Isolation characterization and selection of endophytic bacteria from *Murdannia spectabilis* (Kurz) Faden grown in Zn/Cd contaminated area and affect to promote plant growth under the metal stress

Ladawan Rattanapolsan

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 Ladawan Rattanapolsan , as a partial fulfillment of the requirements for the Doctor of Philosophy Biology at Mahasarakham University

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TITLE Isolation characterization and selection of endophytic bacteria from *Murdannia spectabilis* (Kurz) Faden grown in Zn/Cd contaminated area and affect to promote plant growth under the metal stress

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ABSTRACT

This research aims to study isolation and identification of endophytic bacteria from storage roots, underground stems (tubers), leaves and peduncle of *Murdannia spectabilis* (Kurz) Faden growing in forest area of zinc mine, Mae Sot, Tak Province, Thailand. A total of 52 endophytic bacteria were isolated from the explants. They tolerated various concentrations of Zn and Cd, then 24 isolated surviving on Trypticase Soya Agar (TSA) adding with Zn (250-500 mg L^{-1}) and Cd (20-50 mg L^{-1}) were selected for bacterial identification and tested for plant growth promotion ability. The 16S rDNA gene sequencing indicated that the bacterial isolates were in genera of *Bacillus*, *Pantoea*, *Microbacterium*, *Curtobacterium*, *Chryseobacterium*, *Cupriavidus*, *Siphonobacter* and *Pseudomonas*. In addition, all of 24 isolates were able to produce IAA, the levels of IAA produced by endophytes ranged from 1.6 to 75.6 mg L^{-1}. Only six isolates showing high IAA, phosphate and siderophore production, nitrogen fixation, ACC deaminase activity, and well-adapted to high Zn/Cd concentrations were selected for studying the plant growth promoting properties under Zn (150 mg L^{-1}) plus Cd (30 mg L^{-1}) stress. The results indicated that the Zn and Cd stress affected to decrease the IAA production and nitrogen fixation of RDMSSR04, RDMSP03 and RDMSP06 strains, but no effect on RDMSSR02, RDMSSR07 and RDMSSR05. Interestingly, the bacterial isolates from different parts of plant displayed different levels of Zn and/or Cd tolerances, and they could promote plant growth or confer higher tolerance to plant grown in heavy metal contaminated soil. The inoculation of *Cuprividus plantarum* RDMSSR05 and *Chryseobacterium ureilyticum* RDMSSR07 were investigated on plant growth promoting and Zn/Cd accumulation in *M. spectabilis* under a tissue culture system. The endophytic bacterial inoculations did not significantly affect the growth and Zn/Cd accumulation in plant. The endophytic bacterial inoculants could not survive in plants over the experimental period. Moreover, *Curtobacterium luteum*, an indigenous endophytic bacterium, was found in this study, and the bacterium might correlate to the mechanism of plant tolerance and to metals detoxification mechanisms.

The effects of Zn or Cd tolerance and accumulation in *M. spectabilis* were studied after 4 weeks when treated with Zn (50-1,000 mg L^{-1}) and Cd (5-50) mg L^{-1}.

Fresh weight, dry weight, the number of tubers and the percentage of yellow/pale leaves and stress induction focused on chlorophyll content, protein content, cell death, total phenolic compound and stress enzymes activity (SOD, CAT) were compared between the treated and control plants. The results indicated that the concentrations of Zn 500-1,000 mg L^{-1} or Cd 25-50 mg L^{-1} affected the plant growth, increased chlorosis and stunting and decreased the chlorophyll content. In addition, higher Zn or Cd concentrations slightly caused to protein content, cell death, total phenolic compound and stress enzymes activity. The SDS-PAGE showed the effect of the toxicity of metals on protein expression. From the criteria for a metal accumulative plant, *M. spectabilis* could be classified as a Zn/Cd indicative plant. µ-XRF imaging indicated that the Zn was mainly distributed in the vascular bundle of the leaf tissues. In comparison with the Zn K-edge XANES spectra of the reference materials, the oxidation state of Zn accumulated in the leaves was 2+ (Zn^{2+}). The EXAFS presented the first coordination shell was both Zn-O and Zn-S ligands.

Keyword : Murdannia spectabilis (Kurz) Faden Zn Cd endophytic bacteria

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Ladawan Rattanapolsan

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

1.1 Background

Endophytic bacteria are bacteria living in the internal tissues of plant without causing symptoms of the disease. These bacteria may enhance certain metabolic activities, such as promoting plant growth, control against soil-borne diseases (Mastretta et al., 2006; Ryan et al., 2008). In some cases, endophytic bacteria may help the plants tolerance to biotic and abiotic stress such as heavy metals (Rosenblueth and Martinez-Romero, 2006). Many researches have shown that the relationship between plants and microorganisms significantly increased the mobilization and accumulation of heavy metals and the plants' resistance to the metal stress (Ma et al., 2011b) such as zinc (Zn) (Long et al., 2011), cadmium (Cd) (Chen et al., 2010) copper (Cu) (Sun et al., 2010), nickel (Ni) (Ma et al., 2011a), and lead (Pb) (Sheng et al., 2008) etc. Since endophytic bacteria can multiply inside the plant tissues, they associate more closely with their host than rhizosphere and phyllosphere bacteria. Therefore, endophytic bacteria can directly and/or indirectly promote the growth and health of plants, and likewise the plants provide nutrients and habitat for bacteria (Weyens et al., 2009).

Murdannia spectabilis (Kurz) Faden, Haew-Ka-Tai or Ya-Khon-Kai in Thai, is in the Commelinaceae family. It is a perennial plant with morphology of short underground stem, thin to moderate thickness of roots and swollen roots. Shoot of *M. spectabilis* seem to be dry and dead in the dry season; however, stem and storage root still survive under the ground from season to season. During rainy season, the dry stem and tuber are recovering and new leaves can grow from the refresh underground parts. The growth of *M. spectabilis* from the dormancy period may correlated with endophytic bacteria. In addition, our exploration of forest in a zinc mine area, Phatat Phadaeng sub-district, Mae Sot, Tak Province, Thailand found high amounts of Zn and Cd were accumulated in *M. spectabilis* (Panitlertumpai et al., 2003).

Rattanapolsan et al. (2013) clearly showed that *M. spectabilis* was a Zn and Cd hyperaccumulative plant, and the shoot (stem and leaves) parts accumulated 2,067 mg kg^{-1} dry weight of Zn and 27 mg kg^{-1} dry weight of Cd. In addition, some bacterial isolates colonized within the storage root tissues of *M. spectabilis* growing in the Zn/Cd contaminated soil, and the isolates were able to tolerate high concentrations of Zn (20-250 mg L^{-1}) and Cd (10-50 mg L^{-1}).

Endophytic bacteria have been isolated from many plants since woody plant to herbaceous and crop plants (Lodewyckx et al., 2002a). They were found in many parts of plants such as roots, stems, leaves, flowers as well as fruits and seeds (Lodewyckx et al., 2002b; Sun et al., 2010; Compant et al., 2011; Pereira and Castro, 2014). Moreover, they presented endophytic populations in metals hyperaccumulative plants with genetic diversity, heavy metals resistance properties, and plant growth promoting properties under the metal stresses (Li et al., 2012). Zn and Cd resistant endophytic bacteria were isolated from *Thlaspi caerulescens* (Zn hyperaccumulator) (Lodewyckx et al., 2002b). Group of Cd resistant endophytic bacteria, *Serratia nematodiphila* LRE07, *Enterobacter aerogenes* LRE17, *Enterobacter* sp. LSE04 and *Acinetobacter* sp. LSE06, were isolated from *Solanum nigrum* L., a Cd hyperaccumulative plant (Chen et al., 2010). Another group of Zn and Cd resistant bacteria in genus *Pseudomonas, Bacillus, Stenotrophomonas* and *Acinetobacter* were isolated from *Sedum alfredii*, a Zn/Cd hyperaccumulator (Long et al. (2011). Sun et al. (2010) isolated Cu resistant endophytic bacteria such as *Arthrobacter* sp., *Bacillus* sp., *B. pumilus, Sphingomonas* sp., *Sphingomonas* sp., *Herbaspirillum* sp., and *Microbacterium kitamiense* form *Commelina communis*, a Cu hyperaccumulative plant in the family Commelinaceae. Almost the metals endophytic bacteria covering direct and indirect properties to promote plant growth such as the production of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, indole 3-acetic acid (IAA), siderophores and extracellular enzymes, phosphate solubilization, and nitrogen fixation (Chen et al., 2010; Long et al., 2011; Luo et al., 2011; Rajkumar et al., 2012; Pereira and Castro, 2014). Furthermore, inoculation of the endophytic bacteria promotes phytoremediation efficiency with increasing plant biomass and solubilizing of metals (Ma et al., 2011b). Cd-resistant bacterial endophytes significantly increased Cd solubilization and resulted to a higher amount of Cd accumulated in the increased

biomass (Chen et al. (2010; Luo et al. (2011). Zhang et al. (2011) also reported that the inoculation of Pb-resistant endophytic bacteria promoted dry weight and increased the accumulation of Pb in shoot of plants. In addition, the content of carotenoids, chlorophylls, and photochemical efficiency of *Festuca rubra* plant increased when *Pseudomonas* sp. was inoculated; however, Cd and Zn concentrations in the shoot were significantly lower because of biomass increasing (Burges et al., 2016). From the advantages of endophytic bacteria to promote phytoremediation and support plant growth under metal stress, metal resistance endophytic bacteria should be searched and studied for further application.

Therefore, this research aimed to study isolation and characterization of endophytic bacteria from *M. spectabilis* growing in Zn/Cd contaminated soil in the forest of the zinc mining area. The endophytic bacteria were screened by Zn/Cd resistance and plant growth promoting properties. Effects of Zn and/or Cd on the plant growth and detoxification mechanism of *M. spectabilis* were carried out in a tissue culture system. Finally, inoculation of some selected endophytic bacteria and their effects on promoting growth and Zn/Cd accumulation of *M. spectabilis* were carefully investigated in the in vitro system.

1.2 Objectives

This research aims to:

1.2.1 Isolate and characterize endophytic bacteria from swollen storage roots, underground stems (tubers), leaves and peduncles of *M. spectabilis*, a Zn/Cd hyperaccumulator.

1.2.2 Screen the isolated endophytic bacteria by Zn/Cd resistance and plant growth promoting properties.

1.2.3 Study the effects of Zn and/or Cd on plant growth and detoxification mechanism of *M. spectabilis* in a tissue culture system.

1.2.4 Study the effects of endophytic bacterial inoculation on plant growth promoting of *M. spectabilis* and Zn/Cd accumulation in the plant tissue.

1.3 Advantages of the study

1.3.1 Isolation and characterization of endophytic bacteria from *M. spectabilis* growing in the Zn-mine forest area will give basis information of local bacterial diversity associated with *M. spectabilis*, a Zn/Cd hyperacumulative perennial plant.

1.3.2 Endophytic bacteria screening by Zn/Cd resistance and plant growth promoting properties could be applied in a bioaugmentation process to promote the growth and propagation of *M. spectabilis* in the Zn/Cd contaminated area for recovering forest habitats in the zinc mine.

1.3.3 Colonization of some selected endophytic bacteria and Zn/Cd accumulation in *M. spectabilis* will provide information of plant-endophytic bacteria association.

CHAPTER 2

LITERATURE REVIEW

2.1 *Murdannia spectabilis* (Kurz) Faden

M. spectabilis is in the Commelinaceae family. It is found throughout Thailand and distributes in China, Myanmar, Indo-china and Philippines. Its ecology is wet and open areas in deciduous forest, scrub or grasslands, altitude 100-1,300 meters, and flower from May to August, daily flowering time from the late afternoon to evening (or when there are dark clouds). The botany of the plants is a monocotyledon, perennial plant, basal rosettes with spirally arranged leaves. Leaves; basal leaves; radical leaves, leaf blade linear, apex acute, margin undulate. Stem is erect tall (including inflorescences). The roots are thin to moderate thickness, swollen roots, rhizomes absent. The flower is purple to violet-blue, slightly zygomorphic (Figure 1) (Thitimetharoch, 2004; Shu, 2000; Rattanapolsan et al., 2013). Our exploration of forest in a zinc mine area, Phatat Phadaeng sub-district, Mae Sot, Tak Province, Thailand uncovered high amounts of zinc (Zn) and cadmium (Cd) accumulated in *M. spectabilis* (Panitlertumpai et al., 2003). Rattanapolsan et al. (2013) showed that *M. spectabilis* was a Zn and Cd hyperaccumulative plant with translocation factors greater than 1. Zn and Cd accumulated in plant shoot (stem and leaves) were 2,067 ± 74 mg kg^{-1} dry weight and 26.7 ± 1.2 mg kg^{-1} dry weight, respective, and Zn and Cd accumulated in root were 1,148 ± 71 and 20.2 ± 4.2 mg kg^{-1} dry weight, respectively.

Kingdom: Plantae

Division: Magnoliophyta

Class: Liliopsida

Order: Commelinales

Family: Commelinaceae

Genus: *Mudannia*

Species: *Murdannia spectabilis*

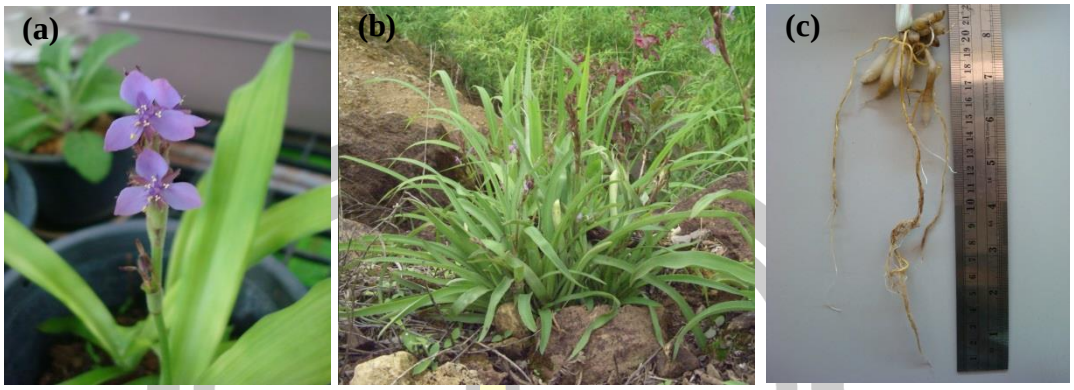


Figure 1 *Murdannia spectabilis*
(a) flowers, (b) leaves and (c) roots and storage roots

2.2 Cd toxicity to plant and microorganism

Cd is a non-essential trace elements for plants. Cd is a one of the most toxic heavy metals for plants and microbes (Benavides et al., 2005, Ayangbenro and Babalola, 2017). In plant, Cd is a divalent heavy metal cation (Cd^{2+}) which is easily taken up and causes phytotoxicity (Hoseini and Zargari, 2013). A high Cd accumulated in plant leaves (5-10 µg g-1 leaf dry weight) usually toxics to the plants (White and Brown, 2010), but Cd-hyperaccumulators can accumulate high amount of Cd (100 µg Cd g-1 leaf dry weight) (Baker, 1981). Cd can be accumulated in all plant parts. The Cd accumulation causes plants to decrease growth and enzyme activities, relating with Calvin cycle, carbohydrate and phosphorus metabolism, and CO^2 fixation (Gill and Tuteja, 2011). Cd can disturb physiological metabolisms in plants like transpiration, photosynthesis, respiration, and nitrogen assimilation (Wang et al., 2008, Chen et al., 2011). In microorganisms, the effects of Cd toxicity in bacteria, algae and fungi are severe inhibition of such physiological processes as growth, photosynthesis, and nitrogen fixation, even at Cd concentrations lower than 2 mg L^{-1}. In addition, Cd causes morphological abnormality in these microorganisms, which is probably relating with deleterious effects on cell division. This may be direct or indirect mechanism due to Cd affecting on protein synthesis and cellular organelles such as mitochondria and chloroplasts (Trevors et al., 1986, Ayangbenro and Babalola, 2017).

2.3 Correlation of microorganism and plant in phytoremediation

Phytoremediation is the use of plants to remove pollutants from the environment (Cunningham and Berti, 1993; Raskin et al., 1994). The success of remediation depends on plant biomass and the ability of high metal concentration transfer to above ground parts of the plant. Advantages of phytoremediation are environmentally friendly method and lower cost than physical and chemical processes, However, major disadvantages of the phytoremediation are small biomass and slow growth of hyperaccumulator plants (Ali et al., 2013). Toxicity of high amount of a metal in soil results in decrease metabolism and growth of plants (Chibuike and Obiora 2014). In addition, bioavailability of metal in soil is an important factor in success of metal translocation (Rajkumar et al., 2012). Many researchers explained the relationship between plant and microorganisms, which the relationship greatly increased metal mobilization in soil and support accumulation of metal in plant tissues (Rajkumar et al., 2012).

In general, the effect of microbial activities in the root or the soils around plant root can increase the efficiency of phytoremediation in metal contaminated soil by two way of direct and indirect mechanisms. In case of direct mechanism, microorganisms can improve metal solubilization by acidification of soil pHs and producing of metal, chelating agents sucha as siderophores and organic acids. Some microorganisms produce biosurfactants to assist in the phytoextraction. In other ways, microorganisms can improve phytostabilization by precipitation or immobilization of metal contaminants in the rhizosphere. Extracellular polymeric substances (EPS) produced from bacteria can complex with the metal and decrease metal uptake. Some microorganisms catalyze the oxidation and reduction of metal resulting in decrease toxicity and immobilization of toxic metals in rhizospheric soil. For indirect methods, bacteria help plant to tolerate toxic metals and/or to promote plant growth under the metal with a high biomass. In addition, bacteria probably affect an increased or decreased uptake of metals. Furthermore, metal-tolerant mycorrhizal fungi colonized in metal hyperaccumulative plants are resistant to heavy metals and play an important role in phytoremediation process (Rajkumar et al., 2012). Plant-associated microbes accelerate the phytoremediation process are shown in Figure 2.

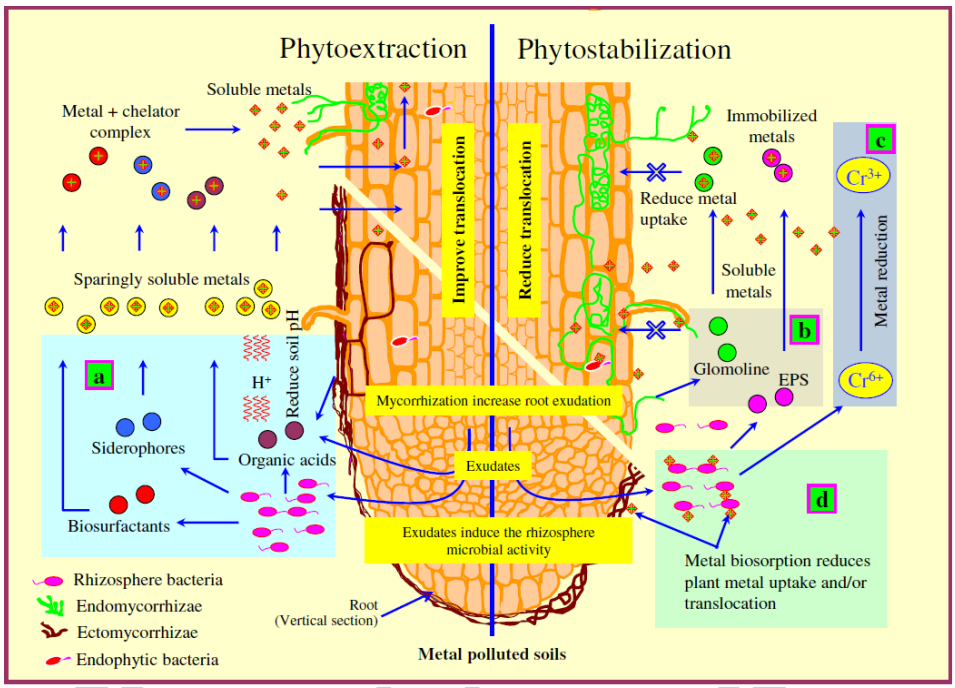


Figure 2 Plant-associated microbes accelerate the phytoremediation process in metal contaminated soils by enhancing metal mobilization/immobilization.

(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 and/or (d) metal biosorption. Abbreviations: extracellular polymeric substances (EPS) (Rajkumar et al., 2012)

2.4 Endophytic bacteria

Bacterial endophytes are bacteria that live inside the internal tissues of a plant without symptoms or disease in the host plant. These bacteria can enhance some metabolic activities to promote plant growth and help control of soil-borne diseases (Mastretta et al., 2006; Ryan et al., 2008). In some cases, endophytic bacteria improve plants tolerance to biotic and abiotic stresses. Several studies have shown that the relationship between plants and microorganisms significantly increased the mobilization and accumulation of heavy metals and the metal stress resistance (Ma et al., 2011b). Since endophytic bacteria can multiply inside the plant tissues, they are probably going to associate more closely with their host than rhizospheric and

phyllosphere bacteria. In which, rhizosphere is the region of soil that is immediately close to the surface of the root and that is affected by root exudates (Kennedy 1999), phyllosphere is the aerial surface of plants including stems, leaves, flowers and fruits that provide a habitat for microorganisms. The interactions among endophytic bacteria and plant explain that a host plant provide nutrients and habitat for the colonized bacteria, and the bacteria can directly or indirectly support growth and health of the host plant (Weyens et al., 2009).

Endophytic bacteria have been isolated from many plant species both monocotyledonous and dicotyledonous plants, ranging from woody plant to herbaceous and crop plants (Ryan et al., 2008). They have been found in various plants' parts such as roots, stem, leaves (Lodewyckx et al., 2002 Sun et al., 2010; Pereira and Castro, 2014), flowers (Compant et al., 2011) as well as fruits(de Melo Pereira et al., 2012) and seeds (Silva et al., 2016). The diversity of plant species show different types of endophytic bacteria (Sessitsch et al., 2002; van der Lelie et al., 2009). Endophytic bacterial may be "facultative" or "obligate" for the host, in accordance with the genotype and life strategy of host plant. Obligate endophytic bacteria can be strictly within the host plant, using the metabolism of the plant for surviving and may be transferred from one generation to the next generation by seeds or vegetative plant tissues. Whereas, facultative endophytic bacteria can live outside the host plant during a certain period of their life cycle, and they are mainly related to the soil surrounding of the plants and in the atmosphere (Abreu-Tarazietal., 2010, Su et al., 2010; Hamilton et al., 2012; Afzal et al., 2014 and Gouda et al., 2016). Inoculation of endophytic bacteria into soils or onto plants could efficiently colonize in the plant tissues and showed high activity in both rhizosphere and endosphere (Afzal et al., 2013).

The mechanisms of endophytic bacteria are helpful to their host plant including the production of phytohormones and enzymes associated with growth regulator metabolism such as indole-3-acetic acid (IAA), 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, the secretion of siderophores and ethylene (Glick et al., 1998; Hardoim et al., 2008; Rajkumar et al., 2009). In addition, during initial colonization, they can enhance plant growth by fixing nitrogen and increasing the availability of phosphate (Kuklinsky-Sobral et al., 2004; Luo et al., 2011). Chen et al.

(2010) proposed the heavy metal-resistant endophytic bacteria of *Serratia nematodiphila* LRE07, *Enterobacter aerogenes* LRE17, *Enterobacter* sp. LSE04 and *Acinetobacter* sp. LSE06. The bacteria were isolated from *Solanum nigru*m L., which was a Cd-hyperaccumulating plant growing in a Cd contaminated soil. Their properties for promoting plant growth have been characterized *in vitro* as the production of ACC deaminase, IAA, siderophores and phosphate solubilization. Moreover, these four bacteria significantly increased Cd solubilization in soil when compared with the non-inoculated soil. Long et al. (2011) isolated and characterized endophytic bacteria from *Sedum alfredii*, a Zn/Cd hyperaccumulator,. Bacterial density studies basis on molecular techniques with 16S rDNA sequencing showed higher bacterial population in roots than in leaves and stems. Most isolates were close to genus *Pseudomonas*, *Bacillus*, *Stenotrophomonas* and *Acinetobacter*. Endophytic bacteria were able to display high Zn and Cd resistance, and all endophytic bacteria had IAA production. Moreover, some bacterial strains had the properties of $Ca_3(PO_4)_2$, $ZnCO_3$, and $Zn_3(PO_4)_2$ solubilization, siderophore production, and nitrogen fixation. In addition, the endophytic bacteria isolated from plants were able to promote plant growth and/or provide their host plants for higher tolerance to heavy metals contaminated soils (Li et al., 2012).

2.5 The diversity of metal resistant bacterial endophyte in hyperaccumulator plants.

Heavy metal pollution can not only affect the parameters related to the quality and performance of the plant, but also cause changes in the size, composition and activity of the plant, which are related to the community of microorganisms (Rajkumar et al., 2009). The abiotic stresses produced by inorganic and organic forms of heavy metals have effect on the growth, morphology and metabolism of microorganisms. Kamnev et al. (2005) presented the effects of heavy metals on the various types of biomass and diversity of endophytic. Bacteria isolated from contaminated sites can resist higher concentrations of metal than bacterial isolated from uncontaminated areas (Diaz-Ravina and Baath, 1996; Rajkumar et al., 2009). Many researchers have been interested in the interactions between endophytes and

hyperaccumulative plants due to biotechnological applications for bioremediation and to discover the community composition of microorganisms living in a naturally contaminated environment (Rajkumar et al., 2009). For example, the metal resistant endophytic bacteria were isolated from varied hyperaccumulating plants as shown in Table 1. Hyperaccumulating plants can be colonized by a high number of different divisions, genera, and species of metal resistant endophytic bacteria.

Table 1 List of hyperaccumulator plants and their associated endophytic bacteria.

Hyperaccumulators Plant	Metal	Organs	Endophytes	Reference
Alyssum bertolonii	Ni	Leaves	*Staphylococcus, Microbacterium*, *Pseudomonas*	Barzanti et al., 2007
		Stem	*Staphylococcus*, *Curtobacterium*, *Microbacterium*, *Curtobacterium*	
		Root	*Staphylococcus*, *Bacillus, Arthrobacter*, *Pseudomonas, Curtobacterium, Microbacterium*, *Paenibacillus, Leifsonia*	
Brassica napus	Pb	Root	*Pseudomonas fluorescens*, *Microbacterium* sp.	Sheng et al., 2008

Table 1 (Cont'd)

Hyperaccumulators Plant	Metal	Organs	Endophytes	Reference
Nicotiana tabacum	Cd	Seed	*Enterobacter* sp., *Xanthomonadaceae*, *Pseudomonas* sp., *P. fulva*, *Stenotrophomonas* sp., *Clostridium aminovalericum*, *Sanguibacter* sp.	Mastretta et al., 2009
Elsholtzia splendens	Cu	Root, Stem, Leaves	*Exiguobacterium aurantiacum*, *Burkholderia* sp., *Bacillus cereus*, *B. firmus*, *B. megaterium*, *Serratia marcescens*, *Acinetobacter calcoaceticus*, *A. junii*, *Micrococcus luteus*, *Moraxella* sp., *Paracoccus* sp.	Sun et al., 2010
Commelina communis	Cu	Root, Stem, Leaves	*Arthrobacter* sp., *Bacillus* sp., *B. pumilus*, *Sphingomonas* sp., *Sphingomonas* sp., *Herbaspirillum* sp., *Microbacterium kitamiense*,	Sun et al., 2010
Sedum alfredii Hance	Zn/Cd	Root	*Pseudomonas fluorescens*, *Bacillus pumilus*, *Acinetobacter calcoaceticus*	Long et al., 2011

Table 1 (Cont'd)

Hyperaccumulators Plant	Metal	Organs	Endophytes	Reference
Sedum alfredii Hance		Stem	*Stenotrophomonas maltophilia*, *Bacillus cereus*, *Pseudomonas synxantha*	Long et al., 2011
		Leaves	*Pseudomonas fluorescens*, *Bacillus pumilus*, *Bacillus subtilis*	
Solanum nigrum L.	Cd	Root, Stem, Leaves	*Bacillus* sp., *Arthrobacter* sp., *A. oxydans*, *Flavobacterium* sp., *Chryseobacterium* sp., *Agrobacterium tumefaciens*, *Sphingomonas* sp., *Pseudomonas oryzihabitants*, *Serratia* sp., *S. marcescens*, *Curtobacterium* sp. *Microbacterium* sp., *M. foliorum*, *M. hydrocarbonoxydans*	Luo et al., 2011
Commelina communis	Pb	Root, Stem, Leaves	*Agrobacterium tumefaciens*, *Acinetobacter sp.*, *Bacillus sp.*, *B. subtilis*, *B. megaterium*	Zhang et al., 2011
Pteris vittata	As	Root, Stem, Leaves	*Bacillus* sp. *Bacillus* sp., *Paenibacillus* sp. *Bacillus* sp.	Zhu et al., 2014

Table 1 (Cont'd)

Hyperaccumulators Plant	Metal	Organs	Endophytes	Reference
Pteris multifida	As	Root,	*Bacillus* sp., *Paenibacillus* sp., *Lysinibacillus* sp., *Sphingomonas* sp.	Zhu et al., 2014
		Stem,	*Bacillus* sp., *Massilia* sp., *Micrococcus* sp., *Curtobacterium* sp., *Roseomonas* sp., *Staphylococcus* sp., *Microbacterium* sp.	
		Leaves	*Bacillus* sp., *Micrococcus* sp., *Brevundimonas* sp., *Paracoccus* sp.,	
Astragalus bisulcatus	Se	Root,	*Bacillus* sp., *B. atrophaeus*, *Pantoea agglomerans*, *Pseudomonas* sp., *P. koreensis*, *Advenella kashmirensis*, *Variovorax* sp.	Jong et al., 2015
		Stem,	*Paenibacillus illinoisensis*, *Bacillus atrophaeus*, *Pseudomonas* sp.	
		Leaves	*Bacillus* sp., *B. atrophaeus*, *B. cereus*, *Pantoea agglomerans*, *Staphylococcus epidermidis*	

Table 1 (Cont'd)

Hyperaccumulators Plant	Metal	Organs	Endophytes	Reference
Stanleya pinnata	Se	Root,	*Bacillus sp., B. atrophaeus, Pantoea* sp., *P. agglomerans, Pseudomonas sp., P. koreensis, P. moraviensis, Arthrobacter* sp., *Staphylococcus* sp.	Jong et al., 2015
		Stem,	*Bacillus atrophaeus, Staphylococcus* sp., *S. condimenti, Pseudomonas sp., P. koreensis,*	
		Leaves	*Bacillus atrophaeus, Pantoea agglomerans,*	

2.6 The use of endophytic bacteria in phytoremediation

Endophytic bacteria play an important role in host plant adaptation to contaminated soils. They can enhance phytoremediation by mobilizing or immobilizing heavy metal contaminants in the soil, promoting the growth of plants, decreasing phytotoxicity and developing metal tolerance of the plants, as well as in different ways (Germaine et al., 2009; Weyens et al., 2010).

2.6.1 Plant growth promotion

The helpful effects of endophytes on their hyperaccumulators seem to occur through similar mechanisms represented for plant growth-promoting rhizobacteria (PGPR). Plant growth-promoting bacteria can affect plant growth in two ways, directly or indirectly. The direct promotion of plant growth by PGPR either facilitating the acquisition of essential nutrient resources and synthesizing plant hormones. The indirect promotion of plant growth occurs when PGPR decrease or

prevent the deleterious effects of one or more phytopathogenic organisms (Glick 2012).

Indole-3-acetic acid (IAA), a phytohormone, production is normal phenomena among numerous genera of soil bacteria and fungi, endophytic bacteria also are able to synthesize IAA (Sessitsch et al., 2004, Sheng et al., 2008; Chen et al., 2010; Zhang et al., 2011). Bacterial endophytic isolated from metal-hyperaccumulator plants such as *Serratia nematodiphila* LRE07, *Enterobacter aerogenes* LRE17, *Enterobacter* sp. LSE04, and *Acinetobacter* sp. LSE06 from *Solanum nigrum* L. (Cd-hyperaccumulator) (Chen et al., 2010). *Rahnella* sp. JN6 from *Polygonum pubescens* (Mn-hyperaccumulator) (He et al., 2013). *Burkholderia* sp. SaZR4, *Burkholderia* sp. SaMR10, *Sphingomonas* sp. SaMR12, *Variovorax* sp. SaNR1, and *Enterobacter* sp. from *Sedum alfredii* Hance. (Zn-hperaccumulator) (Wang et al., 2014). *Bacillus pumilus* E2S2, *Bacillus* sp. E1S2, *Bacillus* sp. E4S1, *Achromobacter* sp. E4L5 and *Stenotrophomonas* sp. E1L from *Sedum plumbizincicola* (Zn/Cd hyperaccumulator) (Ma et al., 2015). They can produce IAA to stimulate plant growth and increase efficiency in the phytoremediation process. In general, concentrations of IAA are different from one microorganism to another: 8 µg mL^{-1} with *Herbaspirillum seropedicae* (Govindarajan et al., 2007) and 28 µg mL^{-1} with *P. fluorescens* G16 (Sheng et al., 2008). Shin et al. (2012) reported that endophytic *Bacillus* sp. MN3-4 can improve the host plant's root elongation by 46.25 % through the production of IAA in comparison to the controls. Furthermore, some endophytic bacterial produce cytokines and/or gibberellins that stimulate the growth of plants under non-stress conditions. (Feng et al., 2006). The effect of IAA has been found to depend on the concentration, A low level of growth regulator produced by microorganism promotes primary root elongation whereas a high level of IAA stimulates lateral and root formation but inhibit primary root growth (Rajkumar et al., 2009). Therefore, endophytes can help plants grow by balancing plant hormones. For example, *Pseudomonas fluorescens* was reported as a bacterium of the rhizosphere that causes elongation and growth of root hairs (Rosenblueth and Martinez-Romero, 2006). Some *P. fluorescens* can also be endophytes, presenting in the roots and stems of some hyperaccumulators (Sheng et al., 2008). The discovered plant growth promotion

under metal stress when inoculation of plant with *P. fluorescens* was assumed to be the results of bacterial IAA production and excretion (Sheng et al., 2008). Therefore any direct influence on phytohormone production by bacteria may in turn affect their phytostimulating efficiency (Rajkumar et al., 2009).

Ethylene (C_2H_4) is an important plant hormone that controlling the growth and metabolism of plant cell (Ping and Boland, 2004), but excess ethylene production promoted by stress can inhibit plant development processes such as root elongation, parallel root growth, and root hair formation (Mayak et al., 2004). Thus, ACC deaminase production is likely an imperative and effective path for endophytes to control their plant. The cleavage of ACC produces in ammonia and α-ketobutyrate which are promptly used by microorganisms. In this case, these microorganism act as a sink for ACC. Madhaiyan et al. (2007) reported the highest potential of *Methylobacterium oryzae* and *Burkholderia* sp. Bacteria isolated from rice tissue, to protect tomato seeds from the toxicity of Ni and Cd under gnotobiotic conditions. In addition, they suggested that the inoculation of endophytes can also help to reduce the phytotoxic effects of metals by dividing the metal charge as its biosorption and bioaccumulation capacity.

Nitrogen is a necessary part of many essential plant compounds. It is a main part of all amino acids, nucleic acids, and chlorophyll. Nitrogen is determined the most limiting plant growth nutrient as a result of atmospheric N_2, that comprise about 78% of the Earth's atmosphere, can't be assimilated by higher plants directly into protein (Havlin et al., 2005). Additionally, to regulating the plant growth regulator levels, some endophytes accelerate the growth of the plant through nitrogen fixation. A well-known example is that nitrogen-fixing endophytes from sugarcane, which promote N to the plant and improve plant growth (Muthukumarasamy et al., 2002). Moreover, the cultivation of sugarcane, several different plants, as well as rice, corn, wheat, poplar and grass, are inhabited by endophytes of nitrogen fixation (Rajkumar et al., 2009). Rai et al. (2004) reported that the inoculation of *Prosopis juliiflora* with a fly ash tolerant rhizobium strain provided tolerance for the plant to grow under fly ash stress conditions with additional translocation of metals to the above-ground parts. Likewise, Long et al. (2011) found that endophytic bacteria,

Bacilli pumilus that isolated from roots and leaves of Zn/Cd *sedum alfredii* had the efficacy of nitrogen fixation.

