POTENTIAL USE OF PINEAPPLE (*ANANAS COMOSUS* L.) AND CADMIUM TOLERANT BACTERIA TO REDUCE CADMIUM TOXICITY IN SOIL

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Declaration

I hereby certify that this thesis is the result of my own investigations and that no part of it has been submitted for any degree other than Doctor of Philosophy at the Newcastle University. All references to the work of others have been duly acknowledged.

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Abstract

Cadmium (Cd) is a toxic metal that occurs in the environment naturally and as a pollutant from industrial and agricultural sources. The Cd concentration of agricultural soils in Thailand has increased during the last few decades as a consequence of mining activities. This toxic metal is allocated to edible plant parts and moves into the food chain via rice grains in some provinces in the Northern part of Thailand. Pineapple (*Ananas comosus* L.) is the most widely cultivated tropical fruit in Thailand and possesses the photosynthetic specialisation of crassulacean acid metabolism (CAM). Since CAM plants are generally tolerant to abiotic stress, it was hypothesised that pineapple would be relatively tolerant to Cd and could potentially be grown as a crop in Cd-contaminated soils. The present study determined the effect of Cd on photosynthetic performance, growth and Cd accumulation in different parts of pineapple. Pineapple plants grown in Cd-contaminated soil did not show any visual symptoms of toxicity and whilst light use efficiency (F_v/F_m) and rates of net dark CO_2 uptake were reduced by Cd treatment, biomass reductions were only 14% of control at the highest concentration of Cd (150 mg Cd kg^{-1}).

Levels of Cd accumulated by pineapple increased as the Cd concentration in soil increased. Pineapple plants took up Cd from the soil, resulting in lower Cd concentrations in the rhizosphere soil compared with the bulk soil. The soil-extractable Cd decreased over time which may have been due to the uptake of Cd by plant and to remaining metal ions becoming more tightly bound with organic materials or root exudates. Calculated as Cd µg per g dry weight, roots accumulated higher amount of Cd than shoots.

Short-term and long-term exposure to Cd resulted in a reduction in the numbers and diversity of soil microbes as indicated using plate count and DGGE techniques. The pineapple rhizosphere exhibited increased numbers of bacteria and fungi and a distinct and more diverse bacterial population when compared to bulk soil. Cd pollution of soil reduced both microbial numbers and bacterial diversity of all soil samples. Certain bacteria in the phyla *Nitrospirae, Verrucomicrobia, Acidobacteria* and *Chloroflexi* were only present in Cd-contaminated soil.

Cd-resistant bacteria were isolated from the rhizosphere of pineapple grown in Cd-contaminated soil and were subsequently used as inoculants in an effort to improve plant growth and reduce Cd toxicity. Nine isolates of Cd-resistant bacteria were isolated from the rhizosphere soil of pineapple. Two bacterial strains, *Bacillus firmus* and *Arthrobacter*

humicola were identified as having the potential to alleviate Cd toxicity in soil since both isolates were found to accumulate Cd at 17.21 and 19.83 mg Cd per g biomass at an initial Cd level of 50 mg Cd l^{-1}. Application of *B. firmus* and *A. humicola* near the root zone of pineapple grown in Cd-contaminated soil resulted in an increased uptake of nutrients compared with non-inoculated plants. In addition, soil inoculation resulted in higher rates of dark CO_2 uptake, reduced Cd accumulation and increased biomass of pineapple plants grown in Cd-contaminated soil as compared with non-inoculated plants.

Overall it would appear that pineapple could be grown commercially in Cd contaminated soils as it is relatively Cd resistant and it would also reduce total and extractable Cd soil levels over time. Bacterial inoculation of soil could be used to improve pineapple growth but the financial viability of this would need to be determined.

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Table of Contents

List of Figures

List of tables

Abbreviations

A	Ampere
ABA	Abscisic acid
ACC-deaminase	1-aminocyclopropane-1-carboxylate-deaminase
Ag^{2+}	Silver ion
Al	Aluminium
APS	Ammonium persulphate
As	Arsenic
ATP	Adenosine 5´-triphosphate
ATPase	Adenosine triphosphatase
BSA	Bovine serum albumin
°C	Degree Celsius
C3	Photosynthesis with primary fixation of CO_2 catalysed by Rubisco
C4	Photosynthesis with primary fixation of CO_2 catalysed by PEPC
CAM	Crassulacean acid metabolism
Cd	Cadmium
Cd^{2+}	Cadmium ion
CdS	Cadmium sulfide
CEC	Cation exchange capacity
CFU	Colony forming units
Cr	Chromium
Chl	Chlorophyll
Cu	Copper
DGGE	Denaturing gradient gel electrophoresis

DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
E-64	Trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetracetic acid
F_m	Maximum fluorescence yield of a dark-adapted leaf
F_o	Minimum fluorescence yield of a dark-adapted leaf
F_v	Variable fluorescence yield of a dark-adapted leaf (F_m-F_o)
F_v/F_m	Maximum quantum yield of photosystem II
Fe	Iron
Fe^{3+}	Ferric ion
G6P	Glucose 6-phosphate
HCl	Hydrochloric acid
Hg	Mercury
Hg^{2+}	Mercuric ion
H^+-PPiase	H^+-Pyrophosphate
H_2S	Hydrogen sulphide
IAA	Indole Acetic Acid
ICP-OES	Inductive coupled plasma optical emission spectroscopy
K	Potassium
LWGB	Lower gel buffer
MDH	Malate dehydrogenase (EC 1.1.1.37)
MT	Metallothionein

mg	Milligrams
mm	Millimetres
Mn	Manganese
Mn^{4+}	Manganese ion
Mn (II)	Manganese (II) oxide
Mn (IV)	Manganese (IV) oxide
N	Nitrogen
NA	Nutrient agar
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NB	Nutrient broth
Ni	Nickel
P	Phosphorus
PAGE	Polyacrylamide gel electrophoresis
Pb	Lead
PC	Phytochelatin
PCS	Phytochelatin syntase
PCR	Polymerase Chain Reaction
PEP	Phosphoenolpyruvate
PEPC	Phosphoenolpyruvate carboxylase
PEPCK	Phosphoenolpyruvate carboxykinase
PFD	Photon flux density
PPFD	Photosynthetic photon flux (area) density
PGA	3-Phosphoglycerate

PGP	Plant growth promoting
PGPR	Plant growth promoting rhizobacteria
PMSF	Phenylmethylsulphonyl fluoride
PSII	Photosystem II
OAA	Oxaloacetate
ROS	Reactive oxygen species
RPM	Rotations per minute
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39)
S_2	Sulfide
SDS	Sodium dodecyl sulphate
Se	Selenium
SH	Sulfhydryl group
TAE	Tris-acetate EDTA buffer
TBE	Tris-borate EDTA buffer
TBS	Tris-buffered saline
TBST	Tris-buffered saline plus tween-20
TE	Tris-EDTA buffer
TEMED	N,N,N′,N´-tetra-methyl-ethylenediamine
TLP	Tris-buffered low-phosphate
TPHs	Total petroleum hydrocarbons
Tris-	2-amino-2-(hydroxymethyl)-1,3-propanediol
Tris-HCl	Tris-Hydrochloride
Tween-20	Polyoxyethylenesorbitan monolaurate
U	Uranium

UPGB	Upper gel buffer
UV	Ultra-violet
Zn	Zinc

Chapter I

General Introduction

1 General Introduction

Metal pollution has become one of the most severe environmental problems today. Excessive accumulation of heavy metals is toxic to most living things (Cosio *et al.*, 2005; Jing *et al.*, 2007) and cadmium (Cd) is a particularly toxic heavy metal with no known biological function. Natural soil properties (Cd may be present in parent rocks) or agricultural, manufacturing, mining, and waste disposal practices may lead to high soil Cd levels (Vig *et al.*, 2003). Cd can cause serious problems to microbes, plants and animals even at low concentrations and can be highly toxic to human beings through its bioaccumulation in the food chain (Florijn and Van Beusichem, 1993b; Das *et al.*, 1997; Liang *et al.*, 2005; Gu *et al.*, 2007). Cd can inhibit plant root and shoot growth, affect nutrient uptake and homeostasis and is frequently accumulated by agriculturally important crops (Sanità Di Toppi and Gabbrielli, 1999). Cadmium-enriched crop products that are consumed by animals and humans can result in high levels of Cd exposure and cause disease. In Japan, Cd-contaminated rice caused Itai-Itai disease near the Jinzu River basin in the middle of the 20th century; even in recent years, rice is the major source of Cd intake of people in Japan (Watanabe *et al.*, 2000). Soil Cd pollution is cumulative with levels increasing over time, the soil may eventually become unusable for crop production. Similarly, contamination of soil with Cd can negatively affect biodiversity and the activity of soil microbial communities (McGrath, 1994). The overall aim of this thesis is to investigate the potential to use microbial and plant combinations to reduce metal toxicity in contaminated soil.

