THE POTENTIAL PLANTS FOR PHYTOREMEDIATION OF CADMIUM AND ZINC CONTAMINATED SOILS AND ENHANCEMENT OF PHYTOREMEDIATION BY PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR)

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ABSTRACT

Plant species grown on heavy metal contaminated soils around Padaeng Zn mine were collected and investigated for Cd and Zn accumulation to find the potential metal hyperaccumulator plants for phytoremediation. *Chromolaena odoratum*, *Gynura pseudochina*, *Justicia procumbens* and *Impatiens violaeflora* met the required hyperaccumulation criteria. They were thus considered as Cd hyperaccumulators while *J. procumbens* was also considered a Zn hyperaccumulator. *C. odoratum* from field collection accumulated 166 and 110 mg Cd kg^{-1} DW in shoots and roots, respectively. Moreover, the hydroponically grown plants could accumulate 266 and 1,670 mg Cd kg^{-1} DW in shoots and roots, respectively without showing any toxicity symptoms at Cd supply level of 2.5 mg l^{-1}. *C. odoratum* was the best candidate for feasible phytoremediation. It was able to accumulate not only high Cd concentration but also retained many favorable characteristics suitable for practical phytoremediation. Metal toxicities in *C. odoratum* exposed to high Cd concentration were seen as having brown colored roots, root fragmentation, and red spots in the veins and petioles of leaves. Tissue damage and organelle deformities were also observed. They possibly resulted from the interference of Cd with the homeostatic pathway for essential metals. In *C. odoratum*'s roots, Cd was mainly found in the cell wall, intercellular space and in cells close to the vascular system. It indicated that Cd transported apoplastically can be immobilized in the cell wall and intercellular space while Cd taken into plant cells will be bound to phytochelatin and transported into vacuole. In plant leaves, Cd was found in mesophyll cells lying along the way of water migration from vascular cylinder to the epidermis, indicating involvement of transpiration in metal transportation.

Normally heavy metal availability in soil is not the only rate limiting factor for phytoextraction, but also the retardation of plant growth due to metal toxicity. The combination between plants and plant growth promoting rhizobacteria (PGPR) is an alternative for improving phytoextraction efficiency since PGPR not only promote plant growth but also enhance metal uptake. In the present study *Chryseobacterium sp*. TKS21, *Comamonas testosteroni* TKS07, *Cupriavidus taiwanensis* TKS05, *Delftia sp*. TKS10, *Pseudomonas aeruginosa* TKS22, and *Serratia marcescens* TKS01 were isolated from the rhizosphere of *C. odoratum* grown on metal contaminated soils. All of them could colonize the roots of *Helianthus annuus*. Moreover, TKS21, TKS07, TKS05, and TKS10 not only promoted plant growth but also enhanced Cd accumulation in the plants grown on non-sterile metal contaminated soil. Plant growth promotion may have resulted from production of IAA synthesized by bacterial inoculants, while Cd accumulation was probably enhanced through metal solubilizing activity by the rhizobacteria. Enhancing metal accumulation in high yielding crop plants without diminishing their yield is fundamental to successful phytoextraction.

KEY WORDS: *CHROMOLAENA ODORATUM* / HYPERACCUMULATOR / Cd / Zn / PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR)

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การศึกษาพืชที่เป็นไปได้เพื่อใช้บำบัดดินที่ปนเปื้อนโลหะแคดเมียมและสังกะสี และการเพิ่มประสิทธิภาพของการบำบัดโดยใช้แบคทีเรียรอบรากพืชที่ส่งเสริมการเติบโตของพืช

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บทคัดย่อ

พืชที่เจริญบนดินที่ปนเปื้อนโลหะหนักรอบเหมืองผาแดงถูกเก็บรวบรวมและตรวจสอบการสะสมโลหะแคดเมียมและสังกะสีเพื่อหาพืชดูดซับโลหะหนักปริมาณสูงไปใช้ในการบำบัดดินปนเปื้อนโลหะ *Chromolaena odoratum*, *Gynura pseudochina*, *Justicia procumbens*, และ *Impatiens violaeflora* ผ่านเกณฑ์การเป็นพืชดูดซับโลหะหนักปริมาณสูง พืชเหล่านั้นถูกพิจารณาให้เป็นพืชดูดซับโลหะแคดเมียมปริมาณสูง ขณะที่ *J. procumbens* ก็เป็นพืชดูดซับโลหะสังกะสีปริมาณสูงด้วย *C. odoratum* ซึ่งรวบรวมมาจากพื้นที่สำรวจ สะสม 166 และ 110 มิลลิกรัมแคดเมียม ต่อกิโลกรัมมวลแห้งในส่วนลำต้น และราก ตามลำดับ พืชที่เพาะเลี้ยงด้วยสารละลายที่มีแคดเมียมผสมอยู่ในความเข้มข้น 2.5 มิลลิกรัมต่อลิตร สามารถสะสมโลหะในส่วนลำต้นและรากได้ 267 และ 1,670 มิลลิกรัมแคดเมียมต่อกิโลกรัมมวลแห้ง ตามลำดับ โดยไม่แสดงอาการความเป็นพิษ *C. odoratum* เป็นพืชที่เหมาะสมในการบำบัดดินที่ปนเปื้อนโลหะโดยใช้พืช นอกจากมีความสามารถสะสมแคดเมียมได้ในปริมาณสูงแล้วยังมีคุณลักษณะหลายอย่างที่เหมาะสมในการบำบัดการปนเปื้อนโลหะโดยใช้พืชในทางปฏิบัติ ความเป็นพิษซึ่งเกิดจากแคดเมียมที่พบใน *C. odoratum* คือ รากพืชเป็นสีน้ำตาล การหักเป็นท่อนของราก จุดแดงตามเส้นใบและก้านใบ การทำลายของเนื้อเยื่อและความผิดปกติของออร์แกเนลล์ การเปลี่ยนแปลงนี้อาจเป็นผลจากการรบกวนของแคดเมียมต่อสมดุลแร่ธาตุที่จำเป็นของพืช ในรากพืชแคดเมียมถูกพบมากในผนังเซลล์ ช่องว่างระหว่างเซลล์ และภายในเซลล์ที่อยู่ใกล้กับระบบท่อลำเลียง แคดเมียมซึ่งถูกลำเลียงโดยไม่ผ่านเซลล์จะถูกตรึงไว้ในผนังเซลล์ และช่องว่างระหว่างเซลล์ ขณะที่แคดเมียมที่ถูกนำเข้าเซลล์จะถูกจับกับไฟโทคีลาทินแล้วถูกลำเลียงไปเก็บไว้ในแวคิวโอล ในใบของพืชแคดเมียมถูกพบในเซลล์ใบซึ่งเรียงตัวอยู่ในแนวทางการลำเลียงน้ำจากระบบ ท่อลำเลียงไปสู่เซลล์ชั้นนอกสุดของใบ แสดงให้เห็นว่าการลำเลียงโลหะในใบพืชเกี่ยวพันกับการคายน้ำของพืช

ความสามารถถูกนำไปใช้ได้ของโลหะหนักในดินและการเจริญเติบโตของพืชที่ลดลงเนื่องจากความเป็นพิษของโลหะล้วนเป็นข้อจำกัดของ phytoextraction การรวมกันระหว่างพืชกับแบคทีเรียที่ส่งเสริมการเจริญของพืช (PGPR) เป็นหนึ่งทางเลือกที่จะนำไปสู่การเพิ่มศักยภาพของ phytoextraction ไม่เพียงแค่ PGPR สามารถส่งเสริมการเติบโตของพืชได้ แล้วยังช่วยการดูดซับโลหะหนักด้วย ในการศึกษาครั้งนี้ *Chryseobacterium sp.* TKS21, *Comamonas testosteroni* TKS07, *Cupriavidus taiwanensis* TKS05, *Delftia sp.* TKS10, *Pseudomonas aeruginasa* TKS22 และ *Serratia marcescens* TKS01 ถูกแยกมาจากบริเวณดินรอบรากของ *C. odoratum* ที่เจริญบนดินปนเปื้อนโลหะหนัก แบคทีเรียทั้งหมดสามารถอยู่ร่วมกับรากของ *Helianthus annuus* ได้ TKS21, TKS07, TKS05 และ TKS10 ส่งเสริมการเติบโตและการดูดซับโลหะหนักของพืชที่ปลูกในดินปนเปื้อนโดยไม่ผ่านกระบวนการปลอดเชื้อ แบคทีเรียอาจส่งเสริมการเติบโตของพืชโดยการผลิต IAA และส่งเสริมการสะสมโลหะหนักของพืชโดยการทำให้โลหะในดินละลายได้ การสะสมโลหะในพืชที่เพิ่มขึ้นโดยมวลพืชไม่ลดลงเป็นหลักพื้นฐานที่จะนำไปสู่ความสำเร็จของ phytoextraction

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LIST OF ABBREVIATIONS

A°	Angstrom
°C	degree Celsius
cm plant^{-1}	centimeter per plant
cm	centimeter
cmol kg^{-1}	centimole per kilogram
dS m^{-1}	decisiemen per meter
DW	dry weight
g	gram
g cm^{-3}	gram per cubic centimeter
g ha^{-1} yr^{-1}	gram per hectare per year
g l^{-1}	gram per liter
g plant^{-1}	gram per plant
h	hour
keV	kiloelectronvolt
kg	kilogram
kV	kilovolt
l	liter
M	Molar
mg	milligram
mg day^{-1}	milligram per day
mg kg^{-1}	milligram per kilogram
mg l^{-1}	milligram per liter
mg m^{-3}	milligram per cubic meter
min	minute
ml	milliliter
mm	millimeter
mM	millimolar

LIST OF ABBREVIATIONS (cont.)

µg mg^{-1}	microgram per milligram
µg g^{-1}	microgram per gram
µg kg^{-1}	microgram per kilogram
µg ml^{-1}	microgram per milliliter
µM	micromolar
µg day^{-1}	microgram per day
µg m^{-3}	microgram per cubic meter
nm	nanometer
nM	nanomolar
ppb	part per billion
ppm	part per million
psi	pound per square inch
rpm	round per minute
s	second
t ha^{-1}	ton per hectare
v/v	volume by volume
w/v	weight by volume

CHAPTER I
INTRODUCTION

Heavy metal pollution, the most seriously environmental problem, has accelerated rapidly since the onset of the industrial revolution (Garbisu & Alkorta, 2001). It has been attracting considerable public attention over the last decades. The entry of heavy metal contaminants into the environment results from either natural processes or human activity. Natural contamination originates from either excessive weathering of mineral from bedrocks or displacement of certain contaminants from groundwater or subsurface layers of the soil. Human activities that release heavy metals into agricultural and non agricultural lands are (1) disposal of industrial effluents, (2) sewage sludges, (3) deposition of air borne industrial wastes, (4) military operations, (5) mining, (6) land-fill operation, (7) industrial solid waste disposal, and 8) use of agricultural chemicals such as pesticides, herbicides and fertilizers (Saxena *et al*., 1999). The metal species commonly found in the soil as a result of the aforementioned human activities include copper (Cu), lead (Pb), zinc (Zn), nickel (Ni), cobalt (Co), mercury (Hg), and cadmium (Cd). Although some of these metals are required in small amounts by living organisms for their normal physiological activities (i.e., they provide essential cofactors for metalloproteins and enzyme), at high concentrations they can act in a deleterious manner by blocking essential functional groups, displacing other metal ions, or modifying the active conformation of biological molecules (Collins & Stotzky, 1989).

Soil contamination by Cd is recognized as an extremely significant pollution due to its deleterious impacts on animals, plants, and microorganisms (Vassilev *et al.*, 2002). In addition high mobility of this metal in soil-plant system allows its easy entering into food network where they may provoke human diseases (Ryan *et al*., 1982; Nogawa *et al*., 1987). Many reports have shown that the long-term consumption of Cd contaminated food has resulted in chronic and/or acute human Cd

disease as manifested by Itai-Itai disease, a form of osteomalacia and proximal tubular renal dysfunction, respectively (Shimada *et al*., 1977; Tohyama *et al*., 1982; Nogawa *et al*., 1983; Kido *et al*., 1988; Cai *et al*., 1990; Hochi *et al*., 1995). Furthermore, Cd-induced renal dysfunction in dietarily exposed individuals is irreversible and progressive despite decreased exposure (Kido *et al*., 1988; Nogawa & Kido 1993).

Reports of health effects of Cd in populations not occupationally exposed to Cd have centered in Japan (Kido *et al*., 1990; Kobayashi *et al*., 2002; Nogawa *et al*., 1983; Shimada *et al*., 1977) and China (Cai *et al*., 1990; Jin *et al*., 2002; Nordberg, 2003; Wu *et al*., 2001) where rice-based agricultural systems are contaminated with Cd from the use of irrigation waters that receive Cd either via natural runoff and/or uncontrolled discharges from non-ferrous mines and smelters. Cd pollution was also found in the surface soils near the metal processing industry all over Europe (Vassilev *et al*., 2002).

Cd never occurs in isolation in natural environments, but rather appears mostly as a "guest" metal in Pb:Zn mineralization (Baker *et al.*, 1990). Simmons *et al.* (2005) revealed that soil total Cd concentrations in Thailand are positively correlated with total soil Zn. Recently, Cd contaminations in paddy fields and potential risks to public health have been discovered in Mae Sot District, Tak Province, Thailand (Simmons *et al*., 2003; 2005). The areas polluted by Cd were those in close proximity to a Zn mine. The soil Zn concentrations in those area ranged from 100 – 8,036 mg Zn kg^{-1} while Cd concentrations ranged from 0.5 – 284 mg Cd kg^{-1} soil which were over both European Economic Community (EEC) Maximum permissible (MP) soil Cd concentration of 3 mg kg^{-1} soil and the Thai standard of 0.141 mg Cd kg^{-1} soil. Moreover, rice samples from contaminated fields were found to be contaminated with Cd ranging from 0.05 – 7.7 mg kg^{-1} rice. Over 90% of the rice samples collected contained Cd at concentrations exceeding the Codex Committee on Food Additives and Contaminants (CCFAC) draft Maximum Permissible Level for rice grain of 0.2 mg Cd kg^{-1}. In addition, as a function of demographic group, estimated Weekly Intake (WI) values ranged from 20 to 82 μg Cd kg^{-1} body. This poses a significant public health risk to local communities (Simmons *et al*., 2005). With regard to health impacts of Cd-exposed populations in Mae Sot, Thailand, the Cd contaminated lands should be

managed to harmless the problem instantaneously (Swaddiwudhipong *et al*., 2007; Teeyakasem *et al*., 2007).

To minimize the entry of Cd into food chain, the appropriate management for cleaning up the contaminated soil and inhibiting further Cd contamination to the other area is very important. The traditional methods for heavy metal decontamination are based upon either extraction of metals physico-chemically, such as acid leaching, or immobilization *in situ*, by e.g. vitrification. All those techniques require specialized equipments and operators. They are therefore costly and only appropriate for the decontamination of small areas. They also remove all biological activity from treated soils resulting in adversely effects on physical structure and fertility of the soils (Baker *et al*., 1994; Yan-de *et al*., 2007). In order to eliminate or control hazardous chemicals in soils, biological processes are being employed. One effective and promising process is phytoremediation, which is the use of green plants to remove pollutants from the environment or to render them harmless (Cunningham & Berti, 1993). Phytoremediation has been reported to be an effective, *in situ*, non-intrusive, low-cost, ecological friendly technology to remediate polluted soils (Garbisu & Alkorta, 2001). It also helps prevent landscape destruction and enhances activity and diversity of soil microorganisms to maintain healthy ecosystems. Phytoremediation is consequently considered to be a more attractive alternative than traditional methods. Metal tolerant plants, which can grow on metal enriched soils, play important role in phytoremediation processes. Especially, hyperaccumulator plants, which can uptake and accumulate exceptionally high concentration of heavy metal in their shoots, are being employed to achieve the effective phytoextraction, one of powerful phytoremediation approaches.

More than 400 hyperaccumulator species have been identified recently (Brooks, 2000). Most of them are associated with metal rich soils. Although hyperaccumulator plants have exceptionally high metal accumulating capacity, most of them have a slow growth rate and often produce limited amounts of biomass when the concentration of available metals in the contaminated soil is very high. For example, *Thlaspi caerulescens* (Brassicaceae) has been identified as Cd hyperaccumulator. In hydroponic experiments, a French and a Prayon populations of *T. caerulescens* were able to accumulate Cd in their shoots over 10,000 mg kg^{-1} and

5,900 mg kg^{-1} DW, respectively. Moreover, in the field trial the French population was able to accumulate up to 500 mg Cd kg^{-1} in its shoot at 12 mg Cd kg^{-1} soil (Lombi *et al*., 2000). *T. caerulescens* has been considered to be one of the promising plants used for phytoextraction of Cd and Zn. However the use of *T. caerulescens* is problematic in term of its slow growth, the rosette characteristics as well as its sensitivity to heat and drought. An alternative way is the use of metal tolerant species that are heat tolerated, although with a lower metal accumulating capacity but higher growth rates, such as Indian mustard (*Brassica juncea*) and sunflower (*Helianthus annuus*). Nevertheless, the application of phytoremediation, employing the hyperaccumulator, has raised some concerns related to invasiveness and disruption of indigenous ecosystems because the introduction of alien plants may alter ecosystem function (Angle *et al*., 2001). Therefore, one alternative option is to find native hyperaccumulator plants from polluted regions and use them for soil remediation in the same region. Because some plants have adapted to grow on polluted sites, it is possible to find and use them to revegetate degraded soils either for extracting or stabilizing the heavy metals (González & González-Chávez, 2006). With regard to aforementioned reasons, there has been continuous interest in searching for native plant species which adapt to local climatic conditions and are able to colonize metal-enriched soils for use in land reclamation (Shu *et al*., 2002).

Although most Cd hyperaccumulators discovered thus far are natives of temperate climates, the relatively high plant species diversity was found in metal mining areas in the tropics (including the Padaeng Zn mine area of Tak Province). Recently, the native plant species, considered as Pb hyperaccumulator, were also found in Bo Ngam Pb mine (Homyog *et al*., 2008; Rotkittikhun *et al*., 2006). They are *Chromolaena odoratum*, *Conyza sumatrensis*, *Buddleja asiatica*, *Sonchus arvensis*, and *Cyperus sp*. So, plant screening around Padaeng Zn mine has a high potential to discover native Cd/Zn hyperaccumulators. In the present study, plant species grown on Cd contaminated soils around Padaeng Zn mine were collected to find the potential Cd hyperaccumulator plants.

Normally, hyperaccumulator plants have high capacity to accumulate exceptionally high concentration of heavy metal, but they cannot uptake the metal up to the maximum level because of the limited metal availability in soils. Metal

availability is controlled by soil associated factors including pH, CEC, soil texture and organic matter (Saxena *et al*., 1999). Another limitation in phytoremediation is that hyperaccumulators often have a slow growth rate and produced limited amounts of biomass. The use of combination between plants and plant growth promoting rhizobacteria (PGPR) are considered to be an alternative in phytoremediation technology (Yan-de *et al*., 2007). Microbial populations inhabiting plant rhizosphere are currently known to affect heavy metal mobility and availability to the plant through release of chelating agents, acidification, phosphate solubilization, and redox changes (Yan-de *et al*., 2007). Especially, some PGPR may also exert some beneficial effects on plant growth and nutrition through a number of mechanisms including N_2 fixation, production of phytohormones and siderophores, and transformation of nutrient elements when they are either applied to seeds or incorporated into the soil (Kloepper *et al*., 1989; Glick *et al*., 1999). The use of PGPR in combination with metal tolerant plants or hyperaccumulator plants is expected to improve the efficiency of phytoremediation (Yan-de *et al*., 2007).

In the present study, the rhizobacteria associated with the roots of hyperaccumulator plants grown on metalliferous soil around Padaeng Zn mine were isolated and identified. The isolated rhizobacteria were further studied on their plant growth promoting characteristics (PGP) under laboratory conditions. Furthermore, the effects of selected rhizobacteria on the growth and metal uptake of the plants were studied. *Helianthus annuus* (sunflower), classified as the member of Asteraceae family where *Chromolaena odoratum* also belongs to, was selected to be a model plant used in the experiment. Seedlings of *H. annuus* inoculated with isolated bacterial strains to investigate the roots colonization by the bacteria were studied through the scanning electron microscope (SEM). Moreover, plant seedlings inoculated with the isolated bacteria were grown in heavy metal contaminated soils to study the effects of those bacteria on the growth and heavy metal uptake of the plant. The results obtained from this experiment are useful for the application of PGPR to improve phytoremediation efficiency.

CHAPTER II
OBJECTIVES

The objectives of the present study were as follows:

1. To determine the uptake and accumulation of heavy metals (Cd and Zn) by plant species found in the Padaeng Zn mine area, Tak province, Thailand.

2. To identify metal tolerant plant species grown on metal enriched soils whether they were accumulator or excluder and the potential application of these plants for phytoremedation of metal contaminated soils.

3. To investigate the capability of *Chromolaena odoratum* in Cd accumulation as well as phytotoxicity caused by this metal under laboratory conditions.

4. To localize Cd deposit in the tissues of *C. odoratum* grown on the media supplemented with Cd.

5. To screen rhizobacteria associated with the roots of *C. odoratum* grown on metal enriched soils.

6. To investigate the metal tolerant capacity and plant growth promoting characteristics of the isolated rhizobacteria under laboratory conditions.

7. To study the association between the isolated bacteria and the roots of *Helianthus annuus* and determine the ability of these bacteria to promote plant growth.

8. To study the influences of the isolated bacteria on heavy metal uptake and growth of *H. annuus* grown on metal contaminated soils.

CHAPTER III
LITERATURE REVIEW

In the last few decades there has been a great deal of concern over environmental pollution mainly due to the direct effects on human health and decrease in agricultural yields (Jarup, 2003). Heavy metals have been considered to be major environmental pollutants which are found in soil, water and toxic gasses formed in the atmosphere by photochemical reactions. They are strongly retained in the soil, with little leaching and may remain in the soil for thousands of years (Selim & Kingery, 2003). Since heavy metals do not break down, they can be leached through the soil layers leading to the contamination of the water table. The increased contamination and subsequent accumulation of heavy metals in the soil may have serious consequences for agriculture, since the concentration of heavy metals may reach high levels in the soil and become a limiting factor for normal plant growth and productivity of field crops. Furthermore, the consumption of agricultural products contaminated by heavy metals can affect human health (Dudka & Miller, 1999).

3.1 Heavy metal abundance

Heavy metals are basically defined as elements with metallic properties (ductility, conductivity, stability as cations, ligand specificity, etc.) and an atomic number more than 20 (Yan-de *et al*., 2007). They include both biologically “essential” and “non-essential” elements. The essential elements, known as “trace elements”, are required in low concentrations for normal growth of plants and animals. They include cobalt (Co), copper (Cu), manganese (Mn), selenium (Se), nickel (Ni), molybdenum (Mo), and zinc (Zn). The non-essential heavy metals, known as “toxic elements”, are phytotoxic and/or toxic to animals. They are, for example, mercury (Hg), lead (Pb),

cadmium (Cd), and arsenic (As), and recognized as health hazards as a result of environmental pollution (Berglund *et al.*, 1984).

Heavy metals are present in the soil as natural components (natural background levels) or as a result of human activities. The most common heavy metal contaminants are Cd, Cr, Cu, Hg, Pb, and Ni. Actually, heavy metals are natural elements that are found at various background levels (Table 3.1) and at different places throughout the world due to their various concentrations in the bedrock. Thus, for example, Ni, Cr and Co are abundant in serpentine soils whereas Zn, Pb and Cd are high in calamine soils. In addition to the background levels, pollution by toxic heavy metals resulted from anthropogenic sources has accelerated dramatically since the beginning of the industrial revolution. The primary sources are mining and smelting of metalliferous ores, gas exhaust, fuel production, fertilizer and pesticide application, and municipal waste generation. Thus, heavy metal pollution has become one of the most severe environmental problems (Greger, 1999; Garbisu & Alkorta, 2001). Heavy metals cannot be destroyed biologically (no degradation) but are only transformed from one oxidation state or organic complex to another. As a consequence of the alteration of its oxidation state, the metal may become either: (i) more water soluble resulting in high bioavailability, (ii) inherently less or more toxic, (iii) less water soluble so that it precipitates resulting in less bioavailability, or (iv) volatilized and removed from the polluted area (Garbisu & Alkorta, 1997).

Table 3.1 Background levels in natural water and sediment (Förstner & Wittman 1979) and the upper limit of non-polluted soil (Timmerman *et al.*, 1984).

Metal	Natural water ($\mu g\ l^{-1}$)		Soil ($\mu g\ l^{-1}$)		Sediment ($\mu g\ l^{-1}$)	
	Sea water	**Fresh water**	**Sandy**	**Loam**	**Lake**	**Sea**
Cd	0.01 – 0.07	0.07	1	1	0.14 – 2.5	0.02 – 0.43
Cr	0.08 – 0.15	0.5	15	30	7 – 77	11 – 90
Co	0.04	0.05	5	15		0.1 – 74
Cu	0.04 – 0.1	1.8	15	25	16 – 44	4 – 250
Hg	0.01	0.01	0.15	0.15	0.004 – 0.2	0.001 – 0.4
Mn	0.2	< 5	500	800		390 – 6700
Mo	10	1	5	5		0.2 – 27
Ni	0.2 – 0.7	0.3	1	1	34 – 55	2 – 225
Pb	0.001 – 0.015	0.2	50	50	14 – 40	7 – 80
Zn	0.01 – 0.62	10	100	150	7 – 124	16 – 165

3.2 Cadmium (Cd)

3.2.1 Chemical and physical properties

Cd (atomic number 48; relative atomic mass 112.40) is a metallic element belonging, together with Zn and Hg, to group IIb in the periodic table. It is a relatively rare metal in nature. It is present in various types of rocks and soils and in water as well as in coal and petroleum. Cd is not found in pure state but occurs as sulfide along with Zn ores and in minor amounts in Pb and Cu ores.

Cd can form a number of salts. Its mobility in the environment and effects on the ecosystem depend on the nature of these salts. Since there is no evidence that organocadmium compounds, where the metal is covalently bound to carbon, occur in nature, only inorganic Cd salts will be discussed. Cd may occur bound to proteins and other organic molecules and form salts with organic acids, but in these forms, it is regarded as inorganic (IPCS, 2006). Cd has a relatively high vapor pressure. The vapor is oxidized quickly to produce Cd oxide in the air. When reactive gases or vapor, such as carbon dioxide, water vapor, sulfur dioxide, sulfur trioxide or hydrogen chloride, are present, the vapor reacts to produce Cd carbonate, hydroxide, sulfite, sulfate or chloride, respectively. These salts may be formed in stacks and emitted to the environment. Some of the Cd salts, such as the sulfide, carbonate or oxide, are practically insoluble in water. However, these can be converted to water soluble salts in nature under the influence of oxygen and acids; the sulfate, nitrate, and halogenates are soluble in water. The physical and chemical properties of Cd and its salts are summarized in Table 3.2 (IPCS, 2006).

The stable state of Cd in nature is Cd^{2+}. This imparts moderate covalency in bonds and high affinity for sulphydryl groups, leading to increased lipid solubility, bioaccumulation and toxicity (Chatterjee & Dube, 2006). Most of the Cd found in mammals, birds, and fish is probably bound to protein molecules. In animals, Cd accumulates in liver and kidney through its strong binding with cysteine residues of metallothionein. Since the metabolism of Cd is closely related to Zn metabolism, metallothionein binds and transports both Cd and Zn (Chatterjee & Dube, 2006). Cd seems to displace Zn in many vital enzymatic reactions, causing disruption or cessation of activity. $Cd(OH)_2$ is more basic than $Zn(OH)_2$, whereas $Hg(OH)_2$ is an

extremely weak base. The halides of Zn and Cd are essentially ionic whereas $HgCl_2$ is covalent and almost undissociated in aqueous solution (Chatterjee & Dube, 2006). Cd has a long biological half life (>10 years) and its concentration in the body increases with age. Cd can react with several macromolecules and organic compounds of biological importance e.g. purine, pyrimidines, nucleosides, nucluotides, RNA, DNA, enzyme etc. It also competes with Zn to inhibit the SHO group of the thiol containing enzymes (Chatterjee & Dube, 2006).

Table 3.2 Physical and chemical properties of Cd and its salt (IPCS, 2006).

	Empirical formula	Atomic or molecular weight	Relative density ($g\ cm^{-3}$)	Melting point (°C)	Boiling point (°C)	Water solubility ($g\ l^{-1}$)
Cadmium	Cd	112.41	8.642	320.9	765	insoluble
Cadmium chloride	$CdCl_2$	183.32	4.047	568	960	1,400 (20 °C)
Cadmium acetate	$C_4H_6CdO_4$	230.50	2.341	256	decomposes	very soluble
Cadmium oxide	CdO	128.40	6.95	< 1426	900-1000 (decomposes)	insoluble
Cadmium hydroxide	$Cd(OH)_2$	146.41	4.79	300 (decomposes)		0.0026 (26 °C)
Cadmium sulfide	CdS	144.46	4.82	1750		0.0013 (18 °C)
Cadmium sulfate	$CdSO_4$	208.46	4.691	1000		755 (0 °C)
Cadmium sulfite	$CdSO_3$	192.46		decomposes		slightly soluble

3.2.2 Occurrence of Cd in the environment

Cd is a relatively rare element in the earth crust and is not found in the pure state in nature. It is present in various types of rocks and soils, especially in calamine soils. Cd occurs mainly in association with the sulfide ores of Zn, Pb and Cu. Cd are released to the environment form the weathering of rocks into river water and

then to the oceans. Volcanic eruptions and forest fires are also natural sources of Cd release to the atmosphere (Nriagu, 1980; 1989). However, Cd emissions from anthropogenic sources considerably exceed those from natural sources. Cd has been produced commercially in the twentieth century. It is a by-product of the Zn industry; its production is thus determined essentially by that of Zn. Cd is utilized for production of electroplating, pigments, stabilizers, Ni-Cd battery, and alloys.

World Cd production and consumption are shown in Figure 3.1. The global production of Cd has increased steadily since 1910 when Cd electroplating was developed commercially. The average annual production of Cd increased from around 20 tons in the 1920s to about 12,000 tons in the period 1960 – 1969 and it has fluctuated around 20,000 tons since 1987 (Sandra, 1986). The pattern of Cd uses has changed in recent years. In the past Cd was mainly used in the electroplating of metals and in pigments or stabilizers for plastics. In 1960, the engineering coatings and plating sector accounted for over half the Cd consumed worldwide, but in 1990 this had declined to less than 8% (ATSDR, 1999). Nowadays, Ni-Cd battery manufacture consumes most of the Cd output and it is expected that this application will expand with the increasing use of rechargeable batteries and their potential use for electric vehicles. For instance, the demand for Cd in Ni-Cd batteries moved from 3,000 tons in 1980 to 9,000 tons in 1990 (ATSDR, 1999). This rapid growth has more than offset declining trends for pigments, plating and stabilizers. In many respects Cd has become a vital component of modern technology, with countless applications in the electronics, communications, power generation and aerospace industries.

Fertilizers often contain some Cd that will enter the soil when fertilizers are applied to crops (ATSDR, 1999). Cd can also enter the soil or water from spills or leaks at hazardous waste sites if large amounts of dissolved Cd are present at the site. The form of Cd at these sites is important since many forms do not easily dissolve in water (ATSDR, 1999).

Cd element itself does not break down in the environment but can be changed into different forms. Most forms of Cd stay for a long time in the same place where they first entered the environment. Some forms of the Cd that goes into the water will bind to soil but some will remain in the water. Some forms of Cd in soil can enter water or is taken up by plants. Several vegetable, fruit and cereal crops have

been reported to accumulate Cd in their tissues (Prasad, 1997). Cd was translocated to the rice grain where 22 to 24% of the total metal content in the rice biomass was concentrated (Wang *et al*., 2003). Fish and mammals can take some forms of Cd into their bodies from air, water, or food. Cd can easily bio-concentrated through the food chain as shown in Figure 3.2. Consumption of food products containing Cd gives rise to deleterious effects on human health. Cd can change forms and accumulate in the body, thus it stays in the body for a very long time (ATSDR, 1999).

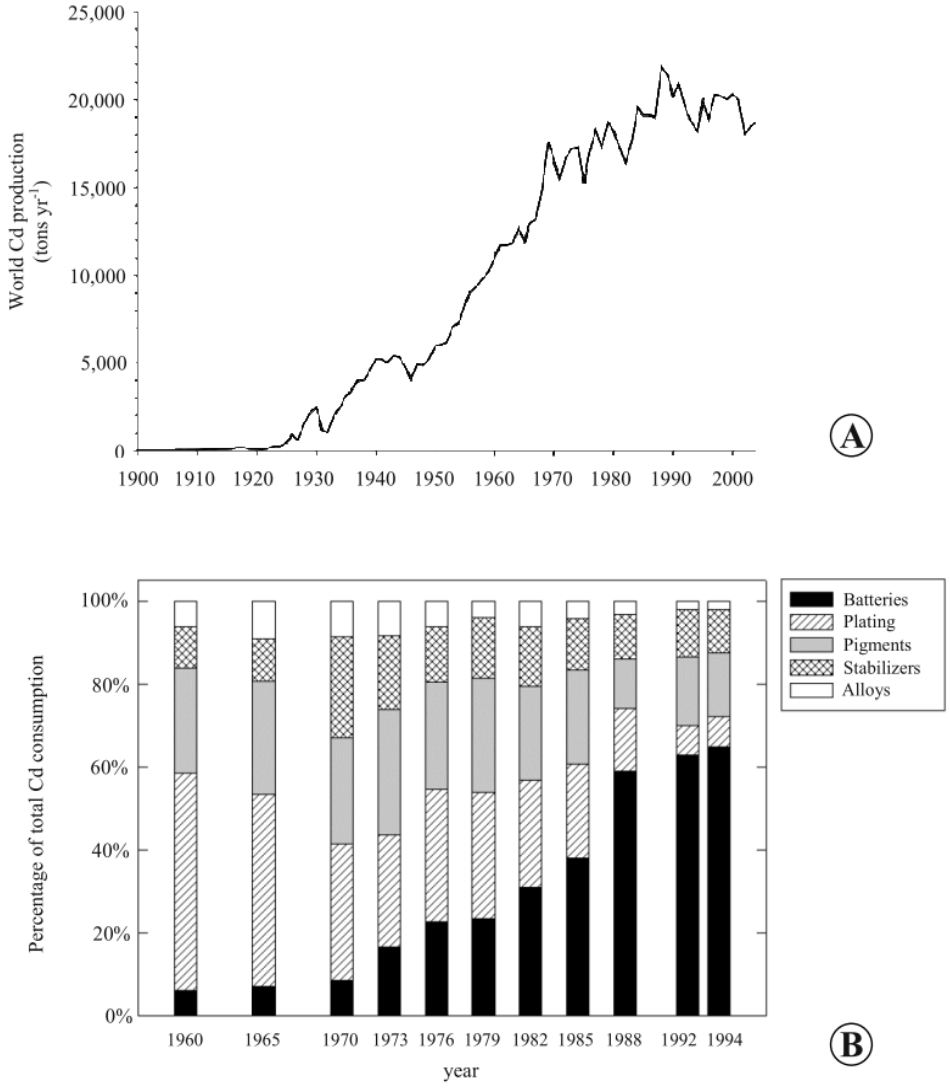


Figure 3.1 (A) World Cd production trend (Wikipedia contributor). **(B)** World Cd consumption (Cadmium Market Update Analysis and Outlook, 1995).

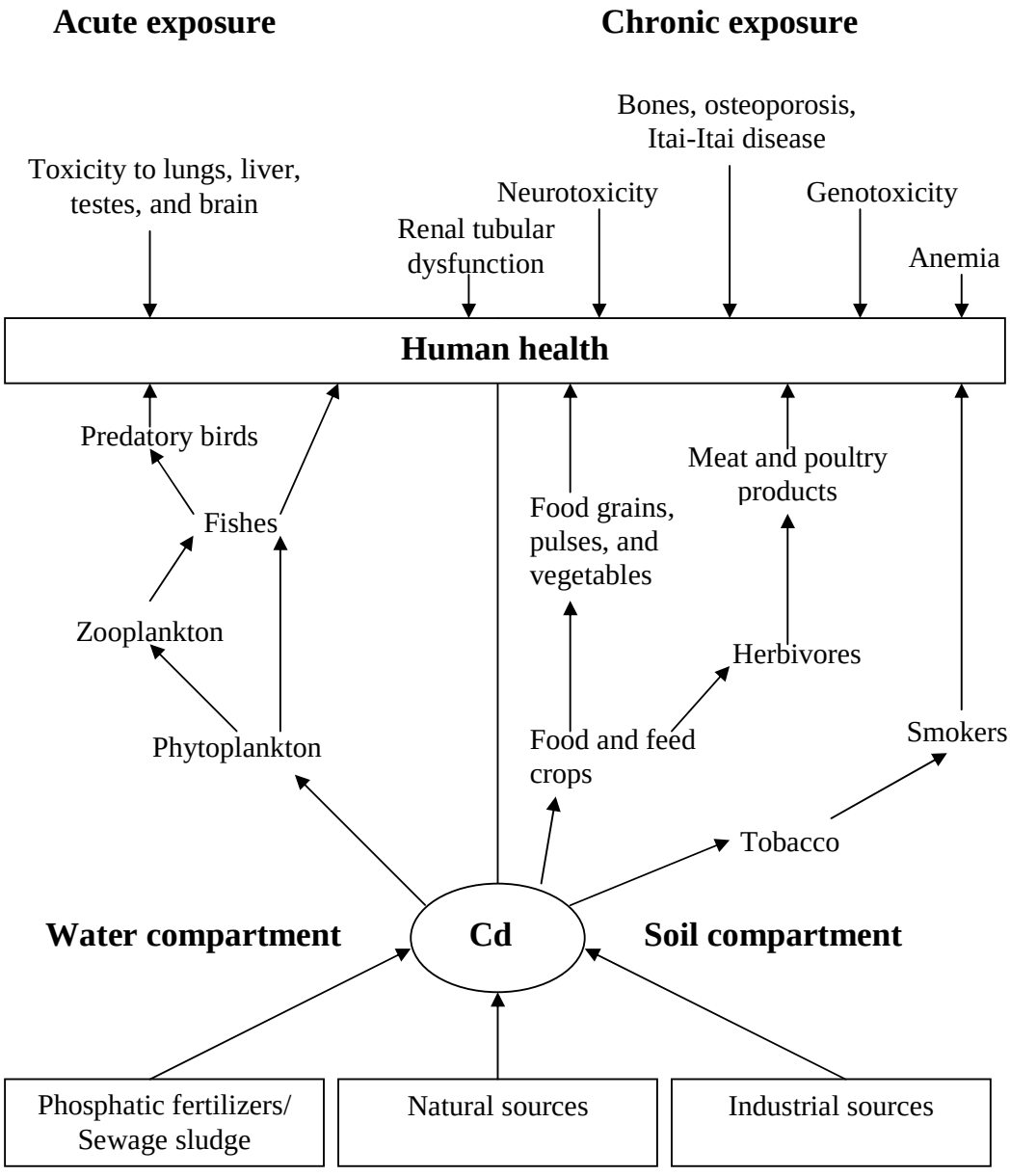


Figure 3.2 Biomagnification of Cd in food chain and its effects on human health (Dudka & Miller, 1999).

3.2.3 Effects of Cd on human health and animals

Cd can enter the human body from food, water, or breath. Very little Cd enters through the skin (Kuriakose & Prasad, 2008). People who smoke the cigarette containing Cd can get this metal by breathing. Other people who breathe in Cd are those who work with Cd and people who live near hazardous waste sites or factories that release Cd into the air. The population who live near hazardous waste sites may eat or drink Cd in food, dust, or water. Cd that enters the body stays in liver and kidneys and it leaves the body slowly, in urine and feces (Kuriakose & Prasad, 2008). The major route for Cd intake is ingestion and is largely due to the presence of trace levels of Cd in foodstuffs (Fig. 3.2). The tolerable daily Cd intake established by WHO is 60 and 70 μg day^{-1} for adult women and men, respectively. The kidney is the critical organ after long-term occupational or environmental exposure to Cd (Friberg *et al*., 1994; Bernard & Lauwerys, 1980). Chronic accumulation in the kidney cortex leads to disfunctions and loss of proteins, amino acids, and glucose in urine. Cd not only impairs kidney but also elevates the risk of osteoporosis, by inhibiting mineralization, vitamin D activation, and calcium uptake (Jarup *et al*., 1998; McLaughlin *et al*., 1999). Extreme cases of chronic Cd toxicity can result in osteomalacia and bone fractures, as characterized by the disease called Itai-Itai in Japan during the 1950s and 1960s, where local populations were exposed to Cd contaminated rice. The consumption of food or drinking water containing high Cd levels severely irritates the stomach, leading to vomiting and diarrhea (Kuriakose & Prasad, 2008). Moreover, Cd toxicity also brings to reproduction problems, cardiovascular diseases and hypertension. Cd is also classified as human carcinogen (IARC, 1993).

The potential toxicity of Cd to human health depends upon the form of Cd present, the amount taken into the body, and whether Cd is eaten or breathed (ATSDR, 1999). There are no known good effects from taking in Cd. Short-term exposure to moderate concentrations (200–500 μg m^{-3}) of freshly generated Cd fume during less than 1 hour may cause symptoms similar to those of the metal fume fever, usually with a complete recovery within a few days. More intense or prolonged exposure may lead, again after a latency period of several hours, to a chemical pneumonitis with death in 15–20% of cases (ATSDR, 1999). Chronic respiratory effects consisting of bronchitis,

obstructive lung disease or emphysema have been described in the past in workers heavily exposed to Cd (more than 20 μg m^{-3} for more than 20 years).

In experimental animals, Cd can produce acute toxic effects on various organs, such as kidney, liver, pancreas, testes and lung (by inhalation). In chronic effects to animals, Cd gives rise to a nephropathy very similar to that described in humans. Its effects are characterized functionally by the appearance of a tubular or mixed-type proteinuria, aminoaciduria, glucosuria and hypercalciuria and, morphologically, by lesions predominantly involving the tubules (ATSDR, 1999). Other chronic effects which have been described in animals treated with Cd include lung emphysema and inflammation (by inhalation), disturbances in calcium and vitamin-D metabolism resulting in bone lesions, hepatic damage and effects on the pancreas, testes or cardiovascular system. Cd can also produce embryotoxic, teratogenic and carcinogenic effects (Lauwerys, 1994).

3.2.4 Effects of Cd on plants

Although Cd is not essential for plant growth, Cd^{2+} ions are readily taken up by roots and translocated into the leaves in many plant species, with Cd depressing growth by inducing physiological, biochemical and ultrastructural changes in plants by affecting photosynthesis, respiration, chlorophyll fluorescence and nutrient uptake (Mendelssohn *et al*., 2001). The morphological symptoms in plants exposed to excess level of Cd have been documented. They include, for example, growth retardation, smaller leaves, curled and chlorotic leaves, and a red-brown coloration on the leaf margins and veins can be observed (Påhlsson, 1989). Cd can also affect germination of seeds, seedling vigor, plant development, and plant metablolism by limiting water transport to growing tissues and leaves, impairing transpiration rate, causing utrastructural changes in cell organelles and altering activities of enzymes of various metabolic pathways (Shah & Dubey, 1997a; Shah *et al*., 2001; Fediuc & Erdei, 2002). Inhibition of cell growth due to Cd may be a result of increased cross-linking of pectin in the middle lamellae. This cross-linking might be responsible for inhibition of cell expansion and its further growth (Poschenrieder *et al*., 1989). The inhibition of cell expansion and its growth might also be due to either direct or indirect effects of Cd on auxin metabolism and auxin carriers (Barcelo & Poschenreider, 1990). In addition, Cd

can induce disorders of cell wall microfibrils in *Chara vulgaris* (Heumann, 1987). Chronic exposure to low levels of Cd decreases a relative cell wall volume in *Clamydomonas bullosa* (Visviki & Rachlin, 1994) and degradation of the wall layer has been observed in *Anabaena flosaquae* exposed to high Cd levels (Rachlin *et al*., 1984; Rai *et al*., 1990). Cd has also been found to cause a severe loss of cohesiveness of the outer polysaccharide layer of the heterocyst envelope of *Nostoc* and this effect was substantially ameliorated by Ca (Mateo *et al*., 1994).

Photosynthesis and transpiration

The decline in net photosynthetic rate in Cd exposed plants might be caused by distorted chloroplast ultrastructure, restrained synthesis of chlorophylls, plastoquinone and carotenoids, disturbed electron transport, inhibited activities of enzymes in the cells (Seregin & Ivanov, 2001). The fine structure of chloroplasts in Cd treated plants degenerate and the affected plants are characterized by the occurrence of large plastoglobuli and disorganized lamellar or grana structures (Krupa *et al*., 1993). The inhibition of photosynthesis may be related to disturbances in the non-cyclic electron transport since Cd^{2+} is known to affect the PSII activity and/or water splitting system at the level of protein (Bazzaz & Govindjee, 1974; Tukendorf, 1993). Cd can form stable complexes with phosphates, thus the formation of ATP is supposed to be affected. The increased respiration rate in Cd-treated plants also suggests an increased demand for ATP production through the oxidative photophosphorylation (Hampp *et al*., 1976; Lee *et al*., 1976). Reduction in transpiration rate is also supposed to be due to lower water content in the plant and stomatal closure. Cd toxicity leading to a decreased root/shoot ratio could result in a reduced water uptake followed by a lowered water content and transpiration rate of the shoots (Schlegel *et al*., 1987).

Water status

Cd has negative effects on growth and performance of roots (Mitchell & Fretz, 1977) and influences the ability of roots to absorb water (Carlson *et al*., 1975). In sunflower leaves Cd caused closure of stomata (Bazzaz *et al*., 1974). Cd can inhibit the transpiration in *Acer saccharinum* because of its effect on stomatal closure (Lamoreaux & Chaney, 1978). With increase in Cd level the sum total of stomatal aperture per unit leaf area decreases. An increase in number of undeveloped and defective stomata was found in Cd treated plants as compared to untreated plants

(Greger & Johansson, 1992). Guard cells are reported to be smaller in Cd-treated plants compared to control plants (Breckle, 1991).

Nutrient uptake

Cd affects the uptake of both cations (K^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{3+}) and anions like NO_3^-. There are at least two mechanisms for decreased uptake of nutrients. The first mechanism, physico-chemical mechanism, depends on the size of metal ion radii. For example Cd^{2+} (1.03 A°) decreases the uptake of Zn^{2+} (0.83 A°) and Ca^{2+} (1.06 A°) (Yang *et al*., 1996). The second mechanism relies on the metal induced disorder in the cell metabolism leading to the changes in membrane enzyme activities and membrane structure (Keck, 1978; Burzyński, 1987). The efflux of K^+ occurs from the roots, apparently due to the extreme sensitivity of K^+-ATPase and –SH groups of cell membrane proteins to Cd (Burzyński, 1987). Cd drastically alters the lipid composition of membranes. The resulting changes in the membrane permeation together with membrane enzymes could shift the ionic balance in the cytoplasm. Nitrate uptake declines in Cd-stressed plants which could also be due to moisture stress induced by Cd. Decreased nitrate uptake in turn results in lower nitrate reductase activity and disturbed nitrogen metabolism (Hernandez *et al*., 1997). Under exposure to Cd, content of K^+ decreases in root and cotyledon, whereas Cd content declines in cotyledons and Fe content declines in roots (Burzyński, 1987). In clover and cabbage plants Cd decreases both uptake and transport of Zn^{2+}, Fe^{3+}, Mn^{2+}, Ca^{2+} and Mg^{2+} (Yang *et al*., 1996).