Phosphate solubilization, Phosphorus (P) is another essential macronutrient for biological growth and development. Although soils have large reserves of total P, the amounts available to plants is usually a little proportion of this total (Stevenson and Cole, 1999). The low availability of P to plants is a result of the vast majority of soil P found in insoluble forms, and plants only absorb P in two soluble forms of the monobasic ($H_2PO_4^-$) and also the diabasic ($H_2PO_4^{2-}$) ions (Glass, 1989, Ae and Shen, 2002). Phosphorous solubilizing bacteria are common in the rhizosphere, and the secretion of organic acids and phosphatases is a common technique to support the exchange of insoluble types of P for use in plants (Kim et al., 1998). Many endophytic strains indicate dissolved mineral phosphates (Verma et al., 2001), suggesting that endophytic bacteria may increase plant phosphate availability during initial formation. Kuklinsky-Sobral et al. (2004) showed that 52% of soluble phosphates are isolated from soybeans. Puente et al. (2009) reported that endophytic bacteria in cacti seeds improve the seedlings in barren rock, improving the elimination of P_2O_5, Fe_2O_3, K_2O, and MgO from the substrate.

2.6.2 Mobilization of metals in phytoextraction

Plant-related microorganisms enhance phytoextraction by modifying the dissolvability, accessibility, and transport of metal and nutrients by produce organic acid, release of chelators, siderophores production, or redox changes and/or the metal mobilization (Saravanan et al., 2007; Sheng et al., 2008).

Siderophores are organic molecules that show high affinity for Fe(III) ions, and the compounds form complexes with different bivalent heavy metal ions like Al, Cd, Cu, Ga, In, Pb and Zn. The binding of the siderophore to a metal expands the metal solubility (Rajkumar et al., 2010), that can be absorbed by the plant as solubilizing operators for press from minerals or organic compounds to enhance its efficient uptake under conditions of iron limitation. Several studies have been reported the siderophore production of endophytes from various plants and they increased plant growth in low nutrition environments (Idris et al., 2004; Barzanti et al., 2007; Sheng et al., 2008; Chen et al., 2010; Ma et al., 2011; Shin et al., 2011;

Zhang et al., 2011). For example, Barzanti et al. (2007) reported that 83% of endophytic bacteria isolated from *Alyssum bertolonii*. They produce siderophores and promote the plant growth under Ni stress. Similarly, Idris et al. (2004) showed the siderophore production in Ni-resistant bacteria isolated from *Thlaspi goesingense*. Lodewyckx et al. (2002) presented that the endophytes isolated from stem and root tissues of *T. caerulescen* did not manufacture siderophores under iron deficient condition.

Moreover, some endophytes have been shown that extend the mobilization of heavy metals through the secretion of organic acids of low molecular mass. Sheng et al. (2008) showed that the water-soluble Pb significantly increased but the pH decreased in a solution with endophytes growth, suggested that this might be due to the production of organic acids by endophytic bacteria. Kuffner et al. (2010) also found that some *Actinobacterium* endophytes could release metabolites that mobilize metals in contaminated soils, mobilized Zn and/or Cd, and a greater accumulation of metals in the leaves of *salix caprea*. Long et al. (2011) indicated that *Pseudomonas fluorescens*, *Bacillus pumilus*, *Acinetobacter calcoaceticus* can effectively solubilize $ZnCO_3$ and $Zn_3 (PO_4)_2$.

2.6.3 Metal accumulation in plants

The efficiency of phytoremediation in metal contaminated soils is particularly dependent on the uptake of metals and the accumulation in the aboveground. They have been proposed that some endophytes are resistant and/or plant growth promoting endophytes can improve the uptake and accumulation of metals in plants. Chen et al. (2010) proposed that four endophytic bacteria resistant to heavy metals increased the accumulation of Cd in roots, stems, and leaves of *Solanum nigrum* L. growing in Cd-contaminated soils, and the amount of accumulated Cd in the soil increased with the concentration of Cd in the soil. Likewise, Mastretta et al. (2009) found that the inoculation of Cd-resistant endophyte (*Sanguibacter* sp.) with *Nicotiana tabacum* accumulated the concentration of Cd in the shoot about three-fold compared with various un-inoculated control. Sheng et al. (2008) found that the inoculation of *Brassica napus* with Pb-resistant endophytic bacteria enhanced Pb uptake into the shoot. However, the presence of metal resistant endophytes decreased

the uptake of metals by the plants and thereby enhanced plant biomass. Madhaiyan et al. (2007) presented that inoculation with *M. oryzae* and *Burkholderia* sp. isolated from rice tissues reduced the Ni and Cd uptake in roots and shoots of tomato and their availability in soil. Chatterjee et al. (2009) reported that the inoculation of Cr-resistant bacteria reduced the toxicity of Cr(VI) to non-toxic (Cr(III) and decreased Cr accumulation in the chilli plants. Huo et al. (2012) showed the effect of inoculation with the Cu-resistant *Pantoea* sp. Jp3-3 significantly decreased Cu absorption and accumulation in guinea grass under Cu stress. The reduced accumulation of metals could be due to bacterial immobilization of metals in rhizosphere.

2.7 Plant colonization and inoculation with endophytes

After the colonization of the rhizosphere and the rhizoplane, some soil microorganisms will enter the roots and establish subpopulations that will vary between 10^5-10^7 CFU g^{-1} FW (Hallmann, 2001). This includes particular characteristics needed for endophytic ability i.e. the potentiality to effectively colonize the plant. Passive infiltration will occur at cracks, like those occurring at emergence of the roots or created by harmful microorganisms, also as by root tips (Figure 3). Expression of lipopolysaccharides, flagella, pili and twitching motility related to the colonization of endophytic and bacterial movement in plants (Böhm et al., 2007). In addition, secretion of cell wall degradation involved in colonization of endophytic bacteria and spreading within the plant (Lodewyckxet al., 2002).

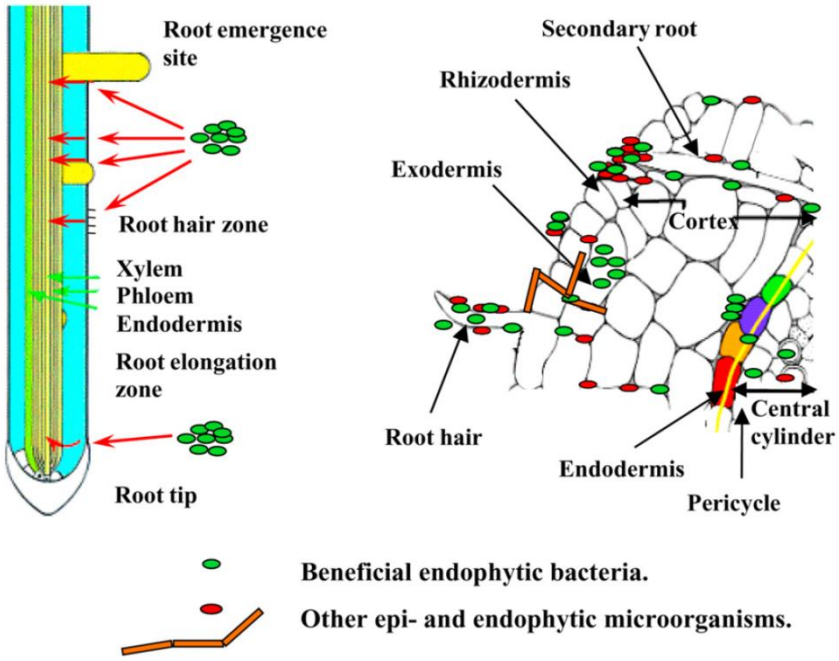


Figure 3 Sites of plant colonization by endophytic bacteria.
Drawing modified from Reinhold-Hurek and Hurek, (1998) and Compant , (2007).

Active or passive mechanisms are explained by the translocation process of the endophytic bacteria inside the host plant, which allows them to be transferred from the rhizobia to the root cortex. Barriers to successful bacteria in the root zone, such as endodermis, can block colonization because only a few bacteria can pass endodermis (Gregory, 2006). It's probably that endophytes can go through the endodermis will secrete cell-wall degrading enzymes permitting them to continue colonization within the endorhiza (James et al., 2002). As an alternative, some bacterial cells into endodermal cell acute because some species are not continuously expanding secondary roots, which caused pericycle which fall within the scope of endodermis barrier (Gregory, 2006). Under natural conditions, some harmful bacteria can damage the endodermis while allowing endophytic bacteria to enter the central cylinder. Passing through the endodermis barrier, endophytic bacteria must penetrate the periphery to separate the vessels of the root tissue of their host vessels (Figure 3).

Furthermore, the correlation between bacterial endophytes colonization and heavy metal distribution may explain the mechanism of hyperaccumulator plant accumulation and heavy metal detoxification. Techniques utilized for mapping

considers in biological tissues was μ-EDX or Synchrotron radiation X-ray fluorescence (West et al., 2009, Majumdar et al., 2012), micro-proton-induced X-ray emission (micro-PIXE) (Lyubenova et al., 2012), also based on a laser-methods, for example, laser ablation and inductively coupled plasma mass spectrometry (LA-ICP-MS) (Guerra et al., 2011, Qin et al., 2011).

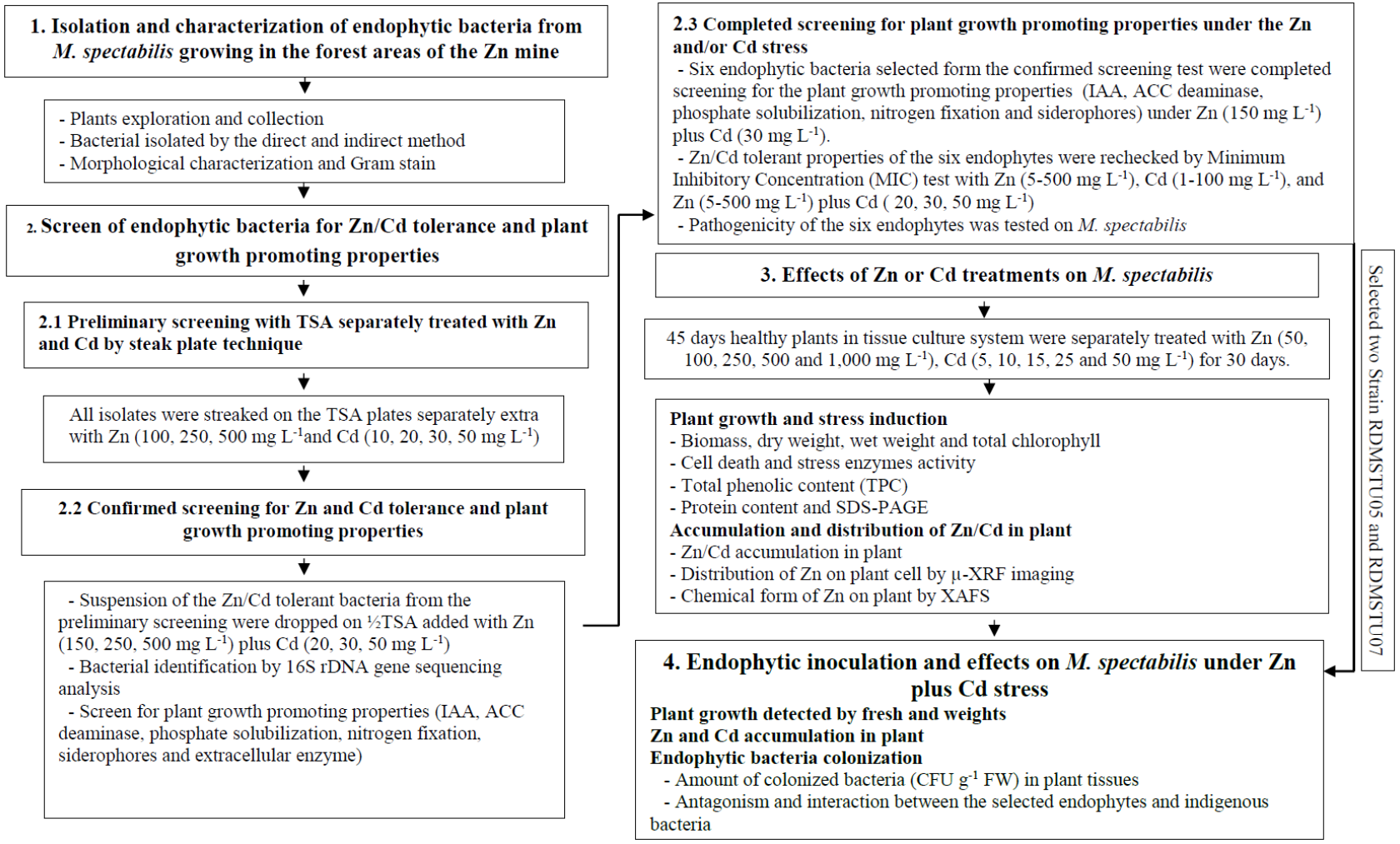
X-ray fluorescence analysis (XRF) is a fast and non-destructive method that has many applications (West et al., 2009). The techniques have been widely used in the analysis of elemental distribution in plant tissues and have demonstrated to be a promising tool to present the *in vivo* mapping of metals in plants (Punshon et al., 2009). For instance, Hokura et al. (2006a) reported that the first time that the 2-D cell distribution of cadmium was obtained in *Arabidopsis halleri* ssp.gemmifera tissue using an X-ray microbeam of high-energy synchrotron radiation. Hokura et al. (2006b) studied Cd distribution in *Nicotiana tabacum* (Cd hyperaccumulator) as determined by μ-XRF imaging. The results showed that Cd accumulated in the sieved tissue of the plant. Mongkhonsin et al. (2011) examined the distribution of chromium (VI) accumulated in *G. pseudochina* (L.) DC using μ-XRF imaging. Cr was found mainly in the periderm and periderm of the tuber, xylem, vein and epidermis, as well as the trichome of leaf tissue. Cr signals confirm that Cr is transported from the stem to the leaf through xylem and vein.

CHAPTER 3

MATERAILS AND METHODS

This research was designed to isolate and characterize culturable endophytic bacteria from *M. spectabilis* growing in forest areas of the Padang Industry, Phatat Phadaeng sub-district, Mae Sot, Tak province, Thailand. The endophytic isolates were screened for Zn/Cd tolerance and plant growth promoting properties of indole-3-acetic acid (IAA) production, ACC deaminase activity, phosphate solubilization, nitrogen fixation, siderophores production and extracellular enzymes. Effects of Zn and Cd stresses on physiology and the metals accumulation of *M. spectabilis* were carried out in a tissue culture system. The threshold concentrations of Zn plus Cd stresses on the plant were selected for study an effect of the endophytic inoculation. In addition, antagonism and interaction between the select endophytic bacteria and indigenous endophytic bacteria in *M. spectabilis* were investigated. Flow chart of the experimental studies is shown in Figures 4. All chemicals used in this research were analytical grade.

Figure 4 Research diagram



3.1 Isolation and characterization of endophytic bacteria

3.1.1 Plant materials

Plants and soil samples were collected from the four sites in the forest area of the Padand Industrial, a zinc mine in Phatat Phadaeng sub-district, Mae Sot, Tak Province, Thailand, in August 2015 (Rainy season). The four locations are following:

1. Site: N 16º 39’ 6.6”E 98º 39’ 41.2”
2. Site: N 16º 39’ 6.4”E 98º 39’ 44.5”
3. Site: N 16º 39’ 3.1”E 98º 39’ 45.8”
4. Site: N 16º 39’ 3.1”E 98º 40’ 11.6”

The soil temperature was determined in that site using a digital thermometer (Hanna HI98501, Romania)

Soil analyzes: Soil samples were carefully removed plant roots before air dried. Then the soil samples were dried at 80 ^{o}C for 24 hours in a hot air oven (Redline-Binder, Germany) until a stable weight was reached to determine the soil moisture. A 1:1 of soil:water suspension was shaken at 150 rpm for 1 hour and determined the value of pH by a pH meter (Denver Instrument Model 215, USA). A dried sample was sieved and ground to a homogenized sample. Total concentrations of Zn and Cd in a soil samples were analyzed by a modified method of ASTM E841-04 (ASTM, 2004). A soil sample was weighted for 0.10 g by a balance (PA214 Ohaus, USA) and put into a borosilicate tube. The soil was digested with 3 mL of aqua regia (mixture 3:1 of 35% (w/v) HCl and 70% HNO_3) at 150 ^{o}C for 3 hours, then 3 mL of deionized water was filled into the cooling tube sample and reheated at 90 ^{o}C for 1 hour. To obtain amounts of extractable Zn and Cd, a soil sample was extracted by shaking with 0.005 M of diethylene triamene penta acetate (DTPA) (Lindsay and Norvell, 1978). The mixture between soil and DTPA at the 1:2 ratio was shaken with an orbital Shaker (PSU-10i, EU) at 150 rpm, room temperature (30 ± 5 ^{o}C) for 2 hours. All samples from the acid digestion and the DTPA extraction were filtrated with Whatman no.50 and no.5, respectively. The filtrated samples were analyzed for Zn and Cd by inductively coupled plasma spectroscopy ICP-OES (PerkinElmer Optima 8000, USA).

Zn and Cd accumulation in plant: Plants were carefully washed with an excess of tap water, then rinsed three times with deionized water before wiped off the remaining water with clean and soft paper. A clean plant sample was separated into storage roots, underground stems (tubers), leaves and peduncles. Each parts was dried at 60 °C for 24 hours in the hot air oven. A dried plant sample was digested with a modified method of Miller (1998). A 0.10 g of the plant sample was socked in 3 mL of HNO_3 (70% v/v) for 24 hours, before heated at 150 °C for 1 hour in a test tube. The sample was further digested with 1 mL of $HClO_4$ (70% v/v) at 215 °C for 2 hours. Then, 3 mL of deionized water was added to the cooling tube and reheated at 90 °C for 1 hour. The samples were filtered by Whatman no. 50 before analyzed with an atomic absorption flame emission spectrophotometry (AAS) (Agilent 280, Australia).

3.1.2 Isolation of culturable endophytic bacteria

The plant samples collected from the Zn mine were washed with an excess of tap water, rinsed three times with distilled water and then separated into storage roots, tubers, leaves and peduncles. In the first step, leaves and storage roots were used as representatives of above ground and underground samples to study an optimum condition for surface sterilization. Table 2 shows various concentrations of Haiter bleach (Kao, Thailand) (6% w/w sodium hypochlorite (NaOCl) as the active ingredient) and soaking at different times for surface-sterilization test. Each plant sample was cut and trimmed into 1 to 3 cm long. The healthy trimmed samples were socked in 75% (v/v) ethanol for 3 minutes, before tested for surface sterilized with the various conditions in Table 2. After the step of soaking in NaOCl, the plant samples were washed three times with sterile distilled water to remove the surface sterilization agents. Success in the surface sterilization processes was investigated by rolling the sterilization plant samples over a plain trypticase soy agar (TSA) (Himedia, India), and spreading 0.1 mL of the third rinse water onto TSA media. The plates were incubated at at 30 ± 5 ºC and observed the growth of any microbial colony for 3-7 days. The surface sterilized conditions that resulted in no growth of any microbes were applied for the isolation of endophytic bacteria. The success conditions for surface-sterilization of storage root and leaves were applied to sterilize surface of tuber and peduncle samples, respectively.

Table 2 Concentrations of 6% w/w sodium hypochlorite (NaOCl) and soaking times for surface-sterilization test.

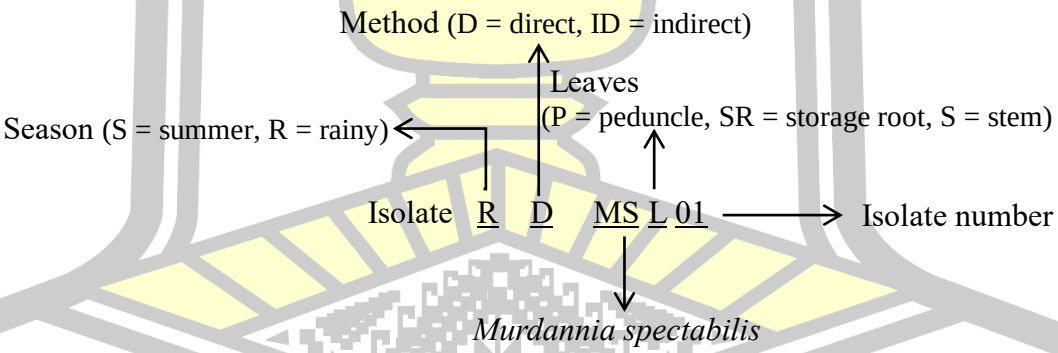
Explants	Treatments	Condition for surface-sterilization	
		NaOCl (% w/w)*	Time (Minutes)
Leaf	1	1.2	10
	2	0.9	10
	3	0.6	10
	4	0.3	10
Storage root	1	1.2	15
	2	0.9	15
	3	0.6	15
	4	(1) 0.9	15
		(2) 0.3	5

* NaOCl solution was prepared from Haiter bleach (Kao, Thailand).

The surface-sterilization of explants were studied for the best condition before applied in the endophytic bacterial isolation. For the above-ground parts (leaves and peduncles), the explants were soaked in 0.9% (v/v) NaOCl solution for 10 minutes, and rinsed three times for 5 minutes in each sterile water (Appendix A1). Whereas, underground part (storage root and tuber), which had a lot of rhizobacteria, had to used two times of disinfectants by NaOCl. The explants from storage root and tuber were soaked in 0.9% (v/v) NaOCl solution for 15 minutes, then soaked in 0.3% (v/v) NaOCl solution for 5 minutes, and rinsed three times for 5 minutes in each sterile water. The successes of surface sterilization processes is shown in Appendix A1 and A3, respectively.

Endophytic bacteria were isolated from the surface-sterilized samples of storage root, tubers, leaves and peduncles by direct and indirect methods. In case of direct method, the explants were directly plated on TSA media. For indirect method (dilution-plate techniques), each explants was weight and ground in a steriled mortar containing sterile phosphate-buffered saline (PBS) (PBS buffer per liter consists of 1.44 g Na_2HPO_4; 0.24 g KH_2PO_4, 0.20 g KCl, 8.00 g NaCl, pH 7.4) under aseptic techniques. The ratios of each explants and PBS buffer were 2.5 g of storage roots in

5 mL PBS buffer, 0.5 g of tubers in 2.5 mL PBS buffer, 1 g of leaves in 5 mL PBS buffer and 1 g of peduncles in 5 mL PBS buffer. Serial dilutions of the homogenized pulp samples were prepared by sterile phosphate-buffered saline to obtain 10^{-1} and 10^{-2} dilutions. Endophytic bacteria from each dilution were isolated by spread plate technique and completed in triplicate. To prevent the growth of endophytic fungi A 100 mg L^{-1} of nystatin (nystatin oral suspension; Continental-Pharm, Thailand) was added to the melting sterilized TSA medium (45 ºC) before agar plate preparation. 0.1 mL of each dilutions was spread onto the TSA suppled with nystatin and incubated at 30 ± 5 °C. Bacterial cultures were observed daily for 2-7 days. Bacterial colonies on the different characteristics of colony such as size, shape, edge or margin, surface, opacity and colour were separated counted to obtain the number of each colony forming units (CFU) per gram fresh weight (FW). The different endophytic bacterial colonies were picked and isolated by quadrant steak plate technique on the plain TSA medium. The bacterial cell morphology was investigated under a light microscope with simple and Gram's staining methods. Every isolates were stored as stock cultures at -20 °C in trypticase soy broth (TSB) containing 15% (v/v) glycerol. The endophytic bacterial isolates were given code names as following:



3.2 Screen of endophytic bacteria for Zn and/or Cd tolerance and plant growth promoting properties

3.2.1 Preliminary screening for Zn or Cd tolerant endophytic bacteria

The bacterial isolates from 3.1.2 were refreshed in control TSA media (without Zn and Cd) for 24-48 hours. Then one loop of each bacterial isolates were streaked on each TSA plates separately supplied with each concentrations of Zn (100,

250 and 500 mg L^{-1}) and Cd (10, 20, 30 and 50 mg L^{-1}). Stock solutions of Zn (5,000 mg L^{-1}) and Cd (500 mg L^{-1}) were separately autoclaved before supplied into the sterilized melting TSA. The Zn and Cd solutions were prepared from $ZnSO_4.7H_2O$ (Ajax Finechem, Australia) and $3CdSO_4.8H_2O$ (Ajax Finechem, Australia), respectively. All streak plates were incubated at 30 ± 5 °C for 24-48 hours. The bacteria survived on the TSA contaminated with Zn in the ranges of 250-500 mg L^{-1} or Cd in the ranges of 20-50 mg L^{-1} were selected for confirmed screening test.

3.2.2 Confirmed screening test for Zn and Cd tolerant endophytic bacteria

The Zn and/or Cd tolerant endophytic bacteria from 3.1.1 were confirmed screening by ½TSA media contaminated with Zn plus Cd conditions. The nine Zn plus Cd conditions were prepared from 3x3 factorial experiments of Zn (150, 250, 500 mg L^{-1}) and Cd (20, 30, 50 mg L^{-1}). Cell suspension was prepared by cultivation a bacterium in TSB and shaking at 150 rpm, 30 ± 5 ºC for 18-24 hours. The bacterial cells were collected by centrifugation, washed two times with the PBS buffer and re-suspended in the PBS buffer to obtain an optical density (OD) of 0.1 units at a wavelength of 600 nm. The approximate cell density was $1.5x10^8$ CFU mL^{-1}, when compared the OD with McFarland standard at 0.5 units. A 5 µl cell suspension of a bacterial isolate was dropped on a ½TSA plate contaminated with a condition of Zn plus Cd and incubated at 30 ± 5 °C for 24-48 hours. This confirmed screening test showed that the bacterial isolates from 3.1.1 were tolerant to both Zn and Cd. Therefore, they were studied for PGPB properties and identified by 16S rDNA sequencing analysis.

3.2.3 Plant growth promoting properties of endophytic bacteria

The PGPB properties of IAA production, ACC deaminase activity, nitrogen fixation and phosphate solubilization were tested with the selected endophytes under Zn/Cd stress-free conditions.

Indole-3-acetic acid (IAA) production: A bacterial cell suspension for inoculation was prepared by centrifugation, washing and resuspending with the PBS buffer to obtain 0.1 unit at OD_{600}. A 50 µl of a bacterial suspension was inoculated into an amber glass bottle containing 5 mL of TSB supplemented with 0.2% (w/v) of

tryptophan, and shaken at 150 rpm, 30 ± 5 ^{o}C for 48 hours. After incubation, bacterial cells and supernatant were separated by centrifugation at 6,000 rpm for 10 minutes. A supernatant was mixed with Salkowski's reagent (2% of 0.5 M $FeCl_3$ in 35% $HClO_4$ solution) at a ratio of 2:1 in the dark for 20 minutes. The absorbance at 530 nm was analyzed by a visible spectrophotometer, (Spectronic 20 Genesys Thermo Scientific, USA). The IAA concentrations in the supernatants were determined using a standard calibration curve of IAA. The IAA stock solutions were prepared from a chemical standard of IAA (Sigma-Aldrich, USA).

ACC deaminase: The bacterial isolate was considered based on the capacity of the individual isolate to utilize ACC as a sole nitrogen source. The 30 µl of each bacterial suspension in the PBS buffer (OD_{600} of 0.1) was inoculated in a 10-mL test tube contained with 3 mL modified DF minimal salts medium. The modified DF minimal salts medium per liter consists of 2.0 g glucose, 2.0 g citric acid, 4.0 g KH_2PO_4, 6.0 g Na_2HPO_4, 0.2 g $MgSO_4.7H_2O$ and 10 mL of micro-nutrient solution. One liter of micronutrient solution was prepared by dissolving 200 mg $CaCl_2$, 200 mg $FeSO_4.7H_2O$, 15 mg H_3BO_3, 20 mg $ZnSO_4.7H_2O$, 10 mg Na_2MoO_4, 10 mg KI, 10 mg $MnCl_2$, 5 mg $CoCl_2$, 5 mg $CuCl_2$, in distilled water in (Dworkin and Foster 1958). The modified DF minimal salts medium was sterilized at 121 ^{o}C for 15 minutes, 3 mM ACC was suppled as the sole source of nitrogen. The 200 mM ACC stock solution was prepared by dissolving ACC (98% purity) (Sigma Aldrich, USA) in sterile distilled water and filtered through a 0.2 µm sterile filter membrane (Minisart Syringe Filters-Sartorius, Germany) and stored at -20 °C. The plain DF minimal salts medium without ACC was used as a negative control, and the medium supplemented with $(NH_4)_2SO_4$ (0.2 % w/v) was used as a positive control. All inoculated tubes were shaken 150 rpm, 30 ± 5 ^{o}C for 72 hours. The growth of endophytic bacterial isolates in the plain and supplemented DF minimal salts medium were observed. An increase turbidity of the bacterial cells in the DF media supplemented with ACC indicated that the bacterial isolate had ACC deaminase activity to digested ACC and used as nitrogen source. The turbidity of bacterial cells were investigated at OD 600 nm.

Nitrogen fixation: A 5 µl of each bacterial suspension in the PBS buffer (OD_{600} of 0.1) was dropped on a N-free malate medium (Ronald, 2005). The N-free malate medium per liter consisted of 5.0 g malic acid, 0.5 g K_2HPO_4, 0.2 g $MgSO.7H_2O$, 0.1 g NaCl, 0.02 g $CaCl_2.2H_2O$, 0.002 g $NaMoO_4.2H_2O$, 0.01 g $FeCl_3.6H_2O$, 0.025 g bromothymol blue and 0.25 g yeast nitrogen base w/o amino acid. The pH of the medium was adjusted to 7.0 with pellet of NaOH before adding 15 g of the agar. The inoculated plates were incubated at 30 ± 5 ºC for 1-7 days. A change of the medium by an isolate from green to blue colour indicated that the isolate had a nitrogen-fixing activity.

Phosphate solubilization: A 5 µl of bacterial suspension in the PBS buffer (OD_{600} of 0.1) was dropped on the National Botanical Research Institute's phosphate growth (NBRIP) agar plate. The NBRIP medium per liter consists of 10.0 g glucose, 5.0 g $Ca_3(PO_4)_2$, 5.0 g $MgCl_2.6H_2O$, 0.25 g $MgSO_4.7H_2O$, 0.2 g KCl and 0.1 g $(NH_4)_2SO_4$. Dissolve ingredients of the first part and adjust the pH to 7.0 before adding 15 g of the agar (Ronald, 2005). The inoculated plates were incubated at 30 ± 5 ºC for 1-7 days. A clear zone around a bacterial colony presented a phosphate solubilizing activity of the bacterium.

Siderophore production: A 5 µl of bacterial suspension in the PBS buffer (OD_{600} of 0.1) was dropped on the TSA and incubated at 30 ± 5 ºC for 24 - 48 hours. To detect siderophore production, a CAS medium was overlaid over the inoculated TSA plate, and the detection was done by observing the colour changes of the medium for 30 minutes. The colour changes around the bacterial colonies presented that the endophytic bacteria was able to produce siderophores. In which, the colour changes depending on the different types of siderophores such as a change from blue to violet colour indicating to catechol type, and a change from blue to orange colour indicating to hydroxamate types (Pérez-Miranda et al., 2007). The CAS medium was prepared by following Schwyn and Neilands (1987). One liter of the medium consisted of 60.5 mg of Chrome azurol S (CAS), 72.9 mg of hexadecyltrimetyl ammonium bromide (HDTMA), 30.24 g of Piperazine-1,4-bis (2-ethanesulfonic acid) (PIPES), 10 mL of 1 mM $FeCl_3 \cdot 6H_2O$ in 10 mM HCl and 0.9% (w/v) of agarose.

Cellulase assay: A 5 µl of bacterial suspension in the PBS buffer (OD_{600} of 0.1) was dropped on a carboxy methylcellulose (CMC) agar medium. One liter of the CMC agar medium consisted of 0.5 g KH_2PO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, 2 g CMC disodium salt and 15 g agar. After 5 days of incubation at 30 ± 5 ºC, the CMC plates were flooded with a Congo red solution (1 mg L^{-1}) for 15 minutes. The Congo red solution was poured out of the plate, then an excess Congo red remaining in the agar plate was washed with 1 M NaCl for 15 minutes. A clear zone around the bacterial colony indicated to extracellular cellulase production (Gupta et al., 2012).

Ligninolytic enzymes assay: A 5 µl of bacterial suspension in the PBS buffer (OD_{600} of 0.1) was dropped on the TSA plate containing methylene blue (0.25 g L^{-1}) as an indicator. The plates were incubated at 30 ± 5 ºC for 1-3 days. The agar plates were monitored daily for bacterial growth and decolorization of the methylene blue dyes. Decolorized zone that appeared around the bacterial colony indicated the presence of ligninolytic enzyme activity (Hooda et al., 2015).

Lignin alkali degradation: A 5 µl of bacterial suspension in the PBS buffer (OD_{600} of 0.1) was dropped on a mineral salt medium (MSM) agar. One liter of the MSM agar consisted of 10 g D-glucose, 5 g peptone, 2.4 g Na_2HPO_4, 2.0 g K_2HPO_4, 0.1 g NH_4NO_3, 0.01 g $MgSO_4$, 0.01 g $CaCl_2$ and 1 mL of trace element solution (5.00 g EDTA, 2.20 g $FeSO_4.7H_2O$, 0.10 g $ZnSO_47H_2O$, 0.03 g $MnCl_24H_2O$, 0.03 g H_3BO, 0.20 g $CoCl_2$ $6H_2O$, 0.03 g $CuCl_22H_2O$, 0.03 g $NiCl_2$ $6H_2O$, 0.03 g $Na_2MoO_42H_2O$ in distilled water 1,000 mL) (Pfenning and Lippert, 1966). The MSM agar containing 0.1% (w/v) lignin alkali (Sigma-Aldrich, USA). The pH of medium was adjusted to 7.6 with 0.1 M NaOH or 1 M HCl. The inoculated plates were incubated at 30 ± 5 ºC for 1-7 day. The growth of bacteria on the plates indicated to the lignin degradation property.

3.2.4 Bacterial identification by 16S rDNA sequencing analysis

The Zn and Cd tolerant endophytes from 3.2.2 were identified by 16S rDNA sequencing analysis. The bacteria cells growing in TSB was prepared by centrifugation and washing with the PBS buffer. Genomic DNA of a bacterium was

extracted by a modified phenol: chloroform procedure of Sambrook and Russel (2001). 16S rDNA gene were amplified by PCR, using 100 ng genomic DNA as template with bacteria universal primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rP2 (5'-ACGGCTACCTTGTTACGACTT-3') (Weisburg et al., 1991). A 50 µl of the PCR mixture consisted of 1 µl DNA template, 5 µl of 10xTap DNA polymerase buffer, 1.5 µl of 50 mM $MgCl_2$, 1 µl of dNTP at 10 mM, 0.5 µl of 5 Unite per µl DNA Taq polymerase (Invitrogen, USA), 2.5 µl of 10 µM for each primer and 36 µl sterile deionized water. The PCR was performed in a Thermal Cycler (Applied Biosystems VeritiTM 96-Well Thermal Cycler, USA). The amplification of the thermal cycling program was carried out by 1 cycle of 94 ºC for 5 minutes (denaturation), 57 ºC for 2 minutes (annealing) and 72 ºC for 2 minutes (extension); 29 cycles of 94 ºC for 2 minutes, 57 ºC for 30 seconds and 72 ºC for 2 minutes; and a final extension cycle of 72 ºC for 10 minutes (Wood et al., 1998). The amplified DNA was purified with the GF-1 AmbiClean Kit (Vivantis, USA), then the DNA sequence was analyzed by Macrogen sequencing service (Seoul, Korea). The 16S rDNA sequences of the bacteria were analyzed by comparison with the database of National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) program.