1.1 Cd problems and toxicity in Thailand

The Cd concentration of agricultural soils in Thailand has increased during the last few decades. This toxic heavy metal is allocated to edible plant parts and moves into the food chain via rice grains in some provinces in the Northern part of Thailand (Honda *et al.*, 2010). In the Mae Sot District of Northern Thailand, Cd contamination of cultivated soil and rice grain was brought to public attention by a joint investigation carried out by the International Water Management Institute (IWMI) and the Department of Agriculture (DOA) in 2003 (National Research Center for Environmental and Hazardous Waste Management, 2005). The area is the biggest Zinc (Zn) production site in Thailand and the estimated mine production capacity is 50000 metric tons (Padaeng Industry Public Company Limited, 2008). Mining actions such as drilling, explosions, material transfer, mine tailings disposal and drainage causes extensive Cd distribution throughout the mined areas (Ministry of Industry, 2006). Soils in this area contain high concentrations of

Cd and Zn. The average Cd level in the sediment of cinder stacks was 228.5 mg Cd kg^{-1} soil (Tunmanee and Thongmarg, 1994). The studies of Simmons *et al.*, (2005) showed that total soil Cd concentrations in rice-based agricultural systems ranged from 0.5 to 284 mg Cd kg^{-1} soil which was upto 94 times European Economic Community (EEC) Maximum Permissible (MP) soil cadmium concentration of 3.0 mg Cd kg^{-1} soil and 1,800 times the Thai standard of 0.15 mg Cd kg^{-1} soil. The estimates of Cd level of contaminated soil in different areas are shown in Table 1.1.

Table 1.1 Typical levels of Cd in soil (non-polluted to polluted soil).

Area	Cd contamination (mg kg^{-1})	Source	Reference
Chaina	33.1	Paddy soils	Herawati *et al.*, (2000)
France	80.2	Sludge	Alloway, (1990)
Hamshire	0.70	Municipal waste incinerators	Abbott *et al.*, (1997)
Indonesia	20.1	Paddy soils	Herawati *et al.*, (2000)
Japan	81.4	Paddy soils	Herawati *et al.*, (2000)
Jintsu Valley (Japan)	3.0	Pb-Zn mine	Alloway, (1990)
Newcastle upon Tyne	0.65	Municipal waste incinerators	Rimmer *et al.*, (2006)
Scotland	0.09	Municipal waste incinerators	Collett *et al.*, (1998)
Shipham			
Garden	134	Zn mine	Alloway, (1990)
Field	365	Zn mine	Alloway, (1990)
Spain	0.40	Municipal waste incinerators	Meneses *et al.*, (1999)
Spain	0.20	Municipal waste incinerators	Llobet *et al.*, (2002)

Moreover, rice grain Cd concentrations in the 524 fields sampled, ranged from 0.05 to 7.7 mg Cd kg^{-1}. In addition, 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}. With this amount of Cd in rice and based on the Thai daily rice consumption, it was estimated that local residents would have been exposed to cadmium 14-30 times higher than that

recommended from Joint FAO/WHO Expert Committee on Food Additives (Padungtod *et al.,* 2011). The prolonged consumption of Cd contaminated rice has potential risks to public health and health impacts of Cd exposed populations in Mae Sot have been demonstrated. Padungtod *et al.,* (2011) suggested that the high concentration of Cd in Mae Sot district poses excessive risk of causing Cd-induced renal failure among the local residents who habitually consume contaminated rice. Even though the Mae Sot district in Tak province is one of the areas best known for rice cultivation in Thailand the high Cd contamination has led to prohibition of rice cultivation in the area in an effort to prevent further exposure. It is clear that the soils in this area either need remediation or would benefit from the growth of other useful crops that do not transfer Cd to the human population. This would also be of enormous benefit to the local economy.

1.2 Soil and metals

1.2.1 Metal availability in soils

Plants cannot usually access the total pool of a metal present in the growth substrate. Instead, the fraction of the metal which plants can absorb is known as the available or bioavailable fraction. Metals present in a soil can be divided into a number of fractions including; the soluble metal in the soil solution, metal-precipitates, metal sorbed to clays, hydrous oxides and organic matter, and metals within the matrix of soil minerals. These different fractions are all in dynamic equilibrium with each other (Norvell, 1991). However, while the soluble metal in the soil solution is directly available for plant uptake other soil metal pools are less available (Davis and Leckie, 1978; Del Castilho *et al.*, 1993). A change in the concentration of metal in the matrix of soil minerals is slow relative to exchange and desorption reactions between clays, hydrous oxides, organic matter and the soil solution (Shuman, 1991; Whitehead, 2000). Metals within the soil solution are the only soil fraction directly available for plant uptake (Fageria *et al.*, 1991; Marschner, 1995; Whitehead, 2000). Hence, factors which affect the concentration and speciation of metals in the soil solution will affect the bioavailability of metals to plants.

1.2.1.1 Factors affecting metal availability (with a focus on Cd)

1.2.1.1.1 Total metal concentration

The total metal concentration of a soil includes all fractions of a metal, from the readily available to the highly unavailable. Other soil factors, such as pH, organic matter, clay

and redox conditions, determine the proportion of total metal which is in the soil solution. Hence, while total metal provides the maximum pool of metal in the soil, other factors have a greater importance in determining how much of this soil pool will be available to plants (Wolt, 1994). In addition, many reports revealed that while total metal correlates with bioavailable soil pools of metal it is inadequate by itself to reflect bioavailability (Lexmond, 1980; Sauvé *et al.*, 1996; McBride *et al.*, 1997b; Sauvé *et al.*, 1997; Peijnenburg *et al.*, 2000).

1.2.1.1.2 pH

The equilibrium between metal speciation, solubility, adsorption and exchange on solid phase sites is intimately connected to solution pH (Olomu *et al.*, 1973; Kalbasi *et al.*, 1978; Cavallaro and McBride, 1984; McBride *et al.*, 1997b; Sauvé *et al.*, 1997). Many studies found that soil pH has a large effect on metal bioavailability (Turner, 1994; McBride *et al.*, 1997b). It has been revealed that some plants can actively or passively change H^+ excretion under heavy metal stress (Dong *et al.*, 2007). Such root-induced changes of rhizosphere pH play a major role in the bioavailability of many pH dependent nutrients but also potentially toxic metals and a range of trace metals (Hinsinger *et al.*, 2006). The solubility of most heavy metals is influenced by soil pH. In general, solubility and heavy metal uptake increases as pH decrease. Xian, (1989) showed that the exchangeable Cd in soil increased when soil pH decreased from 7.0 to 4.55. This is important as many researchers have found that Cd uptake and accumulation are related to the amount of exchangeable Cd rather than the total Cd (Xian, 1989; Wu and Zhang, 2002b; Cheng *et al.*, 2004). Soil pH significantly affected the accumulation of Cd in strawberry (Szteke and Jedrzejczak, 1988) and barley (Wu and Zhang, 2002b); a lower soil pH increased Cd uptake. Therefore, it may possible to reduce Cd toxicity in Cd-contaminated acid soil by lime addition. However, the alteration of pH has different affects in plant Cd absorption in hydroponic experiments. Some studies showed that in hydroponics decreasing pH may result in competition between H^+ and Cd^{2+}, and thereby reduce Cd absorption (Lu *et al.*, 1992).

1.2.1.1.3 Oxidation-reduction condition

Several physical and chemical properties of soils affect the metal solubilisation processes. Among the chemical properties, oxidation-reduction status (Eh) is important for the

solubility of heavy metal in contaminated soils and their availability to plants (Reddy and Patrick, 1977). Redox conditions can affect the availability of metals by affecting the proportion of particular metal species such Mn (II) and Mn (IV) in the soil solution and by affecting the solubility of metals in the soil solution (Patrick and Jugsujinda, 1992; Evangelou, 1998). Generally, Eh of the rhizosphere is lower than that of bulk soil due to the following reasons: (a) roots consume oxygen, (b) roots excrete reducing substances such as phenolics which can react with Fe^{3+} or Mn^{4+}, (c) rhizosphere microorganisms must consume oxygen when they make use of root secretion products. At a low redox potential, hydrogen sulfide (H_2S) is produced by micro-organism and then S_2- forms a complex with Cd forming insoluble cadmium sulfide (CdS) which is not easily absorbed by crops (Dong *et al.*, 2007). Most metal sulphides are highly insoluble even in strongly acid conditions (Lindsay, 1979). The presence of high amounts of Fe^{3+} and Mn^{4+} compete with Cd^{2+}, thereby reducing plant absorption. Lu *et al.*, (1992) showed that a significant decrease in Cd absorption and accumulation when Eh decreased in flooded rice fields. Finally, application of $(NH_4)_2SO_4$ and sulphate fertilizers has been shown to reduce Cd accumulation in plants due to H_2S production and subsequent, formation of CdS (Hassan *et al.*, 2005b).