Enzyme activity

There is some evidence shown that Cd could affect biochemical processes in plants. It might cause protein changes both qualitatively and quantitatively. Proteins perform numerous crucial functions in the cell, primarily in the form of enzymes that mediate biochemical reactions required for cellular functions. Mostly Cd influences the enzyme functions by its interaction with –SH groups of enzymes (Seregin & Ivanov, 2001). It might interact not only with free –SH groups which are essential for enzyme reactions but also with –SH groups which are necessary for the stabilization of enzyme tertiary structure. Moreover, Cd might replace other metals like Zn in metal-activated enzymes. A decline in the activity of carbonic anhydrase, a Zn activated enzyme, is observed in soy bean (Lee *et al*., 1976), following Cd treatment which is

possibly due to the inhibitory effect of Cd on Zn uptake leading to Zn deficiency in plants and thus causing decreased activity of the enzyme. Both stimulations and inhibitions of enzyme activities in Cd exposed plant have been reported. The examples of various plant enzymes affected by Cd are shown in Table 3.3 (Sharma & Dubey, 2006).

Table 3.3 Effect of Cd on activities of enzymes in different metabolism processes (Sharma & Dubey, 2006).

Metabolic Processes	**Enzymes**	**Plant species**	**Effect of Cd**	**References**
Chlorophyll	ALA synthase	*Phaseolus vulgaris*	-	Padmaja *et al*., 1990
Synthesis	ALA dehydratase	*Phaseolus vulgaris*	-	
Protein metabolism	Protease	*Oryza sativa*	-	Shah & Dubey, 1997b
RNA metabolism	RNAase	*Oryza sativa*	-	Shah & Dubey, 1995
Sugar metabolism	Acid invertase	*Oryza sativa*	+	Verma & Dubey, 2001
	Sucrose synthase	*Oryza sativa*	+	
	Sucrose phosphate syntase	*Oryza sativa*	-	
N metabolism	Nitrate reductase	*Sesamum indicum*	-	Singh *et al*., 1994
	Glutamine synthatase	*Pisum sativum*	-	Chugh *et al*., 1992
	Glutamate dehydrogenase	*Phaseolus vulgaris*	+	Gouia *et al*., 2003
Phosphorus	Acid phosphatase	*Oryza sativa*	-	Shah & Dubey, 1997c
Metabolism	Alkaline phosphatase	*Oryza sativa*	-	
	Inorganic pyrophosphatase	*Oryza sativa*	-	
Photosynthesis	RUBP Carboxylase	*Phaseolus vulgaris*	-	Siedlecka *et al*., 1997
	PEP Carboxylase	*Phaseolus vulgaris*	-	
Ion-transport	H+-ATPase	*Avena sativa*	-	Astolfi *et al*., 2003
	K+-ATPase	*Cucumis sativus*	-	Burzyński, 1987
Antioxidative	Catalase	*Helianthus annus*	+	Gallego *et al*., 2002
Metabolism	Ascorbate peroxidase	*Helianthus annus*	+	Gallego *et al*., 2002
	Guaiacol peroxidase	*Oryza sativa*	+	Shah *et al*., 2001
	Glutahione reductase	*Oryza sativa*	+	Shah *et al*., 2001
	Superoxide dismutase	*Oryza sativa*	+	Shah *et al*., 2001

'+' and '-' represent stimulatory and inhibitory effects of Cd on enzymes respectively

Oxidative stress

Cd can disrupt the photosynthetic electron chain, leading to increased production of $O_2^{\cdot -}$ (Asada & Takahashi, 1987). It induced production of ROS within plants which depends on the intensity of stress, repeated stress periods and age of the plants (Shah *et al*., 2001; Singh & Tewari, 2003; Milone *et al*., 2003). Rice plants grown for 20 days in the presence of 500 μM Cd showed about 0.8–1.7 times increase in superoxide anion generation and about 1.4–1.6 times increase in lipid peroxidation products as measured in terms of malondialdehyde (MDA) levels indicating thereby that Cd induces oxidative stress in rice plants (Shah *et al*., 2001). Lipid peroxidation is regarded as an indicator of oxidative damage involving oxidative degradation of polyunsaturated fatty acyl residues of membranes (Girotti, 1990). Like all aerobic organisms plants possess the antioxidative mechanism comprising of antioxidant molecules and enzymes to protect themselves from the oxidative damage caused by harmful oxygen species. Increased activity of the antioxidative enzyme superoxide dismutase and peroxidase is observed when plants are exposed to Cd (Shah *et al*., 2001). The increased activity of antioxidative enzymes in metal exposed plants appears to serve as an important component of antioxidant defense mechanism of plants to combat metal induced oxidative injury (Shah *et al*., 2001). The activity of another antioxidative enzyme catalase increased in rice seedlings grown at moderately toxic Cd (100 μM) level. Whereas with highly toxic Cd (500 μM) level, a marked inhibition in catalase activity was noted (Shah *et al*., 2001). Decline in catalase activity in plants grown under higher levels of Cd appears to be due to inhibition of enzyme synthesis or a change in assembly of enzyme subunits (Shah *et al*., 2001). Glutathione together with ascorbic acid affect plant tolerance to reactive oxygen species by participation in the detoxification of these species in plant cells (Noctor & Foyer, 1998). The adaptation of *Helianthus annuus* plants grown in the presence of Cd has been shown to be due to increased activity of key antioxidative enzymes superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase together with unaltered values of reduced to oxidized glutathione (GSH/GSSG) and reduced to oxidized ascorbic acid (AsA/DHA) ratios (Gallego *et al*., 2002).

3.3 Zinc (Zn)

3.3.1 Chemical and physical properties

Zinc (Zn) occurs in group IIb of the periodic table of the elements together with Cd. Zn is one of the most common elements in the earth's crust. It is found in the air, soil, and water and is present in all foods. Naturally most Zn ore found in the environment is in the form of zinc sulfide. In pure elemental (or metallic) form, Zn is a bluish-white, shiny metal. Powdered Zn is explosive and may burst into flames if stored in damp places. Metallic Zn has many uses in industry. A common use for Zn is to coat steel and iron as well as other metals to prevent rust and corrosion; this process is called galvanization. Metallic Zn is also mixed with other metals to form alloys such as brass and bronze and used to make dry cell batteries. Zn can also combine with other elements, such as chlorine, oxygen, and sulfur, to form Zn compounds. Zn compounds that may be found at hazardous waste sites are Zn chloride, Zn oxide, Zn sulfate, and Zn sulfide. They are widely used in industries. The physical properties of Zn are shown in Table 3.4 (Irwin *et al*., 1997).

Table 3.4 The physical properties of Zn (Irwin *et al*., 1997).

Properties	
Atomic number	30
Atomic weight	65.38 g/mole
Melting point	419.5 ºC
Boiling point	908 ºC
Temperature at 1 mm Hg (vapor pressure)	487 ºC

3.3.2 Occurrence of Zn in environment

Zn enters the air, water, and soil as a result of both natural processes and human activities. Most Zn enters the environment as the result of mining, purifying of Zn, Pb, and Cd ores, steel production, coal burning, and burning of wastes. These activities can increase Zn levels in the atmosphere. Waste streams from Zn and other

metal manufacturing and Zn chemical industries, domestic waste water, and run-off from soil containing Zn can discharge Zn into waterways (ATSDR, 2005).

The level of Zn in soil increases mainly from disposal of Zn wastes from metal manufacturing industries and coal ash from electric utilities. Sludge and fertilizer also contribute to increased levels of Zn in the soil. In air, Zn is present mostly as fine dust particles. This dust eventually settles over land and water. Rain and snow aid in removing Zn from air. Most of the Zn in lakes or rivers settles on the bottom. However, a small amount may remain either dissolved in water or as fine suspended particles. The level of dissolved Zn in water may increase as the acidity of water increases. Fish can collect Zn in their bodies from the water that they swim in and from the food that they eat. Most of the Zn in soil is bound to the soil and does not dissolve in water. However, depending on the type of soil, some Zn may reach groundwater, and contamination of groundwater has occurred from hazardous waste sites. Zn may be taken up by animals eating soil or drinking water containing Zn. Zn is also a trace mineral nutrient and as such, small amounts of Zn are needed in all animals (ATSDR, 2005).

3.3.3 Effects of Zn on human health and animals

Zn is an essential nutrient and present in all tissues of the human body. It is a structural component of over 300 enzymes, important for metabolism of all macromolecules, in the metabolism of nucleic acids and in metabolism of other minerals (EC SCF, 2003). Zn also has an important role for gene expression as a constituent of transcription factor (Zn finger domains). In the human genome, about 10% of proteins have the potential for binding Zn (Andreini *et al*., 2006). Zn deficiency is rare, but suboptimal intake of Zn is frequent. Deficiency therefore impacts many aspects of health, growth, reproduction, susceptibility to infections, and behavior. Acrodermatitis enteropathica is a genetic disease that leads to Zn deficiency. Excess Zn derives from pollution (WHO, 2001). Symptoms of acute poisoning are nausea, vomiting, diarrhea, lethargy, and fever. Chronic exposure should interfere with copper status and immune response interfering with reproduction. Zn and Cu are mutual antagonists, interfering for absorption in the intestine. Zn also interferes with iron absorption.

The large amount of Zn inhaled (as Zn dust or fumes from smelting or welding) can cause a specific short term disease called metal fume fever, which is generally reversible once exposure to Zn ceases. However, very little is known about the long term effects of breathing Zn dust or fumes. The large amount of Zn taken into the body through food, water, or dietary supplements can also affect health. The levels of Zn that produce adverse health effects are much higher than the Recommended Dietary Allowances (RDAs) for Zn of 11 mg day^{-1} for men and 8 mg day^{-1} for women. If large doses of Zn (10–15 times higher than the RDA) are taken by mouth even for a short time, stomach cramps, nausea, and vomiting may occur. The high levels of Zn ingested for several months may cause anemia, damage the pancreas, and decrease levels of high density lipoprotein (HDL) cholesterol (ATSDR, 2005).

The consumption of food containing very large amounts of Zn (1,000 times higher than the RDA) for several months caused many health effects in rats, mice, and ferrets, including anemia and injury to the pancreas and kidney. Rats that ate very large amounts of Zn became infertile or had smaller babies. Putting low levels of certain Zn compounds, such as Zn acetate and Zn chloride, on the skin of rabbits, guinea pigs, and mice caused skin irritation. Skin irritation from exposure to these chemicals would probably occur in humans (ATSDR, 2005).

Consuming too little Zn is at least as important a health problem as consuming too much Zn. Without enough Zn in the diet, people may experience loss of appetite, decreased sense of taste and smell, decreased immune function, slow wound healing, and skin sores. Too little Zn in the diet may also cause poorly developed sex organs and retarded growth in young men. If a pregnant woman does not get enough zinc, her babies may have birth defects (ATSDR, 2005).

3.3.4 Effects of Zn on plants

Generally, Zn is the least toxic among heavy metals. It is an essential nutrient for plant growth, although elevated concentrations resulted in growth inhibition and toxicity symptoms. Zn concentrations in plants are between 15–100 ppm. Zn is a constituent of metalloenzyme or a cofactor for several enzymes such as carbonic anhydrases, alcohol dehydrogenases, superoxide dismutase and plays an important role in regulating nitrogen metabolism. As Zn forms stable complexes with

DNA and RNA it might also affect DNA and RNA stability. Zn deficiency may cause significant changes in plant metabolism, resulting in growth retardation, stunted growth and interveinal chlorosis of leaves and short internodes. Zn deficiency is more widespread than that of any other micronutrient and Zn fertilizers are often used in agriculture (Hagemeyer, 1999).

Naturally, plants may expose to the Zn enriched soils. Several plant species have adaptation for Zn tolerance while sensitive species or genotypes may show toxicity symptoms. Normally, the symptoms of Zn toxicity are similar to those of Zn deficiency. An excess supply of Zn affects both shoot and root growth and the shoot become stunted and chlorotic. Further, the epidermis of roots and the cell in the epidermis may become lignified. The well documented symptoms of Zn toxicity are decreased leaf chlorophyll content and rate of photosynthesis. At some stages in the biosynthesis of chlorophyll, Zn may compete with Fe, leading to chlorosis. The chlorophyll loss was accompanied by a reduced net photosynthetic rate. Moreover, Zn causes callose deposits on sieve tube plates in the phloem resulting in restricted carbohydrates translocation, leading to accumulation of sugars and starch in the leaves and a reduced transport to growing parts. Zn toxicity may increase permeability of root membranes, which will cause nutrients to leak out from the roots. The excess of Zn may also affect enzymes involved in plant metabolisms. For example it was found that phosphoenolpyrovatecarboxylate (PEPC), a key enzyme of photosynthesis in C_4 plants, was more sensitive to Zn. Other enzymes affected by heavy metals are hydrolytic enzymes like phosphatase and ribonuclease. Nitrate reductase enzyme included in nitrogen metabolism is also disturbed by excess of Zn (Påhlsson, 1989).

3.4 Permissible limitation

The standard guidelines for Cd and Zn contaminated soils are shown in Table 3.5. In non-contaminated soils, total concentrations of Cd and Zn generally less than 2 and 900 mg kg^{-1}, respectively (Alloway, 1995; Bowen, 1979). These ranges of concentrations depend predominantly on the parent material of soil and on the degree of weathering. Toxic levels in soils with respect to plant growth are reported as 3–8 mg kg^{-1} and 70–400 mg kg^{-1} for Cd and Zn, respectively (Kabata-Pendias & Pendias, 1984). To protect ecosystems when sewage sludge is applied to agricultural soil, the European Union has set the following range for heavy metal concentrations in soil, according to the soil pH (low value for pH 6, high value for pH 7): 1–3 mg Cd kg^{-1} and 150–300 mg Zn kg^{-1}(EEC, 1986). Most countries have set the standard guidelines or maximum permissible limitation which is applied to protect the agricultural land and human health (Table 3.6).

Table 3.5 Guidelines for Cd and Zn contaminated soils.

Guidelines	Total concentration (mg kg^{-1})		Reference
	Cd	Zn	
Non-contaminated soil	0.02 – 2	1 – 900	Alloway, 1995; Bowen, 1979
The toxic levels with respect to plant growth	3 – 8	70 – 400	Kabata-Pendias & Pendias, 1984
The European Union maximum permissible level for sludge amended soils	1 – 3	150 – 300	European Economic Commission, 1986
Thai background level	0.002 – 0.141	0.1 – 140	Pongsakul & Attajarusit, 1999
Thai investigation level	0.15	70	Zarcinas *et al*., 2004

Table 3.6 The maximum permissible concentration of heavy metals in soils of some countries and areas (Xia, 1996).

Country	pH	Heavy metal concentration (mg kg^{-1})	
		Cd	Zn
China	< 6.5	0.3	200
	6.5 – 7.5	0.3	250
	> 7.5	0.6	300
Germany	≥ 6.0	3.0	300
France	≥ 6.0	2.0	300
Italy	≥ 6.0	3.0	300
Canada (Ontario)	≥ 6.0	1.6	220
England	Field land ≥6.0	3.5	280
Scotland	≥ 5.5	1.6	150

The US federal government develops regulations and recommendations to protect public health which can be enforced by law. The Environmental Protection Agency (EPA), the Occupational Safety and Health Administration (OSHA), and the Food and Drug Administration (FDA) are some federal agencies that develop regulations for toxic substances. Recommendations provide valuable guidelines to protect public health, but cannot be enforced by law. The Agency for Toxic Substances and Disease Registry (ATSDR) and the National Institute for Occupational Safety and Health (NIOSH) are two federal organizations that develop recommendations for toxic substances. Regulations and recommendations can be expressed in not-to-exceed levels in air, water, soil, or food that are usually based on levels that affect animals, then they are adjusted to help protect people.

Some regulations and recommendations for Cd and Zn include the following: EPA has stated that drinking water should contain Cd and Zn no more than 5 ppb and 5 ppm, respectively. Furthermore, any release of more than 1,000 pounds (or in some cases 5,000 pounds) of Zn or its compounds into the environment (i.e., water, soil, or air) must be reported to EPA. The EPA also limits how much Cd can be

put into lakes, rivers, dumps, and cropland, and does not allow Cd in pesticides. The FDA limits the amount of Cd in food colors to 15 ppm. The National Academy of Sciences (NAS) estimates a Recommended Dietary Allowance (RDA) for Zn of 11 mg day^{-1} (men). 11 mg day^{-1} is the same as 0.16 mg kg^{-1} of body weight per day for an average adult male (70 kg). An RDA of 8 mg day^{-1}, or 0.13 mg kg^{-1} of body weight for an average adult female (60 kg), was established for women because they usually weigh less than men. Lower Zn intake was recommended for infants (2–3 mg day^{-1}) and children (5–9 mg day^{-1}) because of their lower average body weights. The RDA provides a level of adequate nutritional status for most of the population. Extra dietary levels of Zn are recommended for women during pregnancy and lactation. An RDA of 11–12 mg day^{-1} was set for pregnant women. Women who nurse their babies need 12–13 mg day^{-1}. To protect workers, OSHA has set an average legal limit of 1 mg m^{-3} for Zn chloride fumes and 5 mg m^{-3} for Zn oxide (dusts and fumes) in workplace air during an 8–hour workday, 40–hour work week. This regulation means that the workroom air should contain no more than an average of 1 mg m^{-3} of Zn chloride over an 8–hour working shift of a 40–hour work week. NIOSH similarly recommends that the level of Zn oxide in workplace air should not exceed an average of 1 mg m^{-3} over a 10–hour period of a 40–hour work week. OSHA now limits the amount of Cd in workplace air to 5 µg m^{-3}.

3.5 Cd contamination in Thailand

Cd contamination in agricultural soil and crops, especially rice, has been discovered in Mae Sot district, Tak province, Thailand (International Water Management Institute, South East Asia Region, 2003; Simmons *et al.*, 2005). Since 1977, Zn mining activities have been started after the Department of Mineral Resources, Ministry of Industry classified this area as the richest source of Zn minerals in Thailand. At present Zn mine has been actively operating by Padeang Industry. There is a report showing that the paddy fields receiving irrigation from the two creeks (Mae Tao and Mae Ku) were found to contain markedly elevated Cd and Zn levels. Both creeks passed through a Zn rich area where the Zn mine had been actively

operated for more than 20 years. About 85% of the paddy soil samples receiving irrigation from both creeks contained Cd content above the acceptable level. Rice grain and soybean grown in the areas were also detected to have elevated Cd content compared with the normal values. The Cd contaminated areas were estimated to be about 13,200 rais (×1,600 m^2) of paddy fields affecting 12 villages with a total population of 12,075 in 2004.

In 2005, Simmons *et al*. conducted a survey study in Mae Sot district, Tak province, Thailand to assess the degree of soil Cd and Zn contamination and associated rice grain Cd contamination downstream of an actively mined zone of Zn mineralization in that area. Total soil Cd and Zn concentrations in the rice-based agricultural system investigated ranged from 0.5 to 284 mg kg^{-1} and 100 to 8,036 mg kg^{-1}, respectively. Further, the results indicate that the contamination is associated with suspended sediment transported to fields via the irrigation water. Consequently, the spatial distribution of Cd and Zn is directly related to a field's proximity to primary outlets from the in-field irrigation channels and inter-field irrigation flows with 60–100% of the Cd and Zn loading associated with the first three fields in irrigation sequence. Rice grain Cd concentrations in the 524 fields sampled ranged from 0.05 to 7.7 mg kg^{-1}. Over 90% of the rice grain samples collected contained Cd at concentrations exceeding the Codex Committee on Food Additives and Contaminants (CCFAC) *draft* Maximum Permissible Level for rice grain of 0.2 mg Cd kg^{-1}. From a public health perspective, Weekly Intake (WI) values from rice consumption alone ranged from 20 to 98 µg Cd kg^{-1} Body. This poses a significant public health risk to local communities (Simmons *et al*., 2005). In 2004, the Pollution Control Department of the Thai Ministry of Natural Resources and Environment also collected rice grain samples from 45 randomly selected house holds within Phatat Pha Daeng, subdistrict. Rice grain Cd concentrations ranged from 0.05 to 5 mg kg^{-1} with a mean of 1.25 mg kg^{-1}. Estimated WI values ranged from 22 to 44 µg Cd kg^{-1} Body (Ministry of Natural Resources 2004). In 2007, Swaddiwudhipong *et al* conducted a survey to determine urinary Cd, a good index of excessive Cd exposure and body burden, among the exposed residents aged 15 years and older in the Cd contaminated areas, Mae sot district, Tak province. Of the 7,697 persons surveyed, 45.6% had urinary Cd levels < 2 µg g^{-1} creatinine and 47.2% had Cd levels between 2 and 4.9 µg g^{-1} creatinine. About

4.9% were between 5 and 10 μg g^{-1} creatinine and 2.3% had Cd concentrations > 10 μg g^{-1} creatinine. The American Conference of Governmental Industrial Hygienists Biological exposure index for urinary Cd is 5 μg g^{-1} creatinine. The proportion of persons who had urinary Cd > 5 μg g^{-1} creatinine significantly increased with increasing age and was also higher among women than men. The persons who mainly consumed rice grown locally in the contaminated areas had higher urinary Cd than those who did not. Since consumption of the contaminated food grown in the areas is the main source of excessive Cd exposure, the production of rice and other crops for human consumption should be prohibited. This measure can prevent further accumulation of Cd in the body of the exposed population. The production of non-food crops in these areas is strongly recommended and supported by the government.

3.6 Remediation technologies for heavy metal contaminated soils

3.6.1 Conventional remediation technologies

Conventional technologies available for water and soil remediation can be broadly classified based on whether they are employed *in situ* or *ex situ* (Saxena *et al*., 1999). The most commonly used technologies for *in situ* and *ex situ* remediation are listed as follows:

Soil flushing: The process involves physical separation by vertical or horizontal leaching using a fluid (water or an aqueous solution containing chelators) which is followed by collection and treatment of the leachates in basins or trench infiltration systems. The average cleanup time for a 20,000 ton contaminated soil site using this approach is more than 3 years.

Pneumatic fracturing: The process involves injection pressurized air into the soil to develop cracks in low permeability areas, thereby enhancing the extraction efficiencies of other *in situ* technologies

Solidification/Stabilization: In these processes the contaminant is physically enclosed in a stabilized mass or through chemical interactions induced between the stabilizing agent and the contaminant. The average clean up time for a 20,000 ton contaminated soil site using this approach is about 1 year

Vitrification: This technology utilizes thermal energy to melt the soil to enable physical or chemical stabilization.

Electrokinetics: The contaminants are mobilized as charged species towards polarized electrodes placed in the soil. The migrated contaminants can be removed or treated *in situ*.

Chemical reduction/oxidation: In this remediation process, the contaminants are chemically converted into less hazardous, more stable, less mobile and/or inert forms.

Soil washing: This process refers to the separation of contaminants adsorbed to fine soil particles using an aqueous solution, through size separation, gravity separation, or attrition scrubbing.

Excavation, retrieval and off-site disposal: This method requires removal and transportation of the contaminated soil to and off-site treatment and disposal-facility.

The conventional technologies are not suitable for practical applications because these techniques are high cost, low efficiency, large destruction of soil structure and fertility and technically limited to relatively small areas. Most of soil characteristics changed after treating with these techniques and thus decreased the soil quality. Thus, the development of phytoremediation strategies for heavy metals contaminated soils is necessary (Saxena *et al*., 1999). The advantages of this technique including the related lower costs compared with conventional techniques, avoiding the excavation of soil, have no adverse effect on the soil quality, and can be used in the contaminated soil of large area.

3.6.2 Phytoremediation

The idea that plants are used to extract metals from soils came from the discovery of different wild plants, usually found on mineralized soils, which can accumulate high concentrations of metals in their shoot (Garbisu & Alkorta, 2001). Phytoremediation can be defined as the process of utilizing plants to absorb, accumulate, detoxify and/or render harmless, contaminants in the growth substrate (soil, water and air) through physical, chemical or biological processes. The phytoremediation can be applied to both organic and inorganic pollutants present in

solid or liquid substrates. It is an emerging low-cost, ecologically friendly alternative to the conventional remediation technologies. Plants grown in metal-enriched substrates take up metal ions to varying degree. This uptake is largely influenced by the bioavailability of the metals in soils which is controlled by soil associated factors such as pH, CEC, soil texture, etc. Additionally, the capability of plant itself to tolerate and/or accumulate metals from soils and rhizospheric processes around plant root also influence on the uptake of plants (Saxena *et al*., 1999).

3.6.3 Advantages of phytoremediation

Phytoremediation has many advantages as follows: (1) large scale application, as plants can be sown or planted in large areas, (2) growing plants is relatively inexpensive, (3) plants provide an aesthetic value to the landscape of contaminated sites, (4) phytoremediation process is environmentally friendly and ecologically safe, (5) some plant species used for phytoremediation can have potential economic returns which would offset the cost of the technology, (6) plants concentrate the contaminants within their tissues, thereby reducing the amount of hazardous waste and (7) concentrated hazardous wastes require smaller reclamation facilities for extraction the heavy metals. Apart from the direct advantages, plants provide indirect benefits to the contaminated sites such as: (1) increased aeration of the soil which in turn enables microbial degradation of organic contaminants and microbe-assisted uptake of metal contaminants, (2) reduced soil erosion due to plant stand, (3) enhancement of rhizospheric micro-fauna and flora for maintaining a healthy ecosystem (Saxena *et al*., 1999).

3.6.4 Phytoremediation approaches

Phytoremediation is accomplished by many approaches as following (Garbisu & Alkorta, 2001).

Phytoextraction: is the use of plants to remove contaminants from soils. Pollutant-accumulating plants are utilized to transport and concentrate toxic contaminants (metals or organics) from the contaminated soil into the above-ground shoots which were then harvested and incinerated and/or buried.

Phytostabilization: The use of plants to reduce the bioavailability of pollutants in the environment. Plants stabilize pollutants in soils, thus rendering them harmless and reducing the risk of further environmental degradation by leaching of pollutants into the ground water or by airborne spread.

Phytovolatilization: The use of plants to volatilize pollutants. Plants extract volatile pollutants, e.g. selenium, mercury, from soil and volatilize them from the foliage.

Phytodegradation: The use of plants and associated microorganisms (plant-assisted bioremediation) to degrade organic pollutants. Plant roots in conjunction with their rhizospheric microorganisms are utilized to remediate soils contaminated with organics; the air purifying uses of some plants.

Rhizofiltration: The use of plant roots to absorb or adsorb pollutants, mainly metals, from water and aqueous-water streams. Plant roots grown in aerated water absorb, precipitate and concentrate toxic metals from polluted effluents.

3.7 Soil associated factors influencing heavy metal availability

Basically, the availability of heavy metals in soil is controlled by: (1) chemical (pH, Eh, CEC, metal speciation), (2) physical (size, texture, clay content, organic matter), and (3) biological (bacteria, fungi) processes and their interactions (Ernst, 1996).

***pH*:** The chemical forms of heavy metals in soil are affected by modification of the soil pH. An increase in pH results in higher adsorption of heavy metals to soil particles and reduces the uptake of heavy metals by plants (Kuo *et al.*, 1985). On the other hand, acidification increases the metal absorption by plants through a reduction of metal adsorption to soil particles (Brown *et al.*, 1994; Chaney *et al.*, 1995).

***Redox potential (Eh)*:** The Eh of the soil is a measure of the tendency of the soil solution to accept or donate electrons. As Eh decreases, heavy metal ions are converted from insoluble to soluble form, thus increasing bioavailability (Kabata-Pendias & Pendias, 1984).

***Cation Exchange Capacity (CEC)*:** The CEC is a measure of the ability of the soil to retain metal ions. The CEC increases with increasing clay content in the soil while the availability of the metal ions decreases (Kabata-Pendias & Pendias, 1984).

***Soil type*:** The bioavailability of heavy metals in the soil also depends on the texture of the soil. A gradient of metal ion availability exists in varying soil types with the availability being lowest in clay soils, followed by clay loam, and finally loams and sand. Similarly, heavy metal concentrations in soil are also dependent on the soil order; Gleysols and Luvisols have the highest concentration, followed by Brunisols and Podzols. However, this observation can also be related to soil texture because Gleysols and Luvisols have higher clay content, compared with Brunisols and Podzols (Webber & Singh, 1995). Normally, a higher level of heavy metal can be retained in fine-textured soils such as clay and clay loam, compared with coarse textured soils such as sand. This is in part due to the low bioavailability of these metal ions, or reduced leaching as metals are bound to the soil matrix in fine-textures soils (Webber & Singh, 1995). The complexation between heavy metals and organic matter, humic acid in particular, has been well documented (Friedland, 1990). The high organic matter content enhances the retention of the metals, drastically reducing the metal availability.

***Chelates*:** An essential component of the bioavailability process is the exudation of metal chelating compounds by plant roots (e.g. phytosiderophore). These chelators are synthesized by plants and can mobilize heavy metals such as Cu, Pb and Cd by formation of stable complexes (Mench *et al*., 1988). Chelators are usually low molecular weight compounds such as sugars, organic acids, amino acids and phenolics that can change the metal speciation, and thus metal bioavailability. Apart from the chelating agents produced by plants, the addition of synthetic chelating agents to contaminated soils was shown to substantially increase the metal solubility in the soil. Nowadays, numerous studies have focused on evaluation the effect of adding synthetic chelates such as ethylene diaminetetracetic acid (EDTA), ethyleneglycoltetracetic adid (EGTA) and citrate on the uptake of metals by plants (Salt *et al*., 1998; Cunningham & Ow, 1996). In contrast, addition of chelates to mineral nutrient solutions has also resulted in decreased metal accumulation as well as in the apparition of phytotoxicity symptoms (Srivastava & Appenroth, 1995).

Cd being a chemical element is dissolved in the soil solution, adsorbed in organic and inorganic colloidal surfaces, occluded into soil materials, precipitated with other compounds and incorporated into biological materials. A shift from solid-phase forms to that of the soil solution is essential to increase plant available chemical constituents in the soil. The factors governing the equilibrium between the solid and liquid phases of Cd in soils are complicated and not fully in the soil system. It is influenced by aforementioned factors, for example soil pH, organic matter content, redox potential, chemical form of Cd, etc. It has been reported that amounts of Cd absorbed by plants tend to increase as the concentration of Cd in the soil increases. A large number of studies suggest that Cd accumulation by plant in relation to Cd concentration in soil deal with soil that has been amended with municipal sewage sludge. There is some evidence shown that plants grown on Cd enriched soils in containers in the green house absorb more Cd than the same plant grown on the same soil amended with identical amounts of Cd in the field. This differential behave is most probably depended on root development (De Vries & Tiller, 1978). Several chemical extractants have been tested to provide an index of Cd phytoavailability of Cd recovery from soils. They include weak acids, neutral salts and chelating agents. The Cd extracted by the NH_4OAC was regarded as an exchangeable form, whereas the HCl and HNO_3 extractions estimated closely the total amounts. Some researches have been suggesting the use of DTPA extracting solution in predicting Cd uptake and yield by crops. The mobility and phytoavailabilty of Cd largely depends on its chemical form and speciation in soils. Cd solubility in soils is decreased as pH increased.

3.8 Metal tolerant plants

Metal tolerance refers to specific individuals of a species which are able to withstand greater amounts of toxicity than their immediate relatives on normal soil (Antonovics *et al*., 1971). Tolerance is therefore conferred by the possession of specific physiological mechanisms which effectively enable it to function normally even in the presence of high concentrations of potential toxic elements (Baker, 1987). The phenomenon of heavy metal tolerance in plants has attracted the interests of

scientists for 50 years since the discovery of Pb-tolerance of *Agrostis tenuis* grass (Bradshaw, 1952), and so far a substantial number of tolerant species or ecotypes have been identified around the world (Baker, 1987).

3.8.1 Basic strategies in metal tolerant plants

A few of the higher plant species have adaptations that enable them to survive and to reproduce in soils heavily contaminated with heavy metals. Such species are divided into two main groups: (1) pseudometallophytes that grow on both contaminated and non-contaminated soils, and (2) absolute metallophytes that grow on metal contaminated and naturally metal-rich soils (Baker, 1987). Primarily two basic strategies are recognized that confer tolerance to the toxicity of heavy metal in plants. There are (i) metal exclusion strategy, comprising avoidance of metal uptake and restriction of metal transport to the shoots and (ii) metal accumulation that the plants have mechanisms to keep toxic metals in their cell and/or tissue (Baker, 1981; 1987).

The pseudometallophytes possessing exclusion or avoidance strategy are currently used to revegetate bare soil area (e.g. in phytostabilization), where the lack of vegetation results from excessively high metal concentrations (Dahmani-Muller *et al*., 2000). There is some evidence shown that plants possess the avoidance mechanisms restricting the entry of toxic metals into their cell or tissues. Seed coat presents the first barrier for Cd absorption by germinating seed, therefore Cd does not enter the embryos even at lethal concentrations (Seregin & Ivanov, 2001). At the plant root, the barriers against Cd uptake include immobilization of Cd by extracellular carbohydrates like mucilage, callose as well as by the constituents of cell wall (Wagner, 1993; Nishizono *et al*., 1987). In roots and leaves of bush bean Cd ions seem to get bound mostly on pectic sites and histidyl groups of the cell wall (Leita *et al*., 1996). After absorption by roots Cd ions accumulate primarily in the rhizodermis and cortex. The multilayer cortex seems to reduce the toxic effects of Cd on other tissues by binding most of the Cd ions in the cell wall (Seregin & Ivanov, 2001). The Casparian strip present in endodermis is also a barrier for Cd entrance into the central cylinder (Seregin *et al*., 2004).

The accumulation strategy consists of highly specialized plant mechanism to keep high concentration of metals in the tissues. Different plant species have

different potentials to bioconcentrate metals in their tissue so it is difficult to know whether the metal uptake of plant is low, normal or high. Therefore, Markert (1994) tried to give normal values of metal concentrations in plants (Table 3.7). The vascular plants which accumulate exceptionally high concentrations of heavy metals in their tissues are called hyperaccumulators.

Table 3.7 Normal composition of trace elements in a plant (Markert, 1994).

Trace element	**µg g^{-1}**
Alumimium	80
Cadmium	0.05
Chromium	1.5
Cobalt	0.2
Copper	10
Gold	0.001
Iron	150
Lead	1.0
Manganese	200
Mercury	0.1
Molybdenum	0.5
Nickel	1.5
Silver	0.2
Zinc	50

There are many mechanisms that the plants employ to tolerate and/or accumulate toxic heavy metal in their cells. Probably, heavy metal ions, such as Cd^{2+}, might be bound to particular proteins and peptides inside the cell. Phytochelatins (PC) represent a group of metal binding peptides. Their common structure is (γ-glutamic acid-cysteine)$_n$-glycine, where n = 2–11 [(γ-Glu-Cys)$_n$-Gly] (Zenk, 1996). Primarily Cd can be bound to the thiol group of the cysteine residues in the phytochelatin peptides and the Cd–PC complex is about 1000 times less toxic to the plant enzymes as compared to free Cd ion (Kneer & Zenk, 1992). So the metal–PC complex has been shown to protect sensitive metabolic enzymes. The production of phytochelatins is a widespread mechanism of Cd detoxification in higher plants. Typically, Cd^{2+} enters the plant cell through the permeable cell wall. The metal ions will immediately

activate the latent, constitutive PC synthase that synthesizes, at the expense of GSH, PC molecules for chelating metal ions. Subsequently, PC–metal (Cd^{2+}) complexes are actively transported across the tonoplast into the vacuole (Salt & Rauser, 1995). The vacuole is most likely the ultimate storage compartment for heavy metal ions. In tobacco leaves and other plant species PC–heavy metal complexes have shown to accumulate in vacuoles (Vögeli-Lange & Wagner, 1990). The vacuolar compartmentalization of PC–Cd complexes prevents the free circulation of Cd ions as well as PC–Cd complexes in the cytosol and forces them to localize within a limited area (Hart *et al*., 1998). However the PC–Cd complexes are not deposited inside vacuoles, they have a rapid turnover (Grill *et al*., 1988). Under the acidic pH condition inside the vacuole, PC–Cd complexes dissociate and Cd may be bound with vacuolar organic acids like citrate, oxalate, malate (Krotz *et al*., 1989). The PC molecules are subsequently degraded by vacuolar enzymes and the individual amino acid can re-enter the cytosol. The diagram showing the biosynthesis and turnover of metal-PC complexes is depicted in Fig 3.3 (Zenk, 1996).

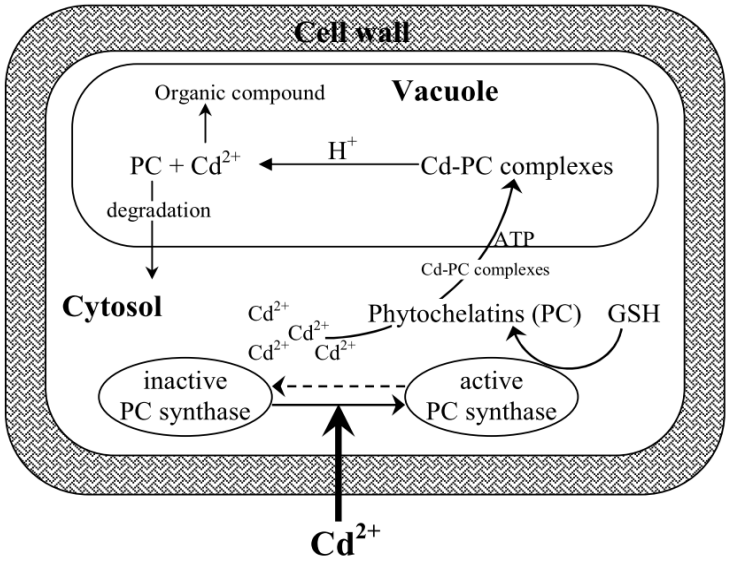


Figure 3.3 Cd detoxification in plant cell. Cd ions entering the cell activate the PC synthase that catalyzes the transformation of GSH to PC. The Cd–PC complexes are actively transported into vacuole. The metal is stored inside the vacuole by binding with organic acid (Zenk, 1996).

3.8.2 Hyperaccumulator plants

Hyperaccumulators are herbaceous or woody plants that accumulate and tolerate, without visible toxicity symptoms, a hundred times or greater metal concentrations in shoots than those usually found in non-accumulators (Baker *et al.*, 2000; Barceló & Poschenrieder, 2003). They have evolved internal mechanisms that allow them to take up and tolerate large metal concentrations that would be extremely toxic to other species (Clemens, 2001; Lasat, 2002). These plants are perfectly adapted to the particular environmental conditions of their habitat and the high uptake and accumulation of metals may contribute to their defense against herbivores and fungal infections (Boyd & Martens, 1998). Usually, the metabolic and energetic costs of their adaptation mechanisms do not allow them to compete efficiently on uncontaminated soil with non-metallophytes. Hyperaccumulators have a low biomass production since they have to use their energy in the mechanisms, which they have evolved, to cope with the high metal concentrations in the tissue.

The hyperaccumulators first characterized were members of the Brassicaceae and Fabaceae families (Salt *et al.*, 1998). At present, over 420 plant species of hyperaccmulators from all over the world that can accumulate high concentrations of metals at contaminated sites were discovered (Baker *et al*., 2000). The standard criteria for hyperaccumulator have been defined. Basically there are at least four criteria as follows:

(1) The concentrations of heavy metals in plant shoots reach hyperaccumulating levels which are shown in Table 3.8 (Baker *et al*., 1994a).

(2) The concentration of heavy metal in above-ground part is 10–500 times more than that in usual plants (1 mg Cd kg^{-1}; 100 mg Zn kg^{-1}; Shen & Liu, 1998).

(3) The metal concentrations in shoots are invariably greater than that in roots or shoot/root quotient should be more than 1, showing an ability of the plant to uptake metals and store them in their above-ground part (Baker & Whiting, 2002).

(4) Extraction coefficient, which is defined as the heavy metal concentration in plant above-ground part divided by that in soil, should be more than 1, indicating the capacity of plants to take up metal from soil (Chen *et al*., 2004).

Table 3.8 The lowest metal concentration in shoots of hyperaccumulators, number of taxa and families which are hyperaccumulators, and examples of hyperaccumulators (Baker *et al*., 1994a; Reeves & Baker, 1998).

Metal	Concentration in shoots (mg g^{-1} dry weight)	Number of Taxa	Number of Families	Example of species
Cd	> 0.1	1	1	*Thlaspi caerulescens*
Pb	> 1.0	14	6	*Minuartia verna*
Co	> 1.0	28	11	*Aeollanthus biformifolius*
Cu	> 1.0	37	15	*Aeollanthus biformifolius*
Ni	> 1.0	317	37	*Berkheya coddi*
Mn	> 10	9	5	*Macadamia neurophylla*
Zn	> 10	11	5	*Thlaspi caerulescens*

3.8.3 Metal uptake and transportation in plants

Metal transportation in plant species with exclusion strategy is frequently based on reduced metal uptake into roots, preferential storage of metals in root vacuoles and restricted translocation to shoots. Hyperaccumulator plants, in contrast, take up more metals, store a lower proportion of them in roots, and export higher amounts to shoots (Greger, 1999). Typically, the plant takes up the Cd from soil via the root system. To a lesser extent Cd gets access into plant system via leaves. The ability of leaves to absorb Cd depends on specific leaf morphology (Godzik, 1993). However, in aquatic plants, Cd is taken up not only through roots but also by shoot system. It appears that the availability of Cd to plants is depended on the pH and ionic strength of the soil medium (Hatch *et al*., 1988; Ahumada & Schalscha, 1993). Free Cd ion (Cd^{2+}) is considered to be the Cd species which is widely available to the plants (Grant *et al*., 1998). Cd interaction at the shoot or root surface is affected by H^+ ions (pH) and Ca^{2+} concentrations; in both cases the availability of Cd is sensitive to the presence of dissolved organic matter in the soil sediment as well as ambient water surrounding the shoots (Ornes & Sajwan, 1993; Campbell *et al*., 1998).

In terrestrial plant species, heavy metals are mostly taken up from soil through root system and transported to shoots. They are first taken into the apoplast of

the root. Then some of the total amount of the heavy metal is transported further into the cell, some is transported further in the apoplast and some becomes bound to cell wall substances. The primary cell walls consist of a network of cellulose, hemicellulose and glycoproteins. This network forms pores of different sizes in which the ions can move. In the smaller pores the positively charged metal ions are attracted to negative charges of the cell wall structure. Depending on the density of negative charges in the cell wall, the metal ions can be concentrated. To be able to reach the xylem vessels of the roots, the metals have to cross the endodermis and the suberinized Casparian strips, which retard the entrance of Cd into the central cylinder (Seregin *et al*., 2004). Consequently, most of the metal uptake is performed by the younger parts of the root where the Casparian strips are not yet fully developed (Hardiman *et al.*, 1984; Marschner, 1995). Part of the heavy metal that has been taken into the apoplast is further transported through the plasma membrane into the cytoplasm. There are some evidences shown that Cd can cross the tonoplast via Cd^{2+}/H^{+}-antiport system (Salt & Wanger, 1993) as well as by phytochelatin–Cd transporter (Vögeli-Lange & Wagner, 1990) that appears to be Mg–ATP dependent (Salt & Rauser, 1995). In cytoplasm, heavy metals bind to negative charges in various macromolecules which are either soluble or part of cellular structures.

Formation of less toxic metal complexes is essential in metal hyperaccumulation in plants. The toxicity of metal cations is mainly due to their tendency to form organic complexes with distinct ligands, which interfere with membrane functions, enzyme reactions, electron transport system. The metal uptake and root-to-shoot transport of high metal concentrations are only possible when these toxic interactions are avoided by the synthesis of strong chelators that efficiently bind the metals in a non-toxic form, thereby allowing flux to and through the xylem up to the leaves. The transport of heavy metals in the phloem is probably difficult since the phloem consists of living cells containing substances and ions easy to bind to (Greger, 1999). The Cd transportation from roots to shoots is probably driven by the transpiration system. Evidence for this was provided by Salt *et al*. (1995) who showed that ABA-induced stomatal closure dramatically reduced Cd accumulation in shoots of Indian mustard.

Studies of Cd uptake by plants have indicated that at lower Cd concentrations (2.5 to 90 nM), its transport is an active, energy requiring H^+-ATPase mediated process whereas at high concentrations of Cd the uptake is a non-metabolic (passive) process, involving diffusion coupled with sequestration. Although the mechanism of Cd transport across the plasmalemma of root cells is not well understood, the electrochemical potential across the membrane appears to be an important factor (Grant *et al*., 1998). It is suggested that the uptake of cationic solutes is likely to be driven largely by the negative membrane potential across the plasma membrane, which is generated in part by metabolically dependent processes such as proton extrusion via the plasma membrane H^+-ATPase (Kochian, 1991). Also, Cd uptake is a transporter mediated process which exhibits Michaelis-Menten kinetics and is controlled by a transport protein present in the membrane (Hart *et al*., 1998). Similar carrier mediated uptake has been reported for a number of divalent cationic micronutrients (Kochian, 1991). Zn competitively inhibits Cd uptake by plant roots, suggesting that Cd is transported across the plasma membrane via a native Zn-transport system. However, the reported kinetic constants of Zn and Cd uptake are quite different. The K_m value for Zn uptake by roots ranges between 2 to 6 μM, and for Cd uptake nearly two orders of magnitude lower. Thus if Zn and Cd share a common influx pathway, the affinity of the transporter appears to be considerably higher for Cd than for Zn (Hart *et al*., 1998).

Absorption of Cd by green microalgae *Chlorella vulgaris, Ankistrodesmus braunii* and *Eremosphera viridis* revealed that Cd is mainly sorbed in the cell wall. The binding sites seem to be the acidic groups in the cell wall structure (Geisweid & Urbach, 1983). In water hyacinth (*Eichhornia crassipes*) Cd was found to accumulate throughout the roots (Hosoyama *et al*., 1994). In *Solidaga altissima*, Cd was found in most parts of the plant, but located mainly in the cambium, cortex and phloem tissues (Hosoyama *et al*., 1994). Within the cell, most of the Cd is accumulated in the vacuole. The fact that Cd is found in Golgi apparatus and endoplasmic reticulum is apparently related to metal secretion through the cell surface and into the vacuole. A small quantity of Cd reaches nuclei, chloroplast, mitochondria and exerts toxic effects on these organelles (Miller *et al*., 1973; Malik *et al*., 1992).

3.8.4 Potential plants for phytoremediation

Cd is a toxic heavy metal associated with Zn mining and industrial opration. Hyperaccumulation of Cd is a rare phenomenon in higher plants. Zn is an essential microelement, but is toxic to animals and plants at high concentrations. So far, *Thlaspi caerulescens* (*Brassicaceae*) has been identified as Cd and Zn hyperaccumulator able to meet Cd and Zn hyperaccumulation criteria of 100 and 10,000 mg kg^{-1} shoot dry weight, respectively (Baker *et al*., 2000). Another possible Cd and Zn hyperaccumulator could be *Arabidopsis halleri* (L) which was found to hyperaccumulate Cd and Zn in hydroponics (Küpper *et al*., 2000). The plant species with high ability to accumulate high concentration of Cd in their tissues are shown in Table 3.9. They might be useful for applying in phytoremedation program.

Table 3.9 Plant species which can accumulate high concentration of Cd in their tissues.