3.2.5 Completed screening for PGPB properties under Zn plus Cd stress

The endophytic bacteria were compared using the properties of Zn and Cd tolerance and PGPB properties. The bacteria that produced a high IAA, fixed nitrogen and product siderophores was selected for completed screening test. In addition, the bacterial isolates that have been reported to be human and plant pathogenicity were not selected for further study. According to the criteria, the six endophytic isolates of RDMSSR02, RDMSSR04, RDMSSR05, RDMSSR07, RDMSP03 and RDMSP06 were completed screening for PGPB properties under Zn plus Cd stress. The Zn and/or Cd tolerant properties of the six isolates were rechecked to obtain minimum inhibitory concentration (MIC). Finally, the six isolates was tested for pathogenicity on *M. spectabilis*.

Plant growth promoting properties under Zn plus Cd: The six endophytic isolates were tested for IAA production, ACC deaminase, phosphate solubilization, nitrogen fixation and siderophore production under Zn plus Cd stress. The methods were following the sub-topic of 3.2.3, in which each media was supplied with 150 mg L^{-1} of Zn plus 30 mg L^{-1} of Cd.

Minimum Inhibitory Concentration (MIC): A modified microtiter plate with resazurin indicator was applied to investigate the MIC (Sarker et al., 2007). The total volume of each well was 120 µl, containing 50 µl of TSB, 50 µl of Zn and/or Cd stock solutions or sterile water (control), 10 µl of resazurin solution and 10 µl of bacterial suspension with the OD_{600} of 0.132. The resazurin solution was prepared by dissolving 135 mg of resazurin in 20 mL of sterile distilled water, then the solution was filtered through a sterile 0.2 µm filter membrane. The final concentration of the resazurin solution was 6.75 mg mL^{-1}. The Zn and/or Cd concentrations were prepared by a two-fold serial dilution to obtain the Zn and Cd ranges of 3.9-500 mg L^{-1} and 0.78-100 mg L^{-1}, respectively. The dual treatments were carried out by using Zn in the range of 3.9-500 mg L^{-1} plus the three concentration of Cd at 20, 30 and 50 mg L^{-1}. The MIC experimental design is shown in Figure 5. The plates were studied in duplicate. The MIC plates were prepared under aseptic conditions and incubated at 30 ± 5 ^{o}C for 3-7 Days. The colour changes from purple to pink or colourless were positive. The colour changes from dark purple to purple (light purple or soft purple) were determined as the minimum inhibitory concentration (MIC) value.

	Zn		Cd		Zn/Cd 50		Zn/Cd 30		Zn/Cd 20			
	1	2	3	4	5	6	7	8	9	10	11	12
A	500		100		500/50		500/30		500/20		+Mo	+Mo
B	250		50		250/50		250/30		250/20		x	x
C	125		25		125/50		125/30		125/20		+Mo	+Mo
D	62.5		12.5		62.5/50		62.5/30		62.5/20		-Mo	-Mo
E	31.25		6.25		31.25/50		31.25/30		31.25/20		x	x
F	15.63		3.13		15.63/50		15.63/30		15.63/20		x	x
G	7.81		1.56		7.81/50		7.81/30		7.81/20		x	x
H	3.9		0.78		3.9/50		3.9/30		3.9/20		x	x

Control (TSB+MO)

Control (½ TSB+MO) (½ TSB-MO)

Figure 5 MIC experimental design.

Pathogenicity test: Pathogenicity of the six isolates were tested by following Kang et al. (2007). The plants were cultured in Murashige and Skoog (MS) (Murashige and Skoog, 1962) medium in plant tissue culture glass bottles (diameter 4.5 cm, height 8.5 cm) at 25 ºC under a 1,500 lux light intensity for a 12 hours photoperiod for 4 weeks before the test. The bacterial inoculation was carried out under aseptic technique. Bacterial cells (10^8 CFU mL^{-1}) suspended in PBS buffer were swabbed on 2-3 healthy leaves by a sterile cotton swab. In addition, bacterial inoculation on rhizosphere was carried out by apply 100 µl of the bacterial inoculum on surface of the MS medium. For a control system, the same amounts of sterilized PBS buffer were applied instead of the bacterial suspension. The treated plants were cultured in the plant tissue culture system. Any symptom, especially bacterial spot, was daily assessed and recorded for two weeks (14 days) after inoculation.

3.3 Effects of Zn or Cd treatments on *M. spectabilis*

Effects of Zn and/or Cd on *M. spectabilis* were investigated to obtain threshold concentrations of Zn and/or Cd that affecting on *M. spectabilis.* The plant samples were evaluated for fresh weight, dry weight, number of storage root and the percentage of yellow/pale leaves (phytotoxicity) and chlorophyll content. The plants cell death were analyzed. Total phenolic content (TPC), total protein including SDS-PAGE and the stress enzymes activities of superoxide dismutase (SOD) and catalase (CAT) were also investigated to obtain any stress effects of Zn and/or Cd. Amounts of Zn and/or Cd accumulated in the plant tissue were analyzed by the AAS. In addition, micro X-ray Fluorescence (µ-XRF) imaging and X-ray Absorption Fine Structure (XAFS) was analyzed to acquire Zn distribution and probable chemical form of Zn complexes accumulated in the plant tissues.

3.3.1 Plant cultivation and Zn and/or Cd treatment

M. spectabilis's shoots were soaked in soap and washed with running water. These samples were immersed in 75% ethanol (v/v) for 3 minutes. The samples were surface sterilized with 0.9% (v/v) NaOCl solution for 10 minutes, and then soaking in 0.3% (v/v) NaOCl solution for 10 minutes, and finally rinsed three times

with sterile distilled water to remove surface sterilization agents. The shoots were cultured on 20 mL MS medium containing 0.1 mg L^{-1} benzylaminopurine (BAP) (Sigma-Aldrich, Germany). The solution pH was adjusted to 5.7 with 0.1 M NaOH or 1 M HCl, then agar was added to obtain 7.0 g of L^{-1} and autoclaving at 121 ^{o}C for 15 minutes. The explants were cultured at 25 ^{o}C under 1,500 lux of light intensity and a 12 hours photoperiod. After 30 days, subculture transferred to 20 mL of the ½ MS medium cultured for 45 days.

The plants for treatment with Zn or Cd were selected from healthy plants with a similar number of leaves and heights grown on ½ MS medium cultured for 45 days. The plant samples were separately treated with 2 mL each of stock solution Zn or Cd. The final concentrations of Zn or Cd in 20 mL of ½ MS medium were 0, 50, 100, 250, 500, and 1,000 mg L^{-1}, Cd concentrations of 0, 5, 10, 15, 25 and 50 mg L^{-1}. The Zn and Cd solutions were prepared from $ZnSO_4.7H_2O$ and $3CdSO_4.8H_2O$ respectively. The plants after the treatment were cultured for 30 days.

3.3.2 Detection of plant growth and stress under Zn and/or Cd induction

Plant growth: After treatment, the toxicity of a high metals concentration was related to the appearance of symptoms on leaves. The phytotoxicity was evaluated by determination amount of plant with necrotic spots and/or chlorosis of leaves (yellowish colour). The plants sample were washed with deionized water and then carefully dried with soft and clean paper. A plant was separated into root and leaves and weighted for fresh weight, then the plant samples were dried at 60 °C for 24 hours and weighed for dry weight.

Chlorophyll content: The chlorophyll content of the leaves was determined by the method of Mosaleeyanon et al. (2004). All leaves were washed with deionized water before wiped off the remaining water with clean and soft paper. The clean leaves were cut into small pieces. A randomly selected 0.2 g of the lower second and third of the basal leaves were mixed and blended in 5 mL of 95.5% (v/v) acetone. The mixture was transform to a closed tube and wrapped with aluminum foil to prevent evaporation and light effect. The mixture was incubated at 4 °C for 48 hours. Extinction of the extraction solution was measured at a wavelength of 662 and

644 nm using a UV-Vis spectrophotometer (Beckman Coulter DU 730 Life Science, USA). A solution of 95.5% (v/v) acetone was used as a blank. The total concentration of chlorophyll (chlorophyll a and b) based on fresh-weight (mg g^{-1}) was calculated according to the following equations:

$$\text{Chlorophyll a} = 9.78\ OD_{662} - 0.99\ OD_{644}$$

$$\text{Chlorophyll b} = 21.420\ OD_{644} - 4.65\ OD_{662}$$

OD_{662} and OD_{644} were the optical density at 662 and 644 nm, respectively.

Estimation of cell death: the death of root cells was stained with Evans blue. The method was determined according to Kumar et al. (2013). The fresh roots were cut into a length of 2-3 cm from the root tips and a 0.1 g randomly selected were stained with 0.25% (w/v) aqueous solution of Evans blue for 15 minutes, then washed three times with deionized water, each for 10 minutes to remove the excess stain. The root samples were immersed in 3 mL of N, N-dimethylformamide (DMF) for 1 hour at room temperature to solubilize the dye bound to the death cells. The absorbance of released Evans blue was measured using absolute DMF as a blank at 600 nm by the UV-Vis spectrophotometer.

Total phenolic content (TPC): For extraction, a randomly selected 0.25 g of fresh leaves were homogenized into liquid nitrogen followed by 5 mL of 50% methanol. Homogenates were centrifuged at 6,000 rpm for 10 minutes at 4 °C, then supernatant were filtered through Whatman No.1 and adjusted the volume to 5 mL. The TPC was analyzed by a modified Folin-Ciocalteau method (Cicco et al., 2009). A 100 µl of leaf extract was pipetted into 1.5 mL Eppendorf tubes, and 500 µl of 10% (v/v) Folin-Ciocalteau reagent was applied. The mixture was incubated in the dark for 3 minutes, then 100 µl of 7.5% (w/v) Na_2CO_3 and 300 µl of deionized water was added and incubated for 2 hours in the dark. After incubation, the absorbance at 731 nm was measured using the UV-Vis spectrophotometer. Gallic acid (GA) was used as a standard for making a calibration curve. The amounts of TPC in the samples were calculated by the standard curve. The results were expressed as milligrams of Gallic acid equivalents per gram of plant fresh weight (mg GA g^{-1} FW).

High performance liquid chromatography (HPLC) analysis: The phenolic compounds in the extracts were investigated by HPLC with a C18 guard column (4.6 mm x 10 mm, 5 µm) (VetiSepTM UPS C-18, Thailand) and a C-18 reversed-phase column (4.6 mm x 250 mm, 5 µm) (GL Science Lab InertSustain C-18, Japan). Each extract was filtered through a 0.2 µm nylon filter (Whatman, GE Healthcare, UK), before 20 µl of a sample was applied. The mobile phase was the gradient elution between 3% (v/v) acetic acid in water (solvent A) and 99.9% (v/v) methanol (solvent B) (Zuo et al., 2002), with a flow rate at 1 mL min^{-1} and the column temperature was 40 ºC. The gradient profile is shown in Table 3. The HPLC chromatograms were detected using a diode array UV detector (SPD-M20A, Shimadzu, Japan) at three wavelengths (254, 280 and 360 nm) for both phenolic acids and flavonoids. The reference chemicals were gallic acid, catechin, caffeic acid, epicatechin, chlorogenic acid, vanillin, p-cumalic acid, rutin and myricetin. Identification of the HPLC peak, the unknown peaks were compared with the retention time (RT) of the standard chemicals.

Table 3 Gradient conditions of mobile phase for HPLC.

Time (minute)	Solvent A	Solvent B
0	100	0
5	90	10
10	80	20
15	70	30
20	60	40
30	50	50
35-40	100	0

Analysis of enzyme activity: To extract enzyme, leaf samples (0.5xx g) were homogenized into liquid nitrogen followed by 1.2 mL of 50 mM sodium phosphate buffer pH 7.0 containing with 2% (w/v) polyvinylpolypyrrolidone (PVPP) and 1 mM ethylenediamminetetraacetic acid (EDTA). Homogenates were centrifuged at 10,000 rpm for 15 minutes at 4 °C. The supernatant was used for the analysis of enzyme activity and determined the protein concentration by the Lowry method

(Lowry et al., 1951) and bovine serum albumin (BSA) as the standard protein. Some effects from the Zn and/or Cd stresses on the plant enzyme activity were determined with superoxide dismutase and catalase.

(i) Superoxide dismutase (SOD, E.C. 1.15.1.1) activity was estimated by recording the decrease in absorbance of superoxide nitro blue tetrazolium complex by the enzyme. SOD activity was determined according to the modified method of Sebastian and Prasad (2014). The required reaction mixture was prepared with 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 75 µm nitroblue tetrazolium (NBT), and 0.1 mM EDTA. The aliquot (2.85 mL) of this mixture were transferred to small glass tubes followed by 50 µl of crude enzyme and 100 µl of 60 µM riboflavin (final concentration of riboflavin was 2 µM), added at last. After mixing, samples were illuminated for 10 minutes using fluorescent tubes (27W) in an aluminum foil-lined box, the distance between the lamp and the sample was 20 cm. The control was the reaction mixture without the raw enzyme (the crude enzyme was replaced by 50 µl of buffer) when the light was exposed under the same conditions as the sample. Whereas, the reaction mixture containing crude enzyme was incubated in the dark as a blank. The reaction was stopped by switching off the light and the tubes stored in the box before measuring. Absorbance of the reaction mixture was recorded at 560 nm using the UV-Vis spectrophotometer. One unit of SOD activity was defined as the amount of enzyme required to inhibit the 50% photochemical reduction of NBT under the test conditions.

(ii) Catalase (CAT, E.C. 1.11.1.6) activity was measured for the decomposition of H_2O_2 (ε = 39.4 $mM^{-1}cm^{-1}$) according to the modified method of Aebi (1984). 1 mL of reaction mixture consisted of 950 µl of 10 mM H_2O_2 in 50 mM sodium phosphate buffer pH 7.0 and 50 µl of crude enzyme. The reaction started by adding crude enzyme. The decrease in absorbance at 240 nm was detected every 30 seconds for 5 minutes, and calculated for the catalase activity.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis: Protein sample was extracted with phenol and aqueous phases. A 400 µl phenol extraction buffer at pH 8.0 (80 % saturated phenol, 0.01% (v/v) 2-mercapto-ethanol in 120 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 100 mM KCl) was added

to fresh leaves (0.1 g) in a mortar and crushed until homogenize on ice. The homogenate was then transferred to 1.5 mL microcentrifuge tube and centrifuged at 13,000 rpm, 4 ºC for 5 minutes. The phenol and aqueous phases were transferred into the new microcentrifuge tube. The total proteins were precipitated by the addition of 5 volume of 100 mM cooled ammonium acetate in methanol and then incubated at -20 ºC for 30 minutes. Then, it was centrifuged at 13,000 rpm at 4 ºC for 20 minutes. The protein pellet was washed with cool acetone buffer, before pour off acetone buffer. The pellet was dried in the laminar flow to avoid contamination. The protein pellet was re-suspended in 50 µl of protein sample buffer (63 mM Tris-HCl pH 6.8, 2% (v/v) SDS, 5% (v/v) mercaptoethanol, 20% (v/v) glycine). Each 30 µg of protein samples were fractionated on 15% acrylamide gel electrophoresis for 2 hours at 20 mA per gel. Staining of the gel with coomassie reagent brilliant blue R-250 staining buffer. The R-250 straining buffer consisted of 0.1% (w/v) coomassie brilliant blue R-250 in 40% methanol and 10% acetic acid. The molecular weight analysis of protein bands was compared to the molecular weight of a protein marker ranging from 10 to 250 kDa (EnzMart Biotech, Thailand). The protein patterns were separated by SDS-PAGE and analyzed using the GeneTools software program (SynGene, England).

3.3.3 Accumulation and distribution of Zn and/or Cd in plant

Zn and Cd accumulation in plant:

After harvesting, the shoots and roots were separated and washed with tap water, then rinsed three times with deionized water. After drying, the plant samples were weighed separately for wet weight and dried at 60 °C in the oven for 24 hours. After the plant samples were dried, weighting for dry weight. The plant was digested with 3 mL of HNO_3 (70% v/v) for 24 hours and heating at 150 °C for 1 hour, then adding 1 mL of $HClO_4$ (70% v/v) heating to 215 °C for 2 hours. Finally, 3 mL of deionized water was added to the samples and boiled at 90 °C for 1 hour (Miller's modified method, 1998). The digestion was analyzed for Zn and Cd concentration by AAS. The data was analyzed by one-way analysis of variation (ANOVA) in a completely randomized design (CRD). The variance and means separation were performed using the Duncan's new multiple range test (DMRT) at $p < 0.01$. Statistical analysis was performed using SPSS Version 14.0, software program. Translocation

factor (TF) was calculated from the ratio of metal concentration of plant shoots (above-ground of plant) to plant roots, and bioaccumulation factor (BF) was calculated from the ratio of metal concentration of the shoots and the extractable concentration of metal in the surrounding soil of the plant roots. (Phaenark et al., 2009).

Distribution of Zn on plant cell by µ-XRF imaging

To investigate the Zn distribution by µ-XRF imaging, the middle parts of the mature leaves were taken as samples for free hand transverse sectioning. The cross sections of the samples were cut to a thickness of 200-300 µm using a clean stainless-steel razor and quickly placed on dry ice. The sections were freeze-dried for 24 hours using a lyophilizer (Heto Power Dry PL3000, Japan). µ-XRF imaging was performed at beamline 6B, Synchrotron Light Research Institute (Public Organization), Thailand. The µ-XRF imaging data was analyzed by PyMca software package (version 5.1.2).

X-ray absorption spectroscopy (XAS) analysis

The X-ray absorption near edge structure (XANES) provides information on atomic oxidation states and local geometries around atoms. The extended X-ray absorption fine structure (EXAFS) presents detailed information about the local environment surrounding the atom; coordination numbers and bond lengths.

(i) *XANES analyzes*: The leaves sample were washed with an excess of running deionised water, and then were freeze-dried using the lyophilizer. For bulk XANES analysis, the samples were ground and mixed to a homogenize using a ball mill grinder (Mini-Mill pulverisette 23 Fritsch, Germany). Each sample was pressed into a pellet diameter 10 mm. The pellet was placed with a Kapton® tape (Lanmar Inc., Northbrook, IL, USA) on a sample holder for XAFS analysis. XANES spectra of the Zn K-edge and S K-edge were performed at beamline 8 (operation energy 1.25 keV) at Synchrotron Light Research Institute (Public Organization), Thailand. XANES spectra were collected by fluorescence X-ray detector of 13-Channel Germanium detector (GeD), at room temperature. Double crystal monochromators for

Zn and S were Ge (222) and InSb (111), respectively. The focusing mirror was a bending magnet, and beam size was 10 mm (h) x 1 mm (v). To obtain a good signal-to-noise ratio, the I0 ion chamber (10-cm-long) and I1 ion chamber (40-cm-long) were filled with specified gas mixtures and pressure for Zn (I0: Ar 93 mbar, I1: Ar 509 mbar) and S (I0: $N_2$37 mbar, I1: N_2 200 mbar). The sample chamber was filled with helium gas. The energy calibration was conducted before operating XAS data of samples by using Zn metal foil and $FeSO_4.7H_2O$ for Zn and S respectively. The reference chemicals were $ZnSO_4$, ZnO, ZnS, $Zn(CH_3OO)_2$ and $Zn(NO_3)_2$ and adsorption techniques were used to prepare the Zn-cysteine, Zn-glutathione and Zn-methionine and Zn-cellulose reference materials (Panitlertumpai et al., 2013). XANES spectra and the linear combination fitting (LCF) were analyzed using Athena software. All spectra of each sample were aligned, normalized and merged spectra before the LCF. The LCF fit of the Zn K-edge spectra was performed as a flattened normalized μ(E) from, under the force weights sum to 1 and weights forced between 0 and 1, 9640-9710 eV.

(ii) *EXAFS analysis*: The data were Fourier transformed and a Hanning window was used with over a k-range of 3-8 Å. The contribution of the first shell was simulated in R space with the r-range of 1-3 Å, depending on the data quality. The XAFS analysis data was analyzed by Athena and Artemis software.

3.4 Effects of endophytic bacterial inoculation on *M. spectabilis*

The aim of this study was to investigate the effect of endophytic bacteria *Cupriavidus plantarum* RDMSSR05 and *Chryseobacterium ureilyticum* RDMSSR07 inoculation on plant growth promoting and Zn/Cd accumulation of *M. spectabilis*. After the treatment, the plant samples were measured for plant growth as fresh weight, dry weight, number of leaves. Zn or Cd accumulations in plant were analyzed by AAS. In addition, bacterial endophytes were detected during the experiment.

3.4.1 Colonization of endophytic bacteria

The plantlets after cultured for 45 days (Figure 6) were immersed in the endophytic bacterial (RDMSSR05 and RDMSSR07) suspension or sterile water PBS

(control) for 30 minutes. The plantlets was placed in sterile petri dish which filled with sterilized soft paper. The plantlets were cultured on 20 mL ½ MS medium for 45 days. To investigate the colonization of the inoculated bacteria in plant tissues, the whole plant was surface sterilized according to the procedure of the above endophytic bacterial isolation, the bacteria was spread on the ½ TSA medium. The re-isolates were checked by morphological characteristics and Gram staining.

Figure 6 *M. spectabilis* culture in MS medium containing 0.1 mg L^{-1} BAP for 45 days.

3.4.2 Plant culture and inoculation

For inoculation, endophytic bacteria were grown overnight in TSB medium at 30 ± 5 ^{o}C on a rotary shaker. Cells were collected by centrifugation, washed and suspended in PBS to obtain a final inoculum density of 10^8 CFU mL^{-1}.

M. spectabilis were cultured in MS medium containing 0.1 mg L^{-1} BAP at 25 ^{o}C under 1,500 lux of light intensity and a 12 hours photoperiod for 45 days. The plantlets after cultured for 45 days were soaked in the bacterial suspension or sterile PBS (control) for 1 hour, and then were placed in sterile petri dish which filled with sterilized soft paper. Plantlets were cultured on 20 mL ½ MS medium for 2 weeks. After inoculation for 2 weeks, the plants for treatment with Zn and Cd were selected from healthy plants with a similar number of leaves and heights. Plants were treated with 2 mL of Zn and Cd, final concentrations of Zn and Cd in 20 mL of ½ MS medium was 500 mg L^{-1} plus 15 mg L^{-1}, respectively, under a tissue culture system for 2 weeks. After 2 weeks, the plants were harvested for measurement the growth parameter and metal accumulation.

Plant growth and metal accumulation: After the treatment, plant samples were washed with tap water, then rinsed three times with deionized water and dried. A whole plant (shoot and root) was weighted for the fresh weight and dried at 60 °C for 24 hours. The plant samples were weighted for dry weight. The dried samples were digested with HNO_3 (70% v/v) and $HClO_4$ (70% v/v) by a modified method of Miller (1998). Amounts of Zn and Cd in plant were measured by AAS.

Endophytic bacteria count: The plant interior colonization was quantified according to the procedure of the above endophytic bacterial isolation. The bacteria was spread on the ½ TSA medium and ½ TSA containing Zn 150 mg L^{-1} plus Cd 30 mg L^{-1}. Endophytic population were collected at days 7, 14, 21 and 28 after inoculation. The identity of re-isolates was checked by morphological characteristics and 16S rDNA sequencing.

3.4.3 Growth curve determination of endophytic bacteria

The results of colonization of endophytic bacteria showed that there was an indigenous endophytic bacterium in *M. spectabilis*. The indigenous endophytic bacteria identified by 16S rDNA sequencing were *Curtobacterium luteum*.

Growth curve determination: For the preparation of the bacterial inoculum, the bacteria of *C. plantarum* RDMSSR05, *C. ureilyticum* RDMSSR07 and *C. luteum* were refreshed from stock culture and was grown into TSB. The bacterial endophytes were cultured by shaking at 150 rpm, 30 ± 5 ºC for 16-18 hours. The bacterial cells were collected by centrifugation, washed two times with PBS buffer and re-suspended in PBS buffer to obtain a final inoculum density of 10^8 CFU mL^{-1}. 1% (v/v) bacterial inoculum was inoculated into TSB medium and shaken at 150 rpm, 30 ± 5 ºC for 48 hours. The bacterial growth was determined by plate count technique on TSA medium. The optical density was observed every 4 hours at 600 nm.

3.4.4 Antagonistic effects of endophytic bacteria

Antagonism was designed to investigate the interaction between the inoculants endophytic bacterial isolated and the indigenous endophytic bacteria. This study were carried out by cultivation in both solid and liquid media. The endophytic bacteria were grown overnight in TSB medium at 30 ± 5 ºC on a rotary shaker. Cells

were collected by centrifugation, washed and suspended in PBS to obtain a final inoculum density of 10^8 CFU mL^{-1}.

Cultivation on solid media: the suspension of each analyzed endophytic bacterium (RDMSSR05 and RDMSSR07) was spread on the TSA plate using sterile cotton swab. Then, the sterile filter paper discs (about 6 mm in diameter) were placed on the agar surface, and added 10 µl of the indigenous endophytic bacterial suspension to paper discs. The petri dishes were incubated at 30 ± 5 ºC for 48 hours. The zone of inhibition produced by endophytic was observed.

Cultivation on liquid media: For the preparation of the bacterial inoculum, the refreshed bacteria from the stock culture and cultivated in the TSB. The bacterial endophytes were cultured at 150 rpm, 30 ± 5 ºC for 16-18 hours. The bacterial cells were collected by centrifugation, washed two times with PBS and re-suspended in PBS to obtain a final inoculum density of 10^8 CFU mL^{-1}. 1% bacterial inoculum was inoculated into TSB medium and shaken at 150 rpm, 30 ± 5 ºC for 10 hours. The treatments of this study were following: (i) Dual culture of *C. luteum* and *C. plantarum* RDMSSR05, each an initial concentration of cells equivalents to 10^3 CFU mL^{-1}. (ii) Dual culture of *C. luteum* and *C. ureilyticum* RDMSSR07, each an initial concentration of cells equivalents to 10^3 CFU mL^{-1}. (iii) Mixed culture of *C. luteum*, *C. plantarum* RDMSSR05 and *C. ureilyticum* RDMSSR07, each an initial concentration of cells equivalents to 10^2 CFU mL^{-1}. In addition, culture of *C. luteum*, which is an initial concentration of cells equivalent to 10^6 CFU mL^{-1}, were used as a control compared with the previous study.

The bacterial growth was determined by plate count technique every 2 hours (0, 2, 4, 6, 8 and 10 hours). A 100 µl aliquot from each dilution were spread on ½ TSA plate and ½ TSA containing Zn 150 mg L^{-1} plus Cd 30 mg L^{-1} plate. Each treatment was collected and done for duplication. A bacteria plate was incubated at 30 ± 5 ºC for 48 hours.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Isolation and characterization of endophytic bacteria from *M. spectabilis*

4.1.1 Plant exploration and collection

During a survey of four sites in forest area of zinc mine, Phatat Phadaeng sub-district, Mae Sot, Tak Province, Thailand, in August 2015, which was in rainy season, *M. spectabilis*' s plants were found at Site: N 16º 39' 6.6"E 98º 39' 41.2", 550-600 meters above sea level. The area was an inactive mining activity that shows a high diversity of native plants (Figure 7).

Figure 7 *M. Spectabilis* (Kurz) Faden growing in the zinc mine, Padaeng industry Public Company Limited, Mae Sot, Tak Province, Thailand.

M. spectabilis growing in the contaminated soil was able to tolerate and accumulate Zn and Cd in the roots and above ground parts of the plant as shown in Table 4. The soil around *M. spectabilis*'s rhizosphere contained high amounts of total Zn and Cd as 40,716 ± 4,839 and 128 ± 16 mg kg^{-1} dry weight, respectively. The concentrations of DTPA extractable Zn and Cd were 1,215 ± 117 and 15.3±1.5 mg kg^{-1} dry weight, respectively. The soil temperature measuring at the site was 25.2 ± 0.3 ºC. The pH and soil moisture content of the rhizospheric soil were neutral pH values (7.07 ± 0.06) and 30 ± 8.1 % moisture.

4.1.2 Isolation and characterization of endophytic bacteria

The endophytic bacterial population in each explants studied by the spread plate method were ranged from 9.9 x 10^2 to 8.8 x 10^5 CFU per gram fresh weight. Table 4 shows the Zn and Cd contents and the endophytic bacterial counts in each parts of *M. spectabilis* growing in the Zn/Cd contaminated soil.

Table 4 Zn and Cd contents and the endophytic bacterial counts in each parts of *M. spectabilis* growing in the Zn/Cd contaminated soil.

Part of plants	Metal accumulation (mg kg^{-1} dry weight)		CFU g^{-1} fresh weight
	Zn	Cd	
Storage root	6,107.46 ± 1,774.34	26.39 ± 3.34	2.3 x 10^3
Tuber	1,484.46 ± 180.80	24.42 ± 1.67	9.9 x 10^2
Leave	1,494.13 ± 310.91	8.14 ± 2.08	8.3 x 10^5
Peduncle	420.81 ± 48.50	2.92 ± 0.56	1.4 x 10^4

The endophytic bacteria were isolated from both direct and indirect methods depending on the morphology of colony and Gram stain (Appendix A6). A total of 52 endophytic bacterial isolates were derived from the four parts of surface-sterilized explants, as 19 isolates from storage roots, 9 from underground stems (tubers), 8 from leaves and 16 isolates from peduncle. The percentages of the isolates collected from, storage roots, tubers, leaves and peduncle were 36.5%, 17.3%, 15.4% and 30.8 %, respectively. The 52 isolates were screened for Zn or Cd tolerant properties by streaking each bacterium on the TSA plates separately supplied with Zn (100-500 mg L^{-1}) or Cd (10-50 mg L^{-1}). There were 24 isolates tolerated Zn to 250-500 mg L^{-1} or Cd to 20-50 mg L^{-1} (Appendix A7). Therefore, the 24 isolates of RDMSP03, RDMSP04, RDMSP05, RDMSP06, RDMSP07, RDMSP11, RIMSP02, RDMSSR02, RDMSSR03, RDMSSR04, RDMSSR05, RDMSSR07, RDMSSR08, RDMSSR12, RDMSSR13, RIDMSSR02, RIDMSS01, RIDMSS04, RIDMSS05, RIDMSS06, RIDMSS07, RIDMSS09 RDMSL03 and RIDMSL01 were selected for bacterial identification.

The 16S rDNA sequence of the 24 endophytic bacterial isolates were matched with the genetic sequence database of GenBank. The 24 bacterial isolates from *M. spectabilis* were belonging to four major groups of Firmicutes (37.5%), Actinobacteria (29.2%), Proteobacteria (20.8%) and Bacteroidetes (12.5%). They were closely related phylogenetically to the genera of *Bacillus, Pantoea, Microbacterium, Curtobacterium, Chryseobacterium, Cupriavidus, Siphonobacter* and *Pseudomonas*, respectively (Figure 8).

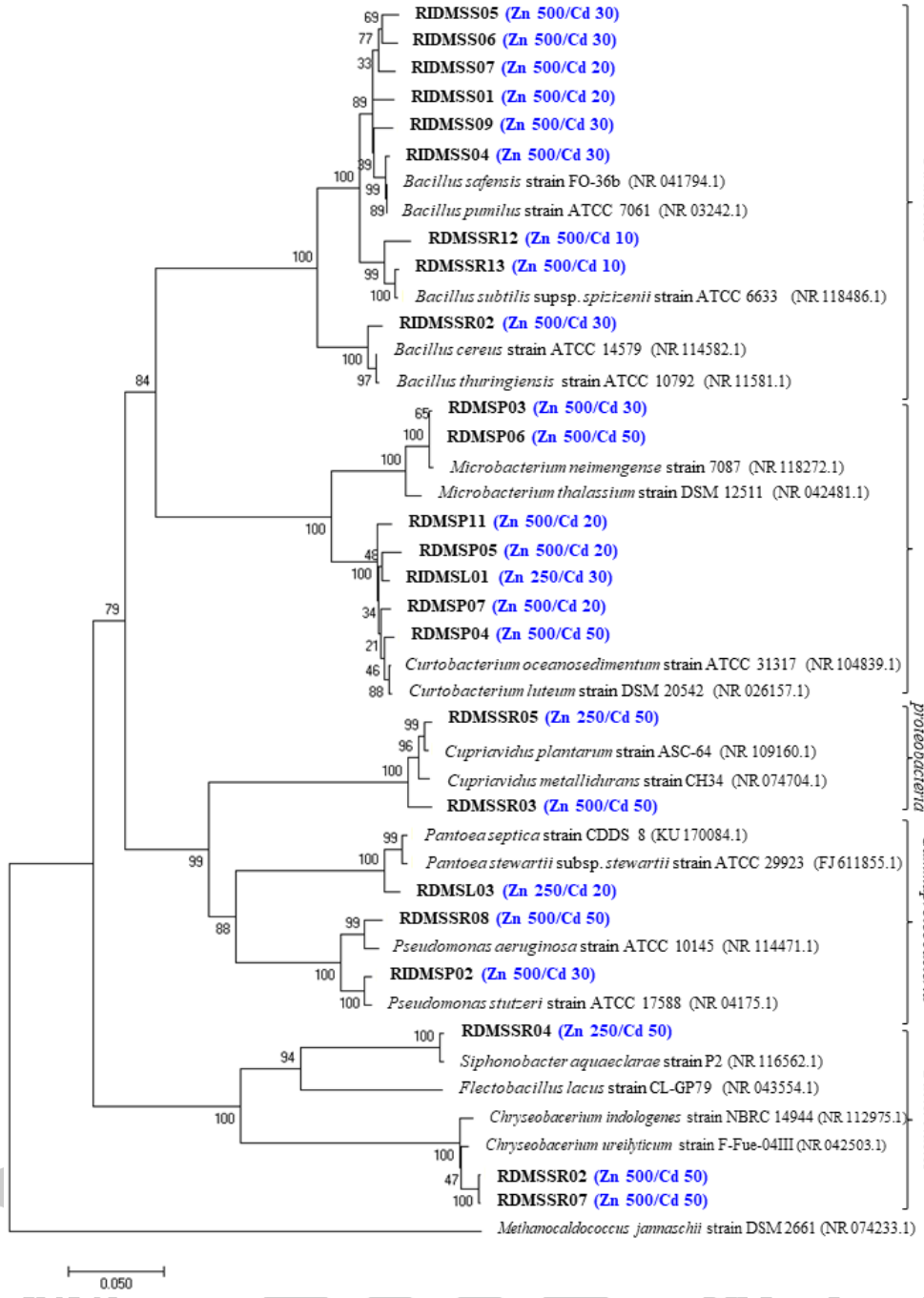


Figure 8 Phylogenetic analysis of 16S rDNA sequences of 24 endophytic bacteria isolated from *M. spectabilis* and sequences from GenBank (indicated by accession number), using the Maximum Likelihood method with 1,000 bootstrap replicates. Strains from this study are in bold font (indicated Zn and Cd tolerance). There were a total of 1043 positions in the final dataset. Evolutionary analyses were conducted in MEGA7. Bootstrap values are indicated at the node. Bar indicates 0.05 substitutions per nucleotide position.

4.2 Screening for Zn and Cd tolerate bacteria and plant growth promoting properties.

The 24 bacterial isolates were screened for Zn plus Cd tolerance in half strength TSA medium. For the screening test with dual Zn and Cd treatment, the half strength TSA medium were applied to avoid probable precipitation of the metals and to limit nutrient to the bacteria. The results showed that almost isolates were able to tolerate Zn 150 mg L^{-1} plus Cd 20 mg L^{-1}, whereas RDMSSR12, RDMSSR13 and RIDMSP02 could not tolerate the concentration at the dual treatment. Table 5 shows that the bacteria isolated from the storage roots had trend to tolerate higher Zn and Cd concentrations than the bacteria from tubers, leaves and peduncles. Moreover, the isolates of RDMSP03 and RDMSP06 clearly showed the clear zone around their colonies on the TSA supplemented with Zn 250 mg L^{-1} plus Cd 50 mg L^{-1} (Appendix A9). On the other hand, the isolates of RDMSSR03 and RDMSSR05 showed the turbid zone around their colonies on the TSA supplemented with Zn 250 mg L^{-1} plus Cd 50 mg L^{-1} (Appendix A8). The clear zone indicated that the bacteria might secrete acid substance to dissolve the precipitate metals. Whereas the turbid zone indicated that the isolates probably secreted substance to precipitate the metals.

Table 5 Growth of endophytic bacteria in the half formula of TSA media supplemented with various concentration of Zn plus Cd.