1.2.1.1.4 Organic matter

Organic matter exerts a major control on the availability of metals to plants (McBride *et al.*, 1997b; Towers and Paterson, 1997) and it is especially responsible for reducing the availability of Cd and Zn to plants. It has been reported that organic matter reduces heavy metal toxicity by adsorbing metals and forming large stable metal-organic matter complexes that plants are unable to absorb (Alloway and Jackson, 1991; Bell *et al.*, 1991; El-Hassanin *et al.*, 1993). For example, MacLean, (1976) and Eriksson, (1988) showed that plant uptake of Cd decreased with an increase in soil organic matter. He and Singh, (1993) showed a positive correlation between soil organic matter and the bioavailability of Cd in contaminated soil. However, they found that plant uptake of Cd was greater in a newly cultivated soil than in adjacent soil that had undergone long-term cultivation, even though the newly cultivated soil had considerably more organic matter and less total Cd.

1.2.1.1.5 Clays and hydrous oxides

Clays and hydrous oxides such as oxides of Al, Fe and Mn, play an important role in the availability of metals. Clays and hydrous oxides reduce metal availability mainly by

specific adsorption onto surface hydroxyl groups (Miller *et al.*, 1987; Pampura *et al.*, 1993), nonspecific adsorption (exchange) (Kalbasi *et al.*, 1978; Basta and Tabatabai, 1992), co-precipitation (Martinez and McBride, 1998), and precipitation as the form of metal oxide or hydroxide (Martinez and McBride, 1998). Therefore, increasing clay and hydrous oxide contents in soils provides more sites for adsorption of metals and thus reduces the directly bioavailable metal (Shuman, 1975; Ghanem and Mikkelsen, 1988; Barrow, 1993; Qiao and Ho, 1996).

1.3 Effect of Cd on soil micro-organisms

Amongst the many heavy metals polluting soil, Cd is of concern not only because of its potentially harmful effects on humans, animals and plants, but also adverse effects on microbes and their roles in maintaining soil fertility (Smith, 1996; Yao *et al.*, 2000; Jose *et al.*, 2002). Some metals such as calcium, cobalt, chromium, copper, iron, potassium, magnesium, manganese, sodium, nickel and zinc are essential, serve as micronutrients and are used for redox-processes, to stabilize molecules through electrostatic interactions, as components of various enzymes and for regulation of osmotic pressure (Bruins *et al.*, 2000). Many other metals such as silver, aluminium, cadmium, gold, lead and mercury have no biological role and are nonessential (Bruins *et al.*, 2000) and are toxic to micro-organisms. The toxicity of nonessential metals occurs through the displacement of essential metals from their native binding sites or through ligand interactions (Nies, 1999; Bruins *et al.*, 2000). Metal ions such as Hg^{2+}, Cd^{2+} and Ag^{2+} tend to bind to SH groups and thus inhibit the activity of sensitive enzymes (Nies, 1999). Furthermore at high levels both essential and nonessential metals can damage cell membranes, alter enzyme specificity, disrupt cellular functions and damage the stucture of DNA (Bruins *et al.*, 2000). Cd can also cause changes in the size, composition and activity of soil microbial community (Giller *et al.*, 1998). The study of microbial population in soil by using plate dilution techniques has shown that bacteria are more sensitive to Cd than fungi. In addition, the effect of Cd on the bacterial population depended on its concentration used and the soil type (Vig *et al.*, 2003). A summary of the effects of Cd on soil micro-organisms is shown in Table 1.2.

Table 1.2 Effect of Cd on microbial populations and their activities.

Soil type	Cd concentration (mg kg^{-1})	Effect on microbes	Reference
Andosol soil	1124	Reduced CO_2 evolution Increase bacterial number	Hattori, (1992)
Agricultural soil	2	Reduced potential nitrification rate 14%	Smolders *et al.*, (2001)
	200	Reduced potential nitrification rate 50-80%	
Brown earth-loamy sand	10-100	Increased fungal numbers Reduced cellulose decomposition	Khan and Frankland, (1984)
Clay-loam	60	Reduced cellulose decomposition by 7% Reduced population of bacteria by 86%, fungi 60%, actinomycetes 86%	Khan and Frankland, (1984)
Clayey loam	2248	Stimulated N-mineralization	Hassen *et al.*, (1998)
Forest humus	1000	Reduced CO_2 16-24%	Fritze *et al.*, (2000)
Forest litter	250	Reduce CO_2 14%	Laskowski *et al.*, (1994)
	400	Reduced CO_2	Niklinska *et al.*, (1998)
Forest soil	500	Reduced CO, dehydrogenase activity, ATP content	Landi *et al.*, (2000)
Grey soil	11.24-1124	Decreased CO_2 Increased fungi number, ATP content No effect on bacterial number	Hattori, (1991)
	1830	Inhibit organic materials decomposition	Hattori, (1996)
Loamy soil	200-4000	Reduced CO_2 15-58%	Fritze *et al.*, (1995)
Montepaldi soil	3-4000	Reduced ATP content, dehydrogenase activity	Moreno *et al.*, (2001)
Red soil	5-100	Reduced biomass 14-91%	Khan *et al.*, (1997)
Sandy loam soil	4.7	Reduce microbial biomass 42-60%	Brookes *et al.*, (1986a)
	8.6	Reduced nitrogen fixation 25%	Brookes *et al.*, (1986b)
	10	Reduced C-mineralization	Gupta *et al.*, (1984)
	50	Reduced bacterial growth, microbial biomass, dehydrogenase enzyme	Dar, (1996)
Sandy luvisol	100	Reduce CO_2, dehydrogenase	Wilke, (1991)
Sewage sludge	815	Reduced dehydrogenase activity	Moreno *et al.*, (1999)
Semiarid soil	3-8000	Reduced ATP content, dehydrogenase activity	Moreno *et al.*, (2001)

1.4 Plant uptake and transport of metals

Plants have developed a range of mechanisms to obtain metals from solution and transport these metals within the plant body. The metal uptake through roots depends on the concentration of the metal in the soil. However, uptake does not increase linearly with the concentration of the metal in the soil. This is because metals are often present under bound conditions. The uptake efficiency is highest at lower concentrations, because the low metal concentration also minimises competition between the metal ions at the absorption (uptake) surface (Greger *et al.*, 1991). The larger the root surface area available, the more effective the uptake of metal ions. Competition for the metal ions between plants at the same location can also occur, reducing uptake efficiency (Marschner, 1995)

1.4.1 Uptake mechanisms

There are different pathways associated with the entry of dissolved substances into plant cells. The cytosol is a barrier between the vacuole and the outside of the plant cell that offers high resistance to the passage of any solution that includes salts and bases (Nultsch, 2001). Plants have a natural tendency to take up metals, and their passage into plant cells will probably be hampered by this barrier.

Soluble metal ions enter the root via either extracellular (apoplastic) or intracellular (symplastic) pathways. In the apoplastic pathway, metal moves through cell walls and any extracellular space (i.e. without crossing any membrane) as it travels across the root cortex. In the symplastic pathway, metals travel across the root cortex by passing from one cell to the next via the plasmodesmata. The ions can reach the endodermis, which is the beginning of the internal space by travelling along this waterway (Nultsch, 2001). To get into the xylem, the ions must pass through the endodermis and the Casparian strip (Figure 1.1). The Casparian strip is the barrier that limits the entry of metal ions into the xylem. Dissociated molecules and ions are transferred relatively easy, whereas substances with higher lipophilicities or strong binding capacities are usually retained. Once the metal ions are taken up by the roots, they can be stored in underground tissues or exported to the shoot. Transport into the shoot involves loading in the xylem sap and translocation to the aerial parts.