Species	Cd concentration (mg kg^{-1} DW)	Cd concentration in culturing media	References
Lemna trisulca	2,300	0.64 mg Cd l^{-1}	Huebert & Shay, 1993
Lemna minor	13,300	10 mg Cd l^{-1}	Zayed *et al*., 1998a
Azolla filiculoides	>10,000	15 mg Cd l^{-1}	Sela *et al*., 1989
Thlaspi caerulescens	>1,000	200 mg Cd l^{-1}	Brown *et al*., 1995
Thlaspi caerulescens	14,100–28,000	500 μM	Lombi *et al*., 2000
Cardaminopsis halleri	281		Dahmani-Muller *et al*., 2000
Taraxacum officinale	20–410 (shoot) 20–1,360 (root)	0.5–50 μM	Simon *et al*., 1996
Teraxacum officinale	1,049		Kuleff *et al*., 1984
Triticum aestivum	479 (shoot)	150 mg Cd l^{-1}	Brown & Thomas, 1983
Thlaspi caerulescens	>1,000		Robinson *et al*., 1998
Thlaspi caerulescens	2,800	500 mg Cd kg^{-1} soil	Lombi *et al*., 2000
Thlaspi caerulescens	164–1,020	Cd-contaminaed soils	Baker *et al*., 1994b Brown *et al*., 1994
Helianthus annuus	114	10 mg Cd kg^{-1} soil	Simon, 1998
Cichorium intybus	10–300 (shoot) 10–890 (root)	0.5–50 μM	Simon *et al*., 1996

T. caerulescens could accumulate Cd in the leaves up to 1600 mg Cd kg^{-1} dry weight without detectable decrease of its dry biomass up to 50 mg extractable Cd kg^{-1} soil (Robinson *et al*., 1998). Recently, in the hydroponics experiment one French population of *T. caerulescens* (Ganges ecotype) was able to accumulate Cd in the shoots more than 10,000 mg kg^{-1} without biomass reduction. This population was able to accumulate up to 500 mg Cd kg^{-1} in the shoot at 12 mg Cd kg^{-1} soil in the field trial, which is encouraging for the Cd phytoextraction from agricultural soils (Lombi *et al*., 2000). Very different values have been reported about dry mass of *T. caerulescens*. Robinson *et al*. (1998) estimated that dry biomass production of this species averaging 2.6 t ha^{-1}, whereas in field trials Kayser *et al*. (2000) found less than 1 t ha^{-1}. On the other hand, the yield of fertilized crop of *T. caerulescens* could be easily increased by a factor of three without significant reduction in Cd tissue concentration (Bennett *et al.,* 1998). Robinson *et al*. (1998) accepting dry mass potential of *T. caerulescens* of about 5 t ha^{-1} calculated that soil contaminated by 10 mg Cd kg^{-1} would be cleaned in only 2 years. Controversially, *T caerulescens* is not suitable for phytoextraction due to its rosette characteristics, making difficult the mechanical harvesting and has low resistance to hot and dry environments (Vassilev *et al*., 2002). As the aforementioned information, many of hyperaccumulator plants have slow growth rate and very low biomass; they therefore need much time to remove contaminants from soils.

The ideal plant for metal phytoextraction has to be highly productive in biomass and to assimilate and translocate high concentrations of heavy metals to its shoots. Additional favorable traits are fast growth, easy propagation and a deep root system. Some tree species, mainly willows (*Salix*) and poplars (*Populus*) exhibit these traits and are in use in phytoremediation programs. Short-rotation coppice of willow has shown particular promise as a renewable energy crop having ability to accumulate higher levels of some heavy metals, for example Zn and Cd (Riddell-Black *et al*., 1997; Rulford *et al*., 2002). In fact, *Salix* species are not metal hyperaccumulators, but it was shown that some clones are able to accumulate Cd up to 70 mg kg^{-1} dry weights in leaves (Landberg & Greger, 1994; 1996). Due to big variation in shoot Cd concentrations found in different *Salix* species and clones, very different values concerning Cd removal have been reported (Vassilev *et al*., 2002). The resistance of

willow plants to excess Cd is very important characteristic, as they have to be able to grow continuously on Cd-contaminated land and to deliver an additional economical value (dry matter for energy, chip wood and paper production), but this aspect is not very well addressed.

The use of non-woody biomass plants has been later introduced in the phytoextraction concept in order to overcome the limitations, such as low dry biomass of hyperaccumulators as well as low phytoavailability of some metals. For the later limitation the chemically assisted approach has been developed (Salt *et al*., 1998). It has been mostly adjusted to Pb phytoextraction (Blaylock *et al*., 1997), but later extended to the other target metals. Firstly, the chemically–assisted metal phytoextraction was based only on EDTA (ethylenediamine tetraacetic acid) application, but it has been criticized for some toxic properties of the chemical as well as for the possible leaching of metals to groundwater (Crèman *et al*., 2001). At present, a number of studies are forwarded to find efficient and environmentally acceptable amendments.

The primarily interest concerning non-woody biomass crops now is focused on not only oilseed rape (*Brassica napus*), tobacco (*Nicotiana tabacum*), flax (*Linium usitatissimum*), peppermint (*Mentha piperita*), cotton (*Gossipium hirsutum*), but also triticale and maize (*Zea mays*), sunflower (*Helianthus annuus*), Indian mustard (*Brassica juncea*) (Vassilev *et al*., 2002). Using garden plants to remove metals from contaminated soils is a popular and satisfactory strategy, because it recovers the contaminated sites and also generates economic value if appropriate flower species are cultivated. When rainbow pink (*Dianthus chinensis*) was grown on a contaminated site in northern Taiwan for five weeks, the Cd concentration in the plant shoot increased from 1.56 (under the control treatment) to 115 mg kg^{-1}, and the Cd uptake was about 100 g ha^{-1} yr^{-1} (Chen & Lee, 1997).

3.8.5 *Chromolaena odoratum*

Chormolaena odoratum (Fig. 3.4) is a perennial shrub that forms dense tangled bushes 1.5–2.0 m in height, occasionally reaching 6 m as a scrambler up trees (McFadyen & Skarratt, 1996). Its stems branch freely, with lateral branches developing in pairs from the axillary buds. The older stems are brown and woody near the base; tips and young shoots are green and succulent. The root system is fibrous and does not penetrate beyond 20–30 cm in most soils. The flower heads are borne in terminal corymbs of 20 to 60 heads on all stems and branches. The flowers are white or pale bluish-lilac, and form masses covering the whole surface of the bush. Moreover, *C. odoratum* can spread very easily due to its fast growth rate, and prolific, wind-dispersed seed production (McFadyen & Skarratt, 1996). It is grown best in areas with a pronounced dry season (McFadyen, 1989).

Classification of *Chromolaena odoratum*

Kingdom – Plantae (Plants)
Subkingdom – Tracheobionta (Vascular plants)
Superdivision – Spermatophyta (Seed plants)
Division – Magnoliophyta (Flowering plants)
Class – Magnoliopsida (Dicotyledons)
Subclass - Asteridae
Order - Asterales
Family – Asteraceae (Aster family)
Genus – Chromolaena DC. (thoroughwort)
Species – *Chromolaena odoratum* (L.) King & H.E. Robins.

C. odoratum has been reported as a Pb hyperaccumulator found in Bo Ngam Pb mine, Kanchanaburi province, Thailand (Rotkittikhun *et al*., 2006). In field survey, it could accumulate Pb in shoots and roots up to 1,377 and 4,236 mg kg^{-1} dry weight, respectively and could tolerate soil Pb concentrations up to 100,000 mg kg^{-1}. Under hydroponic experiments, *C. odoratum* accumulated Pb in shoots and roots up to 1,772 and 60,655 mg kg^{-1}, respectively at the Pb supply level of 10 mg l^{-1}. *C. odoratum* is a potential hyperaccumulator plant, which grows rapidly, has substantial biomass, wide distribution, and is suitable for the phytoremediation of metal contaminated soils (Tanhan *et al*., 2007).

Figure 3.4 *Chromolaena odoratum*

Figure 3.5 *Helianthus annuus*

3.8.6 *Helianthus annuus*

Helianthus annuus or sunflower (Fig. 3.5) is an annual plant in the family Asteraceae, with a large flowering head. The stem of the flower can grow as high as 3 m tall, with the flower head reaching up to 30 cm in diameter with the "large" seeds. It is biomass producing crop of interest in phytoremediation.

Classification of *Helianthus annuus*

Kingdom - Plantae
Subkingdom - Tracheobionta
Superdivision - Spermatophyta
Division - Magnoliophyta
Class - Magnoliopsida
Subclass - Asteridae
Order - Asterales
Family - Asteraceae
Genus - Helianthus
Species - *Helianthus annuus*

Sunflower is used in diverse situations for environmental clean up. At a contaminated wastewater site in Ashtabula, Ohio, 4-week old sunflowers were able to remove more than 95% of uranium in 24 h (Dushenkov *et al.*, 1997a; 1997b). Rhizofiltration has been employed using sunflower in a U.S. Department of Energy (DOE) pilot project with uranium waste at Ashtabula, Ohio, and on water from a pond near the Chernobyl nuclear plant in the Ukraine. Sunflowers accumulated Cs and Sr, with Cs remaining in the roots and Sr moving into the shoots (Dushenkov & Kapulnik, 2002). Chen & Cutright (2001) conducted the experiments to investigate the ability of synthetic chelators for enhancing the heavy metal uptake of *H. annuus*. They found that *H. annuus* could accumulate Cd and Ni in shoots up to 115 and 117 mg kg^{-1}, respectively with the EDTA application rate of 0.5 g kg^{-1} (Chen & Cutright, 2001).

3.9 Rhizosphere

The term “rhizosphere” was first proposed by Hiltner to designate the zone of enhanced microbial abundance in and around the roots. The soil-root interface is generally called the rhizoplane. The rhizosphere is represented by few millimeters of soil surrounding the plant roots and influenced by their activities. Because the rhizosphere is characterized by steep gradients of microbial abundance and chemical characteristics, the boundary between rhizosphere and bulk soil is not accurately determined. The rhizosphere can be defined as a highly dynamic, solar/plant driven microenvironment which is characterized by feedback loops of interactions between plant root process, soil characteristics, and the dynamics of the associated microbial populations. It can also be defined as any volume of soil specifically influenced by plant roots and/or in association with roots and hairs, and plant produced material. This space includes soil bound by plant roots, often extending a few millimeters from the root surface and can include the plant root epidermal layer (Gray & Smith, 2005).

3.9.1 Plant growth promoting rhizobacteria (PGPR)

Plant growth promoting rhizobacteria (PGPR) are a heterogeneous group of bacteria that can be found in the rhizosphere, at root surfaces and in association with roots, which can improve the extent or quality of plant growth. In general, they can be separated into extracellular PGPR (ePGPR), existing in the rhizosphere, on the rhizoplane or in the spaces between cells of the root cortex, and intracellular PGPR (iPGPR), which exist inside root cells, generally in specialized nodular structures (Gray & Smith, 2005). In the last few decades a large array of bacteria including species of *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Enterobacter*, *Brevundimonas*, *Bacillus*, *Rhizobium*, *Kluyvera* and *Mesorhizobium* have been reported to enhance plant growth (Zhuang, *et al*., 2007). The direct promotion by PGPR entails either providing the plant with a plant growth promoting substances synthesized by the bacteria or facilitating the uptake of certain plant nutrients from the environment. The indirect promotion of plant growth occurs when PGPR lessen or prevent the deleterious effects of one or more phytopathogenic microorganisms.

The exact mechanisms by which PGPR promote plant growth are not fully understood, but are thought to include (i) the ability to produce or change the concentration of plant growth regulators like indoleacetic acid and ethylene, (ii) a symbiotic N_2 fixation (iii) antagonism against phytopathogenic microorganisms by production of either antibiotics or cyanide, (iv) solubilization of mineral phosphates and other nutrients (Zhuang *et al*., 2007; Gray & Smith, 2005; Yan-de *et al*., 2007). Plant growth promoting bacteria must be rhizospheric competent, able to survive and colonize in the rhizospheric soil. Unfortunately, the interaction between associative PGPR and plants can be unstable. The results obtained from laboratory scale cannot always be dependably reproduced under field conditions. The variability in the performance of PGPR may be due to various environmental factors that may affect their growth and exert their effect on the plant. The environmental factors include climate, weather conditions, soil characteristics or the composition or activity of the indigenous microbial flora of the soil (Yan-de *et al*., 2007).

3.10 Properties of PGPR

3.10.1 IAA production

Indole-3-acetic acid (IAA) is the most important member of the auxin family. It generates the majority of auxin effects in intact plants, and is the most potent native auxin. Auxins are a class of phytohormone and play important role in coordination of many growth and behavioral processes in the plant life cycle. It regulates almost every aspect of plant growth and development in various biological processes including cell division, elongation, and differentiation; root initiation and elongation; vascular system patterning; somatic embryogenesis; apical dominance; flower development; fruit ripening; and tropisms (Yang *et al*., 2007). In the plant, IAA can be derived from either of two tryptophan-independent pathways, which may utilize indole-3-glycerol phosphate or indole as a precursor, or four tryptophan-dependent pathways including the indole-3-pyruvic acid (IPyA) pathway, the indole-3-acetonitrile pathway, the indole-3-acetaldoxime pathway, and the tryptamine pathway (Hull *et al*., 2000; Zhao *et al*., 2002). IAA can be conjugated to amino acids, sugars,

and carbohydrates. IAA conjugates have been implicated in several important plant processes including IAA storage, transport, protection from enzymatic destruction, and targeting of the IAA for catabolism. The bioactive form of IAA is believed to be free IAA. Plants maintain the IAA concentration by a complex network of pathways through the interplay of IAA biosynthesis, conjugate formation, hydrolysis, and irreversible oxidation (Campanella *et al*., 1996; Ostin *et al*., 1998).

The ability to synthesize phytohormones is widely distributed among plant-associated bacteria. Most of the bacteria isolated from plant rhizospheres are able to produce IAA. Similar to plant IAA production, microorganisms also possess several different IAA biosynthetic pathways. The metabolic routes are classified in terms of their intermediates as the indole-3-acetamide (IAM), IPyA, indole-3-acetonitrile, and tryptamine pathways (Costacurta & Vanderleyden, 1995). One major route, the IAM pathway, is employed mostly by pathogenic bacteria including the gallforming bacterium *Pseudomonas syringae* pv. savastanoi. First, oxidative decarboxylation of tryptophan leading to indole-3-acetamide is catalyzed by IaaM (tryptophan-2-monooxygenase). The conversion of indole-3-acetamide to IAA is catalyzed by IaaH (indole-3-acetamide hydrolase). Both IAM pathway (L-tryptophan→IAM→IAA) and IPyA pathway are shown in Fig. 3.6 (Patten & Glick, 1996). The IPyA pathway is the major IAA biosynthetic pathway used by plant growth-promoting bacteria including *Pseudomonas putida* GR12-2 (Patten & Glick, 2002). In many cases, a single bacterial strain may possess more than one pathway (Manulis *et al*., 1991). The IAM pathway is involved primarily in gall formation, and the IPyA pathway enhances bacterial epiphytic fitness (Yang *et al*., 2007). Although the role of IAA biosynthesis by microorganisms is not fully understood, IAA provides bacteria with a mechanism to influence plant growth by supplementing the host plant's endogenous pool of auxin (Yang *et al*., 2007).

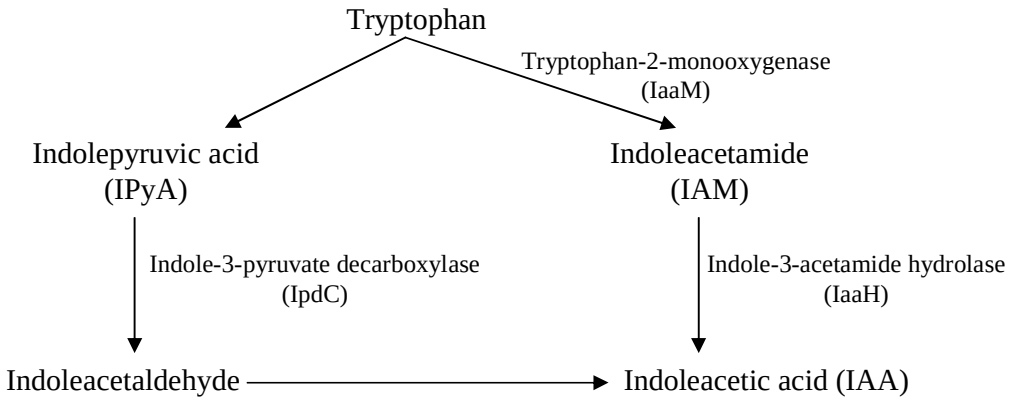


Figure 3.6 IAM and IPyA metabolic routes of IAA biosynthesis pathways (Patten & Glick, 1996).

3.10.2 Phosphate solubilization

Phosphorus (P) is one of the major essential macronutrients for biological growth and development. Its cycle in the biosphere can be described as 'open' or 'sedimentary', because there is no interchange with the atmosphere. Microorganisms play an important role in the P cycle. This cycle occurs by means of the cyclic oxidation and reduction of P compounds. The concentration of soluble P in soil is usually very low. The cell might normally take up P in the forms of HPO_4^{2-} or $H_2PO_4^-$ (Rodríguez & Fraga, 1999). Most agricultural soils contain large reserves of P, a considerable part of which has accumulated as a consequence of regular applications of P fertilizers. A large portion of soluble inorganic phosphate applied to soil as chemical fertilizer is rapidly immobilized after application and becomes unavailable to plants. The phenomena of fixation and precipitation of P in soil is generally highly dependent on pH and soil type. A second major component of soil P is organic matter. Organic P may constitute 30–50% of the total phosphorus in most soils. It is largely in the form of inositol phosphate (soil phytate). It is synthesized by microorganisms and plants and is the most stable of the organic forms of phosphorus in soil, accounting for up to 50% of the total organic P. Other organic P compounds in soil are in the form of phosphomonoesters, phosphodiesters including phospholipids and nucleic acids, and phosphotriesters (Rodríguez & Fraga, 1999).

Although several phosphate solubilizing bacteria occur in soil, usually their numbers are not enough to compete with other bacteria commonly established in the rhizosphere. Thus, the amount of P liberated by them is generally not sufficient for increasing plant growth. Therefore, inoculation of plants by a phosphate solubilizing microorganism is powerful technique to increase plant yield. There are several reports on plant growth promotion by bacteria which can solubilize inorganic and/or organic P from soil after their inoculation in soil or plant seeds. The productions of other metabolites beneficial to the plant, such as phytohormones, antibiotics, or siderophores, have created confusion about the specific role of phosphate solubilization in plant growth. However, there is evidence supporting the role of phosphate solubilizing rhizobacteria in plant growth enhancement (Rodríguez & Fraga, 1999). For example the growths of maize and lettuce were stimulated by several microorganisms capable of mineral phosphate solubilization. Inoculation with *Rhizobium leguminosarum* selected for their P-solubilization ability has been shown to increase significantly the P concentration and to improve growth in maize and lettuce (Chabot *et al*., 1996). A strain of *Pseudomonas putida* also stimulated the growth of roots and shoots and increased ^{32}P-labeled phosphate uptake in canola (Lifshitz *et al*., 1987). Inoculation of rice seeds with *Azospirillum lipoferum* strain 34H increased the phosphate ion content and resulted in significant improvement of root length and fresh and dry shoot weights (Murty & Ladha, 1988). Simultaneous increases in P uptake and crop yields have also been observed after inoculation with *Bacillus firmus* (Datta *et al*., 1982) and *Bacillus polymyxa* (Gaur & Ostwal, 1972).

The mechanism of phosphate solubilization is not currently understood. It is generally accepted that the mechanism of mineral phosphate solubilization is the action of organic acids synthesized and released by soil microorganisms. Organic acids results in acidification of the microbial cell and its surroundings and consequently P may be released from a mineral phosphate (Goldstein, 1994). The production of organic acids by phosphate solubilizing rhizobacteria has been well documented. Among them, gluconic acid seems to be the most frequent agent of mineral phosphate solubilization. It is reported as the principal organic acid produced by phosphate solubilizing bacteria such as *Pseudomonas* sp. (Illmer & Schinner, 1992), *Erwinia herbicola* (Liu *et al*., 1992), *Pseudomonas cepacia* (Goldstein *et al*.,

1993). Another organic acid identified in strains with phosphate-solubilizing ability is 2-ketogluconic acid, which is present in *Rhizobium leguminosarum* (Halder *et al*., 1990), *Rhizobium meliloti* (Halder & Chakrabartty, 1993), *Bacillus firmus* (Banik & Dey, 1982). Strains of *Bacillus liqueniformis* and *Bacillus amyloliquefaciens* were found to produce mixtures of lactic, isovaleric, isobutyric, and acetic acids. Other organic acids, such as glycolic, oxalic, malonic, and succinic acid, have also been identified among phosphate solubilizers (Illmer & Schinner, 1992; Banik & Dey, 1982). Alternative possibilities other than organic acids for mineral phosphate solubilization have been proposed. The release of H^+ to the outer surface in exchange for cation uptake or with the help of H^+ translocation ATPase could be possible ways for phosphate solubilization. Other mechanisms have been considered, such as the production of chelating substances by microorganisms as well as the production of inorganic acids, such as sulphidric, nitric, and carbonic acid (Sperberg, 1958; Duff & Webley, 1959; Rudolfs, 1922; Hopkins & Whiting, 1916).

3.10.3 Siderophore production

Siderophores are organic ligands produced by microorganisms and some plants under Fe-limited conditions, and commonly present in soil systems (Neilands, 1981; Kraemer, 2004). They have a high specificity for Fe^{3+} and also form complexes with other metals, although with a lower affinity (Hu & Boyer, 1996). The ability to form stable metal ligand complexes suggests that siderophores may influence metal mobility in soils by affecting rates of mineral weathering, and by either enhancing or inhibiting metal adsorption (Hepinstall *et al*., 2005). Siderophore formation in response to heavy metal exposure may have both beneficial and detrimental effects. It may lower the free metal concentration and provide a protective effect if the uptake receptor discriminates against the metal-siderophore complex. If the siderophore uptake receptor does not distinguish against the metal-siderophore complex, siderophore formation may also provide a secondary mechanism for metal uptake (Hu & Boyer, 1996). The effects on metal uptake and toxicity are dependent on the siderophore-metal complex being recognized by an uptake receptor. For example, production of the siderophore decreased the toxicity of Cu to the cyanobacterium *Anabaena sp*. (Clarke *et al*., 1987) but increased the toxicity of Cu to *Bacillus*

megaterium (Arceneaux *et al*., 1984). There is evidence shown that phytosiderophores (PS) facilitate not only Fe uptake but also Zn uptake by graminaceous plants (Zhang *et al*., 1991; Römheld *et al*., 1991). If phytosiderophore is efficient metal-mobilizing agents in soil and if they serve as efficient mediators for metal uptake by plants, they might serve as an effective means to achieve phytoextraction of contaminated soils. Since PS release is confined to the small portion of the soil where roots are located, the adverse effects of metal leaching by excess chelators is expected to be negligible. Moreover PS release not only facilitated Fe uptake, but also led to coincidental uptake, translocation, and accumulation of other transition metal ions (Zn, Cu, Mn) in the plant shoot (Shenker *et al*., 2001).

The majority of siderophores are either of the catecholate class or hydroxamate class (Fig. 3.7), but siderophores containing other functional groups have been found. Bacteria produce siderophores containing a variety of functional groups but most fungi produce hydroxamate-type siderophores (Renshaw *et al*., 2002). For example, the strain *Pseudomonas aeruginosa* ATCC 15692 (PAO1) has been shown to synthesize two distinct siderophores, pyochelin and pyoverdin. Pyochelin is a poor water soluble, low molecular weight siderophore which binds Fe^{3+} with a stoichiometry of two molecules per Fe atom and a remarkably low stability constant. Pyoverdin is a very water soluble molecule and a powerful chelator of ferric iron, which is bound with a stoichiometry of 1:1 with the high stability constant (Cox & Adams, 1985; Wendenbaum *et al*., 1983).

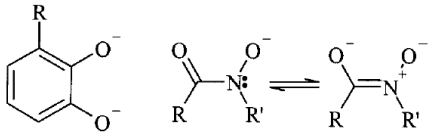


Figure 3.7 The catecholate (left) and hydroxamate (right) functional groups

3.11 Phytoremediation assisted by soil rhizobacteria

Metal tolerant plants play important role in phytoremediation program. The ideal plant for phytoextraction should grow rapidly, produce large biomass, and be able to tolerate and accumulate high concentrations of metals in shoots (Nie *et al*., 2002). Although hyperaccumulator plants have exceptionally high metal accumulating capacity, most of these have a slow growth rate and often produce limited amounts of biomass when the concentration of available metals in the contaminated soils is very high. An alternative is the use of plants with a lower metal accumulating capacity but higher growth rates, such as Indian mustard (*Brassica juncea*). Another way is the use of combination between plants and plant growth promoting rhizobacteria (PGPR), which is recently considered to improve efficiency of phytoremediation (Zhuang *et al*., 2007). Actually, rhizosphere contains a large population of microorganism possessing high metabolic activity compared to bulk soil. Microbial populations are known to affect heavy metal availability to the plant through the release of chelating agents, acidification, phosphate solubilization, and redox changes. Especially, some PGPR may exert some beneficial effects on plant growth through several mechanisms including N_2 fixation, production of phytohormones and siderophores, and transformation of nutrient elements. The use of rhizobacteria in combination with plants is expected to provide high efficiency for phytoremediation (Abou-Shanab *et al*., 2003b; Whiting *et al*., 2001). The examples of the phytoremediation using combination between plants and PGPR are shown in Table 3.10.

Table 3.10 Phytoremediation of heavy metal using combination between plants and PGPR (Zhuang *et al*., 2007).

Bacteria	Plant	Heavy metal	Condition	Role of PGPR	Reference
Azotobacter chroococcum HKN-5 *Bacillus megaterium* HKP-1 *Bacillus mucilaginosus* HKK-1	*Brassica juncea*	Pb, Zn	Pot experiments in greenhouse	- Stimulated plant growth - Protected plant from metal toxicity	Wu *et al*., 2006
Bacillus subtilis SJ-101	*Brassica juncea*	Ni	Pot experiments in growth chamber	- Facilitated Ni accumulation	Zaidi *et al*., 2006
Brevundimonas sp. KR013 *Pseudomonas fluorescens* CR3 *Pseudomonas sp*. KR017 *Rhizobium leguminosarum bv. trifolii* NZP561	None	Cd	Culture media	- Sequestered Cd directly from solution	Robinson *et al*., 2001
Kluyvera ascorbata SUD165 *Kluyvera ascorbata* SUD165/26	Indian mustard Canola tomato	Ni Pb, Zn	Pot experiments in growth chamber	- Both strains decreased plant growth inhibition by heavy metals - No increase of metal uptake	Burd *et al*., 2000
Mesorhizobium huakuii subsp. *rengei* B3	*Astragalus sinicus*	Cd	Hydroponics	- Expression of PCS_{At} gene increased ability of cells to bind Cd^{2+}, approximately 9- to 19-fold	Sriprang *et al*., 2003

3.11.1 Interactions in rhizosphere

Successful phytoremediation is largely depended on the interactions among soils, heavy metals, rhizobacteria, and plants. The complex interactions are affected by a variety of factors, such as characteristics and activities of plants and rhizobacteria, climatic conditions, soil properties.

***Plant – bacteria interactions*:** Soil microbes play significant roles in recycling of plant nutrients, maintenance of soil structure, detoxification of noxious chemicals, and control of plant pests and plant growth. In phytoremediation of heavy

metals, rhizobacteria can improve remediation efficiency of plants or reduce phytotoxicity of heavy metals. In addition, plants and bacteria can form specific associations in which the plant provides the bacteria with a specific carbon source that induces the bacteria to reduce the phytotoxicity caused by metal contaminated soil. Alternatively, plants and bacteria can form nonspecific associations in which normal plant processes stimulate the microbial community, which in the course of normal metabolic activity degrades contaminants in soils. Plant roots can provide root exudates, as well as increase ion solubility. These biochemical mechanisms increase the remediation activity of bacteria associated with plant roots (Yan-de *et al*., 2007).

Heavy metal – bacteria interactions: There is some evidence shown that rhizobacteria possess several mechanisms which can alter heavy metals bioavailability through the release of chelating substances, acidification of the microenvironment, and by influencing changes in redox potential (Smith & Read, 1997). For example, the addition of *Sphingomonas macrogoltabidus*, *Microbacterium liquefaciens*, and *Microbacterium arabinogalactanolyticum* to *Alyssum murale* grown in serpentine soil significantly increased the plant uptake of Ni when compared with the un-inoculated controls as a result of soil pH reduction (Abou-Shanab *et al.,* 2003b). However, heavy metals are known to be toxic to plants and most organisms when present in soils in excessive concentrations (Yan-de *et al*., 2007).

Plant – bacteria – soil interactions: The specificity of the plant-bacteria interactions is dependent upon soil conditions, which can alter contaminant bioavailability, root exudates composition, and nutrient levels. In addition, the metabolic requirements for heavy metal remediation may also dictate the form of the plant-bacteria interaction, either specific or nonspecific. Along with metal toxicity, there are often additional factors that limit plant growth in contaminated soils including arid conditions, lack of soil structure, low water supply and nutrient deficiency (Yan-de *et al*., 2007).

3.11.2 Effects of PGPR on phytoremediation

Plant growth: Plant growth promoting rhizobacteria (PGPR) can improve plant growth and development in heavy metal contaminated soils by mitigating phytotoxicity of heavy metals. It is well known that heavy metals can even be toxic for metal tolerant plants, if the concentration of metals in the environment is sufficiently high. The toxicity is partly attributable to iron deficiency in plants growing on heavy metal contaminated soil (Wallace *et al*., 1992). Furthermore, the low Fe content in plants grown in the presence of high concentrations of heavy metals generally results in these plants becoming chlorotic, since Fe deficiency inhibits both chloroplast development and chlorophyll biosynthesis (Imsande, 1998). PGPR can produce siderophores, which have the effect of chelating Fe^{3+} and significantly increasing the bioavailability of soil bound Fe (Hepinstall, 2005). Consequently, PGPR may help plants obtain sufficient iron (Wallace *et al*., 1992). A number of PGPR containing enzyme ACC deaminase, which hydrolyses and decreases the amount of ethylene precursor, can reduce ethylene level and stimulate plant growth (Glick, 2005). In some plants, ACC is exuded from roots or seeds and then taken up by rhizobacteria and cleaved by ACC deaminase to ammonia and α-ketobutyrate (Glick *et al*., 1998). The lowering of ACC levels results in a reduction in the amount of plant ethylene and a decreased extent of ethylene inhibition of plant seedling and root elongation. In addition the growth of plant root may also be stimulated by IAA produced by PGPR. The low level of IAA produced by rhizobacteria can promote primary root elongation, whereas high level of IAA stimulates lateral and adventitious root formation (Glick, 1995). Generally, PGPR can facilitate plant growth by altering the hormonal balance within the plants.

***Soil properties*:** There is evidence shown that the chemical conditions of the rhizosphere differ from those of the bulk soil, as a consequence of various processes that are induced by plant roots and/or by the rhizobacteria (Hinsinger, 2001; Marschner, 1995). Plant-bacteria interactions could stimulate the production of compounds that could alter soil chemical properties in rhizosphere and enhance heavy metals accumulation in plants. For example, soil acidification in the rhizosphere of *Thlaspi caerulescens* facilitates metal ion uptake by increasing metal ion mobility around the roots (Delorme *et al*., 2001). In addition, microorganisms can remove a

number of metals from the environment by reducing them to a lower redox state (Lovley, 1995). Many of the microorganisms that catalyze such reactions use the metals as terminal electron acceptors in anaerobic respiration. The microbial reduction of Cr^{6+} to Cr^{3+} has been one of the most widely studied forms of metal bioremediation. A wide diversity of heterotrophic organisms is known to carry out this reaction which, depending upon the organism, can take place anaerobically or aerobically (Wang & Shen, 1995).

***Transformation of heavy metals*:** The efficiency of phytoremediation is also influenced by the bioavailability of metals to plants. Rhizobacteria may transform heavy metals to the forms that are more readily taken up by plant roots. For example, bacteria could enhance Se accumulation in plants by reducing selenate to organic Se, and organoselenium forms like SeMet are known to be taken up at faster rates into roots than inorganic forms (Zayed *et al.*, 1998b). Rhizobateria can also directly influence metal bioavailability by altering chemical properties, such as pH, organic matter content, redox state. The pH and organic content are the main factors controlling the bioavailability of heavy metals in soils (Gray *et al.*, 1998). For example, a strain of *Pseudomonas maltophilia* was shown to reduce the mobile and toxic Cr^{6+} to nontoxic and immobile Cr^{3+}, and also to minimize environmental mobility of other toxic ions such as Hg^{2+}, Pb^{2+}, and Cd^{2+} (Blake *et al.*, 1993; Park *et al.*, 1999).

***Plant pathogens*:** PGPR provides different mechanisms for suppressing plant pathogens. The major mechanism is antibiosis by antibiotic substances. The antibiotics produced by bacteria are pyrrolnitrin, pyocyanine, 2,4-diacetyl phloroglucinol (Pierson & Thomashow, 1992) and siderophores. Other important mechanisms include production of lytic enzymes such as chitinases and β-1,3-glucanases which degrade chitin and glucan present in the cell wall of fungi (Fridlender *et al.*, 1993; Velazhahan *et al.*, 1999), HCN production and degradation of toxin produced by pathogen (Yan-de *et al.*, 2007).

CHAPTER IV
MATERIALS AND METHODS

4.1 Plant screening for phytoremediation potential

4.1.1 Instruments

1) GPS
2) Block digester
3) Flame atomic absorption spectrophotometer (FAAS; Variance SpectraA 55B)
4) pH meter: Hanna Instruments, model pH 211 micro process
5) Hot air oven
6) Rotary Shaker
7) Vortex: Genie, model K-550-GE
8) Plant press
9) Fume hood

4.1.2 Chemical preparations

4.1.2.1 DTPA extracting solution

DTPA extracting solution was 0.005 M DTPA (diethylene triamine pentaacetic acid), 0.01 M calcium chloride ($CaCl_2$), and 0.1 M TEA (Triethanolamine; $HOCH_2(CH_2)_3N$) with pH 7.3. To prepare 10 liters of this solution, 19.67 g of DTPA, 14.7 g of $CaCl_2.2H_2O$ and 149.2 g of TEA were dissolved in approximately 200 ml of deionized water. After all ingredients were sufficiently dissolved, deionized water was added to bring the volume to 9 liters. The pH of the solution is adjusted to 7.30±0.05 with 6 M HCl while stirring. The solution was added by deionized water to final volume of 10 liters. This solution was stable for several months (Lindsay & Norvell, 1978).

4.1.2.2 Aqua Regia solution (3:1 HCl:HNO_3)

This solution must be prepared fresh for each experiment. 300 ml of HCl (37% w/v; Merck) was placed into a beaker. 100 ml of HNO_3 (65% w/v; Merck) was then slowly added to mix with HCl in the beaker. The beaker was covered by a watch glass and left in the fume hood for 6 h for the reaction to subside. Finally Aqua Regia was transferred to an appropriate storage bottle.

4.1.2.3 5 N HCl

5 N HCl was prepared by adding 414 ml of HCl (37% w/v; Merck) into a beaker containing 586 ml of deionized water. The solution was gently mixed and transferred to an appropriate storage bottle.

4.1.2.4 2:1 HNO_3 and $HClO_4$ mixture

The mixture was prepared by slowly adding 100 ml of $HClO_4$ (72% w/v; Merck) into a beaker containing 200 ml of HNO_3 (65% w/v; Merck). The solution was then mixed and transferred to an appropriate storage bottle.

4.1.3 Site description

Padaeng Zn mine and Baan Pha Tae village are located in Mae Sot district, Tak province, Thailand (Figure 4.1). The annual temperature is 27.03 °C and annual rainfall is 1797.9 mm. Zn in this area is in the form of Hemimorphite ore ($Zn_4(Si_2O_7)(OH)_2.H_2O$). The following five sampling sites were selected to collect the plant and soil samples;

Site 1 Tailing pond area (N 16º 39′ 13.2″ E 98º 40′ 43.2″)

Site 2 Open pit area (N 16º 39′ 12.3″ E 98º 39′ 42.3″)

Site 3 Stockpile area (N 16º 39′ 42.8″ E 98º 39′ 56.1″)

Site 4 Forest area (N 16º 39′ 40.7″ E 98º 38′ 52.7″)

Site 5 Cd contaminated rice field (N 16º 40′ 18.2″ E 98º 37′ 58.5″)

Actually, these areas were around Zn mine, they were contaminated with Cd because Cd and Zn naturally coexist. Site 1 – 3 were located in Padaeng Zn mine that have actively operated for more than 20 years. Site 4 was the future Zn mine area. The area was covered with undisturbed native plant species. Site 5 was a former rice paddy area which had been contaminated with Cd by irrigation water from the Mae

Tao creek, the upper stretches of which pass through an actively mined Zn-mineralized zone.

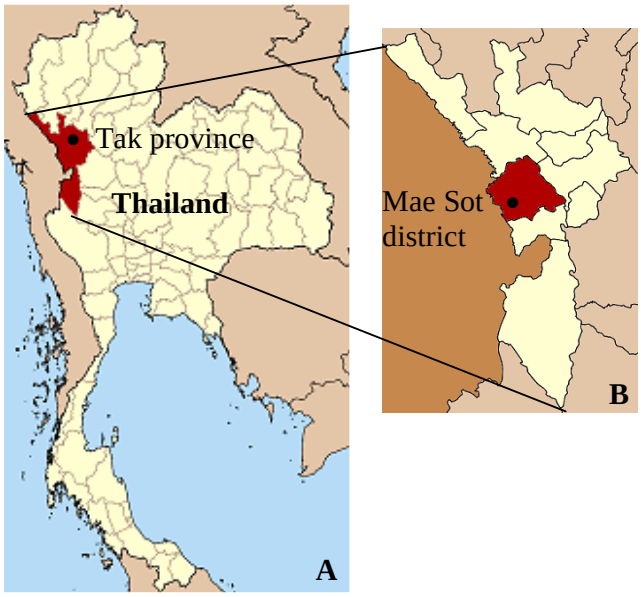


Figure 4.1 (A) The map of Thailand showing Tak province.
(B) The Mae Sot district where the Padaeng Zn mine and Baan Pha Te village are located. Wikipedia contributor; http://en.wikipedia.org.

4.1.4 Soil sampling and analyses

Surface soil samples (0–20 cm in depth) were collected from five sites during February 2006 to February 2007. For each site, 10–20 sub-samples were randomly collected and combined into a composite sample, and a 1 kg sub-sample of the composite sample was taken back to the laboratory for preparation and analysis. The samples were oven dried at 70ºC for 72 h, then ground into fine powder and sieved through a 2-mm nylon mesh sieve. The samples were characterized by the Department of Soil Science, Faculty of Agriculture, Kasetsart University, Thailand for pH and electrical conductivity (EC) in a 1:5 soil:water suspension using pH and EC meters, respectively (Rayment & Higginson, 1992); organic matter was measured by the Walkley-Black titration method (Walkley & Black, 1934); total N by the Kjedhal method (Black, 1965); available P by the Bray II method (Bray & Kurtz, 1945); available K by an atomic absorption spectrophotometer after extraction with NH_4OAc

at pH 7 (ICARDA, 2001); and cation exchange capacity (CEC) by leaching with NH_4OAc at pH 7 followed by distillation (Rayment & Higginson, 1992). The soils around plant roots where the plants were taken were also collected. They were prepared and analyzed to determine heavy metal concentrations.

Analysis for total metal concentrations in soil: To determine the total concentration of Cd and Zn in collected soils, soil samples were digested with Aqua Regia using an open tube digestion method (Simmons *et al*., 2005). 5 ml of Aqua Regia was added into test tube containing 0.5 g of soil samples. The tubes were heated in block digester at 60 ℃ for 1 h. The samples were mixed at intervals using a vortex mixer. The tubes were then heated at 105 ℃ for 1 h. After that the temperature was increased to 140 ℃. The tubes were left in the block digester until approximately 1 ml of Aqua Regia remained. The samples were mixed by vortex mixer at low speed. If sample contained a large amount of organic matter and/or iron/manganese oxides repeated 5 ml additions of Aqua Regia may be required. Digestion was complete when the sediment remaining at the base of the digestion tube demonstrated an overall grey bleached appearance. When digestion was complete, all tubes were removed from digestion block and 5 ml of 5 N HCl was added into those tubes. All tubes were then heated in the block digester again at 140 ℃ for 10 minutes. After that they were removed from the block digester, placed in a rack and allowed to cool. The samples were mixed by hand and filtered into 25 ml volumetric flasks by filter paper (Whatman© No.42). The filtrate volume of each sample was made up to 25 ml and the solution was then transferred to plastic bottles. The Cd and Zn concentrations were determined by flame atomic absorption spectrophotometer (FAAS). To assess the analytical precision, three replicates of samples and an appropriate Standard Reference Material (SRM) were performed in each analytical batch.

Analysis for extractable metal concentrations in soil: The extractable Cd and Zn in soils were determined by using DTPA extracting solution with a soil:extractant ratio of 1:5 (Simmons *et al*., 2005). 5 g of soil samples were placed into a glass bottles. 25 ml of DTPA solution was added into those bottles. The bottles were shaken on a rotary shaker at 120 rpm for exactly 2 h. After that the samples were filtered through filter paper (Whatman© No.42). Filtrates of each sample were collected in plastic tubes. The heavy metal concentrations were determined by flame

atomic absorption spectrophotometer (FAAS). To assess the analytical precision, three replicates of samples and an appropriate Standard Reference Material (SRM) were performed in each analytical batch.

4.1.5 Plant sampling and analyses

Plant samples were collected from the same sites as the soil samples. At least five individuals of each species were randomly collected within the sampling areas, then mixed to give a composite whole plant samples. Plant samples for identification were kept in a plant press for identification. The plant identification was accomplished by the Department of Botany, Kasetsart University, Thailand.

After collection, the plants were thoroughly washed in running tap water to remove any surface soil contamination, then rinsed with deionized water twice and separated into shoots and roots. The samples were oven dried at 70 ºC for 72 h, subsequently ground into fine powder and sieved through a 2-mm. nylon sieve.

Analysis for heavy metal concentrations in plant: To determine the Cd and Zn concentrations in plant shoots and roots, plant samples were digested in mixed acid using an open tube digestion technique (Simmons *et al.*, 2005). 1 g of each plant sample was placed into the glass test tubes. 10 mL of mixed HNO_3:$HCLO_4$ (2:1, v/v) was added into those tubes. All tubes were heated in block digester at 100 ℃ for 1 h. Then the temperature was increased to 150 ℃ for 1 h. After that it was increased to 200 ℃. The tubes were left in the block digester until approximately 1 ml of solution remained. When digestion was complete, all tubes were removed from block digester and 1 ml of HCl was added into those tubes. The samples were gently mixed. They were further heated in the block digester at 200 ℃ for 10 minutes. After that the tubes were removed from the block digester, placed in a rack and allowed to cool. Deionized water was added into those tubes. The samples were mixed and filtered by filter paper (Whatman© No. 42). The filtrate volume of each sample was made up to 25 ml and the solution was then transferred to plastic bottles. The Cd and Zn concentrations were determined by FAAS. To assess the analytical precision, three replicates of samples and an appropriate Standard Reference Material (SRM) were performed in each analytical batch.

4.1.6 Data and statistical analyses

The heavy metal concentrations in plant and soil samples were presented as mg kg^{-1} plant dry weight and mg kg^{-1} soil, respectively. Data were expressed as means with standard deviation (SD). Analysis of variance (one way ANOVA; SPSS 11.5 computer software) was used to asses the differences of soil characteristics of five study sites. If the F-value showed significant differences ($p \leq 0.05$), means were compared with least significant difference method (LSD).

Various hyperaccumulator definitions and concepts were adopted in the analysis. They were listed as follows:

Translocation factor or shoot/root quotient is described as the ratio of heavy metals in plant shoot to that in plant root. Translocation factor >1 indicates preferential partitioning of metals to the shoot (Baker & Whiting, 2002; Yanqun *et al*., 2005; Branquinho *et al*., 2007; González & González-Chávez, 2006).

Extraction coefficient is described as the heavy metal concentration in shoot devided by the heavy metal concentration in soil. It can be used to evaluate the ability of plant to accumulate the heavy metal (Chen *et al*., 2004; Yanqun *et al*., 2005; Branquinho *et al*., 2007)

Bioaccumulation factor, defined as a ratio of metal concentration in plant shoot to the extractable concentration of metal in the soil, is used for quantitative expression of accumulation (Deram *et al*., 2006; Branquinho *et al*., 2007)

4.2 Accumulation, toxicity and localization of Cd in *Chromolaena odoratum*

4.2.1 Instruments

1) Flame Atomic Absorption Spectrophotometer: (FAAS; Variance SpectrAA 55B
2) Hot air oven
3) pH meter: Hanna Instruments, model pH 211 micro process
4) Transmission electron microscope: (TEM; Phillips CM10) equipped with energy dispersive X-ray microanalyser (EDAX)
5) Ultracut microtome
6) Light microscope: (Olympus CH40) equipped with Digital camera (Olympus DP12)

4.2.2 Chemical preparations

4.2.2.1 Hogland's solution

The composition of the Hoagland's solution in 1 liter was as follows: 0.1 ml of 1M KH_2PO_4; 1 ml of 1M KNO_3; 1 ml of 1M $Ca(NO_3)_2$; 0.4 ml of 1 M $MgSO_4$; 0.2 ml of 5 ppm $FeCl_3 \cdot 6H_2O$; 0.2 ml of Micronutrients. Micronutrients were prepared by dissolving 2.86 g of H_2BO_3; 1.81 g of $MnCl_2 \cdot 4H_2O$; 0.22 g of $ZnSO_4 \cdot 7H_2O$; 0.08 g of $CuSO_4 \cdot 5H_2O$ and 0.02 g of H_2MoO_4 in 1 liter of deionized water (Hoagland & Arnon, 1950). The pH of the Hoagland's solution was adjusted to 5.5±0.5 with HCl or NaOH.

4.2.2.2 Fixative solution (6% gultaraldehyde)

It was prepared by mixing 6 ml of 50% gulturaldehyde, 25 ml of 0.2 M cacodylate buffer and 10 ml of distilled water in beaker. The pH of the solution was adjusted to 7.2 – 7.4 by 6 M HCl. Finally, the solution was transferred to 50 ml volumetric flask and distilled water was then added to bring to 50 ml. The solution must be freshly prepared. The fixative solution used for tissue preparation to localize Cd was similar to that of fixative previously mentioned but 1% (w/v) of tannic acid was added.

4.2.2.3 Post fixative solution (1% OsO_4)

It was prepared by thoroughly mixing 5 ml of 4% OsO_4, 10 ml of 0.2 M cacodylate buffer and 5 ml of distilled water in disposable plastic tube. The tube was covered by aluminum foil to protect the solution from light. The solution must be freshly prepared before use.

4.2.2.4 Araldite epoxy resin

Araldite 502 kit (Electron Microscopy Science: EMS) was an epoxy resin embedding medium which yields a light gold color block. Araldite resin was prepared by adding 20 ml of Araldite 502 into the disposable plastic beaker containing 22 ml of DDSA. The mixture was thoroughly mixed to achieve uniformity. After that 0.63 ml of DMP-30 was added. The solution was thoroughly mixed. Araldite was freshly prepared before use.

4.2.3 Plant species and propagation

Chromolaena odoratum was used in this experiment. Commercial seeds of this plant were not available and the percentage of germination of seeds obtained from the field collection was very low. Thus the plants were propagated by stem cutting and cultured by hydroponic system. The plant stems were clipped with shears approximately 2–3 cm long, cleaned with tap water and grown on the vessels containing Hoagland's solution. All vessels were put on shelves equipped with lighting (4600 lx) in the laboratory with controlled conditions (25±2 ℃, 12 h day/night photoperiod). The solutions were aerated and changed every week to prevent depletion of nutrients. Plants were grown for 2 months until they produced new leaves, stems and roots.

4.2.4 Cd uptake and accumulation

Uniform plants were selected and transferred to conical flask containing 180 ml of culture media supplemented with Cd. The concentrations of Cd were varied from 0.5 to 10 mg l^{-1}. Plants grown in the culture solution without metal served as control. The media were renewed every 7 days, and Cd concentrations in the culture media were determined. There were 12 replicates for each treatment. Plants were harvested after 2 weeks of treatments. The fresh weight, shoot and root length of each

plant were measured at the beginning and at the end of the experiments. The toxicity symptoms were assessed by eye throughout the experiment. At the end of the experiment plants were collected, washed thoroughly with tap water and followed by deionized water. They were then oven dried at 70 °C for 72 h and their dry weights were determined. Plant samples were ground into fine powder. To determine the Cd concentration in plant tissues, plant samples were processed by the procedures described in the section 4.1.5.

4.2.5 Data and statistical analyses

The relative growth rate (RGR) was calculated according to Hunt (1982). $RGR = [\ln(W_2) - \ln(W_1)]/(t_2 - t_1)$; W_1 and W_2 were plant fresh weight (g) at time t_1 and t_2.

The tolerance index (TI) was calculated by dividing the root length at the different metal concentrations by that obtained in the control solution as suggested by Wilkins (1978). The following equation was used: TI (%) = 100×(root length in metal treatment)/(root length in the control).

