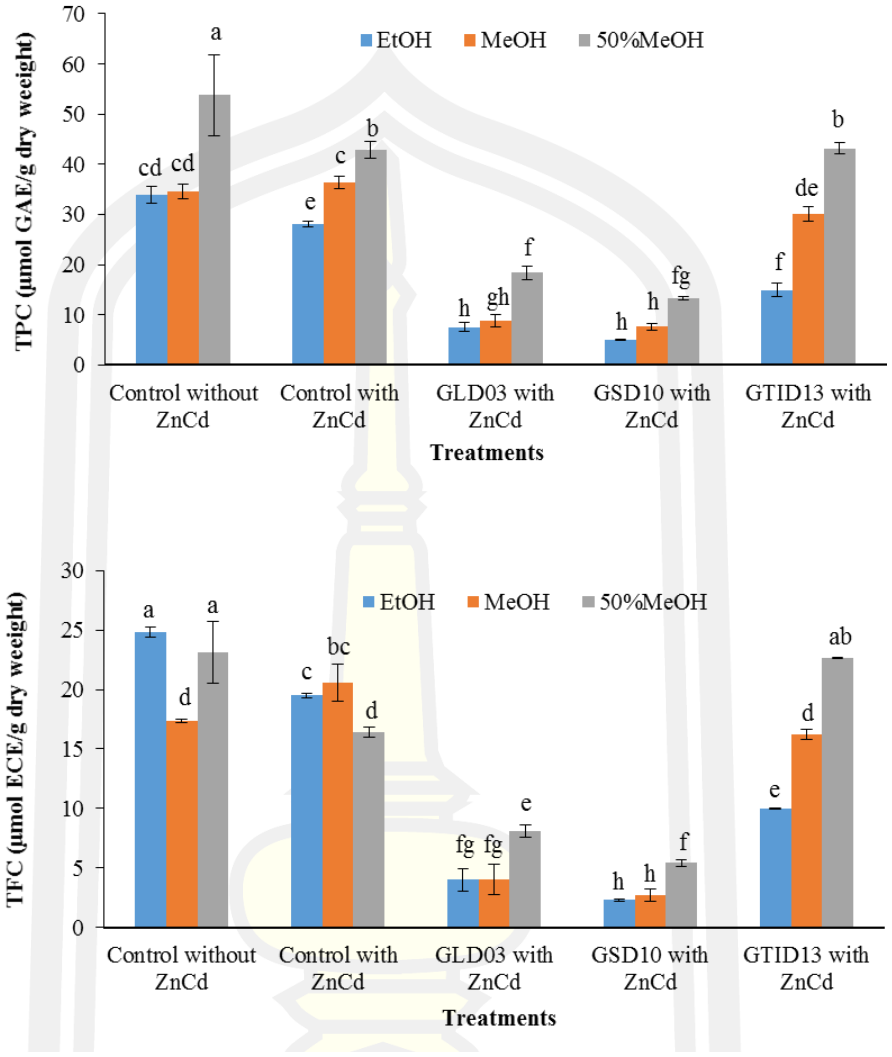


Figure 25 TPC and TFC in sum of all fractions (1, 2 and 3) from the leaf extracts of the treated *G. pseudochina* plants in pot system. The percolation and partition method were performed by 99.9% (v/v) ethanol, 99.9% (v/v) methanol and 50% (v/v) methanol. Results shown with different letters on error bars are significantly different ($p < 0.01$, Duncan's new multiple range test). Data are given as means ± SD ($n = 3$).

Table 13 Summary of TPC and TFC in *G. pseudochina* leaf extract affected by different treatments in pot system.

Treatments	TPC (μmol GAE/ g dry weight)	TFC (μmol ECE/g dry weight)
Control	122.18 ± 11.36[a]	65.25 ± 3.14[a]
Control with Zn+Cd	107.43 ± 3.50[b]	56.49 ± 1.76[b]
GLD03 with Zn+Cd	34.80 ± 3.44[d]	16.13 ± 2.80[d]
GSD10 with Zn+Cd	26.07 ± 0.14[d]	10.42 ± 0.13[e]
GTID13 with Zn+Cd	88.24 ± 1.20[c]	48.88 ± 0.61[c]

Note: Difference the letters (a-e) in the same column are significant differences according to Duncan's test ($p<0.01$). Data are given as means ± SD (n=3)

(3) Antioxidant activity

The antioxidant activities of the leaf extracts are shown in Figure 26. Table 14 indicated that TPC and TFC of plant inoculated with *Chryseobacterium* sp. GTID13 were no significant to the extracts from the control plants under metal stress. The IC_{50} of leaf extract from *G. psuedochina* inoculated with *Chryseobacterium* sp. GTID13 had DPPH radical scavenging activity similar to control plant under the metal stress.

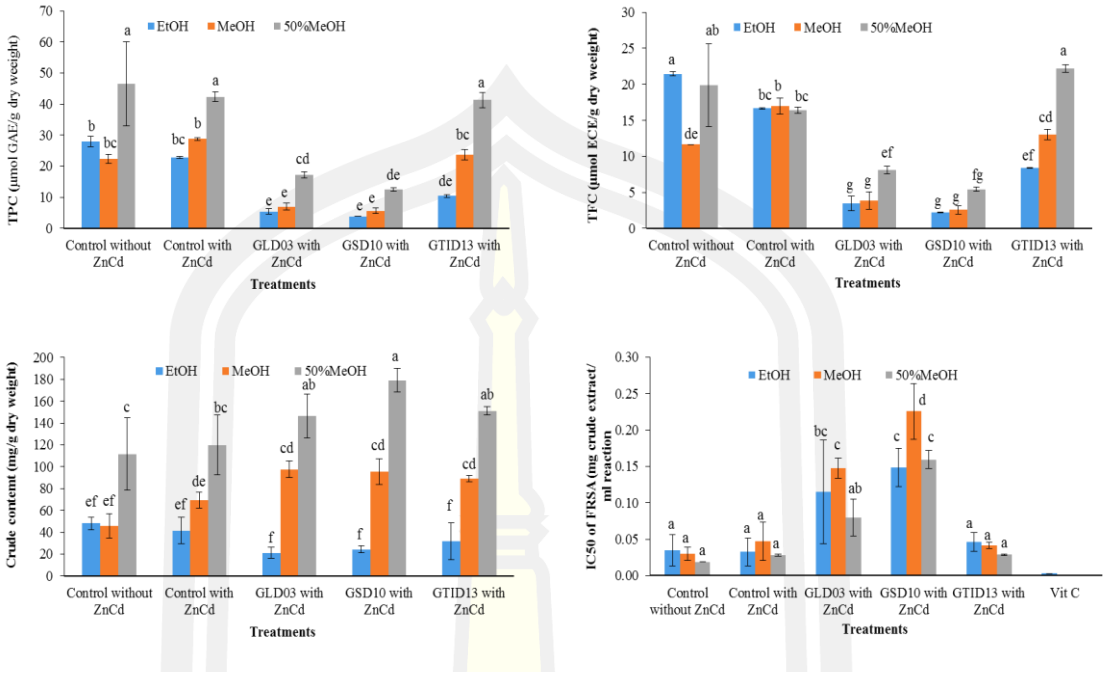


Figure 26 TPC, TFC, crude content and IC_{50} of FRSA in the 1st fraction from the leaf extracts of the treated *G. pseudochina* plants in pot system.

The percolation and partition methods were performed by 99.9% (v/v) ethanol, 99.9% (v/v) methanol and 50% (v/v) methanol. Results shown with different letters on error bars are significantly different ($p < 0.01$, Duncan's new multiple range test). Data are given as means ± SD ($n = 3$).

Table 14 Sum totals of TPC, TFC, crude content and IC_{50} in *G. pseudochina* leaf extracts of the 1st fraction in pot system.

Treatments	TPC (µmol GAE/ g dry weight)	TFC (µmol ECE/ g dry weight)	Crude content (mg/g dry weight)	IC_{50} (mg crude extract/ ml reaction)
Control	96.86 ± 16.66^{a}	53.01 ± 6.03^{a}	205.46 ± 38.36^{c}	0.08 ± 0.03^{a}
Control with Zn+Cd	93.88 ± 2.37^{ab}	50.07 ± 1.40^{ab}	230.91 ± 47.05^{ab}	0.11 ± 0.05^{a}
GLD03 with Zn+Cd	29.50 ± 3.00^{c}	15.43 ± 2.78^{c}	265.50 ± 17.36^{ab}	0.34 ± 0.08^{b}
GSD10 with Zn+Cd	21.90 ± 0.36^{c}	10.24 ± 0.25^{c}	298.80 ± 25.66^{a}	0.53 ± 0.05^{c}
GTID13 with Zn+Cd	75.39 ± 3.80^{b}	43.63 ± 1.29^{b}	272.15 ± 18.22^{ab}	0.12 ± 0.01^{a}

Note: Difference the letters (a-c) in the same column are significant differences according to Duncan's test (p<0.01). Data are given as means ± SD (n=3)

(3) Phenolic compounds analysis by HPLC

The HPLC chromatogram of extracts exhibited similar patterns and 4 major peak positions (Figure 27). Inoculation of *Curtobacterium* sp. GLD03 and *Stenotrophomonas* sp. GSD10 decreased level of major peaks that found in all extracts. Whereas, *Chryseobacterium* sp. GTID13 showed similar to the peak level of the extracts from the control plant. Table 15 also indicated that inoculation with *Chryseobacterium* sp. GTID13 increased level of CCA, Unknown and RUT when compared with the control plants.

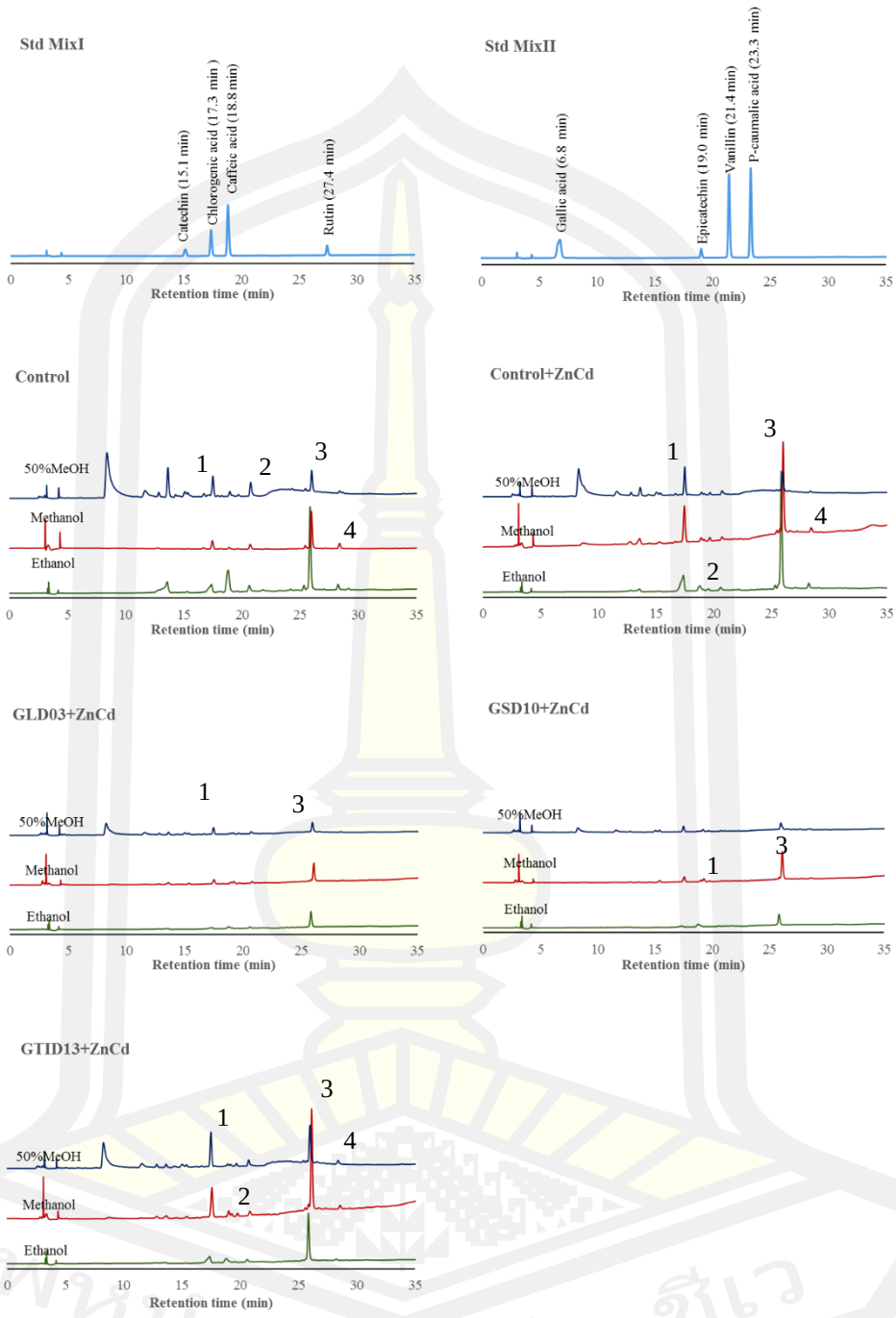


Figure 27 HPLC chromatograms of solvent extracts obtained from *G. pseudochina* inoculated with endophytic bacteria and treated with Zn plus Cd in pot system.
1= Chlorogenic acid (CCA), 2=Caffeic acid (CA), 3=Unknown and 4=Rutin (RUT).

Table 15 Concentration of Chlorogenic acid (CCA), Caffeic acid (CA) and Rutin (RUT) and peak area of unknown from HPLC chromatogram of *G. pseudochina* leaves extract at 280 nm in pot system.

Treatments	CGA (mg/g dry weight)	CA (mg/g dry weight)	RUT (mg/g dry weight)	Unknown (Area)
Control	1.34 ± 0.01^{c}	0.49 ± 0.01^{a}	1.04 ± 0.04^{a}	3,656,563 ± 122,344^{b}
Control with Zn+Cd	1.77 ± 0.01^{a}	0.27 ± 0.02^{b}	0.99 ± 0.01^{a}	4,308,547 ± 7,246^{a}
GLD03 with Zn+Cd	0.32 ± 0.05^{d}	0.11 ± 0.01^{d}	0.45 ± 0.00^{b}	762,286 ± 201,649^{d}
GSD10 with Zn+Cd	0.29 ± 0.00^{d}	0.17 ± 0.00^{c}	0.43 ± 0.01^{b}	1,130,462 ± 8,2136^{c}
GTID13 with Zn+Cd	1.53 ± 0.10^{b}	0.27 ± 0.02^{b}	1.01 ± 0.07^{a}	3,844,917 ± 99,834^{b}

Note: Difference the letters (a-d) in the same column are significant differences according to Duncan's test (p<0.01). Data are given as means ± SD (n=3).

CHAPTER 5
DISCUSSIONS AND CONCLUSIONS

5.1 Discussions

The results obtained were discussed in the term characterization of endophytic bacteria isolated from *G. pseudochina*, which were collected from the conserved forest area of the Padaeng zinc mine. The bacteria were screened for Zn and Cd tolerant endophytic bacteria, plant growth promoting properties under the Zn plus Cd stress and no reported of plant and human pathogen. Then, the effects of selected bacterial inoculation on *G. pseudochina* plants growing under Zn/Cd stress in both tissue culture and pot systems were examined considering in plant growth, bacterial colonization, Zn and Cd accumulation, and phenolic compounds. Consequently, a possible mechanism of the bacterial inoculation on *G. pseudochina* was proposed.

5.1.1 Isolation and characterization of endophytic bacteria from *G. pseudochina*

Interactions between endophytic bacteria and hyperaccumulative plants have attracted several investigators with applications for bioremediation and for studying bacterial communities living in contaminated environment (Rajkumar et al., 2009). Endophytic bacteria have been found in various plant part such as roots, stems, leaves, flowers, fruits and seeds, and it can be isolated from surface sterilized plant tissue (Lodewyckx et al., 2002; Rajkumar et al., 2009). As the results, 20, 27 and 26 endophytic bacteria were isolated from the leaves, stems and tubers of *G. pseudochina*, respectively. There were 17 isolates out of 73 isolates that could resist high Zn (200 mg/l) and Cd (50 mg/l) concentration. They belonged to three major groups of Actinobacteria (41%), Proteobacteria (53%), and Bacteroidetes (1%). The phylogenetic analysis indicated that they were closely related to 13 genera of *Acinetobacter*, *Curtobacterium*, *Yokenella*, *Cupriavidus*, *Cellulomonas*, *Rhizobium*, *Enterobacter*, *Stenotrophomonas*, *Methylobacterium*, *Beijerinckia*, *Chryseobacterium*, *Klenkia* and *Marmoricola*. These genera were reported as common soil bacteria and endophytic bacteria of several plants growing in metals contaminated soil (Idris et al., 2004;

Barzanti et al., 2007; Sheng et al., 2008; Mastretta et al., 2009; Chen et al., 2010; Shin et al., 2012; Zhang et al., 2011; Ma et al., 2015)

Plant-growth promoting abilities were presented beneficial of endophytic bacteria to their host plants such as indole-3-acetic acid (IAA), siderophore production, nitrogen fixation and phosphate solubilization (Glick et al., 1998; Hardoim et al., 2008; Rajkumar et al., 2009). The 17 endophytic bacteria were screened for their Zn plus Cd tolerance properties and plant-growth promoting abilities. This study found that only 9 bacterial strains maintained their plant-growth promoting properties of IAA production, N_2-fixation, siderophore secretion and phosphate solubilization under Zn (200 mg/l) plus Cd (50 mg/l) contamination. Interestingly, siderophore and indole-3-acetic acid (IAA) production by *S. maltophilia* GSD10 and *Chryseobacterium* sp. GTID13 were induced by the Zn plus Cd stress. The plant host benefit from the endophytes by their natural resistance to soil contaminants, which substantially assist phytoremediation (Ryan et al., 2008). Many endophytic bacteria were able to produce IAA to stimulate plant growth and enhance phytoremediation of metals (Chen et al., 2010; Ma et al., 2011; Shin et al., 2012; Zhang et al., 2011). In addition, siderophore-producing endophytes were able to enhance plant growing in low nutrition environments (Chen et al., 2010; Ma et al., 2011; Shin et al., 2012; Zhang et al., 2011).