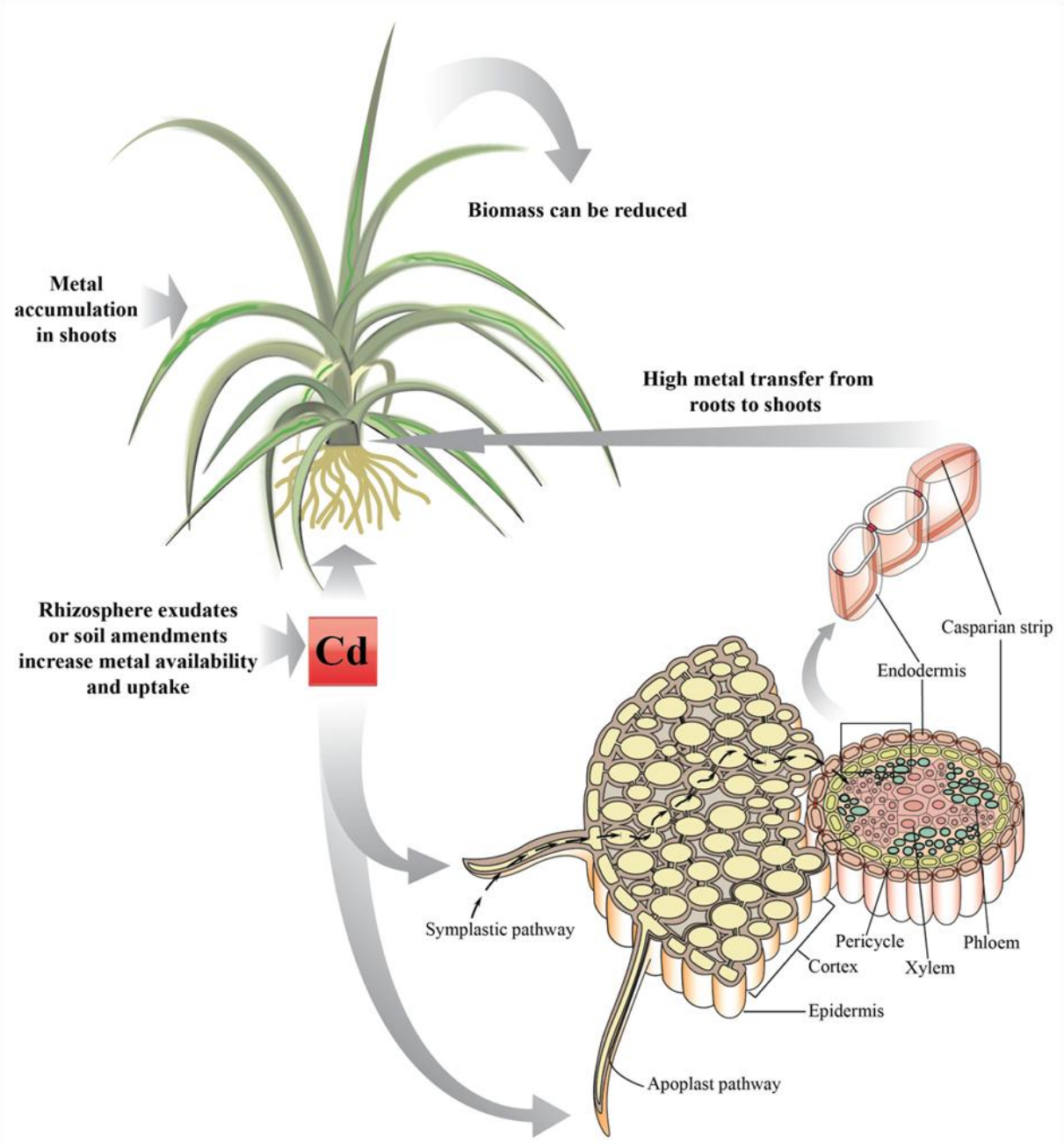

Figure 1.1 Pathways for metal uptake by the root. Free diffusion in the rhizosphere (1); Through the cortex, metal ions may travel via the apoplastic pathway and symplastic pathway (2); metal ions transfer to the symplast in the endodermis (3); transpiration-stream-driven transport to the shoot. The diagram is modified from (Do Nascimento and Xing, 2006; Taiz and Zeiger, 2006).

1.4.2 Metal transport in shoots

Two major transport mechanisms for metals are via the xylem and phloem. The effects of metals on the rate of movement and composition of the xylem and phloem sap may impact on plant response to metal toxicity. Translocation effects include the relative proportions of metals in roots and shoots, potential sites of toxic action of metals and the translocation of other nutrients within the plant. Xylem transport of metal ions is essentially driven by mass upward flow of water created by the transpiration stream (Kochian, 1991; Welch, 1995). Transport of metals within the phloem is thought to occur via the positive hydrostatic pressure gradient developed from the loading of sucrose into the phloem from mature actively photosynthesizing leaves and unloading of sucrose into the sink tissues such as rapidly growing tissues, apical root zones and reproductive organs (MacRobbie, 1971; Hocking, 1980; Welch, 1995). The mechanism of metal transport is shown as Figure 1.2.

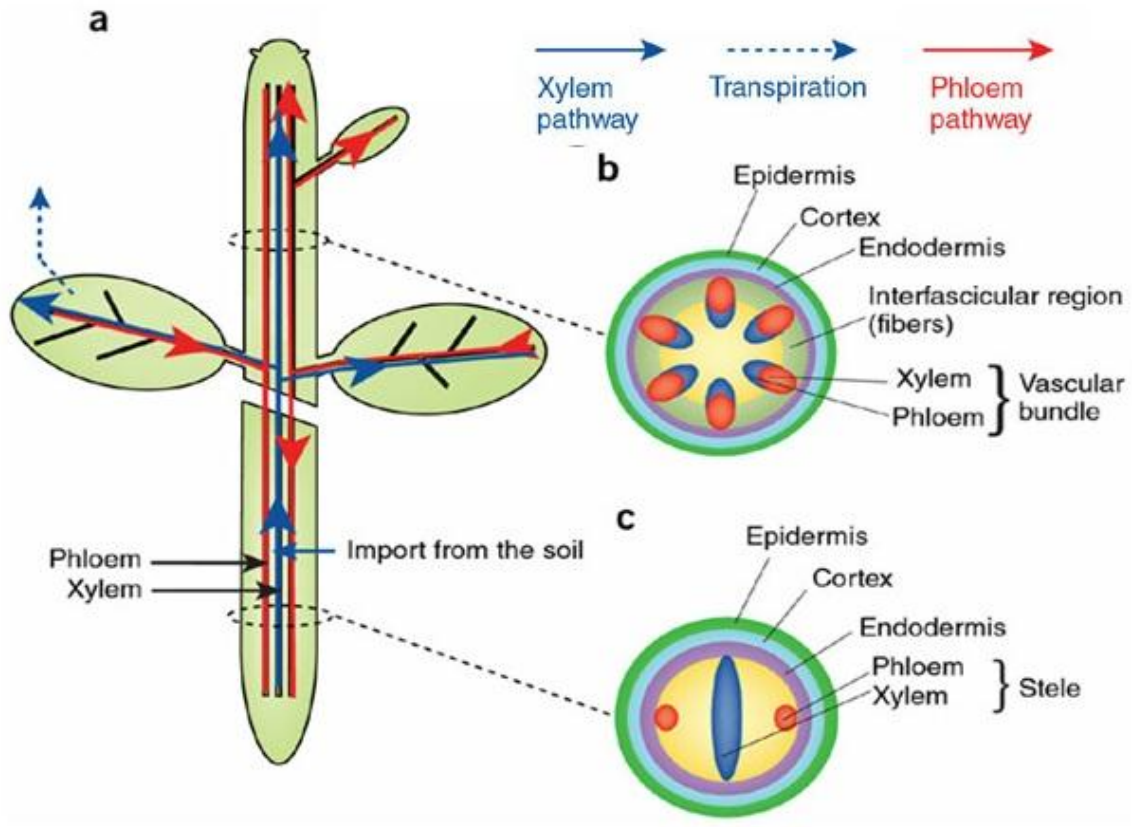

Figure 1.2 Routes of long-distance transport in plants (a). The xylem transport (blue) conducts metal ions from the root to shoot. The phloem flow (red) redistributes the metal ions from the leaves to roots and other sink tissues. Shoot stem (b) and root (c). Section schemes show the disposition of the vascular tissues. The diagram is modified from Robert and Friml, (2009).

1.5 Effect of Cd on plants

Cd does not have any beneficial physiological role in plants, but when accumulated, it affects all aspects of growth and development (Figure 1.3). In plants, Cd is one of the most readily absorbed and most rapidly translocated heavy metals, and can cause numerous morphological and physiological changes even at low concentrations (Seregin and Ivanov, 1998). At the morphological level, an excessive amount of Cd causes inhibition of plant growth, photosynthesis, enzyme function and cell metabolism in addition to disturbances in plant-water relationships (Mobin and Khan, 2007; Shi and Cai, 2008; Shi *et al.*, 2010). These effects are strongly dependent upon the plant species and the level of Cd applied.