The percentage metal uptake was calculated from $\%\ uptake = [(C_0 - C_1)/C_0]\times 100$, where C_0 and C_1 were initial and remaining concentrations of metal in media (mg l^{-1}), respectively (Abdel-Halim *et al*., 2003).

The bioaccumulation coefficient (BC) was described as the heavy metal concentration in plant divided by heavy metal concentration in the solution (Nanda-Kumar *et al*., 1995)

Data were expressed as means with standard deviation. Analysis of variance (one way ANOVA; SPSS 11.5 computer software) was used to test the effects of Cd on plant growth and on metal accumulation by plant. If the F-value showed significant differences ($p \leq 0.05$), means were compared with least significant difference method (LSD).

4.2.6 Phytotoxicity symptoms

Similar experiments were conducted as described in section 4.2.4. Plant samples were randomly collected and processed for TEM analysis. Fragments of roots, stems and leaves were first fixed in 6% glutaraldehyde at 4 ℃ for 6 h. After that samples were post-fixed in 1% OsO_4 for 6 h. They were then rinsed by fresh cacodylate buffer three times and dehydrated in ascending series of ethanol (ranging from 30% to 100%). After dehydration, samples were transferred to propylene oxide. They were gradually saturated with Araldite epoxy resin. Finally, the plant tissues were embedded in fresh Araldite and kept at 45 ℃ for 2 days and then 60 ℃ for 2 days. Samples were cut into thin sections (80 nm) with diamond knives or glass knives, mounted on copper grids, and observed through the TEM at an accelerating voltage of 80 kV.

4.2.7 Localization of Cd

Plant tissues were processed by the procedures described in section 4.2.6, but they were first preserved by fixative solution added with 1% tannic acid and were not post-fixed with OsO_4. Cd localization was performed in the TEM equipped with an energy dispersive X–ray analyzer, at an accelerating voltage of 80 kV, with a take–off angle of 45°. The spectra from 0 to 10 keV with Cd peaks were recorded after 200 s.

4.3 Isolation of rhizobacteria to find the possible strains beneficial for improving phytoremediation

4.3.1 Instruments

1) Autoclave: Isuzu, model 20-5030/5040, and Iwaki, model ACV-3167N
2) Centrifuge: IEC, model B-22M Programmable centrifuge
3) Electronic balance: Mettler Toledo, model AX205
4) Flame Atomic Absorption Spectrophotometry: (FAAS; Variance SpectrAA 55B)
5) Incubator shaker: Lab-Line, model 3530-2
6) Laminar flow hood: Issco, model Bvt124
7) Light microscope: (Olympus, model CH40) equipped with digital camera: (Olympus, model DP12)
8) pH meter: EUTECH Instruments, model pH 510
9) Spectrophotometer: Aquarius Cecil, model CE 7200
10) Vortex: Genie, model K-550-GE

4.3.2 Chemical preparations

4.3.2.1 Nutrient broth (NB) and agar (NA)

NB contained the following compounds in 1 liter; 3 g of beef extract (Criterion), 5 g of peptone (Criterion) and 5 g of yeast extract (Criterion). NA contained the same ingredients as NB with the addition of 15 g of agar (Bacto). NB and NA were sterilized by autoclave at 121 °C, 15 psi for 15 minutes. NA was dispensed into sterile Petri dishes after autoclaving. The media were freshly prepared before use.

4.3.2.2 Stock solutions of heavy metal

Stock solution of Cd (10,000 mg l^{-1}) was prepared by dissolving 2.031 g of $CdCl_2.0.5H_2O$ in 100 ml deionized water. Stock solution of Zn (20,000 mg l^{-1}) was prepared by dissolving 4.939 g of $ZnSO_4$ in 100 ml deionized

water. The stock solutions were sterilized by filtering through 0.45 μm millipore membrane before use.

4.3.2.3 Salkowski's reagent

50 ml of 35% perchloric acid (Merck) was mixed with 1 ml of 0.5 M $FeCl_3.6H_2O$.

4.3.2.4 Fe-deficient mineral salt medium

The medium contained the following compounds in 1 liter; 0.36 g of KH_2PO_4, 1.40 g of K_2HPO_4, 0.25 g of $MgSO_4.7H_2O$, 0.2 g of NaCl, 0.02 g of $CaCl_2.2H_2O$, 15 mg of EDTA, 0.16 mg of $ZnSO_4$, 0.25 mg of H_3BO_3, 0.2 mg of Na_2MoO_4, 0.2 mg of $MnSO_4.4H_2O$ and 0.02 mg of $CuSO_4.5H_2O$. The medium was supplemented with mannitol and NH_4Cl at the concentrations of 20 mM and 10 mM, respectively. It was autoclave sterilized before use (Rajkumar *et al.*, 2006).

4.3.2.5 Modified chrome azurol S (CAS) assay solution

The solution was prepared as follows:

Solution 1: 21.9 mg of hexadecyltrimethylammonium bromine (HDTMA, Fluka) was dissolved in 25 ml deionized water while stirring constantly over low heat.

Solution 2: 1.5 ml of 1 mM $FeCl_3.6H_2O$ (in 10 mM HCl) was mixed with 7.5 ml of 2 mM chrome azurol S (CAS, Sigma). This solution was slowly added to the solution 1. The mixture was then transferred to a 100 ml volumetric flask.

Solution 3: 50 ml of 1 M MES buffer solution (Sigma). The pH of the solution was adjusted to 5.6 with 50% KOH. This solution was added to the volumetric flask containing the previous mixture and then the deionized water was added to bring the volume to 100 ml.

A faster reacting assay solution, termed a "shuttle" solution was prepared by adding 87.3 mg of 5-sulfosalicylic acid (Sigma) to the solution immediately before use (Alexander & Zuberer, 1991).

4.3.2.6 National Botanical Research Institute's phosphate growth medium (NBRIP)

NBRIP contained the following ingredients in 1 liter; 10 g of glucose, 5 g of $Ca_3(PO_4)_2$, 5 g of $MgCl_2.6H_2O$, 0.25 g of $MgSO_4.7H_2O$, 0.2 g of KCl and 0.1 g of $(NH_4)_2SO_4$. The pH of the media was adjusted to 7.0 before autoclaving (Mehta & Nautiyal, 2001).

4.3.2.7 0.2 % aminonaphtholsulphonic acid

The solution was prepared by dissolving 0.5 g of the 1,2,4-aminonaphtholsulphonic acid (Sigma), 30 g of sodium bisulphate ($NaHSO_3$, Himedia) and 6 g of sodium sulphite (Na_2SO_3, Merck) in 250 ml of deionized water. If the solution did not filter clear, it would be left overnight and again filtered. A fresh solution should be prepared every 2 weeks.

4.3.3 Isolation and identification of rhizobacteria

The bacteria were isolated from the root zone of *Chromolaena odoratum* growing on Cd contaminated soil near Padaeng Zn mine, Mae Sot district, Tak Province, Thailand. The whole plants with soil around their roots were randomly collected, kept in plastic bag and carried to the laboratory. The whole roots of plants (about 10 g fresh weight) were removed from the soils, carefully washed in sterile water, and homogenized in 1 ml of sterile water using a sterile mortar and pestle. The homogenate was added to 500 ml conical flask containing 100 ml of NB. The soil (1 g) associated with plant root was placed into 100 ml of NB in 500 ml conical flask. The flasks were incubated at 30°C, 150 rpm for 24 h. To isolate the Cd tolerant rhizobacteria, 1 ml of culture was transferred to 100 ml of fresh NB supplemented with 25 mg Cd l^{-1} in 500 ml conical flask. The flasks were then incubated at 30°C, 150 rpm for 48 h. After that, aliquots of culture were serially diluted and then spread over nutrient agar (NA) plates. The plates were incubated at 30 °C overnight. Different strains of bacteria were picked and streaked on NA. The isolated bacterial strains were identified on the basis of their 16S rDNA sequencing which was done at the Mahidol University-Osaka University: Cooperative Research Center (MU-OU: CRC), Faculty of Science, Mahidol University. They were kept in glycerol at -80°C as a stock culture for further study.

The growth of isolated bacterial strains was determined. The bacteria were grown overnight in NB at 30 ℃, 150 rpm. The bacterial cultures were then transferred to the conical flask containing 100 ml of NB with the initial absorbance at 600 nm of 0.05. The absorbance at 600 nm was measured every 1 h to determine the growth and the growth curve was also established. (Dell'Amico *et al*., 2008; Belimov *et al*., 2005; Rajkumar *et al*., 2006).

4.3.4 Determination of minimum inhibitory concentrations (MIC) of heavy metals

To determine the MIC of heavy metals, 10 ml of NB supplemented with heavy metals (Cd or Zn) was prepared in 50 ml sterile plastic tubes. The concentrations of Cd and Zn were varied from 0 to 200 mg Cd l^{-1} and 0 to 400 mg Zn l^{-1}, respectively. The isolated bacteria grown overnight in NB were added into those tubes with the initial absorbance at 600 nm of 0.05. All tubes were incubated at 30 ℃, 150 rpm. The absorbance at 600 nm was measured at log phase and stationary phase to determine the growth of those isolated bacteria exposed to heavy metals. The diagram presenting the relationship between heavy metal concentrations and the bacterial growth (absorbance at 600 nm) was established. The lowest metal concentration that prevented growth was considered the Minimum inhibitory concentrations (MIC) (Abou-Shanab *et al*., 2007; Hassen *et al*., 1998).

4.3.5 Solubilization of heavy metals in soil by isolated bacteria

The isolated bacteria were grown overnight in NB at 30 ℃, 150 rpm. They were harvested by centrifugation at 6,000 rpm for 10 min and then washed twice with sterile water. The bacteria were finally suspended in sterile water. An aliquot of 1 ml of bacterial suspension (with an absorbance at 600 nm of 0.5) was used as an inoculum for 3 g of sterile heavy metal contaminated soils placed in 50 ml plastic tubes. 1 ml of sterile deionized water was added to 3 g of sterile soils as the (axenic) control. All tubes were incubated at room temperature for 14 days (Abou-Shanab *et al*, 2006; Chen *et al*., 2005). At the end of the experiment, the extractable heavy metal concentrations in soil were determined by the DTPA extraction procedures described in section 4.1.4.

4.3.6 Biosorption of heavy metals by isolated bacteria

The isolated bacteria were grown overnight in NB at 30°C, 150 rpm. The bacterial cultures were transferred to the conical flasks containing NB supplemented with either Cd or Zn at the initial concentrations of 10 mg l^{-1}. The initial absorbance of the bacteria at 600 nm was 0.05. The flasks containing only NB supplemented with heavy metals were also prepared as control. All flasks were incubated at 30°C, 150 rpm. Five replications were prepared. The concentrations of heavy metal retained in the culture media and the bacterial growth were monitored at early stationary phase. Bacterial growth was monitored by measuring both an absorbance at 600 nm and bacterial dry weight. To determine the dry weight, 10 ml of culture was filtered through 0.45 μm millipore membranes and then the filter membranes were dried at 105°C to constant weight. The final concentrations of heavy metals retained in the filtrates were measured by FAAS (Robinson *et al*., 2001; Congeevaram *et al*., 2007).

4.3.7 Indole acetic acid (IAA) production

The isolated bacteria were grown overnight in NB at 30 °C, 150 rpm. The bacterial cultures were then transferred to sterile plastic tubes containing 10 ml of NB supplemented with L-tryptophan at the concentration of 1,000 μg ml^{-1}. The initial absorbance of bacteria at 600 nm was 0.05. All tubes were incubated at 30 °C, 150 rpm. The IAA production was determined at late log phase. To determine the IAA production, the bacterial cultures were centrifuged at 10,000 rpm for 15 min. The supernatants (2 ml) were mixed with 2 drops of orthophosphoric acid and 4 ml of the Salkowski's reagent. The development of pink color would be observed if the sample contained IAA (Fig 5.24A). The absorbance was taken at 530 nm. The IAA concentration was determined using a calibration curve of pure IAA served as a standard following the linear regression (Zaidi *et al*., 2006).

4.3.8 Siderophore production

The majority of siderophore falls into either hydroxamate classes or catechol classes. Both functional groups are chemically detectable by colorimetric assays. A sensitive chemical method for the detection of siderophores is based on their affinity for Fe^{3+} and is therefore independent of the structure. The following chemical equation explains the principle:

Fe-Dye + Ligand $\rightarrow$ FeL + Dye

A strong ligand (e.g., siderophore) is added to a highly colored Fe dye complex. When the Fe ligand complex is formed, the release of the free dye is accompanied by a color change. The ternary complex chrome azurol S/iron (III)/hexadecyltrimethylamonium bromide, reported to have an extremely high sensitivity, was chosen to determine siderophore production. (Schwyn & Neilands, 1987)

The amount of siderophore produced by the isolated bacterial strains was determined using the modified chrome azurol S (CAS) assay (Alexandere & Zuberer, 1991). The isolated bacteria were grown overnight in NB at 30 °C, 150 rpm. They were harvested by centrifugation at 6,000 rpm for 15 min and then washed with sterile water. Bacterial cells were suspended in Fe-deficient mineral salt medium and incubated overnight at 30 °C, 150 rpm. The bacterial cultures were centrifuged at 10,000 rpm for 15 minute to collect the supernatants. To determine the siderophore, 0.5 ml of the supernatant was mixed by 0.5 ml of modified CAS assay solution. Positive reaction was qualitatively estimated by changes in color of assay reagent from blue to orange and quantitatively determined by measuring an absorbance at 630 nm (Fig. 5.24C). The assay was considered as negative when no change in blue color was observed. A reference solution (no siderophore) was prepared using the uninoculated medium. Zero absorbance was calibrated with a mixture of CAS solution and 1.5 mM deferoxamine mesylate. A standard curve was prepared by analyzing the absorbance at 630 nm of ascending concentration of standard solutions (deferoxamine mesylate or pseudobactin).

4.3.9 Phosphate solubilizing activity

The isolated bacteria were grown overnight in NB at 30 °C, 150 rpm. The bacterial cultures were transferred to sterile plastic tubes containing 10 ml of NBRIP medium. The tubes containing uninoculated medium served as controls. All tubes were incubated on a rotary shaker at 30 °C, 150 rpm for 72 h. The bacterial cultures were centrifuged to collect the supernatant. The phosphate in the supernatant was quantitatively estimated using the colorimetric method (King, 1932). The supernatant was measured into test tubes and deionized water was added to about 10 ml. 1 ml of 72% perchloric acid, 1 ml of 5% ammonium molybdate and 0.5 ml of 0.2% aminonaphtholsulphonic acid were then added. After that, deionized water was added to obtain the final volume of 15 ml. The contents in the test tube were gently shaken between each addition, and finally mixed by inverting and shaking. After 5 min, the absorbance was measured by spectrophotometer. The standards containing an appropriate amount of phosphate were analyzed at the same time and in the same way as samples to establish a standard curve (Fig. 5.24E).

4.4 Bioinoculation of plant by PGPR to promote plant growth and enhance metal uptake

4.4.1 Instruments

1) Autoclave: Isuzu, model 20-5030/5040, and Iwaki, model ACV-3167N
2) Critical point dryer (CPD)
3) Flame Atomic Absorption Spectrophotometer (FAAS; Variance SpectrAA 55B)
4) Incubator: Lab-Line, model Ami-Hi-Lo Chamber
5) Incubator shaker: Lab-Line, model 3530-2
6) Laminar flow hood: Issco, model Bvt124
7) Hot air oven
8) pH meter: EUTECH Instruments, model pH 510
9) Scanning Electron Microscope (SEM)
10) Spectrophotometer: Aquarius Cecil, model CE 7200
11) Vortex: Genie, model K-550-GE

4.4.2 Chemical preparations

4.4.2.1 Plant culture medium

Murashige and Skoog' medium (MS) was used in this experiment. It was prepared from 4 chemical stock solutions described as follows:

Macroelement stock solution contains the following ingredients in 1 liter of deionized water; 33 g of NH_4NO_3, 38 g of KNO_3, 8.8 g of $CaCl_2.2H_2O$, 7.4 g of $MgSO_4.7H_2O$, and 3.4 g of KH_2PO_4.

Microelement stock solution contains the following ingredients in 1 liter of deionized water; 166 mg of KI, 1.24 g of H_3BO_3, 4.46 g of $MnSO_4.4H_2O$, 1.72 g of $ZnSO_4.7H_2O$, 50 mg of $Na_2MoO_4.2H_2O$, 5 mg of $CuSO_4.5H_2O$, and 5 mg of $CoCl_2.6H_2O$.

Iron source stock solution was prepared by dissolving 5.56 g of $FeSO_4.7H_2O$ and 7.46 g of $Na_2EDTA.2H_2O$ in 1 liter of deionized water.

Organic supplement stock solution contains the following ingredients in 1 liter of deionized water; 20 g of Myoinositol, 100 mg of Nicotinic acid, 100 mg of Pyridoxine-HCl, 100 mg of Thiamine-HCl, and 400 mg of Glycine. This stock solution was freshly prepared every week and kept in –20 ℃.

To prepare MS medium, 50 ml of macroelement stock was placed into beaker. Deionized water was added. 5 ml of microelement, iron source, and organic supplement were then added. After that 30 g of sucrose was thoroughly mixed. Deionized water was added to bring the 1 liter volume. The pH of the medium was adjusted to 5.50±0.05. Finally, 15 g of agar was added and mixed. The medium was autoclave sterilized and dispensed to plant culturing glass bottles. They were left in room temperature to solidify. MS medium was freshly prepared before use.

4.4.3 Plant species and plant propagation

The model plant used in this experiment was sunflower (*Helianthus annuus*). It was a member of Asteraceae family. There was some evidence shown that *H. annuus* was a metal tolerant plant with low accumulation of Cd (Chen & Cutright, 2001). It was a useful plant used as a model to investigate the effects of PGPR on plant growth and metal uptake by plant. Seedling propagation was achieved because commercial seeds were available. The seeds obtained from CHIA TAI CO., LTD. Thailand had purity more than 95% and germination rate more than 85%.

4.4.4 Axenic seed

Plant seeds were surface sterilized by shaking in sterile 10% Clorox solution (filter sterilization) supplemented with detergent for 1 h. After that, the sterile seeds were washed by shaking in sterile deionized water for 30 min, three times. The sterile seeds were dried by placing on sterile filter paper (Abou-Shanab *et al*., 2006).

4.4.5 Bacterial inoculums

The isolated bacterial strains used in the experiments were *Chryseobacterium sp.* TKS21, *Comamonas testosteroni* TKS07, *Cupriavidus taiwanensis* TKS05, *Delftia sp.* TKS10, *Pseudomonas aeruginosa* TK S22, *Serratia marcesens* TKS01, and *Citrobacter sp*. BKS01. Bacterial inoculums were prepared by

growing isolated bacteria in NB overnight at 30 °C, 150 rpm. Bacterial cells were then harvested by centrifugation at 6000 rpm, 4 °C and washed by sterile water twice. They were suspended in sterile water. The absorbance at 600 nm of the bacterial suspensions served as inoculums were adjusted to 0.1.

4.4.6 Heavy metal contaminated soils

Cd/Zn contaminated soil used in the pot experiments was collected from Cd contaminated paddy fields near the Padaeng Zn mine. The soil was characterized by the Department of Soil Science, Kasetsart University, Thailand. Characteristics of soil are described in Table 4.1.

Table 4.1 Characteristics of soil used in the pot experiments.

Soil characteristics		
pH	6.5	
Organic matter (OM)	1.65 %	
Soil texture	Loam	
Total-N	0.10 %	
Phosphorus	248	mg kg^{-1}
Potassium	700	mg kg^{-1}
Calcium	2,600	mg kg^{-1}
Magnesium	300	mg kg^{-1}
EC (1:5)	2.71	dS m^{-1}
CEC	14.40	cmol kg^{-1}
Total Cd	39.35	mg kg^{-1}
DTPA-extractable Cd	5.91	mg kg^{-1}
Total soil Zn	1,651.31	mg kg^{-1}
DTPA-extractable Zn	83.84	mg kg^{-1}

4.4.7 Association between isolated bacteria and plant roots

The axenic seeds of *Helianthus annuus* were put into the MS media prepared in the culturing glass bottles. 4 seeds were put in each bottle. All bottles were incubated to geminate the plant at room temperature in the dark for 3 days. Germinated seeds were inoculated by the bacterial inoculums. The seeds without inoculation served as control. Fifteen bottles were conducted for each inoculum and 7 inoculums were prepared. All bottles were put on the shelves equipped with artificial light (12 h light/dark photo periods) at room temperature. After 15 days, plant roots were randomly taken and prepared for analysis by the scanning electron microscope (SEM). They were cut into small pieces, fixed in 6% glutaraldyhyde for 6 h. and post-fixed in 1% OsO_4. Samples were then rinsed by cacodylate buffer three times, dehydrated by ascending alcohol series (30%–100%) and dried in the critical point dryer (CPD). Finally, root samples were coated by gold particle and observed under the SEM at an accelerating voltage of 80 kV.

4.4.8 Plant growth promoted by the isolated bacteria

The axenic seeds of *Helianthus annuus* were put into the MS medium prepared in the culture glass bottles (4 seeds/bottle). All bottles were incubated to germinate the plant at room temperature in the dark for 3 days. Germinated seeds were inoculated by the bacterial inoculums. The seed without inoculation served as control. 20 bottles were conducted for each inoculum and 7 inoculums were prepared. All bottles were incubated on the shelves equipped with artificial light (12 h light/dark photo periods) at room temperature for 15 days. Plants were harvested and the shoot and root length and fresh weight were measured. Then they were dried at 70 °C and the dry weight determined.

4.4.9 Pot experiments

4.4.9.1 Pot experiment under laboratory conditions

A pot experiment was conducted to investigate the influences of PGPR on growth and metal uptake of plants, *Helianthus annuus*. Heavy metal contaminated soil was autoclave sterilized before use. 250 g of sterile soil was placed in plastic pots, and axenic seeds were put into the soil. Sterile deionized water was

then added. Plants were germinated and grown in closed shelf at 25±2 °C, 12 h light/dark photoperiod. 50 ml of bacterial inoculums were added into the soil after plant germination. Four isolated bacterial strains (*Chryseobacterium sp.* TKS21, *Comamonas testosteroni* TKS07, *Cupriavidus taiwanensis* TKS05, and *Delftia sp.* TKS10) were selected. 12 pots were conducted for each bacterial strain. The pots without bacterial inoculation served as control. Plants were grown for 60 days and then harvested for analysis. Plant growth and heavy metal accumulation influenced by isolated bacteria were determined. Bacterial population on plant roots was also determined by dilution plate method.

4.4.9.2 Pot experiment under environmental conditions

The experiment was conducted similar to that described in section 4.4.9.1 but the soil was not autoclave sterilized. Plants were germinated and grown in the greenhouse. Four isolated bacterial strains (*Chryseobacterium sp.* TKS21, *Comamonas testosteroni* TKS07, *Cupriavidus taiwanensis* TKS05, and *Delftia sp.* TKS10) were selected. 12 pots were conducted for each bacterial strain. The pots without bacterial inoculation served as control.

4.4.9.3 Plant analysis for heavy metal concentration

Whole plants were thoroughly washed and rinsed by deionized water. Plant growth was monitored by measuring shoot length, root length, plant fresh weight and plant dry weight. Heavy metal concentrations in plant samples were determined by the procedures described in section 4.1.5. Briefly, plant sample (1 g dry weight) was digested in HNO_3:$HClO_4$ using an open tube digestion technique. The concentrations of Cd and Zn were determined by FAAS.

4.4.10 Data and statistical analyses

The translocation factor (TF) and extraction coefficient (EC) described in the section 4.1.6 were also used for the data analysis.

Data were expressed as means with standard deviation. Analysis of variance (one way ANOVA; SPSS 11.5 computer software) was used to evaluate the effects of the isolated bacteria on the plant growth and the metal accumulation in plants. If the F-value showed significant differences ($p \leq 0.05$), means were compared with least significant difference method (LSD).

CHAPTER V
RESULTS

5.1 Plant screening for phytoremediation potential

5.1.1 Soil characterizations

The soil characteristics from five sampling sites were compared (Table 5.1). The soils from all five sampling sites had neutral pH values (7.1 – 7.6). The pH values in soils from sites 1, 2 and 3 were significantly greater than from those in sites 4 and 5 ($p<0.05$). The electrical conductivity (EC) values in soils from all sites were not significantly different ($p>0.05$). The percentage of total nitrogen in soil from site 5 was significantly greater than that in sites 1, 2 and 3 ($p<0.05$) but similar to that in soil from site 4. The available P concentrations in soils from site 1 and 2 were similar ($p>0.05$) but significantly different from those in sites 3 and 5 ($p<0.05$). However, the available P in soils from sites 4 and 5 were also similar. There was no significant difference in the available K concentrations among the sites. The organic matter (OM) in soil from site 5 was significantly different from that in all other sites ($p<0.05$). The organic matters of soil samples from sites 1 and 3; 2 and 4; and 5 were classified as very low, low and medium, respectively. The cation exchange capacity (CEC) in soil from site 5 was the highest (12.70±1.70 cmol kg^{-1}) and significantly different from those in other sites ($p<0.05$). Most characteristics of soils from sites 1, 2 and 3 were generally similar, except the available P concentrations, while the characteristics of soils from sites 4 and 5 were also similar, except OM and CEC.

Table 5.1 Soil characterization from five sampling areas.

Characteristics	**Site 1** *Tailing Pond area*	**Site 2** *Open pit area*	**Site 3** *Stockpile area*	**Site 4** *Forest area*	**Site 5** *Cd contaminated rice field*
pH	7.85±0.35[a]	7.60±0.00[a]	7.55±0.07[a]	7.10±0.00[c]	7.05±0.21[c]
EC (dS m^{-1})	0.97±0.92[a]	0.69±0.45[a]	0.58±0.37[a]	1.31±1.18[a]	0.93±0.59[a]
Total N (%)	0.03±0.01[a, b]	0.05±0.02[a, b]	0.03±0.01[a]	0.07±0.01[b, c]	0.11±0.02[c]
Available P (ppm)	5.50±2.12[a]	7.50±0.71[a]	19.5±4.95[b]	11.5±0.71[a, c]	17.0±2.83[b, c]
Available K (ppm)	29.0±14.1[a]	34.0±5.7[a]	23.5±0.7[a]	51.0±17.0[a]	56.5±3.5[a]
OM (%)	0.50±0.28[a]	0.80±0.42[a, b]	0.40±0.00[a]	1.40±0.14[b]	1.55±0.07[c]
CEC (cmol kg^{-1})	5.50±2.26[a]	6.60±0.99[a]	4.50±0.57[a]	8.00±0.71[a]	12.70±1.70[b]
Total Cd (mg kg^{-1})	596.0±136.0[a]	542.6±98.5[a]	894.0±341.1[a]	1,458.4±308.1[b]	63.5±2.0[c]
Extractable Cd (mg kg^{-1})	28.3±10.8[a]	31.0±5.6[a]	64.5±52.4[a]	47.6±29.0[a]	11.8±9.0[a]
Total Zn (mg kg^{-1})	20,672.5±6,752.3[a]	20,271.7±3,611.4[a]	31,319.0±9,504.1[a]	57,011.8±7,005.0[b]	2,732.7±91.7[c]
Extractable Zn (mg kg^{-1})	338.7±49.8[a]	453.7±135.0[a]	790.2±610.7[a]	639.0±409.4[a]	220.3±57.4[a]

Means followed by a common letter in the same row for each characteristic are not significantly different from each other using LSD test (p>0.05).
EC, electrical conductivity. OM, organic matter. CEC, Cation exchange capacity.

5.1.2 Cd and Zn concentrations in collected soils

Total and DTPA-extractable concentrations of heavy metals in surface soils and in soils around plant roots from five study sites are shown in Tables 5.1, 5.2, and 5.3. The total Cd concentration in soils was significantly and positively correlated (R^2 = 0.9143, n = 216) with total Zn concentrations (Fig. 5.1) indicating that Cd and Zn naturally coexist. The total Cd and Zn concentrations in surface soil from sites 1, 2 and 3 were significantly different (p<0.05) from those in soils from sites 4 and 5 while those in soils from sites 4 and 5 were also significantly different from each other (p<0.05). The Cd and Zn concentrations in surface soils were extremely high at site 4, moderately high at sites 1 – 3 and low at site 5 (Table 5.1). For the five study sites, the mean total concentrations of Cd in surface soils ranged from 64 – 1,458 mg kg^{-1} while that of Zn ranged from 2,733 – 57,012 mg kg^{-1} (Table 5.1).

The DTPA-extractable concentrations of Cd and Zn in soils from five sites were not significantly different (Table 5.1). The mean extractable concentrations of Cd ranged from the highest to the lowest as follows; site 3 (65 mg kg^{-1}) > site 4 (48 mg kg^{-1}) > site 2 (31 mg kg^{-1}) > site 1 (28 mg kg^{-1}) > site 5 (12 mg kg^{-1}). The mean extractable concentrations of Zn were ordered similarly; site 3 (790 mg kg^{-1}) > site 4 (639 mg kg^{-1}) > site 2 (454 mg kg^{-1}) > site 1 (339 mg kg^{-1}) > site 5 (220 mg kg^{-1}).

5.1.3 Cd and Zn concentrations in collected plants

A total of 36 plant species from 16 families (26 herbs, 2 shrubs, 2 undershrubs and 6 grasses) were collected from five sampling sites. The Cd and Zn concentrations in plant shoots and roots are presented in Table 5.2 and 5.3, respectively. Five plant species (*Justicia procumbens, Gynura pseudochina, Impatiens violaeflora, Chromolaena odoratum* and *Brachiaria sp*.) had Cd concentrations greater than 100 mg kg^{-1} dry weight in their shoots (the Cd hyperaccumulating level proposed by Baker *et al.* 1994) with the highest concentration (548 mg kg^{-1} dry weight) in *J. procumbens* (Fig. 5.2). Only *J. procumbens* had Zn concentration in its above surface biomass (11,071 mg kg^{-1} dry weight) greater than the 10,000 mg kg^{-1} dry weight proposed by Baker *et al*. (1994) as the Zn hyperaccumulating level.

Table 5.2 Cd concentrations (mg kg^{-1} dry weight) in soils around plant root, and in the shoot and root of plants collected from five sampling sites.

Family	*Species*	Type	Sites	Cd concentration (mg kg^{-1} dry weight)			
				Soil		Plant	
				Total Cd	Extractable Cd	Shoot	Root
Acanthaceae	*Justicia procumbens*	Herb	2	771.9±39.7	174.1±1.0	548.0±4.2	527.4±13.3
	Justicia sp.	Herb	5	116.6±3.23	17.5±0.6	1.8±0.1	18.8±0.4
Amaranthaceae	*Aerva sanguinolenta*	Herb	4	1,145.1±20.3	81.2±3.5	30.4±0.2	185.8±15.8
Araceae	*Colocasia esculenta*	Herb	5	51.9±2.0	6.4±0.5	6.7±0.1	16.4±0.3
Asteraceae	*Ageratum conyzoides*	Herb	5	104.2±0.4	14.6±0.3	20.5±0.4	14.3±0.7
	Bidens biternata	Herb	5	73.5±2.4	8.3±0.2	4.8±0.2	7.9±0.8
	Blumea mollis	Herb	5	69.9±1.7	18.7±1.0	8.3±0.1	14.3±0.7
	Blumea napifolia	Herb	5	53.5±1.2	15.7±0.6	13.0±0.2	8.0±0.8
	Chromolaena odoratum	Shrub	3	1,266.0±7.6	124.9±4.1	166.0±10.3	110.3±7.7
	Conyza sumatrensis	Herb	1	421.4±12.2	79.1±2.8	89.8±1.9	63.7±7.3
	Crassocephalum crepidioides	Herb	5	75.0±3.3	12.4±0.5	17.0±0.5	13.2±1.3
	Grangea maderaspatana	Herb	5	50.3±4.3	8.8±0.2	13.9±0.1	11.5±1.2
	Gynura pseudochina	Herb	2	184.4±4.5	22.4±0.6	457.7±8.8	76.3±2.0
	Laggera pteradonta	Herb	1	634.3±14.9	81.6±1.8	65.6±0.7	47.4±1.7
	Sonchus arvensis	Herb	1	61.0±3.3	17.1±0.7	47.8±1.3	18.9±2.1
	Spilanthes iahadicensis	Herb	5	58.5±1.0	6.8±0.1	0.5±0.1	2.7±0.5
	Wedelia trilobata	Herb	5	64.9±1.6	8.8±0.2	5.0±0.1	6.3±0.2
Balsaminaceae	*Impatiens violaeflora*	Herb	4	345.6±17.9	164.7±1.2	212.3±2.9	185.0±15.8
Boraginaceae	*Heliotropium indicum*	Herb	5	27.1±1.2	17.1±0.7	1.4±0.1	2.2±0.2
Buddlejaceae	*Buddleja asiatica*	Shrub	3	1,260.6±7.4	90.3±1.8	71.9±1.5	29.4±1.1
Cyperaceae	*Kylinga brevifolia*	Grass	5	34.6±0.1	4.6±0.4	24.1±0.2	17.5±3.4
Euphorbiaceae	*Euphorbia hirta*	Herb	5	54.7±2.5	12.5±0.1	15.1±0.3	22.6±2.3
Fabaceae	*Aeschynomene americana*	Herb	1	30.5±1.1	13.7±0.2	13.2±0.3	7.5±0.3
	Crotalaria montana	Herb	2	300.5±3.9	84.5±1.0	79.1±13.6	33.1±0.8
Lamiaceae	*Hyptis suaveolens*	Under shrub	5	11.7±0.3	6.8±0.1	1.2±0.3	10.0±0.6
Malvaceae	*Sida rhombifolia*	Herb	4	117.8±2.5	55.4±0.5	34.4±0.7	58.2±1.5
Onagraceae	*Ludwigia hyssopifolia*	Herb	5	60.0±2.5	6.4±0.5	0.4±0.2	3.9±0.4
Poaceae	*Brachiaria sp.*	Grass	4	537.8±32.5	168.9±6.4	137.3±6.9	647.3±67.5
	Eleusine indica	Grass	1	383.2±8.0	34.7±1.5	36.9±4.0	150.0±15.7
	Imperata cylindrica	Grass	1	521.1±6.6	40.6±3.4	53.0±3.7	133.2±16.6
	Neyraudia arundinacea	Grass	2	232.0±5.5	15.7±0.4	29.7±1.3	35.8±0.4
	Thysanolaena maxima	Grass	1	831.5±19.6	96.6±3.3	20.3±1.7	175.6±36.5
Rubiaceae	*Spermacoce remota*	Herb	5	65.6±2.0	19.1±1.5	2.0±0.1	5.0±0.5
	Rubia sp.	Herb	5	58.8±1.6	8.1±0.2	3.1±0.4	4.1±0.3
Scrophulariaceae	*Lindenbergia philippensis*	Under shrub	3	1,294.4±27.0	118.1±3.0	31.7±1.9	54.9±2.1
	Scoparia dulcis	Herb	5	99.0±2.8	15.5±0.1	4.2±0.2	12.5±2.0

Table 5.3 Zn concentrations (mg kg^{-1} dry weight) in soils around plant root, and in the shoot and root of plants collected from five sampling sites.

Family	*Species*	Type	Sites	Zn concentration (mg kg^{-1} dry weight)			
				Soil		Plant	
				Total Zn	Extractable Zn	Shoot	Root
Acanthaceae	***Justicia procumbens***	Herb	2	51,793.5±2,864.1	1,495.3±5.7	11,071.1±107.1	10,741.1±83.9
	Justicia sp.	Herb	5	3,541.9±118.3	409.5±8.8	205.1±49.0	6,529.2±146.0
Amaranthaceae	***Aerva sanguinolenta***	Herb	4	63,115.9±1,920.0	1,169.3±65.2	842.1±86.4	5,793.1±935.8
Araceae	***Colocasia esculenta***	Herb	5	1,851.1±69.6	184.5±5.8	316.7±8.9	5,029.2±31.5
Asteraceae	***Ageratum conyzoides***	Herb	5	3,129.2±62.5	312.3±9.4	148.0±2.9	1,625.0±335.9
	Bidens biternata	Herb	5	2,957.6±49.6	289.6±2.1	64.9±4.8	1,637.5±163.8
	Blumea mollis	Herb	5	3,099.4±49.1	260.5±13.2	64.6±1.0	885.0±46.0
	Blumea napifolia	Herb	5	2,763.3±38.5	224.8±14.2	77.3±1.5	432.5±43.3
	Chromolaena odoratum	Shrub	3	41,216.5±495.9	1,111.7±63.6	1,773.3±159.6	1,494.8±46.6
	Conyza sumatrensis	Herb	1	5,689.6±54.3	253.3±8.6	943.1±32.5	545.7±55.7
	Crassocephalum crepidioides	Herb	5	2,680.3±180.2	263.0±14.0	155.8±6.0	1,480.0±148.0
	Grangea maderaspatana	Herb	5	1,887.2±66.0	209.8±5.4	261.3±1.2	425.0±42.5
	Gynura pseudochina	Herb	2	16,740.7±697.9	1,682.3±113.0	6,171.6±179.6	3,579.3±116.0
	Laggera pteradonta	Herb	1	12,454.3±536.4	414.3±12.6	650.2±19.5	497.3±14.4
	Sonchus arvensis	Herb	1	1,243.1±134.5	91.8±4.3	210.1±9.3	479.2±47.9
	Spilanthes iahadicensis	Herb	5	1,966.7±62.0	178.6±4.0	97.1±6.7	1,636.7±209.3
	Wedelia trilobata	Herb	5	2,533.3±32.7	273.8±6.6	150.3±1.8	1,600.0±296.6
Balsaminaceae	***Impatiens violaeflora***	Herb	4	21,957.6±2,022.1	1,557.0±15.9	3,164.8±61.9	3,431.4±343.1
Boraginaceae	***Heliotropium indicum***	Herb	5	1,540.2±63.1	156.5±0.7	118.3±4.3	133.0±5.6
Buddlejaceae	***Buddleja asiatica***	Shrub	3	47,888.9±571.1	1,018.7±64.3	2,999.8±92.3	759.1±12.0
Cyperaceae	***Kylinga brevifolia***	Grass	5	1,191.2±12.7	137.3±2.8	442.3±2.2	3,186.3±415.4
Euphorbiaceae	***Euphorbia hirta***	Herb	5	1,951.2±37.0	127.3±3.2	155.3±7.3	282.7±28.3
Fabaceae	***Aeschynomene americana***	Herb	1	1,397.7±75.3	72.3±1.0	277.1±55.0	165.6±2.4
	Crotalaria montana	Herb	2	19,549.1±305.0	1,822.7±14.5	4,883.9±160.3	4,211.2±137.4
Lamiaceae	***Hyptis suaveolens***	Under shrub	5	318.5±2.1	49.8±2.0	46.4±1.7	117.3±5.8
Malvaceae	***Sida rhombifolia***	Herb	4	9,305.8±319.7	898.0±7.0	582.7±52.1	907.6±50.7
Onagraceae	***Ludwigia hyssopifolia***	Herb	5	3,750.4±65.5	284.5±2.5	132.6±4.5	1,862.5±186.2
Poaceae	***Brachiaria sp.***	Grass	4	33,339.5±3,151.1	1,550.0±28.2	2,494.8±22.5	44,029.1±1,310.2
	Eleusine indica	Grass	1	9,751.8±474.1	390.0±23.4	2,051.0±84.2	2,085.7±422.3
	Imperata cylindrica	Grass	1	14,739.3±296.4	392.2±29.9	1,019.7±34.8	4,603.1±340.7
	Neyraudia arundinacea	Grass	2	6,309.9±251.7	246.8±4.3	968.8±11.2	1,759.7±23.7
	Thysanolaena maxima	Grass	1	15,599.4±182.8	408.8±16.8	600.5±14.1	2,717.5±675.4
Rubiaceae	***Spermacoce remota***	Herb	5	2,639.6±98.9	158.0±13.4	129.4±3.4	129.9±13.0
	Rubia sp.	Herb	5	2,103.9±69.8	181.3±12.0	91.9±2.6	1,286.7±76.3
Scrophulariaceae	***Lindenbergia philippensis***	Under shrub	3	55,655.2±4,052.3	1,670.7±12.7	1,015.9±89.5	1,019.1±73.8
	Scoparia dulcis	Herb	5	3,280.8±54.9	288.2±3.9	158.4±31.1	2,582.5±367.2

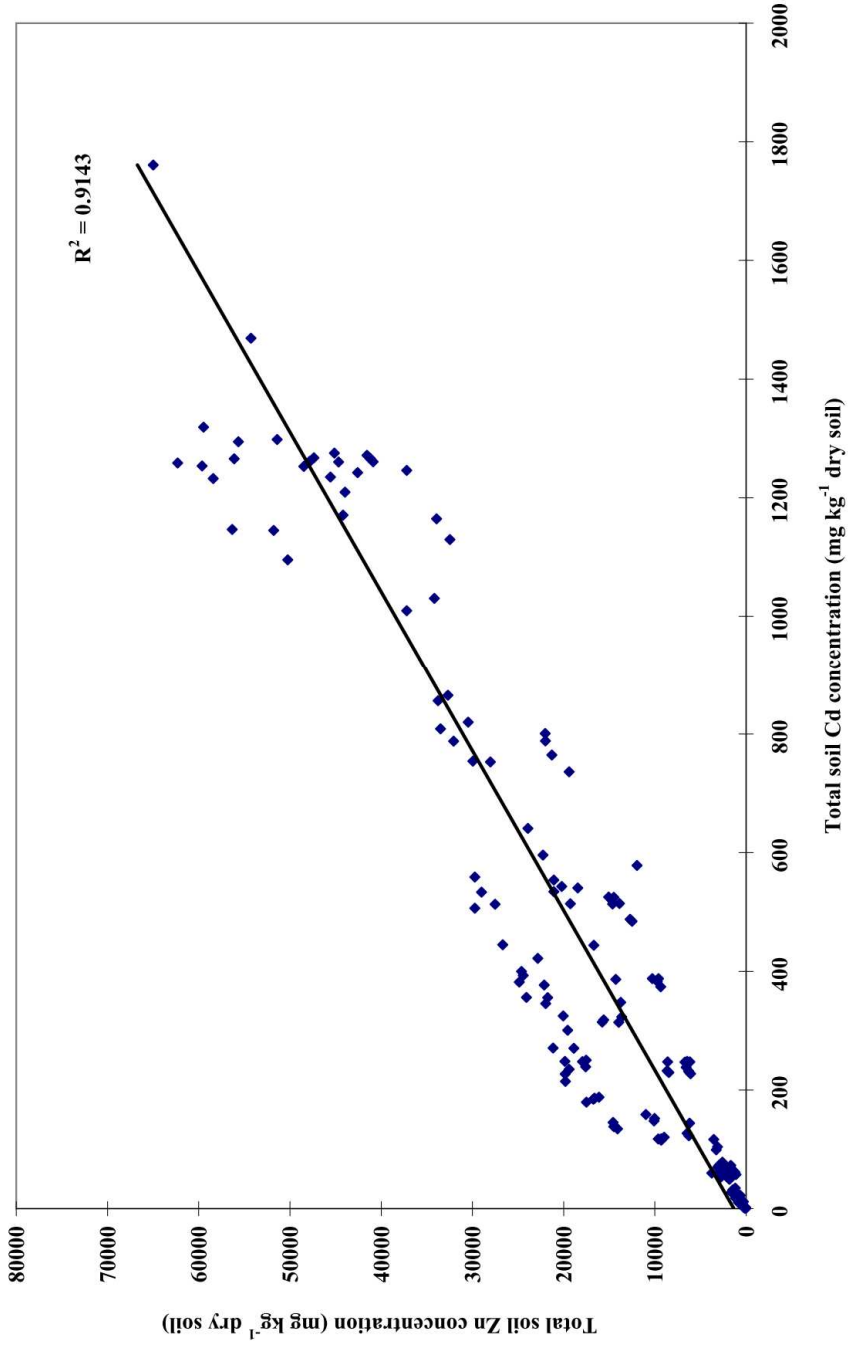


Figure 5.1 The correlation between total Cd and Zn concentrations in soil samples collected from five sampling sites.

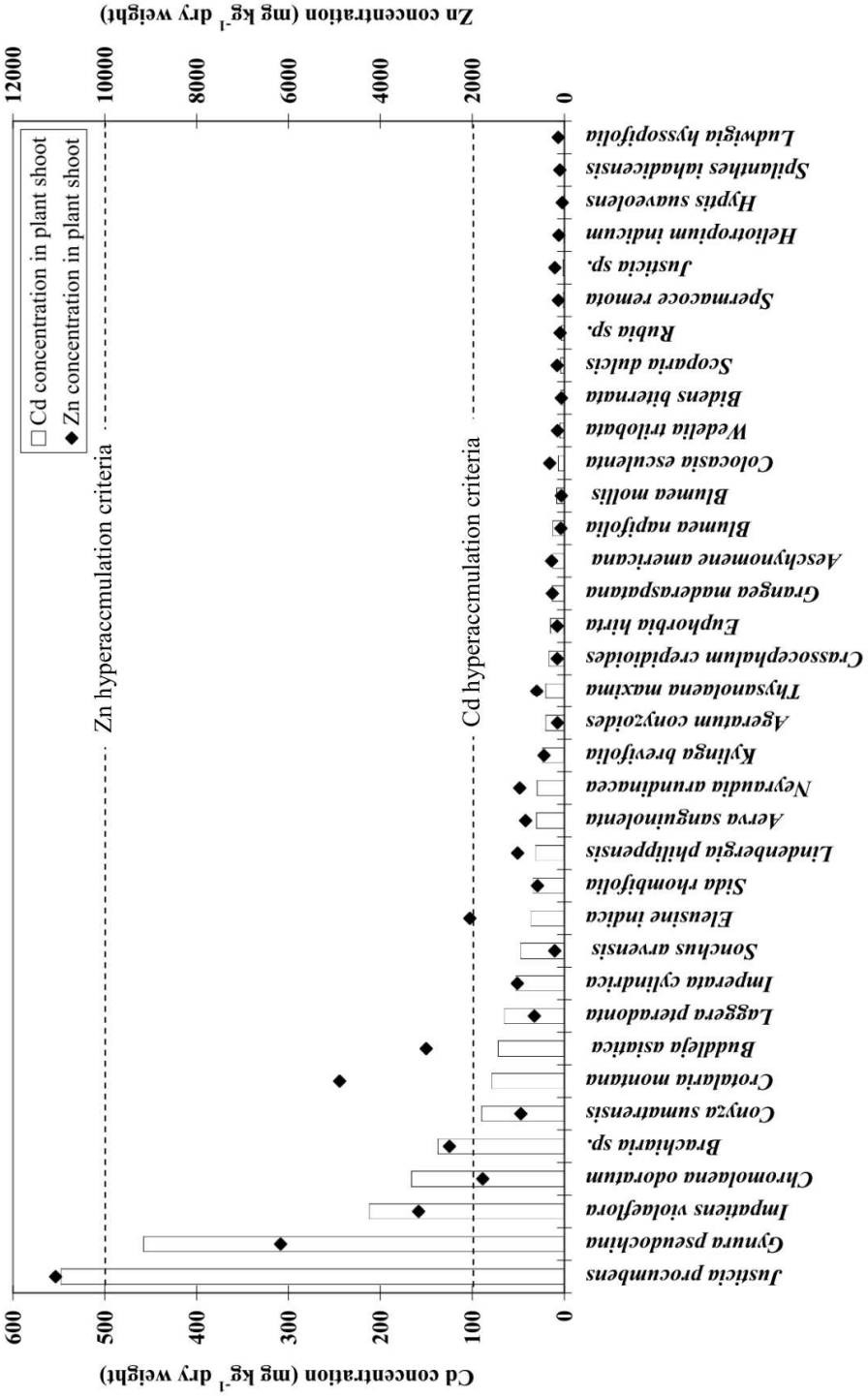


Figure 5.2 Cd and Zn concentrations in the shoots of plants collected from five sampling sites.

5.1.4 Potential hyperaccumulator plants based on different criteria

The translocation factors of the collected plants for Cd and Zn are shown in Table 5.4. The highest translocation factors for Cd (6.0) and Zn (3.95) were found in *Gynura pseudochina* and *Buddleja asiatica*, respectively. Fifteen plant species (for Cd) and eight plant species (for Zn) were found to have translocation factors greater than 1 (Fig. 5.3).