Curtobacterium sp. GLD03, *S. maltophilia* GSD10 and *Chryseobacterium* sp. GTID13 were non-pathogenic on *G. pseudochina*, which was their host plant. In which, *Chryseobacterium* sp. PMSZPI and *Stenotrophomonas* spp. were reported to have *czc* genes, which was responsible for the efflux of three metal cations (Co^{2+}, Zn^{2+} and Cd^{2+}) and the metals resistance (Jain & Bhatt, 2014; Nongkhlaw & Joshi, 2019). *Stenotrophomonas* sp. Was isolated from seed of *Nicotiana tabacum*, a Cd hyperaccumulator (Mastretta et al., 2009), and stem of *Sedum alfredii*, a Zn/Cd hyperaccumulator (Xinxian et al., 2011). In addition, *Stenotrophomonas* sp. isolated from ginger rhizome, a medicinal herbal plant, had ability to produce siderophore (Jasim et al., 2014). *Chryseobacterium* sp. and *Curtobacterium* sp. isolated from stem of *Solanum nigrum*, a Cd hyperaccumulator, were classified as Cd resistance endophytic bacteria (Luo et al., 2011).

5.1.2 Effect of bacterial inoculation on *G. pseudochina* under Zn/Cd stress

The experiment in *in vitro* study of a tissue culture system maintained high humidity level and controlled lighting and nutrients. Therefore, Under humid and nutrient-rich conditions, the bacterial inoculation did not cause changes in the amounts of Zn and Cd accumulated in plants. Rattanapolsan et al. (2021) studied the effect of endophytic bacterial inoculation on *Murdannia spectabilis* growing under Zn and Cd stress in the tissue culture system, and their results showed that inoculation and uninoculation did not make a difference in metal accumulation and plant growth. Therefore, *in vivo* system of the pot experiment was studied further to obtain an inoculation effect.

Under the pot experiment, Zn and Cd stress affected to decrease the chlorophyll content in both uninoculated and inoculated plants. The inoculation of *S. maltophilia* GSD10 increased the accumulation of Zn and Cd in roots. Whereas, the inoculation of *Curtobacterium* sp. GLD03 and *Chryseobacterium* sp. GTID13 increased the accumulation of Zn and Cd in the shoots. Chen et al. (2010) reported that the inoculation with Cd resistance endophytic bacteria influenced the accumulation of Cd in root, stem, and leaf tissues of *Solanum nigrum*. Ma et al. (2016) also reported that *Achromobacter piechaudii* E6S, which was a metal resistant endophytic bacterium, significantly stimulated plant biomass, uptake and bioaccumulation of Cd, Zn, and Pb in *Sedum plumbizincicola*. Luo et al. (2011) showed the inoculation of *Chryseobacterium* sp. decreased Cd toxicity and increased the root dry weight of *S. nigrum*. Further experimental results showed that the inoculation of *Curtobacterium* sp. GLD03 and *S. maltophilia* GSD10 increased the percentage of chlorosis and necrotic leaves in the *G. pseudomonas* plants. The chlorosis and necrotic leaves might be caused by increased metal accumulation, causing increased abiotic stress that generated reactive oxygen species (ROS), leading to oxidative stress and cell damage (Díaz et al., 2001). In addition, the toxic of Cd caused the reduction in nutrient uptak and photosynthesis, leading to growth inhibition, chlorosis and browning of root tips (Wójcik & Tukiendorf, 2004; Mohanpuria et al., 2007; Mongkhonsin et al., 2016). Panitlertumpai et al. (2013) also reported that treatment of *G. pseudochina* with a high concentration of Zn and Cd caused chlorosis and the metal accumulation in roots and shoots.

A higher rate of survival and colonization ability is essential to obtain benefits, it is important to analyze the survival and colonization potential of the inoculated strains in the host cells (Brehm-stecher & Johnson, 2004). Endophytic bacteria should have the capability to overcome plant defense responses mediated through the production of ROS, which cause stress to invading bacteria (Reinhold-Hurek & Hurek, 1998). This research showed that *Curtobacterium* sp. GLD03, *S. maltophilia* GSD10 and *Chryseobacterium* sp. GTID13 were able to colonize in all plant parts, especially stem and root tissues. The GFP-labelled *S. maltophilia* GSD10 cells investigated under a confocal microscope clearly showed the colonization of the endophytic bacterium inside the root tissues. Elbeltagy et al. (2001) also presented the distribution of GFP-tagged *Herbaspirillum* sp. strain B501*gfp1* in 7-day-old seedlings of rice. Root hairs offer a logical point of entry for these endophytes as many cases of intracellular plant-microbe interactions begin with the colonization of the microbe through intracellular access to root hairs (Kandel et al., 2017). Moreover, the plant parts occupied by the endophytic bacteria might differ in that *Curtobacterium* sp. lived in roots, stems and leaves while *Chryseobacterium* sp. found only in stem (Luo et al., 2011).

An extreme adaptation of endophytes to their host and it thereby contributes to the coproduction of bioactive molecules compounds that can be used by plants for protection from biotic and abiotic stress factor (Joseph & and Mini, 2011; Nair & Padmavathy, 2014; Aswani et al., 2020). Therefore, the leaf extracts of *G. pseudochina* plants inoculated with the endophytic bacteria were studied for preliminary phytochemical screening. The leaf extracts obtained from both control and treated plants contained tannins, sponins, betacyanins, terpenoids, glycosides, sponins, steroids and carbohydrates. In addition, steroids were only found in the leaf extracts obtained from plants grown in potted systems. Moektiwardoyo et al. (2014) also presented that the phytochemical screening of *G. pseudochina* leaves showed tannins, flavonoids, monoterpenenes, sesquiterpenes and steroids.

In addition, bioactive compounds in plants might be different due to several factors such as plant material, plant treatment, cultivation method, and the extraction methods (Aswani et al., 2020). For the *in vitro* of tissue culture system, the results indicated that the leaf extracts obtained from the plants treated with Zn plus Cd

and inoculated with *Curtobacterium* sp. GLD03 and *Chryseobacterium* sp. GTID13 had a higher content of TPC and TFC than those obtained from the plants given only from Zn plus Cd. In which, the metanolic leaves extract of the plants inoculated with *Chryseobacterium* sp. GTID13 had a high DPPH radical scavenging activity, with the lowest IC_{50} values. On the other hand, the results from the potted experiments showed that the high Zn and Cd accumulated in plants caused the decrease of TPC and TFC in their leaf extracts, both uninoculated and inoculated with the endophytes. Because of the high metal accumulation and lower stresses under the *in vitro* experiments, the TPC and TFC content of the leaf extracts from the tissue culture system were higher than the leaf extracts from the pot system. The reduction of phenolic composition and antioxidant activities due to excess concentrations of Zn and/or Cd accumulated in plants was reported in *G. pseudochina* (Mongkhonsin et al., 2016), *Erica andevalensis* (Márquez-García et al., 2012) and *Camellia sinensis* (Zagoskina et al., 2007). Purwanto et al. (2010) used thin-layer chromatography (TLC) to indicate that *in vitro* cultures of *G. pseudochina* produced a higher amount and variety of secondary metabolites. Therefore, this research studied the chemical profile of the treated plants by HPLC. HPLC chromatograms of the leaf extracts from *G. pseudochina* plants, both uninoculated and inoculated with the bacterial strains of GLD03, GSD10 and GTID13 were similar patterns and 4 main peaks containing chlorogenic acid (CGA), caffeic acid (CA) and rutin (RUT) and unknown. Interestingly, the HPLC peaks indicated that inoculation with *Chryseobacterium* sp. GTID13 increased level of CGA, RUT and the unknown peak when compared with control plants. Kumar et al. (2014) reported that *Azotobacter chroococcum* CL13 inoculated on turmeric rhizomes was able to enhance the production of curcumin. The production of vindoline, ajmalicine, and serpentine in *Catharanthus roseus* explants were improved by the endophytic inoculation of *Staphylococcus sciuri* and *Micrococcus* sp. Maggini et al. (2017) also reported that alkamide biosynthesis of Echinacea *purpurea* might be modulated with a pool of endophytic bacterial inoculation.

5.2 A possible mechanism of the selected bacterial inoculation on *G. pseudochina*

A possible mechanism of bacterial inoculation on *G. psuedochina* were presented using the results of *in vivo* experiment of the pot experiments. The fertile soil

contaminated with Zn 1,000 mg/kg and Cd 50 mg/kg, which were the Zn and Cd levels in the zinc mine, was used in this study. *Curtobacterium* sp. GLD03, which was an endophytic actinobacterium isolated from leaf part of *G. pseudochina*, modulated the increase Zn and Cd accumulation in the shoot. Although the GLD03 strain had the IAA production ability under the stress of Zn plus Cd, the plant growth and the amounts of TPC, TFC, CA, CGA, RUT and unknown in the leaf extracts were decreasing. That might cause by the high Zn and Cd accumulation in the plants. The effects of *Curtobacterium* sp. GLD03 inoculation on *G. pseudochina* growing under high Zn and Cd stress was concluded as shown in Figure 28 (a). Gram negative and Proteobacteria group presented by *S. maltophilia* GSD10, an endophyte in Proteobacteria group, resulted to increase the amounts of Zn and Cd accumulated in roots part. This bacterium had the plant growth promoting properties of N_2-fixation, IAA and siderophore production under the Zn plus Cd stress. However, the amounts of TPC, TFC, CA, CGA, RUT and unknown in the leaf extracts were decreased due to the high Zn and Cd stress. The effects of *S. maltophilia* GSD10 inoculation on *G. pseudochina* growing in the Zn plus Cd contaminated soil was summarised in Figure 28 (b). *Chryseobacterium* sp. GTID13, a gram negative in Bacteroidetes group, also promoted the increase amounts of Zn and Cd accumulated in the shoots. The GTID13 strain had siderophore production ability under the high Zn plus Cd. Although the leaf extracts obtained from *G. pseudochina* plants inoculated with *Chryseobacterium* sp. GTID13 slightly decreased in TPC, TFC, CA, the peaks of CGA, RUT and the unknown peak compound increase. The effects of *Chryseobacterium* sp. GTID13 inoculation on its host plant growing under the stress of Zn plus Cd was presented in Figure 28 (c).

These results indicated that the inoculation with the Zn/Cd tolerant endophytic bacteria were able to promote the accumulation of Zn and Cd into the root or shoot parts. However, the high Zn and Cd deposits caused so much stress on plants that they reduce their phenolic compound production. Although the three endophytes maintained the plant growth promoting properties under the high Zn and Cd contamination, only *Chryseobacterium* sp. GTID13 inoculation affected by the increase of CGA, RUT and unknown compound, which should be defined further.

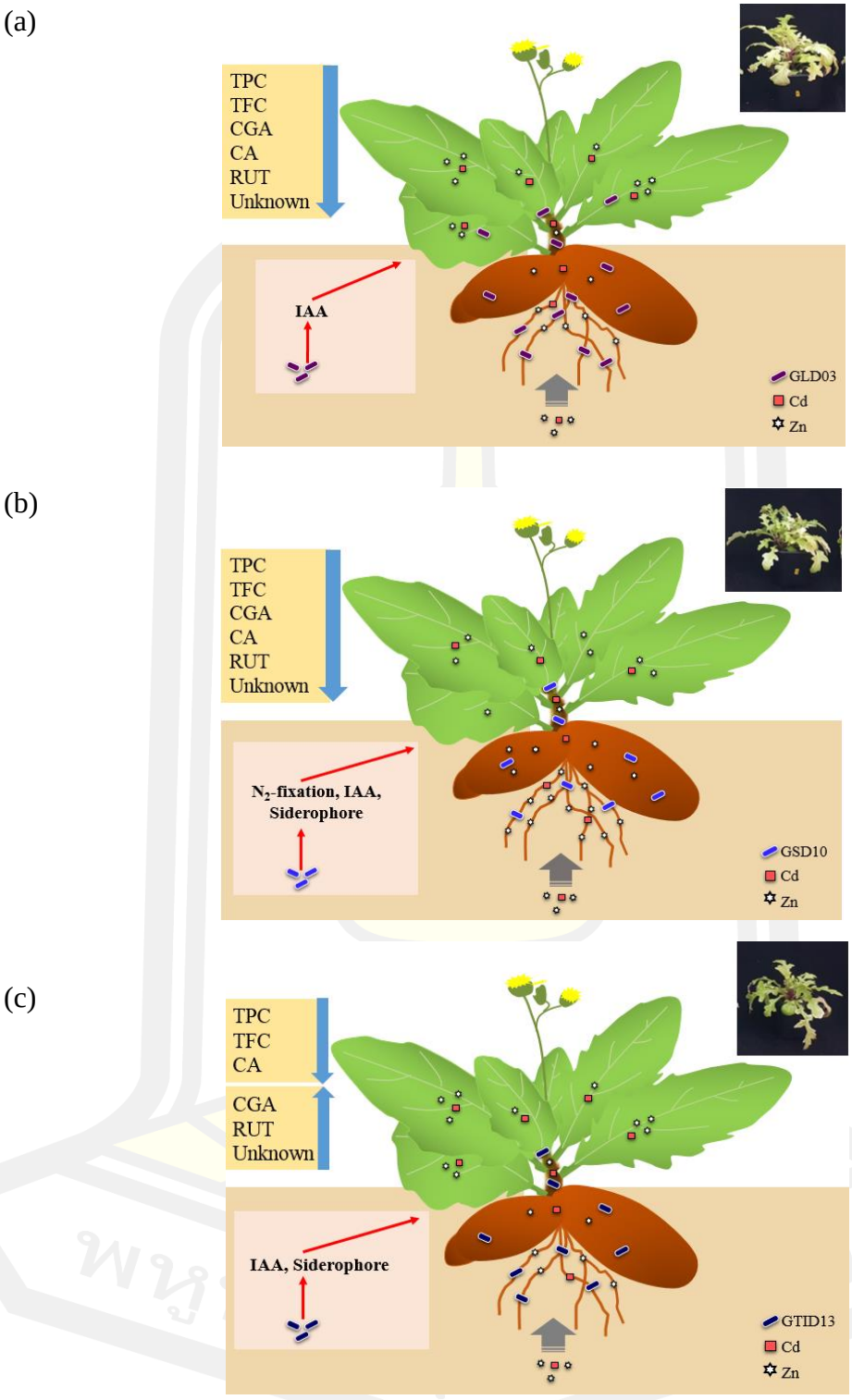


Figure 28 Effects of endophytic inoculation on *G. pseudochina* growing under high Zn and Cd stress, (a) *Curtobacterium* sp. GLD03 (b) *S. maltophilia* GSD10 and (c) *Chryseobacterium* sp. GTID13.

5.3 Conclusions

The 20, 27 and 26 endophytic bacteria (total 73 isolates) were isolated from leave, stem and tuber of healthy *G. pseudochina* plants, which grew in Zn and Cd contaminated soil in a zinc mining area, respectively. The 17 bacterial isolates were able to tolerate on TSA plate containing high concentrations of Zn (200 mg/l) and Cd (50 mg/l). In which, 9 isolates tolerated Zn (200 mg/l) plus Cd (50 mg/l), and maintained plant-growth promoting abilities. In addition, siderophore and indole-3-acetic acid (IAA) on the production of *S. maltophilia* GSD10 and *Chryseobacterium* sp. GTID13 were induced due to the Zn plus Cd stress. *Curtobacterium* sp. GLD03, *S. maltophilia* GSD10 and *Chryseobacterium* sp. GTID13, which have had no reports of pathogenicity, were selected for monitoring the *G. pseudochina* plants' inoculating effects. The plants were treated with high Zn and Cd concentration under the *in vitro* of tissue culture and the *in vivo* of pot experiment, with and without separately endophytic inoculation. In comparison with uninoculated control plants, *S. maltophilia* GSD10 increased the accumulation of Zn and Cd in the roots, whereas *Curtobacterium* sp. GLD03 and *Chryseobacterium* sp. GTID13 increased the amounts of Zn and Cd deposited in the shoots. The three endophytic bacterial strains were able to colonize all parts of *G. pseudochina*, especially stem and root tissues. The GFP-labelled *S. maltophilia* GSD10 cells clearly showed the colonization in root tissues. In comparison with the phenolic compounds of the leaf extracts from the plants treated with Zn plus Cd, the inoculation of *Curtobacterium* sp. GLD03 and *S. maltophilia* GSD10 in the plants under the Zn plus Cd stress resulted to decrease TPC and TFC in their leaf extracts. Whereas, TPC and TFC of the leaf extracts from the plants inoculated with *Chryseobacterium* sp. GTID13 were similar to the extracts from the uninoculated plants. In addition, the leaf extracts of the plant inoculated with *Chryseobacterium* sp. GTID13 had the highest DPPH radical scavenging activity as the lowest IC_{50}. The antioxidant activities were correlated with TPC and TFC. HPLC chromatograms of all extracts were similar patterns with 4 main peaks of CA, CGA, RUT and an unknown compound. The plants inoculated with *Curtobacterium* sp. GLD03 and *S. maltophilia* GSD10 affected to decrease all 4 main peaks in the leaf extracts. Whereas, the inoculation by *Chryseobacterium* sp. GTID13 might modulate the increase of CGA, RUT and unknown compound, which should be identified further by Liquid

chromatography–mass spectrometry (LC-MS/MS). Consequently, the results obtained demonstrated that *Chryseobacterium* sp. GTID13 could be applied in a bioaugmentation process to decrease Zn/Cd phytotoxicity and support phytoextraction of the metals by growing *G. pseudochina.*

5.4 Suggestions

5.4.1 This research study at the threshold high concentrations of Zn and Cd, in which the effect of endophytics inoculation could not express their plant growth promoting properties. Therefore, the effect of inoculation of endophytic bacteria should be re-examined in an in vivo system with lower Zn and/or Cd concentrations.

5.4.2 For clearer mechanism, the chemical profile of the leaf extracts of the plant treated with Zn plus Cd stress, with and without the bacterial inoculation should be studied further by LC-MS/MS

5.4.3 Application of *G. psuedochina*, a Zn/Cd hyperaccumulative herbaceous herbal plant, for phytoremediation and phytomining should be studied further in a field experiment.