Strain	Concentrations of Zn plus Cd in ½ TSA medium (Zn + Cd, mg L^{-1})								
	150+ 20	150+ 30	150+ 50	250+ 20	250+ 30	250+ 50	500+ 20	500+ 30	500+ 50
RDMSSR02	+++	+++	++	++	++	++	-	-	-
RDMSSR03	+++	+++	++	++	++	++	-	-	-
RDMSSR04	+++	+++	++	++	++	+	+/-	-	-
RDMSSR05	+++	+++	+++	++	++	++	++	++	++
RDMSSR07	+++	+++	++	++	++	++	+	-	-
RDMSSR08	+++	+++	++	++	++	++	++	++	++
RDMSSR12	-	-	-	-	-	-	-	-	-
RDMSSR13	-	-	-	-	-	-	-	-	-
RIDMSSR02	++	+++	++	+	+	+	-	-	-
RIDMSS01	++	++	+	+	+	-	-	-	-
RIDMSS04	++	++	+	+	-	-	-	-	-
RIDMSS05	++	++	+	+	+	-	-	-	-
RIDMSS06	++	++	+	+/-	-	-	-	-	-
RIDMSS07	++	++	+	+/-	-	-	-	-	-
RIDMSS09	++	++	+	-	-	-	-	-	-
RDMSL03	++	-	-	+	-	-	+	-	-
RIDMSL01	+	+	+	+/-	+/-	+/-	-	-	-
RDMSP03	+++	+++	++	++	+	+/-	+	-	-
RDMSP04	++	-	-	+	-	-	+	-	-
RDMSP05	+++	-	-	+	-	-	-	-	-
RDMSP06	+++	+++	++	++	+	+/-	+	-	-
RDMSP07	+++	-	-	+	-	-	+	-	-
RDMSP11	+++	-	-	+	-	-	+	-	-
RIDMSP02	-	-	-	-	-	-	-	-	-

(–) indicates no growth; (+/-) growth/weak, (+) low growth; (++) moderate growth; (+++) high growth. Control; all strains were shown high growth (+++) on ½ TSA medium.

The 24 isolates were tested for their abilities on the production of IAA and siderophores as well as, ACC deaminase activity, nitrogen fixation and phosphate solubilization. The results are shown in Table 6. Although the 24 isolates was able to produce IAA, the quantity produced varied widely from 1.6 to 75.6 mg L^{-1}. Almost the isolates had nitrogen fixation property. Only six isolates of RDMSSR02, RDMSSR07, RDMSSR08, RDMSSR12, RDMSSR13 and RIDMSSR02 was able to produce siderophores as indicating with orange halo around their colonies (Appendix A10). The three isolates of RDMSSR07, RDMSSR08 and RDMSL03 had the ability of phosphate solubilization. The isolates of RDMSSR03 and RDMSSR05 belonging to *Cupriavidus* could utilize ACC as nitrogen source, which indicated to ACC deaminase enzyme activity. Table 7 shows the properties of the 24 isolates on the extracellular enzymes secretion to degrade cellulose and lignin. The isolates of RDMSSR12, RDMSSR13, RIDMSSR02, RIDMSS01, RIDMSS04, RIDMSS05, RIDMSS06, RIDMSS07 and RIDMSS09 that belonged to the genus *Bacillus* could produce cellulase. The ten isolates that decolorized methylene blue probably secreted ligninolytic enzyme. However, only two isolates of RDMSSR05 and RDMSSR08 clearly showed the ability of lignin degradation.

Table 6 Plant growth promoting properties of the endophytic bacteria.

Strain	Plant growth promoting activities				
	IAA (mg L^{-1})	ACC[a]	N_2 fixation[b]	Phosphate[b]	Siderophore[b]
RDMSSR02	37.72 ± 7.12	-	-	-	+
RDMSSR03	4.66 ± 0.64	+	++	-	-
RDMSSR04	20.21 ± 7.21	-	-	-	-
RDMSSR05	3.17 ± 0.92	+	+++	-	-
RDMSSR07	42.06 ± 6.77	-	-	+	+
RDMSSR08	8.29 ± 0.88	-	+++	+	++
RDMSSR12	14.52 ± 5.28	-	++	-	+
RDMSSR13	16.12 ± 2.12	-	++	-	+
RIDMSSR02	10.25 ± 1.00	-	-	-	+
RIDMSS01	10.40 ± 0.79	-	+++	-	-
RIDMSS04	9.46 ± 1.09	-	+++	-	-
RIDMSS05	8.60 ± 0.12	-	+++	-	-
RIDMSS06	11.53 ± 0.75	-	+++	-	-
RIDMSS07	8.03 ± 0.36	-	+++	-	-
RIDMSS09	12.81 ± 0.90	-	+++	-	-
RDMSL03	23.56 ± 3.36	-	+++	++	-
RIDMSL01	1.64 ± 0.46	-	+	-	-
RDMSP03	40.29 ± 18.98	-	++	-	-
RDMSP04	30.52 ± 2.83	-	-	-	-
RDMSP05	25.33 ± 2.50	-	-	-	-
RDMSP06	33.91 ± 21.86	-	++	-	-
RDMSP07	13.42 ± 1.56	-	-	-	-
RDMSP11	29.39 ± 3.83	-	-	-	-
RIDMSP02	75.57 ± 7.04	-	+++	-	-

IAA is expressed as means ± SD (n=3-6). [a] ACC deaminase + = Positive, - = Negative

[b] Nitrogen fixation, Phosphate solubilization and Siderophore (−) negative, (+) positive/weak, (++) intermediate, (+++) strong production

Bold stain indicated endophytic strains further used test plant growth promoting property under Zn and Cd stress.

Table 7 Extracellular enzyme of the endophytic bacteria.

Strain	Extracellular enzyme		
	Cellulase [a]	Lignin degradation [a]	Ligninolytic enzymes[b]
RDMSSR02	-	-	-
RDMSSR03	-	-	+
RDMSSR04	-	-	-
RDMSSR05	-	+	+
RDMSSR07	-	-	-
RDMSSR08	-	+	+
RDMSSR12	+	-	-
RDMSSR13	+	-	-
RIDMSSR02	+	-	+
RIDMSS01	+	-	+
RIDMSS04	+	-	+
RIDMSS05	+	-	+
RIDMSS06	+	-	+
RIDMSS07	+	-	+
RIDMSS09	+	-	+
RDMSL03	-	-	-
RIDMSL01	-	-	-
RDMSP03	-	-	-
RDMSP04	- (ND)	- (ND)	- (ND)
RDMSP05	-	-	-
RDMSP06	-	-	-
RDMSP07	-	-	-
RDMSP11	-	-	-
RIDMSP02	- (ND)	- (ND)	- (ND)

[a] + = Positive, - = Negative

[b] + zone of decolourization present; - no zone of decolourization

ND = not detected

Bold stain indicated endophytic strains further used test plant growth promoting property under Zn and Cd stress.

From the five criteria of (i) no reports about human and plant pathogenicity, (ii) a high IAA production (iii) nitrogen fixation, (iv) siderophore production, and especially (v) greater tolerance to dual Zn and Cd treatment, the six bacterial isolates of RDMSSR02, RDMSSR04, RDMSSR05, RDMSSR07, RDMSP03 and RDMSP06 (bold stain in table 7) were selected for the ability test of plant growth promoting properties under the Zn (150 mg L^{-1}) plus Cd (30 mg L^{-1}) treatment.

Table 8 shows that RDMSSR02 and RDMSSR07 belonged to *Chryseobacterium ureilyticum* with 98% similarity, RDMSSR04 belonged to *Siphonobacter aquaeclarae* with 99% similarity, RDMSSR05 belonged to *Cupriavidus plantarum* with 99% similarity, and both isolates RDMSP03 and RDMSP06 belonged *Microbacterium neimengense* with 99% similarity. In addition, IAA production and nitrogen fixation of RDMSSR04, RDMSP03 and RDMSP06 were affected by the Zn plus Cd treatment. Whereas, the abilities of RDMSSR02, RDMSSR07 and RDMSSR05 remained under the Zn plus Cd treatment.

Table 8 Plant growth promoting activities of the six endophytic bacteria under Zn and Cd stresses.

Strain	Closest NCBI match	% Identity	Plant growth promoting activities under the Zn and Cd stresses			
			IAA (mg L^{-1})	Nitrogen fixation	Siderophore	ACC deaminase
RDMSSR02	*Chryseobacterium ureilyticum* strain F-Fue-04IIIaaaa	98	40.33 ± 14.02	-	+	-
RDMSSR04	*Siphonobacter aquaeclarae* strain P2	99	3.80 ± 1.43	-	-	-
RDMSSR05	*Cupriavidus plantarum* strain ASC-64	99	2.85 ± 0.84	++	-	+
RDMSSR07	*Chryseobacterium ureilyticum* strain F-Fue-04IIIaaaa	98	49.42 ± 9.76	-	+	-
RDMSP03	*Microbacterium neimengense* strain 7087	99	2.00 ± 0.20	-	-	-
RDMSP06	*Microbacterium neimengense* strain 7087	99	1.78 ± 0.34	-	-	-

Plant growth promoting activities under the presence of Zn 150 mg L^{-1} plus Cd 30 mg L^{-1}. IAA is expressed as means ± SD (n=6). All strain absences phosphate solubilization activity (−) negative, (+) positive/weak, (++) intermediate, (+++) strong production.

The six isolates were confirmed screening for Zn and Cd tolerance in the liquid medium by minimum inhibitory concentration (MIC) test. Table 9 showed that RDMSSR05 tolerated to 500 mg L^{-1} of Zn and also Zn (500 mg L^{-1}) plus Cd (20, 30 and 50 mg L^{-1}) treatments. In addition, the isolated of RMDSSR02 and RDMSSR07 tolerated to 25 mg L^{-1} of Cd and Zn (250 mg L^{-1}) plus Cd (50 mg L^{-1}). While, the isolates of RDMSSR04 tolerated to 100 mg L^{-1} of Cd. The MIC test showed that the Zn and Cd tolerant ability of some isolated decreased under dual Zn and Cd treatment in the liquid state.

Table 9 Heavy metal tolerance of the six endophytic bacteria.

Strain	Minimum inhibitory concentration (MIC) (mg L^{-1})				
	Zn	Cd	Zn/ Fixed Cd 50 mg L^{-1}	Zn/ Fixed Cd 30 mg L^{-1}	Zn/ Fixed Cd 20 mg L^{-1}
RDMSSR02	500	25	250/50	250/30	>500/20
RDMSSR04	125	>100	62.5/50	62.5/30	125/20
RDMSSR05	>500	>100	>500/50	>500/30	>500/20
RDMSSR07	500	25	250/50	500/30	>500/20
RDMSP03	125	3.13	62.5/50	62.5/30	250/20
RDMSP06	7.81	0.78	< 3.9/50	< 3.9/30	< 3.9/20

After incubated 7 days

The six isolates of RDMSSR02, RDMSSR04, RDMSSR05, RDMSSR07, RDMSP03 and RDMSP06 were also studied the pathogenicity on *M. spectabilis* for 7-14 days. The results showed no symptom of disease on the plant leaves after inoculation of each bacteria for 14 days. Figure 9 shows the healthy plant after inoculation with RDMSSR05 and RDMSSR07 isolates by swapping on leaves and spreading on the MS media.

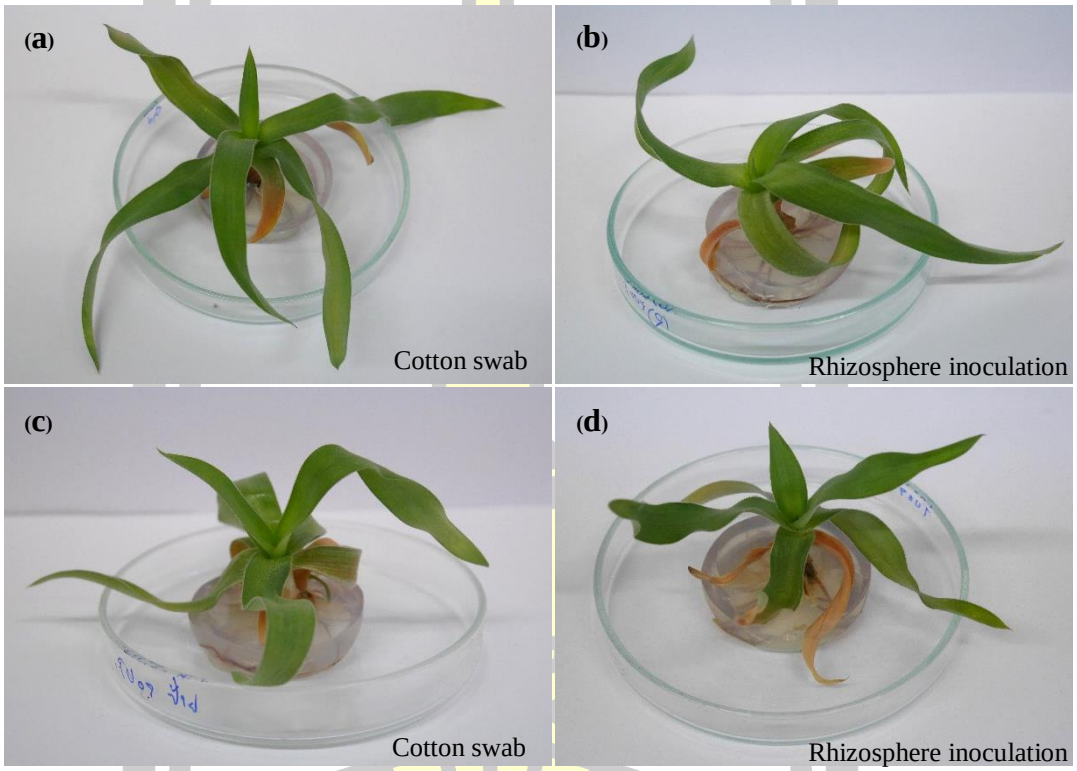


Figure 9 Pathogenicity test of endophytic bacteria on *M. spectabilis*, 14 after days inoculation. (a-b, RDMSSR05, c-d RDMSSR07).

From all results of bacterial screening tests, *C. plantarum* RDMSSR05 and *C. ureilyticum* RDMSSR07 were selected for studying the effects of endophytic bacterial inoculation on the growth of *M. spectabilis* under Zn and Cd stress.

4.3 Effects of metals tolerance and accumulation in *M. spectabilis*

The growth of *M. spectabilis* under various Zn and/or Cd concentrations were investigated to obtain a threshold Zn and Cd stress. Then, effect of the bacterial inoculation on the plant growth under the threshold stress was observed.

4.3.1 Effects of Zn or Cd on plant growth and stress induction

The effects of Zn or Cd on the growth of *M. spectabilis* after 4 weeks of treatment were determined by the fresh weight, dry weight, number of tuber and the percentage of yellow/pale leaves (phytotoxicity). The morphological changes in the *M. spectabilis* plants treated with Zn or Cd showed the presenting of leaf chlorosis and growth inhibition, especially in the treatments of 500-1,000 mg L^{-1} Zn (Figure 10 a) and 15-50 mg L^{-1} Cd (Figure 10 b).

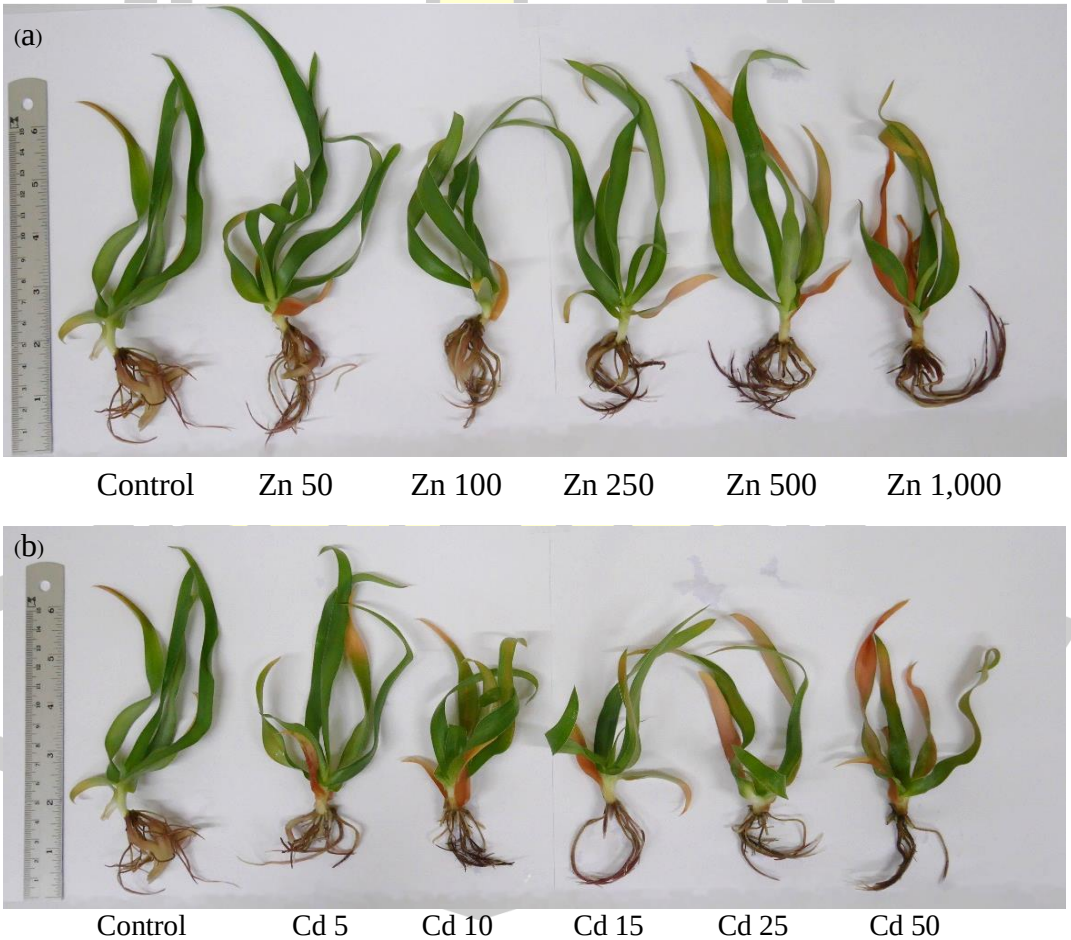


Figure 10 The morphological changes of *M. spectabilis* treated with Zn (50-1,000 mg L^{-1}) or Cd (5-50 mg L^{-1}) in a tissue culture system.

Figure 11 (a) shows that the increase of Zn in the MS medium from 50 to 500 mg L^{-1} did not significantly affect for the fresh weight of leaves when compared with control, whereas higher Zn concentrations of 1,000 mg L^{-1} significantly decreased the fresh weight of leaves. The results of fresh weights of leaves were related to the percentage of yellow/pale leaves (Figure 11 (e)). In addition, the high Zn concentrations of 500 and 1,000 mg L^{-1} resulted to yellow/pale leaves and less number of storage roots. The results of storage roots were related to the fresh weight of root. The Zn concentrations of 500 and 1,000 mg L^{-1} were significantly decreased the fresh weight of root (Figure 11 c). In case of Cd treatments, the Cd treatments affected to the plant growth (b, d, f), especially fresh weight. The fresh weight of plants' leaves under 50 mg L^{-1} Cd treatments were lower than the fresh weight of the control plants' leaves (Figure 11 (b)).

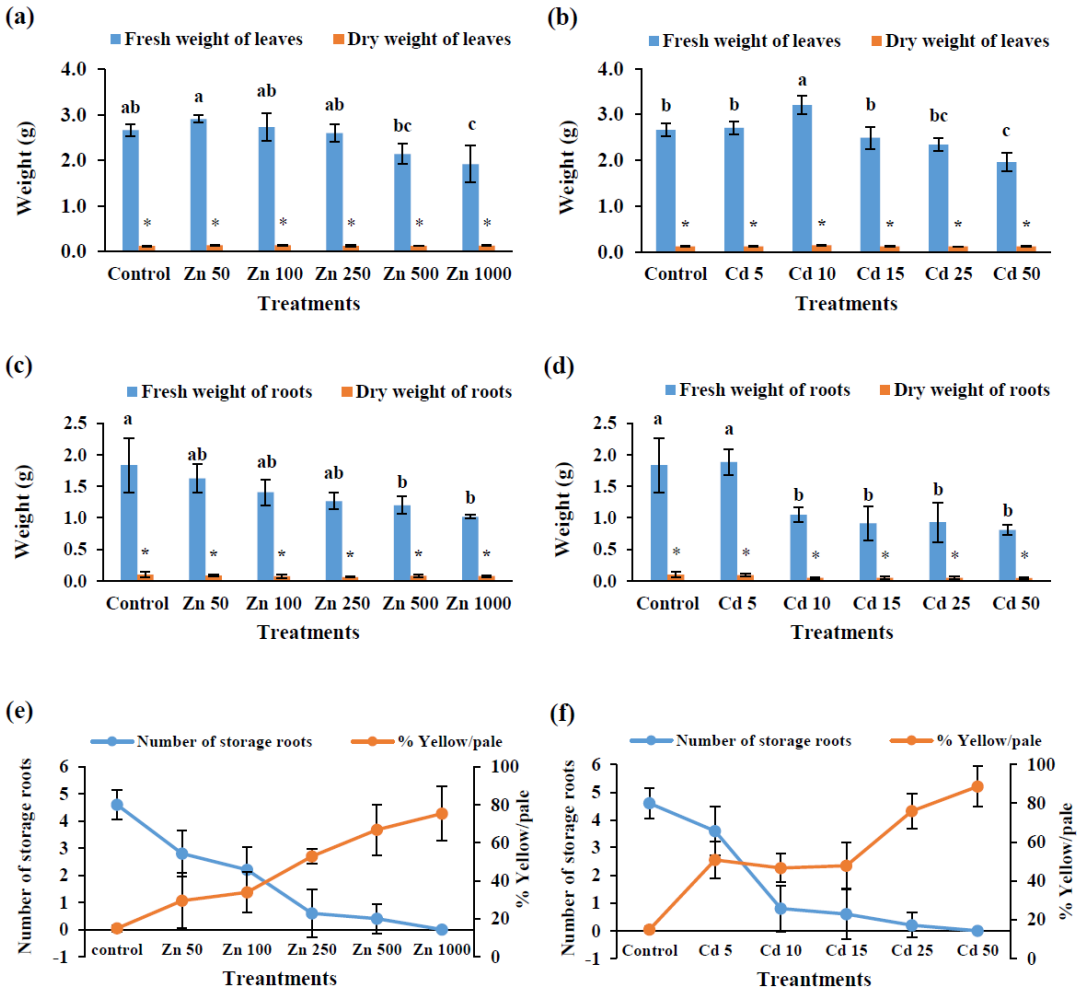


Figure 11 Effect of Zn and Cd on growth of *M. spectabilis* separately treated with various concentrations of Zn and Cd (a, b fresh weight and dry weight of leaves, c, d fresh weight and dry weight of roots, e, f number of storage root and the percentages of yellow/pale leaves). The results shown with different letters (a-c) on the error bars are significantly different ($P < 0.01$, Duncan's new multiple range test). The data are given as the means ± SD (n = 3-5).

The leaf chlorophyll concentrations after treated with various Zn or Cd concentration are shown in Figure 12 (a, b). The results indicated that the higher Zn and Cd concentrations significantly decreased the chlorophyll contents. Figure 12 (c, d) shows the Zn or Cd effecting on cell death in the roots. In comparison with the control plants, Zn and Cd treatments results in increased cell death. However, there were not significantly different within the groups of Zn or Cd treatments.

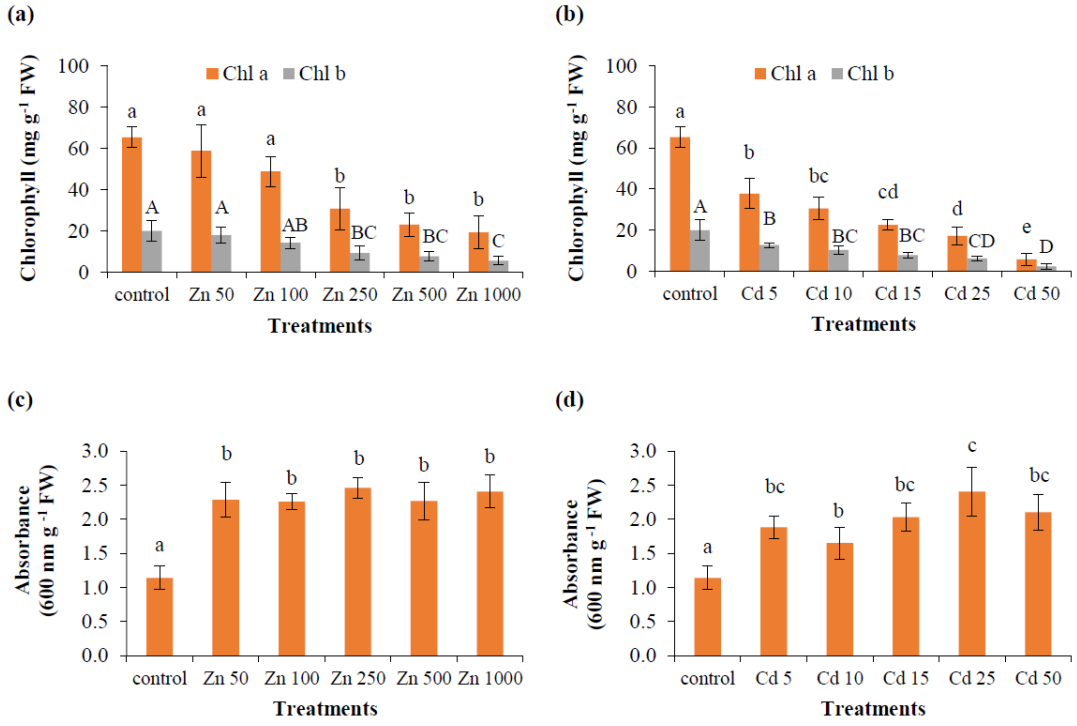


Figure 12 Chlorophyll content in the leaves (a and b) and cell death measurement in the roots (c and d) of *M. spectabilis* after treated with Zn or Cd. The results shown with different letters (a-e) and (A-D) on the error bars are significantly different ($P < 0.01$, Duncan's new multiple range test). The data are given as the means ± SD (n = 4).

The effect of Zn or Cd on total phenolic content (TPC) and protein content in the leaves of *M. spectabilis* are shown in Figure 13. The results indicated that the higher Zn (1,000 mg L^{-1}) and Cd (50 mg L^{-1}) concentrations significantly affected to increase the TPC content when compared with the control (Figure 13 (a, b). The plants treated with Zn concentrations of 50 to 500 mg L^{-1} had tread to decreased their protein content when compared with the control plant (Figure 13(c)). However, the protein content extracted from the plant treated with 1000 mg L^{-1} of Zn was not significantly differ from the control plant. In case of Cd treatments, the protein content extracted from the Cd treated plants had trend to increase, but not significant, when compared with control (Figure 13d).

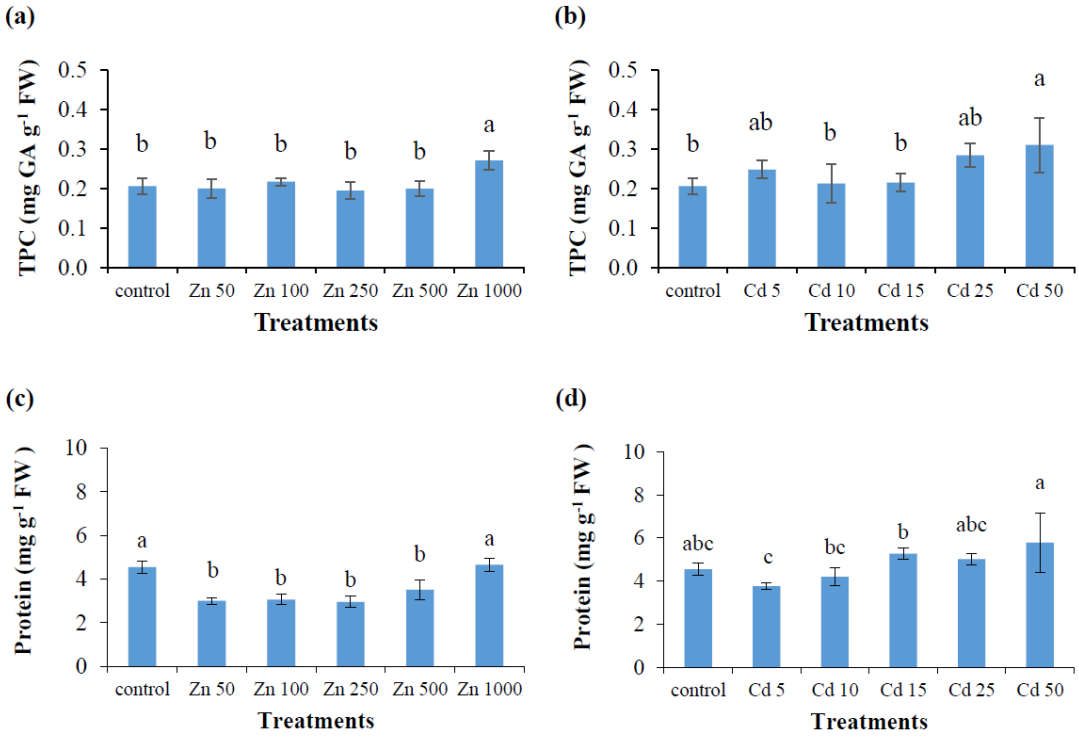


Figure 13 Total phenolic content (TPC) (a and b) and total protein content (c and d) extracted from the leaves of *M. spectabilis* after separately treated with various concentrations of Zn or Cd. The results shown with different letters on the error bars are significantly different ($P < 0.01$, Duncan's new multiple range test). The data are given as the means ± SD (n = 4).

The effects of Zn or Cd on activities of the antioxidant enzymes (SOD, CAT) in the leaves of *M. spectabilis* are shown in Figure 14. The Zn concentrations of 100 mg L^{-1} and Cd concentrations of 5 to 25 mg L^{-1} induced the SOD activity when compared with control plants (Figure 14 (a, b)). In comparison with the control plants, the Zn concentrations 50 to 100 mg L^{-1} also induced the CAT activity (Figure 14 (c)). Whereas, the Cd treatments (5-50 mg L^{-1}) did not significantly affect the CAT activity (Figure 14 (d)).

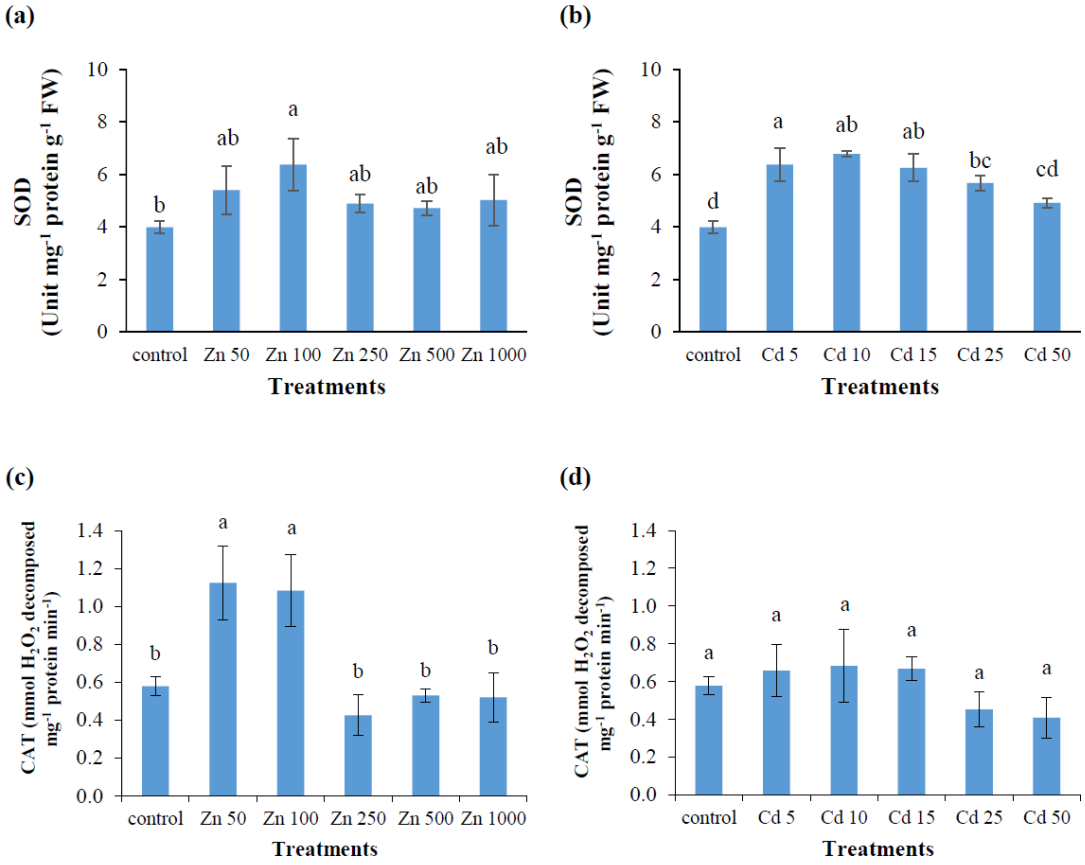


Figure 14 Enzymes activities in the leaves of *M. spectabilis* after treated with Zn or Cd. (a, b) superoxide dismutase (SOD), (c, d) catalase (CAT). The results shown with different letters on the error bars are significantly different ($P < 0.01$, Duncan's new multiple range test). The data are given as the means ± SD (n = 3).

Pearson correlation coefficients (Table 10) were performed to compare the correlations between TPC, SOD activity, CAT activity, protein content and the Zn or Cd accumulated in shoot (Zn Shoot or Cd Shoot). For the Zn treatments; TPC had the positive correlation between with SOD (r=0.529), protein content (r = 0.564) and the Zn accumulation in shoot (r = 0.779). For Cd treatment, TPC had a positive correlation with the protein content (r = 0.681) and the Cd accumulation in shoot (r = 0.723). Especially, the protein content had positive correlation with the Cd accumulated in shoot (r=0.759).

Table 10 Correlation coefficients (r) for relationships between TPC, SOD, CAT, protein and Zn or Cd accumulation in shoot.

Zn-Treatment	TPC	SOD	CAT	Protein	Zn-Shoot
TPC (mg GA g^{-1} FW)	1	0.529*	-0.004	0.564*	0.779**
SOD (Unit g^{-1} FW)		1	0.349	0.454	0.328
CAT (mmol H_2O_2 g^{-1} FW min^{-1})			1	0.102	-0.333
Protein (mg g^{-1} FW)				1	0.305
Zn accumulation in shoot (mg g^{-1} FW)					1
Cd-Treatment	TPC	SOD	CAT	Protein	Cd-Shoot
TPC (mg GA g^{-1} FW)	1	0.194	-0.050	0.681**	0.723**
SOD (Unit g^{-1} FW)		1	0.156	0.260	0.150
CAT (mmol H_2O_2 g^{-1} FW min^{-1})			1	0.266	-0.017
Protein (mg g^{-1} FW)				1	0.759**
Cd accumulation in shoot (mg g^{-1} FW)					1

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

The protein patterns of *M. spectabilis* leaf extracts were studied by SDS-PAGE due to the correlation between the metals accumulation and the total protein content. Figure 15 shows that the highest Zn treatment (1,000 mg L^{-1}) decreased the protein bands of 27, 46 and 60 kDa, but increased the protein bands of 12, 22 and 35 kDa. Moreover, the protein band of 13 kDa significantly occurred after the plants treated with 25 mg L^{-1} of Cd and Zn (500 mg L^{-1}) plus Cd (15 mg L^{-1})

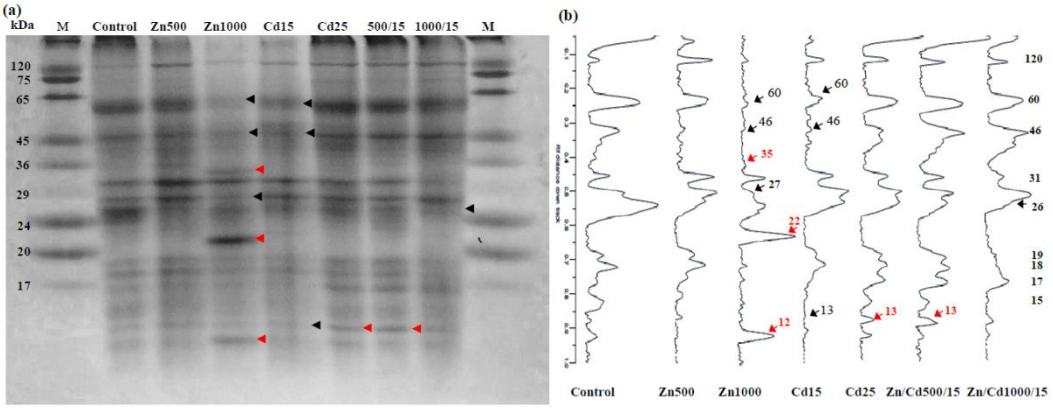


Figure 15 SDS-PAGE (15% w/v) of total protein extracts from the leaves of *M. spectabilis* treated with Zn (500 and 1,000 mg L^{-1}) and Cd (15 and 25 mg L^{-1}), dually treated with Zn (500 and 1,000 mg L^{-1}) and Cd 15 mg L^{-1} and the protein extract from leaves of the control plants (Control). The molecular weights of the proteins were calculated based on the molecular weights of the protein marker (M). ((a) SDS-PAGE, (b) densitometry analysis, arrows indicate different polypeptide bands; red arrows show increased intensity, black arrows show decreased intensity).