The extraction coefficients of the collected plants are shown in Table 5.4. The highest extraction coefficient for Cd (2.48) was found in *G. pseudochina* while the highest for Zn (0.37) was found in both *G. pseudochina* and *Kylinga brevifolia* (Table 5.4). Only *G. pseudochina* had the extraction coefficient more than 1 which is one of the criteria for identifying hyperaccumulator plants (Chen *et al.*, 2004).

The bioaccumulation factors are shown in Table 5.4. When the bioaccumulation factor was taken into account, the DTPA-extractable concentrations of metals were considered instead of total metal concentrations. Several plant species had a bioaccumulation factor greater than 1 (Fig. 5.3) and the highest bioaccumulation factors for Cd (20.48) and Zn (7.40) were found in *G. pseudochina* and *J. procumbens*, respectively.

In this study, 4 plants species (*Chromolaena odoratum*, *Gynura pseudochina*, *Justicia procumbens* and *Impatiens violaeflora*) were considered as the potential plants suitable for phytoremediation of Cd contaminated areas. They not only met many hyperaccumulating criteria but also possessed the characteristics useful for phytoremediation including high biomass production, good distribution and propagation (Table 5.5 and Figure 5.4).

Table 5.4 Translocation factor, extraction coefficient and bioaccumulation factor for Cd and Zn in plants collected from five sampling sites.

Family	*Species*	Translocation factor		Extraction coefficient		Bioaccumulation factor	
		Cd	Zn	Cd	Zn	Cd	Zn
Acanthaceae	***Justicia procumbens***	1.04	1.03	0.71	0.21	3.15	7.40
	Justicia sp.	0.10	0.03	0.02	0.06	0.10	0.50
Amaranthaceae	***Aerva sanguinolenta***	0.16	0.15	0.03	0.01	0.37	0.72
Araceae	***Colocasia esculenta***	0.41	0.06	0.13	0.17	1.04	1.72
Asteraceae	***Ageratum conyzoides***	1.43	0.09	0.20	0.05	1.41	0.47
	Bidens biternata	0.61	0.04	0.07	0.02	0.58	0.22
	Blumea mollis	0.58	0.07	0.12	0.02	0.44	0.25
	Blumea napifolia	1.61	0.18	0.24	0.03	0.82	0.34
	Chromolaena odorata	1.51	1.19	0.13	0.04	1.33	1.60
	Conyza sumatrensis	1.41	1.73	0.21	0.17	1.14	3.72
	Crassocephalum crepidioides	1.29	0.11	0.23	0.06	1.38	0.96
	Grangea maderaspatana	1.21	0.61	0.28	0.14	1.59	1.25
	Gynura pseudochina	6.00	1.72	2.48	0.37	20.48	3.67
	Laggera pteradonta	1.39	1.31	0.10	0.05	0.80	1.57
	Sonchus arvensis	2.52	0.44	0.78	0.17	2.80	2.29
	Spilanthes iahadicensis	0.17	0.06	0.01	0.05	0.07	0.54
	Wedelia trilobata	0.80	0.09	0.08	0.06	0.57	0.55
Balsaminaceae	***Impatiens violaeflora***	1.15	0.92	0.61	0.14	1.29	2.03
Boraginaceae	***Heliotropium indicum***	0.64	0.89	0.05	0.08	0.08	0.76
Buddlejaceae	***Buddleja asiatica***	2.45	3.95	0.06	0.06	0.80	2.94
Cyperaceae	***Kylinga brevifolia***	1.37	0.14	0.70	0.37	5.30	3.21
Euphorbiaceae	***Euphorbia hirta***	0.67	0.55	0.28	0.08	1.21	1.22
Fabaceae	***Aeschynomene americana***	1.77	1.67	0.43	0.20	0.97	3.84
	Crotalaria montana	2.39	1.16	0.26	0.25	0.94	2.68
Lamiaceae	***Hyptis suaveolens***	0.12	0.40	0.10	0.15	0.17	0.93
Malvaceae	***Sida rhombifolia***	0.59	0.64	0.29	0.06	0.62	0.65
Onagraceae	***Ludwigia hyssopifolia***	0.09	0.07	0.01	0.04	0.06	0.47
Poaceae	***Brachiaria sp.***	0.21	0.06	0.26	0.07	0.81	1.61
	Eleusine indica	0.25	0.98	0.10	0.21	1.06	5.26
	Imperata cylindrica	0.40	0.22	0.10	0.07	1.31	2.60
	Neyraudia arundinacea	0.83	0.55	0.13	0.15	1.89	3.92
	Thysanolaena maxima	0.12	0.22	0.02	0.04	0.21	1.47
Rubiaceae	***Spermacoce remota***	0.41	1.00	0.03	0.05	0.11	0.82
	Rubia sp.	0.75	0.07	0.05	0.04	0.38	0.51
Scrophulariaceae	***Lindenbergia philippensis***	0.58	1.00	0.02	0.02	0.27	0.61
	Scoparia dulcis	0.34	0.06	0.04	0.05	0.27	0.55

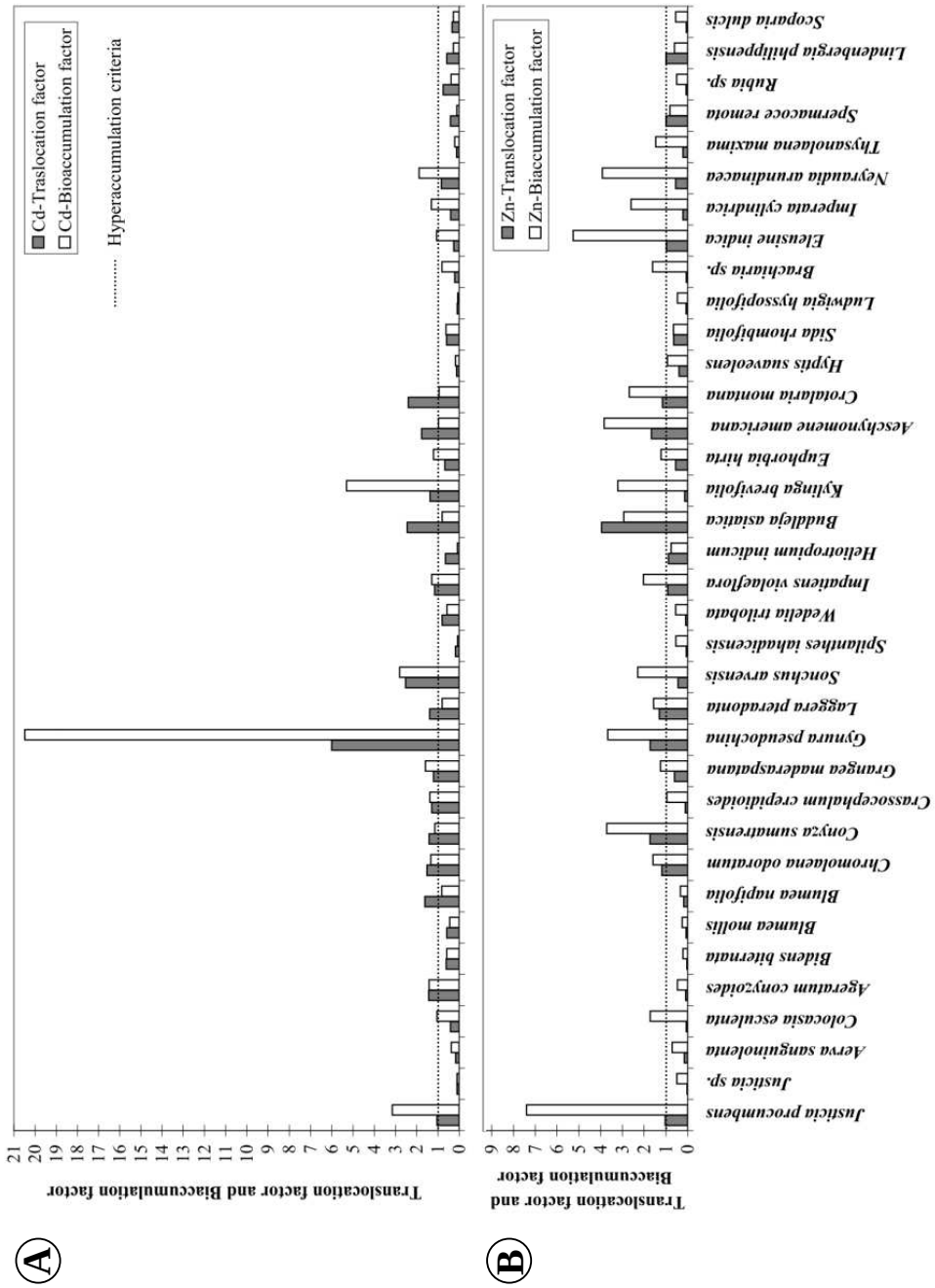


Figure 5.3 The translocation factors and bioaccumulation factors for Cd **(A)** and Zn **(B)** in plants collected from five sampling sites.

Table 5.5 The plant species considered as the potential candidates for phytoremediation of Cd contaminated soils.

Criteria	Plant species			
	Chromolaena odoratum	*Gynura pseudochina*	*Justicia procumbens*	*Impatiens violaeflora*
Type	Shrub	Herb	Herb	Herb
Cd concentration in shoot (mg kg^{-1} dry mass)	166.0±10.3	457.7±8.8	548.0±4.2	212.3±2.9
Translocation factor	1.51	6.00	1.04	1.15
Extraction coefficient	0.13	2.48	0.71	0.61
Bioaccumulation factor	1.33	20.48	3.15	1.29
Height (m)	1.0 – 2.0	0.4 – 1.0	0.3 – 0.5	< 0.3
Shoot dry mass (g/individual)	26.7±9.2 (< 1m)	5.7±2.4	3.3±0.9	2.4±1.1
Season	Rainy and drought	Rainy	Rainy	Rainy
Distribution	Excellent	Good	Good	Good
Propagation (natural habitat)	Excellent	Fair	Excellent	Excellent

Figure 5.4 Cd hyperaccmulators from the Padaeng Zn mine.
(A) *Chromolaena odoratum*, **(B)** *Gynura pseudochina*,
(C) *Justicia procumbens*, and **(D)** *Impatiens violaeflora*.

5.2 Accumulation, toxicity and localization of Cd in *Chromolaena odoratum*

5.2.1 Plant growth and morphologically toxicity symptoms

The growth of *Chromolaena odoratum* cultured in Hoagland's solution supplemented with various Cd concentrations is shown in Table 5.6 and Figure 5.5. The differential fresh weight, shoot and root length of Cd treated plants were significantly different ($p<0.05$) from that of control plants. They were decreased when Cd concentrations in culture media were increased (Fig. 5.5). The relative growth rates (RGR) of Cd treated plants (% as control) were obviously decreased with increasing Cd concentrations (Fig. 5.5). Similarly the tolerance indices (TI) of Cd treated plants were also declined (Table 5.6). The RGR of the plants treated with Cd at the concentrations of more than 2.5 mg l^{-1} were found to be less than 50% compared with control. Plants treated with 10 mg Cd l^{-1} has the minimum RGR (5.24±0.96), differential shoot length (1.31±0.70 cm $plant^{-1}$), and root length (0.56±2.44 cm $plant^{-1}$).

The external morphology of roots and leaves of Cd treated plants are shown in Figures 5.6 and 5.7, respectively. No visible toxicity symptoms were observed in the roots of the plants treated with Cd at the concentrations less than 2.5 mg l^{-1} (Fig. 5.6). Browning in roots was observed in plants exposed to Cd at more than 5 mg l^{-1}, some showed root fragmentation (at 10 mg Cd l^{-1}) (Fig. 5.6E). Morphologically toxicity symptom observed in leaves of plants treated with Cd at 10 mg l^{-1} was occurrence of many red spots in the vein as well as in the petioles (Fig. 5.7).

Table 5.6 Dry weight, differential fresh weight and shoot and root length of *Chromolaena odoratum* hydroponically cultured in Hoagland's solution supplemented with various Cd concentrations for 14 days.

Cd treatment ($mg\ l^{-1}$)	Dry weight ($g\ plant^{-1}$)			Fresh weight* ($g\ plant^{-1}$)	Shoot length** ($cm\ plant^{-1}$)	Root length*** ($cm\ plant^{-1}$)	Relative growth rate (RGR) (% as control)	Tolerance index (TI) (%)
	leaf	stem	root					
0	0.47 ± 0.20^{a}	0.55 ± 0.21^{a}	0.17 ± 0.14^{a}	2.15 ± 0.88^{a}	6.19 ± 2.70^{a}	8.50 ± 2.38^{a}	100.00 ± 0.00	100.00
0.5	0.24 ± 0.10^{b}	0.56 ± 0.18^{a}	$0.15 \pm 0.04^{a,b}$	1.15 ± 0.78^{b}	3.38 ± 1.83^{b}	7.63 ± 3.34^{a}	69.42 ± 7.24	89.76
2.5	0.23 ± 0.06^{b}	0.62 ± 0.21^{a}	$0.10 \pm 0.04^{a,b}$	$0.94 \pm 0.52^{b,c}$	$2.88 \pm 1.81^{b,c}$	4.25 ± 2.51^{b}	37.38 ± 3.07	50.00
5.0	0.15 ± 0.04^{b}	0.35 ± 0.13^{b}	0.08 ± 0.07^{b}	$0.44 \pm 0.32^{c,d}$	$1.81 \pm 1.65^{b,c}$	1.44 ± 2.72^{c}	26.01 ± 2.35	16.94
10.0	0.17 ± 0.02^{b}	0.28 ± 0.10^{b}	0.08 ± 0.07^{b}	0.09 ± 0.14^{d}	1.31 ± 0.70^{c}	0.56 ± 2.44^{c}	5.24 ± 0.96	6.59

* Fresh weight = the fresh weight after treatment – the fresh weight before treatment

** Shoot length = the shoot length after treatment – the shoot length before treatment

*** Root length = the root length after treatment – the root length before treatment

The data is presented as Mean±SD. Means followed by a common letter in the same column are not significantly different from each other using LSD test ($p>0.05$).

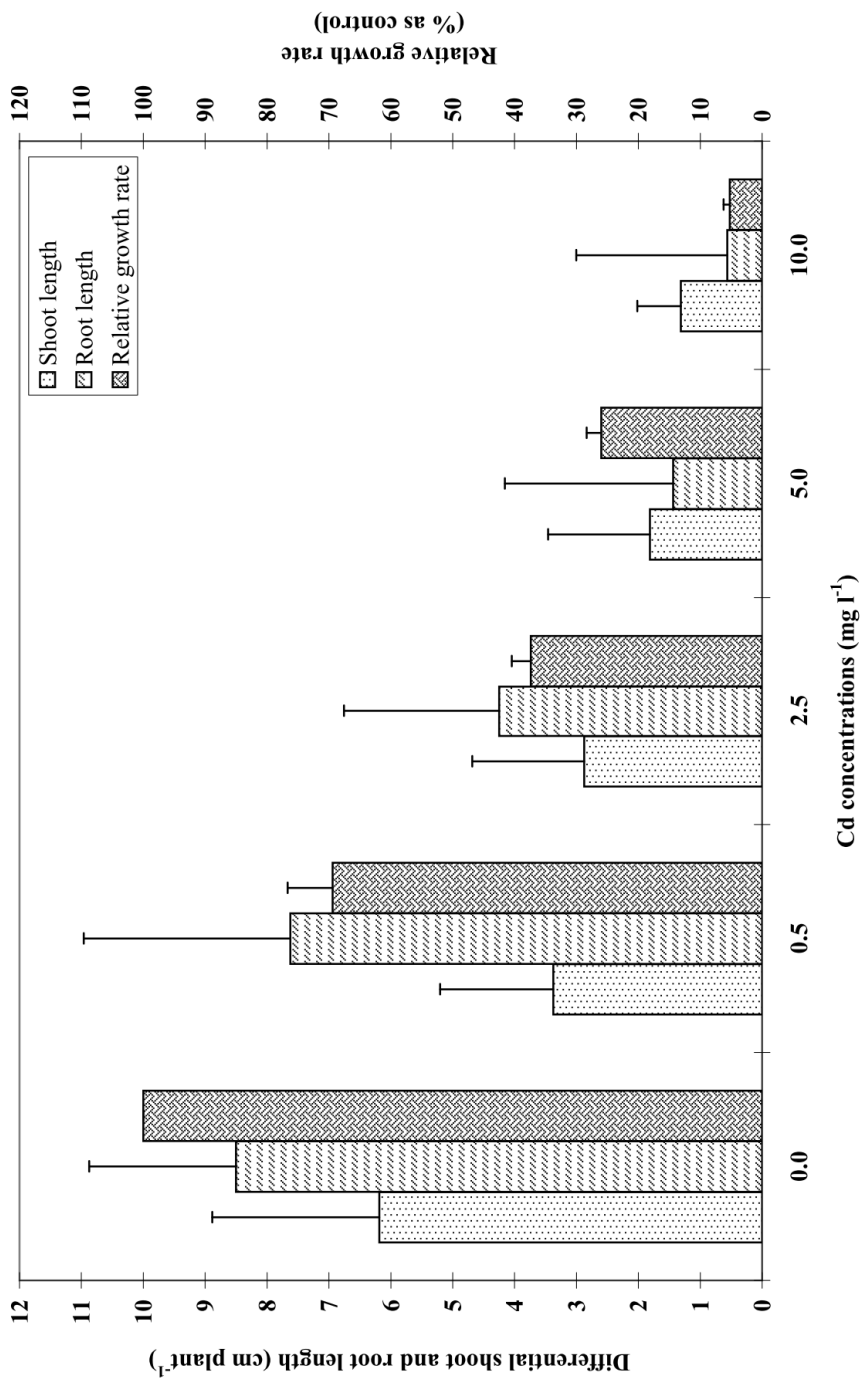
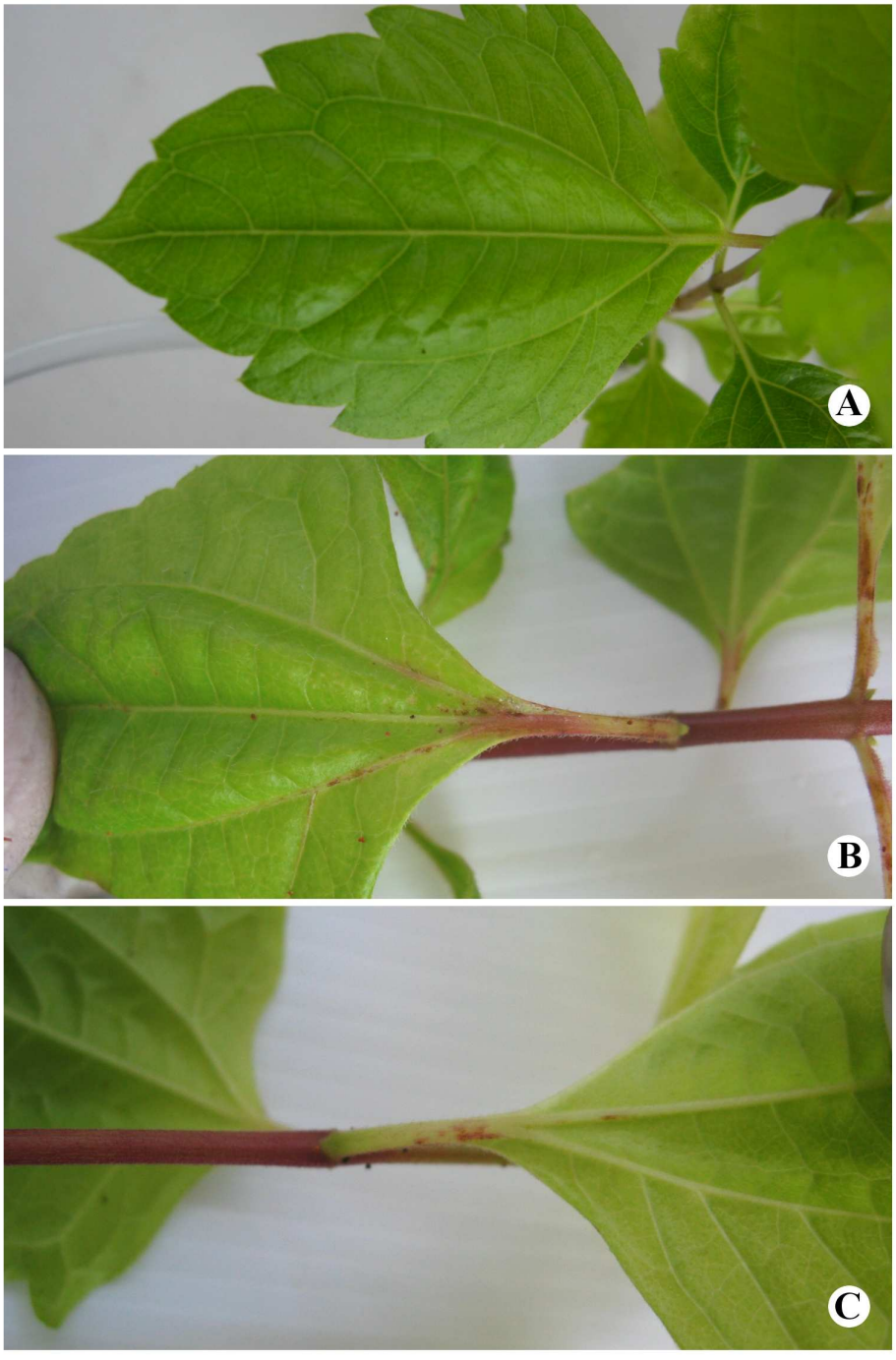


Figure 5.5 Growth of *Chromolaena odoratum* hydroponically cultured in Hoagland's solutions supplemented with various Cd concentrations for 14 days.

Figure 5.6 Root morphology of *Chromolaena odoratum* cultured in Hoagland's solution supplemented with various Cd concentrations for 14 days.
(A) Control
(B) 0.5 mg Cd l^{-1}
(C) 2.5 mg Cd l^{-1}
(D) 5.0 mg Cd l^{-1}
(E) 10 mg Cd l^{-1}
Obviously, the plant roots were changed in color from white to dark brown when they were exposed to elevated Cd concentrations.


A
B
C
D
E

Figure 5.7 Leaf morphology of *Chromolaena odoratum* cultured in Hoagland's solution supplemented with Cd for 14 days.
(A) Control
(B), (C) 10 mg Cd l^{-1}
Many red spots were found in the veins of the leaves and in the petioles of plants exposed to high Cd concentration.


A
B
C

5.2.2 Cd accumulation and removal capacity

Cd accumulations in *Chromolaena odoratum* cultured in Hoagland's solution supplemented with various Cd concentrations are presented in Table 5.7 and Figure 5.8. The Cd concentrations in plant tissues (leaves, stems and roots) were significantly increased when the increased Cd concentration in culture media ($p<0.05$). Exceptionally low Cd concentrations were found in leaves and stems of the plants grown in 10 mg Cd l^{-1} solution (Fig. 5.8). The percentage metal uptake by plant was declined with increased Cd concentrations. The maximum (79.42) and minimum (17.24) percentages of metal uptake were found in plants treated with 0.5 and 10.0 mg Cd l^{-1}, respectively. Similarly the bioaccumulation coefficients (BC) were also decreased when the Cd concentrations in culture media were increased with the minimum BC (441.54) found in plants treated with 10 mg Cd l^{-1} (Table 5.7).

Table 5.7 Cd concentrations in plant tissues (leaves, stems, and roots) of *Chromolaena odoratum* cultured in Hoagland's solution supplemented with various Cd concentrations together with percentage metal uptake and bioaccumulation coefficient.

Cd treatment ($mg\ l^{-1}$)	Percentage metal uptake (%)	Cd concentration in plant tissues ($mg\ kg^{-1}$ dry weight)				Bioaccumulation coefficient (BC)
		Leaf	Stem	Shoot	Root	
0	0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	-
0.5	79.42	33.19 ± 15.18^{b}	42.80 ± 13.92^{b}	75.99 ± 16.86^{b}	484.56 ± 98.58^{b}	1121.10
2.5	50.85	103.26 ± 27.95^{c}	162.81 ± 49.73^{c}	266.07 ± 51.94^{c}	1669.71 ± 392.56^{c}	774.31
5.0	31.18	148.45 ± 43.18^{d}	268.06 ± 85.28^{d}	416.51 ± 83.73^{d}	3535.06 ± 1231.58^{d}	790.31
10.0	17.24	2.31 ± 0.67^{a}	24.07 ± 11.45^{a}	26.39 ± 11.84^{a}	4389.04 ± 715.38^{e}	441.54

The data is presented as Mean±SD. Means followed by a common letter in the same column are not significantly different from each other using LSD test ($p>0.05$)

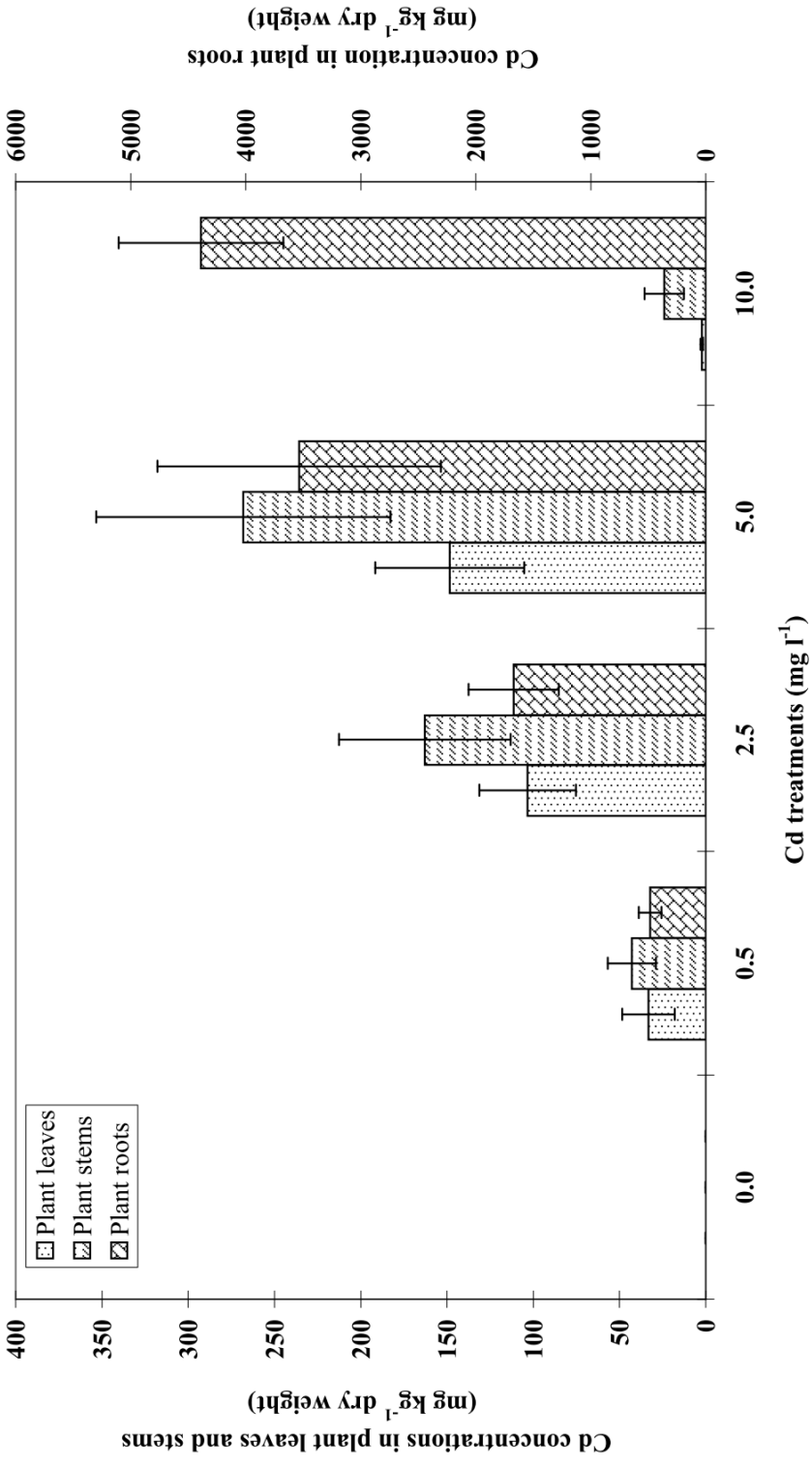


Figure 5.8 The Cd concentrations in the tissues of *Chromolaena odoratum* cultured in medium supplemented with various Cd concentrations for 14 days.

5.2.3 Ultrastructural changes in plant tissues caused by Cd toxicity

The structural changes in root and leaf tissues of *Chromolaena odoratum* hydroponically grown in Hoagland's solution supplemented with Cd were investigated using both light microscope and transmission electron microscope (TEM). The light micrograph showing the cross sections of root and leaf tissues are shown in Figure 5.9 and 5.10, respectively. In plants grown in media without Cd (designed as control plants), the root tissues typically contained epidermis, cortex, endodermis and vascular cylinder (Fig. 5.9A and B). While leaf tissues were composed of epidermis, mesophyll and vascular bundle (vein). Mesophyll layer differentiated into palisade and spongy parenchyma with abundant chloroplasts (Fig. 5.10A and B).

When compared with control plants, no structural changes were found in root tissues of the plants treated with Cd at the concentration less than 2.5 mg l^{-1} (Fig. 5.9C and D). While the loss of common structures of cortex and vascular system as results of the destruction of parenchyma cells were obviously found in the root tissues of plants treated with Cd at the concentrations more than 5.0 mg l^{-1}, especially in plants treated with 10 mg Cd l^{-1} (Fig. 5.9E and F).

As compared with control plants, no obvious changes were found in the leaf tissues of plants treated with Cd at the concentrations less than 2.5 mg l^{-1} (Fig. 5.10C and D). Chloroplasts in leaf tissues of plants treated with elevated Cd concentrations were swollen, especially those in plants treated with 10 mg Cd l^{-1} (Fig. 5.10 F).

The ultrastructural changes, caused by Cd, in plant organelles were also investigated through the TEM. The TEM micrographs of mitochondria and chloroplasts are shown in Figure 5.11 and Figure 5.12, respectively. Normal mitochondria were bound by two membranes and the inner membranes were cristae (Fig. 5.11A). Normal chloroplasts were usually found with their broad surfaces parallel to the cell wall. The internal compartment was composed of stroma (matrix) which was transversed by an elaborate system of thylakoids (Fig. 5.12A). Chloroplast often contained starch grains and lipids called plastoglobuli. When compared with normal mitochondria in control plants (Fig. 5.11A), the mitochondria in plants treated with Cd at the concentrations less than 2.5 mg l^{-1} were found to be normal (Fig. 5.11B and C). While the outer membrane of mitochondria in plants treated with 5 mg Cd l^{-1} was damaged (Fig. 5.11 D) and the loss of normal mitochondrial structure with disruption of internal cristae was

found in plants treated with 10 mg Cd l^{-1} (Fig. 5.11 E). When compared with the normal chloroplasts in control plants (Fig. 5.12A), the chloroplasts of the plants treated with Cd at the concentrations less than 2.5 mg l^{-1} were found to be normal (Fig. 5.12B and C). While the chloroplasts of plants treated with higher Cd concentrations were swollen and had an increased number of starch grains (Fig. 5.12D). Disruption of thylakoid membranes and disappearance of chloroplast membranes were also observed, especially severe damages were found in chloroplast of the plants exposed to 10 mg Cd l^{-1} (Fig 5.12E).

Chromolaena odoratum could grow in the media supplemented with Cd at the concentrations of less than 2.5 mg l^{-1} without any visible morphological changes in both leaves and roots. Moreover no structural changes in leaf and root tissues as well as no structural deformities in plant organelles (mitochondria and chloroplasts) were found in plants grown in media with Cd concentration less than 2.5 mg l^{-1}. The tissue damages and organelle deformities were found in plants treated with Cd at the concentrations more than 5 mg l^{-1}. Especially severe damages were found in plants treated with 10 mg Cd l^{-1}. Based on these results, *C. odoratum* could grow and tolerate Cd when they were cultured in the media with Cd at the concentrations less than 2.5 mg l^{-1}.

Figure 5.9 Light micrographs showing cross sections of root tissues of *Chromolaena odoratum* grown in Hoagland's solution supplemented with Cd for 14 days.
(A), (B) are low and high magnification of root tissues in the plants grown in media without Cd (control)
(C) 0.5 mg Cd l^{-1}
(D) 2.5 mg Cd l^{-1}
(E) 5.0 mg Cd l^{-1}
(F) 10 mg Cd l^{-1}
The loss of normal structure in root tissues was found in the plants treated with Cd at the concentration more than 5 mg l^{-1}.
Ep = Epidermis, C = Cortex, En = Endodermis, VS = Vascular System

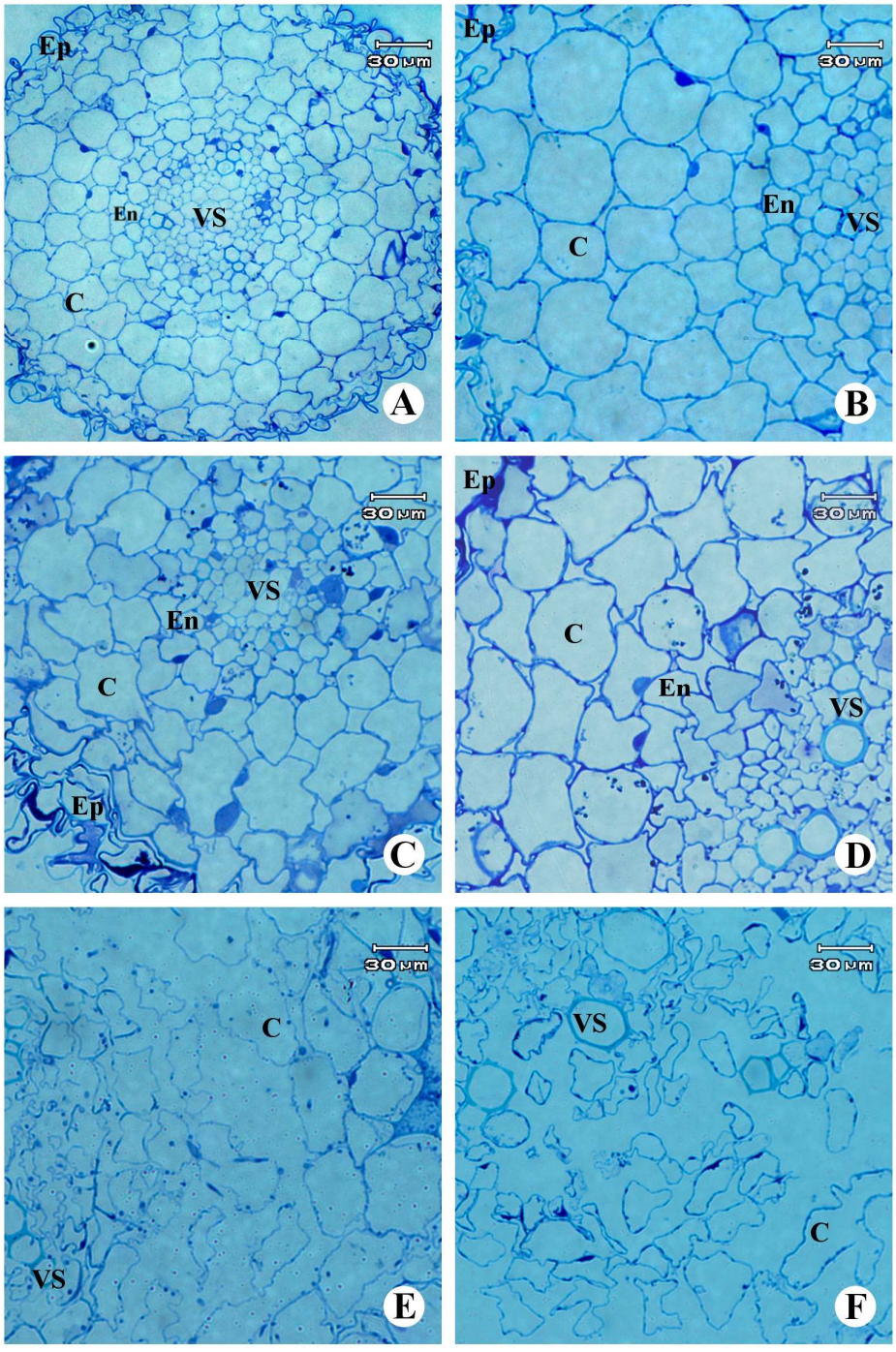

Ep
30 µm
En
VS
C
A
Ep
30 µm
En
VS
C
B
30 µm
VS
En
C
Ep
C
Ep
30 µm
C
En
VS
D
30 µm
C
VS
E
30 µm
VS
C
F

Figure 5.10 Light micrographs showing cross sections of leaf tissues of *Chromolaena odoratum* grown in Hoagland's solution supplemented with Cd for 14 days.
(A), (B) are low and high magnification of leaf tissues in control plants
(C) 0.5 mg Cd l^{-1}
(D) 2.5 mg Cd l^{-1}
(E) 5.0 mg Cd l^{-1}
(F) 10 mg Cd l^{-1}
The chloroplasts in the plants treated with Cd more than 5 mg l^{-1} were found to be swollen, especially in 10 mg Cd l^{-1} treatment.
Ep = Epidermis, PM = Palisade mesophyll, SM = Spongy mesophyll, VS = Vascular system, Arrows indicate abnormal chloroplast

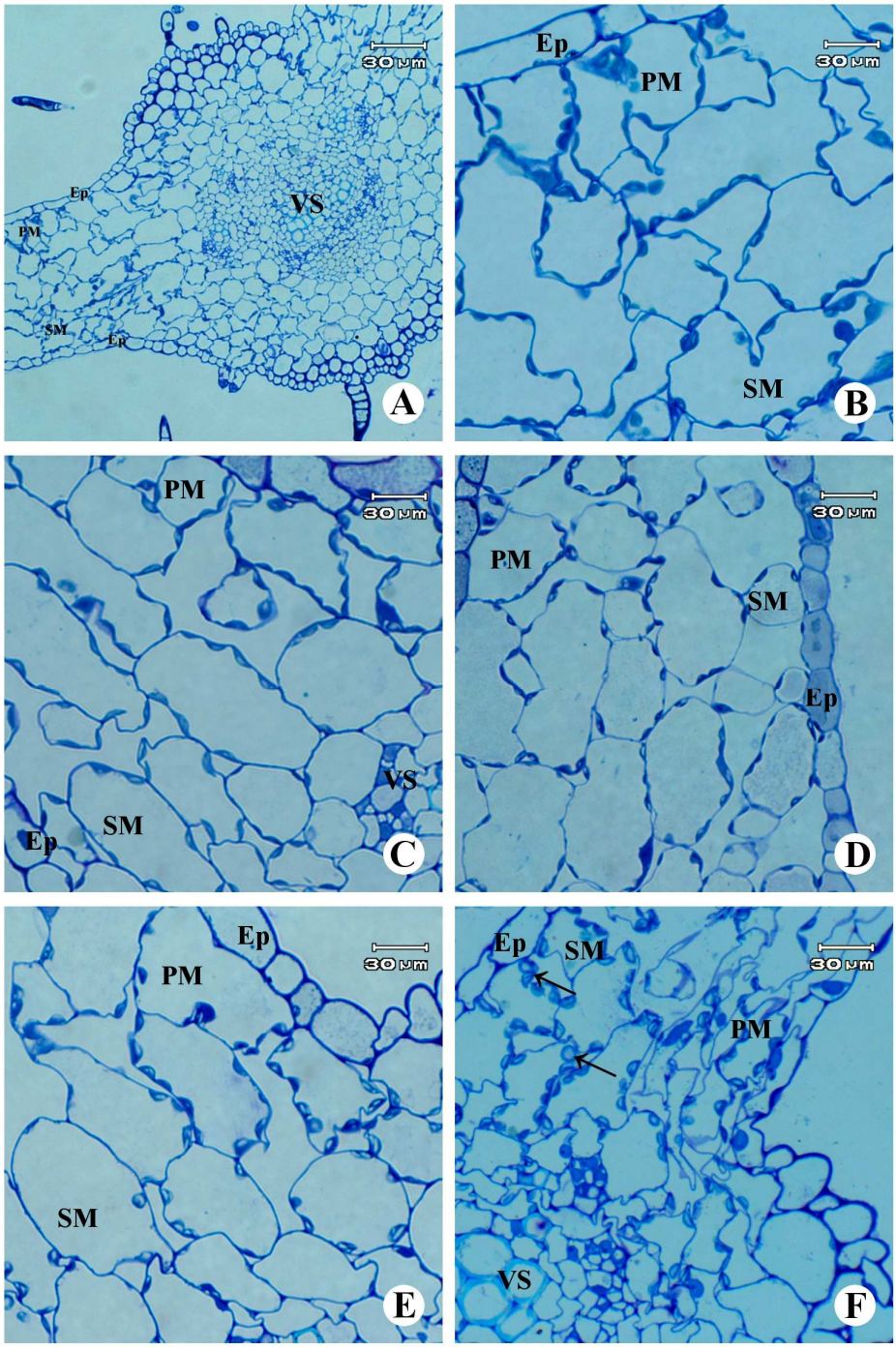

30 µm
Ep
PM
VS
SM
A
B
C
D
E
F

Figure 5.11 TEM micrograph showing mitochondria of *Chromolaena odoratum* grown in Hoagland's solution supplemented with Cd for 14 days.
(A) Control
(B) 0.5 mg Cd l^{-1}
(C) 2.5 mg Cd l^{-1}
(D) 5.0 mg Cd l^{-1}
(E) 10 mg Cd l^{-1}
Arrows indicate ultrastructural changes in mitochondria.
M = Mithochondria, Th = Thylakoid membrane, CW = Cell wall,
Ch = Chloroplast, ICS = Intercellular space

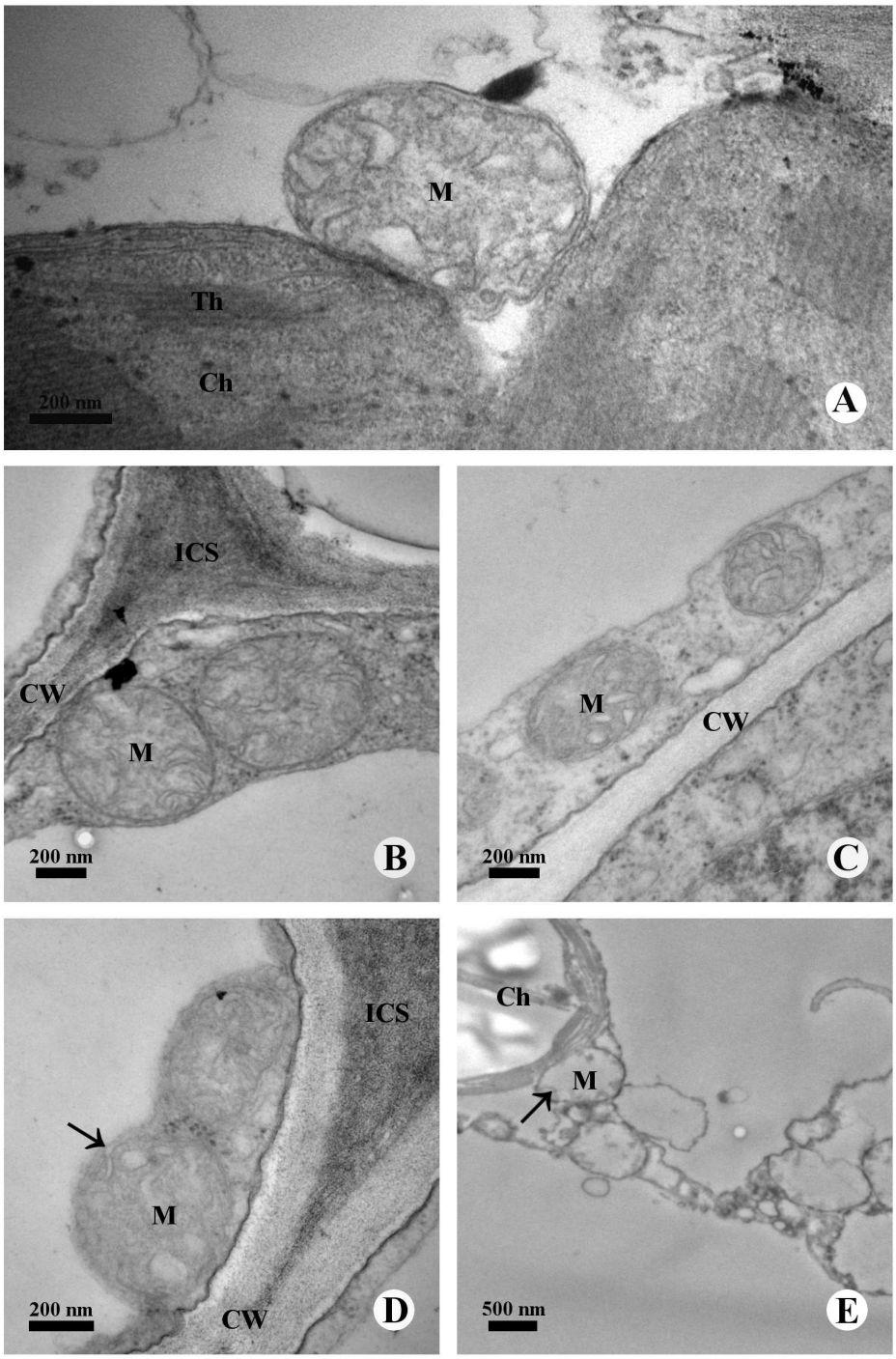
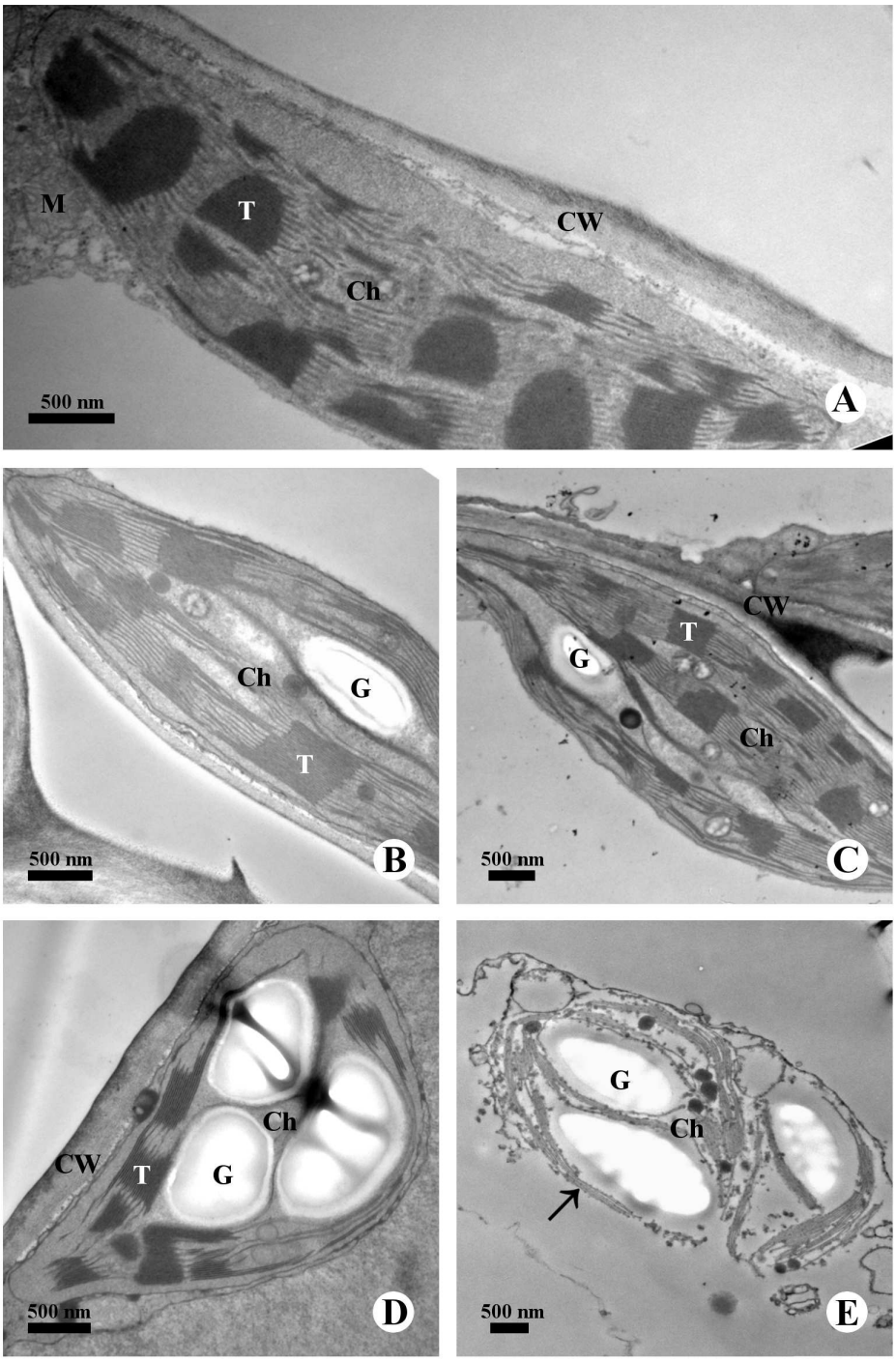

M
Th
Ch
200 nm
A
ICS
CW
M
200 nm
B
M
CW
200 nm
C
ICS
M
200 nm
CW
D
Ch
M
500 nm
E

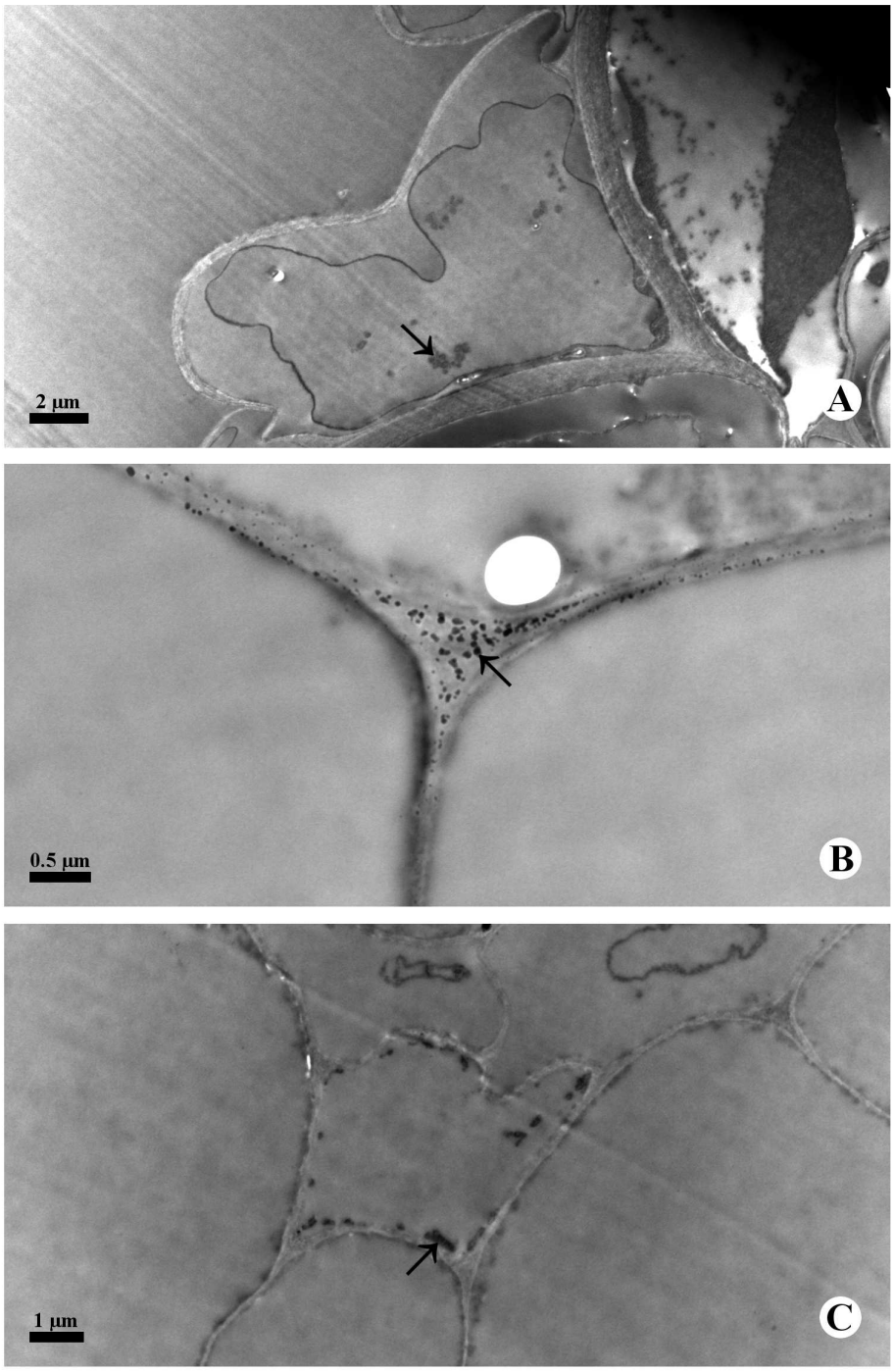
Figure 5.12 TEM micrograph showing chloroplast of *Chromolaena odoratum* grown in Hoagland's solution supplemented with Cd for 14 days.
(A) Control
(B) 0.5 mg Cd l^{-1}
(C) 2.5 mg Cd l^{-1}
(D) 5.0 mg Cd l^{-1}
(E) 10 mg Cd l^{-1}
Arrow indicates ultrastructural changes in chloroplast. Swollen starch grains were found in chloroplasts of plants treated with elevated Cd concentration.
M = Mithochondria, Ch = Chloroplast, CW = Cell wall,
T = Thylakoid membrane, G = starch grain


M
T
CW
Ch
500 nm
A
Ch
G
T
500 nm
B
CW
T
G
Ch
500 nm
C
CW
T
G
Ch
500 nm
D
G
Ch
500 nm
E

5.2.4 Cd localization

Cd localization was performed in root and leaf tissues of *Chromolaena odoratum* grown in Hoagland's solution supplemented with Cd at the concentration of 2.5 mg l^{-1}. TEM micrographs of root and leaf tissues showing dark deposits where Cd was accumulated are shown in Figures 5.13 and 5.15, respectively. While the EDAX spectra of Cd confirmed the dark deposits found in root and leaf tissues are shown in Figures 5.14 and 5.16. In root tissues, Cd deposits were found in epidermal cells, cell wall as well as intercellular spaces and parenchyma cells close to the vascular system. In leaf tissues, Cd deposits were found in mesophyll cells and in epidermal cells.