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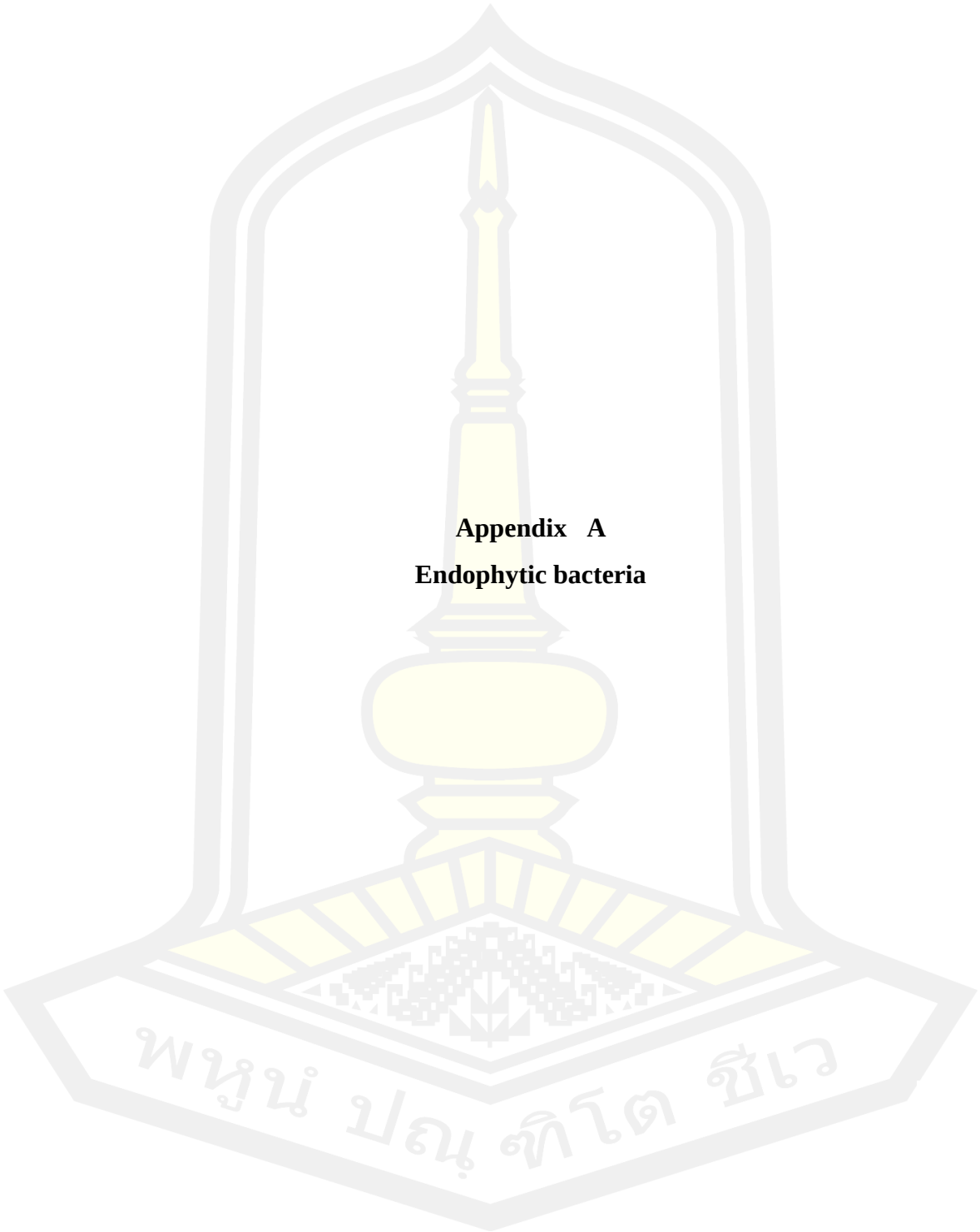
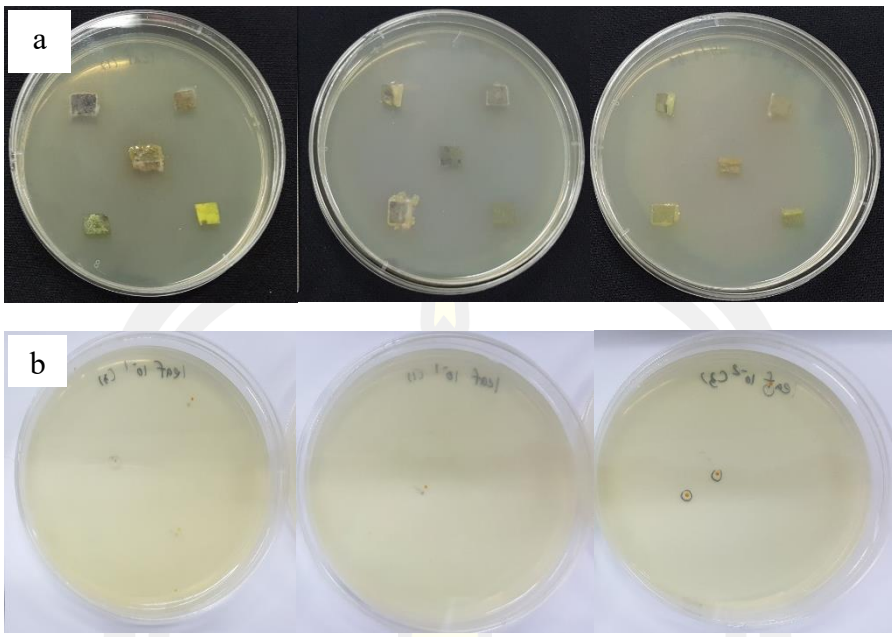
APPENDIXES

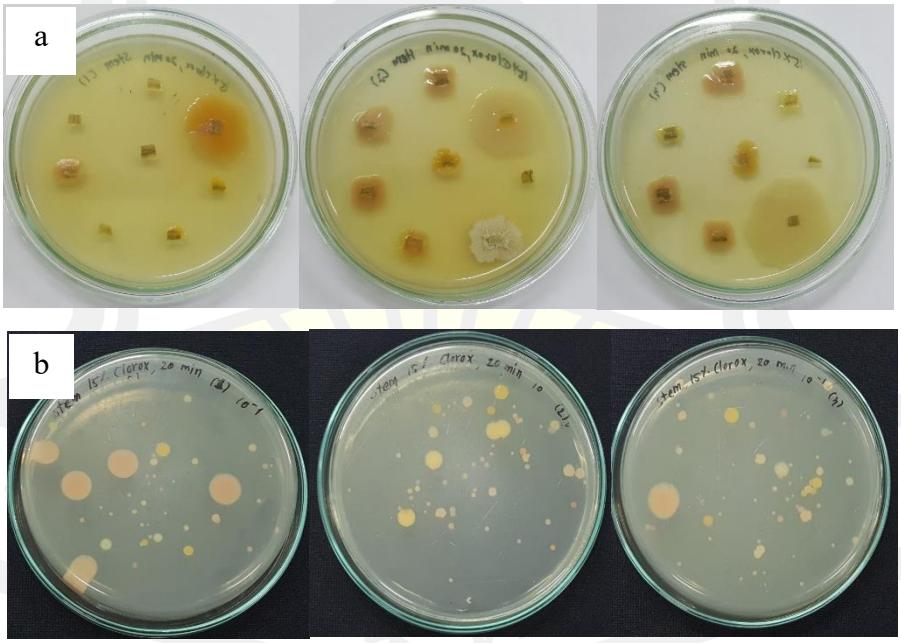


Appendix A

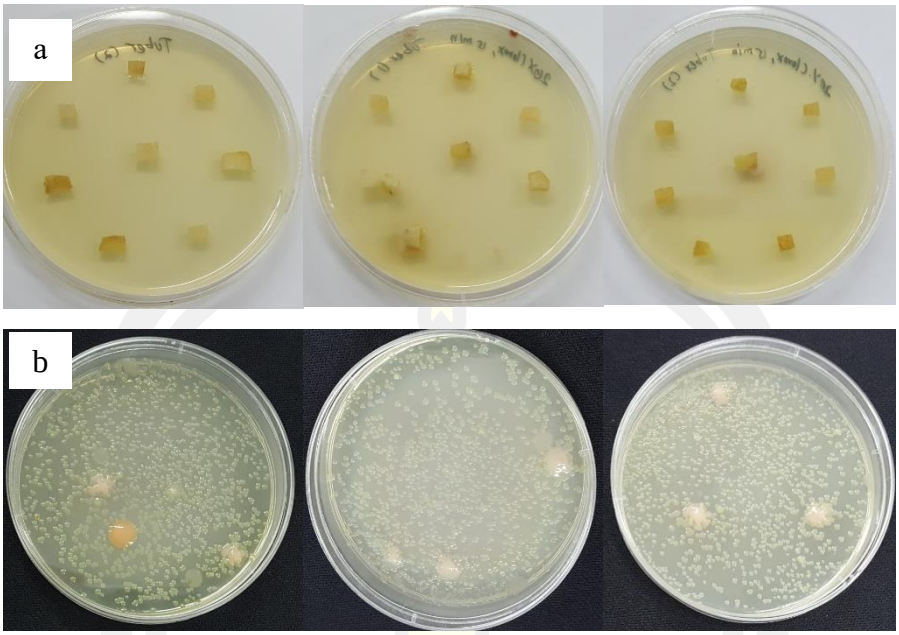
Endophytic bacteria



Appendix A1 Endophytic bacteria were isolated from healthy leaves tissue of *G. pseudochina* (L.) DC. by (a) direct and (b) indirect method.



Appendix A2 Endophytic bacteria were isolated from healthy stem tissue of *G. pseudochina* (L.) DC. by (a) direct and (b) indirect method.



Appendix A3 Endophytic bacteria were isolated from healthy tuber tissue of *G. pseudochina* (L.) DC. by (a) direct and (b) indirect method.

Appendix A4 Colony morphology and Gram's stain of endophytes bacteria isolated from leaves tissue by direct and indirect method.

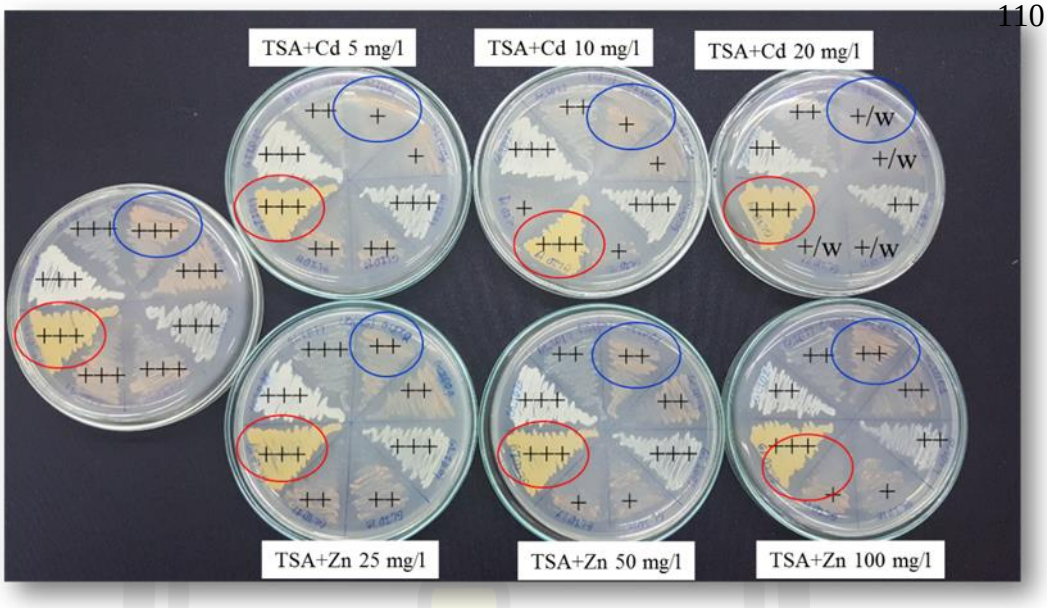
Isolates	Color	Shapes	Margin	Elevation	Surface	Optical	Gram stain,	Shape cell
GLD01	Dark yellow	Circular	Entire	Umbonate	Smooth/glistening	Translucent	Positive/endospore	Rod
GLD02	Yellow	Circular	Entire	Raised	Smooth/glistening	Translucent	Negative	Rod
GLD03	Dark yellow	Circular	Entire	Raised	Smooth/glistening	Translucent	Positive/endospore	Rod
GLD04	Dark yellow	Circular	Entire	Umbonate	Glistening	Translucent	Positive/endospore	Rod
GLD05	White	Circular	Entire	Umbonate	Smooth/glistening	Opaque	Positive/endospore	Rod
GLD06	White	Circular	Entire	Convex	Smooth	Opaque	Negative	Rod
GLD07	White	Irregular	Erose	Flat	Wrinkled	Opaque	Positive/endospore	Rod
GLD08	White	Irregular	Undulate	Pulvinate	Wrinkled	Opaque	Positive/endospore	Rod
GLD09	White	Irregular	Undulate	Flat	Smooth	Translucent	Positive/endospore	Rod
GLID01	Orange-red	Circular	Entire	Umbonate	Smooth/glistening	Translucent	Positive	Rod
GLID02	Red	Circular	Entire	Convex	Smooth	Opaque	Positive/endospore	Rod
GLID03	White-yellow	Circular	Undulate	Raised	Rough	Translucent	Positive/negative	Coccus
GLID04	White	Circular	Entire	Raised	Smooth	Translucent	Negative	Coccus
GLID05	Orange-red	Circular	Entire	Convex	Smooth	Translucent	Positive	Rod
GLID06	Orange-red	Circular	Entire	Convex	Glistening	Translucent	Positive	Rod
GLID07	Orange-red	Circular	Entire	Raised	Smooth	Translucent	Positive	Rod
GLID08	Yellow	Circular	Entire	Convex	Smooth	Opaque	Positive	Rod
GLID09	White	Circular	Entire	Flat	Smooth	Transparent	Positive	Coccus
GLID10	Yellow	Circular	Entire	Raised	Rough	Opaque	Negative	Rod
GLID11	Yellow	Circular	Entire	Raised	Rough	Transparent	Negative	Rod

Appendix A5 Colony morphology and Gram's stain of endophytes bacteria isolated from stem tissue by direct and indirect metho

Isolates	Color	Shapes	Margin	Elevation	Surface	Optical	Gram stain,	Shape cell
GSD01	White	Circular	Entire	Convex	Smooth	Translucent	Negative	Rod
GSD02	Yellow	Circular	Entire	Umbonate	Smooth/glistening	Transparent	Positive/endospore	Rod
GSD03	Yellow	Circular	Entire	Raised	Glistening	Transparent	Positive	Rod
GSD04	White	Circular	Entire	Convex	Smooth	Transparent	Positive	Rod
GSD05	Yellow	Circular	Entire	Raised	Mucoid	Translucent	Positive	Rod
GSD06	White	Circular	Entire	Convex	Smooth	Opaque	Negative	Rod
GSD07	Non	Circular	Erose	Convex	Glistening	Translucent	Negative	Rod
GSD08	Red	Circular	Entire	Umbonate	Glistening	Translucent	Positive	Rod
GSD09	Yellow	Circular	Entire	Raised	Glistening	Transparent	Negative	Rod
GSD10	Yellow	Circular	Entire	Raised	Glistening	Transparent	Negative	Rod
GSD11	Yellow	Circular	Undulate	Raised	Glistening	Transparent	Negative	Rod
GSD12	White	Irregular	Undulate	Pulvinate	Wrinkled	Opaque	Positive/endospore	Rod
GSD13	White	Circular	Entire	Convex	Smooth/glistening	Opaque	Positive/endospore	Rod
GSD14	White	Irregular	Lobate	Flat	Wrinkled	Opaque	Positive/endospore	Rod
GSD15	White	Circular	Entire	Pulvinate	Smooth/glistening	Translucent	Negative	Rod
GSD16	Dark yellow	Circular	Entire	Convex	Smooth/glistening	Transparent	Negative	Rod
GSD17	Cream	Circular	Entire	Convex	Mucoid	Translucent	Negative	Rod
GSD18	White-yellow	Circular	Entire	Pulvinate	Smooth/glistening	Translucent	Negative	Rod
GSD19	White	Irregular	Lobate	Flat	Wrinkled	Opaque	Positive/endospore	Rod
GSD20	Non	Irregular	Erose	Raised	Smooth	Transparent	Positive	Rod
GSID01	White	Circular	Entire	Raised	Smooth/glistening	Translucent	Positive/endospore	Rod
GSID02	Orange-red	Circular	Entire	Convex	Smooth	Opaque	Positive/endospore	Rod
GSID03	Pink	Circular	Entire	Convex	Smooth	Translucent	Negative	Rod
GSID04	Pink-red	Irregular	Undulate	Umbonate	Wrinkled	Opaque	Positive	Rod
GSID05	Yellow	Circular	Entire	Raised	Smooth/glistening	Transparent	Negative	Rod
GSID06	Yellow	Circular	Entire	Flat	Smooth/glistening	Transparent	Negative	Rod
GSID07	Orange-cream	Irregular	Lobate	Umbonate	Wrinkled	Opaque	Positive	Coccus

Appendix A6 Colony morphology and Gram's stain of endophytes bacteria isolated from tuber tissue by direct and indirect method

Isolates	Color	Shapes	Margin	Elevation	Surface	Optical	Gram stain,	Shape cell
GTD01	Cream	Circular	Entire	Raised	Glistening	Translucent	Negative	Rod
GTD02	White	Irregular	Undulate	Flat	Smooth	Opaque	Positive	Rod
GTD03	Yellow	Circular	Entire	Convex	Smooth	Translucent	Negative	Rod
GTD04	Cream	Circular	Entire	Convex	Smooth	Opaque	Positive	Rod
GTD05	White	Circular	Entire	Raised	Rough	Opaque	Negative	Rod
GTD06	White	Circular	Entire	Raised	Smooth	Opaque	Positive	Rod
GTID01	Cream	Circular	Entire	Convex	Smooth/glistening	Translucent	Negative	Rod
GTID02	Yellow	Circular	Entire	Umbonate	Glistening	Transparent	Positive	Rod
GTID03	White	Circular	Entire	Convex	Glistening	Transparent	Positive/endospore	Rod
GTID04	White	Circular	Undulate	Umbonate	Smooth	Translucent	Positive/endospore	Rod
GTID05	White	Circular	Entire	Convex	Glistening	Translucent	Positive/endospore	Rod
GTID06	White	Circular	Entire	Convex	Smooth/glistening	Translucent	Negative	Rod
GTID07	Yellow	Circular	Entire	Flat	Smooth/glistening	Transparent	Positive/endospore	Rod
GTID08	White	Irregular	Lobate	Flat	Rough	Translucent	Positive/endospore	Rod
GTID09	Non	Circular	Entire	Flat	Glistening	Transparent	Negative	Rod
GTID10	Non	Circular	Entire	Umbonate	Glistening	Transparent	Negative	Rod
GTID11	Cream	Circular	Entire	Raised	Glistening	Transparent	Positive/endospore	Rod
GTID12	Yellow	Circular	Entire	Convex	Glistening	Transparent	Negative	Rod
GTID13	Dark yellow	Circular	Entire	Convex	Mucoid	Translucent	Negative	Rod
GTID14	White	Circular	Entire	Raised	Smooth/glistening	Opaque	Positive	Coccus
GTID15	Non	Circular	Entire	Flat	Glistening	Transparent	Negative	Rod
GTID16	Yellow	Circular	Entire	Flat	Glistening	Transparent	Negative	Rod
GTID17	Yellow	Circular	Entire	Raised	Glistening	Transparent	Negative	Rod
GTID18	Yellow	Circular	Entire	Umbonate	Smooth	Transparent	Positive/endospore	Rod
GTID19	Yellow	Circular	Entire	Raised	Glistening	Transparent	Positive	Rod
GTID20	Cream	Circular	Entire	Convex	Smooth/glistening	Translucent	Negative	Rod



Appendix A7 Example recording of bacterial isolates were streaked on TSA plate containing Zn concentrations (25, 20, 100 and 200 mg/l) and Cd concentrations (5, 10, 20 and 50 mg/l). (Note: – = no growth, += poor growth, ++ = moderate growth, +++ = high growth and w=weak)

Appendix A8 Metal resistance of endophytes bacterial isolated from leaves tissue

Isolates	Control	Zn (mg/l)				Cd (mg/l)			
		25	50	100	200	5	10	20	50
GLD01	+++	+++	+++	++	-	+++	+++	++	+/w
GLD02	+++	+++	+++	+++	+++	+++	++	++	++
GLD03	+++	++	++	++	++	++	++	++	++
GLD04	+++	+++	++	++	+/w	+++	+++	++	+/w
GLD05	+++	+++	++	+	-	+++	+++	++	+
GLD06	+++	+++	+++	++	++	+++	+++	++	++
GLD07	+++	++	++	+	-	+/w	-	-	-
GLD08	+++	++	++	+	-	+/w	-	-	-
GLD09	+++	+++	+++	++	++	++	++	++	++

Note: Control (TSA no metal), – = no growth, += poor growth, ++ = moderate growth, +++ = high growth and w=weak

Appendix A8 (Cont.)