Figures 16 shows HPLC chromatogram of phenolic compounds in the leaf extracts. The HPLC profiles of the leaf extracts that were obtained from the plants treated with Zn (1,000 mg L^{-1}) or Cd (50 mg L^{-1}) and the control plants showed similar patterns. The main peak positions at retention time (RT) of 20.3 minutes was increased in the extracts from 50 mg L^{-1} of Cd treatment. Identification of the peaks was performed based on comparison with the RT of the phenolic compound standards. Therefore, the compounds occurring at 18.5 and 28.4 minutes might be caffeic acid and rutin, respectively. The interesting unknown peaks at the retention times of 16.4, 20.3, 23.5 and 25.4 minutes could not be identified by this HPLC analysis.

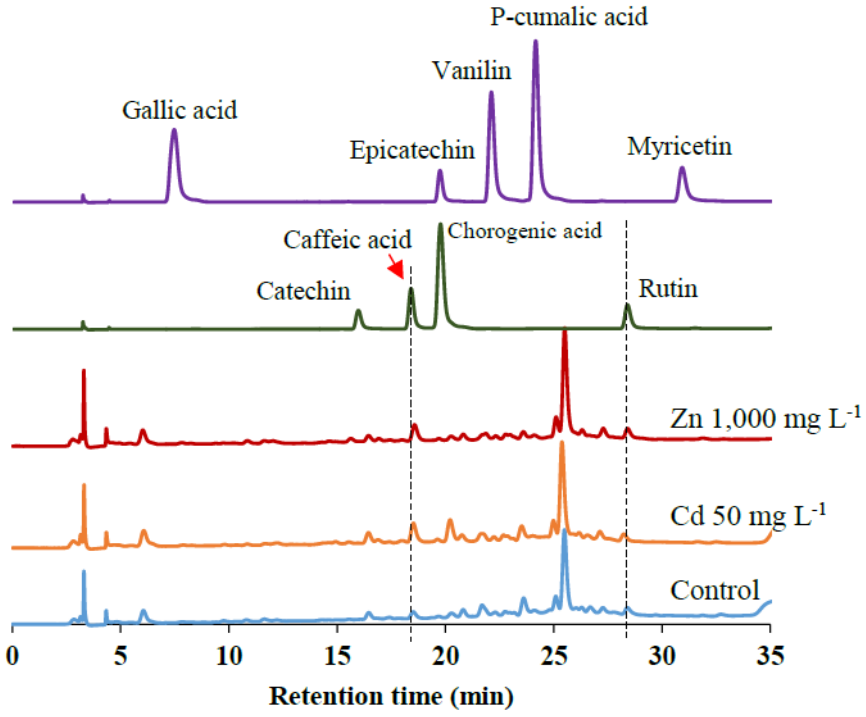


Figure 16 HPLC chromatograms with retention times of phenolic compound standards and leaf extracts from *M. spectabilis* treated with Zn or Cd and control plant detected at wavelengths 280 nm.

4.3.2 Accumulation of Zn/Cd in plant

M. spectabilis treated with various concentrations of Zn (0.04, 50, 100, 250, 500, and 1,000 mg L^{-1}) and Cd (0, 5, 10, 15, 25 and 50 mg L^{-1}) for 4 weeks in the tissue culture system were performed for measuring the Zn and Cd accumulation and calculating the translocation factor (TF) and bioaccumulation factor (BF). In the control treatment, which was 0.04 mg L^{-1} of Zn contained in the MS medium. Zn accumulation in root and shoot of control plants were 0.12±0.04 and 0.09±0.01 mg g^{-1} dry weight, respectively. Table 11 shows that the TF of Zn increased from 0.11 to 0.36 when the plants were treated with increasing Zn concentration from 100 mg L^{-1} to 1,000 mg L^{-1}. The TF of Cd trended to increase when the plants were treated with Cd from 5 to 50 mg L^{-1}; however, the TF of Cd was not significantly different in the values of 0.1 to 0.24. In addition, the highest BF values obtained from the plant treated with the lowest concentrations of Zn (50 mg L^{-1}) and Cd (5 mg L^{-1}) were 1.16 ± 0.19 and 1.28 ± 0.14, respectively.

Table 11 Zinc and cadmium accumulation, translocation factor and bioaccumulation factor of *M. spectabilis*, separately treated with various concentrations of zinc and cadmium.

Treatments	Zn or Cd concentration in tissue culture system (mg L^{-1})[a]	Zn or Cd accumulation (mg g^{-1} dry wt.)		Translocation factor (TF)	Bioaccumulation factors (BF)
		Root	Shoot		
Zn	0	0.12±0.04	0.09±0.01	0.82±0.28	3.09±0.95
	50	5.89±1.56^{b}	1.42±0.22^{c}	0.26±0.07^{a}	1.16±0.19^{a}
	100	12.68±3.79^{a}	1.29±0.20^{c}	0.11±0.05$_{b}$	0.30±0.05^{b}
	250	13.37±1.62^{a}	1.71±0.17^{c}	0.13±0.02^{b}	0.11±0.02^{b}
	500	15.95±2.54^{a}	4.26±0.24^{b}	0.27±0.03^{a}	0.11±0.02^{b}
	1,000	16.10±2.54^{a}	5.74±1.01^{a}	0.36±0.03^{a}	0.09±0.02^{b}
Cd	0	ND	ND	ND	ND
	5	0.46±0.09^{c}	0.07±0.01^{b}	0.15±0.05^{a}	1.28±0.14^{a}
	10	1.24±0.36^{bc}	0.13±0.04^{b}	0.11±0.01^{a}	0.51±0.22^{b}
	15	1.33±0.43^{bc}	0.12±0.02^{b}	0.10±0.06$_{a}$	0.24±0.07^{b}
	25	1.76±0.36^{b}	0.18±0.07^{b}	0.10±0.04^{a}	0.22±0.06^{b}
	50	3.23±0.70^{a}	0.74±0.11^{a}	0.24±0.07^{a}	0.36±0.10^{b}

Data are means ± SD (n = 3). The results shown with different letters (a-c) in the same column are significantly different ($P < 0.01$, Duncan's new multiple range test). The data of Zn and Cd treatment was separately analyzed. [a] The metal concentration (mg L^{-1}) in 20 mL of the MS agar medium.

4.3.3 Distribution of Zn on plant cell by XRF

Figure 17 shows the μ-XRF images of the leaf cross sections of *M. spactabilis* treated with Zn 1,000 mg L^{-1}. The distributions of Zn, Fe, S, Mn, K, Cl and Ca in the leaf cross-section were observed in the vascular bundle and hypodermis. Fe, S, Mn, K Cl and Ca were situated in the same area as the Zn.

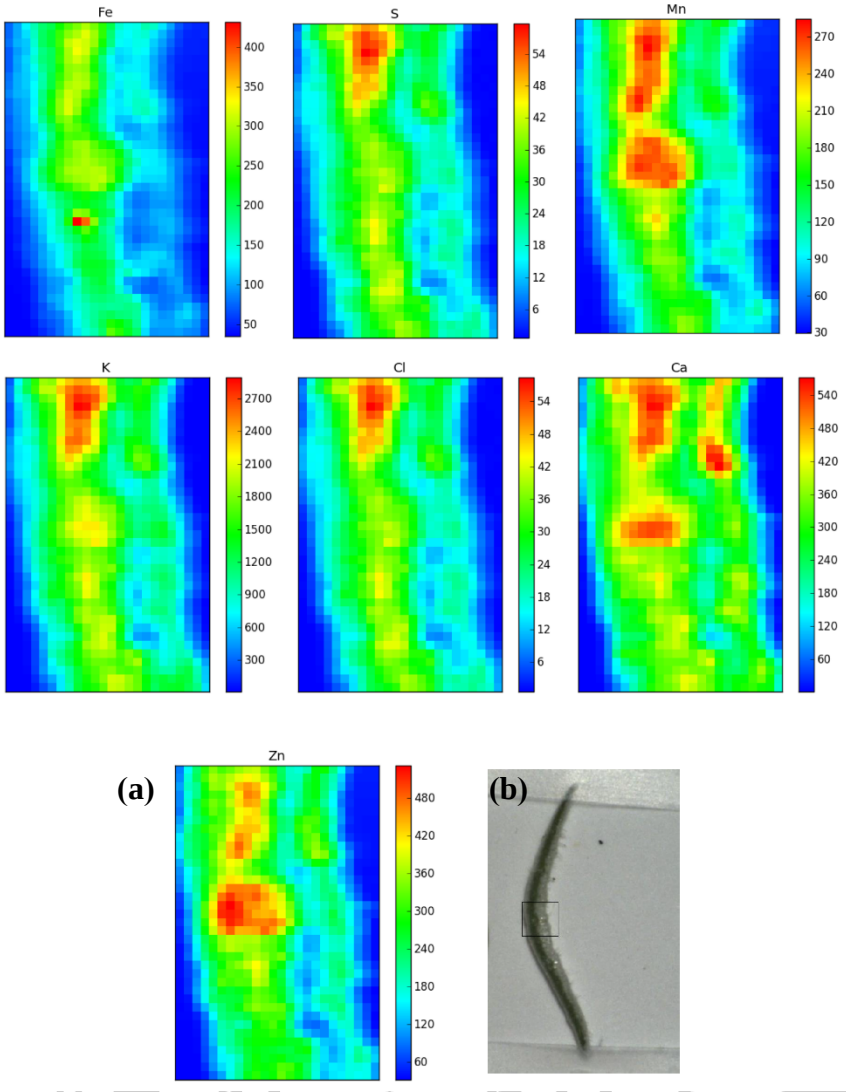


Figure 17 μ-XRF imaging of leaf cross-section of *M. spactabilis* treated with Zn 1,000 mg L^{-1}. The XRF signal intensity is shown as color scale, which blue indicates to the lowest signal and red indicates to the highest signal. (a) shows the XRF mapping of Zn, Fe, S, Mn, K, Cl and Ca, and (b) are photographs of the leaf cross-sections.

4.3.4 X-ray absorption spectroscopy (XAS) analysis

X-ray absorption near-edge structure (XANES) and X-ray absorption fine structure (EXAFS) were applied to obtain information on the oxidation state and coordination of Zn accumulated in the plant tissues, respectively.

The normalized XANES spectra of the S K-edge in the plant samples and the reference materials are shown in Figure 18-19. Figure 18 (A) and (B) present the leaves of *M. spectabilis* treated with various concentrations of Zn and Cd, respectively. The various concentration of Zn and Cd did not effects on the shapes of the XANES spectra, the shapes were similar and had triple peaks. However, the height and shape of the S K-edge XANES spectra of plants treated with Zn and/or Cd tended to decrease, except the plant treated with Cd 15 mg L^{-1}, when compared with the spectrum obtained from the control plant. In this study, the second derivative of the spectra were applied to define the positions of adsorption edge energy as shown in Figure 19. The adsorption edge energy of the S K-edge XANES spectra of all samples were 2472.3 eV, and the shapes of the XANES spectra had triple peaks of 2472.3 eV, 2475 eV and 2481 eV. The triple peaks of sample could be related to the mixtures of $ZnSO_4$ and Zn-glutathione, in which the double peaks at 2472.3 eV and 2475 eV might indicate the Zn-glutathione.

The Zn K-edge XANES spectra of the bulk plant samples demonstrated the oxidation state as Zn^{2+}. The adsorption edge energy was closed at the edge of the Zn-cysteine and $ZnSO_4$ (Figure 20). Table 12 shows linear combination fitting (LCF) was interpreted the spectrum from a sample of unknown with some chemical Zn standards. The results showed that the spectra of samples from *M. spectabilis* treated with Zn (500 mg L^{-1}) and dually treated with Zn (500 mg L^{-1}) and Cd (15 mg L^{-1}) were the best fit with the mixture spectra of ZnS, $ZnSO_4$ $7H_2O$, Zn-cysteine and Zn-cellulose. Therefore, the fitting probably indicated to the mixed interaction or ligation between Zn and O and Zn and S.

Table 13 shows the fitting of EXAFS oscillation yielded the coordination number (N), atomic radius (R) and Debye-Waller factor (σ^2) and and R-factor values. EXAFS were studied to investigate the local structure of Zn on the leaf samples of *M. spectabilis* treated with the highest Zn concentration (Zn 1,000 mg L^{-1}) and dually treated with Zn (1,000 mg L^{-1}) and Cd (15 mg L^{-1}). The leaves treated with Zn 1,000

mg L^{-1} and the leaves treated with Zn (1,000 mg L^{-1}) plus Cd (15 mg L^{-1}) showed similar the nearest neighboring atom. For the Zn 1,000 mg L^{-1} treatment, the fitting for coordination only O showed that the Zn-O coordination in the first shell was 6 (N) and the 2.02 distances Å. For dually treated with Zn (1,000 mg L^{-1}) and Cd (15 mg L^{-1}), the fitting for only O coordination showed that Zn-O coordination in the first shell was 5 (N) and 2.00 Å for the distances. From reducing the R-factor values, assembly of the first shell has been improved by observing both O and S ligands (Table 13).

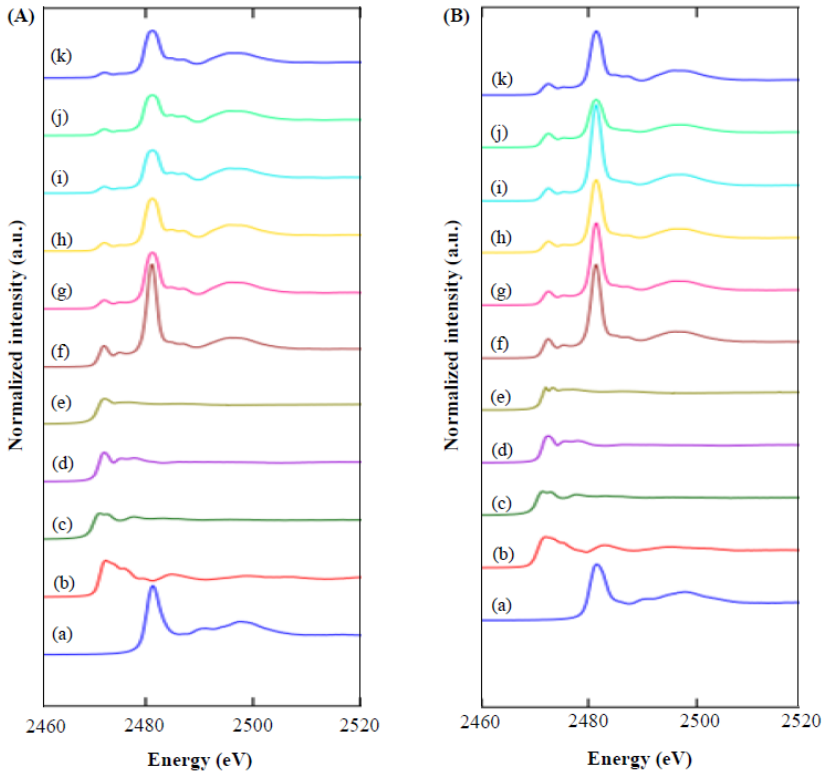


Figure 18 S K-edge XANES spectra of the leaves of *M. spectabilis* treated with various concentrations of Zn, (A) and Cd, (B).

In (A), Zn reference materials are (a) $ZnSO_4$, (b) ZnS, (c) Zn-cysteine, (d) Zn-Glutathione and (e) Zn-Methionine and (f) the leaves of control plant, (g)-(k) are leaves treated with Zn ((g) Zn 50 mg L^{-1} , (h) Zn 100 mg L^{-1} , (i) Zn 250 mg L^{-1} , (j) Zn 500 mg L^{-1} and (k) Zn 1000 mg L^{-1}). In (B), Cd reference materials are (a) $CdSO_4$, (b) CdS, (c) Cd-cysteine, (d) Cd-Glutathione, (e) Cd-Methionine and (f) the leaves of Control plant, (g)-(k) are leaves treated with Cd ((g) Cd 5 mg L^{-1} , (h) Cd 10 mg L^{-1}, (i) Cd 15 mg L^{-1}, (j) Cd 25 mg L^{-1} and (k) Cd 50 mg L^{-1}).

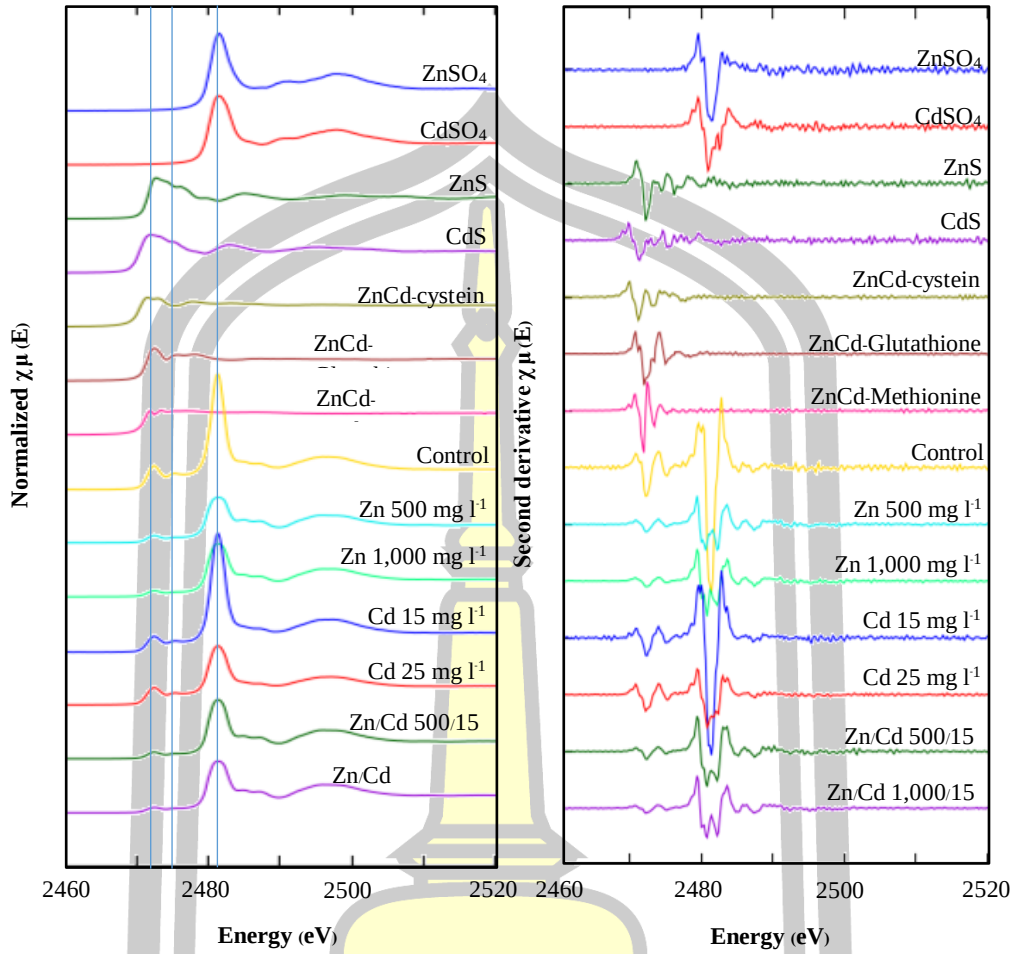


Figure 19 Normalized S K-edge XANES spectra and second derivative of the leaves of *M. spectabilis* treated with Zn (500 and 1,000 mg L^{-1}), Cd (15 and 25 mg L^{-1}), dually treated with Zn (500 and 1,000 mg L^{-1}) and Cd (15 mg L^{-1}) and the leaves of control plant. The reference materials are shown ($ZnSO_4$, $CdSO_4$, ZnS, CdS, ZnCd-cysteine, ZnCd-glutathione and ZnCd-methionine).

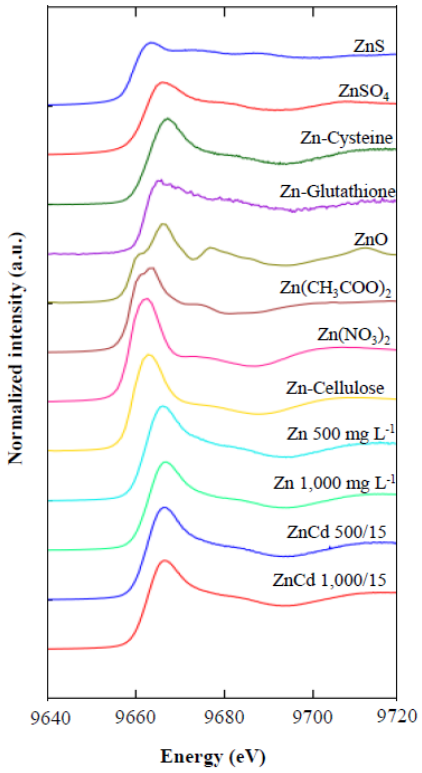


Figure 20 Zn K-edge XANES spectra of the leaves of *M. spectabilis* treated with Zn (500 and 1,000 mg L^{-1}) and dually treated with Zn (500 and 1,000 mg L^{-1}) and Cd (15 mg L^{-1}). The Zn reference materials are shown (ZnS, $ZnSO_4$, Zn-cysteine, Zn-glutathione, ZnO, $Zn(CH_3OO)_2$, $Zn(NO_3)_2$ and Zn-cellulose).

Table 12 Linear combination fitting of Zn K-edge XANES spectra of *M. spectabilis* treated with Zn and Zn plus Cd with the XANES spectra of their reference materials; R-factor, Reduced chi-square and Chi-square are the results of fitting groups, LCF fit of Zn K-edge XANES spectra as flattened μ(E) from 9640 to 9710.

Zn K-edge spectra	Fitting				R-factor	Reduced chi-square	Chi-square
	ZnS	$ZnSO_4$ $7H_2O$	Zn-cysteine	Zn-cellulose			
Zn (500 mg L^{-1})	0.225 ±0.033	0.691 ±0.030	0.007 ±0.052	0.078 ±0.018	0.007	0.002	0.794
Zn (1,000 mg L^{-1})	0.140 ±0.018	0.368 ±0.017	0.456 ±0.034	0.037 ±0.010	0.003	0.001	0.248
Zn + Cd (500+15 mg L^{-1})	0.165 ±0.019	0.553 ±0.018	0.219 ±0.035	0.063 ±0.010	0.003	0.001	0.275
Zn + Cd (1,000+15 mg L^{-1})	0.217 ±0.015	0.456 ±0.014	0.311 ±0.030	0.015 ±0.008	0.002	0.000	0.165

Table 13 EXAFS fitting of the samples and references compounds showing the bond, coordination number (N), atomic radius R (Å), Debye-Waller factor (σ^2), energy shift (ΔE_0) and R-factor values.

Sample	Bond	First shell				
		N	R (Å)	σ^2	ΔE_0 (ev)	R-factor
ZnS	Zn-S	4	2.33±0.04	0.014	3.42	0.038
$ZnSO_4$ $7H_2O$	Zn-O	5	2.03±0.04	0.015	1.19	0.025
Zn-cysteine	Zn-S	4	2.25±0.03	0.011	-6.23	0.030
Zn-cellulose	Zn-O	6	2.05±0.01	0.011	4.36	0.025
Zn (1,000 mg L^{-1})	Fit 1-Zn-O	6	2.02±0.02	0.010	4.15	0.028
	Fit 2-Zn-O	6	2.02±0.08	0.009	4.55	0.015
	Zn-S	1	2.57±0.33	0.015	4.55	
Zn + Cd (1,000+15 mg L^{-1})	Fit 1-Zn-O	5	2.00±0.02	0.008	3.61	0.016
	Fit 2-Zn-O	5	2.00±0.05	0.005	4.31	0.005
	Zn-S	1	2.54±0.21	0.014	4.31	

4.4 The effects of endophytic bacteria inoculation on *M. spectabilis* under Zn/Cd stress

Endophytic strain *C plantarum* RDMSSR05 and *C. ureilyticum* RDMSSR07 were selected for study the effects of endophytic bacteria inoculation on the plant growth and the Zn and/or Cd accumulation in *M. spectabilis* under a tissue culture system. *C. plantarum* RDMSSR05 had properties of nitrogen fixation, ACC-deaminase activity and lignin degradation. *C. ureilyticum* RDMSSR07 had properties of IAA production and siderophore production.

Preliminary colonization of endophytic bacteria showed that the endophytic stain *C. plantarum* RDMSSR05 could colonize the internal tissue of plants and stay for 45 days after inoculation. Wheras, *C. ureilyticum* RDMSSR07 was not detected from the plant tissue after 45 days of inoculation. Furthermore, the detailed study indicated that *M. spectabilis* plant samples growing in the tissue culture still had an indigenous endophytic bacterium (Figure 21). The half formula of TSA plate containing 150 mg L^{-1} of Zn plus 30 mg L^{-1} of Cd was applied as a selective media for study number of the bacterial inoculation, because the indigenous endophytic bacteria was not able to grow in this selective medium (Appendix A13).

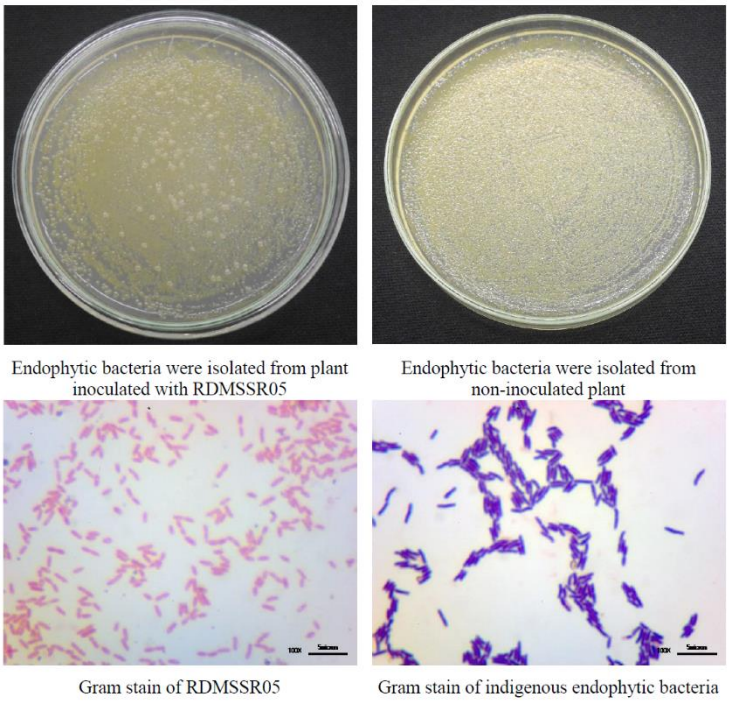


Figure 21 Colonization of *C. plantarum* RDMSSR05, 45 days after inoculation.

The two endophytic bacterial strains were inoculated into one-month old *M. spectabilis* plants. Number of the endophytic bacteria and their colonized ability were investigated on days 7, 14, 21 and 28 after the inoculation. In case of *C. ureilyticum* RDMSSR07, a few colonies were remained in the plant tissue after 7 days (Figure 22). The *C. plantarum* RDMSSR05 was able to colonize into the plant. Although the endophytic bacteria were detected on day 28, but it was found only 17% of all plant samples (Figure 23b). After 14 days of the bacterial inoculation, the bacterial colonized plants were treated with Zn 500 mg L^{-1}plus Cd 15 mg L^{-1} and cultured for 14 days in the tissue culture system. The number of indigenous endophytic bacteria and *C. plantarum* RDMSSR05 re-isolated tended to decrease over the time as shown in Figure 23 (a) and (b), respectively.

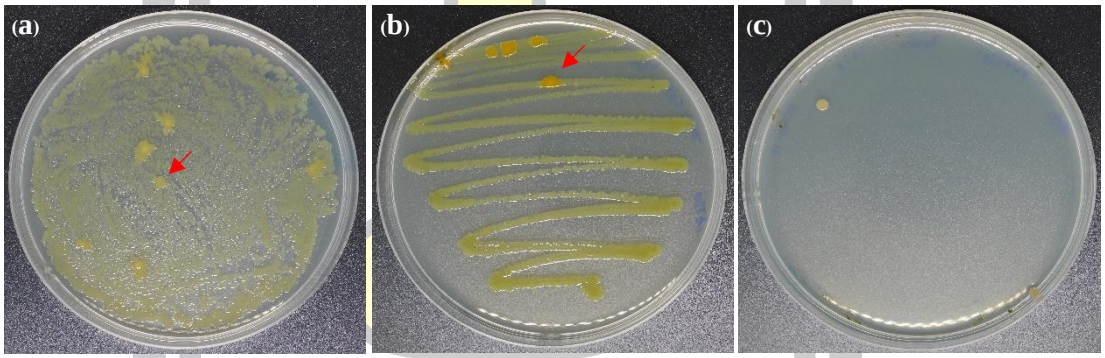


Figure 22 Re-isolated stain *C. ureilyticum* RDMSSR07 from *M. spectabilis* at 7 days after inoculation. (a, b) spreading and steaking on ½ TSA plates, and (c) ½ TSA plates supplement with Zn 150 mg L^{-1} plus Cd 30 mg L^{-1} for 48 hours.

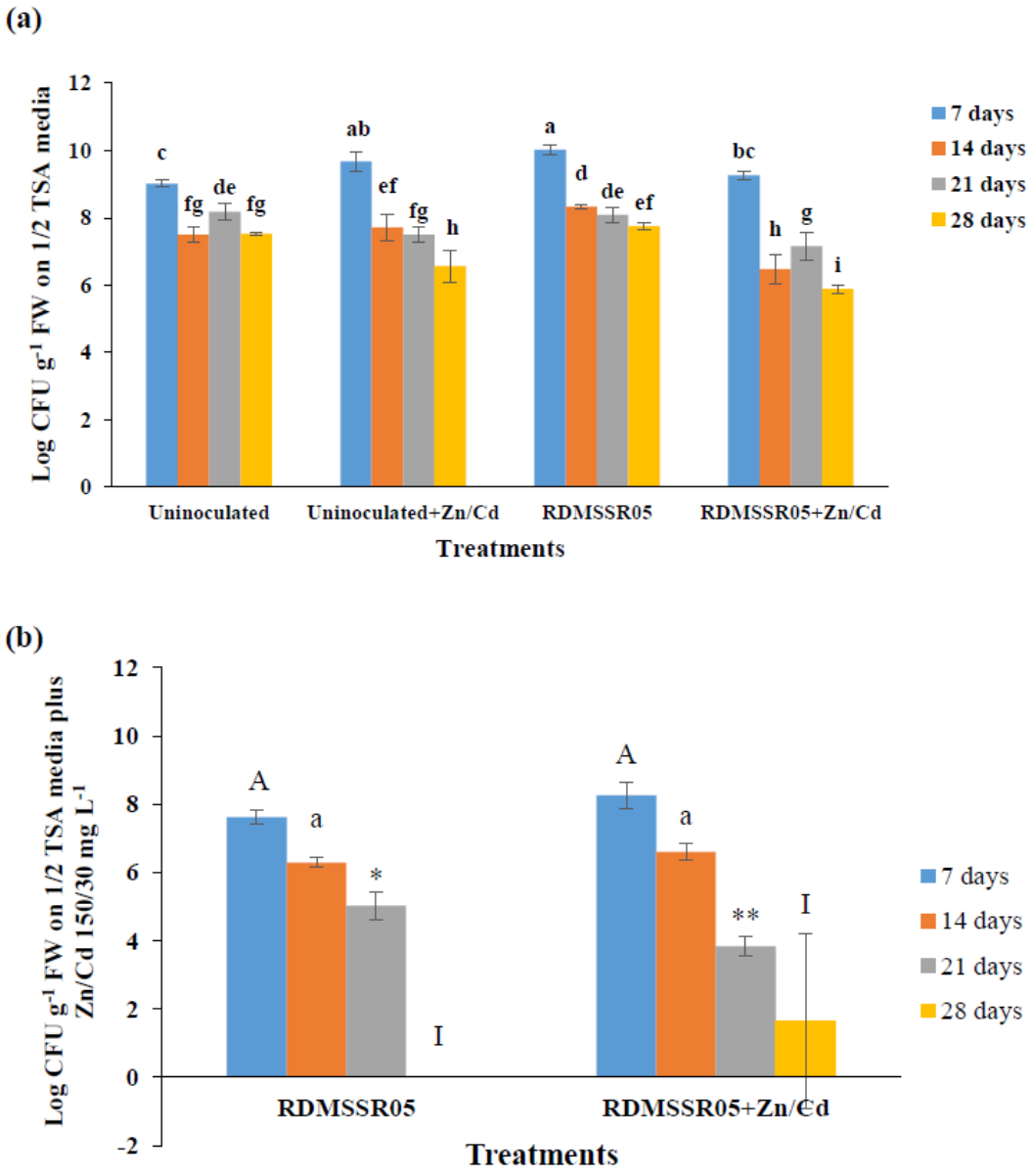


Figure 23 Population of endophytic bacteria isolated from tissues of *M. spectabilis* after inoculation, with and without Zn/Cd. (a) Total bacteria including *C. plantarum* RDMSSR05 and unknown bacteria, The results shown with different letters (a-i) on the error bars are significantly different ($P < 0.01$, Duncan's new multiple range test). The data are given as the means ± SD (n = 4). (b) the number of *C. plantarum* RDMSSR05, The results shown with different letters (symbols (*, **)) on the error bars are significantly different ($P < 0.01$, Mann Whitney U-test)). The data are given as the means ± SD (n = 6).

The identification of re-isolated endophytic bacteria was verified by morphological characteristics, Gram staining and 16S rDNA sequencing. The results confirmed that the endophytic bacterial strains RDMSSR05 and RDMSSR07 were the original culture, and also indicated to short time colonization of the inoculated bacteria. The 16S rDNA sequence showed a 99% similarity to *C. plantarum* and *C. ureilyticum*, respectively. Moreover, an indigenous endophytic bacterium was belonged to *Curtobacterium luteum* with 99% similarity and it was closely related phylogenetically to the original culture (RDMSP05, RDMSP07 and RDMSP11) (Figure 24).