Figure 5.13 TEM micrographs showing root cells of *Chromolaena odaratum* grown in media supplemented with Cd.
(A) Epidermal cell.
(B) Intercellular space among cells located in cortex tissue
(C) Parenchyma cell located close to the vascular system
Arrows indicate the Cd rich sites which were confirmed by EDAX spectra shown in Fig. 5.14


2 µm
A
0.5 µm
B
1 µm
C

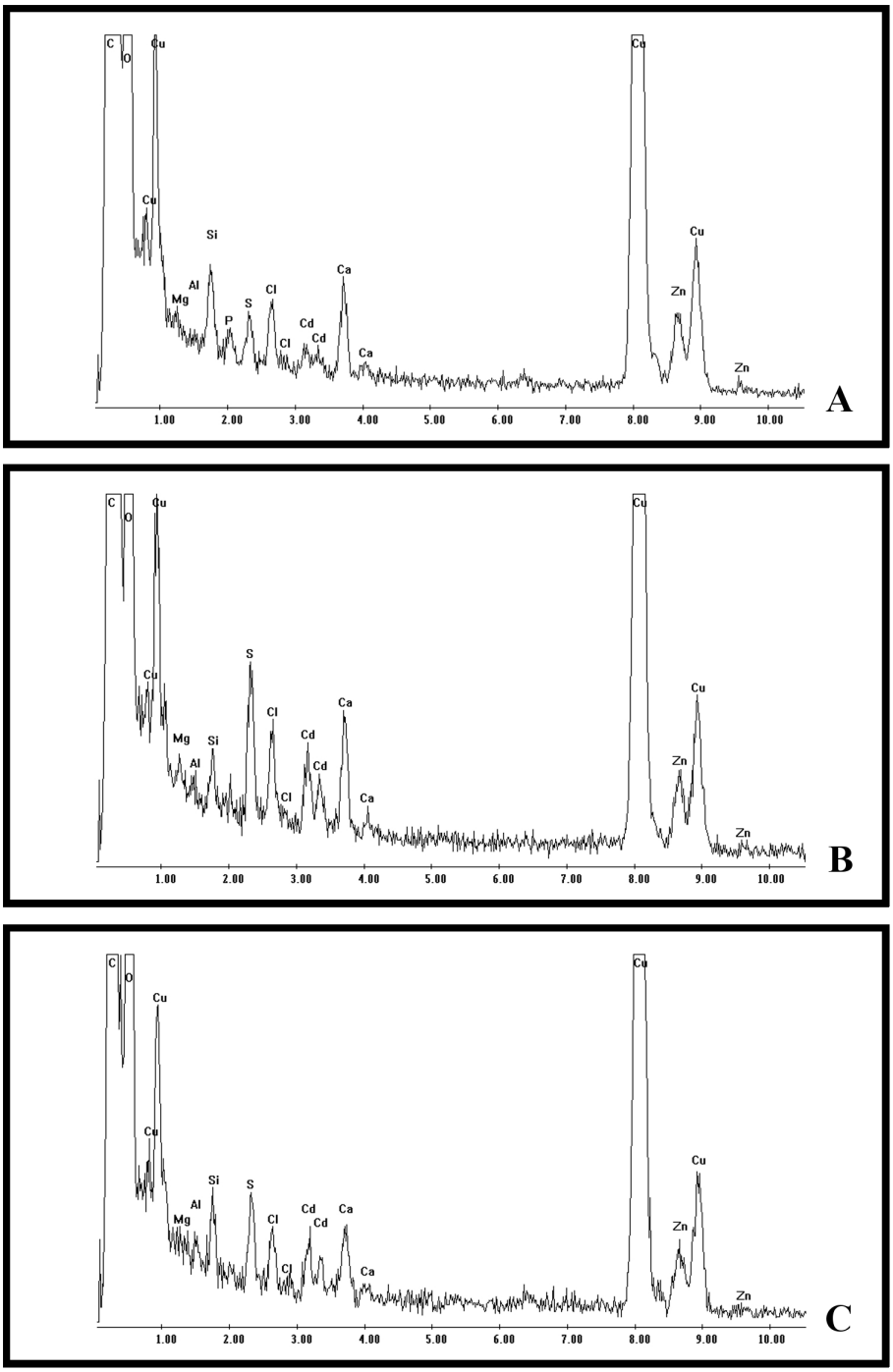
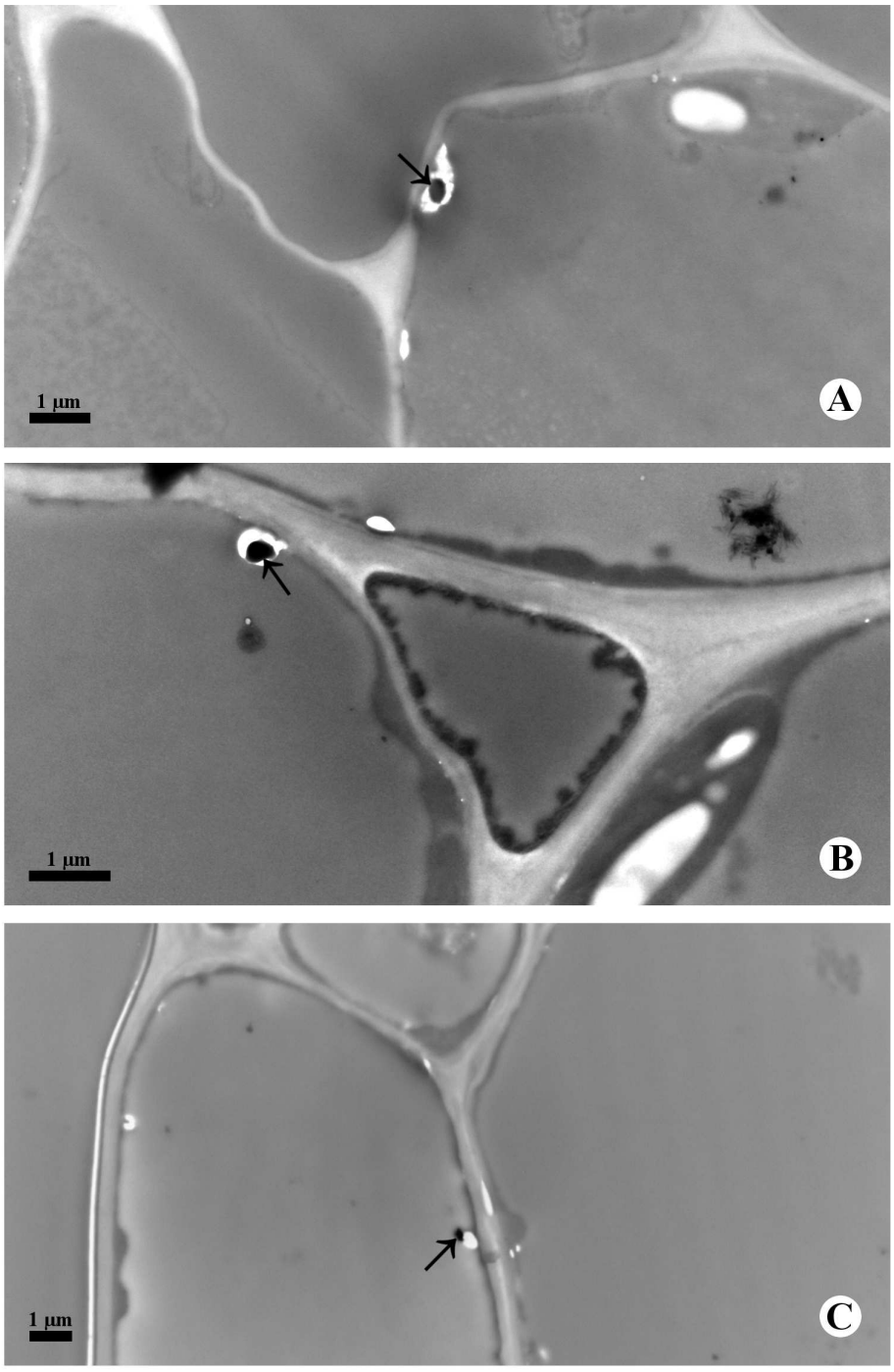


Figure 5.14 EDAX spectra confirming that the dark deposits present in root tissues (Fig. 5.13) were Cd rich sites.

Figure 5.15 TEM micrographs showing leaf cells of *Chromolaena odoratum* grown in media supplemented with Cd.
(A) Mesophyll cells located close to the vascular system
(B) Mesophyll cell
(C) Epidermal cell
Arrows indicate the Cd rich sites which were confirmed by EDAX spectra shown in Fig. 5.16.


1 µm
A
1 µm
B
1 µm
C

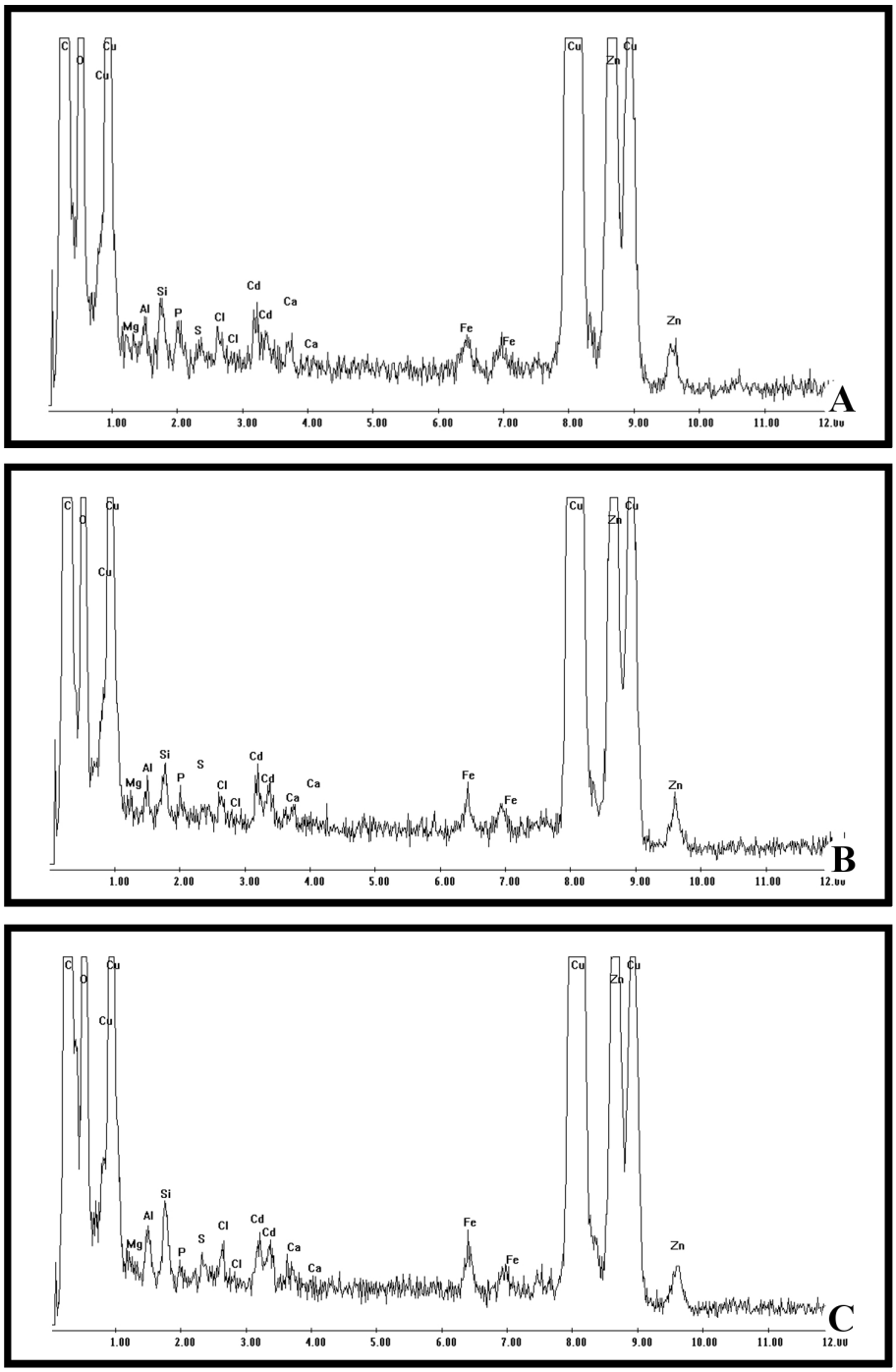


Figure 5.16 EDAX spectra confirming that the dark deposits present in leaf tissues (Fig. 5.15) were Cd rich sites.

5.3 Rhizobacteria screening to find potential strains used to improve phytoremediation

5.3.1 Isolation and identification of rhizobacteria

Rhizobacteria were isolated from Cd/Zn contaminated soil associated with roots of *Chromolaena odoratum*. Six bacterial strains showing different morphological appearance on NA were selected. They were designated as TKS01, TKS05, TKS07, TKS10, TKS21 and TKS22. One bacterial strain was isolated from uncontaminated soil and designated as BKS01. The external morphology of those isolated bacteria is shown in Figures 5.17 and 5.19 and the colonial characteristics of the bacteria are described in Table 5.8. All bacterial strains were Gram-negative (Figs. 5.18 and 5.19).

The isolated bacterial strains were identified based on the analysis of 16S rDNA sequence. The closest strain in 16S rDNA gene sequence for the TKS01, TKS05, TKS07, TKS10, TKS21, TKS22 and BKS01 were *Serratia marcesens*, *Cupriavidus taiwanensis*, *Comamonas testosteroni*, *Delftia sp.*, *Chryseobacterium sp.*, *Pseudomonas aeruginosa* and *Citrobacter sp.*, respectively.

The growth curves of these isolated bacterial strains in NB at 30 °C, 150 rpm are presented in Figure 5.20. All isolated bacteria could grow to stationary phase within 24 h.

Table 5.8 The colonial and cellular characteristics of the isolated bacterial strains.

Bacterial strain	Characteristics
Chryseobacterium sp. TKS21	**Colonies** were circular with entire edges, opaque, convex, bright yellow-pigmented by a non diffusible pigment, becoming mucous after prolonged incubation on NA. **Cells** were Gram-staining-negative short rods.
Comamonas testosteroni TKS07	**Colonies** were circular, convex with a smooth to wavy margin and a smooth to granular surface. They were white with no diffusible pigments. **Cells** were Gram-staining-negative, and rods that were straight or slightly curved.
Cupriavidus taiwanensis TKS05	**Colonies** were white, glistering, mucous, smooth, and convex with an entire edge. **Cells** were Gram-staining-negative rods.
Delftia sp. TKS10	**Colonies** were circular, low convex, entire edges with smooth surface. **Cells** were Gram-staining-negative, straight to slightly curved rods, single or in pairs.
Pseudomonas aeruginosa TKS22*	**Colonies** were circular, smooth with diffusible green fluorescent pigment. **Cells** were Gram-staining-negative, rod shape.
Serratia marcescens TKS01*	**Colonies** were opaque, circular, convex, margins entire, and white, pink, or red (pigment were usually observed in at least some colonies) **Cells** were Gram-staining-negative, short rod.
Citrobacter sp. BKS01	**Colonies** were circular white, convex, entire edge, and smooth surface with non diffusible pigment. **Cells** were Gram-staining-negative, rod shape.

* indicates opportunistic strains of bacteria

Figure 5.17 Colonial morphology of isolated bacterial strains from Cd/Zn contaminated soils associated with roots of *Chromolaena odoratum.* All bacterial strains were grown on NA and incubated at 30 °C.
(A) *Chryseobacterium sp.* TKS21
(B) *Comamonas testosteroni* TKS07
(C) *Cupriavidus taiwanensis* TKS05
(D) *Delftia sp.* TKS10
(E) *Pseudomonas aeruginosa* TK S22
(F) *Serratia marcesens* TKS01.

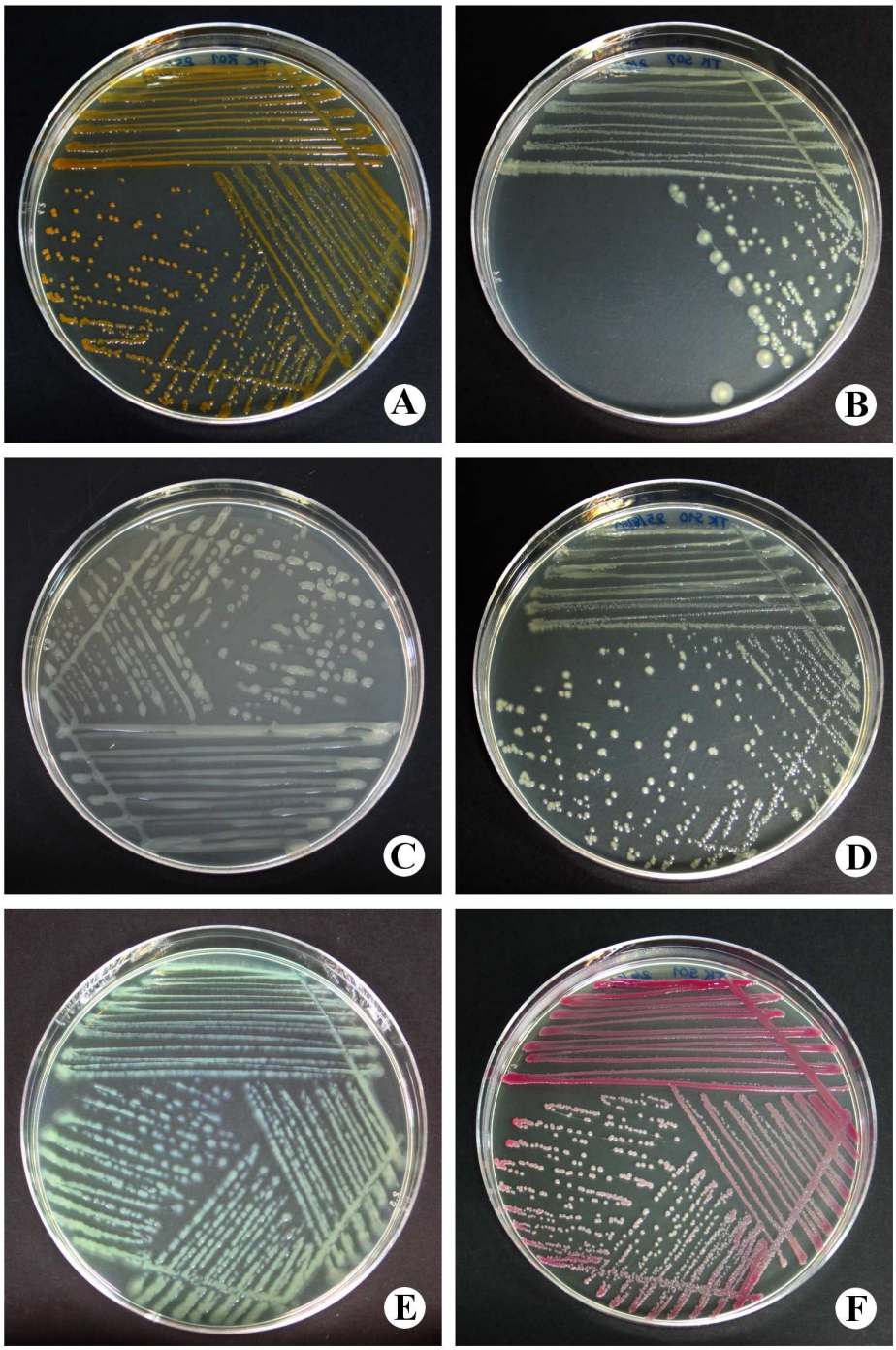

A
B
C
D
E
F

Figure 5.18 Gram staining of isolated bacterial strains.

(A) *Chryseobacterium sp.* TKS21

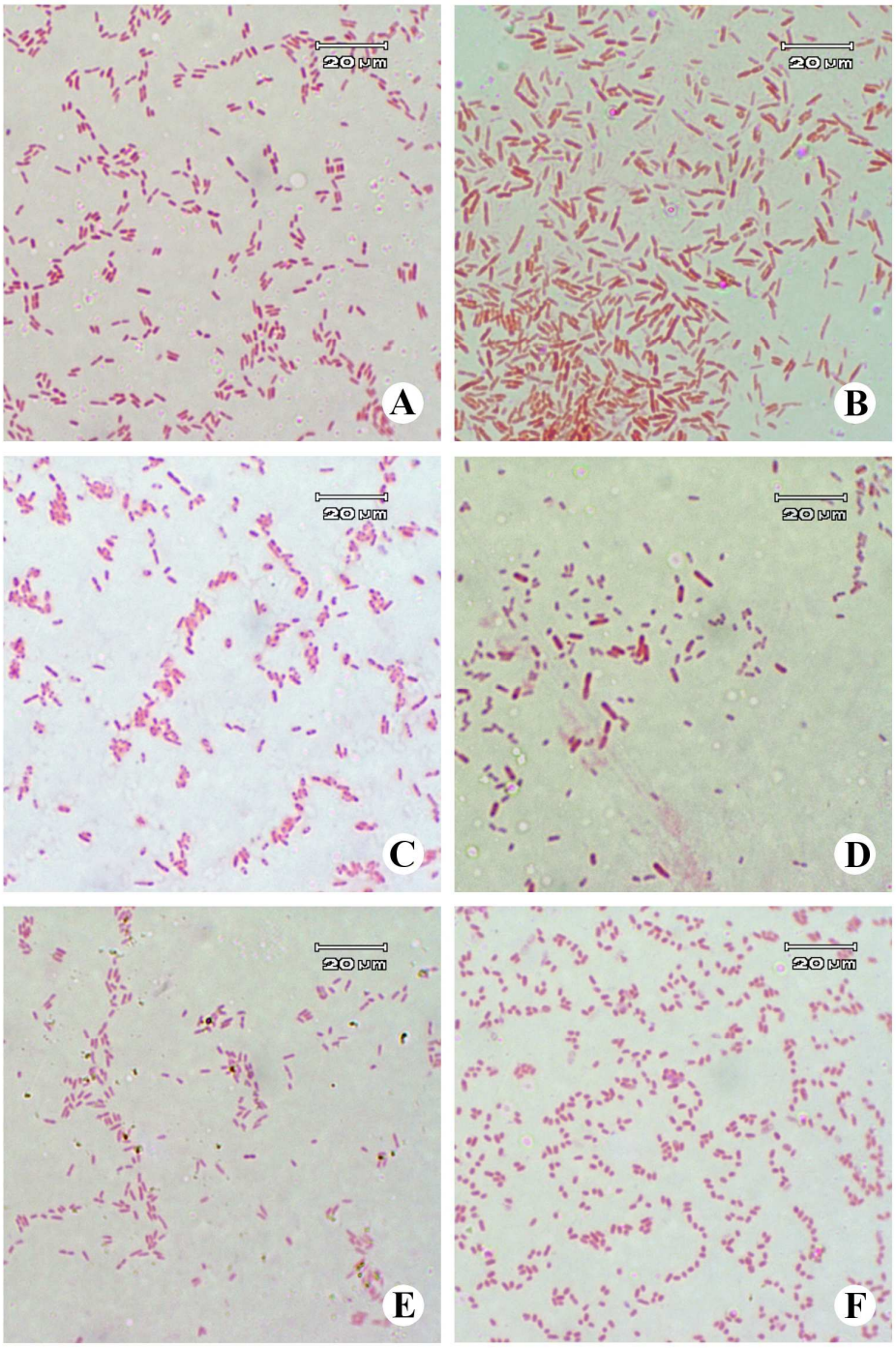
(B) *Comamonas testosteroni* TKS07

(C) *Cupriavidus taiwanensis* TKS05

(D) *Delftia sp.* TKS10

(E) *Pseudomonas aeruginosa* TK S22

(F) *Serratia marcesens* TKS01.


20 µm
A
20 µm
B
20 µm
C
20 µm
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20 µm
F

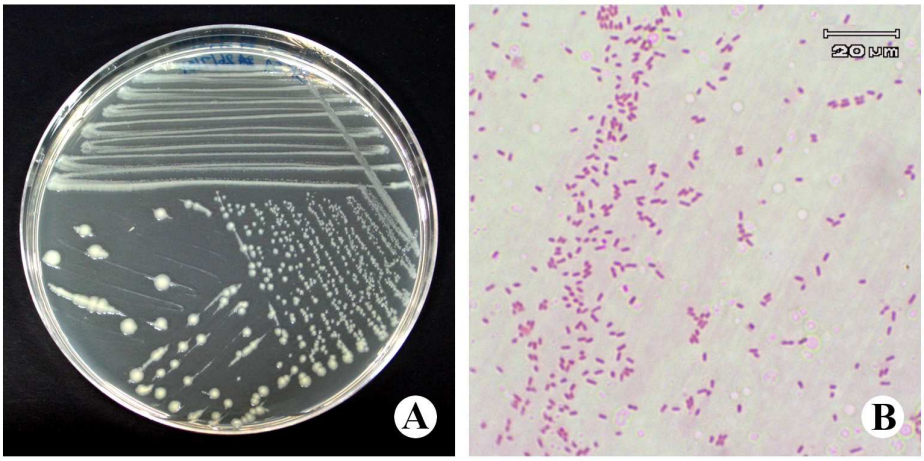


Figure 5.19 Colonial morphology **(A)** and Gram staining **(B)** of *Citrobacter sp.* BKS01 isolated from uncontaminated soils, and grown on NA and incubated at 30 °C.

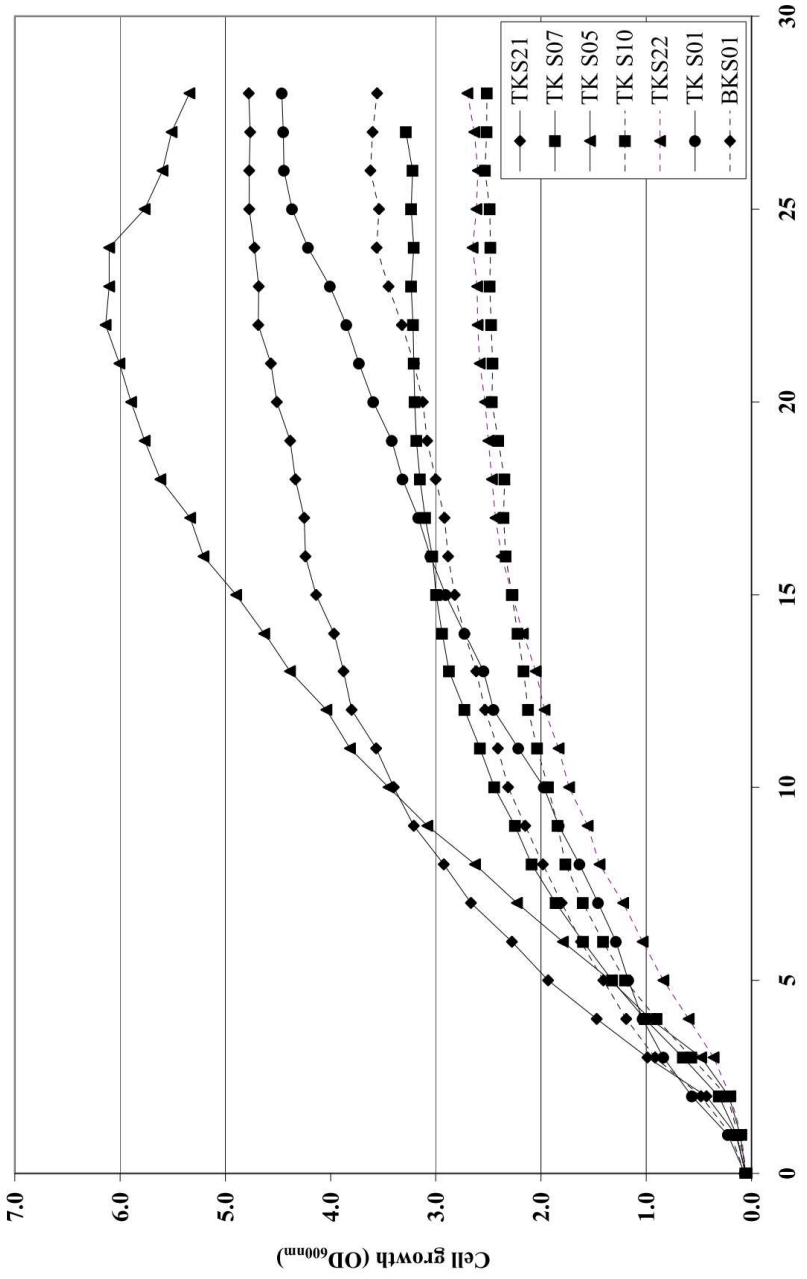


Figure 5.20 Growth of the isolated bacterial strains in NB at 30 °C, 150 rpm: *Chryseobacterium sp.* TKS21, *Comamonas testosteroni* TKS07, *Cupriavidus taiwanensis* TKS05, *Delftia sp.* TKS10, *Pseudomonas aeruginosa* TK S22, *Serratia marcesens* TKS01, *Citrobacter sp.* BKS01.

5.3.2 Determination of minimum inhibitory concentrations (MIC) of heavy metals

All isolated bacterial strains were grown in NB supplemented with various concentrations of either Cd or Zn and incubated at 30 °C, 150 rpm. The growth of each bacterium at log phase and stationary phase was determined by measuring the absorbance at 600 nm. Growth of bacteria in NB without metals was designated as control or normal growth. The growth of isolated bacteria exposed to Cd or Zn is presented in Figures 5.21 and 5.22, respectively. Compared with normal growth, growth of all isolated bacterial strains at log phase was declined with increasing Cd or Zn concentrations. Although the growth of bacteria exposed to heavy metals was delayed at log phase, most bacteria still grew to stationary phase. The bacterial growth at stationary phase also decreased with increasing metal concentrations. The metal concentrations that absolutely inhibited the growth of bacteria were designated as MIC. The MIC of Cd and Zn for the isolated bacterial strains are shown in Table 5.9. The highest MIC was found in *Comamonas testosteroni* TKS07 and *Cupriavidus taiwanensis* TKS05. The MIC of Cd and Zn for TKS07 were >200 and >400 mg l^{-1}, respectively while that for TKS05 were >200 and 400 mg l^{-1}, respectively. They had high capability to tolerate heavy metals. The lowest MIC was found in *Citrobacter sp.* which was isolated from uncontaminated soils and the MIC of Cd and Zn for this strain were 40 and 120 mg l^{-1}, respectively (Table 5.9).

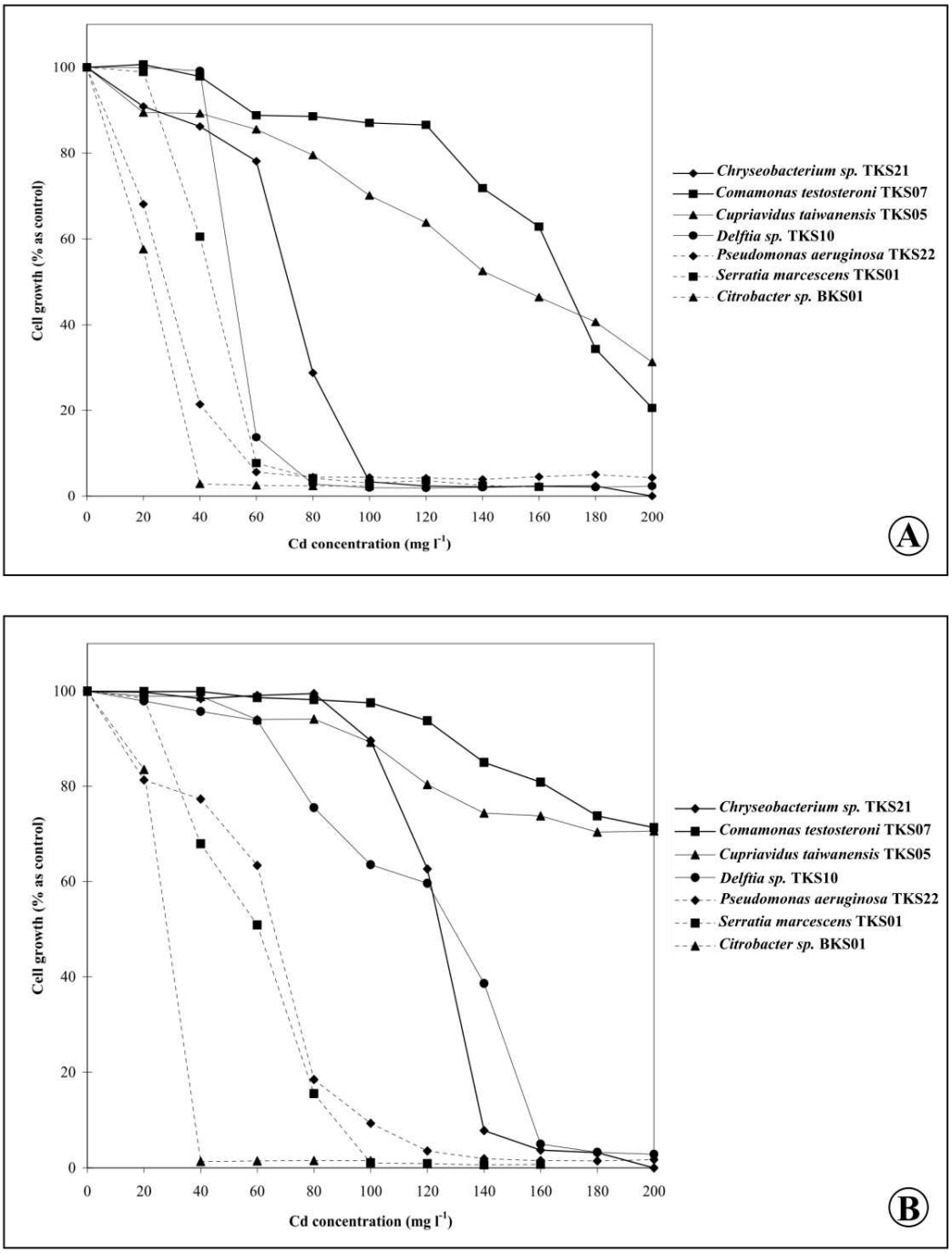


Figure 5.21 Cell growth at log phase **(A)** and stationary phase **(B)** of isolated bacterial strains cultured in NB media supplemented with various Cd concentrations and incubated at 30 °C, 150 rpm.

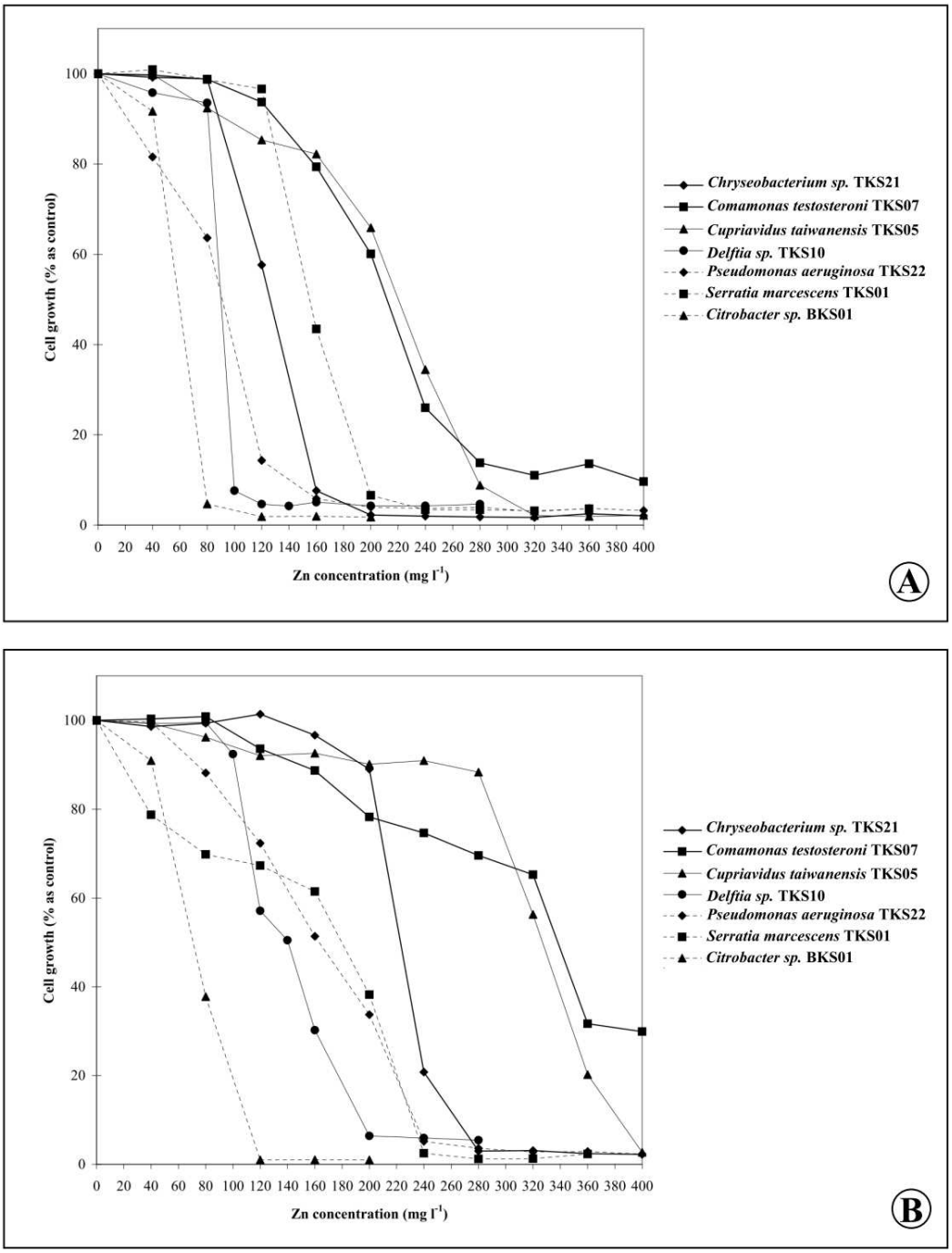


Figure 5.22 Cell growth at log phase **(A)** and stationary phase **(B)** of isolated bacterial strains cultured in NB media supplemented with various Zn concentrations and incubated at 30 °C, 150 rpm.

5.3.3 Solubilization of heavy metals in soil

Metal solubilization in soil by isolated bacteria was evaluated by determining extractable metal concentrations of soils inoculated with those bacteria compared with that of uninoculated soils. The bacterial strains employed in this experiment were *Chryseobacterium sp*. TKS21, *Comamonas testosteroni* TKS07, *Cupriavidus taiwanensis* TKS05, *Delftia sp*. TKS10, *Pseudomonas aeruginosa* TKS22, *Serratia marcescens* TKS01 and *Citrobacter sp*. BKS01. The extractable Cd and Zn concentrations in soils inoculated with isolated bacteria are shown in Table 5.9. The extractable Cd concentrations in soils inoculated with TKS07, TKS05, TKS10 and TKS22 were significantly higher (p<0.05) than that in uninoculated control soils (Fig. 5.23A). These four bacterial strains could increase the Cd availability in metal contaminated soils. The extractable Zn concentrations in soils inoculated with TKS21, TKS07, TKS22 and BKS01 were significantly lower (p<0.05) than that in uninoculated control soils (Fig. 5.23A).

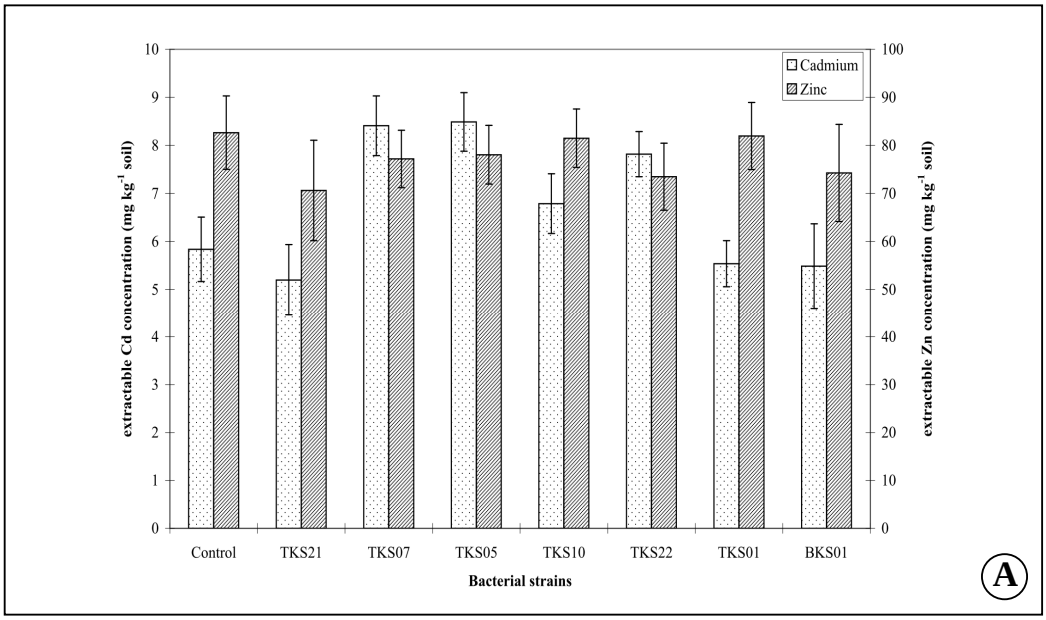
5.3.4 Biosorption of heavy metals

Cd and Zn biosorption by isolated bacterial strains is presented in Table 5.9 and Figure 5.23B. Seven bacterial stains employed in the experiment were *Chryseobacterium sp*. TKS21, *Comamonas testosteroni* TKS07, *Cupriavidus taiwanensis* TKS05, *Delftia sp*. TKS10, *Pseudomonas aeruginosa* TKS22, *Serratia marcescens* TKS01 and *Citrobacter sp*. BKS01. Cd biosorption by these bacterial strains ranged from the highest to the lowest was in the following order: TKS22>BKS01>TKS07>TKS05>TKS01>TKS10>TKS21 (Fig. 5.23B). While the Zn biosorption were ranged in the following order: TKS22>BKS01>TKS05>TKS21> TKS01>TKS10>TKS07 (Fig. 5.23B).