Isolates	Control	Zn (mg/l)				Cd (mg/l)			
		25	50	100	200	5	10	20	50
GLID01	+++	++	++	++	++	+	++	+	+/w
GLID02	+++	++	++	++	++	++	++	++	+
GLID03	+++	+++	+++	++	+	+++	++	++	++
GLID04	+++	+++	+++	++	+/w	+++	+++	++	++
GLID05	+++	++	+	+	+	++	+	+/w	+/w
GLID06	+++	++	+	+	+/w	+	+	+	+/w
GLID07	+++	++	++	++	++	+++	+	+	+/w
GLID08	+++	++	++	++	+/w	+++	+++	++	++
GLID09	+++	+	+	+/w	+/w	++	+	+	+/w
GLID10	+++	+	+	+/w	+/w	+	+	+	+/w

Note: Control (TSA no metal), – = no growth, += poor growth, ++ = moderate growth, +++ = high growth and w=weak

Appendix A9 Metal resistance of endophytes bacterial isolated from stem tissue

Isolates	Control	Zn (mg/l)				Cd (mg/l)			
		25	50	100	200	5	10	20	50
GSD01	+++	+++	+++	+++	++	+++	+++	+++	++
GSD02	+++	+++	+++	+++	+++	+++	+++	+++	+++
GSD03	+++	++	++	++	+	++	++/w	+/w	+/w
GSD04	+++	+++	+++	+++	+++	+++	+++	+++	+++
GSD05	+++	++	+	+/w	-	+/w	+/w	+/w	+/w
GSD06	+++	+++	+++	+++	++/w	+++	+++	+++	++
GSD07	+++	+++	+++	+++	++	+++	+++	+++	+++
GSD08	+++	++	++	++	+	+++	++	++	+/w
GSD09	+++	++	++	++	+	++	++	+	-
GSD10	+++	+++	+++	+++	++	+++	+++	+++	++
GSD11	+++	++	+	+	+/w	++	+	+/w	+/w

Note: Control (TSA no metal), – = no growth, += poor growth, ++ = moderate growth, +++ = high growth and w=weak

Appendix A9 (Cont.)

Isolates	Control	Zn (mg/l)				Cd (mg/l)			
		25	50	100	200	5	10	20	50
GSD12	+++	+++	+++	++/w	-	+	+/w	-	-
GSD13	+++	++	++	+/w	+/w	+	+	-	-
GSD14	+++	++	++	+	-	+	+/w	+/w	-
GSD15	+++	+/w	+/w	+/w	+/w	+/w	+/w	-	-
GSD16	+++	+++	+++	+++	+/w	+++	++	++	+/w
GSD17	+++	++	++	+	+/w	+++	++	++/w	+/w
GSD18	+++	++	++	++	+	++	+	+/w	+/w
GSD19	+++	++	+	+	+/w	+	+/w	-	-
GSD20	+++	++	++	+	-	+	+/w	-	-
GSID01	+++	++	++	++	+/w	++	++	++	+/w
GSID02	+++	++	++	++	+/w	++	+	+/w	+/w
GSID03	+++	+++	+++	+++	++	++	++	++	++
GSID04	+++	+++	++	+	+	++	++	+	+
GSID05	+++	+++	+++	+++	+	+++	+++	+++	+++
GSID06	+++	++	++	++	++	+++	+++	++	+
GSID07	+++	+/w	+/w	+/w	+/w	+/w	+/w	+/w	+/w

Note: Control (TSA no metal), – = no growth, += poor growth, ++ = moderate growth, +++ = high growth and w=weak

Appendix A10 Metal resistance of endophytes bacterial isolated from tuber tissue

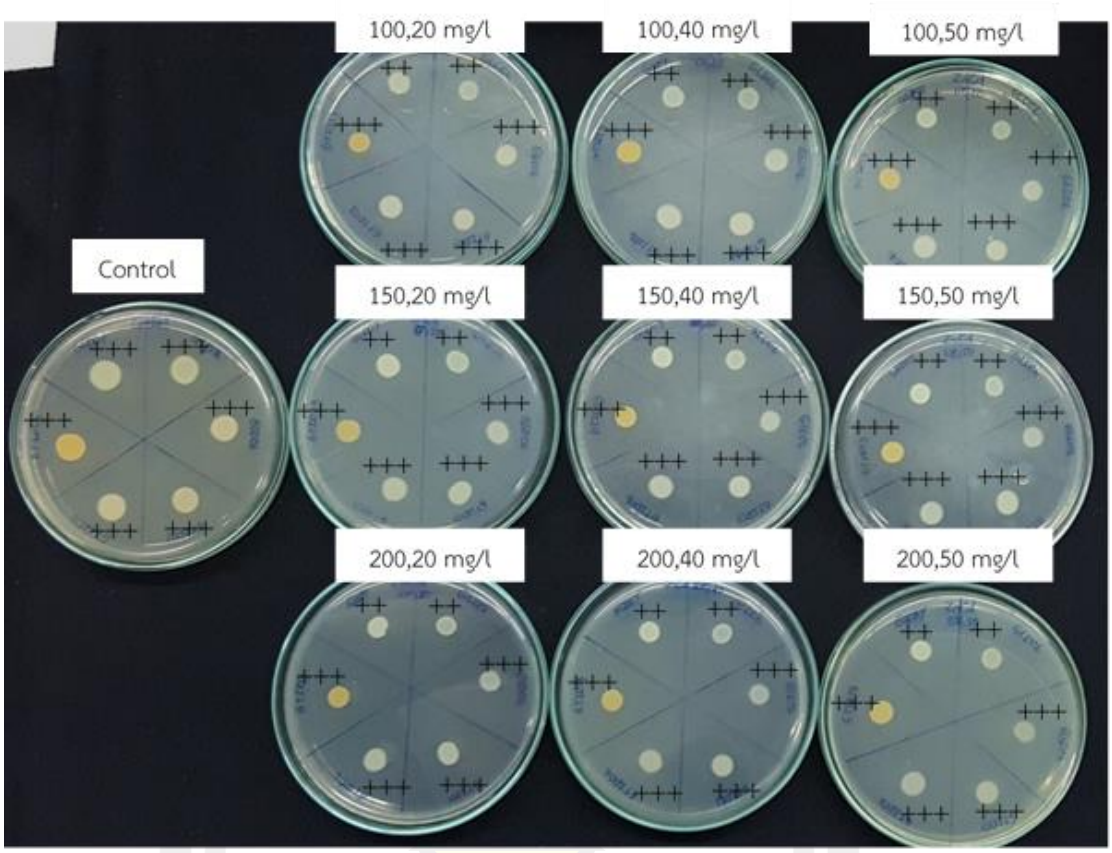
Isolates	Control	Zn (mg/l)				Cd (mg/l)			
		25	50	100	200	5	10	20	50
GTD01	+++	++	++	++	++	+++	+++	++	++
GTD02	+++	+++	+++	-	-	+++	-	-	-
GTD03	+++	++	++	++	++	+++	+++	+++	+++
GTD04	+++	+/w	+/w	-	-	+++	-	-	-
GTD05	+++	+++	+/w	+/w	+/w	-	-	-	-
GTD06	+++	+++	+/w	+/w	+/w	+++	+/w	-	-
GTID01	+++	+++	+++	+++	+++	+++	+++	+++	+++
GTID02	+++	+++	++	++	++	++	++	++	++
GTID03	+++	+++	++	++	++	++	++	++	++
GTID04	+++	+++	++	++	++	+++	++	++	++
GTID05	+++	++	++	++	++	++	++	++	++
GTID06	+++	+++	+++	+++	++	+++	+++	+++	+++
GTID07	+++	++	++	+	+	++	++	++	+

Note: Control (TSA no metal), – = no growth, += poor growth, ++ = moderate growth, +++ = high growth and w=weak

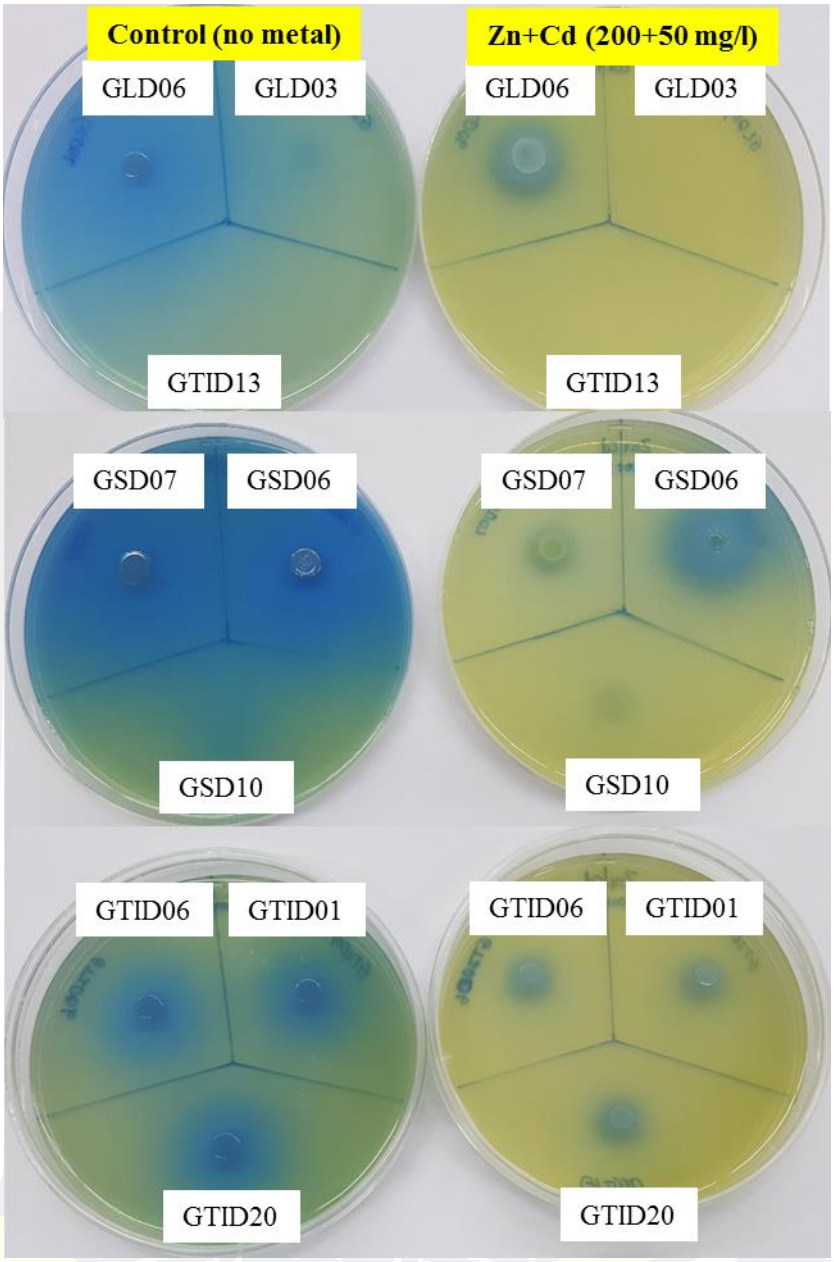
Appendix A10 (Cont.)

Isolates	Control	Zn (mg/l)				Cd (mg/l)			
		25	50	100	200	5	10	20	50
GTID08	+++	++	++	++	+/w	++	++	++	+/w
GTID09	+++	+	+	+	+	+	+	+	+
GTID10	+++	+++	+	+	+/w	+/w	+	+	+
GTID11	+++	+	+	+	+	+	+	+	+
GTID12	+++	+++	++	++	+	++	++	++	+
GTID13	+++	+++	+++	+++	+++	+++	+++	+++	++
GTID14	+++	+++	+++	++	+	+++	+++	+++	++
GTID15	+++	+++	++	++	+	+++	+++	++	+
GTID16	+++	+++	+++	+++	+++	+++	++	++	++
GTID17	+++	++	++	++	+	+++	+++	++	+
GTID18	+++	+++	+++	+++	+++	+++	+++	+++	+++
GTID19	+++	+	+	+	+	+	+	+	+
GTID20	+++	+++	+++	+++	+++	+++	+++	+++	+++

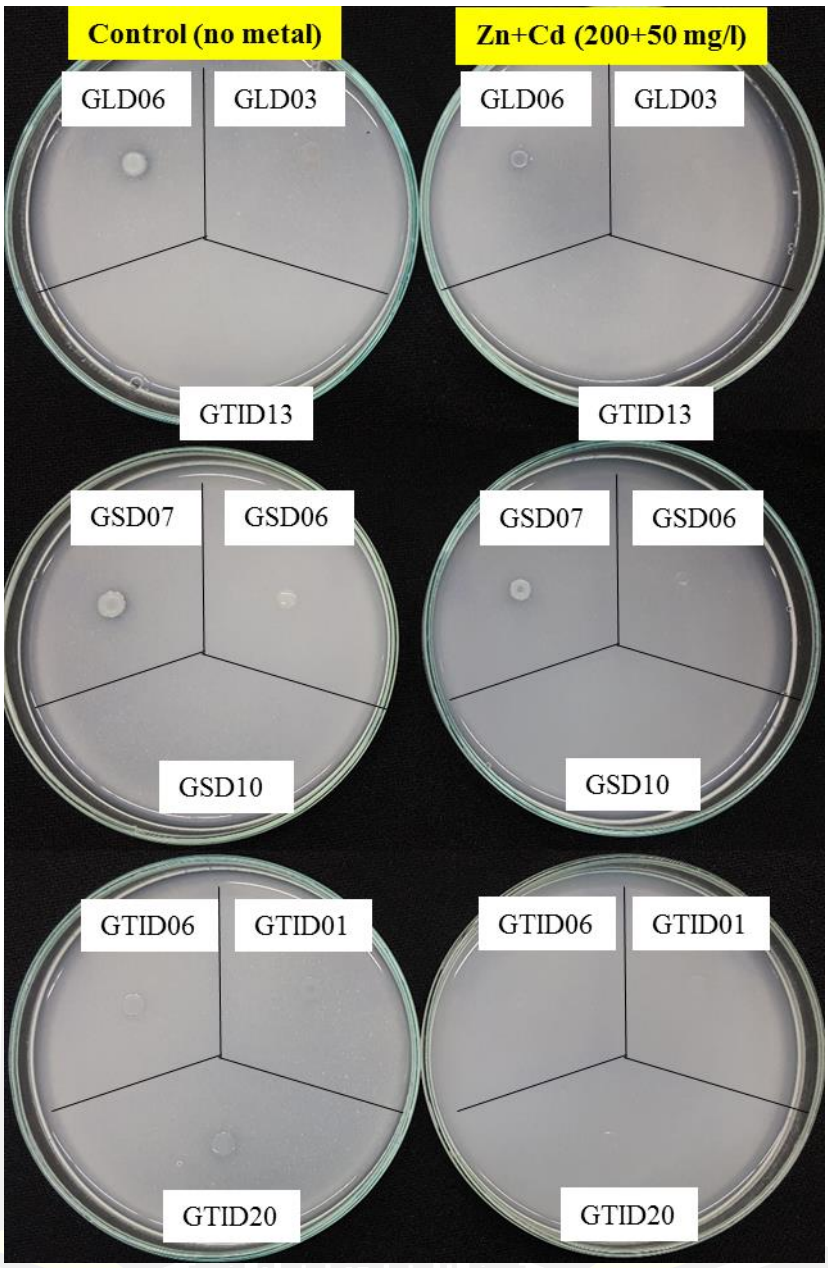
Note: Control (TSA no metal), – = no growth, += poor growth, ++ = moderate growth, +++ = high growth and w=weak