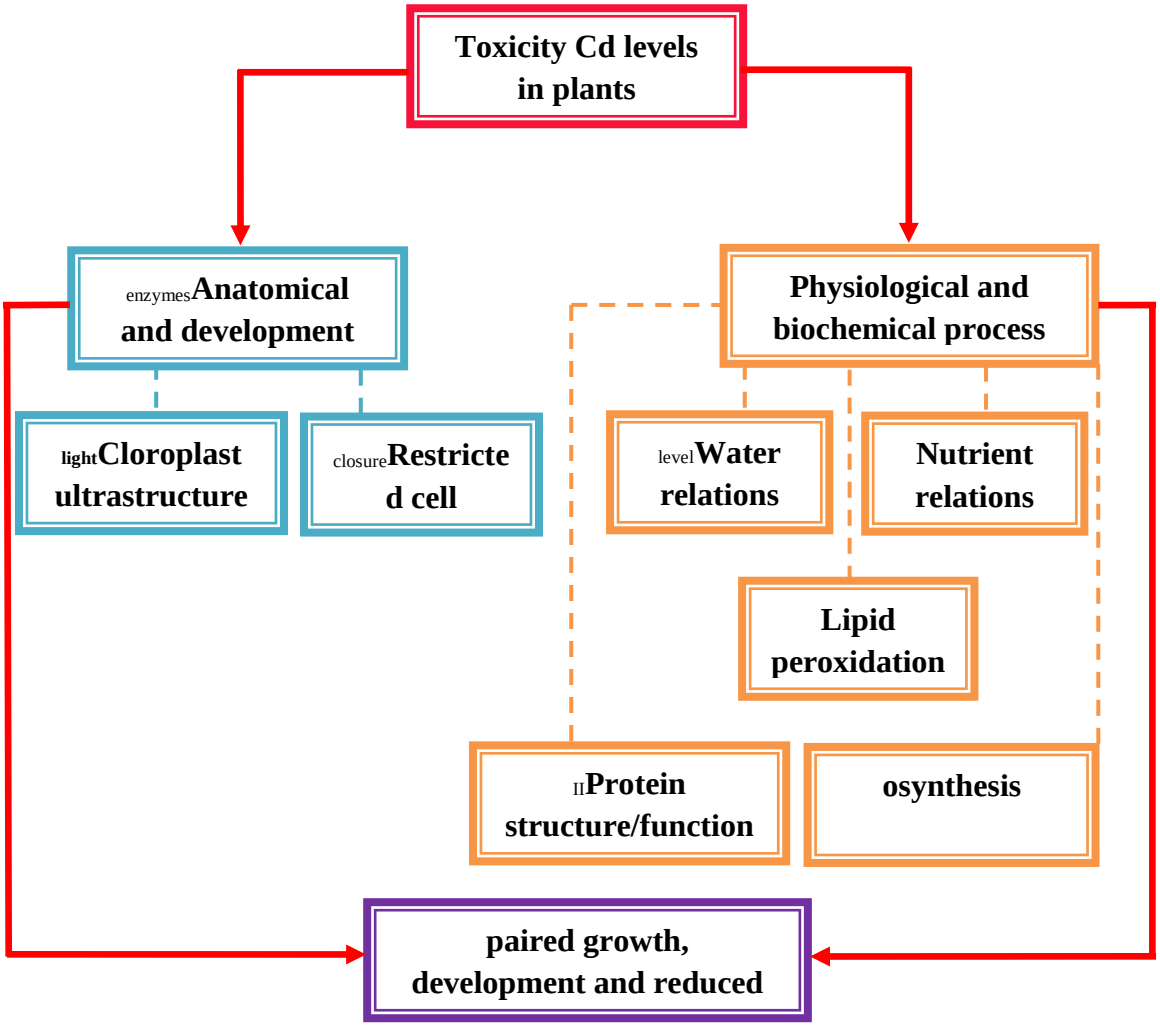

Figure 1.3 Proposed mechanisms of damage by toxic levels of Cd in plants. The Cd adversely affects the plant growth and development and these effects are evident on the physiological, biochemical and development phenomena of plants.

1.5.1 Symptoms of effects of Cd on plants

Plants exposed to Cd can show many symptoms such as chlorosis, leaf roll, and necrosis (Benavides *et al.*, 2005). Chlorosis, leaf roll and stunting are the main and most easily observed toxic symptoms in the presence of excessive amounts of heavy metals. Toxicity may result from the binding of metals to sulphydryl groups in proteins leading to disruption of structure, inhibition of activity and deficiency of essential elements (Van Assche and Clijsters, 1990). The excess amount of heavy metal may stimulate free radical production and reactive oxygen species resulting in oxidative stress (Dietz *et al.*, 1999). Some research has indicated that the effect of cadmium toxicity depends on the applied concentration, the age of leaves, phenological development, plant species and cultivar (Baszynski *et al.*, 1980; Becerril *et al.*, 1989; Costa and Morel, 1994; Lang *et al.*, 1995; Vassilev *et al.*, 1995). Table 1.3 illustrates the incidence of visible toxicity symptoms of Cd in different species.

Table 1.3 Visible effects of Cd toxicity to different plant species.

Plant species	Cd concentration (mg kg^{-1})	Symptoms	Reference
Brassica juncea	0.6	Chlorosis	Salt *et al.*, (1995c)
Catharanthus roseus	5	Chlorosis on youngest and older leaves	Kumar *et al.*, (2010)
Chickpea (*Cicer arietnum* L.)	100	Chlorosis, necrosis	Faizan *et al.*, (2011)
Fenugreek	5	Leaf chlorosis	Singal *et al.*, (1995)
Mungbean (*Vigna radiata*)	12	Enhanced leaf senescence and death of older leaves	Ghani, (2010)
		Producing injury symptoms on the younger leaves	
Populus cultivars	18	Chlorosis, Necrotic spots	Gu *et al.*, (2007)
Salix viminalis	1	Chlorosis	Cosio *et al.*, (2006)
	10	Chlorosis, necrosis, leaf rolling	
Sunflower (*Helianthus annuus*)	4	Chlorosis on the youngest leaves	Di Cagno *et al.*, (2001)
Pteris vittata	15, 30	Chlorosis, necrosis	Drava *et al.*, (2012)
Sedum alfredii	175	Induced leaf senescence	Zhou and Qiu, (2005)
	140, 175	Lower leaves fall off, root became brown and black	
Thlaspi caerulescens	10	Necrotic spots	
Tomato	2	Leaf necrosis, chlorosis, a reddish-brown discoloration of leaf blades, browning of root	Dong *et al.*, (2005)
Tomato	2	Chlorosis, root browning	López-Millán *et al.*, (2009)

1.5.2 Effect of Cd on plant growth

The most general visible, but nonspecific symptom of Cd stress is growth inhibition (Pal *et al.,* 2006). Differences in the degree of expressed phytotoxicity are due to various Cd-concentrations applied to the roots, the duration of treatment, as well as the characteristics of species and cultivars. Cd toxicity on plant growth can be seen clearly in roots because of their significantly higher heavy metal accumulation (Breckle, 1991). The inhibition of root growth can be attributed in part to the inhibition of mitosis, the reduced synthesis of cell-wall components, damage to the Golgi apparatus and changes in the polysaccharide metabolism (Punz and Sieghardt, 1993). Cd also inhibits germination processes and the development of seedlings (Stiborová *et al.*, 1987; Rascio *et al.*, 1993). The effects of Cd on plant growth are shown in Table 1.4.

Table 1.4 Effect of Cd on plant growth.

Plant species	Cd concentration (mg kg^{-1})	Symptoms	Reference
Faba bean plants	9	Significantly inhibited plant growth	Moussa, (2004)
Mungbean (*Vigna radiata*)	12	Reduced shoot length, root length and plant biomass Reduced number of green leaf Reduced leaf area per plant	Ghani, (2010)
Mustard	9	Significantly suppressed shoot weight	Chen *et al.,* (2011b)
	24	Both shoot and root weight decreased progressively	
Pakchoi	6	Marked decreased in root weight	Chen *et al.,* (2011b)
	12	Significantly reduced shoot weight	
	24	Both shoot and root weight decreased progressively	
Pea (*Pisum sativum*)	1	Decreased two-fold dry biomass	Januškaitiene, (2012)
Pfaffia glomerata	15	Significantly reduced total plant dry weight Reduced number of leaves per plant by 26%	Skrebsky *et al.*, (2008)
Pteris vittata	15, 30	Significantly reduced plant growth	Drava *et al.,* (2012)
Sedum alfredii	175	Inhibit root growth, altered root morphology, decreased leaf area, suppressed leaf expansion	Zhou and Qiu, (2005)
Winter barley	25-45	Reduced the productivity by 14-18%	Vassilev *et al.*, (1996)

1.5.3 Effect of Cd on photosynthesis

The negative impacts of Cd on plant growth have been attributed to detrimental effects of the heavy metal on photosynthesis (Sheoran *et al.*, 1990; Stoyanova and Chakalova, 1990; Vassilev *et al.*, 1995; Chen *et al.*, 2011b). Photosynthesis is dependent upon a suite of integrated physiological and biochemical processes (Figure 1.4). Key factors influencing the rate of photosynthesis are: (a) stomatal and mesophyll conductance; (b) the light reactions; (c) Rubisco activity and (d) Calvin cycle and associated carbohydrate processing activities.