Table 5.9 Minimal inhibitory concentrations of Cd and Zn, metal biosorption and metal solubilization (presented as extractable metal concentrations) by isolated bacteria.

Bacterial strain	Minimal inhibitory concentration (mg l^{-1})		Metal biosorption (µg g^{-1} dry mass)		Extractable metal concentrations (mg kg^{-1} soil)	
	Cd	Zn	Cd	Zn	Cd	Zn
Control (uninoculation)	-	-	-	-	5.84 ± 0.67^{a}	82.67 ± 7.63^{a}
Chryseobacterium sp. TKS21	140	280	332.79 ± 43.98	366.95 ± 27.59	5.20 ± 0.74^{b}	70.65 ± 10.44^{e}
Comamonas testosteroni TKS07	>200	>400	1860.22 ± 45.07	66.00 ± 6.04	8.41 ± 0.62^{c}	$77.20 \pm 5.97^{b, c, d}$
Cupriavidus taiwanensis TKS05	>200	400	1211.64 ± 26.94	760.07 ± 31.88	8.49 ± 0.61^{c}	$78.07 \pm 6.10^{a, c, d}$
Delftia sp. TKS10	160	200	433.68 ± 23.49	88.83 ± 8.37	6.79 ± 0.62^{d}	$81.51 \pm 6.08^{a, d}$
Pseudomonas aeruginosa TKS22	120	240	5493.45 ± 24.40	1263.26 ± 53.92	7.82 ± 0.47^{e}	$73.49 \pm 6.97^{c, e}$
Serratia marcescens TKS01	100	240	688.40 ± 24.15	235.23 ± 31.29	$5.54 \pm 0.48^{a, b}$	$81.98 \pm 6.98^{a, b}$
Citrobacter sp. BKS01	40	120	3979.88 ± 44.24	1251.63 ± 47.19	$5.49 \pm 0.88^{a, b}$	$74.28 \pm 10.11^{c, e}$

The data is presented as Mean±SD. Means followed by a common letter in the same column for each bacterial strains are not significantly different from each other using LSD test (p>0.05).



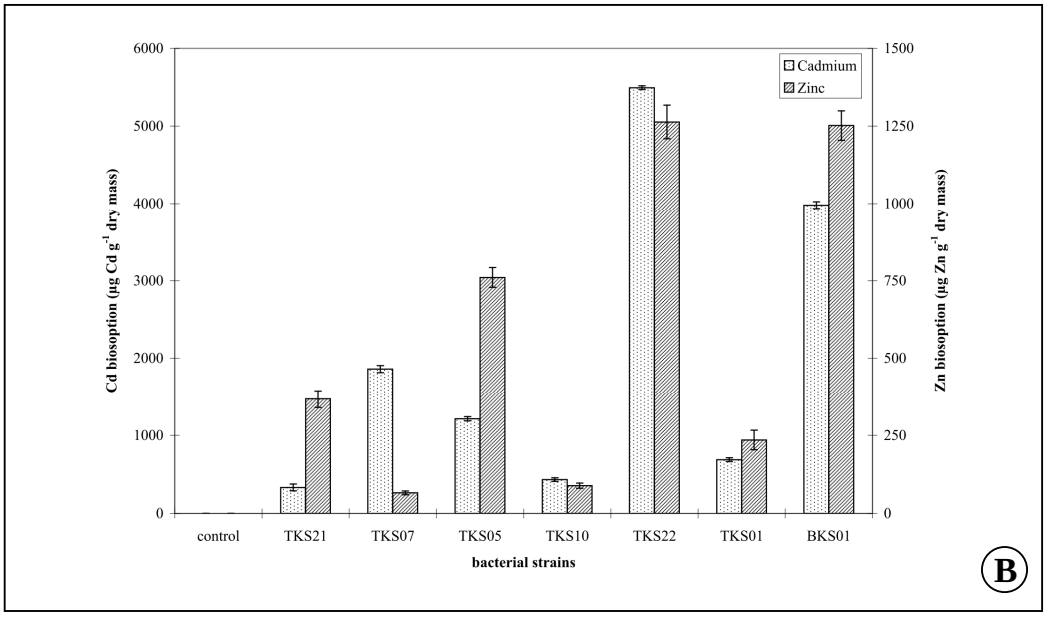


Figure 5.23 (A) Extractable metal concentrations in soils inoculated with isolated bacterial strains. **(B)** Cd and Zn biosorption by isolated bacteria. Control (uninoculated), *Chryseobacterium sp.* TKS21, *Comanonas testosteroni* TKS07, *Cupriavidus taiwanensis* TKS05, *Delftia sp.* TKS10, *Pseudomonas aeruginosa* TKS22, *Serratia marcesens* TKS01, *Citrobacter sp.* BKS01

5.3.5 Idole acetic acid (IAA) production

IAA production by isolated bacteria (*Chryseobacterium sp.* TKS21, *Comamonas testosteroni* TKS07, *Cupriavidus taiwanensis* TKS05, *Delftia sp.* TKS10, *Pseudomonas aeruginosa* TKS22, *Serratia marcescens* TKS01 and *Citrobacter sp.* BKS01) determined by colorimetric method are shown in Figure 5.24B. The concentrations of IAA produced by these bacterial strains are shown in Table 5.10. All bacterial strains could produce IAA in the presence of tryptophan. The maximum IAA production was found in TKS10 (111.3±26.4 µg IAA mg^{-1} dry mass) followed by TKS21 (103.3±8.0 µg IAA mg^{-1} dry mass). Other bacterial strains could also produce IAA with the concentrations ranged from the lowest to the highest in the following order: TKS01<BKS01<TKS07≤TKS22<TKS05 (Table 5.10).

5.3.6 Siderophore production

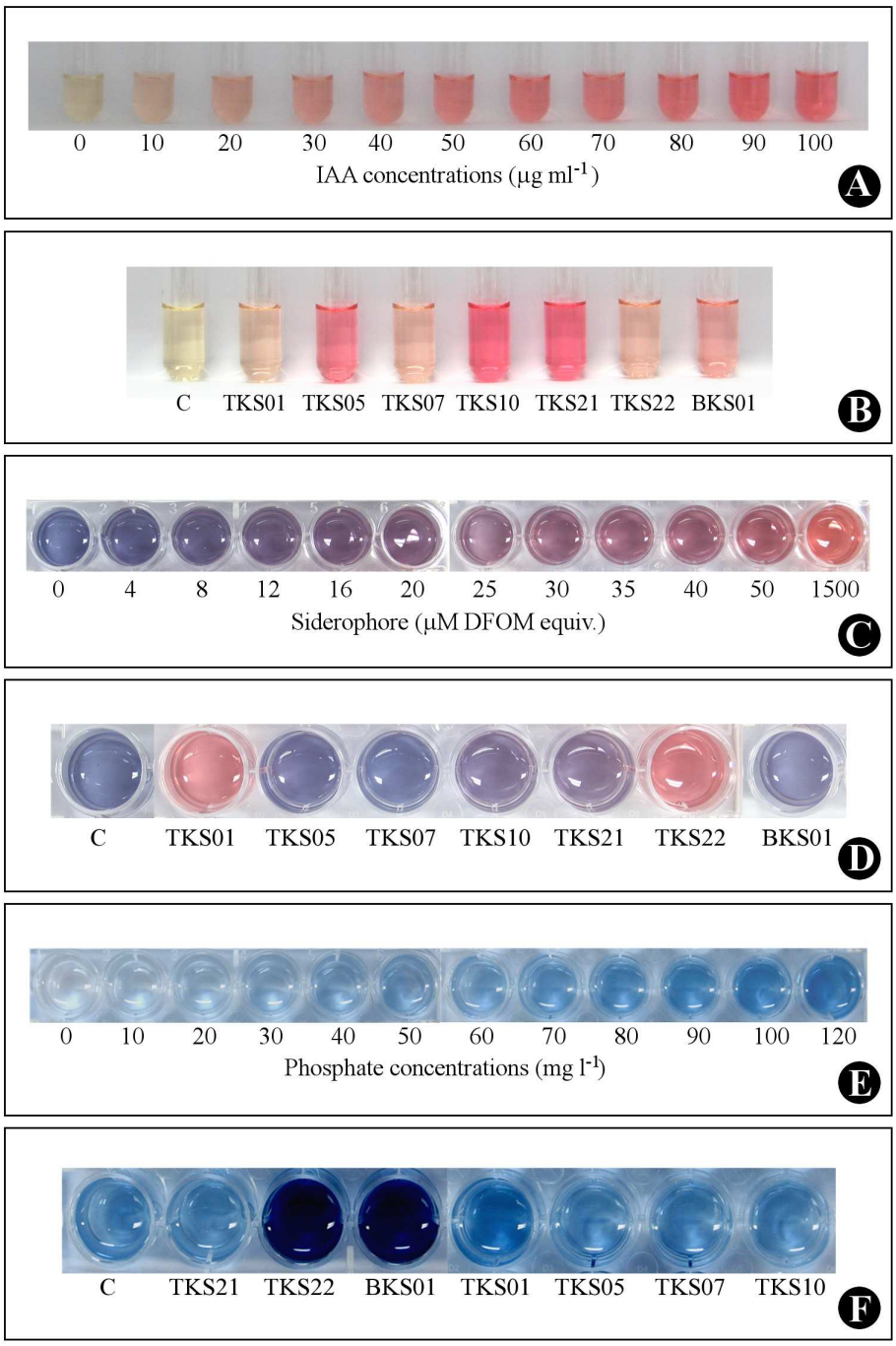
Siderophore production by isolated bacteria (*Chryseobacterium sp.* TKS21, *Comamonas testosteroni* TKS07, *Cupriavidus taiwanensis* TKS05, *Delftia sp.* TKS10, *Pseudomonas aeruginosa* TKS22, *Serratia marcescens* TKS01 and *Citrobacter sp.* BKS01) determined by colorimetric method are presented in Figure 5.24D. The concentrations of siderophore produced by these bacterial strains are shown in Table 5.10. Two bacterial strains, TKS22 and TKS01, could produce high concentrations of siderophore (24.6 and 12.14 µM DFOM mg^{-1} dry mass). TKS21 and TKS10 could also produce siderophore with lower concentrations while TKS07, TKS05 and BKS01 could not produce siderophore.

Table 5.10 IAA, siderophore production, and phosphate solubilization by isolated bacterial strains.

Bacterial strains	IAA production (µg IAA mg^{-1} dry mass)	Siderophore production (µM DFOM mg^{-1} dry mass)	Phosphate solubilization		
			Growth (CFU ml^{-1})	pH	Phosphate concentration (mg l^{-1})
Control (uninoculation)	-	-	0.00	6.35 ± 0.03^{a}	101.76 ± 0.20^{a}
Chryseobacterium sp. TKS21	103.28 ± 8.02	4.07 ± 0.97	3.00×10^{22}	6.85 ± 0.11^{b}	81.33 ± 3.42^{a}
Comamonas testosteroni TKS07	30.10 ± 5.42	0.00 ± 0.00	1.02×10^{12}	8.03 ± 0.66^{c}	88.40 ± 2.97^{a}
Cupriavidus taiwanensis TKS05	47.90 ± 15.35	0.00 ± 0.00	4.08×10^{12}	8.24 ± 0.03^{c}	79.69 ± 2.21^{a}
Delftia sp. TKS10	111.29 ± 26.35	2.30 ± 0.48	9.58×10^{18}	7.99 ± 0.01^{c}	88.22 ± 1.96^{a}
Pseudomonas aeruginosa TKS22	30.21 ± 9.80	24.58 ± 1.28	2.42×10^{20}	3.97 ± 0.10^{d}	544.14 ± 81.94^{c}
Serratia marcescens TKS01	19.78 ± 1.58	12.14 ± 1.38	2.65×10^{22}	7.10 ± 0.10^{b}	158.74 ± 19.87^{b}
Citrobacter sp. BKS01	20.51 ± 0.79	0.00 ± 0.00	1.31×10^{18}	4.26 ± 0.03^{d}	510.52 ± 23.07^{c}

The data is presented as Mean±SD. Means followed by a common letter in the same column for each bacterial strains are not significantly different from each other using LSD test (p>0.05).

Figure 5.24 The colorimetric determination of IAA production, siderophore production and phosphate solubilization by isolated bacterial strains.
(A) Standard IAA solution
(B) IAA production by isolated bacterial strains
(C) Standard siderophore (deferoxamine mesylate)
(D) Siderophore production by isolated bacterial stains
(E) Standard phosphate solution
(F) Phosphate solubilization by isolated bacterial strains
Control (uninoculation), *Chryseobacterium sp*. TKS21, *Comanonas testosteroni* TKS07, *Cupriavidus taiwanensis* TKS05, *Delftia sp.* TKS10, *Pseudomonas aeruginosa* TKS22, *Serratia marcesens* TKS01, *Citrobacter sp*. BKS01**.**


0 10 20 30 40 50 60 70 80 90 100
IAA concentrations (µg ml-1)
A
C TKS01 TKS05 TKS07 TKS10 TKS21 TKS22 BKS01
B
0 4 8 12 16 20 25 30 35 40 50 1500
Siderophore (µM DFOM equiv.)
C
C TKS01 TKS05 TKS07 TKS10 TKS21 TKS22 BKS01
D
0 10 20 30 40 50 60 70 80 90 100 120
Phosphate concentrations (mg l-1)
E
C TKS21 TKS22 BKS01 TKS01 TKS05 TKS07 TKS10
F

5.3.7 Phosphate solubilizing activity

The phosphate solubilized by isolated bacteria (*Chryseobacterium sp.* TKS21, *Comamonas testosteroni* TKS07, *Cupriavidus taiwanensis* TKS05, *Delftia sp.* TKS10, *Pseudomonas aeruginosa* TKS22, *Serratia marcescens* TKS01 and *Citrobacter sp.* BKS01) determined by colorimetric method are shown in Figure 5.24F. The concentrations of solubilized phosphate and the pH of culture media (NBRIP media) are shown in Table 5.10. TKS22, TKS01 and BKS01 could solubilize phosphate since the phosphate concentrations in their media culture were significantly higher ($p<0.05$) than that in media without inoculation (control). Moreover the pH of the media culture of TKS22 and BKS01 were significantly ($p<0.05$) lower than that of control (Fig. 5.25).

Other isolated bacterial strains, TKS21, TKS07, TKS05 and TKS10, could not solubilize phosphate since the phosphate concentrations in their media were not significantly different ($p>0.05$) from that in uninoculated control media. The pH of the media culture of those bacterial strains were significantly higher ($p<0.05$) than that of control (Fig. 5.25).

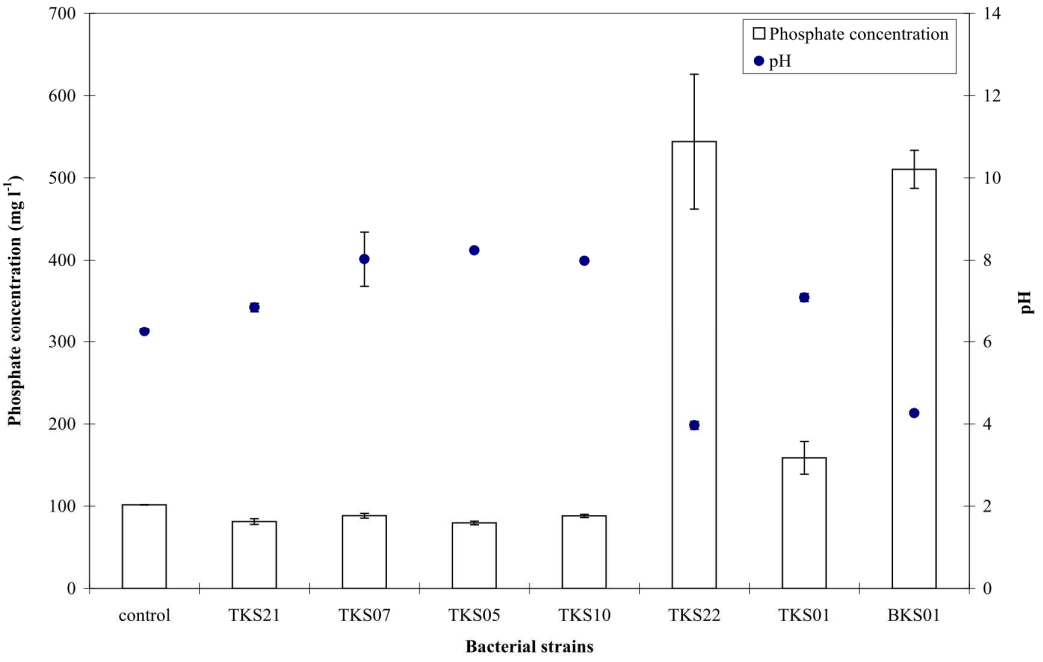


Figure 5.25 Phosphate concentrations and pH of NBRIP media used to culture the isolated bacterial strains for 72 h. at 30 °C, 150 rpm.

Control (NBRIP medium without inoculation)

TKS21 = *Chryseobacterium sp.*

TKS07 = *Comanonas testosteroni*

TKS05 = *Cupriavidus taiwanensis*

TKS10 = *Delftia sp.*

TKS22 = *Pseudomonas aeruginosa*

TKS01 = *Serratia marcesens*

BKS01 = *Citrobacter sp.*

5.4 Bioinoculation of plant by isolated bacterial strains to promote plant growth and enhance metal uptake

5.4.1 Root colonization by isolated bacterial strains

To investigate the root colonization by isolated bacterial strains, the seedlings of *Helianthus annuus* were inoculated with bacteria and grown on MS media in culture bottles. Plant roots colonized by isolated bacteria were observed by the SEM and the SEM micrographs are shown in Figure 5.26. No bacteria were found on the roots of control plants. All isolated bacterial strains could colonize plant roots with or without promoting plant growth.

5.4.2 Plant growth promoted by isolated bacterial strains

Growth of *Helianthus annuus* inoculated with isolated bacterial strains are shown in Table 5.11 and Figure 5.27. Plant seedlings were inoculated with isolated bacteria and grown on MS media in culture bottles for 15 days (Fig. 5.28). The shoot and root length, shoot fresh weight and dry weight were measured to determine the effects of isolated bacteria on plant growth. Compared with control plants, the plant seedlings inoculated with *Chryseobacterium sp.* TKS21, *Comamonas testosteroni* TKS07, *Cupriavidus taiwanensis* TKS05 and *Delftia sp.* TKS10 could grow well while those inoculated with *Pseudomonas aeruginosa* TKS22, *Serratia marcescens* TKS01 and *Citrobacter sp*. BKS01 could not grow (Figs. 5.27 and 5.28).

The shoot and root lengths of the plants inoculated with *Chryseobacterium sp.* TKS21, *C. testosteroni* TKS07, *C. taiwanensis* TKS05 and *Delftia sp*. TKS10 were significantly longer ($p<0.05$) than that of control plants. These bacterial strains had positive effect on the root and shoot lengths of the plant in the following order: TKS05 > TKS21 > TKS10 > TKS07 (Table 5.11). *P. aeruginosa* TKS22, *S. marcescens* TKS01 and *Citrobacter sp*. BKS01 had negative effects on the shoot and root length (Table 5.11). The shoot dry weights of the plants inoculated with *C. testosteroni* TKS07, *C. taiwanensis* TKS05 were significantly ($p<0.05$) higher than that of the control plants (Table 5.11).

H. annuus inoculated with isolated bacterial strains and grown on MS media in the culture bottles are shown in Figure 5.28. *Chryseobacterium sp.* TKS21, *Comamonas testosteroni* TKS07, *Cupriavidus taiwanensis* TKS05 and *Delftia sp.* TKS10 were able to promote plant growth so they were selected to be used in pot experiment to investigate the effects of bacterial strains on metal uptake by plant.

Figure 5.26 SEM micrograph showing roots of *Helianthus annuus* colonized by isolated bacterial strains.

(A) Control plant

(B) *Chryseobacterium sp*. TKS21

(C) *Comamonas testosteroni* TKS07

(D) *Cupriavidus taiwanensis* TKS05

(E) *Delftia sp.* TKS10

(F) *Pseudomonas aeruginosa* TKS22

(G) *Serratia marcesens* TKS01

(H) *Citrobacter sp*. BKS01

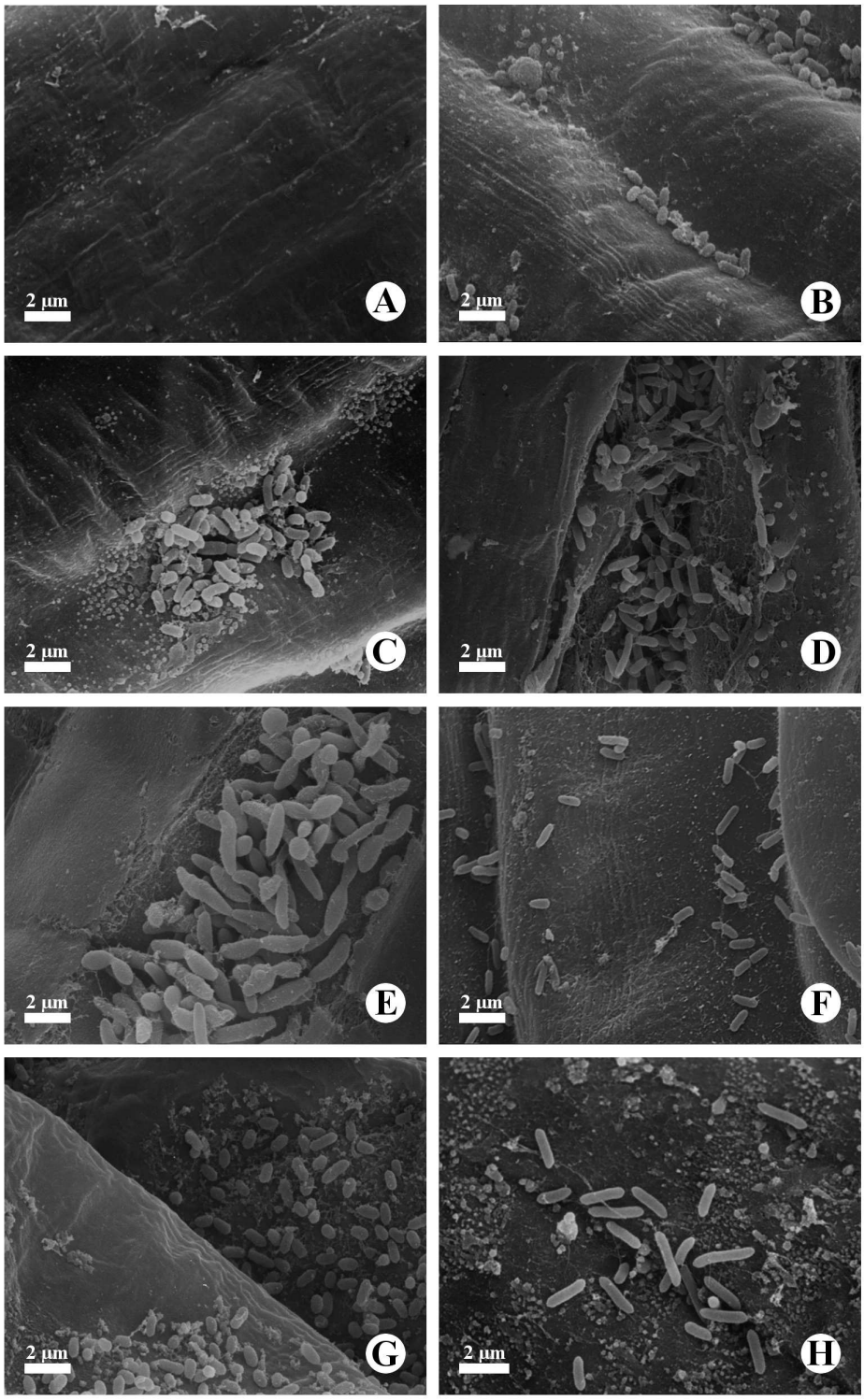

2 µm
A
2 µm
B
2 µm
C
2 µm
D
2 µm
E
2 µm
F
2 µm
G
2 µm
H

Table 5.11 Growth of *Helianthus annuus* seedlings inoculated with isolated bacterial strains and grown on MS media in the culture bottles for 15 days.

Bacterial strains	Root length		Shoot length		Shoot fresh weight (mg plant^{-1})	Shoot dry weight (mg plant^{-1})
	(cm plant^{-1})	Bacterial effect	(cm palnt^{-1})	Bacterial effect		
Control (uninoculation)	1.30 ± 0.15^{a}	-	2.68 ± 0.19^{a}	-	147.0 ± 9.37^{a}	17.50 ± 1.08^{a}
Chryseobacterium sp. TKS21	5.35 ± 0.57^{d}	+ 3.12	7.33 ± 0.81^{e}	+ 1.74	256.0 ± 36.33^{d}	22.70 ± 2.39^{a}
Comamonas testosteroni TKS07	3.33 ± 0.55^{c}	+ 1.56	4.60 ± 0.65^{d}	+ 0.72	206.4 ± 22.86$^{a, d}$	33.70 ± 9.10^{c}
Cupriavidus taiwanensis TKS05	8.50 ± 0.53^{b}	+ 5.54	9.98 ± 0.80^{c}	+ 2.72	476.0 ± 62.92^{c}	34.10 ± 3.70^{c}
Delftia sp. TKS10	4.83 ± 0.76^{d}	+ 2.72	6.80 ± 0.89^{e}	+ 1.54	271.8 ± 48.93^{d}	24.20 ± 3.99$^{a, c}$
Pseudomonas aeruginosa TKS22	0.54 ± 0.11^{a}	- 0.59	0.00 ± 0.00^{b}	- 1.00	0.0 ± 0.00^{b}	0.00 ± 0.00^{b}
Serratia marcescens TKS01	0.42 ± 0.05^{a}	- 0.68	0.35 ± 0.11^{b}	- 0.87	0.0 ± 0.00^{b}	0.00 ± 0.00^{b}
Citrobacter sp. BKS01	0.45 ± 0.02^{a}	- 0.65	0.20 ± 0.09^{b}	- 0.93	0.0 ± 0.00^{b}	0.00 ± 0.00^{b}

The data is Mean±SD. A common letters in the same column for plant seedling inoculated with each bacterial strain are not significantly different from each other using LSD test (p>0.05).

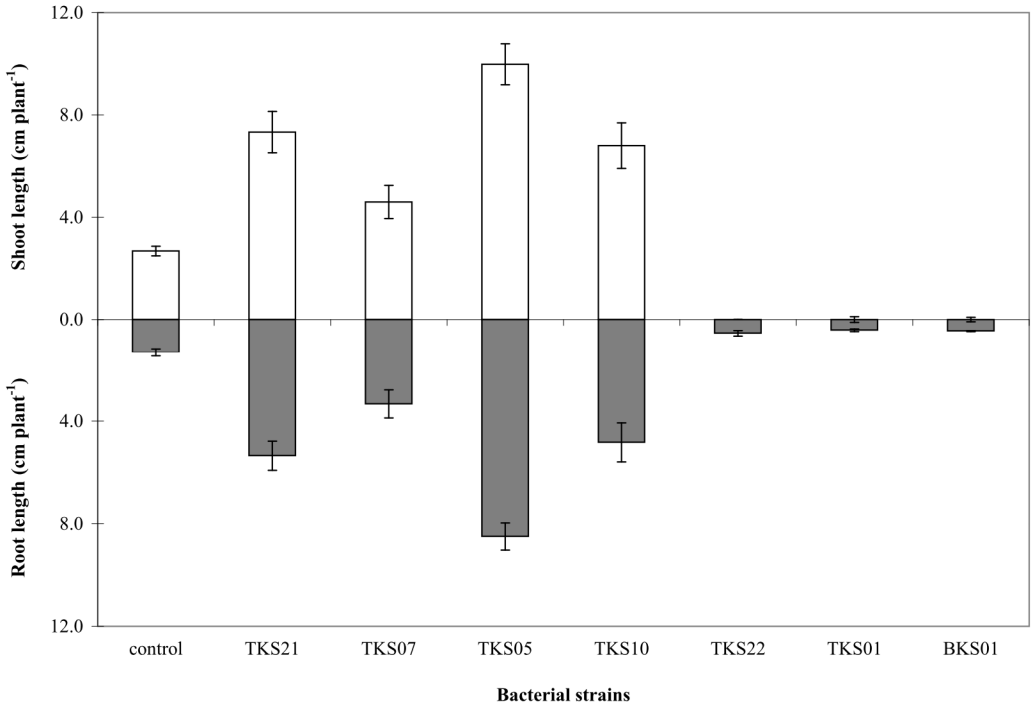


Figure 5.27 Growth of *Helianthus annuus* inoculated with isolated bacterial strains and grown on MS media in culture bottles.

Control = seedlings without inoculation

TKS21 = seedlings inoculated with *Chryseobacterium sp.* TKS21

TKS07 = seedlings inoculated with *Comamonas testosteroni* TKS07

TKS05 = seedlings inoculated with *Cupriavidus taiwanensis* TKS05

TKS10 = seedlings inoculated with *Delftia sp.* TKS10

TKS22 = seedlings inoculated with *Pseudomonas aeruginosa* TKS22

TKS01 = seedlings inoculated with *Serratia marcesens* TKS01

BKS01= seedlings inoculated with *Citrobacter sp.* BKS01

Figure 5.28 *Helianthus annuus* seedlings inoculated with isolated bacterial strains and grown in MS media for 15 days.

(A) Seedlings without inoculation (control)

(B) Seedlings inoculated with *Chryseobacterium sp.* TKS21

(C) Seedlings inoculated with *Comamonas testosteroni* TKS07

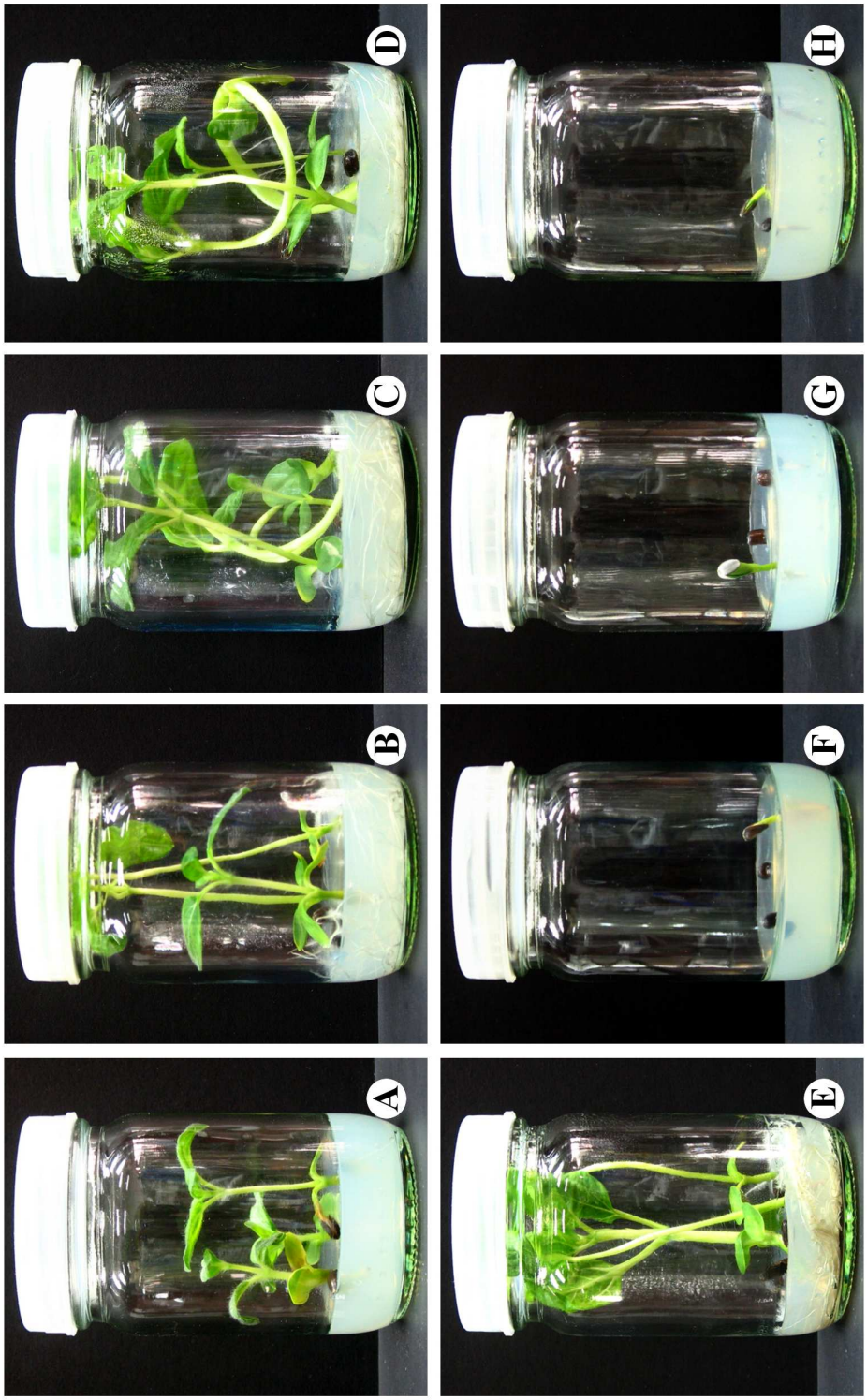
(D) Seedlings inoculated with *Cupriavidus taiwanensis* TKS05

(E) Seedlings inoculated with *Delftia sp.* TKS10

(F) Seedlings inoculated with *Pseudomonas aeruginosa* TKS22

(G) Seedlings inoculated with *Serratia marcesens* TKS01

(H) Seedlings inoculated with *Citrobacter sp*. BKS01


D
H
C
G
B
F
A
E

5.4.3 Growth and heavy metal accumulation of plants assisted by isolated bacteria on sterile soils under laboratory conditions

Seedlings of *Helianthus annuus* were inoculated with selected bacterial strains (*Chryseobacterium sp*. TKS21, *Comamonas testosteroni* TKS07, *Cupriavidus taiwanensis* TKS05 and *Delftia sp*. TKS10) and grown on the sterile heavy metal contaminated soils in the laboratory for 60 days to investigate the effects of these bacteria on growth and metal accumulation of plants. The growth and metal accumulation of plants inoculated with these selected bacteria are shown in Tables 5.12 and 5.13, respectively.

Compared with seedlings without bacterial inoculation (control), the whole plant fresh weight as well as dry weight of the plants inoculated with TKS21, TKS07 were significantly (p<0.05) higher than that of control (Table 5.12 and Fig. 5.29). Additionally, growth (determined by % of control plant) of seedlings inoculated with TKS21 and TKS07 were approximately 307% and 187%, respectively (Table 5.12)

Cd concentrations in the shoots of plants inoculated with TKS07 and TKS05 were 43.39±14.58 and 46.41±13.43 mg kg^{-1} dry weight, respectively and they were significantly different (p<0.05) from that of control (26.62±6.67) (Table 5.13A). Cd extraction coefficient (EC) of plants inoculated with TKS07 (1.12) and TKS05 (1.13) were approximately two times higher than that of control (0.66). Zn concentrations in the shoots of the plants inoculated with isolated bacteria (TKS21, TKS07 and TKS10) were not significantly different (p>0.05) from that of control (Table 5.13B). Exceptionally, Zn concentration in the shoots of plants inoculated with TKS05 (391.04±57.48 mg kg^{-1} dry weight) was significantly higher (p<0.05) than that of control (264.80±38.52 mg kg^{-1} dry weight) with their extraction coefficients of 0.24 and 0.16, respectively.

On the sterile metal contaminated soils, TKS21 and TKS07 were found to promote growth of *H. annuus* while TKS07 and TKS05 could enhance Cd accumulation in plant shoots (Fig. 5.29) and only TKS05 enhanced Zn accumulation in plant shoot.

Table 5.12 Growth of *Helianthus annuus* inoculated with selected bacterial strains and grown on sterile heavy metal contaminated soils in the laboratory.

Bacterial strain	Fresh weight (g plant^{-1})	Dry weight (mg plant^{-1})			Plant growth (% of control)
		Whole plant	Plant shoot	Plant root	
Control (uninoculated)	2.08 ± 0.33^{a}	134.20 ± 29.53^{a}	124.33 ± 25.14^{a}	9.87 ± 5.70^{a}	100.00
Chryseobacterium sp. TKS21	4.45 ± 1.07^{c}	411.35 ± 126.65^{c}	353.60 ± 119.35^{c}	57.75 ± 17.60^{b}	306.52
Comamonas testosteroni TKS07	3.01 ± 0.97^{b}	250.55 ± 101.22^{b}	235.17 ± 98.23^{b}	15.38 ± 5.59^{a}	186.70
Cupriavidus taiwanensis TKS05	2.65 ± 0.48$^{a, b}$	188.93 ± 48.21$^{a, b}$	176.58 ± 44.57$^{a, b}$	12.35 ± 5.46^{a}	140.78
Delftia sp. TKS10	1.88 ± 0.36^{a}	137.97 ± 8.68^{a}	127.32 ± 10.76^{a}	10.65 ± 6.59^{a}	102.81

The data are Mean±SD. A common letters in the same column for the plant inoculated with each bacterial strain are not significantly different from each other using LSD test (p>0.05).

Table 5.13 Cd **(A)** and Zn **(B)** concentrations in shoots and roots of *Helianthus annuus* inoculated with selected bacterial strains and grown on sterile soils in the laboratory.

(A)

Bacterial strain	Cd concentration (mg kg^{-1} dry weight)			Translocation factor	Extraction coefficient
	Soil	Plant shoot	Plant root		
Control (uninoculated)	40.42 ± 4.38[a]	26.62 ± 6.67[a]	52.75 ± 24.89[a]	0.50	0.66
Chryseobacterium sp. TKS21	33.06 ± 1.56[b]	25.62 ± 2.72[a]	35.27 ± 6.21[a]	0.73	0.77
Comamonas testosteroni TKS07	38.88 ± 0.83[a]	43.39 ± 14.58[b, c]	79.68 ± 5.88[b]	0.54	1.12
Cupriavidus taiwanensis TKS05	40.95 ± 1.78[a]	46.41 ± 13.43[b]	100.45 ± 42.46[b]	0.46	1.13
Delftia sp. TKS10	33.67 ± 1.82[b]	31.45 ± 8.81[a, c]	81.50 ± 3.04[b]	0.39	0.93

(B)

Bacterial strain	Zn concentration (mg kg^{-1} dry weight)			Translocation factor	Extraction coefficient
	Soil	Plant shoot	Plant root		
Control (uninoculated)	1672.86 ± 146.55[a]	264.80 ± 38.52[a]	728.57 ± 242.93[a]	0.36	0.16
Chryseobacterium sp. TKS21	1457.27 ± 27.80[b]	229.82 ± 36.44[a]	377.63 ± 79.28[b]	0.61	0.16
Comamonas testosteroni TKS07	1603.61 ± 86.46[a]	320.52 ± 36.57[a]	635.28 ± 227.40[a, b]	0.50	0.20
Cupriavidus taiwanensis TKS05	1657.70 ± 70.28[a]	391.04 ± 57.48[b]	873.23 ± 355.49[a]	0.45	0.24
Delftia sp. TKS10	1487.64 ± 53.37[b]	288.96 ± 87.27[a]	702.22 ± 228.52[a]	0.41	0.19

The data are Mean±SD. A common letters in the same column for the plant inoculated with each bacterial strain are not significantly different from each other using LSD test (p>0.05).

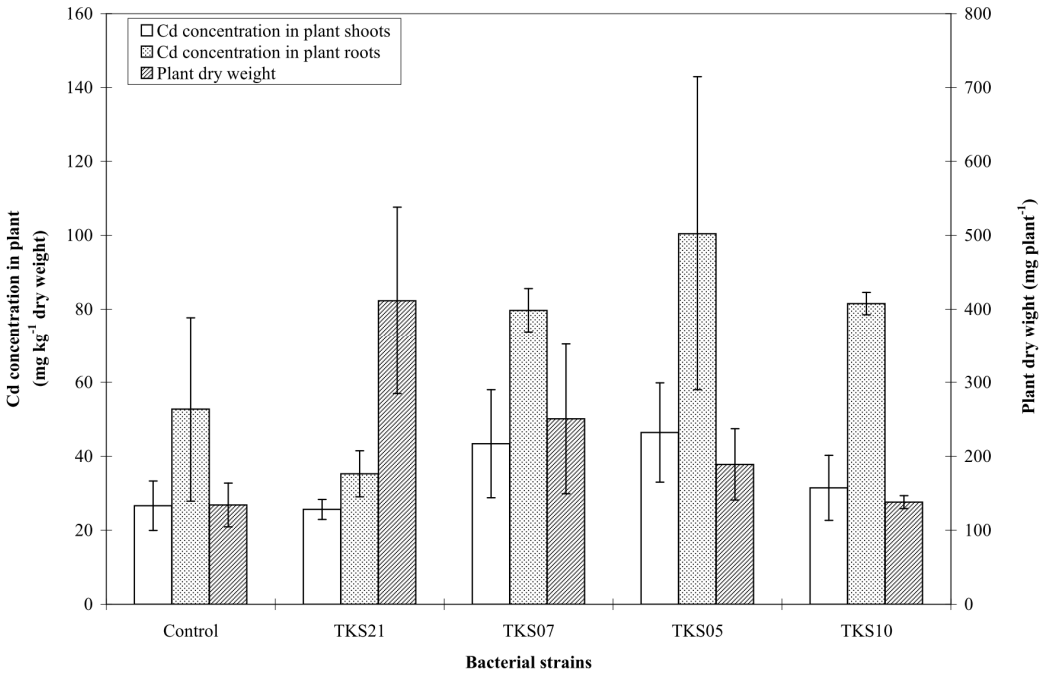


Figure 5.29 Cd accumulation and dry weight of *Helianthus annuus* inoculated with selected bacterial strains and grown on sterile metal contaminated soils in the laboratory.

Control = plants without bacterial inoculation

TKS21 = plants inoculated with *Chryseobacterium sp.* TKS21

TKS07 = plants inoculated with *Comamonas testosteroni* TKS07

TKS05 = plants inoculated with *Cupriavidus taiwanensis* TKS05

TKS10 = plants inoculated with *Delftia sp.* TKS10

5.4.4 Growth and heavy metal accumulation of plants assisted by isolated bacteria on non-sterile soils under greenhouse conditions

Seedlings of *Helianthus annuus* were inoculated with selected bacterial strains (*Chryseobacterium sp*. TKS21, *Comamonas testosteroni* TKS07, *Cupriavidus taiwanensis* TKS05 and *Delftia sp*. TKS10) and grown on the non-sterile heavy metal contaminated soils in greenhouse for 60 days to investigate the effects of bacteria on growth and metal accumulations in the plants. The results are shown in Tables 5.14 and 5.15, respectively.

Compared with the control plant seedlings, the shoot and root lengths of the plants inoculated with selected bacteria were significantly longer ($p<0.05$), except for the shoot length of plants inoculated with TKS21 ($p>0.05$). Moreover whole plant fresh weight as well as dry weight of the plants inoculated with bacteria were significantly ($p<0.05$) higher than that of the control (Table 5.14 and Fig. 5.30), except for that of plants inoculated with TKS21 ($p>0.05$). Growth (determined by % of control plant) of plants inoculated with TKS21, TKS07, TKS05 and TKS10 was approximately 141%, 181%, 152% and 174%, respectively (Table 5.14).

Cd concentrations in the shoots of plants inoculated with selected bacteria were significantly different ($p<0.05$) from that of control. They were 10.97±1.27, 11.09±0.94, 13.11±1.41 and 11.57±1.53 in plants inoculated with TKS21, TKS07, TKS05 and TKS10, respectively while Cd concentration in shoots of control plants was 9.52±0.52 (Table 5.15A). The maximum translocation factor (0.59) and extraction coefficient (0.40) for Cd were found in plants inoculated with TKS05 (Table 5.15A). Zn concentration in the shoots of the plants inoculated with TKS07 (236.79±18.20 mg kg^{-1} dry weight) was significantly different ($p<0.05$) from that of control (204.81±10.49 mg kg^{-1} dry weight) (Table 5.15B).

H. annuus inoculated with selected bacterial strains and grown on non-sterile metal contaminated soils are shown in Figure 5.31. These bacteria were found to promote growth as well as Cd accumulation in the shoots of *H. annuus*.

Table 5.14 Growth of *Helianthus annuus* inoculated with selected bacterial strains and grown in heavy metal contaminated soils in the greenhouse.

Bacterial strain	Shoot length (cm plant^{-1})	Root length (cm plant^{-1})	Fresh weight (g plant^{-1})	Dry weight (g plant^{-1})			Plant growth (% of control)
				Whole plant	Shoot	Root	
Control (uninoculation)	26.25 ± 1.99^{a}	11.00 ± 0.63^{a}	8.01 ± 1.62^{a}	1.07 ± 0.14^{a}	0.88 ± 0.10^{a}	0.19 ± 0.05^{a}	100.00
Chryseobacterium sp. TKS21	27.00 ± 4.73^{a}	16.17 ± 0.75^{c}	13.92 ± 2.73^{b}	1.51 ± 0.54$^{a, b}$	1.17 ± 0.41$^{a, c}$	0.34 ± 0.14^{b}	141.12
Comamonas testosteroni TKS07	32.83 ± 3.92^{b}	14.50 ± 2.59$^{b, c}$	15.17 ± 2.76^{b}	1.94 ± 0.36^{b}	1.70 ± 0.33^{b}	0.24 ± 0.06$^{a, b}$	181.31
Cupriavidus taiwanensis TKS05	33.83 ± 4.17^{b}	14.17 ± 2.32^{b}	12.52 ± 1.82^{b}	1.63 ± 0.27^{b}	1.42 ± 0.19$^{b, c}$	0.21 ± 0.10^{a}	152.34
Delftia sp. TKS10	32.83 ± 6.46^{b}	15.67 ± 0.82^{b,c}	14.54 ± 2.68^{b}	1.86 ± 0.41^{b}	1.53 ± 0.31^{b}	0.33 ± 0.12^{b}	173.83

The data are Mean±SD. A common letters in the same column for the plant inoculated with each bacterial strain are not significantly different from each other using LSD test (p>0.05).

Table 5.15 Cd **(A)** and Zn **(B)** concentrations in shoots and roots of *Helianthus annuus* inoculated with selected bacterial strains and grown in heavy metal contaminated soils in the greenhouse.

(A)

Bacterial strain	Cd concentration (mg kg^{-1} dry weight)			Translocation factor	Extraction coefficient
	Soil	Plant shoot	Plant root		
Control (uninoculation)	39.56 ± 2.09[a]	9.52 ± 0.52[a]	22.30 ± 2.20[a]	0.43	0.24
Chryseobacterium sp. TKS21	37.19 ± 2.14[a, c]	10.97 ± 1.27[c]	23.90 ± 3.29[a]	0.46	0.29
Comamonas testosteroni TKS07	34.78 ± 3.37[b, c]	11.09 ± 0.94[c]	22.13 ± 2.64[a]	0.50	0.32
Cupriavidus taiwanensis TKS05	33.06 ± 1.46[b]	13.11 ± 1.41[b]	22.25 ± 4.00[a]	0.59	0.40
Delftia sp. TKS10	36.75 ± 0.90[c]	11.57 ± 1.53[c]	21.87 ± 4.29[a]	0.53	0.31

(B)

Bacterial strain	Zn concentration (mg kg^{-1} dry weight)			Translocation factor	Extraction coefficient
	Soil	Plant shoot	Plant root		
Control (uninoculation)	1610.73 ± 82.01[a]	204.81 ± 10.49[a]	175.90 ± 19.82[a]	1.16	0.13
Chryseobacterium sp. TKS21	1565.70 ± 80.23[a]	215.46 ± 7.10[a, b, c]	189.84 ± 13.34[a]	1.13	0.14
Comamonas testosteroni TKS07	1586.29 ± 83.62[a]	236.79 ± 18.20[c]	183.40 ± 16.55[a]	1.29	0.15
Cupriavidus taiwanensis TKS05	1512.68 ± 72.67[a]	199.52 ± 12.20[a, b]	179.37 ± 26.39[a]	1.11	0.13
Delftia sp. TKS10	1507.92 ± 91.01[a]	226.43 ± 36.28[a, c]	168.85 ± 31.05[a]	1.34	0.15

The data are Mean±SD. A common letters in the same column for the plant inoculated with each bacterial strain are not significantly different from each other using LSD test (p>0.05).