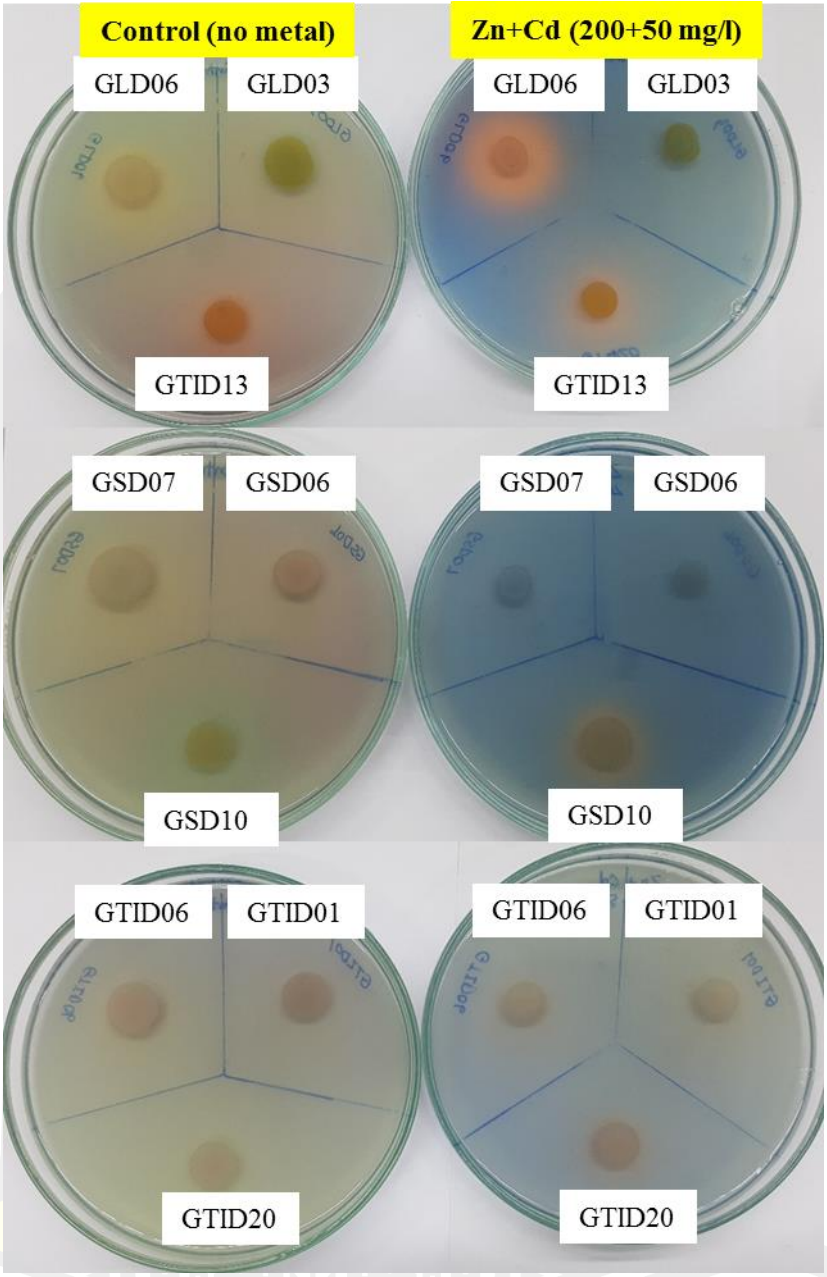
Appendix A11 Example recording of bacterial isolates were streaked on TSA plate containing 20, 40 and 50 mg/l Cd plus 100, 150 and 200 mg/l Zn. (Note: Diameter colony; Control (TSA no metal), ≤ 0.2 mm = +++, Control > 0.2 mm = ++, Control >0.4 mm = +, no growth= -, w = weak growth)



Appendix A12 N_2-fixation properties of endophytes bacterial isolated from *G. pseudochina* under control (no metal) and Zn and Cd contamination (200+50 mg/l)

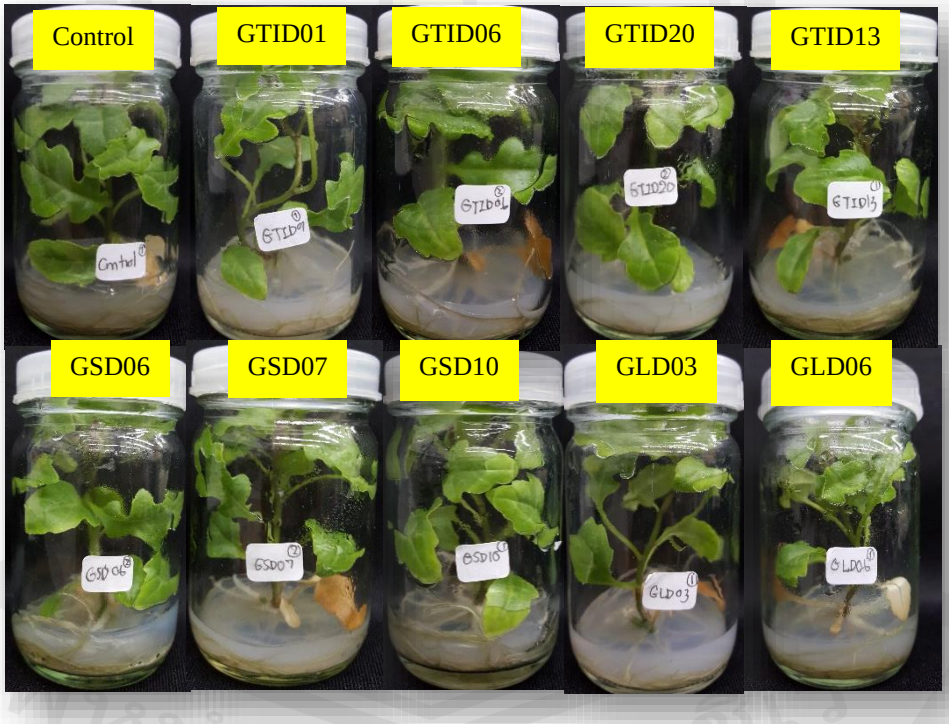


Appendix A13 P-solubilization properties of endophytes bacterial isolated from *G. pseudochina* under control (no metal) and Zn and Cd contamination (200+50 mg/l)

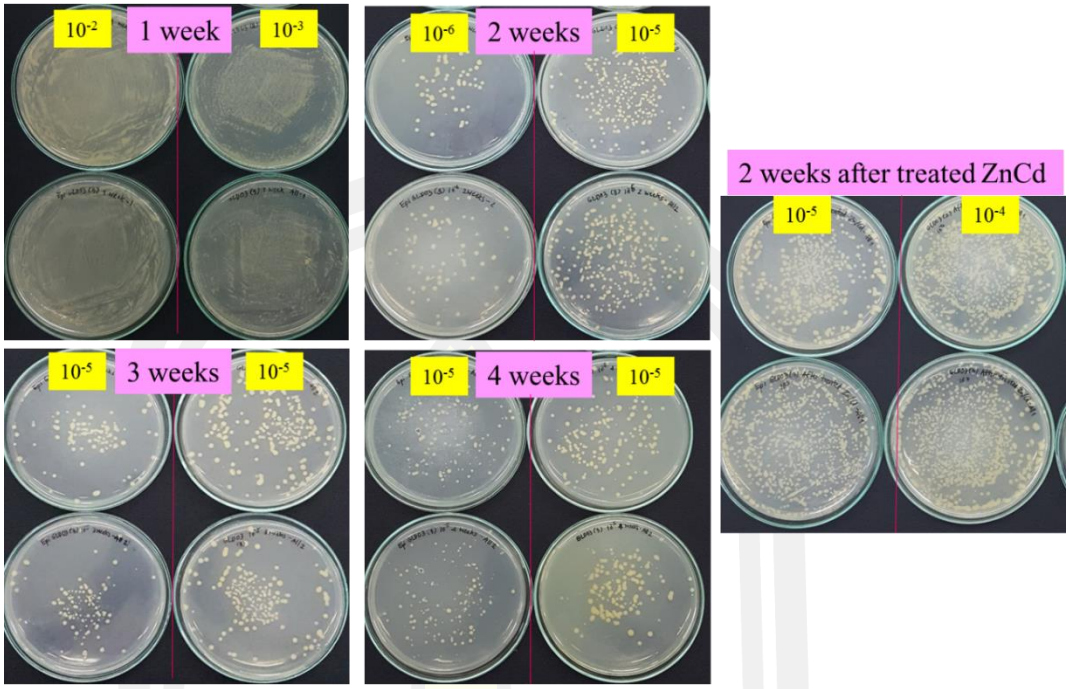


Appendix A14 Siderophore production properties of endophytes bacterial isolated from *G. pseudochina* under control (no metal) and Zn and Cd contamination (200+50 mg/l)

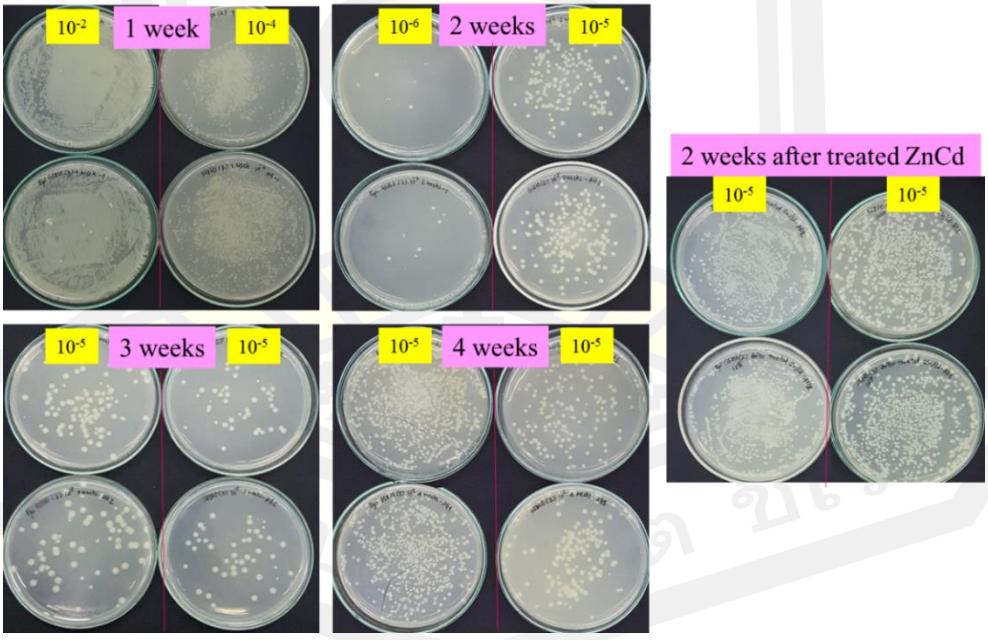
Appendix A15 Pathogenic test with leaf of *G. pseudochina* plants.



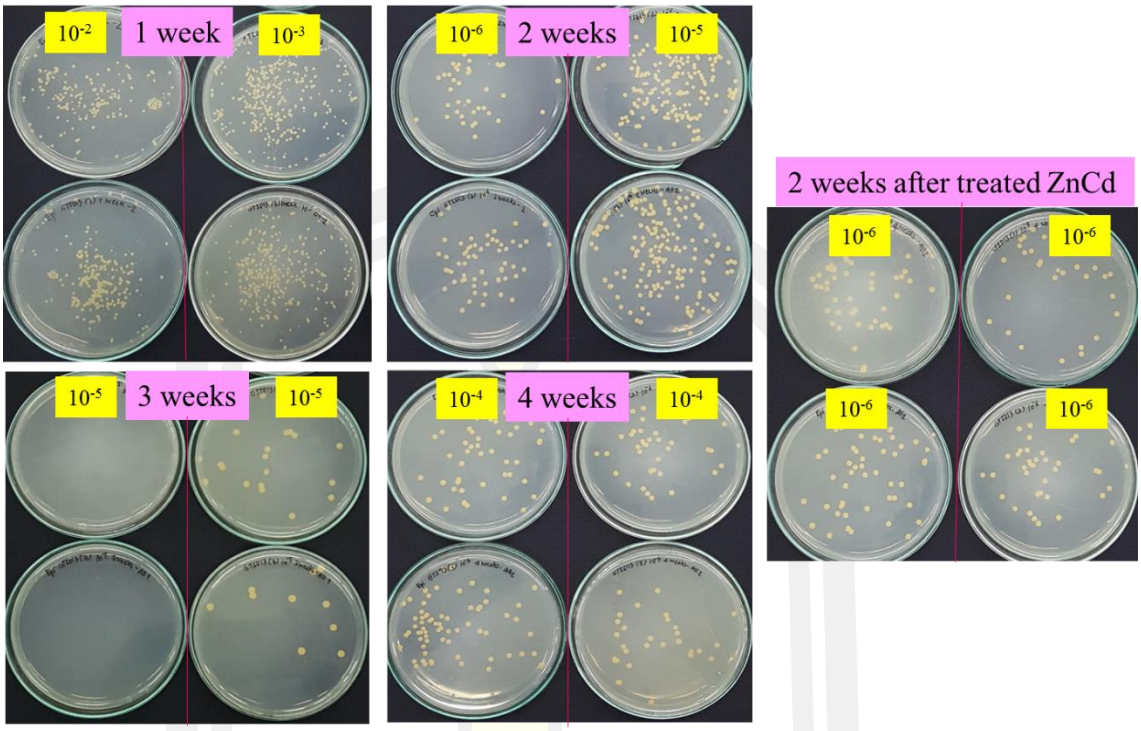
Appendix A16 Pathogenic test with *G. pseudochina* plants in tissue culture system.



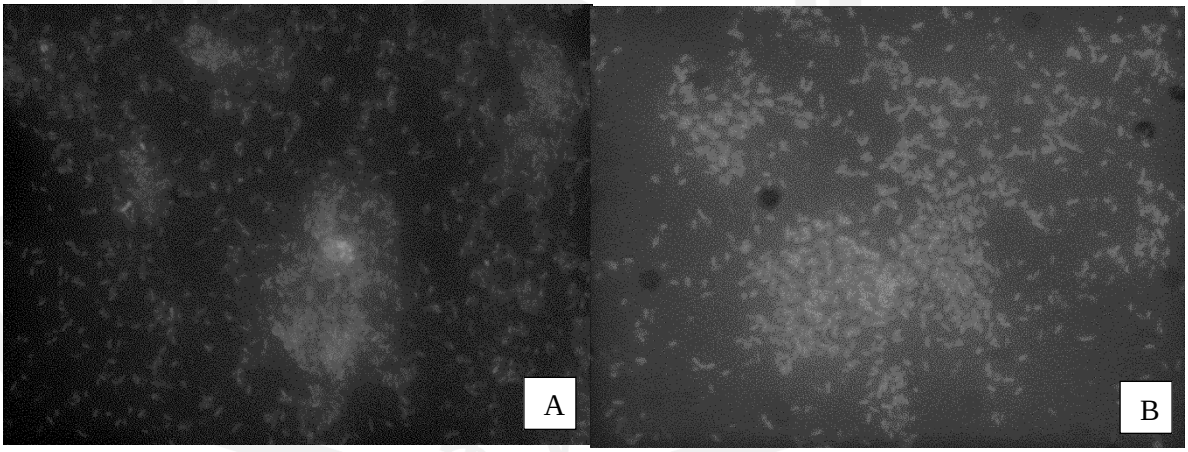
Appendix A17 Colonization of *Curtobacterium* sp. GLD03 for 1, 2, 3, 4 and 2 weeks after treated Zn 100 mg/l and Cd 15 mg/l in one-month old *G. pseudochina* plants.



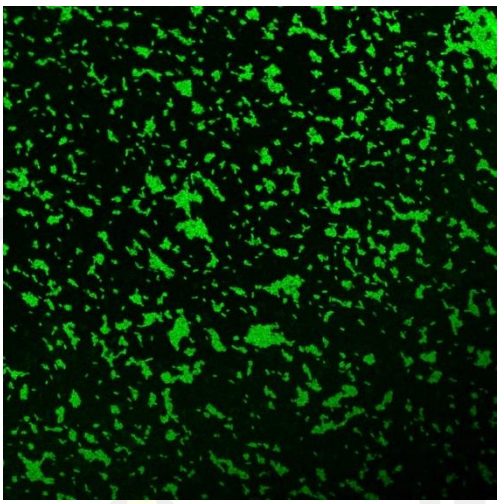
Appendix A18 Colonization of *Stenotrophomonas sp.* GSD10 for 1, 2, 3, 4 and 2 weeks after treated Zn 100 mg/l and Cd 15 mg/l in one-month old *G. pseudochina* plants.



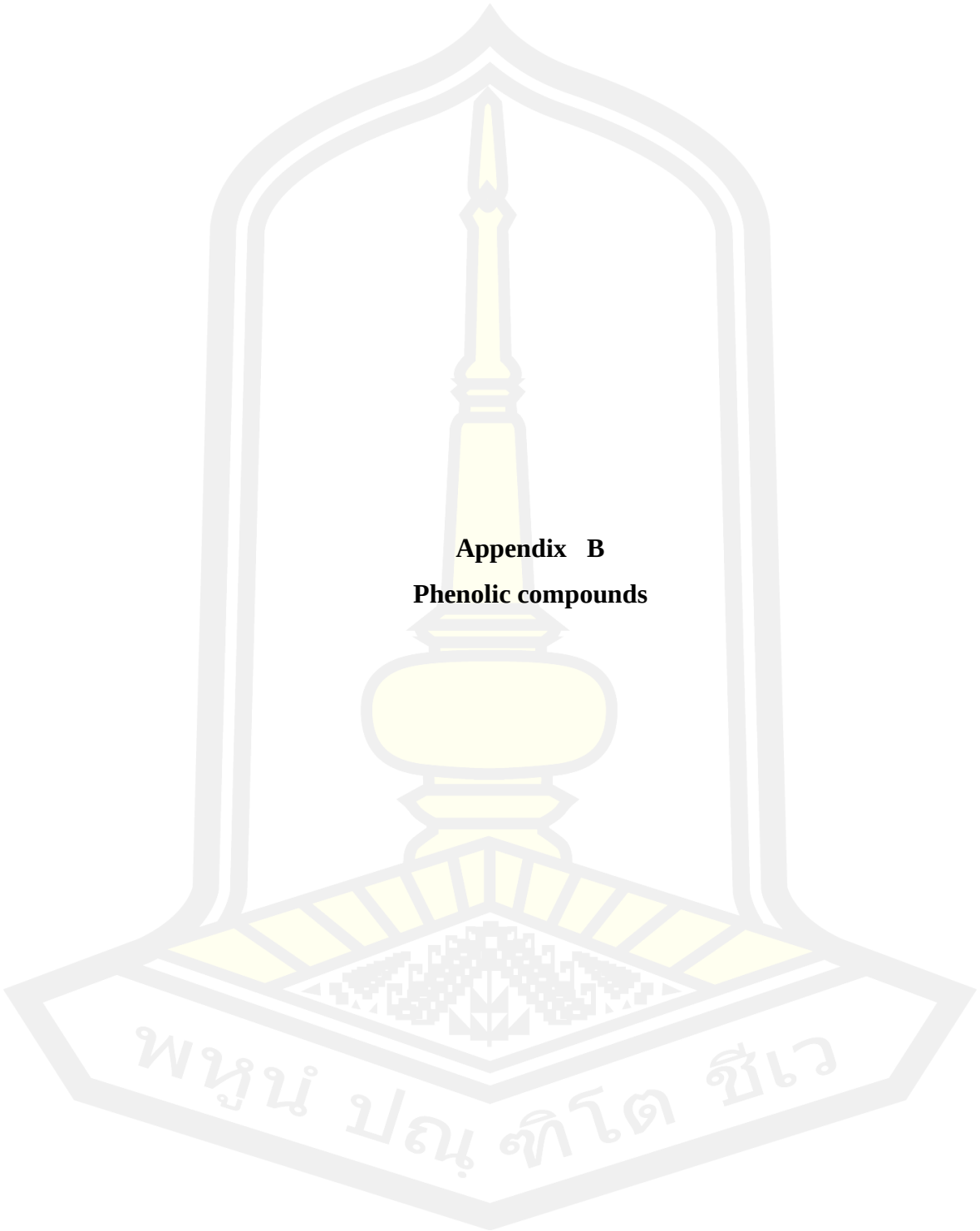
Appendix A19 Colonization of *Chryseobacterium* sp. GTID13 for 1, 2, 3, 4 and 2weeks after treated Zn 100 mg/l and Cd 15 mg/l in one-month old *G. pseudochina* plants.