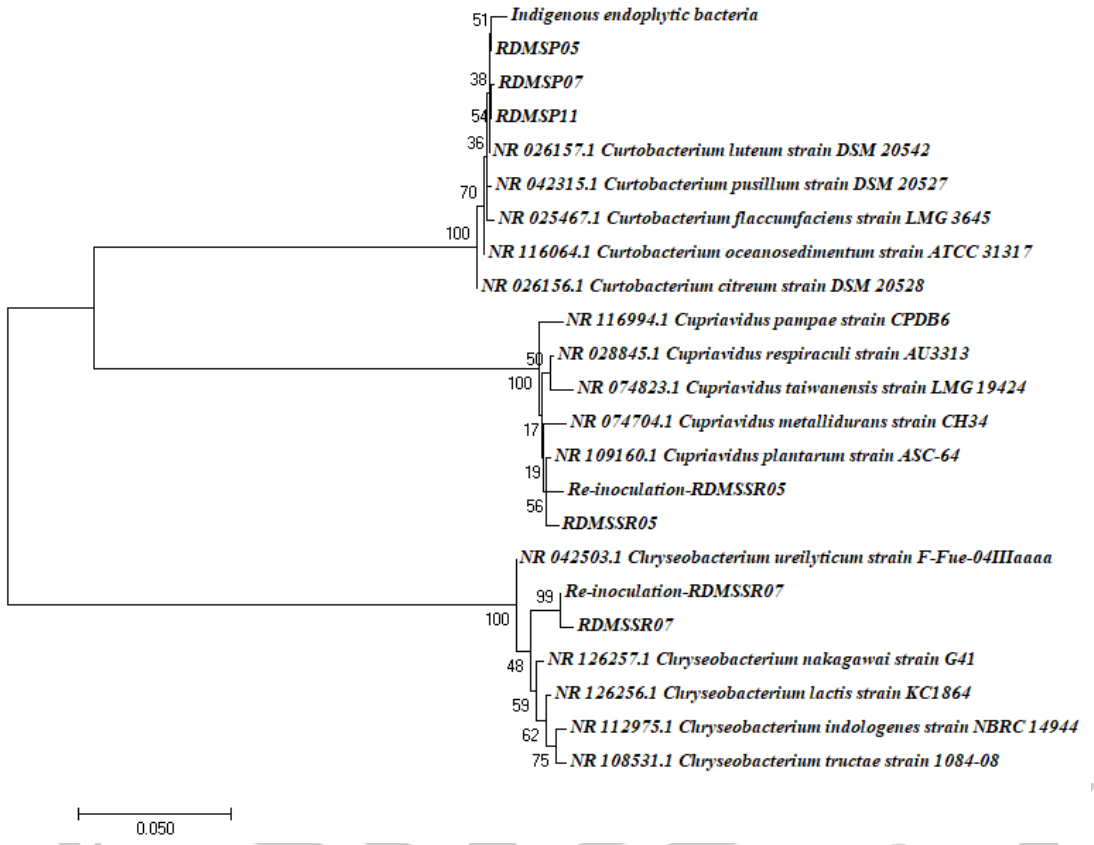


Figure 24 Phylogenetic analysis of 16S rDNA sequences of re-inoculation endophytic bacteria and original culture (RDMSP05, RDMSP07, RDMSP11, RDMSSR05 and RDMSSR07) isolated from *M. spectabilis* and sequences from NCBI (indicated by accession number), using the Maximum Likelihood method with 1,000 bootstrap replicates. There were a total of 1,229 positions in the final dataset. Evolutionary analyses were conducted in MEGA7. Bootstrap values are indicated at the node. Bar indicates 0.05 substitutions per nucleotide position.

Figure 25 shows the effects of the endophytic bacterial strain *C. plantarum* RDMSSR05 on the growth of *M. spectabilis* compared to the un-inoculated (control), which contained the indigenous bacterium, after 28 days of inoculation. The colour and number of leaves, wet weight and dry weight are shown in Figure 26. Plants inoculated with endophytic bacteria showed a non-significant value in all growth parameters. In the same way, endophytic bacterial inoculation did not affect the Zn and Cd accumulation in the plant samples (Figure 27).

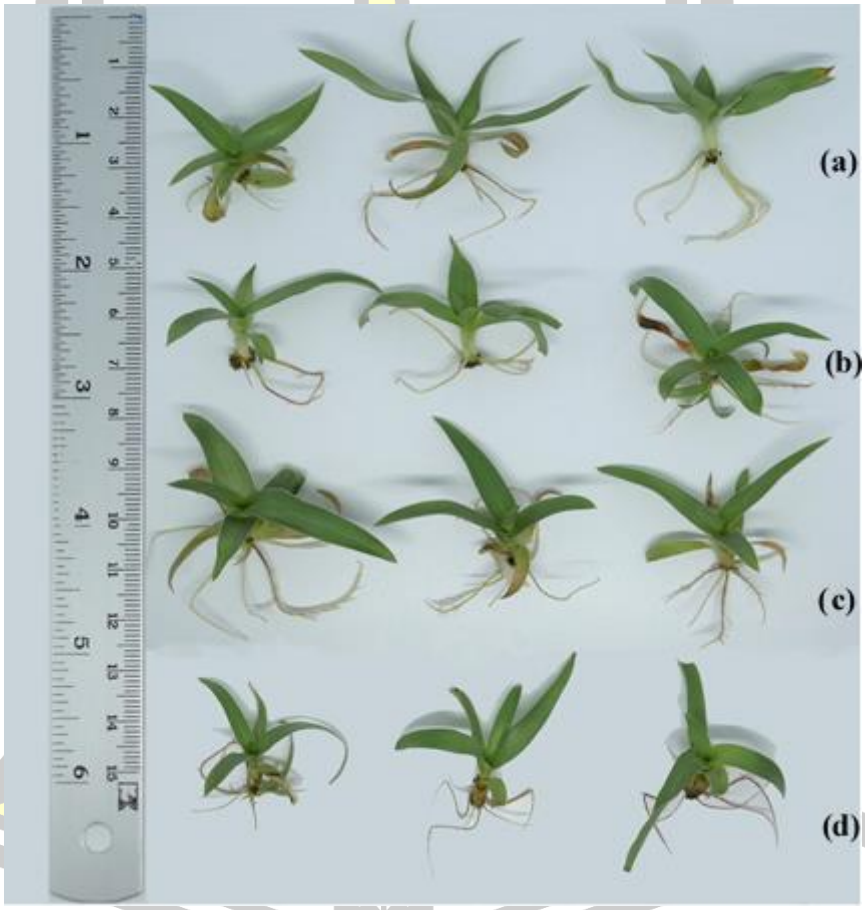


Figure 25 The morphological changes of *M. spectabilis* inoculated with *C. plantarum* RDMSSR05 under the metal stress. (a) plant inoculated bacteria and treat Zn 500 mg L^{-1}plus Cd 15 mg L^{-1}, (b) plant inoculated bacteria, (c) plant without bacteria and treat Zn 500 mg L^{-1}plus Cd 15 mg L^{-1}, (d) plant without bacteria and without metal, 28 days after inoculation.

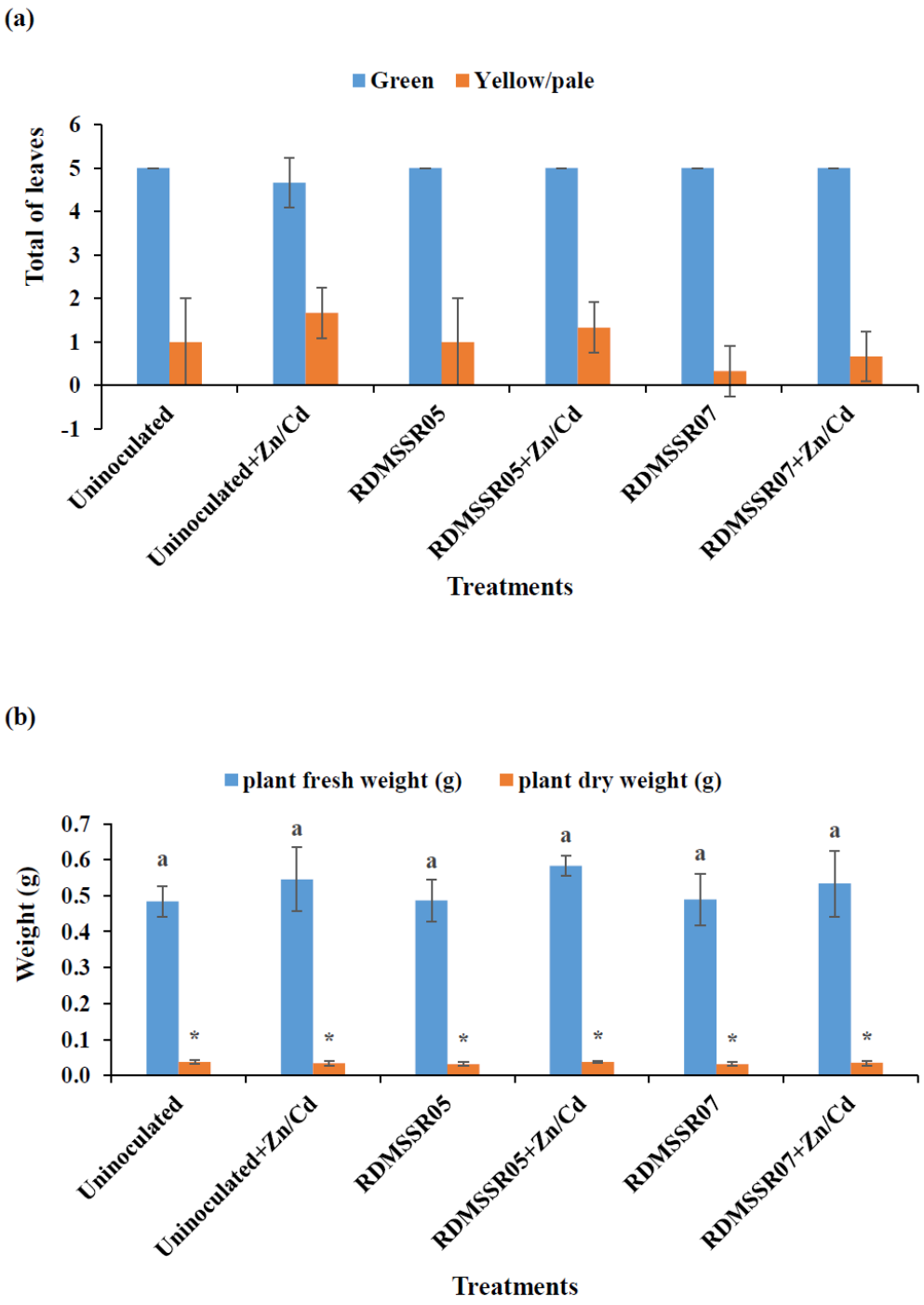


Figure 26 Effect of endophytic bacterial inoculation on growth of *M. spectabilis* under the metal stress. (a) number of leaves, (b) fresh weight and dry weight of plant), 28 days after inoculation. The results shown with different letters on the error bars are significantly different ($P < 0.01$, Duncan's new multiple range test). The data are given as the means ± SD (n=3).

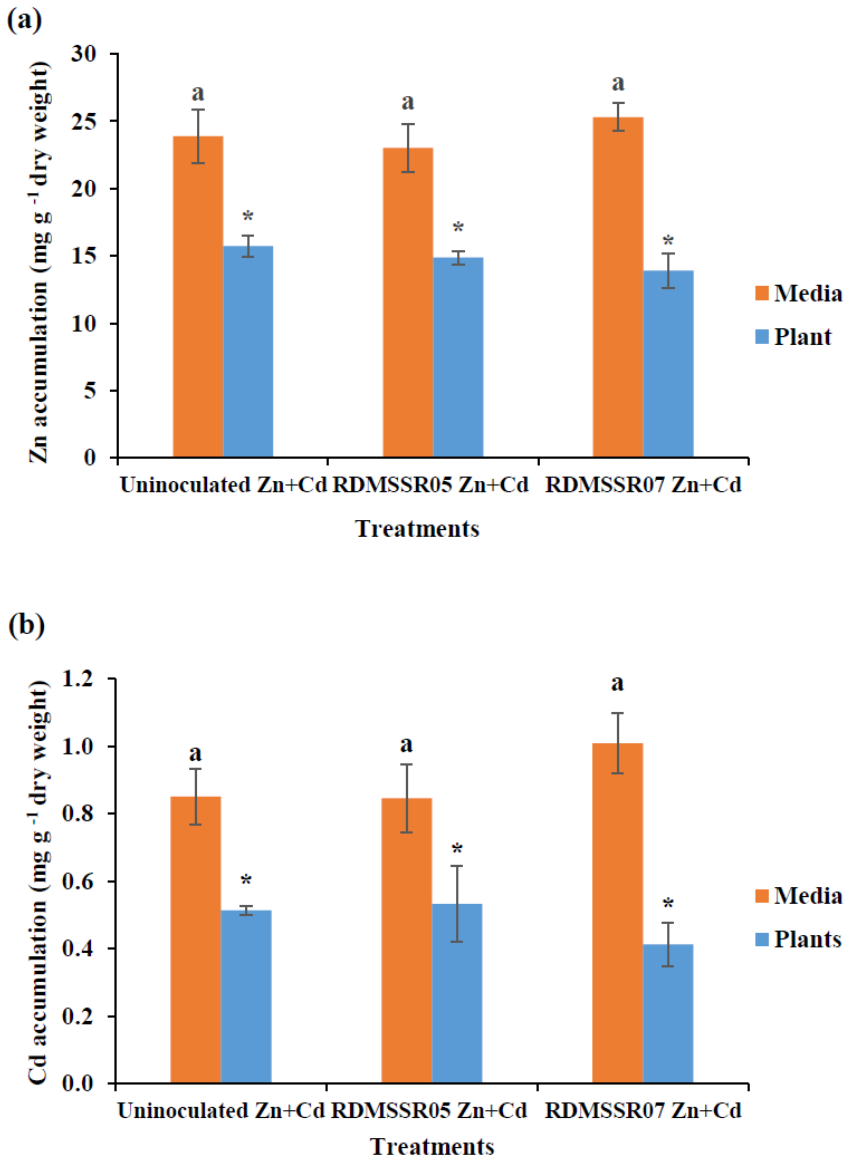


Figure 27 Accumulation of Zn and Cd in *M. spectabilis* and media of the tissue culture system, 28 days after inoculation. The results shown with different letters on the error bars are significantly different ($P < 0.01$, Duncan's new multiple range test). The data are given as the means ± SD (n = 3).

Growths of the selected endophytic isolates and the indigenous bacterium were investigated to obtain some interaction and/or antagonism among the bacteria cultivation. The bacterial growth curve showed that *C. ureilyticum* RDMSSR07 reached the logarithmic growth phase within 4-10 hours and the stationary phase after 16 hours. *C. ureilyticum* RDMSSR07 showed faster growth and had a shorter lag

phase than *C. plantarum* RDMSSR05 and *C. luteum* (indigenous endophytic bacteria). *C. plantarum* RDMSSR05 and *C. luteum* reached the logarithmic growth phase and the stationary phase within 6-10 hours and after 16 hours, respectively. However, after 36 hours, *C. plantarum* RDMSSR05 was in the death phase. (Figure 28).

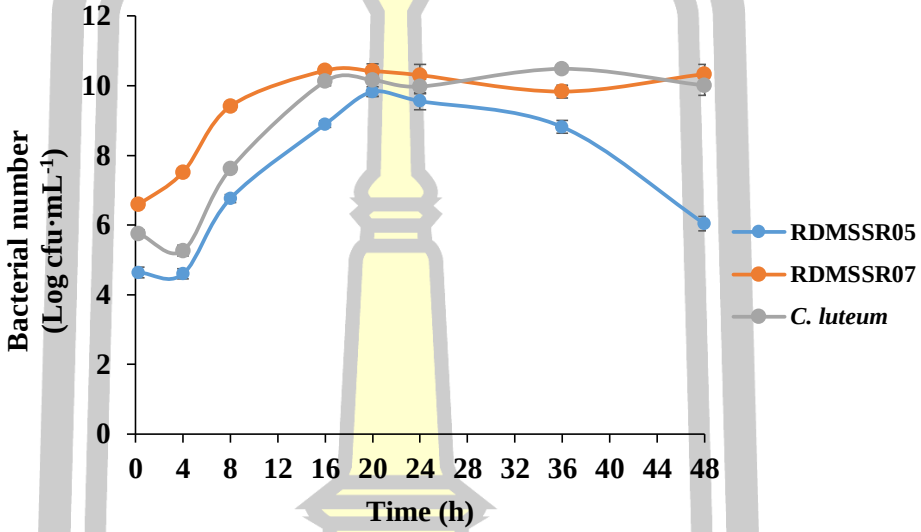


Figure 28 Growth curves of *C. plantarum* RDMSSR05, *C. ureilyticum* RDMSSR07 and *C. luteum* (indigenous endophytic bacteria) in TSB medium for 48 hours.

The antagonism test was carried out by an agar plate technique to investigate the ability of *C. luteum* to control the growth of RDMSSR05. No activity of the inhibition zone presenting in Figure 29 indicated that there were no antagonism between *C. luteum* and *C. plantarum* RDMSSR05 or *C. ureilyticum* RDMSSR07. In addition, co-culture between *C. luteum* and *C. plantarum* RDMSSR05 or *C. ureilyticum* RDMSSR07 were performed in a batch culture system. In addition, dual culture in a batch system between *C. luteum* and *C. plantarum* RDMSSR05 or *C. ureilyticum* RDMSSR07 did not show any effects on their growth as shown in Figure 30 (a) and (b), respectively. In comparison with Figure 30 (d), Figure 30 (c) shows no competition effects in the mix culture of *C. luteum*, *C. plantarum* RDMSSR05 and *C. ureilyticum* RDMSSR07.

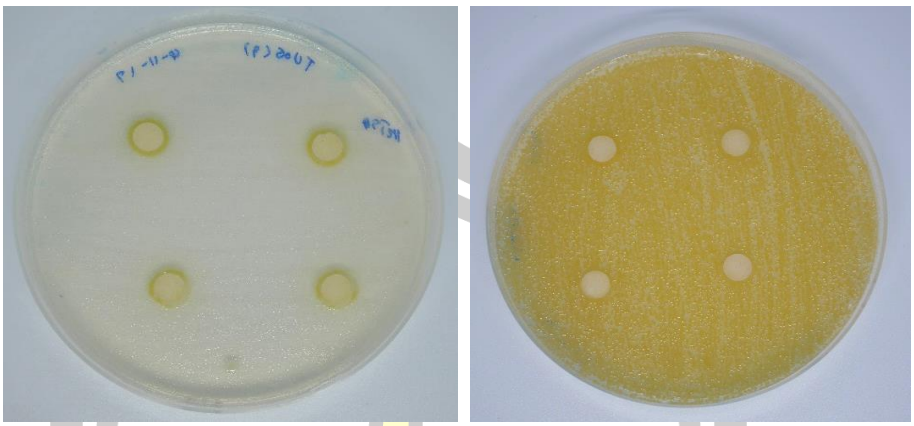


Figure 29 Analysis of the antagonist activity of *C. luteum* (indigenous endophytic bacteria) in *C. plantarum* RDMSSR05 and *C. ureilyticum* RDMSSR07 by agar plate method for 72 hours.

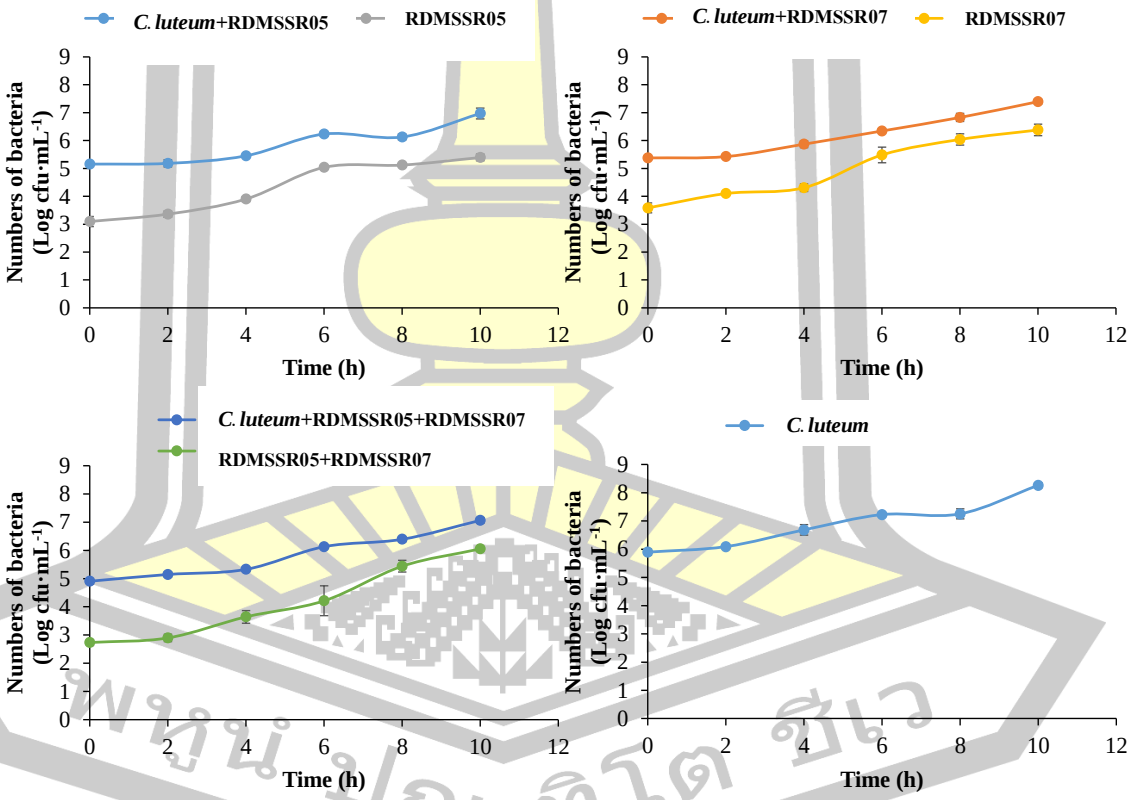


Figure 30 Growth curves of bacterial endophyte in TSB medium. (a) dual culture test of *C. luteum* and *C. plantarum* RDMSSR05, (b) dual culture test of *C. luteum* and *C. ureilyticum* RDMSSR07, (c) mixed culture of *C. luteum*, *C. plantarum* RDMSSR05 and *C. ureilyticum* RDMSSR07 and (d) growth curves of *C. luteum*.

CHAPTER 5

DISCUSSIONS AND CONCLUSIONS

5.1 Discussions

5.1.1 Isolation and characterization of endophytic bacteria from *M. spectabilis*

M. spectabilis is in the Commelinaceae family and is found throughout Thailand (Thitimetharoch, 2004). Panitlertumpai et al. (2003) reported that *M. spectabilis* growing in a Zn mining area, Phatat Phadaeng sub-district, Mae Sot, Tak Province, accumulated high levels of zinc (Zn) and cadmium (Cd). In addition, Rattanapolsan el al. (2013) clearly showed that *M. spectabilis* was a Zn and Cd hyperaccumulative plant from the criteria of translocation factor (TF), and there were Zn and Cd tolerant bacteria colonizing within the storage root tissue. For this study *M. spectabilis*'s plants were collected from the forest areas with no mining activity of the Zn mine. The Zn and Cd concentrations in the rhizospheric soil of the plants were higher than the classified levels of Zn and Cd in non-contaminated soil (Kabata-Pendias and Pendias, 1992). *M. spectabilis* is a perennial plant, and the growth of *M. spectabilis* from the dormancy period might correlate with bacterial endophytes. Therefore, the aims of this research were isolation, characterization and selection of culturable endophytic bacteria from *M. spectabilis* growing in the Zn and Cd contaminated area. To dispose of epiphytic bacteria, the best conditions for surface-sterilization of the explants had to be acquired before isolation of the endophytic bacteria. Endophytic bacteria have been isolated from many plants since woody plant to herbaceous and crop plants (Lodewyckx et al., 2002a; Ryan et al., 2008). The concentrations of Zn and Cd accumulated in each parts of *M. spectabilis* were higher than the critical concentrations of Zn and Cd in non-tolerant plants (Zn 100-300 μg g^{-1} leaf dry weight, Cd 5-10 μg g^{-1} leaf dry weight) (White and Brown, 2010). In this study, we found 52 isolates of endophytic bacteria (9.9×10^2 to 8.8×10^5 CFU g^{-1} of plant tissue (fresh weight)) from the storage roots, underground stems (tubers), leaves and peduncles of *M. spectabilis*, hence the plant's part contained high concentration

of Zn and Cd. The 24 endophytic isolates that tolerated to Zn (250-500 mg L^{-1}) and/or Cd (20-50 mg L^{-1}) were identified by 16S rDNA sequencing analysis. They belonged to genera of *Bacillus*, *Pantoea*, *Microbacterium*, *Curtobacterium*, *Chryseobacterium*, *Cupriavidus*, *Siphonobacter* and *Pseudomonas.* These genera were reported as common soil bacteria and endophytes of several plant species growing in metal-contaminated soils (Idris et al., 2004, Barzanti et al., 2007, Ryan et al., 2008, Sheng et al., 2008, Mastretta et al., 2009, Sun et al., 2010, Long et al., 2011, Chen et al., 2012). The bacterial endophytes isolated from storage roots and tubers were in the same genera, therefore, the bacteria found might relate to the underground parts exposing to soil.

The 24 bacterial isolates were screened for Zn and Cd tolerance and the plant growth promoting properties. Most of all isolates were able to resist Zn 150 mg L^{-1} and Cd 20 mg L^{-1}. The results presented that the endophytic bacteria isolated from the storage roots were greater tolerated to Zn and Cd than the tubers, leaves and peduncles, respectively. Moreover, the two strains, *Microbacterium neimengense* RDMS03 and *Microbacterium neimengense* RDMS06 were able to produce clearing zone around the colony. On the other hand, the strains *Cupriavidus plantarum* RDMSSR03 and *Cupriavidus plantarum* RDMSSR05 were produced turbid zone around their colonies on the TSA media supplemented with Zn and Cd. The clear zone indicated that the bacteria might secrete acid substance to dissolve the precipitate metals. Whereas the turbid zone indicated that the isolates probably secreted substance to precipitate the metals. These isolates may have potential to improve the efficiency of phytoextraction and phytostabilization, respectively. Some bacteria in the genus of *Microbacterium* was isolated from rhizosphere of maize in China (Gao et al., 2013) . *Microbacterium* played a role in plant growth and improved phytoextraction of heavy metal from contaminated soil (Corretto et al., 2015; Khan et al., 2015). Long et al. (2011) reported the effects of endophytic bacteria that was able to solubilize $ZnCO_3$ and $Zn_3(PO_4)_2$ in Zn-contaminated soil. Moreover, Siripornadulsil and Siripornadulsil (2013) reported that Cd-tolerant bacteria increased Cd tolerance in rice and decreased the accumulation of Cd in rice, and *Cupriavidus taiwanensis* KKU2500-3 could possibly change toxic of soluble $CdCl_2$ into non-toxic of insoluble CdS.

The beneficial effects of endophytic bacteria on plant growth promoting and metal uptake depend on plant growth-promoting properties of indole-3-acetic acid (IAA) production, phosphate solubilization, siderophore production, ACC deaminase activity and nitrogen fixation (Glick 2012). The 24 bacterial isolates were able to produce IAA and almost the isolates had nitrogen fixation property. Six strains (RDMSSR02, RDMSSR07, RDMSSR08, RDMSSR12, RDMSSR13 and RIDMSSR02) in the genus of *Chryseobacterium, Pseudomonas* and *Bacillus* were able to produce siderophores. The three strains of *Chryseobacterium ureilyticum* RDMSSR07, *Pseudomonas aeruginosa* RDMSSR08 and *Pentoea* sp. RDMSL03 had the ability of phosphate solubilization. The strains of RDMSSR03 and RDMSSR05 belonging to *Cupriavidus plantarum* was able to produce ACC deaminase enzyme. In addition, the strains of RDMSSR12, RDMSSR13, RIDMSSR02, RIDMSS01, RIDMSS04, RIDMSS05, RIDMSS06, RIDMSS07 and RIDMSS09 that belonged to the genus *Bacillus* could produce cellulase. Only two strains of *Cupriavidus plantarum* RDMSSR05 and *Pseudomonas aeruginosa* RDMSSR08 had the ability of lignin degradation. IAA production is generally a common phenomenon in many bacteria and fungi in the soil (Fu et al., 2015). Endophytic bacteria can also synthesize IAA (Sessitsch et al., 2004, Sheng et al., 2008; Chen et al., 2010; Zhang et al., 2011). Some of the endophytic isolates also demonstrated to solubilize mineral phosphates. Verma et al. (2001) reported that endophytic bacteria could enhance the initial colonization process by phosphate solubilization. Production of extracellular enzymes that degrade the cell wall were an ability of endophytic bacteria for plant colonization (Jha and Kumar, 2007; Pereira and Castro, 2014).

Several research reported that the ability of endophytic bacteria to protect plants from the harmful effects of heavy metals related with their ability to promote plant growth (Sheng et al., 2008; Sun et al., 2010; Long et al., 2011; Luo et al., 2011; Zhang et al. 2011;). In this study, the bacterial stain *C. plantarum* RDMSSR05 had properties of nitrogen fixation, ACC-deaminase activity and lignin degradation. *C. ureilyticum* RDMSSR07 had properties of IAA production and siderophore production. Therefore, the two strains were applied to study the effects of endophytic bacterial inoculation on plant growth promoting and Zn/Cd accumulation in plant. Luo et al. (2011) reported that incoculation of *Chryseobacterium* sp., which is an

endophytic bacterium isolated from *Solanum nigrum* L., helped the plant to increase root dry weight and to promote Cd accumulation. On the other hand, inoculation of *Chrysiobacterium humi* decreased Zn and Cd accumulation in *Helianthus annuus* (Marques et al., 2013).

5.1.2 Effects of metals tolerance and accumulation in *M. spectabilis*

M. spectabilis was extremely tolerant to high Zn and Cd exposure. However, the results indicated that the high concentrations of Zn 500-1,000 mg L^{-1} or Cd 25-50 mg L^{-1} affected the plant growth, increased chlorosis and stunting, and decreasing of the chlorophyll concentration. In addition, higher Zn or Cd concentrations slightly caused to protein content, cell death, total phenolic compound and stress enzymes activity. The separated protein band pattern of SDS-PAGE showed the effects of Zn and Cd on the protein expression. These results might be due to mechanisms of the plant for metals detoxification. The 12 and 13 kDa proteins might be metallothioneins (8-14 kDa), which the plant responded to metal tolerance (Grill et al., 1989, Nakbanpote et al., 2010). The expression of 22-27 kDa and 60 kDa bands under the Zn and Cd stress might indicate heat shock proteins. In which, heat shock proteins are divided into several families, namely: Hsp100, Hsp90, Hsp70, Hsp60 (or chaperonins), 17-30 kDa small hsps (shsps) and ubiquitin (8, 5 kDa) (Al-Whaibi et al., 2011, Joseph et al., 2012). Moreover, the nutrients in MS medium could reduce the metal toxicity to the plants (Yadav et al., 2010). Although Zn is one of the essential trace elements for plants, plants require Zn in small amounts for the regulation of transcription and translation, the structural stability of proteins, the function of oxidoreductases and hydrolytic enzymes, and also the control of enzyme activities (Broadley et al., 2007; Clemens et al., 2010; White, 2012). Therefore, the excessive concentration of Zn is toxic for plants. White and Broadley (2011) reviewed the different plant species differ in both their necessities of Zn and their high Zn concentrations tolerance in plant tissue (Broadley et al.,2007; Fageria, 2009). Cd is a non-essential trace element that contrarily effect on plant growth and development. The various symptoms of Cd toxicity in plants affect morphological and physiological characteristics change such as stomatal opening, chlorosis, leaf rolls, stunting are, transpiration and photosynthesis (Sandalio et al., 2001). Many researches have

suggested that the effect of Cd toxicity on decreasing enzymatic and non-enzymatic antioxidants and inducing oxygen free radical production which causing an oxidative stress (Sandalio et al., 2001, Sytar et al., 2013). Baker (1981) described the criterion for plants growing on metal contaminated soils can be classified into three groups including excluders or non-accumulators, accumulators, and indicators. The relationship between the concentration of metal in the plant and soil is generally linear. From the criteria for a metal accumulation, *M. spectabilis* studied in the tissue culture system could be classified as an indicators plant. The distributions of Zn, Fe, S, Mn, K, Cl and Ca in the leaf cross-section were observed in the vascular bundle and hypodermis. μ-XRF imaging of leaf cross-section of *M. spactabilis* presents the mineral elements (Fe, S, Mn, K, Cl and Ca) was obtained from the growth culture medium. The results suggest that the necessary nutrients Mn can replace or be in the same position as Zn and Cd due to the transition metal properties and Mn^{2+} is likely to be absorbed by negatively charged cell walls (Millaleo et al., 2010, Mongkhonsin et al., 2016). Ca can be stored in leaf vacuoles to avoid too much apoplastic accumulation (White and Broadley, 2003, Mongkhonsin et al., 2016). Cd distribution in *Nicotiana tabacum* studied with μ-XRF imaging was in the sieve tissues (Hokura et al. (2006). Mongkhonsin et al. (2011) investigated the distribution of chromium (Cr) (VI) accumulated in *G. pseudochina* (L.) DC. using μ-XRF imaging found that the Cr was mainly distributed in the vascular bundle and periderm of the tuber, the stem xylem, the vein and the epidermis, including the trichome of the leaf tissues. In addition, Mongkhonsin et al. (2016) proposed that the role of flavonoids and cell wall immobilization in increasing the tolerance of valerian for Zn and Cd. Accumulation of Zn and Cd in the epidermis and vascular bundles is a positive detoxification process (Hu et al., 2009; Fukuda et al., 2008; Vollenweider et al., 2006; Hernandez-Viezcas et al., 2011, 2013). The S K-edge XANES spectra of the tuber and leaves of *M. spectabilis* growing in Zn/Cd contaminated soil were 2473.3 eV. The shapes of XANES spectra had triple peaks, which might relate to the mixtures of $ZnSO_4$ and Zn-cysteine (Rattanapolsan et al., 2013). The Zn K-edge XANES spectra indicated Zn ion (Zn^{2+}) in the plant samples, the EXAFS spectra detailed information about the local environment surrounding the atom; coordination numbers and bond lengths. The best fit of the first shell was both O and S ligands. Isaure et al. (2015) reported that Cd

accumulated in *A. lyrata* and non-tolerant plant was mainly coordinated with S atoms only and slightly involved with O groups. The proportion of O ligands also increased in *A. halleri* and tolerant progenies, while the S ligand was still function. Thus, the bonding with both O and S ligands relate to the Cd tolerance of *M. spectabilis*.

5.1.3 The effects of endophytic bacterial inoculation on *M. spectabilis* under Zn/Cd stress.

Endophytic stain *C. plantarum* RDMSSR05 and *C. ureilyticum* RDMSSR07 were selected for study the effects of the bacterial inoculum on the plant growth and Zn/Cd accumulation in *M. spectabilis* under a tissue culture system. The results obtained for this case showed that the plant growth and metal accumulation were not significantly affected by the endophytic bacterial inoculation. The problems were the decrease number of RDMSSR05 and RDMSSR07over time after the inoculation. Only small amounts of RDMSSR07 were detected after 7 days of inoculation. Furthermore, there was an indigenous endophytic bacterium prolonged colonization within the plant. The 16S rDNA sequence of the indigenous endophytic bacteria was belonged to *Curtobacterium luteum*. The habitat of *Curtobacterium* was related with plants, especially phyllosphere (Behrendt et al., 2002). Some species of *Curtobacterium* were isolated from soil (Chase et al., 2016) such as *Curtobacterium luteum* (Kuddus and Ramteke, 2008). *Curtobacterium* sp. NM1R1, the rhizobacterial strains could be used as an efficient bioinoculant in phytoremediation of many metals contaminated in soil (Roman-Ponce et al., 2017). The indigenous endophytic bacteria had a larger population in *M. spectabilis* than the inoculated bacteria. Probable competition effects between the inoculated bacteria and the indigenous endophyte on the plant growth under the Zn and Cd stress were investigated. The results showed that the metal accumulated in the plant has an effect on decrease the number of the endophytic bacteria. From behavior of the colonization of bacterial endophytes, some endophytic bacteria can enter through endoderm, and xylem vascular system is the main transport route for the systemic colonization of the internal compartments (James et al., 2002) and others locally colonize intercellular spaces (Hardoim et al., 2015). According to this results, the endophytic bacteria might be colonized in the vascular bundles, which was the same area of the metals distribution. The antagonism

test was conducted to investigate the ability of the indigenous endophytic bacteria (*C. luteum*) to control the growth of strain RDMSSR05 and RDMSSR07. The results showed that *C. luteum* was not an antagonist of both species. Meng et al. 2014 suggested that decrease of inoculated endophytic bacteria in root, stem and leaf tissues of *Jerusalem artichoke* after inoculation might occur from plants develop oxidative nitrogen scavenging (ONS) strategy by secreting reactive oxygen species to oxidize and extract nitrogen from symbiotic nitrogen fixing bacteria (White et al., 2012).