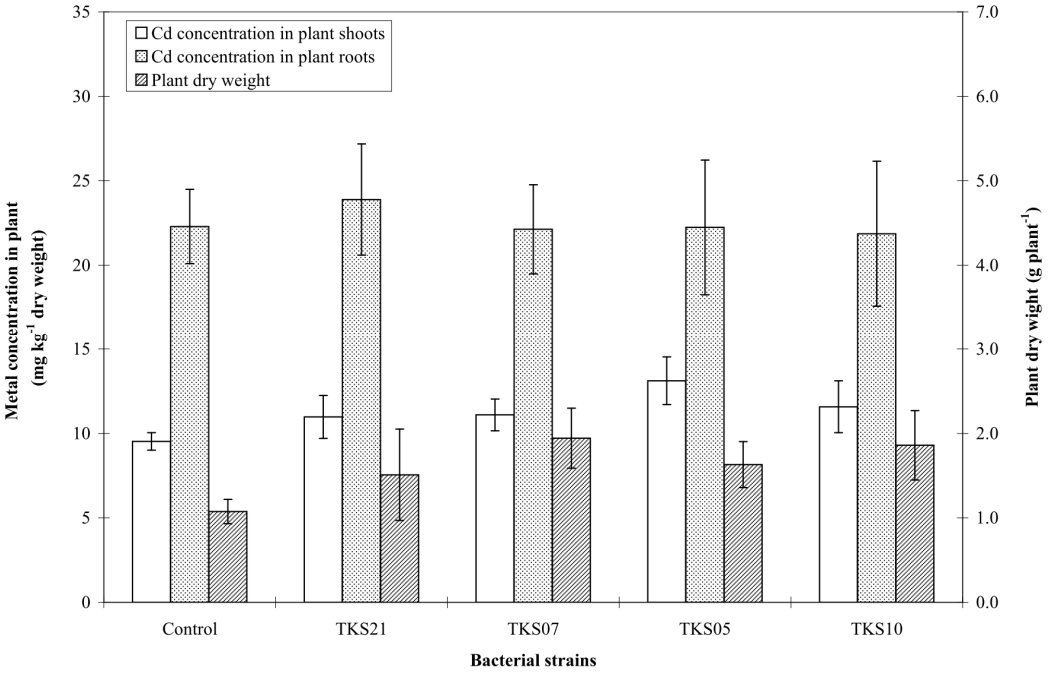


Figure 5.30 Cd accumulation and dry weight of *Helianthus annuus* inoculated with selected bacterial strains and grown in metal contaminated soils in the greenhouse.

Control = plants without bacterial inoculation,

TKS21 = plants inoculated with C*hryseobacterium sp*. TKS21

TKS07 = plants inoculated with *Comamonas testosteroni* TKS07

TKS05 = plants inoculated with *Cupriavidus taiwanensis* TKS05

TKS10 = plants inoculated with *Delftia sp.* TKS10

Figure 5.31 (A) *Helianthus annuus* inoculated with selected bacterial strains and grown in heavy metal contaminated soil for 30 days in the greenhouse. **(B)** *Helianthus annuus* inoculated with selected bacterial strains and grown in heavy metal contaminated soil for 60 days in the greenhouse. Control = plants without bacterial inoculation
TKS21 = plants inoculated with *Chryseobacterium sp.* TKS21
TKS07 = plants inoculated with *Comamonas testosteroni* TKS07
TKS05 = plants inoculated with *Cupriavidus taiwanensis* TKS05
TKS10 = plants inoculated with *Delftia sp.* TKS10


A
Control
TKS05
TKS07
TKS10
TKS21
B
Control
TKS05
TKS07
TKS10
TKS21

CHAPTER VI
DISCUSSION

6.1 Plant screening for phytoremediation potential

In metal-contaminated areas around Padaeng Zn mine, Cd and Zn concentrations in soils had significantly positive correlation. The correlative occurrence of these two metals was already confirmed in agricultural fields by Simmons *et al.* (2005). The total Cd and Zn concentrations in soils at the five study sites were obviously higher than non-contaminated soils, where the concentrations of Cd and Zn were generally less than 2 and 900 mg kg^{-1}, respectively (Alloway, 1995; Bowen, 1979). The Thai background levels for Cd and Zn were less than 0.1 and 140 mg kg^{-1}, respectively (Pongsakul & Attajarusit, 1999). Furthermore they were much greater than the levels reported as toxic with respect to plant growth by Kabata-Pendias & Pendias (1984) (3 – 8 mg Cd kg^{-1} and 70 – 400 mg Zn kg^{-1}). The maximum permissible levels set by the European Union were only 1 – 3 mg Cd kg^{-1} and 150 – 300 mg Zn kg^{-1} for low (pH 6) and high (pH 7) respectively (EEC, 1986). Thus, the high concentrations of metals in soils would be expected to strongly restrict plant growth. However at least 36 plant species collected in this survey grew well in those areas without obvious symptoms of toxicity, indicating they could tolerate elevated concentrations of the two metals. Many plant species were collected from Cd contaminated rice field (site 5) in which the soil had the lowest metal concentrations compared with other sites. The lower metal contamination might have little effects to plant growth and plants growing on this site could potentially accumulate heavy metals in their shoots. Crop plants such as rice and soy bean had also been cultivatable in this area with potentially high yield but their products were contaminated by heavy metals (Simmons *et al.*, 2003; 2005). High mobility of Cd in soil-plant system allows its easy entering into food network resulting in adverse health effects (Ryan *et al.*, 1982).

To date in an international context, over 45 families have been identified as hyperaccumulators (Salt *et al*., 1998; Dushenkov, 2003; Reeves & Baker, 2000), among which were those collected in this study (Acanthaceae, Amaranthaceae, Araceae, Asteraceae, Balsaminaceae, Boraginaceae, Buddlejaceae, Cyperaceae, Euphorbiaceae, Fabaceae, Lamiaceae, Malvaceae, Onagraceae, Poaceae, Rubiaceae and Scrophulariaceae). Most plants that are tolerant to heavy metals, including those collected during this survey, are herbaceous due to their inherent ability to adapt to stressful environments (Landberg & Greger, 1996; Yanqun *et al*., 2005). At least 12 species found in this survey were also found in Bo Ngam Pb mine, Thailand (Rotkittikhun *et al*., 2006) where the climate and environmental conditions were similar to the Padaeng Zn mine. They included *Chromolaena odoratum*, *Conyza sumatrensis*, *Sonchus arvensis*, *Buddleja asiatica*, all of which were considered hyperaccumulator plants suitable for Pb–contaminated soil phytoremediation schemes (Rotkittikhun *et al*., 2006).

A standard definition for Cd and/or Zn hyperaccumulator plants is yet to be agreed. At present, there are four criteria: (1) the concentration of heavy metal in plant shoots reaches hyperaccumulating level (Cd > 100 mg kg^{-1} dry mass; Zn > 10,000 mg kg^{-1} dry mass; Baker *et al*., 1994); (2) the concentration of Cd and Zn in above-ground biomass is 10-500 times greater than that in non-metallophytes (Cd 1 mg kg^{-1} dry mass; Zn 100 mg kg^{-1} dry mass; Shen & Liu, 1998); (3) the Cd and Zn concentrations in shoots are invariably greater than that in roots (namely a translocation factor > 1; Baker & Whiting, 2002); and (4) an extraction coefficient > 1 (Chen *et al*., 2004). According to the first criterion (Cd >100 mg kg^{-1} and Zn >10,000 mg kg^{-1}), 5 species qualified as potential Cd hyperaccumulators (*Brachiaria sp*., *Chromolaena odoratum*, *Gynura pseudochina*, *Impatien violaeflora* and *Justicia procumbens*) and only 1 species (*Justicia procumbens*) as a Zn hyperaccumulator. If translocation factor >1 is used as the selection criteria, 15 species could be considered as Cd hyperaccumulator and 8 species as Zn hyperaccumulator. Only *G. pseudochina* could be regarded as a potential Cd hyperaccumulator if an extraction coefficient >1.0 was considered. However total concentration of chemical elements in soils cannot be considered a good indicator of bioavailability (Adriano *et al*, 2004; Ure, 1996: Wang *et al*, 2004). The evaluation of bioavailable metals is considered of crucial importance

since it allows the assessment of the plant's potential to mobilize or to accumulate metals from soil (Braquinho *et al*., 2007). Branquinho *et al*. (2007) and González & González-Chávez (2006) further suggest that the bioaccumulation factor may be used for the assessment of potential hyperaccumulator. It is however necessary to be cautious when applying definitions of extraction coefficients and bioaccumulation factors because expressing the soil concentration as total soil composition or bioavailable composition will return very different results. The extraction method used to assess the bioavailability of metals in soils is also important when the bioaccumulation factor is mentioned as different extraction methods may give the dissimilar bioaccumulation values. However, the best extraction methods reflecting the actual metal bioavailability have not been defined presently. To evaluate the bioaccumulation factor both EDTA-extraction method (Branquinho *et al*., 2007) and DTPA-extraction method (González & González-Chávez, 2006) have been used. When the extraction coefficient was considered, only one plant was found to be a potential Cd hyperaccumulator, *G. pseudochina*. In contrast, when the bioaccumulation factor was taken into account, most plants had bioaccumulation > 1 indicating their potential as hyperaccumulator (Braquinho *et al*., 2007; González & González-Chávez, 2006).

In the present study, four plant species, *Chromolaena odoratum* (shurb), *Gynura pseudochina* (herb), *Impatiens violaeflora* (herb) and *Justicia procumbens* (herb) could be considered as Cd hyperaccumulators as they met most required criteria. They might be potential plants suitable for phytoextraction of Cd contaminated soils. Moreover *J. procumbens* also considered as a Zn hyperaccumulator was suitable for phytoextraction of Zn contaminated soils. These four hyperaccumulator plants accumulated Cd in their shoots although at considerably lower than *T. caerulescens* which can accumulate Cd in leaves up to 1,600 mg Cd kg^{-1} dry mass (Robinson *et al*., 1998). Nevertheless, they could be potential tools for Cd phytoextraction as they are adapted to the local climatic conditions, have high above-ground biomass and high propagation rates. Additionally, their shoots are upright making easy harvest. Furthermore the potential hyperaccumulator plants from the five selected study areas have an advantage over biomass producing crops as they are tolerant to highly elevated soil Cd and Zn concentration. The high biomass producing

crops, such as *Brassica juncea*, *Helianthus annuus*, *Nicotiana tabacum*, are also considered as suitable species for phytoremediation as they might compensate lower Cd accumulation with much higher biomass yields (Vassilev *et al*., 2002). However, the phytoextraction efficiency of the biomass crops is limited due to the low Cd concentration in the harvestable parts. If higher shoot Cd concentration would be achieved by means of induced phytoextration, the phytoextraction might be limited by phytotoxicity problem (Vassilev *et al*., 2002). Among the likely hyperaccumulator plants identified in this study, *J. procumbens* has the greatest potential to be used in a phytoextraction program if Cd concentration in shoots is the selection criterion adopted. However, Cd phytoextraction by *J. procumbens*, *G. pseudochina* and *I. violaeflora* could be limited as they have seasonal growth periods growing well in the rainy season only. The *C. odoratum*, although with lower Cd concentrations in its shoots as compared with the other three species, may have greater potential in phytoextraction programs due to its high biomass production, good distribution, rapid growth and profuse root system (McFadyen & Skarratt, 1996). Furthermore, *C. odoratum* is a perennial shrub that forms dense tangled bushes 1.5 – 2.0 m in height (McFadyen & Skarratt, 1996). In addition, it is generally preferable to use a perennial since phytoremediation is unlikely to take a single year. Further, the use of a perennial will prevent the need for annual planting (Rothittikhun *et al*., 2006). Due to these factors, and its wind-dispersed seeds, overall it appears to be the best candidate for a practical phytoextraction program.

6.2 Accumulation, toxicity and localization of Cd in *Chromolaena odoratum*

Chromolaena odoratum has been considered a Cd and Pb hyperaccumulator from the field surveys at Padaeng Zn mine, Tak province and Bo Ngam Pb mine, Kanchanaburi province, Thailand, respectively (Phaenark *et al*., 2009; Rotkittikhun *et al*., 2006; Tanhan *et al*., 2007). Plants from field collection accumulated Cd in their shoots and roots up to 166 and 110 mg kg^{-1} DW, respectively (Phaenark *et al*., 2009). They could also accumulate 1,377 and 4,236 mg Pb kg^{-1} DW in their shoots and roots, respectively (Tanhan *et al*., 2007). In hydroponic experiments, the maximum Pb concentrations in shoots and roots of *C. odoratum* were 1,772 and 60,656 mg kg^{-1} DW, respectively, at a Pb supply level of 10 mg l^{-1} (Tanhan *et al*., 2007). In the present study, Cd accumulations by *C. odoratum* were proportionally increased with increased Cd concentrations in culture media while the plant growth was gradually reduced. The maximum Cd concentration in shoots (416.51 mg kg^{-1} DW) was found in plants exposed to 5 mg Cd l^{-1} while that in roots (4,389.04 mg kg^{-1} DW) was found in plants treated with 10 mg Cd l^{-1}. Cd accumulated in roots was higher than that in shoots in all treatments. A number of reports also indicated that Cd was accumulated more in roots than in shoots (Meuwly & Rauser, 1992; Rauser & Meuwly, 1995; Lozano-Rodríguez *et al*., 1997; Seregin & Ivanov, 1997; Lagriffoul *et al*., 1998; Wójcik & Tukiendorf, 1999). *C. odoratum* accumulated Cd in shoots and roots up to 266 and 1,670 mg kg^{-1} DW without showing any toxicity symptoms at a Cd supply level of 2.5 mg l^{-1}. The results confirmed that this plant was a potential Cd hyperaccumulator since Cd concentrations in shoots exceed the hyperaccumulating criteria of 100 mg kg^{-1} DW (Baker *et al*., 1994). *C. odoratum* was a moderate hyperaccumulator with lower Cd concentrations in shoots compared with *Thalspi caerulescense*, the well-known Cd/Zn hyperaccumulator. In a French population of *T. caerulescense* (Ganges population), Cd concentration in shoots was exceptionally high (up to 10,000 µg g^{-1} DW) in hydroponically grown plants (Lombi *et al*., 2000). In addition, *T. caerulescens* from Plombières could accumulate 707 and 602 mg Cd kg^{-1} DW in their roots and shoots, respectively (Wójcik *et al*., 2005a). Although *T. caerulescens* is very efficient in taking up high amounts of Zn, Cd and Ni from the soil, as well as accumulating these metals in shoots, the use of this species for

phytoremediation on a commercial scale is limited due to its small size, slow growth rate and a rosette growth habit, making mechanical harvesting difficult (Wójcik *et al*., 2005a). *C. odoratum* was the good candidate possible for cleaning up Cd contaminated soils since it not only tolerates high Cd level but also retains many above favorable characteristics suitable for practical phytoremediation. It had rapid growth rate, large biomass production, abundant seed production, wide distribution (McFadyen & Skarratt, 1996). Moreover, *C. odoratum* could adaptively grow in drought areas with their numerous roots and upright shoots make them easy to harvest mechanically (Phaenark *et al*., 2009; Tanhan *et al*., 2007). As aforementioned reasons, *C. odoratum* was the promising plant for phytoextraction technology.

Hyperaccumulators can adaptively tolerate to heavy metals; however, the phytoxicity would be found if the plants were exposed to extreme levels of metals. Symptoms of Cd toxicity depend both on the metal content in plant and the efficiency of its detoxification (Wójcik *et al*., 2005a). The most spectacular symptoms of Cd toxicity are: growth retardation, chlorosis and necrosis of leaves, red-brown coloration of leaf margins or veins. Cd changes root morphology, root and leaf anatomy, damages cell structures. It disturbs water balance, mineral nutrition, photosynthesis, respiration and plant development (Prasad 1995). Although, *C. odoratum* has good capability for Cd tolerance, plant growth reduction and phytotoxicity were noticed in plants exposed to high Cd level. In hydroponic experiment, *C. odoratum* could tolerate Cd up to 2.5 mg l^{-1} without any toxicity symptoms as well as alterations in the tissues. When plants were exposed to elevated Cd concentrations, phytoxicity could be found. The toxicity symptoms observed morphologically were brown color of roots and/or root fragmentation. Moreover chlorosis of leaves and red spots in veins and petioles were found in plants treated with 10 mg Cd l^{-1}. Furthermore, tissue damages as well as organelle deformities were obviously seen in plants exposed to Cd at the concentrations more than 5 mg l^{-1}, especially severe damages found in plants treated with 10 mg l^{-1}. Most parenchyma cells in the cortex, endodermis, as well as in the vascular system of roots were destroyed.

The toxicity symptoms seen in the presence of excessive amount of Cd may be due to the destroying of the defense systems of cells (Benavides *et al*., 2005). Wójcik & Tukiendorf (2005) also found damages of root ultrastructure in *Zea mays*

exposed to Cd. Most cortical cells were destroyed as well as many cells of the endodermis and pericycle. Electron-dense droplets were visible in the space surrounding the destroyed membranes and nuclei. Similar phenomenon was also found by Seregin & Ivanov (1997) and Doncheva (1998) in Cd- and Cu-stressed maize, respectively. Cd-caused structural changes in root apices, rhizodermis and cortex were observed by Lunáčková *et al*. (2003) in cuttings of *Salix alba* and *Populus euroamericana* treated with Cd. Cd toxic symptoms of root cells of *Allium sativum* were mainly continued to disintegration of cell organelles, disruption of membranes, withdrawal of plasma membrane from cell wall, and formation of multivesicular bodies in the cytoplasm (Liu & Kottke, 2003). The changes of leaf ultrastructure were much less apparent probably due to much lower Cd content in these organs.

In the present study, organelles associated with plant metabolism (mitochondria and chloroplast) were affected by Cd toxicity. Chloroplasts were swollen with an increased number of starch grains, disarrangement of thylacoid membrane, and destruction of chloroplast membrane. Cristae as well as membrane of mitochondria were also affected. Baszynski *et al*. (1980) also found disorganization of grana and an increase in the number and size of plastoglobuli in Cd-treated tomato plants. Moreover, *Euglena gracilis* exposed to Cd showed distortion of chloroplast, irregularly arranged thylakoids and more plastoglobuli (Duret *et al*., 1986). Ghoshroy & Nadakavukaren (1990) also observed retardation of chloroplast development and severe disruption of grana thylakoids in soybean seedlings grown in Hoagland's solution containing Cd. The toxicity of non–essential metals such as Cd is, at least in part, a consequence of interference of these metals with homeostatic pathways for essential metals (Gzyl *et al*., 2009). It is well established that Cd and other heavy metals may interfere with nutrition balance (Burzyński, 1987; 1988) and hydration of plants (Pal *et al*., 2006), thereby resulting in ultrastructural degradation. The changes of mitochondrial structures may be induced as a result of deficit of biologically active Fe and other essential elements (Vázquez *et al*., 1992b; Čiamporová & Mistrík, 1993). It is suggested that there is a relationship between ion deficiencies, such as those of Mg, P, S, K and Ca, in the cytoplasm and ultrastructural changes, including degradation of mitochondria (Čiamporová & Mistrík, 1993; Koyro, 1997).

Heavy metal tolerance in plants is the result of different processes which prevent excess and toxic heavy metal concentrations in the cytoplasm and organelles (Liu & Kottke, 2003). Subcellular comparmentation of heavy metals can reflect the mechanism that the plants employed to reduce metal toxicity. Cd transported apoplastically can be immobilized in cell wall and/or intercellular space (Nishizono *et al*., 1989) by binding with some extracellular carbohydrates (mucilage, callose) (Verkleij & Schat, 1990; Wagner, 1993). In bush bean, Cd ions were mostly bound by pectic sites and hystidyl groups of the cell wall (Leita *et al*., 1996). Cd localization in maize roots showed that the largest amounts of the metal were usually detected in apoplast including cell walls of the cortex parenchyma, endodermis, pericycle and sieve tubes, and much smaller amounts were detected inside protoplasts (Khan *et al*., 1984; Lozano-Rodríguez *et al*., 1997; Seregin & Ivanov, 1997). Also in other plant species, apoplast and especially cell wall appeared to be the main site of Cd accumulation in roots, e.g. in *Arabidopsis halleri* (Küpper *et al*., 2000) or in *Thlaspi caerulescens* (Vázquez *et al*., 1992a). In the present study, Cd was mainly found in cell wall as well as intercellular spaces among parenchyma cells in the cortex of the root tissues. However, Cd deposits were also found in cells located close to the vascular system. There is some reports revealed that Cd was located in cytoplasm, vacuoles and nuclei of maize roots (Rauser & Ackerley, 1987; Wójcik & Tukiendorf, 2005). Vázquez *et al*. (1992b) showed the presence of Cd in vacuoles and nuclei of bean roots. Cd taken into plant cell might be detoxified by binding with phytochelatin and transported into the vacuoles as non-toxic compound (Zenk, 1996). Phytochelatins (PCs), small metal binding peptides, are essential for normal constitutive tolerance to several non-essential metals, particularly Cd (Clemens *et al*., 1999). Cd ions bind phytochelatins and form stable PC/Cd complexes which are transported and sequestered in the plant vacuoles (Saxena *et al*., 1999). Cd detoxification is achieved by the accumulation Cd, associated with phytochelatins, within the vacuole (Salt *et al*., 1995). A number of Cd hypersensitive *Arabidopsis* mutants appeared to be impaired in PC synthesis (Howden *et al*., 1995; Cobbett *et al*., 1998). Tomato cell lines selected for hypertolerance to Cd exhibited enhanced PC synthesis under Cd exposure (Chen & Goldsbrough, 1994).

In the present study, Cd accumulated in plant leaves was found in palisade and spongy mesophyll cells and epidermal cell. Wójcik *et al*. (2005b) conducted an experiment to localize Cd in *Thlaspi caerulescens*. They also revealed the presence of Cd in vacuoles of mesophyll cells and epidermal cells. Specific Cd localization in mesophyll cells lying on the way of water migration from vascular cylinder to the epidermis and stomata distinctly indicates involvement of transpiration in metal transportation in the leaves (Wójcik *et al*., 2005b). If plants lose the normal function in transpiration system as a result of Cd toxicity, plants cannot transport metals as well as water to the shoot. Little Cd accumulation in shoots of plants exposed to high Cd level might be caused not only by the alterations of normal plant metabolism as a result of improper functions of enzyme caused by Cd ion (Benavides *et al*., 2005) but also the lack of normal transpiration system resulted from severe destruction of plant cell and/or tissues. Plant exposed to high Cd concentrations over a long period of time may die from acute metal toxicities; hence, it will lose its hyperaccumulating potential.

6.3 Rhizobacteria screening to find potential strains used to improve phytoremediation

Microorganisms are ubiquitous in soils to which hyperaccumulators are native, even in those soils containing high concentrations of metals (Schlegel *et al*., 1991; Ghaderian *et al*., 2000). There are many reports shown that the heavy metal resistant bacterial strains could be isolated from the roots of plants grown on metal contaminated soils. Belimov *et al*. (2005) showed that several Cd-tolerant bacterial strains were isolated from the root zone of Indian mustard (*Brassica juncea*) seedling grown in Cd supplemented soils as well as sewage sludge and mining waste highly contaminated with Cd. A high proportion of metal resistant bacteria persist in the rhizosphere of the hyperaccumulators *Thlaspi caerulescens* (Delorme *et al*., 2001) and *Alyssum bertolonii* (Mengoni *et al*., 2001) or *A. murale* (Abou-Shanab *et al*., 2003a) grown on soil contaminated with Zn and Ni or Ni, respectively. Cr^{6+} resistant plant growth promoting bacteria (PGPR), *Pseudomonas sp*. PsA4 and *Bacillus sp*. Ba32 were isolated from heavy metal contaminated soils (Rajkumar *et al*., 2006).

In the present study, 6 bacterial strains were isolated from the rhizosphere of *Chromolaena odoratum*, Cd hyperaccmulator, grown on Cd/Zn contaminated soils. They were *Chryseobacterium sp.* TKS21, *Comamonas testosteroni* TKS07, *Cupriavidus taiwanensis* TKS05, *Delftia sp.* TKS10, *Pseudomonas aeruginosa* TKS22, and *Serratia marcesens* TKS01. Moreover, *Citrobacter sp.* BKS01 the representative bacterium from non-polluted area was isolated from uncontaminated soils. Heavy metal tolerant capabilities of these isolated bacteria were studied in nutrient broth supplemented with various concentrations of Cd and Zn, in order to ensure the success of inoculation and colonization by the isolated bacteria after being introduced into the metal contaminated soils. The minimal inhibitory concentrations (MIC) of all bacterial strains isolated from metal contaminated soils ranged from 100 to >200 and 200 to > 400 mg l^{-1} for Cd and Zn, respectively while that from non-polluted soils was 40 mg Cd l^{-1} and 120 mg Zn l^{-1}. *C. testosteroni* TKS07 and *C. taiwanensis* TKS05 had the highest tolerant capability to heavy metals with the MIC of >200 mg Cd l^{-1} and 400 mg Zn l^{-1}. The results of metal resistance in these isolated bacterial strains were in agreement with those of earlier researchers. Gachhui *et al*. (1989) isolated 43 Hg resistant bacteria from different agricultural fields, out of which

25.5% tolerated up to 271.5 µg ml^{-1} concentration of $HgCl_2$. Malik *et al*. (2002) isolated bacterial strains from industrial soil and reported maximum MIC of 100 µg ml^{-1} for Hg and MIC values up to 2,400 µg ml^{-1} for the other metals (Cu^{2+}, Cd^{2+}, Zn^{2+}, Ni^{2+}, Pb^{2+}, Cr^{3+} and Cr^{6+}). Ansari & Malik (2007) reported that 40 bacterial strains isolated from heavy metal contaminated soils exhibited a maximum MIC of 32 µg ml^{-1} for Hg^{2+}, 200 µg ml^{-1} for Cd^{2+}, 400 µg ml^{-1} for Zn^{2+} and Cu^{2+}, 800 µg ml^{-1} for Ni^{2+} and 1600 µg ml^{-1} for Pb^{2+}.

In the present study, isolated bacterial strains had the ability to absorb heavy metals. Metal biosorption by isolated strains ranged from 333 – 5,493 and 89 – 1,263 µg g^{-1} dry mass for Cd and Zn, respectively with the maximum biosorption found in *P. aeruginosa* TKS22. Actually, intact microbial cells, live or dead, and their products can be highly efficient bioaccumultors of both soluble and particulate forms of metals (Lovley *et al*., 1993; Niu *et al*., 1993; Norberg & Persson, 1984; Silver & Phung, 1996; Silver, 1996). The cell surfaces of all microorganisms are negatively charged owing to the presence of various anionic structures. This gives bacteria the ability to bind metal cations. Various microbial species, mainly *Pseudomonas*, have been shown to be relatively efficient in bioaccumulation of different heavy metals from polluted effluents (Hussein *et al*., 2001; 2005). Hussein *et al*. (2005) revealed that Ni and Cd resistant strains of *P. putida* accumulated Cd up to 182.37 and 160.17 mg g^{-1} biomass, respectively when they were grown in liquid medium with Cd concentration of 2 mmol l^{-1}. They could also accumulate other heavy metals including Cr, Cu and Ni.

In the present study, *C. testosteroni* TKS07, *C. taiwanensis* TKS05, *Delftia sp*. TKS10 and *P. aeruginosa* TKS22 had a pronounced effect on increasing the extractable Cd concentration in autoclaved metal contaminated soils. These microbial enhancements of Cd solubility in soils could have potential for improvement of phytoextraction by plants. Soil microorganisms have been shown to possess several methods that are able to alter metal bioavailability in the soils (McGrath *et al*., 2001; Whiting *et al*., 2001; Lasat, 2002). Chen *et al*. (2005) revealed that the addition of rhizosphere bacteria to the autoclaved soils could increase the availability of Cu in the soils and Cu accumulation by *Elsholtzia splendens*. Abou-Shanab *et al*. (2003b) reported that concentration of extractable Ni was increased from a high Ni soil of 2.2 to

2.6 mg kg^{-1} when the soil was inoculated with *Microbacterium arabinogalactanolyticum* AY509224. The presence of rhizosphere bacteria increased concentrations of Zn (Whiting *et al*., 2001), Ni (Abou-Shanab *et al*., 2003b) and Se (de-Souza *et al*., 1999) in *T. caerulescens, A. murale* and *B. juncea*, respectively.

The exact mechanisms by which PGPR promote plant growth are not fully understood, but are thought to include (i) the ability to produce or change the concentration of phytohormones like IAA, gibberellic acid, cytokinins and ethylene (Arshad & Frankenberger, 1993; Glick, 1995), (ii) a symbiotic N_2 fixation (Boddey & Döbereiner, 1995), (iii) antagonism against phytopathogenic microorganisms by production of siderophores (Scher & Baker, 1982), antibiotics (Shanahan *et al*., 1992) and cyanide (Flaishman *et al*., 1996), (iv) solubilization of mineral phosphates and other nutrients (De Freitas *et al*., 1997; Gaur, 1990). In this study, all isolated bacterial strains were studied on their plant growth promoting properties (PGPs) including, IAA and siderophore production, phosphate solubilization.

All isolated bacterial strains which can produce IAA might be beneficial for phytoremediation by promoting growth of plants stressed by heavy metals. Fässler *et al*. (2010) reported that IAA can alleviate toxic effects of Pb and Zn on plant growth. Growth of metal-stressed plants was effectively increased with 10^{-10} M IAA, and also the extraction of both metals was significantly increased. Dell' Amico *et al*. (2008) showed that *Pseudomonas fluorescens* ACC9 and *P. tolaasii* ACC23 with IAA and siderophore production and ACCD activity could promote the growth of *Brassica napus* grown on either Cd contaminated or non-contaminated soils. In addition IAA productions by these two bacterial strains were enhanced in the presence of Cd^{2+}. Some IAA producing strains of *Pseudomonas putida*, *Xanthomonas maltophilia* and *Bacillus cereus* could promote symbiotic germination of orchid *Pterostylis vittata* (Wilkinson *et al*., 1994). Low levels of IAA produced *in situ* within tissue were sufficient for the growth responses of the colonized tissue (Wilkinson *et al*., 1994). IAA produced by bacteria promotes root growth by directly stimulating plant cell elongation of cell division (Glick *et al*., 1998). All isolated bacterial strains could produce IAA in the presence of tryptophan confirming that they used tryptophan as a precursor for IAA production (Yang *et al*., 2007). Bent *et al*. (2001) reported that the production of indole compounds by *Paenibacillus polymyxa* L6, *P. polymyxa* PW-2,

and *Pseudomonas fluorescens* M20 increased in concentration with increasing concentrations of tryptophan (0–200 mg ml^{-1}). Asghar *et al*. (2002) also showed that PGPR stains produced 24.6 µg ml^{-1} of auxins in the presence of L-tryptophan in medium, which was 184-fold more than that without tryptophan.

Chryseobacterium sp. TKS21, *Delftia sp*. TKS10, *P. aeruginosa* TKS22 and *S. marcescens* TKS01 were found to be siderophore producer. They were possibly used to enhance phytoremediation. Siderophore productions by bacteria possibly affect bioavailability of heavy metals and they can also be stimulated by the presence of metals (van der Lelie *et al*., 1999). Siderophore productions by *P. fluorescens* ACC9 and *P. tolaasii* ACC23 were increased under Cd-stress (Dell'Amico *et al*., 2008). Siderophore production in *Azobacter vinelandii* was increased in the presence of Zn(II) (Huyer & Page, 1988). Abou-shanab *et al*. (2006) confirmed that rhizobacteria could facilitate the release of Ni from the non-soluble phases in soil, thus enhancing Ni availability to *A. murale* through acid, siderophore production and phosphate solubilization. The use of phosphate solubilizing bacteria as inoculants simultaneously increases P uptake by the plant and crop yield. *Rhizobium leguminosarum* with P-solubilization ability significantly increases the P concentration and improves growth in maize and lettuce (Chabot *et al*., 1996). A strain of *Pseudomonas putida* stimulated plant growth and increased ^{32}P-labeled phosphate uptake in canola (Lifshitz *et al*., 1987). In the present study, *P. aeruginosa* TKS22, *S. marcescens* TKS01 and *Citrobacter sp*. BKS01 could solubilize phosphate. Moreover, TKS22 and BKS01 reduced the pH of the culture media from neutral to acidic. The decrease in pH clearly indicates the production of acids, which is considered to be responsible for P solubilization (Rodríauez & Fraga, 1999; Cunningham & Kuiack, 1992; Bano & Mussarat, 2003).

All isolated bacterial strains with some beneficial properties including metal tolerance and solubilization, IAA and siderophore production and phosphate solubilization may be useful for improving phytoremediation of Cd contaminated soil. However, the exact mechanisms by which the rhizobacteria promote plant growth and enhance metal uptake were not clearly understood. Plant inoculations by these isolated bacterial strains are necessary to confirm the potential of these bacteria for improvement of phytoremediation.

6.4 Bioinoculation of plant by PGPR to enhance metal uptake and promote plant growth

The use of plant growth promoting rhizobacteria (PGPR) in phytoremediation is now being considered because PGPR not only promote plant growth on contaminated sites but also enhance metal uptake by plants (Khan, 2005; Yan-de *et al*., 2007). Whiting *et al*. (2001) found that the addition of a mixed inoculum of *Microbacterium saperdae*, *Pseudomonas monteilii* and *Enterobacer cancerogenes* to surface sterilized seeds of *Thlaspi caerulescens* grown in autoclaved soils increased Zn concentration in shoots 2–fold compared with uninoculated control; the total accumulation of Zn was enhanced 4–fold. So *et al*. (2003) demonstrated that bacterial strains resistant to Cu^{2+}, Ni^{2+}, and Zn^{2+} were isolated from water hyacinths (*Eichhornia crassipes*), and the inoculation of some strains could increase the Cu^{2+} removal capacity of the plant. Xiong *et al*. (2008) also showed that rhizospheric bacteria not only protected the hydroponically grown *Sedum alfredii* against heavy metals (Pb, Zn, Cu and Cd), but also enhanced metal uptake by the plant.

In the present study, *Chryseobacterium sp*. TKS21, *Comamonas testosteroni* TKS07, *Cupriavidus taiwanensis* TKS05 and *Delftia* sp. TKS10, *Pseudomonas aeruginosa* TKS22, *Serratia marcescens* TKS01, and *Citrobacter sp*. BKS01 could colonize the roots of *Helianthus annuus*. Successful colonization and persistence in the plant rhizosphere are required for PGPR to exert their beneficial effect on plants (Elliot & Lynch, 1984). Rhizosphere colonization is considered to be a crucial step in the application of microorganisms for beneficial purposes such as biofertilizer, phytostimulation, biocontrol and phytoremediation (Lugtenberg *et al*. 2002). Root colonization, which is a complex process, is under the influence of various parameters such as bacterial traits, root exudates, biotic and abiotic factors (Benizri *et al*., 2001).

Chryseobacterium sp. TKS21, *C. testosteroni* TKS07, *C. taiwanensis* TKS05 and *Delftia* sp. TKS10 could promote the growth of *H. annuus* seedlings grown on media in culture bottles. The shoot and root length of plant seedlings inoculated by these strains were significantly increased compared with non-inoculated plants. Plant growth promotion might be caused by IAA produced by the selected bacterial strains since TKS21, TKS07, TKS05 and TKS10 were found to produce

IAA. The growth promoting phytohormone, IAA, is known to induce root growth by enhancing cell division, cell extension and inducing lateral root growth (Fässler *et al*., 2010). Rhizosphere bacteria, such as some strains of *Pseudomonas* and *Acinetobacter*, were found to produce IAA and thereby stimulate root elongation and lateral root production (Lippmann *et al*., 1995). Inoculation of seedling hypocotyls and roots of canola, wheat, tomato and sunflower with *Azotobater paspali* altered plant growth and development and significantly increased shoot and root weight and root surface area (Abbas & Okon, 1993). Plant growth promotion effects were hypothetically resulted from production of phytohormone (auxins, cytokinins or gibberellins) synthesized by colonizing bacterial cells or by a response of plant to bacterial elicitors of hormonal metabolism in the inoculated plant (Abbas & Okon, 1993).

Nevertheless, growth of *H. annuus* inoculated with *Pseudomonas aeruginosa* TKS22, *Serratia marcescens* TKS01 and *Citrobacter* sp. BKS01 was inhibited although these bacteria could also produce IAA. The inhibition of plant growth might be resulted from not only bacterial infection to plant roots (Walker *et al*., 2004) but also toxic compounds produced by bacterial inoculants (Wilkinson *et al*., 1994). Walker *et al*. (2004) reported that *Pseudomonas aeruginosa* PAO1 and PA14 could infect the roots of *Arabidopsis* and sweet basil (Bais *et al*., 2002). Bolton *et al*. (1990) also showed that some pseudomonas produce a toxin which specifically inhibits wheat (*Triticum aestivum*) root growth. Wilkinson *et al*. (1994) have revealed that symbiotic germination of *Pterostylis vittata* inhibited by IAA producing bacteria was possibly resulted from other growth regulators or toxins in culture which may be deleterious to mycorrhizal synthesis. Gibberellins, cytokinins and IAA have often been detected simultaneously in the culture fractions of many bacterial genera (Barea *et al*., 1976; Cacciari *et al*., 1989; Tien *et al*., 1979). Similarly, soil bacteria are also known to be capable of producing substances toxic to plant growth (Alström & Gerhardson, 1989; Fredrickson *et al*., 1987).

The use of plants for phytoextraction of heavy metals from contaminated soil is limited by the ability of the plants to grow on these soils and take up the target metals, as well as by the availability of the metals for plant uptake in the soil solution (Fässler *et al*., 2010). There are many reports shown that the rhizobacterial inoculation could protect the plants from metal toxicity as well as promote plant growth and also

enhance metal uptake by plants. Inoculation of Indian mustard and canola (*Brassica campestris*) seeds with the PGPR strain *Kluyvera ascorbata* SUD165 protected the plant against Ni toxicity (Burd *et al*., 1998). Belimov *et al*. (2001) revealed that metal resistant PGPR stimulate the growth of *B. napus* cultivated in Cd contaminated soil. Metal resistant strains *Azotobacter chroococcum* HKN-5, *Bacillus megaterium* HKP-1, *B. mucilaginosus* HKK-1 were found to increase growth of *B. juncea* and altered metal bioavailability in soil (Wu *et al*., 2006). In the present study, *Chryseobacterium sp*. TKS21, *C. testosteroni* TKS07, *C. taiwanensis* TKS05 and *Delftia sp*. TKS10 were selected to inoculate *H. annuus*' seedlings grown on sterile heavy metal contaminated soils in laboratory and non–sterile soils in the greenhouse. Obviously, all selected bacterial strains could increase growth of the plants grown on non-sterile metal contaminated soils in the greenhouse while TKS21 and TKS07 also promoted the growth of plants grown on sterile soils. It was likely that bacterial inoculation and environmental factors together influence growth of the metal stressed plants. Plant growth promotion may be resulted from IAA production. Abbass & Okon (1993) suggested that IAA and other plant hormones were responsible for increased growth of canola, tomato (*Lycopersicon esculentum* Mill.), and wheat (*Triticum turgidum* L.) in non-sterile soil inoculated with *Azotobacter paspali*. IAA produced by rhizobacteria can influence plant growth, including root development which improves the uptake of essential nutrients thus increasing plant growth (Vikram, 2007). In particular IAA increased root and sometimes also shoot growth of plants that were stressed by salinity or heavy metals (Chaudhry & Rasheed, 2003; Sheng & Xia, 2006; Egamberdieva, 2009). Leinhos & Bergmann (1995) suggested that exogenously applied IAA may serve in mediating morphological reactions of plants in response to stresses, particularly by increasing root growth. Fässler *et al*. (2010) showed that the addition of IAA (10^{-10} M) to nutrient solution can alleviate Zn and Pb stress in sunflowers by promoting root growth.

Soil condition can influence plant growth promotion by inoculated bacteria. Gholami *et al*. (2009) showed that inoculation with *Pseudomona putida* R-168 and DSM 291, *P. fluorescens* R-93 and DSM50090 and *Azospirillium lipoferum* DSM1691 had more stimulating effect on maize growth in non–sterile soil than sterile soil. It is likely that rhizobacteria, exerting their beneficial effects on plants, had more

competitive ability to survive and affect the growth of inoculated plants in the presence of indigenous microflora (Khalid *et al*., 2004). On the other hand, Roesti *et al*. (2006) suggested that inoculums of the PGPR strains on the seeds may have shifted the bacterial community equilibrium and favored for growth of beneficial population.

In the present study, *Chryseobacterium sp*. TKS21, *C. testosteroni* TKS07, *C. taiwanensis* TKS05 and *Delftia sp*. TKS10 could enhance Cd accumulation in the shoots of *H. annuus*'s seedlings grown on non-sterile soils while TKS07 and TKS05 also increased Cd accumulation in the shoots of the plants grown on sterile soils. The enhancement of Cd accumulation might be mainly resulted from metal solubilizing activity by inoculated bacteria since TKS07, TKS05 and TKS10 were found to be Cd solubilizer. *Chryseobacterium* sp. TKS21, siderophore producer but not Cd solubilizer, could also enhance Cd accumulation. It might increase metal uptake of plant through siderophore production. Wu *et al*. (2006) showed that the higher DTPA extractable Cd and Cu induced by bacterial inoculation resulted in a correspondingly higher Cu and Cd concentration in both shoots and roots of *B. juncea*, suggesting that the bioavailability of these two metals was increased through bacterial metabolic activities or their interactions with the plants. Inoculation with PGPR may facilitate plant growth and thus increase phytoremediation efficiency. Enhancing metal accumulation in high yielding crop plants without diminishing their yield is fundamental to successful phytoextraction (Blaylock *et al*., 1997).

The efficiency of revegetation and phytoremediation of heavy metal contaminated sites is closely related to the presence of higher proportions of metal resistant microbial populations in the soil, which likely conferred a better nutritional assimilation and protection effect on plants (Doelman, 1985). These beneficial effects indicate that microbial inoculation might have some potential in aiding plant growth in the revegation of mine tailing pools or other heavy metal contaminated sites (Doelman, 1985; Wu *et al*., 2006). In the present study, *Chryseobacterium sp*. TKS21, *C. testosteroni* TKS07, *C. taiwanensis* TKS05 and *Delftia sp*. TKS10 might be eventually applied in the development of Cd phytoextraction. All of them could not only promote growth of metal–stressed plants but also enhance metal accumulation of the plants grown on metal contaminated soils. Although these PGPR are able to survive and colonize plant rhizosphere, the interaction between associative PGPR and

plants can be unstable. The results obtained *in vitro* cannot always be dependably reproduced under field conditions (Chanway & Hall, 1993; Zhender *et al*., 1999). The variability in the performance of PGPR may be due to various environmental factors that may affect the growth of PGPR or plant. The environmental factors are, for example, climate, weather conditions, soil characteristics or the composition or activity of indigenous microbial flora of the soil (Joseph *et al*., 2007).

CHAPTER VII
CONCLUSIONS

In the field survey, *Chromolaena odoratum*, *Gynura pseudochina*, *Impatiens violaeflora* and *Justicia procumbens* were considered as Cd hyperaccumulator and *Justicia procumbens* was also considered a Zn hyperaccumulator because they met required hyperaccumulation criteria which are high metal concentrations in their shoots, translocation and bioaccumulation factor more than 1. *C. odoratum* was the best candidate for phytoextraction because it accumulated not only high metal concentration but also retained many favorable characteristics suitable for phytoextraction including high biomass production, good distribution, profuse root system and upright shoot making easy to harvest.

In hydroponic experiment, Cd accumulation in *C. odoratum* was proportionally increased with increased Cd concentrations in media while plant growth was gradually reduced. The plants accumulated 266 and 1,670 mg Cd kg^{-1} DW in shoots and roots, respectively without showing any toxicity symptoms. Phytotoxicities were noticed in plants exposed to high Cd level. There were browning of roots, root fragmentation, chlorosis of leaves and red spots in veins and petioles. Tissue damages and organelle deformities were also observed. Most parenchyma cells in the cortex, endodermis, and vascular system of roots were destroyed. Chloroplasts in leaf mesophyll cells were swollen, increased number of starch grains, disarrangement of thylakoid membrane, and destruction of chloroplast membrane. Cristae and membrane of mitochondria were also affected. These degradations were possibly resulted from the interference of Cd with homeostatic pathway for essential metals.

Localization of heavy metals would reflect mechanisms by which the plants reduce metal toxicity. In *C odoratum*'s roots, Cd was mainly found in cell wall, intercellular space and in cells close to vascular system indicating that Cd transported apoplastically can be immobilized in cell wall and intercellular space while Cd taken into plant cells will be bound to phytochelatin and transported into vacuole. In plant

leaves, Cd was found in mesophyll cells lying on the way of water migration from vascular cylinder to the epidermis indicating involvement of transpiration in metal transportation in leaves.

Chryseobacterium sp. TKS21, *Comamonas testosteroni* TKS07, *Cupriavidus taiwanensis* TKS05, *Delftia sp*. TKS10, *Pseudomonas aeruginosa* TKS22 and *Serrtia marcescens* TKS01 were isolated from the rhizosphere of *C. odoratum* grown on heavy metal contaminated soils. *Citrobacter sp*. BKS01, the representative bacterium from non-polluted area was isolated from uncontaminated soil. All bacterial strains could tolerate Cd and Zn. Their MIC ranged from 40 to >200 and 120 to >400 mg l^{-1} for Cd and Zn, respectively. Bacterial strains isolated from contaminated soil could be tolerant to heavy metals more than the strain isolated from uncontaminated soil. Metal biosorptions by the isolated bacterial strains ranged from 333 – 5,493 and 89 – 1,263 µg g^{-1} dry mass for Cd and Zn, respectively. Normally, cell surface of bacteria is negatively charged owing to the presence of various anionic structures. This gives bacteria the ability to bind metal cations. The properties of isolated bacteria possibly enhance phytoremediation including IAA and siderophore production, and solubilization of phosphate and heavy metals were investigated. The bacterial strains with some beneficial properties might have a potential to be used in phytoremediation.

Successful colonization in the plant rhizosphere is required for plant growth promoting rhizobacteria (PGPR) to exert their beneficial effects on plants. *Chryseobacterium sp.* TKS21, *C. testosteroni* TKS07, *C. taiwanensis* TKS05, *Delftia sp*. TKS10, *P. aeruginosa* TKS22, *S. marcescens* TKS01, and *Citrobacter sp*. BKS01 could colonize the roots of *Helianthus annuus* grown in culture bottles. Moreover, TKS21, TKS07, TKS05, TKS10 could promote plant growth. Plant growth promotion may be resulted from IAA synthesized by colonizing bacteria or by a response of the plant to bacterial elicitors of hormonal metabolism in the inoculated plants. TKS21, TKS07, TKS05 and TKS10 not only promoted the growth but also enhanced Cd accumulation in the shoots of *H. annuus*' seedlings grown on non-sterile soils in the greenhouse. Cd accumulation enhanced by bacterial inoculants was possibly resulted from metal solubilizing activity by the bacteria. Enhancing metal accumulation in high yielding crop plants without diminishing their yield is fundamental to successful phytoextraction.

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