Appendix A20 *Stenotrophomonas* sp. GSD10 cells transform with AKN100 plasmid growth on LB broth supplemented 34 µg/ml Cm, under fluorescence microscope. (A) exposed 5.6 ms and (B) exposed 10.1 ms.

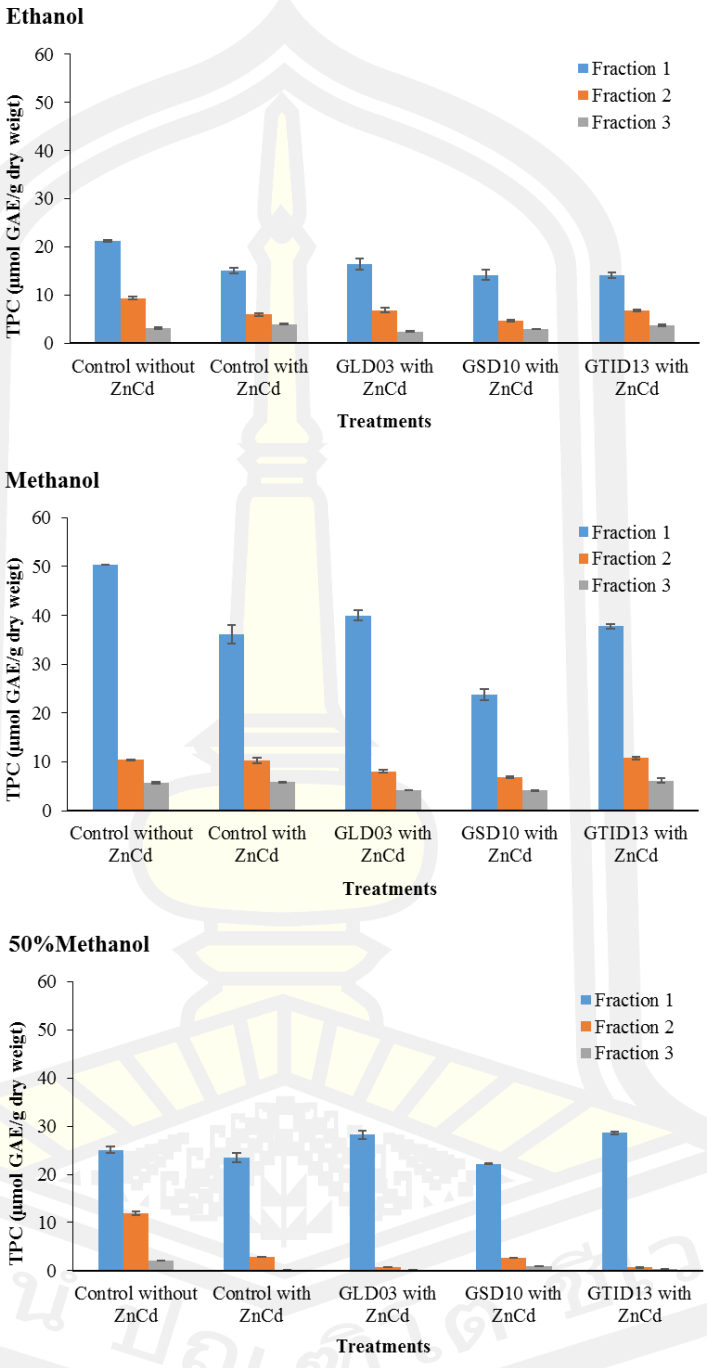


Appendix A21 Confocal image of GSD10 cells transform with AKN100 plasmid under confocal microscope (60X), 524-594nm.

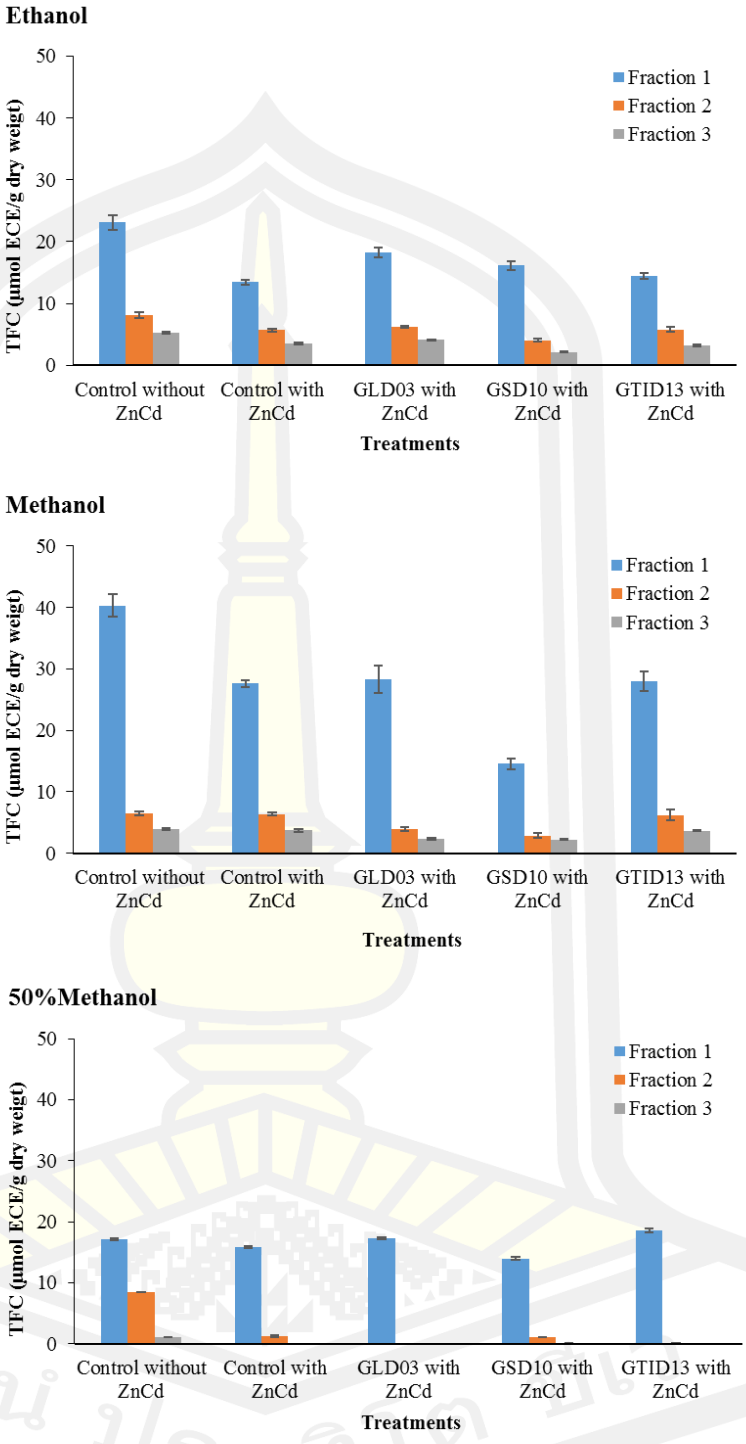


Appendix B

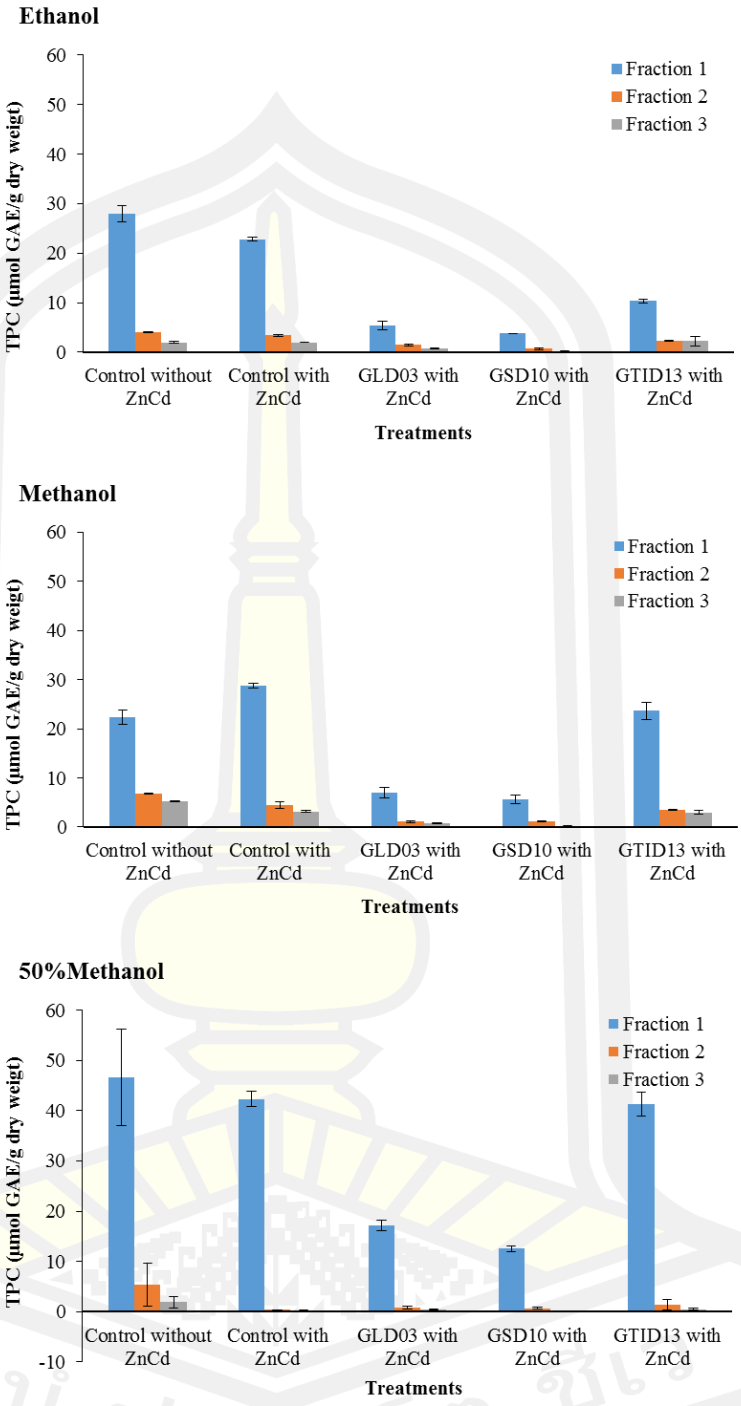
Phenolic compounds



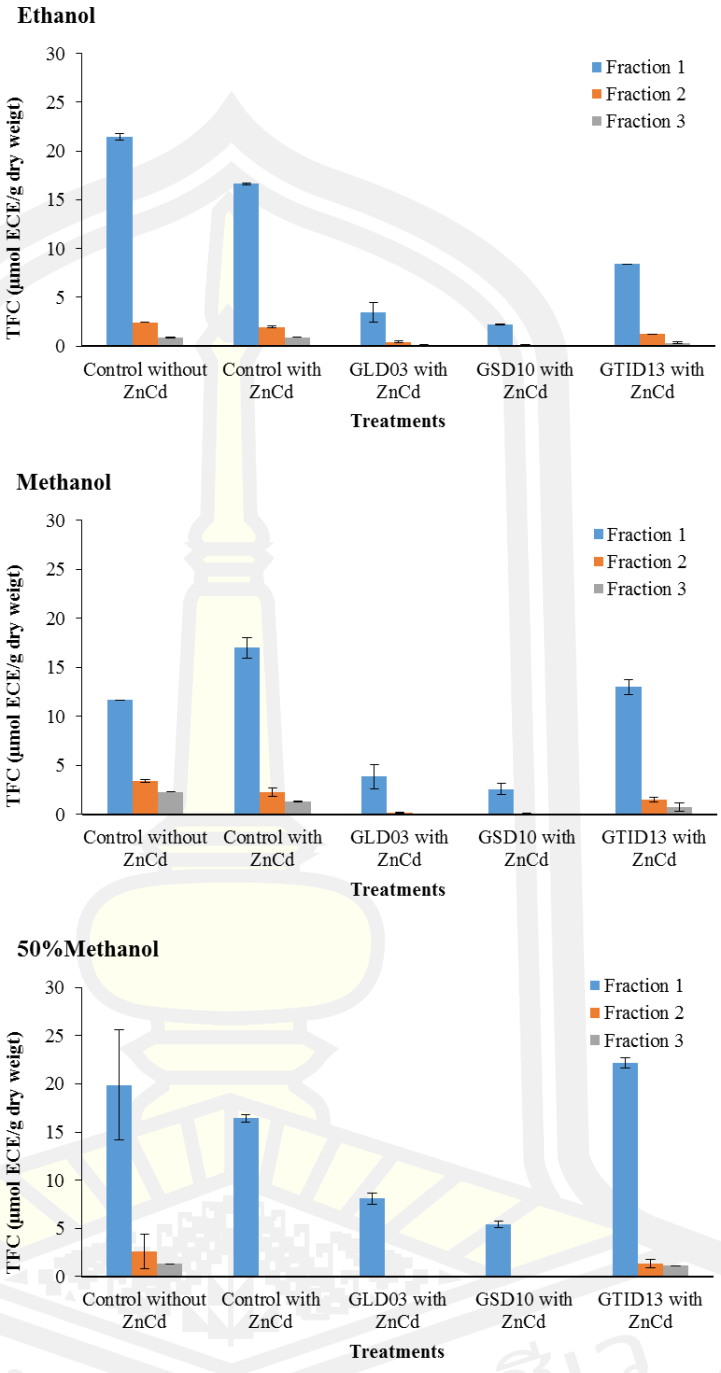
Appendix B1 Total phenolic content (TPC) in each fraction, collected from the fraction order of three solvents of 99.9% (v/v) ethanol, 99.9% (v/v) methanol, and 50% (v/v) methanol in leaf extracts of *G. pseudochina* in plant tissue system. Fractions 1, 2 and 3 mean 1st, 2nd and 3rd repeat extractions with the same solvent. The data are given as the means ± SD (n = 3).



Appendix B2 Total flavonoid content (TFC) in each fraction, collected from the fraction order of three solvents of 99.9% (v/v) ethanol, 99.9% (v/v) methanol, and 50% (v/v) methanol in leaf extracts of *G. pseudochina* in plant tissue system. Fractions 1, 2 and 3 mean 1st, 2nd and 3rd repeat extractions with the same solvent. The data are given as the means ± SD (n = 3).



Appendix B3 Total phenolic content (TPC) in each fraction, collected from the fraction order of three solvents of 99.9% (v/v) ethanol, 99.9% (v/v) methanol, and 50% (v/v) methanol in leaf extracts of *G. pseudochina* in pot system. Fractions 1, 2 and 3 mean 1st, 2nd and 3rd repeat extractions with the same solvent. The data are given as the means ± SD (n = 3).



Appendix B4 Total flavonoid content (TFC) in each fraction, collected from the fraction order of three solvents of 99.9% (v/v) ethanol, 99.9% (v/v) methanol, and 50% (v/v) methanol in leaf extracts of *G. pseudochina* in pot system. Fractions 1, 2 and 3 mean 1st, 2nd and 3rd repeat extractions with the same solvent. The data are given as the means ± SD (n = 3).

Appendix C
Statistical analysis

Appendix C1 One-way ANOVA analysis for fresh and dry weight of *G. pseudochina* leaves after treatment with Zn plus Cd for two weeks in tissue culture system.

ANOVA

Fresh weight

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.163	4	.291	2.815	.084
Within Groups	1.033	10	.103		
Total	2.195	14			

Duncan

Treatments	N	Subset for alpha = .01
		1
Control with ZnCd	3	.5007
GTID13 with ZnCd	3	.5412
GLD03 with ZnCd	3	.5978
GSD10 with ZnCd	3	.6033
Control without ZnCd	3	1.2503
Sig.		.025

ANOVA

Dry weight

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.012	4	.003	.711	.603
Within Groups	.043	10	.004		
Total	.056	14			

Appendix C1 (Cont.)

Duncan

Treatments	N	Subset for alpha = .01
		1
Control with ZnCd	3	.0797
GTID13 with ZnCd	3	.0992
GSD10 with ZnCd	3	.1026
GLD03 with ZnCd	3	.1113
Control without ZnCd	3	.1649
Sig.		.174

Appendix C2 One-way ANOVA analysis for fresh and dry weight of *G. pseudochina* stem after treatment with Zn plus Cd for two weeks in tissue culture system.