5.2 Conclusions

A total of 52 endophytic bacteria were isolated from storage roots, stems, leaves and peduncle of *M. spectabilis* (Kurz) Faden growing in Zn mining area, Mae Sot, Tak Province, Thailand. 19 isolates were from storage root, 9 from stems, 8 from leaves and 16 isolates from peduncle. The 24 isolates surviving on TSA adding with Zn (250-500 mg L^{-1}) and Cd (20-50 mg L^{-1}) were selected for bacterial identification. The 16S rDNA gene sequencing indicated that the bacterial isolates were in genera of Bacillus, *Lapillicoccus*, *Pantoea*, *Microbacterium*, *Curtobacterium*, *Chryseobacterium*, *Cupriavidus*, *Siphonobacter* and *Pseudomonas*. The 24 endophytic bacteria were able to resist Zn 150 mg L^{-1} and Cd 20 mg L^{-1} except RDMSSR12, RDMSSR13 and RIDMSP02. Some Zn-Cd resistant endophytes produced multiple plant growth promoting traits such as IAA production, nitrogen fixation, phosphate solubilization, siderophores production, ACC deaminase activity, extracellular enzymes as a cellulases, ligninolytic enzymes and lignin degradation. Some isolates seemed well-adapted to high Zn/ Cd concentrations (RDMSP03, RDMSP06 and RDMSSR05) . The six isolates of RDMSSR02, RDMSSR04, RDMSSR05, RDMSSR07, RDMSP03 and RDMSP06 were selected for test plant growth promoting properties under Zn and Cd stress. The results indicated that the Zn and Cd decreased the plant growth promoting ability of strain RDMSSR04, RDMSP03 and RDMSP06, but less affected on RDMSSR02, RDMSSR07 and RDMSSR05.

The effects of Zn or Cd tolerance and accumulation in *M. spectabilis* occurred after 4 weeks growth when treated with Zn (50-1,000 mg L^{-1}) and Cd (5-50) mg L^{-1}. Fresh weight, dry weight, number of storage root and the percentage of yellow/ pale leaves (phytotoxicity) and stress induction focused on chlorophyll content, protein content, cell death, protein content, total phenolic compound and stress enzymes activity (SOD, CAT) were compared between the treated and control plants. The results indicated that the Zn or Cd stress affected the plant growth and decreased the chlorophyll concentration, whereas higher Zn concentrations of 500 and 1,000 mg L^{-1} caused a slightly lower protein content, cell death, total phenolic compound and stress enzymes activity. From the criteria for a metal accumulation, *M. spectabilis* could be classified as an indicative plant. μ-XRF imaging indicated that the Zn was mainly distributed in the vascular bundle of the leaf tissues. The Zn K-edge indicated that the oxidation state of Zn accumulated in the leaves was 2+ (Zn^{2+}). In comparison with the Zn K-edge spectra of the reference materials. The first shell the EXAFS presents detailed information about the local environment surrounding the atom; coordination numbers and bond lengths. The best fit in the first shell was both Zn-O and Zn-S ligands, where the sulfur might be in the form of sulfur proteins.

The selected endophytic stain C. plantarum RDMSSR05 and C. ureilyticum RDMSSR07 were inoculated in *M. spectabilis* treated with Zn plus Cd 500 and 15 mg L^{-1} respectively. In this experiment under tissue culture system, the endophytic bacterial inoculation did not significantly affect the growth and Zn/Cd accumulation in plant.

5.3 Suggestions

5.3.1 According to the obtained results from the effects of metal tolerance and accumulation in *M. spectabilis* in the *in vitro* system, this plant could be extremely tolerate to high levels of Zn and Cd. Therefore, the mechanisms for metal detoxification in pot experiment should be studied further.

5.3.2 In this case, the indigenous endophytic bacteria might be related to the mechanisms of plant tolerance and metals detoxification. Therefore, the relationship

between indigenous endophytic bacteria and plant should be investigated for more clearly information.

5.3.3 The endophytic bacterial population colonizing in plant tissues was carried out by the cell counting technique. However, the distribution and location of these bacteria could not be detected. Therefore, the fluorescent labeling techniques should be studied to understand the plant-endophytic bacteria association.

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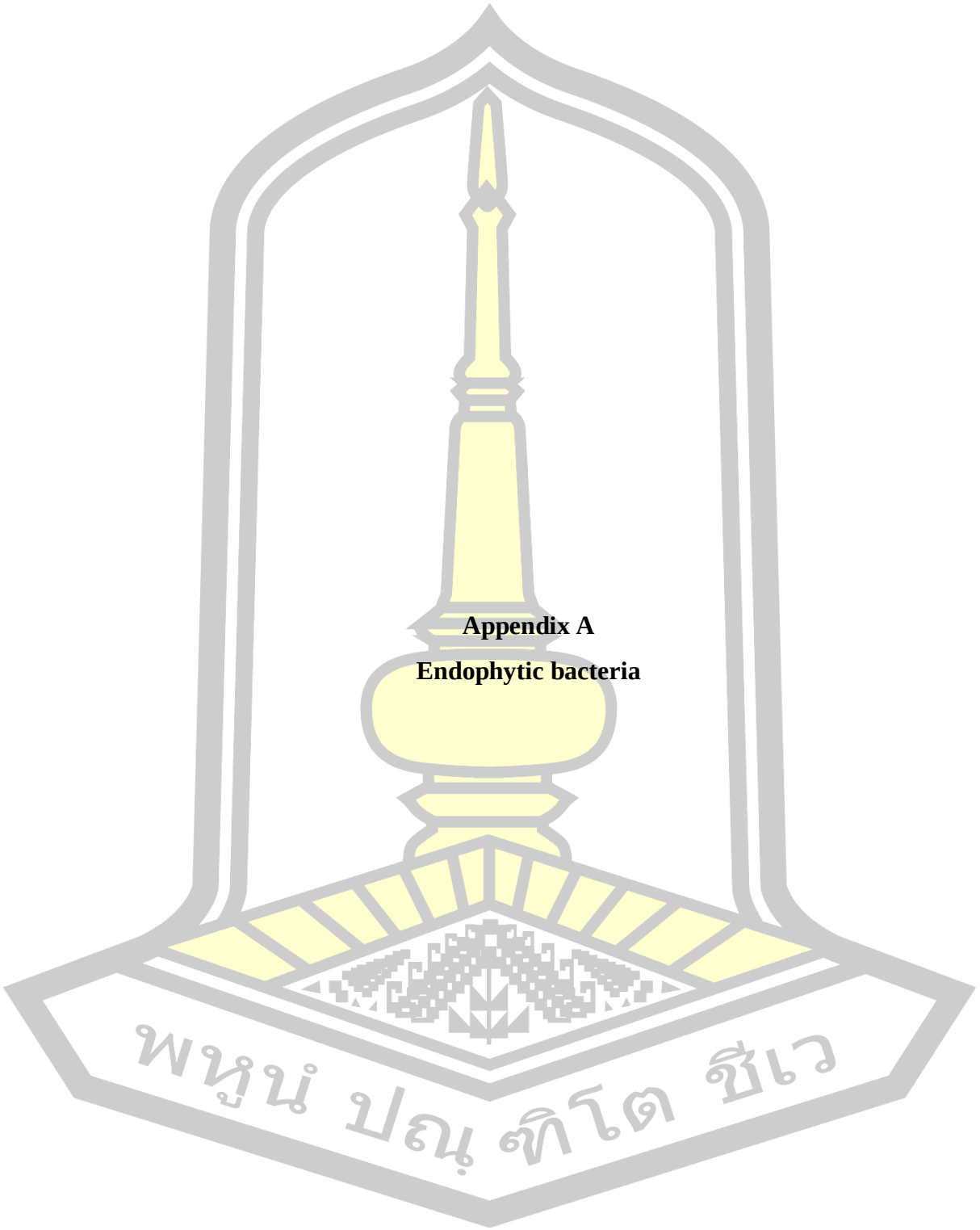
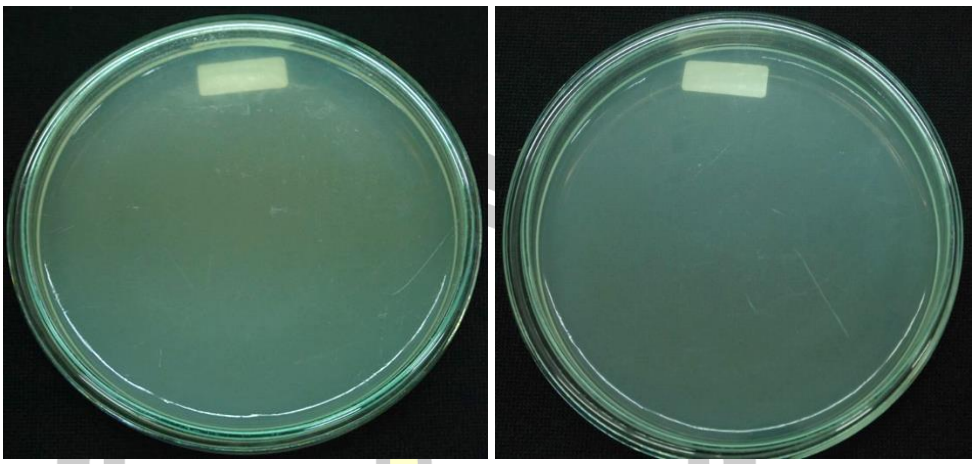
APPENDIXES

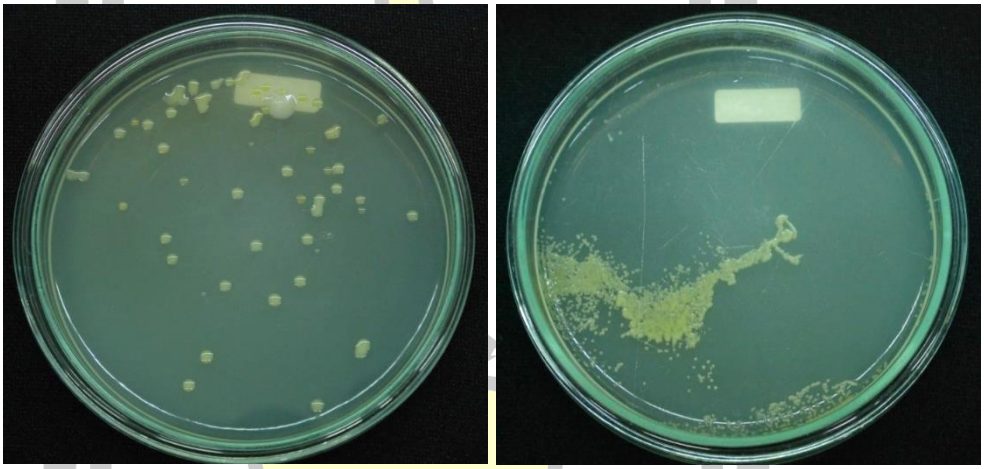


Appendix A

Endophytic bacteria

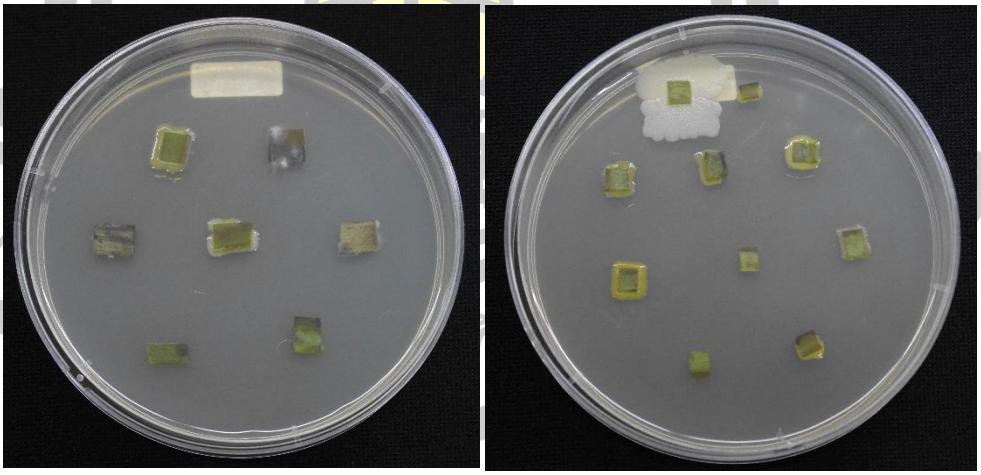


(1) 1.2% w/w NaOCl for 10 minutes (2) 0.9% w/w NaOCl for 10 minutes



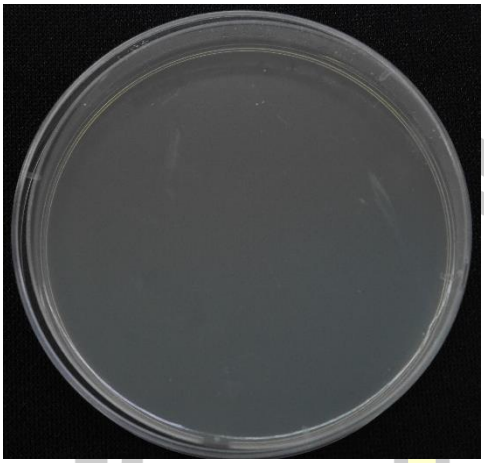
(3) 0.6% w/w NaOCl for 10 minutes (4) 0.3% w/w NaOCl for 10 minutes

Appendix A1 The results of surface sterilization of the leaves by spreading 0.1 L of the final rinse water onto TSA media.

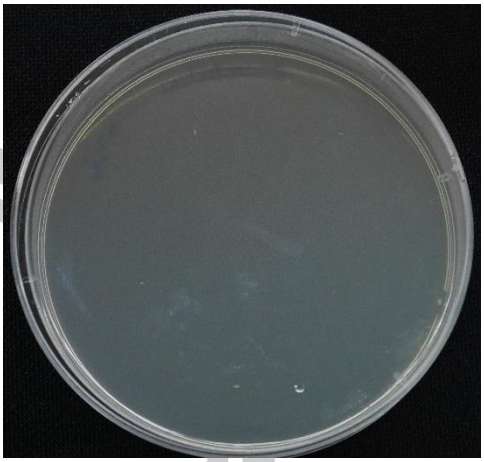


(1) 1.2% w/w NaOCl for 10 minutes (2) 0.9% w/w NaOCl for 10 minutes

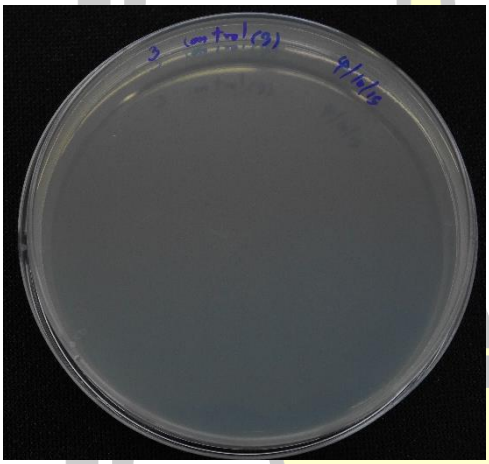
Appendix A2 Growth of endophytic bacteria from cut pieces of leaves of *M. spectabilis* on TSA media.



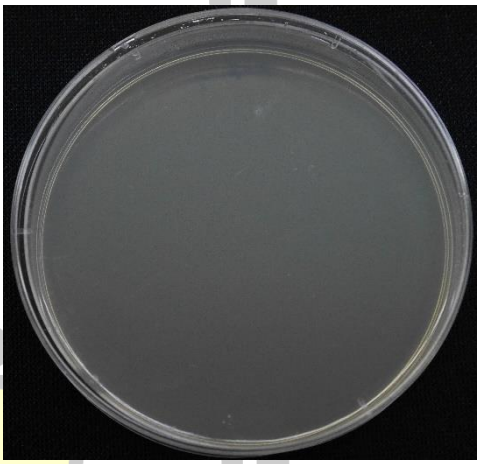
(1) 1.2% w/w NaOCl for 10 minutes



(2) 0.9% w/w NaOCl for 10 minutes

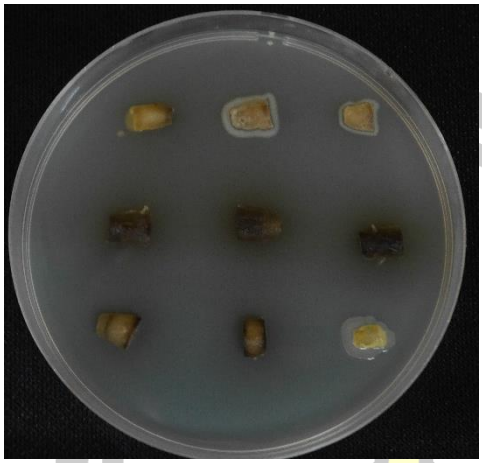


(3) 0.6% w/w NaOCl for 10 minutes

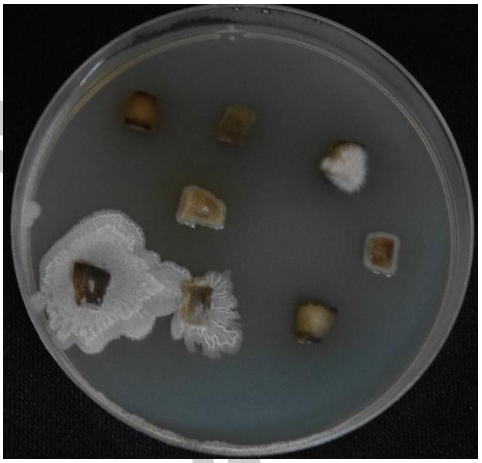


(4) 0.9% w/w NaOCl for 10 minutes then 0.3% w/w NaOCl for 10 minutes

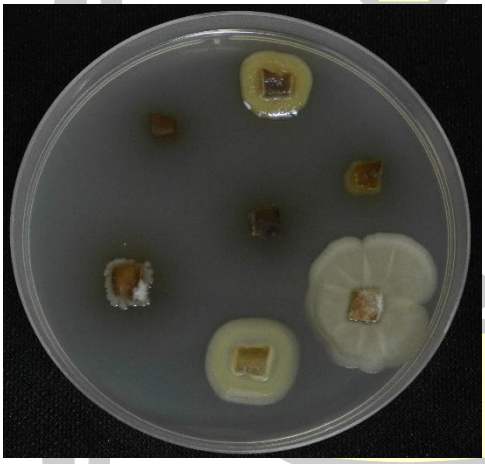
Appendix A3 The results of surface sterilization of the storage roots by spreading 0.1 mL of the final rinse water onto TSA media.



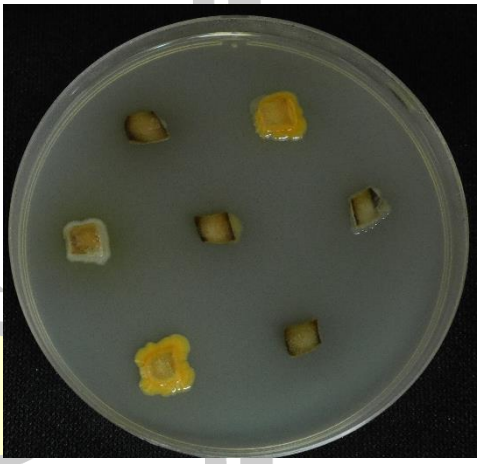
(1) 1.2% w/w NaOCl for 10 minutes



(2) 0.9% w/w NaOCl for 10 minutes

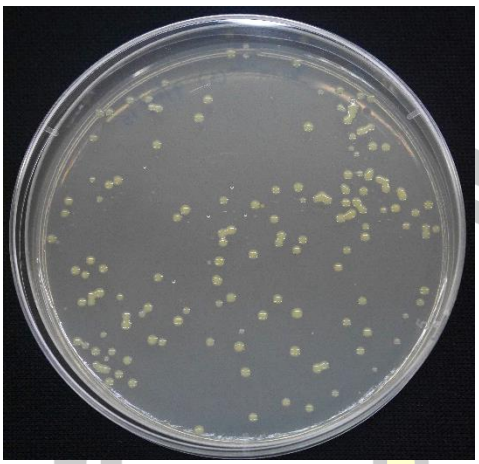


(3) 0.6% w/w NaOCl for 10 minutes

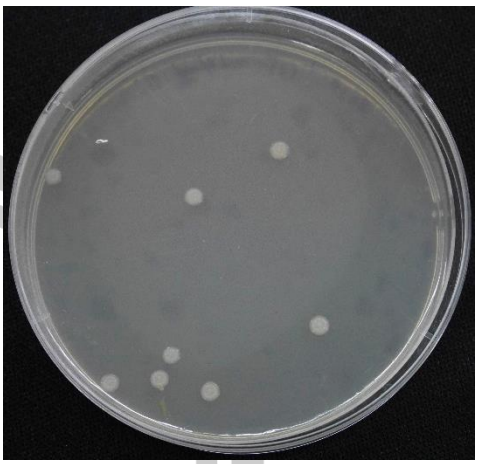


(4) 0.9% w/w NaOCl for 10 minutes then 0.3% w/w NaOCl for 10 minutes

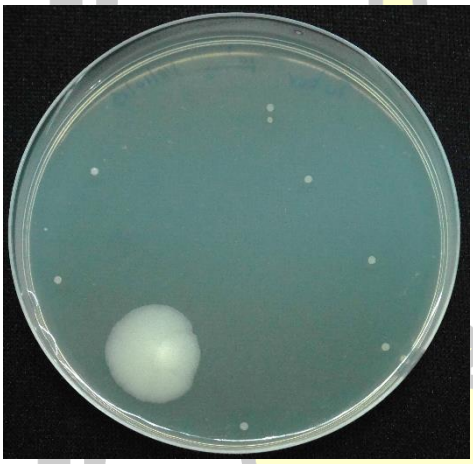
Appendix A4 Growth of endophytic bacteria from cut pieces of storage roots of *M. spectabilis* on TSA media.



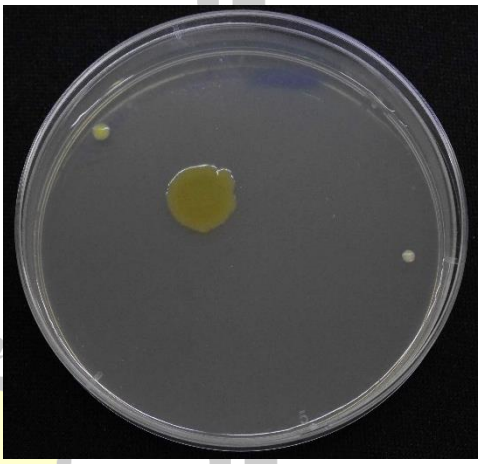
Leaves



Peduncles



Storage roots



Tubers

Appendix A5 Growth of endophytic bacteria from indirect method of *M. spectabilis* on TSA media (Serial dilution 10^{-1}).

Appendix A6 Morphological characteristics and Gram staining of endophytic bacteria

Plant portion	Isolate	Colony colour	Morphological characteristics					Gram staining
			form	margin	elevation	surface	Optical properties	
Leaves	RDMSL01	Yellow to orange	circular	entrie	convex	glistening	opaque	-
	RDMSL02	White	circular	entrie	convex	mucoid	opaque	-
	RDMSL03	Yellow-clear	circular	entrie	convex	glistening	translucent	-
	RDMSL04	Yellow	circular	entrie	convex	glistening	opaque	-
	RDMSL05	Clear	circular	entrie	flat	glistening	translucent	-
	RIDMSL01	Yellow	circular	entire	convex	glistening	translucent	-
	RIDMSL02	Yellow to orange	circular	entire	umbonate	glistening	opaque	-
	RIDMSL03	White to grayish	circular	entire	convex	glistening	translucent	-
Peduncle	RDMSP01	white	circular	entire	convex	rough	opaque	-
	RDMSP02	white	circular	entire	raised	glistening	opaque	-
	RDMSP03	Yellow	circular	entire	convex	mucoid	opaque	-
	RDMSP04	Yellow	circular	entire	convex	mucoid	translucent	-
	RDMSP05	Yellow	circular	entire	convex	glistening	translucent	-
	RDMSP06	white	circular	entire	raised	glistening	opaque	-
	RDMSP07	Yellow	circular	entire	convex	glistening	translucent	-
	RDMSP08	Dark yellow	circular	entire	convex	glistening	opaque	-
	RDMSP09	Yellow	circular	entire	convex	mucoid	opaque	-
	RDMSP10	Cream	circular	entire	convex	mucoid	opaque	-
	RDMSP11	Yellow	circular	entire	convex	glistening	translucent	-
	RDMSP12	Cream	circular	entire	convex	mucoid	opaque	-
	RIDMSP01	white	circular	entire	umbonate	wrinkled/rough	translucent	-
	RIDMSP02	Yellow to orange	circular	entire	convex	glistening	opaque	-
	RIDMSP03	Cream	circular	entire	convex	glistening	opaque	-
	RIDMSP04	white	circular	entire	umbonate	wrinkled/rough	translucent	-

Appendix A6 (Cont')

Plant portion	Isolate	Colony colour	Morphological characteristics					Gram staining
			form	margin	elevation	surface	Optical properties	
Stem	RIDMSS01	Yellow	circular	entire	convex	glistening	opaque	-
	RIDMSS02	White to yellow	circular	entire	flat	glistening	opaque	-
	RIDMSS03	Cream-Yellow	circular	entire	convex	glistening	opaque	-
	RIDMSS04	White	circular	entire	convex	glistening	opaque	-
	RIDMSS05	Cream	circular	undulate	flat	smooth	opaque	-
	RIDMSS06	Cream	irregular	undulate	flat	glistening	opaque	+
	RIDMSS07	clear	irregular	curled	flat	smooth	translucent	-
	RIDMSS08	White	punctiform	entire	convex	glistening	translucent	-
	RIDMSS09	White to grayish	punctiform	entire	convex	glistening	translucent	-
Storage root	RDMSSR01	White to grayish	circular	entire	convex	glistening	opaque	-
	RDMSSR02	Yellow	circular	entire	convex	glistening	opaque	-
	RDMSSR03	White to grayish	circular	entire	convex	glistening	translucent	-
	RDMSSR04	Cream	circular	entire	convex	mucoid	opaque	-
	RDMSSR05	White	circular	entire	convex	glistening	opaque	-
	RDMSSR06	Yellow-clear	punctiform	entire	raised	glistening	translucent	-
	RDMSSR07	Yellow	circular	entire	convex	glistening	opaque	-
	RDMSSR08	White	circular	entire	raised	glistening	opaque	-
	RDMSSR09	White	circular	entire	convex	glistening	translucent	-
	RDMSSR10	Yellow-clear	punctiform	entire	raised	glistening	translucent	-
	RDMSSR11	White	punctiform	entire	raised	glistening	translucent	-
	RDMSSR12	White	irregular	curled	flat	dry	opaque	+
	RDMSSR13	White	irregular	curled	umbonate	wrinkled	opaque	+
	RIDMSSR01	White	circular	entire	umbonate	wrinkled/rough	translucent	-
	RIDMSSR02	White	circular	entire	convex	glistening	opaque	+

Appendix A6 (Cont')

Plant portion	Isolate	Colony colour	Morphological characteristics					Gram staining
			form	margin	elevation	surface	Optical properties	
Storage root	RIDMSSR03	White and slimy	circular	undulate	flat	smooth	opaque	+
	RIDMSSR04	White	circular	entire	umbonate	wrinkled/rough	translucent	-
	RIDMSSR05	White	circular	entire	convex	wrinkled/rough	translucent	-
	RIDMSSR06	White	circular	entire	convex	glistening	translucent	-

Appendix A7 Metal resistance of endophytic bacteria.

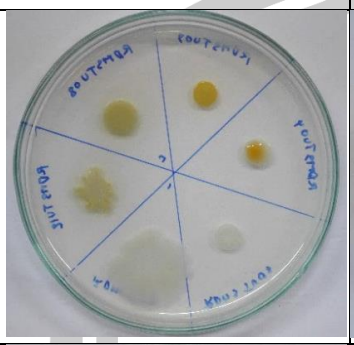
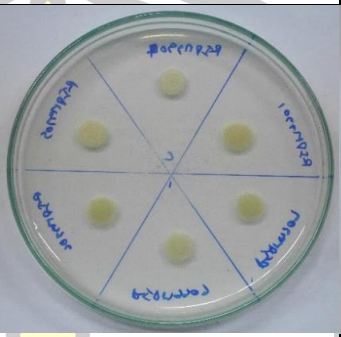
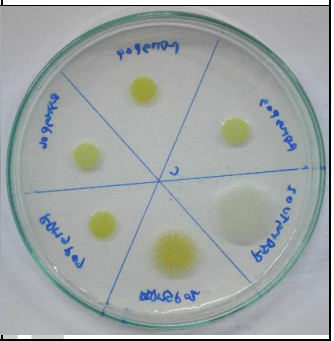
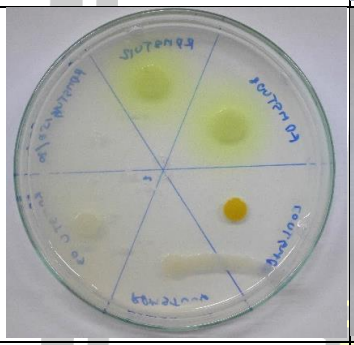
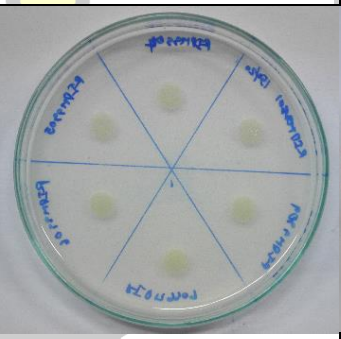
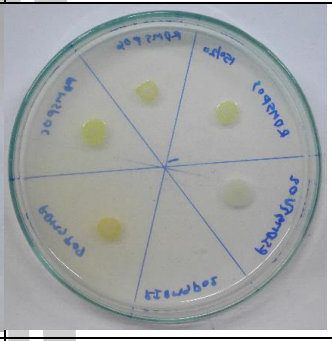
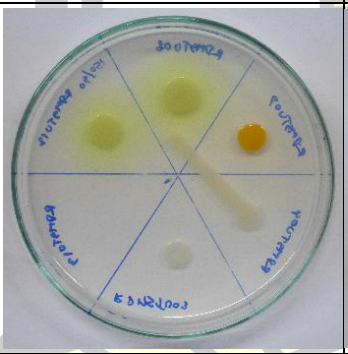
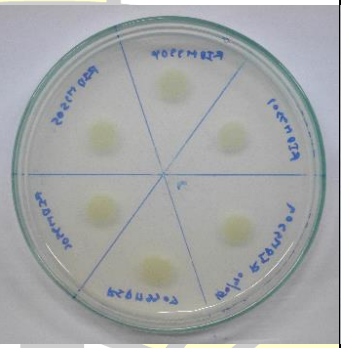
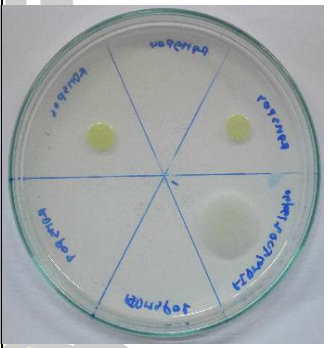
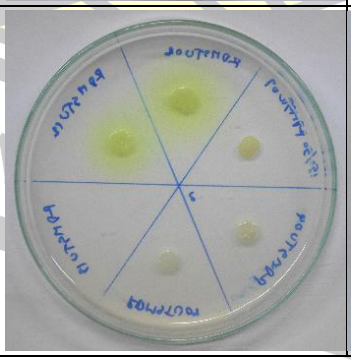
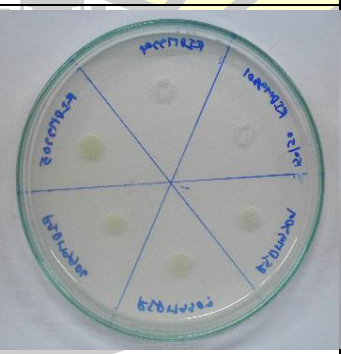
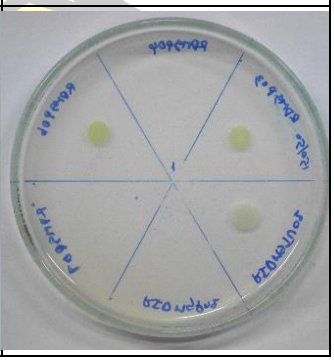
Isolate	Control	Concentration of Zn (mg L^{-1}) in media			Concentration of Cd (mg L^{-1}) in media				
		100	250	500	5	10	20	30	50
RDMSP08	+++	++	++	+	+++	++	++	+	+
RDMSP09	+++	+++	+++	+	+++	+++	++	++	++
RDMSP10	+++	+++	+++	+	+++	+++	++	++	++
RDMSP11	+++	+++	+++	++	+++	++	-	-	-
RDMSP12	+++	+++	+++	++	+++	+++	+++	++	++
RIDMSP01	+++	+++	+++	++	+++	++	+	+	-
RIDMSP02	+++	+++	+++	+++	+++	+++	+++	++	-
RIDMSP03	+++	-	-	-	+	-	-	-	-
RIDMSP04	++	-	-	-	+	-	-	-	-
RIDMSS01	+++	+++	+++	+++	+++	+++	+++	++	-
RIDMSS02	+++	+	+	+	++	+	+	-	-
RIDMSS03	+++	+	+	++	++	+	+	-	-
RIDMSS04	+++	+++	+++	+++	+++	+++	+++	+++	++
RIDMSS05	+++	+++	+++	+++	+++	+++	+++	+++	++
RIDMSS06	+++	+++	+++	+++	+++	+++	+++	+++	++
RIDMSS07	+++	++	+++	+++	++	++	+	+	-

*(–) indicates no growth; (+) low growth; (++) moderate growth; (+++) high growth

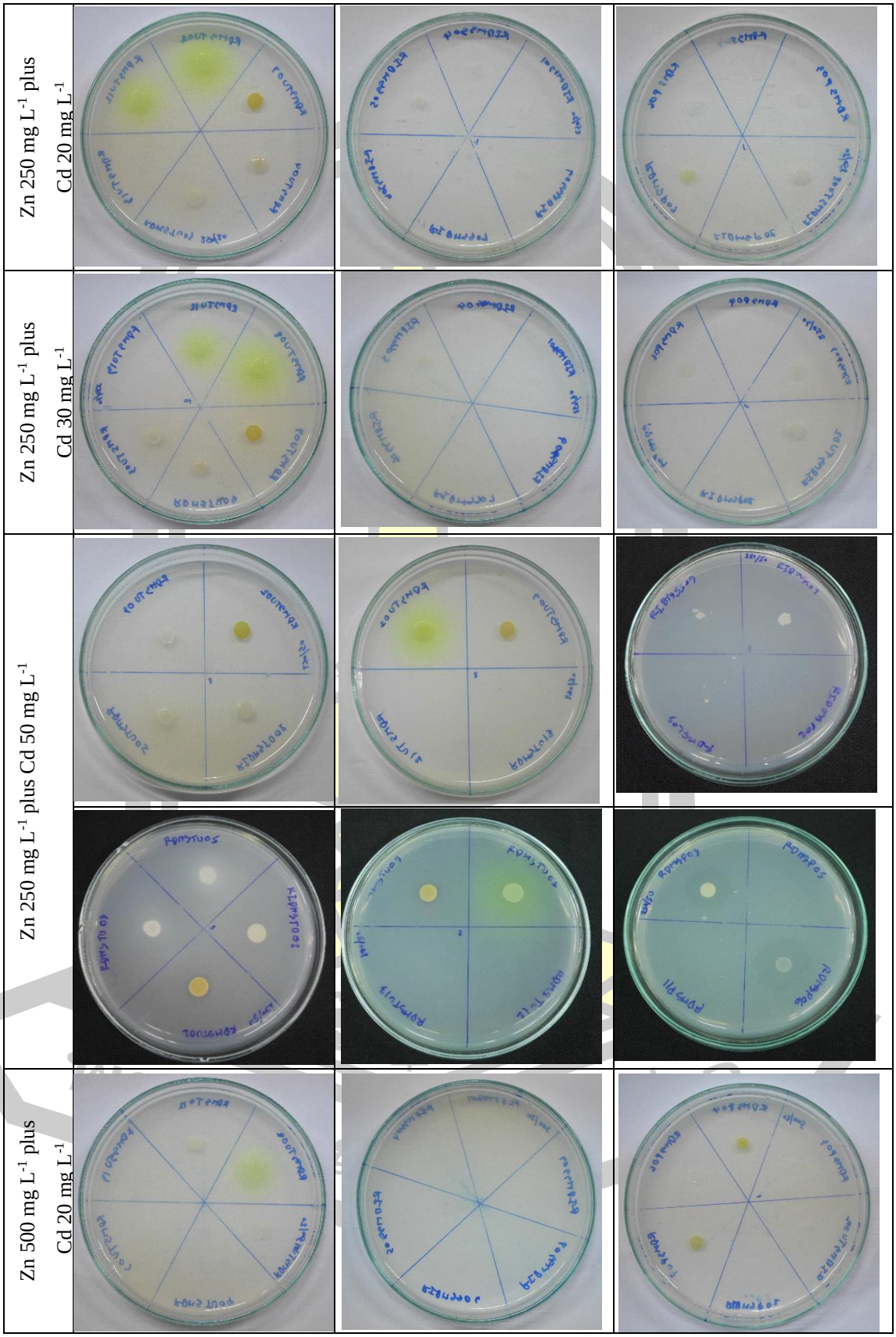
Appendix A7 (Cont')

Isolate	Control	Concentration of Zn (mg L^{-1}) in media			Concentration of Cd (mg L^{-1}) in media				
		100	250	500	5	10	20	30	50
RIDMSS08	+++	+	+	+	+++	+++	+++	+++	++
RIDMSS09	+++	+++	+++	+++	+++	+++	+++	+++	+
RDMSSR01	+++	-	+++	++	+++	+++	++	++	-
RDMSSR02	+++	+++	+++	+++	+++	+++	+++	+++	+++
RDMSSR03	++	+++	+++	+++	+++	+++	+++	+++	+++
RDMSSR04	+++	++	+	+	+++	+++	+++	+++	+++
RDMSSR05	++	+++	+++	++	+++	+++	+++	+++	+++
RDMSSR06	++	+++	++	+	++	++	++	+	-
RDMSSR07	+++	+++	+++	+++	+++	+++	+++	+++	+++

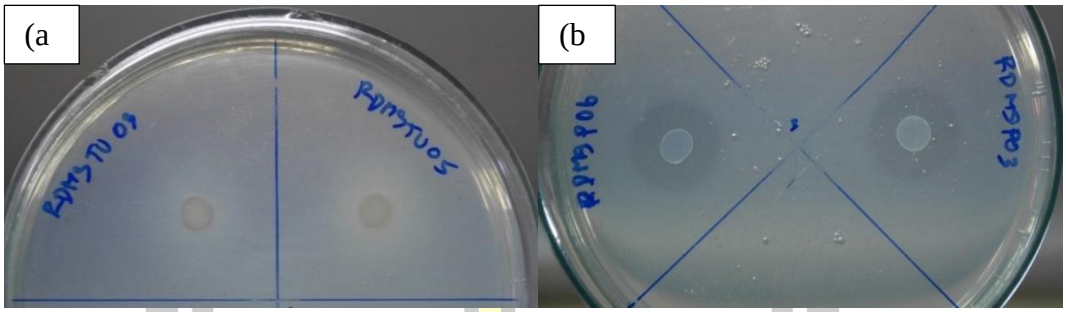
*(–) indicates no growth; (+) low growth; (++) moderate growth; (+++) high growth

Isolates	RDMSSR02, RDMSSR03, RDMSSR04, RDMSSR05, RDMSSR07, RDMSSR08, RDMSSR12, RDMSSR13	RISMSS01, RIDMSS04, RIDMSS05, RIDMSS06, RIDMSS07, RIDMSS09	RDMSP03, RDMSP04, RDMSP06, RDMSP07, RIDMSP02, RIDMSSR02
½ TSA medium			
Zn 150 mg L^{-1} plus Cd 20 mg L^{-1}			
Zn 150 mg L^{-1} plus Cd 30 mg L^{-1}			
Zn 150 mg L^{-1} plus Cd 50 mg L^{-1}			

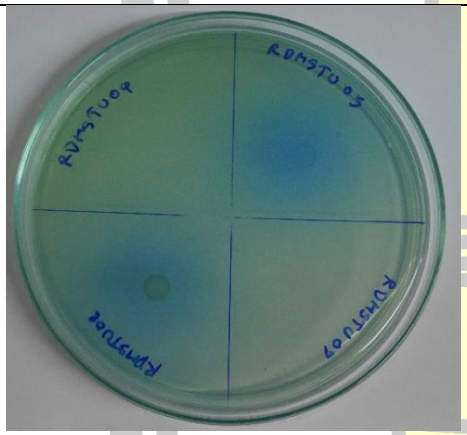
Appendix A8 Growth of endophytic bacteria in a half formula of TSA medium supplemented with Zn and Cd mg L^{-1}.