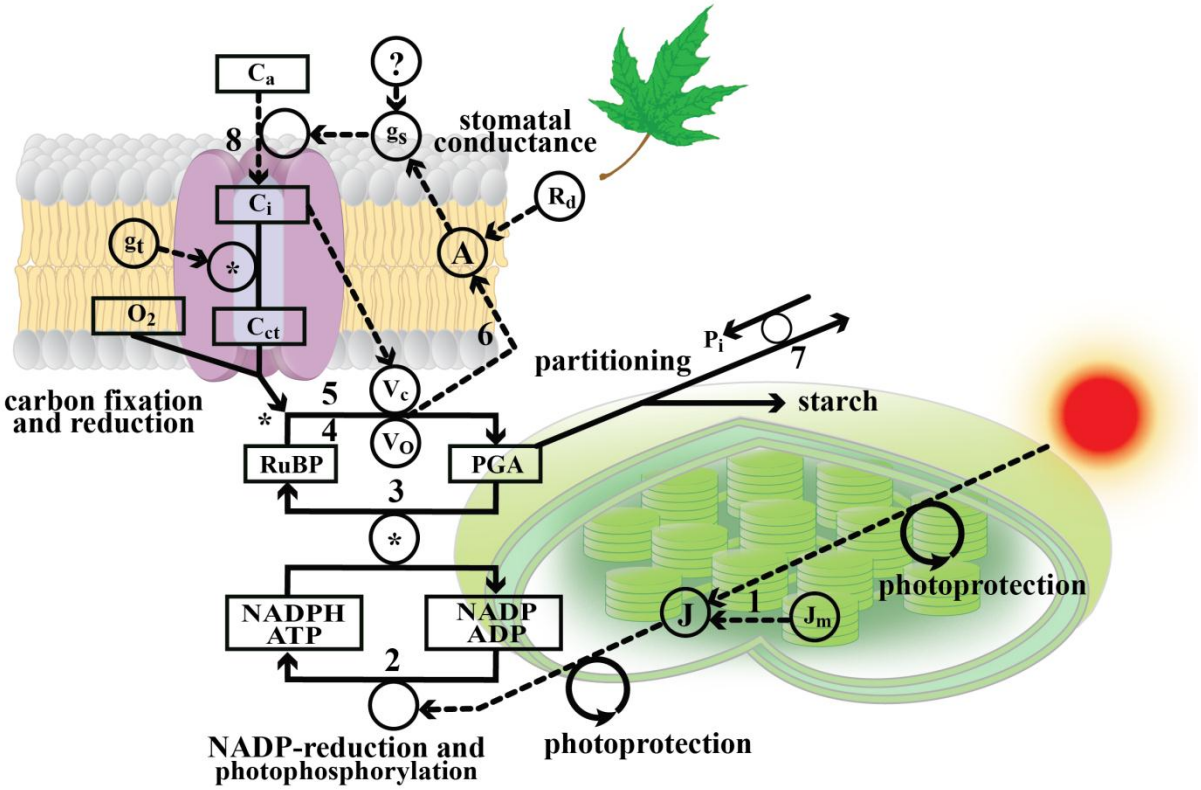

Figure 1.4 Diagram of factors affecting the process of photosynthesis. The reactions are as follows; (1) Light reactions; (2) NADP-reduction and photophosphorylation; (3) Calvin-cycle; (4) Oxygenation of Rubisco (leading to photorespiration); (5) Carboxylation of Rubisco leading to protection of triose phosphate; (6) Mesophyll conductance of CO_2 for fixation by Rubisco; (7) Carbohydrate production; (8) Stomatal conductance of CO_2. External atmospheric CO_2 concentrations (Ca); internal CO_2 concentration (Ci); cytosolic/chloroplastic CO_2 concentration (Cct); stomatal conductance (gs); internal/mesophyll conductance (gt); radiation (Rd); net CO_2 assimilation (A); Carboxylation (Vc); Oxygenation (Vo); electron transport (J); flux of electron transport rate (Jm). The diagram is modified from Taiz and Zeiger, (2006).

1.5.3.1 Stressful conditions and photosynthesis

The common stressful conditions of the environment to which plants are exposed include excess of soluble salts in the soil, water stress, heat, chilling, excessive light, heavy metals etc. Under these conditions, plant growth metabolism and photosynthesis are severely affected. The extent of effect depends on the plant species, the developmental stage of the plant as well as the type, intensity and duration of stress (Dubey, 2005). Effect of different stresses including heavy metal on photosynthetic components and related processes are shown in Figure 1.5

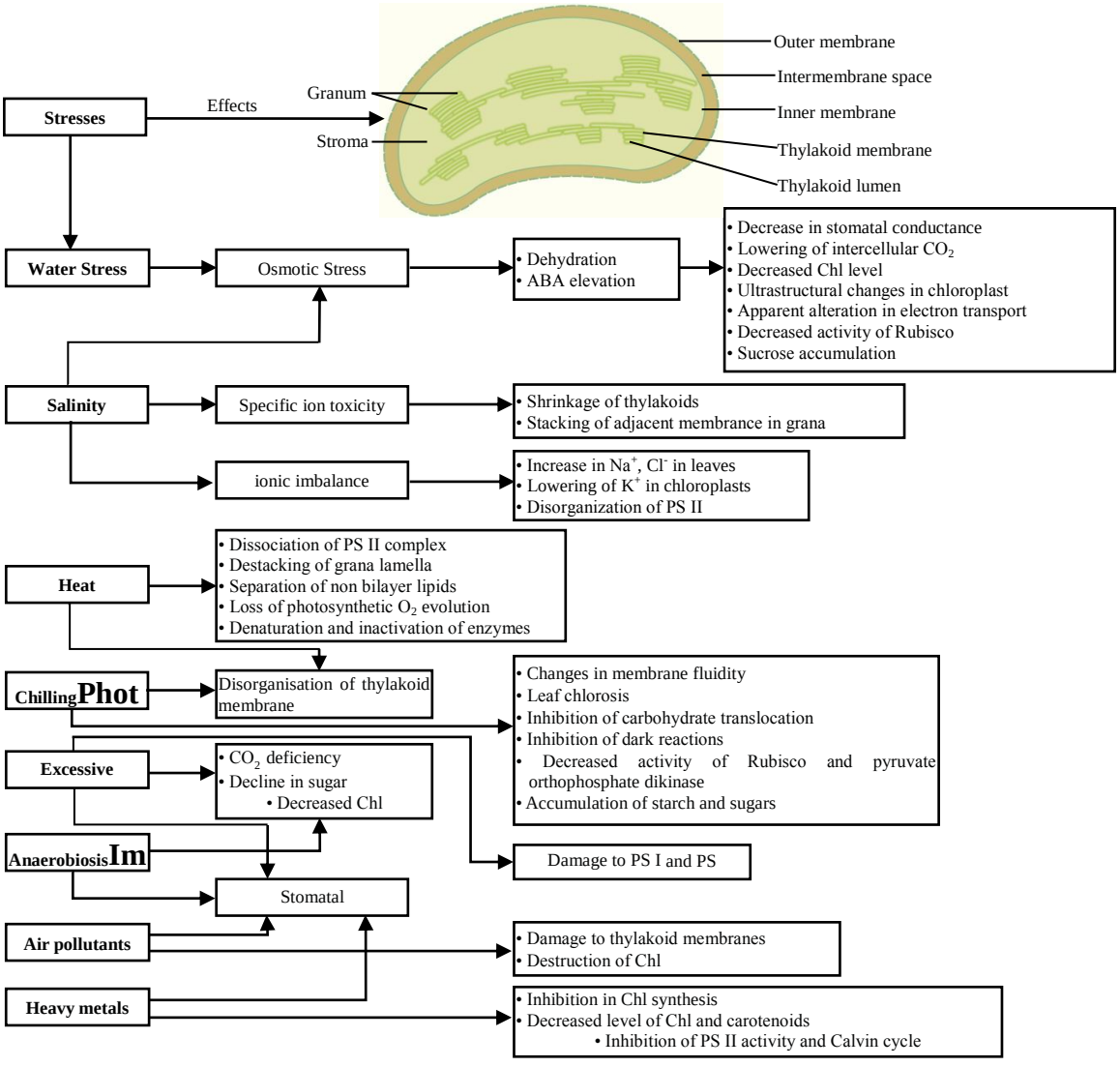

Figure 1.5 Effect of different stresses on photosynthetic components and related processes. Heavy metals have many effects in common and can cause stomatal closure, inhibition in chlorophyll synthesis, decreased level of Chl and carotenoids and inhibition of PSII activity and Calvin cycle enzymes. The diagram is modified from Dubey, (2005).