ANOVA

Fresh weight

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.007	4	.002	.278	.886
Within Groups	.062	10	.006		
Total	.069	14			

Duncan

Treatments	N	Subset for alpha = .01
		1
GLD03 with ZnCd	3	.1328
GSD10 with ZnCd	3	.1401
Control with ZnCd	3	.1474
GTID13 with ZnCd	3	.1534
Control without ZnCd	3	.1943
Sig.		.399

Appendix C2 (Cont.)

ANOVA

Dry weight

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	4	.000	.496	.739
Within Groups	.002	10	.000		
Total	.002	14			

Duncan

Treatments	N	Subset for alpha = .01
		1
Control without ZnCd	3	.0160
GSD10 with ZnCd	3	.0204
GLD03 with ZnCd	3	.0222
Control with ZnCd	3	.0239
GTID13 with ZnCd	3	.0300
Sig.		.238

Appendix C3 One-way ANOVA analysis for fresh and dry weight of *G. pseudochina* root after treatment with Zn plus Cd for two weeks in tissue culture system.

ANOVA

Fresh weight

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.979	4	.245	1.120	.400
Within Groups	2.184	10	.218		
Total	3.163	14			

Appendix C3 (Cont.)

Duncan

Treatments	N	Subset for alpha = .01
		1
GLD03 with ZnCd	3	.6891
GSD10 with ZnCd	3	.7393
Control with ZnCd	3	.7901
GTID13 with ZnCd	3	.8523
Control without ZnCd	3	1.3918
Sig.		.121

ANOVA

Dry weight

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.001	4	.000	.253	.901
Within Groups	.006	10	.001		
Total	.006	14			

Duncan

Treatments	N	Subset for alpha = .01
		1
GLD03 with ZnCd	3	.0510
GTID13 with ZnCd	3	.0613
Control with ZnCd	3	.0651
GSD10 with ZnCd	3	.0662
Control without ZnCd	3	.0683
Sig.		.425

Appendix C4 One-way ANOVA analysis for Zn accumulation of *G. pseudochina* leaves, stems and roots after treatment with Zn plus Cd for two weeks in tissue culture system.

ANOVA

Leaves

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.692	4	.173	8.653	.003
Within Groups	.200	10	.020		
Total	.891	14			

Duncan

Treatments	N	Subset for alpha = .01	
		1	2
Control without ZnCd	3	.0373	
Control with ZnCd	3		.4092
GLD03 with ZnCd	3		.4383
GTID13 with ZnCd	3		.5695
GSD10 with ZnCd	3		.6677
Sig.		1.000	.063

ANOVA

Stems

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	61.406	4	15.351	8.142	.003
Within Groups	18.855	10	1.885		
Total	80.260	14			

Appendix C4 (Cont.)

Duncan

Treatments	N	Subset for alpha = .01	
		1	2
Control without ZnCd	3	.1784	
GTID13 with ZnCd	3		4.0482
Control with ZnCd	3		4.5605
GLD03 with ZnCd	3		4.8860
GSD10 with ZnCd	3		6.1833
Sig.		1.000	.106

ANOVA

Roots

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	83.933	4	20.983	27.610	.000
Within Groups	7.600	10	.760		
Total	91.532	14			

Duncan

Treatments	N	Subset for alpha = .01		
		1	2	3
Control without ZnCd	3	.1189		
GLD03 with ZnCd	3		3.7768	
Control with ZnCd	3		5.2475	5.2475
GSD10 with ZnCd	3			6.2729
GTID13 with ZnCd	3			6.6441
Sig.		1.000	.066	.090

Appendix C5 One-way ANOVA analysis for Cd accumulation of *G. pseudochina* leaves, stems and roots after treatment with Zn plus Cd for two weeks in tissue culture system.

ANOVA

Leaves

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.004	4	.001	7.418	.005
Within Groups	.001	10	.000		
Total	.005	14			

Duncan

Treatments	N	Subset for alpha = .01	
		1	2
Control without ZnCd	3	.0023	
GTID13 with ZnCd	3		.0329
Control with ZnCd	3		.0373
GSD10 with ZnCd	3		.0436
GLD03 with ZnCd	3		.0438
Sig.		1.000	.281

ANOVA

Stems

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.321	4	.080	5.068	.017
Within Groups	.158	10	.016		
Total	.479	14			

Appendix C5 (Cont.)

Duncan

Treatments	N	Subset for alpha = .01	
		1	2
Control without ZnCd	3	.0120	
Control with ZnCd	3	.3184	.3184
GTID13 with ZnCd	3	.3392	.3392
GSD10 with ZnCd	3		.3987
GLD03 with ZnCd	3		.4116
Sig.		.012	.416

ANOVA

Roots

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.531	4	.883	13.249	.001
Within Groups	.666	10	.067		
Total	4.197	14			

Duncan

Treatments	N	Subset for alpha = .01	
		1	2
Control without ZnCd	3	.0029	
GLD03 with ZnCd	3		.9007
Control with ZnCd	3		1.0098
GSD10 with ZnCd	3		1.2999
GTID13 with ZnCd	3		1.3482
Sig.		1.000	.076

Appendix C6 One-way ANOVA analysis for TPC and TFC in sum all fraction (1, 2 and 3) extract of *G. pseudochina* leaves in tissue culture system.

ANOVA

TPC

		Sum of Squares	df	Mean Square	F	Sig.
TPC	Between Groups	5001.019	4	1250.255	179.257	.000
	Within Groups	69.747	10	6.975		
	Total	5070.766	14			

Duncan

Treatments	N	Subset for alpha = 0.01		
		1	2	3
GSD10 with ZnCd	3	82.320		
Control with ZnCd	3		103.633	
GLD03 with ZnCd	3		107.283	
GTID13 with ZnCd	3		109.063	
Control without ZnCd	3			139.423
Sig.		1.000	.037	1.000

ANOVA

TFC

		Sum of Squares	df	Mean Square	F	Sig.
TFC	Between Groups	4943.530	4	1235.883	131.220	.000
	Within Groups	94.184	10	9.418		
	Total	5037.714	14			

Appendix C6 (Cont.)

Duncan

Treatments	N	Subset for alpha = 0.01		
		1	2	3
GSD10 with ZnCd	3	57.280		
Control with ZnCd	3		77.470	
GTID13 with ZnCd	3		79.976	
GLD03 with ZnCd	3		80.373	
Control without ZnCd	3			113.776
Sig.		1.000	.295	1.000

Appendix C7 One-way ANOVA analysis for TPC and TFC in sum all fraction (1, 2 and 3) extract of *G. pseudochina* leaves in tissue culture system. The percolation and partition methods were performed by 99.9% (v/v) ethanol, 99.9% (v/v) methanol and 50% (v/v) methanol.

ANOVA

TPC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7987.491	14	570.535	496.371	.000
Within Groups	34.482	30	1.149		
Total	8021.973	44			

ANOVA

TFC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4357.235	14	311.231	170.786	.000
Within Groups	54.670	30	1.822		
Total	4411.905	44			

Appendix C7 (Cont.)

TPC

Duncan

Treatments	N	Subset for alpha = .01							
		1	2	3	4	5	6	7	8
GSD10 with ZnCd ethanol	3	21.793							
GTID13 with ZnCd ethanol	3		24.753						
Control with ZnCd ethanol	3		24.936						
GSD10 with ZnCd 50methanol	3		25.770						
GLD03 with ZnCd ethanol	3		25.810						
Control with ZnCd 50methanol	3		26.513						
GLD03 with ZnCd 50methanol	3			29.173					
GTID13 with ZnCd 50methanol	3			29.583					
Control without ZnCd ethanol	3				33.840				
GSD10 with ZnCd methanol	3				34.756				
Control without ZnCd 50methanol	3					39.060			
Control with ZnCd methanol	3						52.180		
GLD03 with ZnCd methanol	3						52.296		
GTID13 with ZnCd methanol	3							54.726	
Control without ZnCd methanol	3								66.530
Sig.		1.000	.081	.643	.303	1.000	.895	1.000	1.000

Appendix C7 (Cont.)

TFC

Duncan

Treatments	N	Subset for alpha = .01							
		1	2	3	4	5	6	7	8
GSD10 with ZnCd 50methanol	3	15.226							
Control with ZnCd 50methanol	3	17.063	17.063						
GLD03 with ZnCd 50methanol	3	17.276	17.276						
GTID13 with ZnCd 50methanol	3		18.600						
GSD10 with ZnCd methanol	3		19.646	19.646					
GSD10 with ZnCd ethanol	3			22.403	22.403				
Control with ZnCd ethanol	3			22.623	22.623				
GTID13 with ZnCd ethanol	3				23.503				
Control without ZnCd 50methanol	3					26.610			
GLD03 with ZnCd ethanol	3					28.556			
GLD03 with ZnCd methanol	3						34.536		
Control without ZnCd ethanol	3						36.490	36.490	
Control with ZnCd methanol	3							37.780	
GTID13 with ZnCd methanol	3							37.876	
Control without ZnCd methanol	3								50.673
Sig.		.088	.038	.015	.355	.088	.087	.245	1.000

Appendix C8 One-way ANOVA analysis for TPC, TFC, crude content and IC50 in sum 1st fraction of *G. pseudochina* leaves extract in pot system.

ANOVA

TPC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2175.084	4	543.771	120.579	.000
Within Groups	45.097	10	4.510		
Total	2220.181	14			

Duncan

Treatments	N	Subset for alpha = .01			
		1	2	3	4
GSD10 with ZnCd	3	60.1033			
Control with ZnCd	3		74.6467		
GTID13 with ZnCd	3			80.5733	
GLD03 with ZnCd	3			84.6400	
Control without ZnCd	3				96.7567
Sig.		1.000	1.000	.041	1.000

ANOVA

TFC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2012.704	4	503.176	101.434	.000
Within Groups	49.606	10	4.961		
Total	2062.311	14			

Appendix C8 (Cont.)

Duncan

Treatments	N	Subset for alpha = .01			
		1	2	3	4
GSD10 with ZnCd	3	44.6933			
Control with ZnCd	3		56.8933		
GTID13 with ZnCd	3		60.9400	60.9400	
GLD03 with ZnCd	3			63.8133	
Control without ZnCd	3				80.5167
Sig.		1.000	.050	.145	1.000

ANOVA

Crude contents

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	19797.865	4	4949.466	1.226	.360
Within Groups	40387.130	10	4038.713		
Total	60184.996	14			

Duncan

Treatments	N	Subset for alpha = .01
		1
GSD10 with ZnCd	3	230.2633
Control with ZnCd	3	284.6567
GTID13 with ZnCd	3	295.1367
Control without ZnCd	3	311.7533
GLD03 with ZnCd	3	340.3200
Sig.		.080

Appendix C8 (Cont.)

ANOVA

IC50

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.004	4	.001	28.471	.000
Within Groups	.000	10	.000		
Total	.004	14			

Duncan

Treatments	N	Subset for alpha = .01	
		1	2
Control with ZnCd	3	.1120	
GLD03 with ZnCd	3	.1127	
Control without ZnCd	3	.1170	
GTID13 with ZnCd	3		.1460
GSD10 with ZnCd	3		.1477
Sig.		.345	.736

Appendix C9 One-way ANOVA analysis for TPC, TFC, crude content and IC50 of FRSA in 1st fraction of *G. pseudochina* leaves extract in tissue culture system. The percolation and partition methods were performed by 99.9% (v/v) ethanol, 99.9% (v/v) methanol and 50% (v/v) methanol.

ANOVA

TPC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4747.146	14	339.082	460.331	.000
Within Groups	22.098	30	.737		
Total	4769.244	44			

ANOVA

TFC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2407.189	14	171.942	168.647	.000
Within Groups	30.586	30	1.020		
Total	2437.776	44			

ANOVA

Crude contents

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	78617.198	14	5615.514	4.673	.000
Within Groups	36050.433	30	1201.681		
Total	114667.631	44			

Appendix C9 (Cont.)

ANOVA

IC50

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.016	14	.001	70.537	.000
Within Groups	.000	30	.000		
Total	.016	44			

Appendix C9 (Cont.)

TPC

Duncan

Treatments	N	Subset for alpha = .01								
		1	2	3	4	5	6	7	8	9
GSD10 with ZnCd ethanol	3	14.143								
GTID13 with ZnCd ethanol	3	14.193								
Control with ZnCd ethanol	3	15.013	15.013							
GLD03 with ZnCd ethanol	3		16.440							
Control without ZnCd ethanol	3			21.320						
GSD10 with ZnCd 50methanol	3			22.213	22.213					
Control with ZnCd 50methanol	3				23.463	23.463				
GSD10 with ZnCd methanol	3				23.743	23.743				
Control without ZnCd 50methanol	3					25.046				
GLD03 with ZnCd 50methanol	3						28.200			
GTID13 with ZnCd 50methanol	3						28.603			
Control with ZnCd methanol	3							36.166		
GTID13 with ZnCd methanol	3							37.776		
GLD03 with ZnCd methanol	3								40.003	
Control without ZnCd methanol	3									50.390
Sig.		.251	.051	.212	.046	.040	.569	.029	1.000	1.000

Appendix C9 (Cont.)

TFC

Duncan

Treatments	N	Subset for alpha = .01						
		1	2	3	4	5	6	7
Control with ZnCd ethanol	3	13.433						
GSD10 with ZnCd 50methanol	3	13.963	13.963					
GTID13 with ZnCd ethanol	3	14.460	14.460					
GSD10 with ZnCd methanol	3	14.550	14.550					
Control with ZnCd 50methanol	3	15.800	15.800	15.800				
GSD10 with ZnCd ethanol	3		16.180	16.180	16.180			
Control without ZnCd 50methanol	3			17.106	17.106			
GLD03 with ZnCd 50methanol	3			17.270	17.270			
GLD03 with ZnCd ethanol	3				18.296			
GTID13 with ZnCd 50methanol	3				18.516			
Control without ZnCd ethanol	3					23.090		
Control with ZnCd methanol	3						27.660	
GTID13 with ZnCd methanol	3						27.963	
GLD03 with ZnCd methanol	3						28.246	
Control without ZnCd methanol	3							40.316
Sig.		.014	.021	.113	.015	1.000	.509	1.000

Appendix C9 (Cont.)

Crude contents

Duncan

Treatments	N	Subset for alpha = .01		
		1	2	3
Control with ZnCd ethanol	3	46.073		
Control without ZnCd 50methanol	3	49.483		
GLD03 with ZnCd ethanol	3	50.966		
GSD10 with ZnCd ethanol	3	50.986		
Control with ZnCd 50methanol	3	57.590		
GSD10 with ZnCd 50methanol	3	70.723	70.723	
GTID13 with ZnCd methanol	3	87.390	87.390	
GTID13 with ZnCd ethanol	3	90.680	90.680	
GSD10 with ZnCd methanol	3	108.553	108.553	108.553
GTID13 with ZnCd 50methanol	3	117.066	117.066	117.066
Control without ZnCd ethanol	3	128.660	128.660	128.660
GLD03 with ZnCd methanol	3	133.170	133.170	133.170
Control without ZnCd methanol	3	133.610	133.610	133.610
GLD03 with ZnCd 50methanol	3		156.186	156.186
Control with ZnCd methanol	3			180.996
Sig.		.013	.013	.03

Appendix C9 (Cont.)

IC50

Duncan

Treatments	N	Subset for alpha = .01						
		1	2	3	4	5	6	7
Control without ZnCd 50methanol	3	.0167						
Control without ZnCd methanol	3	.0190						
GTID13 with ZnCd methanol	3	.0227	.0227					
Control with ZnCd 50methanol	3	.0250	.0250					
GLD03 with ZnCd methanol	3		.0307	.0307				
GLD03 with ZnCd ethanol	3			.0363	.0363			
GSD10 with ZnCd methanol	3			.0380	.0380			
Control with ZnCd methanol	3				.0417			
Control with ZnCd ethanol	3				.0450	.0450		
GLD03 with ZnCd 50methanol	3				.0457	.0457		
GTID13 with ZnCd 50methanol	3				.0460	.0460		
GSD10 with ZnCd ethanol	3					.0540	.0540	
GSD10 with ZnCd 50methanol	3						.0557	
GTID13 with ZnCd ethanol	3							.0777
Control without ZnCd ethanol	3							.0817
Sig.		.024	.026	.040	.012	.015	.612	.228

Appendix C10 One-way ANOVA analysis for concentration of Chlorogenic acid (CCA), Caffeic acid (CA) and Rutin (RUT) and peak area of unknown from HPLC chromatogram of *G. pseudochina* leaves extract at 280 nm in tissue culture system.

ANOVA

CGA

		Sum of Squares	df	Mean Square	F	Sig.
CGA	Between Groups	28.713	4	7.178	90.407	.000
	Within Groups	.794	10	.079		
	Total	29.507	14			

Duncan

Treatments	N	Subset for alpha = 0.01		
		1	2	3
GSD10 with ZnCd	3	3.8160		
GLD03 with ZnCd	3		5.3040	
Control with ZnCd	3		5.8760	
GTID13 with ZnCd	3			7.4663
Control without ZnCd	3			7.4670
Sig.		1.000	.032	.998

ANOVA

CA

		Sum of Squares	df	Mean Square	F	Sig.
CA	Between Groups	.026	4	.006	186.288	.000
	Within Groups	.000	10	.000		
	Total	.026	14			

Appendix C10 (Cont.)

Duncan

Treatments	N	Subset for alpha = 0.01			
		1	2	3	4
GLD03 with ZnCd	3	.1710			
GSD10 with ZnCd	3		.2000		
Control without ZnCd	3			.2453	
Control with ZnCd	3				.2690
GTID13 with ZnCd	3				.2793
Sig.		1.000	1.000	1.000	.056

ANOVA

RUT

		Sum of Squares	df	Mean Square	F	Sig.
RUT	Between Groups	2.622	4	.655	111.772	.000
	Within Groups	.059	10	.006		
	Total	2.680	14			

Duncan

Treatments	N	Subset for alpha = 0.01		
		1	2	3
GSD10 with ZnCd	3	.7070		
GLD03 with ZnCd	3	.7130		
Control with ZnCd	3		1.1610	
Control without ZnCd	3			1.5990
GTID13 with ZnCd	3			1.6863
Sig.		.925	1.000	.193

Appendix C10 (Cont.)