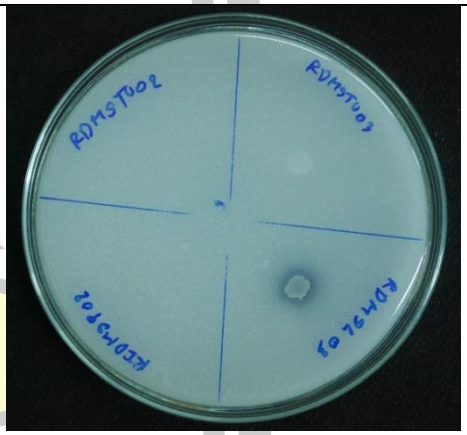
Appendix A8 (Cont')



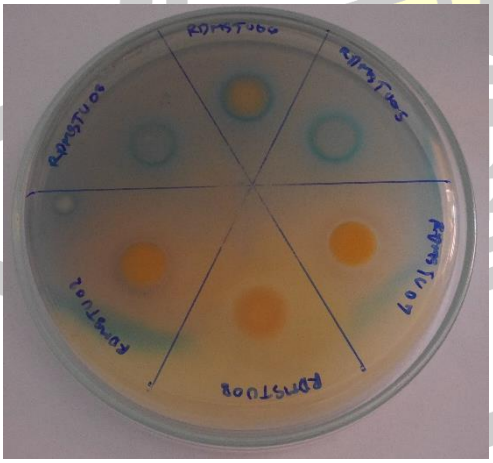
Appendix A9 Endophytic bacteria growing on TSA plates supplement with Zn 250 mg L^{-1} plus Cd 50 mg L^{-1} for 48 hours (a) RDMSSR03 and RDMSSR05 (b) RDMSP06 and RDMSP03



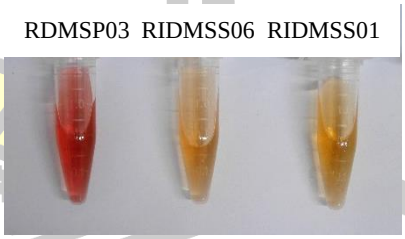
Nitrogen fixation



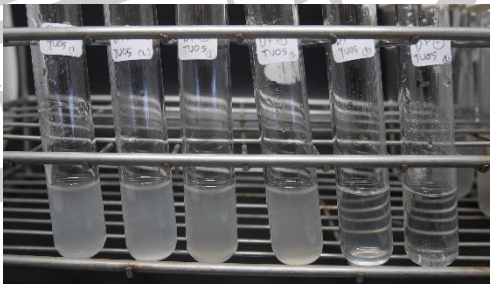
Phosphate solubilization



Siderophore production

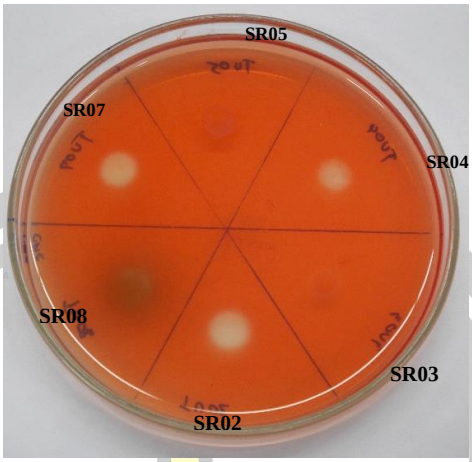


IAA production

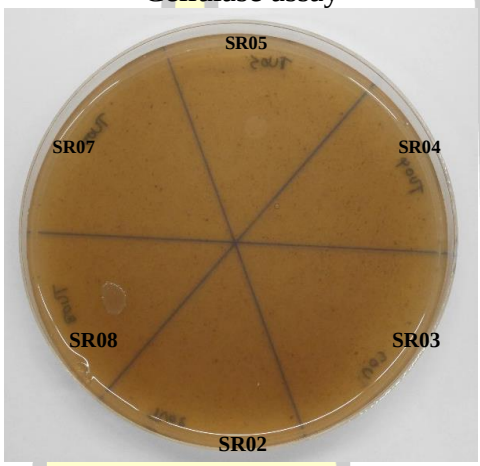


ACC deaminase activity of RDMSSR05

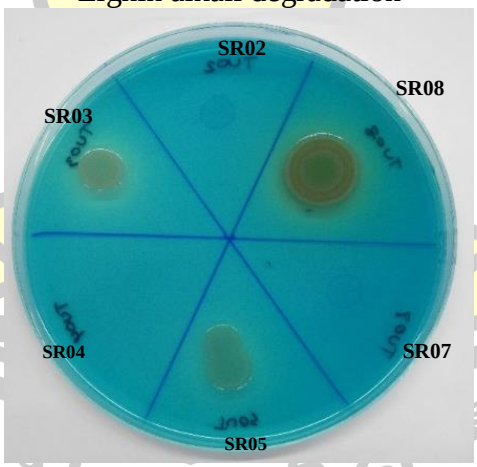
Appendix A10 Plant growth promoting properties of some endophytic bacteria.



Cellulase assay

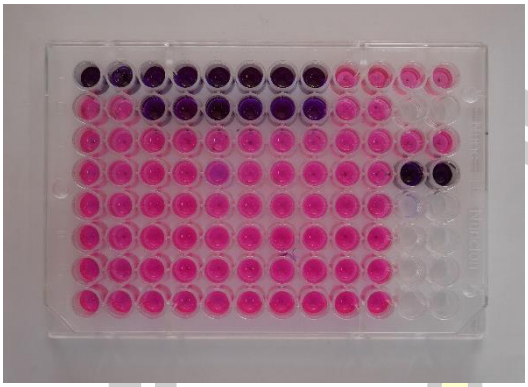


Lignin alkali degradation

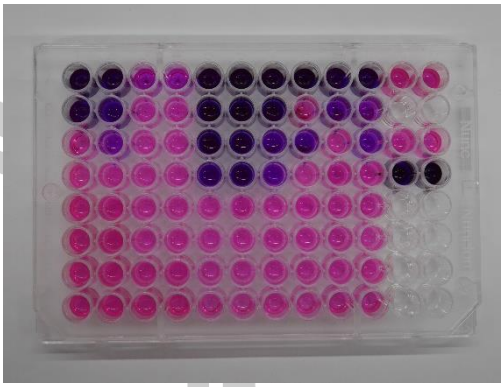


Ligninolytic enzymes

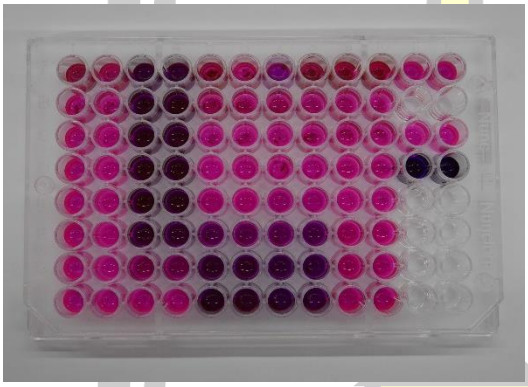
Appendix A11 Extracellula enzymes test of some endophytic bacteria.



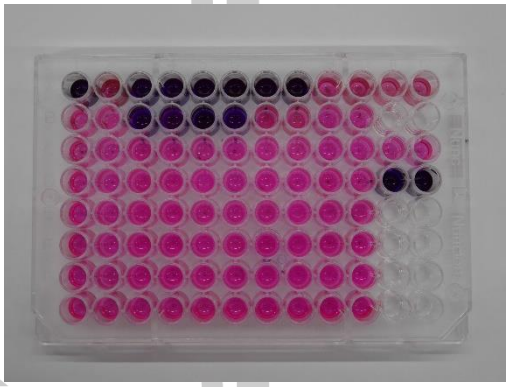
RDMSSR02



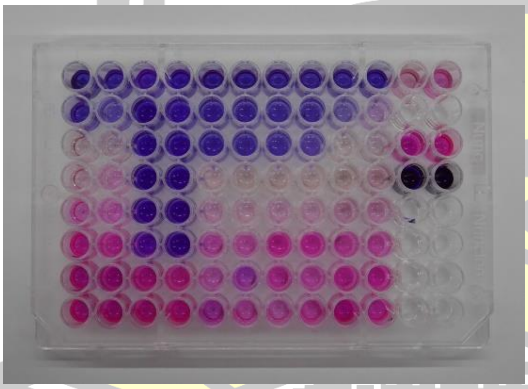
RDMSSR04



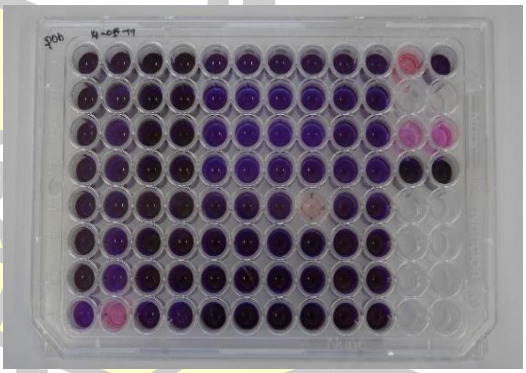
RDMSSR05



RDMSSR07

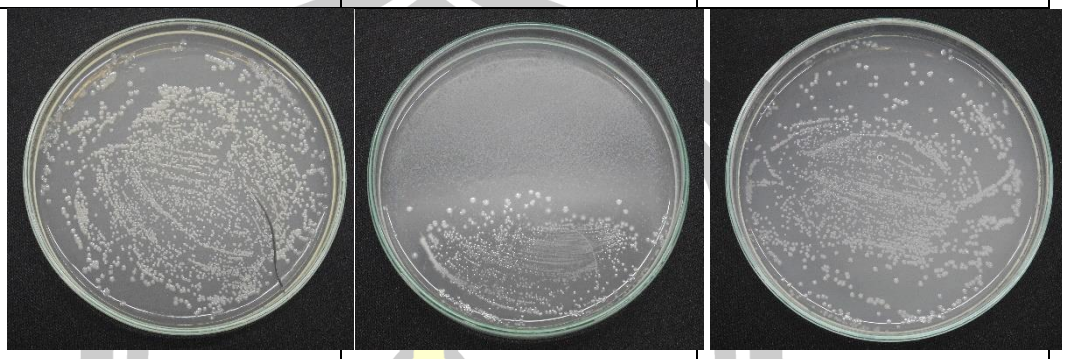
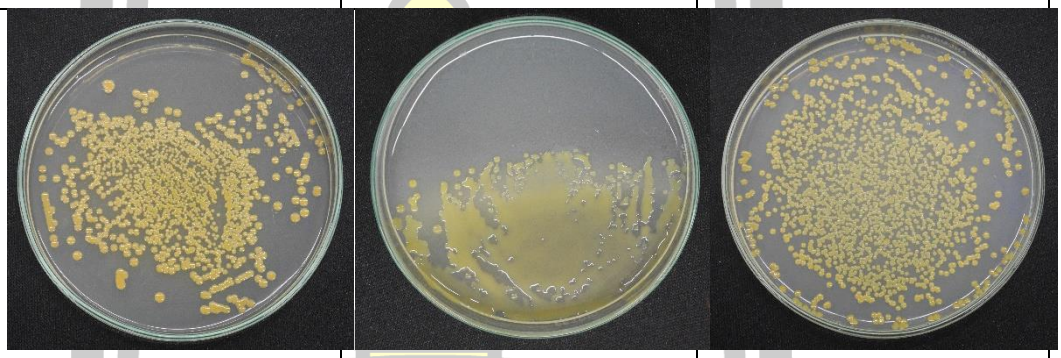
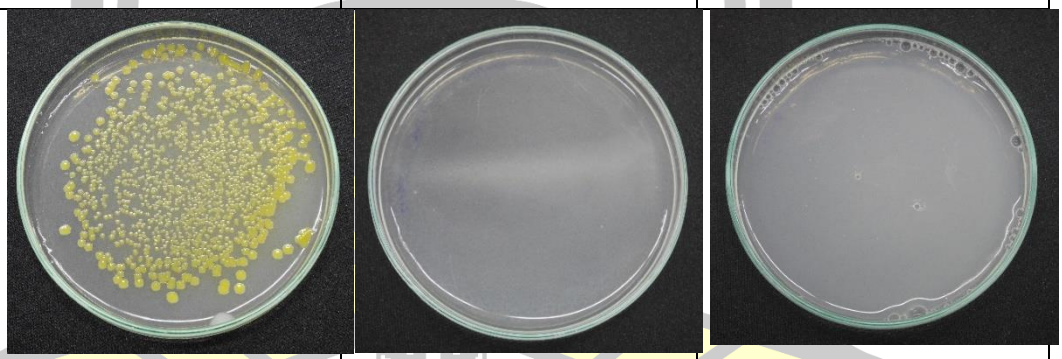


RDMSP03

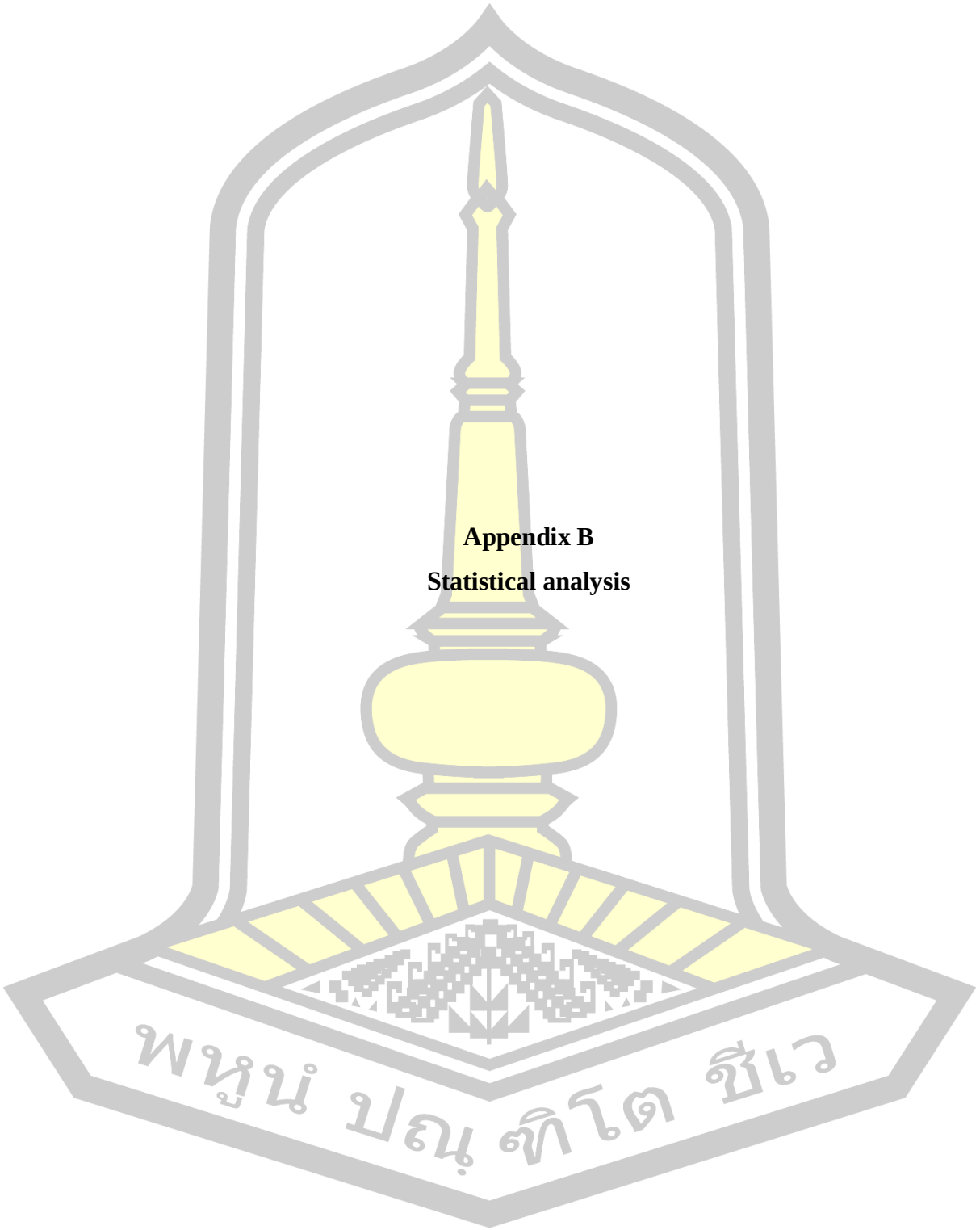


RDMSP06

Appendix A12 96-well plate for minimum inhibitory concentration (MIC) values of endophytic bacteria.

Strain	Media		
	TSA	TSA plus Zn 500+Cd 100 mg L^{-1*}	TSA plus Zn 150+Cd 30 mg L^{-1}
RDMSSR05			
RDMSSR07			
Indigenous endophytic bacteria			

Appendix A13 Morphology of endophytic bacteria isolated from tissues of *M. spectabilis* after inoculation.* Gradient plate



Appendix B
Statistical analysis

Appendix B1 One way ANOVA analysis for fresh weight of leaves treated with various concentration of Zn.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.171	5	.434	7.567	.002
Within Groups	.689	12	.057		
Total	2.860	17			

Duncan

Treatment	N	Subset for alpha = .01		
		1	2	3
Zn 1000	3	1.9163		
Zn 500	3	2.1413	2.1413	
Zn 250	3		2.6293	2.6293
Control	3		2.6513	2.6513
Zn 100	3		2.7280	2.7280
Zn 50	3			2.9037
Sig.		.272	.016	.218

Appendix B2 One way ANOVA analysis for dry weight of leaves treated with various concentration of Zn.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.001	5	.000	1.107	.406
Within Groups	.002	12	.000		
Total	.003	17			

Duncan

Treatment	N	Subset for alpha = .01
		1
Control	3	.1213
Zn 250	3	.1247
Zn 1000	3	.1250
Zn 500	3	.1257
Zn 100	3	.1383
Zn 50	3	.1400
Sig.		.138

Appendix B3 One way ANOVA analysis for fresh weight of roots treated with various concentration of Zn.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.504	5	.101	.650	0.667
Within Groups	1.861	12	.155		
Total	2.364	17			

Duncan

Treatment	N	Subset for alpha = .01
		1
Zn 250	3	1.1450
Zn 1000	3	1.2970
Zn 500	3	1.3460
Zn 100	3	1.3493
Zn 50	3	1.4180
Control	3	1.7000
Sig.		.144

Appendix B4 One way ANOVA analysis for dry weight of roots treated with various concentration of Zn.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.004	5	.001	1.010	0.453
Within Groups	0.009	12	.001		
Total	0.012	17			

Duncan

Treatment	N	Subset for alpha = .01
		1
Zn 250	3	0.0537
Zn 100	3	0.0730
Zn 1000	3	0.0747
Zn 500	3	0.0767
Zn 50	3	0.0800
Control	3	0.1027
Sig.		0.068

Appendix B5 One way ANOVA analysis for fresh weight of leaves treated with various concentration of Cd.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.039	5	.408	7.299	0.002
Within Groups	0.670	12	.056		
Total	2.709	17			

Duncan

Treatment	N	Subset for alpha = .01	
		1	2
Cd 50	3	2.1273	
Cd 25	3	2.3423	
Cd 15	3	2.4883	
Control	3	2.6513	2.6513
Cd 5	3	2.7063	2.7063
Cd 10	3		3.2037
Sig.		0.068	

Appendix B6 One way ANOVA analysis for dry weight of leaves treated with various concentration of Cd.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.001	5	.000	2.298	0.110
Within Groups	0.002	12	.000		
Total	0.003	17			

Duncan

Treatment	N	Subset for alpha = .01
		1
Cd 25	3	0.1167
Cd 15	3	0.1180
Cd 50	3	0.1207
Cd 5	3	0.1210
Control	3	0.1213
Cd 10	3	0.1433
Sig.		0.023

Appendix B7 One way ANOVA analysis for fresh weight of roots treated with various concentration of Cd.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.574	5	.715	6.979	0.003
Within Groups	1.229	12	.102		
Total	4.803	17			

Duncan

Treatment	N	Subset for alpha = .01		
		1	2	3
Cd 50	3	0.8103		
Cd 15	3	0.9113	0.9113	
Cd 25	3	0.9320	0.9320	
Cd 10	3	1.0510	1.0510	
Control	3		1.7000	1.7000
Cd 5	3			1.9863
Sig.		0.410	0.016	0.295

Appendix B8 One way ANOVA analysis for dry weight of roots treated with various concentration of Cd.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.004	5	.001	1.010	0.453
Within Groups	0.009	12	.001		
Total	0.012	17			

Duncan

Treatment	N	Subset for alpha = .01
		1
Cd 50	3	0.0453
Cd 10	3	0.0470
Cd 15	3	0.0487
Cd 25	3	0.0490
Cd 5	3	0.0910
Control	3	0.1027
Sig.		0.020

Appendix B9 One way ANOVA analysis for chlorophyll a in the leaves treated with various concentration of Zn.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7477.441	5	1495.488	20.482	0.000
Within Groups	1314.236	18	73.013		
Total	8791.678	23			

Duncan

Treatment	N	Subset for alpha = .05		
		1	2	3
Zn 1000	4	19.3598		
Zn 500	4	23.0820		
Zn 250	4	30.7955		
Zn 100	4		48.8395	
Zn 50	4		58.8695	58.8695
Control	4			65.3950
Sig.		.089	.114	.294

Appendix B10 One way ANOVA analysis for chlorophyll b in the leaves treated with various concentration of Zn.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	673.226	5	134.645	11.781	.000
Within Groups	205.722	18	11.429		
Total	878.949	23			

Duncan

Treatment	N	Subset for alpha = .05			
		1	2	3	4
Zn 1000	4	5.6178			
Zn 500	4	7.7505			
Zn 250	4	9.3685	9.3685		
Zn 100	4		14.2765	14.2765	
Zn 50	4			17.9223	17.9223
Control	4				19.9848
Sig.		.154	.055	.145	.400

Appendix B11 One way ANOVA analysis for chlorophyll a in the leaves treated with various concentration of Cd.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	10135.315	5	2027.063	54.954	.000
Within Groups	663.957	18	36.887		
Total	10799.272	23			

Duncan

Treatment	N	Subset for alpha = .05				
		1	2	3	4	5
Cd 50	4	5.7115				
Cd 25	4		17.1338			
Cd 15	4		22.5230	22.5230		
Cd 10	4			30.6728	30.6728	
Cd 5	4				37.8120	
Control	4					70.7993
Sig.		1.000	.226	.074	.114	1.000

Appendix B12 One way ANOVA analysis for chlorophyll b in the leaves treated with various concentration of Cd.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	740.219	5	148.044	24.729	.000
Within Groups	107.760	18	5.987		
Total	847.979	23			

Duncan

Treatment	N	Subset for alpha = .05				
		1	2	3	4	5
Cd 50	4	2.3493				
Cd 25	4		6.1580			
Cd 15	4		7.8630	7.8630		
Cd 10	4			10.3133	10.3133	
Cd 5	4				12.7313	
Control	4					19.9848
Sig.		1.000	.337	.174	.179	1.000

Appendix B13 One way ANOVA analysis for cell death in the roots treated with various concentration of Zn.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	219.602	5	43.920	6.779	.001
Within Groups	116.619	18	6.479		
Total	336.222	23			

Duncan

Treatment	N	Subset for alpha = .05		
		1	2	3
Control	4	14.8975		
Zn 500	4		19.2875	
Zn 1000	4		20.0775	
Zn 100	4		20.5150	
Zn 250	4		22.8350	22.8350
Zn 50	4			24.5650
Sig.		1.000	.086	.349

Appendix B14 One way ANOVA analysis for cell death in the roots treated with various concentration of Cd.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	84.064	5	16.813	3.140	.033
Within Groups	96.386	18	5.355		
Total	180.450	23			

Duncan

Treatment	N	Subset for alpha = .05	
		1	2
Control	4	14.8975	
Cd 10	4	15.0075	
Cd 15	4	16.1600	
Cd 50	4	17.4875	17.4875
Cd 5	4	17.5500	17.5500
Cd 25	4		20.3800
Sig.		.161	.110

Appendix B15 One way ANOVA analysis for Zn accumulation treated with various concentration of Zn.

Descriptives

Tretments		N	Mean	Std. Deviation
Zn accumulation in shoot	Control	3	.0910	.00721
	Zn 50	3	1.4237	.23427
	Zn 100	3	1.3057	.21948
	Zn 250	3	1.6867	.16265
	Zn 500	3	4.1443	.18226
	Zn 1000	3	5.8313	1.01591
	Total	18	2.4138	2.04090
Zn accumulation in root	Control	3	.1180	.03897
	Zn 50	3	6.2037	1.49655
	Zn 100	3	12.3840	2.91712
	Zn 250	3	15.5050	1.89506
	Zn 500	3	16.6270	3.12573
	Zn 1000	3	16.6000	2.53662
	Total	18	11.2396	6.59781

Descriptives

Zn accumulation in media	Control	3	.0317	.01097
	Zn 50	3	1.2670	.29691
	Zn 100	3	4.3230	.67673
	Zn 250	3	15.2227	1.30007
	Zn 500	3	39.1960	4.68539
	Zn 1000	3	64.9757	5.18169
	Total	18	20.8360	24.63635

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Zn accumulation in shoot	Between Groups	68.420	5	13.684	68.715	.000
	Within Groups	2.390	12	.199		
	Total	70.810	17			
Zn accumulation in root	Between Groups	678.936	5	135.787	26.671	.000
	Within Groups	61.093	12	5.091		
	Total	740.029	17			
Zn accumulation in media	Between Groups	10216.064	5	2043.213	240.193	.000
	Within Groups	102.078	12	8.507		
	Total	10318.142	17			

Duncan

Zn accumulation in shoot

Treatments	N	Subset for alpha = .01			
		1	2	3	4
Control	3	.0910			
Zn 100	3		1.3057		
Zn 50	3		1.4237		
Zn 250	3		1.6867		
Zn 500	3			4.1443	
Zn 1000	3				5.8313
Sig.		1.000	.340	1.000	1.000

Zn accumulation in root

Treatments	N	Subset for alpha = .01		
		1	2	3
Control	3	.1180		
Zn 50	3		6.2037	
Zn 100	3			12.3840
Zn 250	3			15.5050
Zn 1000	3			16.6000
Zn 500	3			16.6270
Sig.		1.000	1.000	.053

Zn accumulation in media

Treatments	N	Subset for alpha = .01			
		1	2	3	4
Control	3	.0317			
Zn 50	3	1.2670			
Zn 100	3	4.3230			
Zn 250	3		15.2227		
Zn 500	3			39.1960	
Zn 1000	3				64.9757
Sig.		.111	1.000	1.000	1.000

Appendix B16 One way ANOVA analysis for Cd accumulation treated with various concentration of Cd.

Descriptives

		N	Mean	Std. Deviation
Cd accumulation in shoot	Control	3	.0010	.00000
	Cd 5	3	.0663	.01159
	Cd 10	3	.1337	.03758
	Cd 15	3	.1170	.02330
	Cd 25	3	.1787	.06728
	Cd 50	3	.7423	.10516
	Total	18	.2065	.25720
Cd accumulation in root	Control	3	.0017	.00115
	Cd 5	3	.4607	.09022
	Cd 10	3	1.2363	.36260
	Cd 15	3	1.3297	.43160
	Cd 25	3	1.7590	.35598
	Cd 50	3	3.2307	.70144
	Total	18	1.3363	1.10787
Cd accumulation in media	Control	3	.0023	.00153
	Cd 5	3	.0517	.00643
	Cd 10	3	.2687	.03459
	Cd 15	3	.4910	.05311
	Cd 25	3	.7890	.09824
	Cd 50	3	2.0773	.34269
	Total	18	.6133	.73774

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Cd accumulation in shoot	Between Groups	1.089	5	.218	73.955	.000
	Within Groups	.035	12	.003		
	Total	1.125	17			
Cd accumulation in root	Between Groups	18.976	5	3.795	24.106	.000
	Within Groups	1.889	12	.157		
	Total	20.865	17			
Cd accumulation in media	Between Groups	8.990	5	1.798	82.260	.000
	Within Groups	.262	12	.022		
	Total	9.252	17			

Duncan

Cd accumulation in shoot

Treatments	N	Subset for alpha = .01		
		1	2	3
Control	3	.0010		
Cd 5	3	.0663	.0663	
Cd 15	3	.1170	.1170	
Cd 10	3	.1337	.1337	
Cd 25	3		.1787	
Cd 50	3			.7423
Sig.		.016	.036	1.000

Cd accumulation in root

Treatments	N	Subset for alpha = .01			
		1	2	3	4
Control	3	.0017			
Cd 5	3	.4607	.4607		
Cd 10	3		1.2363	1.2363	
Cd 15	3		1.3297	1.3297	
Cd 25	3			1.7590	
Cd 50	3				3.2307
Sig.		.182	.025	.150	1.000

Cd accumulation in media

Treatments	N	Subset for alpha = .01			
		1	2	3	4
Control	3	.0023			
Cd 5	3	.0517			
Cd 10	3	.2687	.2687		
Cd 15	3		.4910	.4910	
Cd 25	3			.7890	
Cd 50	3				2.0773
Sig.		.057	.090	.030	1.000

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Research grants & awards
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2016 Research Grants from Mahasarakham University in the research topic "Isolation and characterization of endophytic bacteria from Murdannia spectabilis (Kurz) Faden from Zn/Cd contaminated area and a potential to promote plant growth under the metal stress"

Research output
Nakbanpote, W., Prasad, M. N.V., Mongkhonsin, B., Panitlertumpai, N., Munjit, R. and Rattanapolsan, L. (2018) "Strategies for rehabilitation of mine waste/leachate in Thailand" in Bio-Geotechnologies for Mine Site Rehabilitation 1st Edition, Elsevier, ISBN: 978-0-12-812986-9 (Book Chapter)

Rattanapolsan, L. and Nakbanpote, W. Sangdee, A (2016) "Isolation and identification of endophytic bacteria from Murdannia spectabilis (Kurz) Faden from Zn/Cd contaminated area" the 11th Science and Technology Conference for Youths: Conference on Science and Technology for Youths, 10-11 June, Bangkok International Trade and Exhibition Centre (BITEC) Bangna, Bangkok, Thailand (Abstract)

Rattanapolsan L, Nakbanpote W, and Saensouk, P (2013) Metals accumulation and leaf surface anatomy of Murdannia spectabilis growing in Zn/Cd contaminated

Soil. EnvironmentAsia, 6: 71-82.

Rattanapolsan, L., Nakbanpote,W. and Saensouk, P. (2013) “Tissue culture of Murdannia spectabilis (Kurz) Faden and the effect of zinc and cadmium on leaf surface anatomy” the 8th Science and Technology Conference for Youths: Conference on Science and Technology for Youths, 21-23 March, Bangkok International Trade and Exhibition Centre (BITEC) Bangna, Bangkok, Thailand (Abstract)