1.5.3.2 Effect of Cd on stomatal conductance

Cd induced reduction in photosynthesis has been related to reduced uptake of CO_2 from stomatal closure which may be linked to structural changes and to water stress imposed by the heavy metal (Bazzaz *et al.*, 1974; Lamoreaux and Chaney, 1977; Schlegel *et al.*, 1987; Krupa *et al.*, 1993). The effects of Cd on stomatal conductance are documented in Table 1.5.

Table 1.5 Effect of Cd on stomatal conductance.

Plant species	Cd concentration (mg kg^{-1})	Symptoms	Reference
Bacopa monniera L.	9	Increased stomatal closure	Ali *et al.*, (2000)
Bean plants (*Phaseolus vulgaris* L.)	10	Increased stomatal and mesophyll resistance Reduced CO_2 fixation	Krupa *et al.*, (1993)
Cajanus cajan (L.)	50	Reduced stamatal conductance Significantly reduced CO_2 uptake	Khudsar *et al.*, (2001)
Pakchoi	24	Stomatal conductance was depressed by 12.26%	Chen *et al.*, (2011b)
Mustard	12	Stomatal conductance was depressed by 12.26%	Chen *et al.*, (2011b)
Faba bean plants	9	Reduced transpiration rate Reduced CO_2 fixation	Moussa, (2004)
Populus cultivars	18	Reduced stomatal conductance	Gu *et al.*, (2007)
Phragmites australis	9	Decreased CO_2 fixation by 28%	Pietrini *et al.*, (2003)
	18	Decreased CO_2 fixation 60%	
	18	Induced stomatal closure Increase internal CO_2 concentration	
Safflower (*Carthamus tinctorius*)	5	Reduced transpiration rate by 48%	Sayed, (1997)
Soybean (*Glycine max* L.)	12	Decreased leaf relative water content Induced stomatal closure Decreased in transpiration rate	Leita *et al.*, (1995)
Sunflower (*Helianthus annuus*)	4	Reduced CO_2 fixation by 16%	Di Cagno *et al.*, (2001)
Tomato	0.2-2.0	Induced stomatal closure	Dong *et al.*, (2005)
White lupin (*Lupinus albus*)	25	Significant reduced CO_2 uptake Decreased water potential	Costa and Spitz, (1997)

1.5.3.3 Effect of Cd on light reactions

The light reactions are the first stage of photosynthesis. Light reactions are dependant upon many indices such as chlorophyll content, chlorophyll fluorescence parameters and the activity of Mg^{2+}-ATPase etc. (Govindjee and Van Rensen, 1978). Many results indicate that Cd decreases the absorption of light energy, electron transport conversion of light energy and photophosphorylation (Shumejko *et al.*, 1996; Fan and Wang, 2000; Wang *et al.*, 2009a). The effects of Cd on aspects important to the light reactions are shown in Table 1.6.

Table 1.6 Effect of Cd on light reactions.

Plant species	Cd concentration (mg kg^{-1})	Symptoms	Reference
Barley plants (*Hordeum vulgare* L.)	10	Decreased in photochemical efficiency (F_v/F_m) of PS(II)	Vassilev and Manolov, (1999)
Canavalia ensiformis	0.9	Reduced chlorophyll content	Lee and Roh, (2003)
Catharanthus roseus	5	Decreased chl a and b	
Mustard	6	Decreased photosynthetic rate 9.02% Decreased carotenoid content	Chen *et al.*, (2011b)
	24	Reduced chl a by 20.56% Reduced chl b by 21.55%	
Pakchoi	12	Decreased photosynthetic rate by 9.33%	Chen *et al.*, (2011b)
	24	Reduced chl a by 32.29% Reduced chl b by 21.54% Decreased carotenoid content	
Pea (*Pisum sativum* L.)	1	Decreased chl a and b	Januškaitiene, (2012)
Pteris vittata	15, 30	Decreased in photochemical efficiency (F_v/F_m) of PS(II) Decreased chl a	Drava *et al.*, (2012)
Rice (*Oryza sativa* L.)	1.4-13.7	Decreased in photochemical efficiency (F_v/F_m) of PS(II) Decreased in chlorophyll content and carotenoids	Pagliano *et al.*, (2006)
Sunflower (*Helianthus annuus*)	4	Slightly affected photochemical efficiency (F_v/F_m) of PS(II) Decreased 61% of Chl a and 79% chl b content	Di Cagno *et al.*, (2001)
Zea mays L.	4	Decrease total chlorophyll content Decreased in photochemical efficiency (F_v/F_m) of PS(II)	Wang *et al.*, (2009a)

1.5.3.4 Effect of Cd on Rubisco and C-processing enzymes

A common change noted in plant exposed to Cd-stress is the modulation of carbon metabolism enzymes (Gouia *et al.*, 2003; Houda *et al.*, 2008). Phosphoenolpyruvate carboxylase (PEPC) plays an anaplerotic role in the provision of C skeletons for amino acid synthesis. An increase in PEPC activity enhances the C flow through the anaplerotic pathways by providing C skeletons for the tricarboxylic acid cycle and for amino acid synthesis. Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), a bifunctional enzyme with the capacity to competitively use CO_2 or O_2 is the key enzyme responsible for overall CO_2 fixation during photosynthesis. Rubisco is considered to be the main target of heavy metal toxic influence on the photosynthetic apparatus (Krupa and Baszyński, 1995). Cd reduces the efficiency of photosynthesis by inhibiting Rubisco (Stiborová *et al.*, 1986a). Decreases in Rubisco activity is due to damaged protein structure and/or Cd-Mg replacement in the active centre (Stiborová, 1988; Malik *et al.*, 1992; Krupa and Baszyński, 1995). Contrastingly, Cd can enhance or inhibit the activity of PEPC in different plant species (Gouia *et al.*, 2003; Wang *et al.*, 2009a). The effect of Cd on Rubisco and C-processing enzymes are shown in Table 1.7.

Table 1.7 The effect of Cd on Rubisco and C-processing enzyme.

1.6 Plant tolerance to Cd toxicity

Plant species	Cd concentration (mg kg^{-1})	Symptoms	Reference
Bean plants (*Phaseolous vulgaris* L.)	4	Enhanced PEPC activity	Gouia *et al.*, (2003)
Canavalia ensiformis	0.9	Decreased Rubisco activity	Lee and Roh, (2003)
Catharanthus roseus	5	Decreased Rubisco activiy	Kumar *et al.*, (2010)
Faba bean plants	9	Decreased Rubisco activity	Moussa, (2004)
Nicotiana rustica	18	Decreased Rubisco activity Reduced soluble sugar content Enhanced PEPC activity	Afef *et al.*, (2011)
Phragmites australis	9	Decreased Rubisco activity by 58%	Pietrini *et al.*, (2003)
	18	Decreased Rubisco activity by 70%	
Sunflower (*Helianthus annuus*)	4	Decreased Rubisco activity	Di Cagno *et al.*, (2001)
	12	Significantly decreased Rubisco activity Weakly stimulated PEPC activity Significantly reduced malate dehydrogenase (MDH) activity	Hammami *et al.*, (2004)
Zea mays L.	4	Decreased PEPC activity Decreased Rubisco activity	Wang *et al.*, (2009a)

In order to survive soil metal pollution, plants have had to develop efficient and specific heavy metal detoxification mechanisms and these appear to vary in different plant species (Punz and Sieghardt, 1993). Below is a concise appraisal of the various metal resistance mechanisms observed in plants.

1.6.1 The cell wall and root exudates

The metal binding properties of the cell wall and its role in metal tolerance has been discussed (Ernst *et al.*, 1992). The root cell wall is directly in contact with metal in the soil solution, and once the metal binding capacity of the root cell wall has been reached the plasma membrane may be affected by metals (Ernst *et al.*, 1992). Bringezu *et al.*, (1999) demonstrated that the heavy metal tolerant *Silene vulgaris* accumulated metals in the epidermal cell walls by binding to a protein or as silicates thereby reducing metal access to the plant cell membrane. In addition, root exudates may precipitate pollutants outside the roots by absorbing and embedding them. Lin *et al.*, (1998) found that Cd complexed with oxides of Fe and Mn, and with some organic acids and this caused greater levels of Cd accumulation in the rice rhizosphere than that obtained in non-rhizosphere soil.