ANOVA

Unknown

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2,2094,459,521,852	4	552,361,488,0463	6.136	.009
Within Groups	9,001,659,808,860	10	90,016,598,0886		
Total	31,096,119,330,712	14			

Duncan

Treatments	N	Subset for alpha = 0.01	
		1	2
GLD03 with ZnCd	3	5,175,238.00	
GSD10 with ZnCd	3	5,199,736.67	
Control with ZnCd	3	5,851,183.67	
GTID13 with ZnCd	3	6,261,833.67	6,261,833.67
Control without ZnCd	3		8,477,914.00
Sig.		.220	.017

Appendix C11 One-way ANOVA analysis for total chlorophyll content of *G. pseudochina* after treatment with Zn plus Cd for 2 months in pot system.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	24338.162	4	6084.541	386.066	.000
Within Groups	236.405	15	15.760		
Total	24574.568	19			

Appendix C11 (Cont.)

Duncan

Treatment	N	Subset for alpha = .01		
		1	2	3
GLD03 with ZnCd	4	7.67200		
GSD10 with ZnCd	4	10.9750	10.9750	
Control with ZnCd	4	14.3920	14.3920	
GTID13 with ZnCd	4		17.2277	
Control without ZnCd	4			99.4070
Sig.		.037	.051	1.000

Appendix C12 One-way ANOVA analysis for Zn accumulation of *G. pseudochina* shoot and root after treatment with Zn plus Cd for two months in pot system.

ANOVA

Shoots

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	15709664.914	4	3927416.229	76.980	.000
Within Groups	1020377.656	20	51018.883		
Total	16730042.570	24			

Appendix C12 (Cont.)

Duncan

Shoots

Treatments	N	Subset for alpha = .05		
		1	2	3
Control without ZnCd	5	12.5364		
GSD10 with ZnCd	5		1631.6003	
Control with ZnCd	5		1801.5431	
GTID13 with ZnCd	5			2117.0718
GLD03 with ZnCd	5			2171.8386
Sig.		1.000	.248	.705

ANOVA

Roots

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	22685644.882	4	5671411.220	8.906	.000
Within Groups	12736448.273	20	636822.414		
Total	35422093.154	24			

Duncan

Treatments	N	Subset for alpha = .05		
		1	2	3
Control without ZnCd	5	14.6645		
GTID13 with ZnCd	5	1017.1484	1017.1484	
GLD03 with ZnCd	5		1350.1781	
Control with ZnCd	5		1551.8324	
GSD10 with ZnCd	5			2964.5856
Sig.		.061	.329	1.000

Appendix C13 One-way ANOVA analysis for Cd accumulation of *G. pseudochina* shoot and root after treatment with Zn plus Cd for two months in pot system

ANOVA

Shoots

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	290000.180	4	72500.045	47.138	.000
Within Groups	30760.944	20	1538.047		
Total	320761.125	24			

Duncan

Treatments	N	Subset for alpha = .05		
		1	2	3
Control without ZnCd	5	.6432		
GSD10 with ZnCd	5		200.7428	
Control with ZnCd	5		251.6605	251.6605
GLD03 with ZnCd	5			283.0800
GTID13 with ZnCd	5			294.1749
Sig.		1.000	.053	.119

ANOVA

Roots

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	37754.399	4	9438.600	12.844	.000
Within Groups	14696.868	20	734.843		
Total	52451.267	24			

Appendix C13 (Cont.)

Duncan

Treatments	N	Subset for alpha = .05		
		1	2	3
Control without ZnCd	5	.4028		
Control with ZnCd	5		53.4097	
GLD03 with ZnCd	5		70.2972	
GTID13 with ZnCd	5		72.7054	
GSD10 with ZnCd	5			121.2663
Sig.		1.000	.300	1.000

Appendix C14 One-way ANOVA analysis for TPC and TFC in sum all fraction (1, 2 and 3) extract of *G. pseudochina* leaves in pot system. The percolation and partition methods were performed by 99.9% (v/v) ethanol, 99.9% (v/v) methanol and 50% (v/v) methanol.

ANOVA

TPC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	10070.955	14	719.354	124.997	.000
Within Groups	172.649	30	5.755		
Total	10243.604	44			

ANOVA

TFC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2841.705	14	202.979	232.565	.000
Within Groups	26.184	30	.873		
Total	2867.889	44			

Appendix C14 (Cont.)

TPC

Duncan

Treatments		Subset for alpha = .01							
	N	1	2	3	4	5	6	7	8
GSD10 with ZnCd ethanol	3	5.080							
GLD03 with ZnCd ethanol	3	7.566							
GSD10 with ZnCd methanol	3	7.650							
GLD03 with ZnCd methanol	3	8.850	8.850						
GSD10 with ZnCd 50methanol	3		13.340	13.340					
GTID13 with ZnCd ethanol	3			14.910					
GLD03 with ZnCd 50methanol	3			18.376					
Control with ZnCd ethanol	3				28.130				
GTID13 with ZnCd methanol	3				30.153	30.153			
Control without ZnCd ethanol	3					33.926	33.926		
Control without ZnCd methanol	3					34.500	34.500		
Control with ZnCd methanol	3						36.393		
Control with ZnCd 50methanol	3							42.910	
GTID13 with ZnCd 50methanol	3							43.173	
Control without ZnCd 50methanol	3								53.750
Sig.		.087	.029	.020	.310	.043	.244	.894	1.000

Appendix C14 (Cont.)

TFC

Duncan

Treatments		Subset for alpha = .01						
	N	1	2	3	4	5	6	7
GSD10 with ZnCd ethanol	3	2.303						
GSD10 with ZnCd methanol	3	2.693						
GLD03 with ZnCd ethanol	3	4.020	4.020					
GLD03 with ZnCd methanol	3	4.020	4.020					
GSD10 with ZnCd 50methanol	3		5.423					
GLD03 with ZnCd 50methanol	3			8.090				
GTID13 with ZnCd ethanol	3			9.986				
GTID13 with ZnCd methanol	3				16.240			
Control with ZnCd 50methanol	3				16.420			
Control without ZnCd methanol	3				17.350			
Control with ZnCd ethanol	3					19.513		
Control with ZnCd methanol	3					20.556	20.556	
GTID13 with ZnCd 50methanol	3						22.650	22.650
Control without ZnCd 50methanol	3							23.096
Control without ZnCd ethanol	3							24.803
Sig.		.046	.091	.019	.179	.182	.010	.011

Appendix C15 One-way ANOVA analysis for TPC and TFC in sum all fraction (1, 2 and 3) extract of *G. pseudochina* leaves in pot system.

ANOVA

TPC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	22380.757	4	5595.189	180.923	.000
Within Groups	309.259	10	30.926		
Total	22690.015	14			

Duncan

Treatments	N	Subset for alpha = 0.01			
		1	2	3	4
GSD10 with ZnCd	3	26.0733			
GLD03 with ZnCd	3	34.8000			
GTID13 with ZnCd	3		88.2400		
Control with ZnCd	3			107.4333	
Control without ZnCd	3				122.1800
Sig.		.084	1.000	1.000	1.000

ANOVA

TFC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7294.516	4	1823.629	430.311	.000
Within Groups	42.379	10	4.238		
Total	7336.895	14			

Appendix C15 (Cont.)

Duncan

Treatments	N	Subset for alpha = 0.01				
		1	2	3	4	5
GSD10 with ZnCd	3	10.4200				
GLD03 with ZnCd	3		16.1333			
GTID13 with ZnCd	3			48.8800		
Control with ZnCd	3				56.4900	
Control without ZnCd	3					65.2533
Sig.		1.000	1.000	1.000	1.000	1.000

Appendix C16 One-way ANOVA analysis for TPC, TFC, crude content and IC50 of FRSA in 1st fraction of *G. pseudochina* leaves extract in pot system. The percolation and partition methods were performed by 99.9% (v/v) ethanol, 99.9% (v/v) methanol and 50% (v/v) methanol.

ANOVA

TPC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8450.503	14	603.607	44.269	.000
Within Groups	409.052	30	13.635		
Total	8859.554	44			

Appendix C16 (Cont.)

ANOVA

TFC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2142.454	14	153.032	59.901	.000
Within Groups	76.643	30	2.555		
Total	2219.098	44			

ANOVA

Crude contents

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	104579.432	14	7469.959	34.289	.000
Within Groups	6535.500	30	217.850		
Total	111114.932	44			

ANOVA

IC50

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.174	14	.012	16.879	.000
Within Groups	.022	30	.001		
Total	.196	44			

Appendix C16 (Cont.)

TPC

Duncan

Treatments	N	Subset for alpha = 0.01				
		1	2	3	4	5
GSD10 with ZnCd ethanol	3	3.7900				
GLD03 with ZnCd ethanol	3	5.3700				
GSD10 with ZnCd methanol	3	5.5833				
GLD03 with ZnCd methanol	3	6.9633				
GTID13 with ZnCd ethanol	3	10.3933	10.3933			
GSD10 with ZnCd 50methanol	3	12.5233	12.5233			
GLD03 with ZnCd 50methanol	3		17.1700	17.1700		
Control without ZnCd methanol	3			22.3800	22.3800	
Control with ZnCd ethanol	3			22.7667	22.7667	
GTID13 with ZnCd methanol	3			23.6600	23.6600	
Control without ZnCd ethanol	3				27.8933	
Control with ZnCd methanol	3				28.7900	
GTID13 with ZnCd 50methanol	3					41.3333
Control with ZnCd 50methanol	3					42.3300
Control without ZnCd 50methanol	3					46.5800
Sig.		.014	.041	.056	.065	.110

Appendix C16 (Cont.)

TFC

Duncan

Treatments	N	Subset for alpha = 0.01						
		1	2	3	4	5	6	7
GSD10 with ZnCd ethanol	3	2.2267						
GSD10 with ZnCd methanol	3	2.5867						
GLD03 with ZnCd ethanol	3	3.4833						
GLD03 with ZnCd methanol	3	3.8533						
GSD10 with ZnCd 50methanol	3	5.4233	5.4233					
GLD03 with ZnCd 50methanol	3		8.0900	8.0900				
GTID13 with ZnCd ethanol	3		8.4000	8.4000				
Control without ZnCd methanol	3			11.6467	11.6467			
GTID13 with ZnCd methanol	3				13.0200	13.0200		
Control with ZnCd 50methanol	3					16.4200	16.4200	
Control with ZnCd ethanol	3					16.6500	16.6500	
Control with ZnCd methanol	3						17.0000	
Control without ZnCd 50methanol	3						19.8900	19.8900
Control without ZnCd ethanol	3							21.4700
GTID13 with ZnCd 50methanol	3							22.2100
Sig.		.034	.038	.014	.301	.012	.019	.102

Appendix C16 (Cont.)

Crude contents

Duncan

Treatments	N	Subset for alpha = 0.01					
		1	2	3	4	5	6
GLD03 with ZnCd ethanol	3	21.1900					
GSD10 with ZnCd ethanol	3	24.4200					
GTID13 with ZnCd ethanol	3	31.8400					
Control with ZnCd ethanol	3	41.6233	41.6233				
Control without ZnCd methanol	3	45.6467	45.6467				
Control without ZnCd ethanol	3	48.1600	48.1600				
Control with ZnCd methanol	3		69.3400	69.3400			
GTID13 with ZnCd methanol	3			89.1000	89.1000		
GSD10 with ZnCd methanol	3			95.2433	95.2433		
GLD03 with ZnCd methanol	3			97.6733	97.6733		
Control without ZnCd 50methanol	3				111.6533		
Control with ZnCd 50methanol	3				119.9500	119.9500	
GLD03 with ZnCd 50methanol	3					146.6400	146.6400
GTID13 with ZnCd 50methanol	3					151.2100	151.2100
GSD10 with ZnCd 50methanol	3						179.1400
Sig.		.056	.042	.038	.027	.019	.015

Appendix C16 (Cont.)

IC50

Duncan

Treatments	N	Subset for alpha = 0.01			
		1	2	3	4
Control without ZnCd 50methanol	3	.0200			
Control with ZnCd ethanol	3	.0300			
Control without ZnCd methanol	3	.0300			
Control with ZnCd 50methanol	3	.0300			
GTID13 with ZnCd 50methanol	3	.0300			
Control without ZnCd ethanol	3	.0333			
GTID13 with ZnCd methanol	3	.0433			
GTID13 with ZnCd ethanol	3	.0467			
Control with ZnCd methanol	3	.0467			
GLD03 with ZnCd 50methanol	3	.0800	.0800		
GLD03 with ZnCd ethanol	3		.1167	.1167	
GLD03 with ZnCd methanol	3			.1467	
GSD10 with ZnCd ethanol	3			.1500	
GSD10 with ZnCd 50methanol	3			.1600	
GSD10 with ZnCd methanol	3				.2267
Sig.		.027	.108	.082	1.000

Appendix C17 One-way ANOVA analysis for TPC, TFC, crude content and IC50 in sum 1st fraction of *G. pseudochina* leaves extract in pot system.

ANOVA

TPC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	15192.572	4	3798.143	61.940	.000
Within Groups	613.199	10	61.320		
Total	15805.771	14			

Duncan

Treatments	N	Subset for alpha = 0.01		
		1	2	3
GSD10 with ZnCd	3	21.9000		
GLD03 with ZnCd	3	29.5000		
GTID13 with ZnCd	3		75.3900	
Control with ZnCd	3		93.8833	93.8833
Control without ZnCd	3			96.8633
Sig.		.262	.016	.651

ANOVA

TFC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4861.454	4	1215.363	127.098	.000
Within Groups	95.624	10	9.562		
Total	4957.078	14			

Appendix C17 (Cont.)

Duncan

Treatments	N	Subset for alpha = 0.01		
		1	2	3
GSD10 with ZnCd	3	10.2367		
GLD03 with ZnCd	3	15.4300		
GTID13 with ZnCd	3		43.6267	
Control with ZnCd	3		50.0667	50.0667
Control without ZnCd	3			53.0067
Sig.		.067	.029	.271

ANOVA

Crude contents

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	16069.338	4	4017.335	4.036	.033
Within Groups	9954.643	10	995.464		
Total	26023.981	14			

Duncan

Treatments	N	Subset for alpha = 0.01	
		1	2
Control without ZnCd	3	205.4600	
Control with ZnCd	3	230.9100	230.9100
GLD03 with ZnCd	3	265.4967	265.4967
GTID13 with ZnCd	3	272.1533	272.1533
GSD10 with ZnCd	3		298.8000
Sig.		.036	.034

Appendix C17 (Cont.)

ANOVA

IC50

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.461	4	.115	41.837	.000
Within Groups	.028	10	.003		
Total	.488	14			

Duncan

Treatments	N	Subset for alpha = 0.01		
		1	2	3
Control without ZnCd	3	.0800		
Control with ZnCd	3	.1100		
GTID13 with ZnCd	3	.1200		
GLD03 with ZnCd	3		.3433	
GSD10 with ZnCd	3			.5333
Sig.		.394	1.000	1.000

Appendix C18 One-way ANOVA analysis for concentration of Chlorogenic acid (CCA), Caffeic acid (CA) and Rutin (RUT) and peak area of unknown from HPLC chromatogram of *G. pseudochina* leaves extract at 280 nm in pot system.

ANOVA

CGA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5.806	4	1.452	430.706	.000
Within Groups	.034	10	.003		
Total	5.840	14			

Appendix C18 (Cont.)

Duncan

Treatments	N	Subset for alpha = 0.01			
		1	2	3	4
GSD10 with ZnCd	3	.29400			
GLD03 with ZnCd	3	.31800			
Control without ZnCd	3		1.34400		
GTID13 with ZnCd	3			1.53000	
Control with ZnCd	3				1.76500
Sig.		.624	1.000	1.000	1.000

ANOVA

CA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.249	4	.062	410.132	.000
Within Groups	.002	10	.000		
Total	.250	14			

Duncan

Treatments	N	Subset for alpha = 0.01			
		1	2	3	4
GLD03 with ZnCd	3	.11033			
GSD10 with ZnCd	3		.16833		
GTID13 with ZnCd	3			.26733	
Control with ZnCd	3			.27367	
Control without ZnCd	3				.48767
Sig.		1.000	1.000	.543	1.000

Appendix C18 (Cont.)

ANOVA

RUT

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.208	4	.302	238.200	.000
Within Groups	.013	10	.001		
Total	1.221	14			

Duncan

Treatments	N	Subset for alpha = 0.01	
		1	2
GSD10 with ZnCd	3	.42600	
GLD03 with ZnCd	3	.44567	
Control with ZnCd	3		.99133
GTID13 with ZnCd	3		1.00833
Control without ZnCd	3		1.04233
Sig.		.514	.125

ANOVA

Unknown

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	33069706182027.59	4	8267426545506.89	570.984	.000
Within Groups	144792500567.33	10	14479250056.73		
Total	33214498682594.93	14			

Appendix C18 (Cont.)

Duncan

Treatments	N	Subset for alpha = 0.01			
		1	2	3	4
GLD03 with ZnCd	3	762,286.00			
GSD10 with ZnCd	3		1,130,462.00		
Control without ZnCd	3			3,656,563.67	
GTID13 with ZnCd	3			3,844,917.67	
Control with ZnCd	3				4,308,547.00
Sig.		1.000	1.000	.084	1.000

BIOGRAPHY

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Research output	1. Munjit, R., Nakbanpote, W. and Sangdee, A. (2015) "Screening of zinc and cadmium tolerance and plant-growth-promoting rhizobacteria from Zea mays L. Rhizosphere" The TSB 2015 "The 27th Annual Meeting of the Thai Society for Biotechnology and International Conference", 17-19 November 2015, Mandarin Hotel by Centre Point, Bangkok Thailand. pp 159-166 (proceeding), pp 150 (poster presentation) 2. Nakpanpote, W., Prasad, MNV., Mongkonsin, B., Panitlertumpai, N., Munjit, R., and Lattanapolsan, L. (2018) Strategies for rehabilitation of mine waste/leachate in Thailand. Chapter 33, In Bio-Geotechnologies for Mine Site Rehabilitation, Eds. M.N.V. Prasad, S.K. Maiti, P.J. Campos Favas, Massachusetts, Elsevier, pp 617-643. (ISBN: 978-0-12-812986-9). 3. Nakbanpote, W., Munjit. R., Sukadeetad, K., Sakkayawong, N., and Damrianant, S. (2019) Effects of drying and extraction methods on phenolic compounds and in vitro assays of Eclipta prostrate Linn leaf extracts, ScienceAsia, 45(127-137). 4. Munjit, R., Nakbanpote, W., Sangdee, A., and Arnold D. (2019) "Effect of Zn/Cd tolerant endophytic bacterial

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