1.6.2 Plasma membrane

The plant plasma membrane may be the first structure that is a target for heavy metal toxicity. Plasma membrane function may be rapidly affected by metals as seen by an increased leakage from cells in the presence of high concentrations of metals (Hall, 2002). Heavy metal cause membrane damage through various mechanisms, including the oxidation of and cross-link with protein thiols, inhibition of key enzyme protein such as H^+-ATPase, or causing changes in the composition and fluidity of membranes lipids (Meharg, 1993). Cd treatments have been shown to reduce the ATPase activity of the plasma membrane fraction of wheat and sunflower roots (Fodor *et al.*, 1995). Thus, metal tolerance may involve the protection of plasma membrane integrity against heavy metal damage (De Vos *et al.*, 1991). The cell wall-plasma membrane interface accumulates large portions of heavy metals and it is therefore believed that this could be the potential site of heavy metal tolerance. In Italian ryegrass (*Lolium multiflorum*), 60% of metal in the roots was bound by the cell wall and plasma membrane (Iwasaki *et al.*, 1990). Plant cation exchange capacity (CEC) is largely determined by the exchange sites in cell walls (Horst and Marschner, 1978). Sensitive wheat cultuvars have much lower cell wall CECs than

tolerant cultivar (Masion and Bertsch, 1997) indicating that tolerant cultivars use a high CEC to complex heavy metals at the cell wall and prevent entry to the cell.

1.6.3 Phytochelatin

Chelation of metals in the cytosol by high-affinity ligands is potentially a very important mechanism of heavy metal detoxification and tolerance in plants. The ligands that have high potential to detoxify the effect of heavy metal are phytochelatins (PC) and metallothioneins (Clemens, 2001). The PCs have been widely studied in plants, particularly in relation to Cd tolerance (Cobbett, 2000). The PC are a family of sulphur rich peptides which are able to bind Cd and some other heavy metals (Cobbett and Goldsbrough, 2002). They are rapidly induced in plants by heavy metal treatment (Rauser, 1995; Zenk, 1996) and are synthesized using glutathione as a substrate by using phytochelatin synthase (PCS); an enzyme activated in the presence of metal ions (Cobbett, 2000). The study of Haag-Kerwer *et al.*, (1999) found that the Cd accumulation of *Brassica juncea* is accompanied by a rapid induction of PC biosynthesis and that the PC content was sufficient to chelate all Cd taken up; this protected photosynthesis but did not prevent a decline in transpiration rate. Transgenic tobacco plants over-expressing cysteine synthase in the cytosol or chloroplasts, had elevated concentrations of PCs, were more tolerant to Cd, Se and Ni but did not accumulate the metal in the leaves (Harada *et al.*, 2001).

1.6.4 Complexing by metallothioneins

Metallothioneins (MT) are a group of low molecular mass, cysteine rich, metal-binding proteins (Robinson and Jackson, 1986; Tomsett and Thurman, 1988). It has been suggested that they function in the regulation of essential metals and in the detoxification of all metals (Steffens, 1990). The MT protect guard cell chloroplasts from degradation upon exposure to Cd, by reducing the presence of reactive oxygen species. It was concluded that the Cd stays bound to the MT in the cytoplasm and is not sequestered into the vacuole, as occurs when Cd is detoxified by PC (Evans *et al.*, 1992).

1.6.5 Organic acids and amino acids

Plants produce several ligands for Cd, Cu, Ni and Zn including citric, malic and histidine and these ligands may play an important role in tolerance and detoxification (Clemens, 2001). Cd-and Zn-citrate complexes are prevalent in leaves, even though malate is more abundant (Rauser, 1995). In the xylem sap moving from roots to leaves, citrate and histidine are the principle ligands for Cu, Ni and Zn (Rauser, 1999). Kramer *et al.*, (1996) showed that the histidine content of the xylem sap on the exposure to Ni was increased 36-fold in the Ni-hyperaccumulating plant *Alyssum lesbiacum*. Specific organic acids can sequester heavy metals and protect the roots from toxicity effects (Jones *et al.*, 2003; Jung *et al.*, 2003; Liao and Xie, 2004; Schwab *et al.*, 2005). Accordingly, the composition and quantity of root secretion may affect the form of heavy metals present. Some plant species such as paddy rice secrete phytosiderophores (amino acids) that can form much more stable complexes than carboxylates with Fe, Cd, Zn and Cu (Römheld, 1991; Hinsinger, 1998; Chaignon *et al.*, 2002; Xu *et al.*, 2005). Mench and Martin, (1991) reported that low molecular weight organic acids secreted by roots played an important role in solubility and availability of heavy metals, and Cd^{2+} availability would be reduced if Cd^{2+} was bound into a Cd chelate complex with root secretion. The roots of some plants, such as wheat and buckwheat, excrete organic acids such oxalic acid, malic acid and citric acid that can chelate with Cd^{2+} to prevent its entrance into roots. Tang, (1998) observed that amino acids could also reduce the toxicity of metal ions. In addition, the combination of organic phosphate acids and Cd ions would produce Cd complexes unavailable to plants.

1.7 Phytoremediation

In order to maintain good quality of soils and waters and keep them free from contamination, continuous efforts have been made to develop technologies that are easy to use, sustainable and economically feasible. The use of plant species for cleaning polluted soils and waters named as phytoremediation has gained increasing attention since last decade, as an emerging cheaper technology (Cunningham and Berti, 1993; Salt *et al.*, 1995a). Numerous plant species have been identified and tested for their traits in the uptake and accumulation of different heavy metals as shown in Table 1. Several types of phytoremediation can be defined according to Schwitzguébel, (2000) as shown in the Figure 1.6.

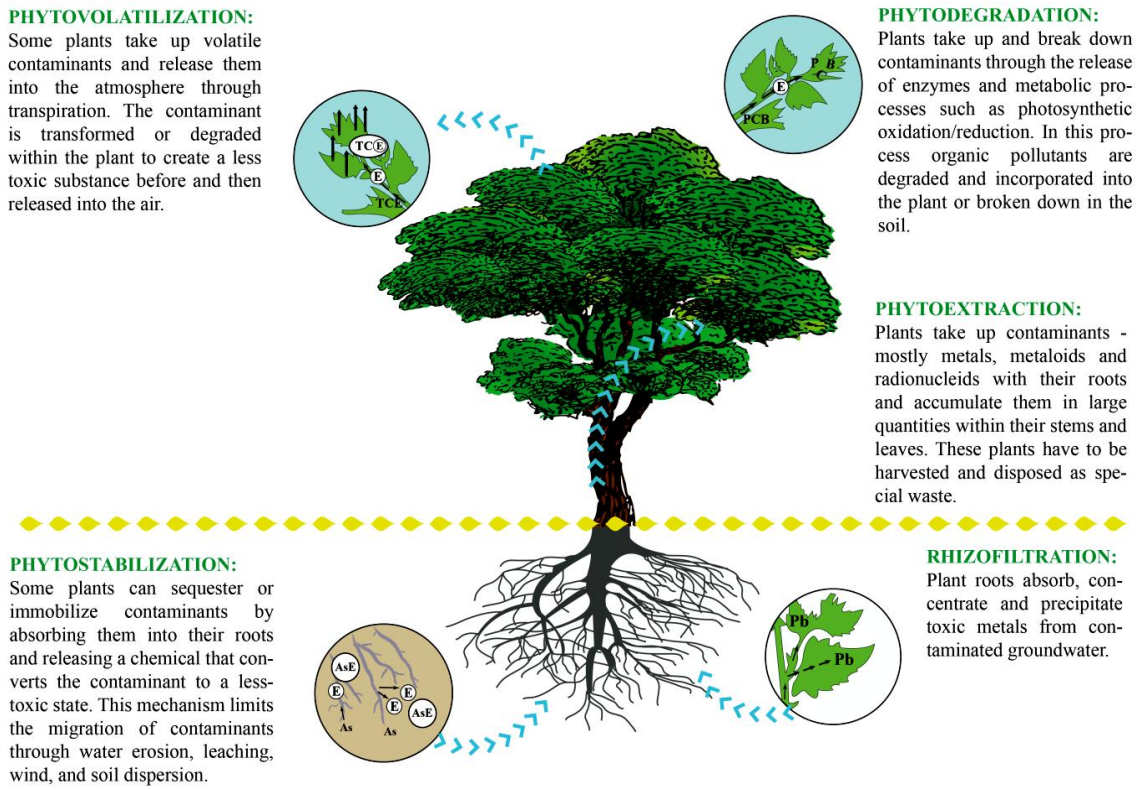

Figure 1.6 Phytoremediation process to remove contaminants from the environments. The diagram is modified from Tsai *et al.*, (2010)

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Table 1.8 Overview of some phytoremediation processes.

Plant species	Mechanism	Metal	Reference
Agrostis tenuis	Phytostabilization	Pb, Zn, Cu	Smith and Bradshaw, (1972)
Arabidopsis halleri	Phytoextraction	Cd	McGrath *et al.*, (2006)
		Cu, Pb	Dahmani-Muller *et al.*, (2000)
Arabidopsis thaliana L.	Phytovolatilization	Hg	Heaton *et al.*, (1998)
Astragalus adsurgens	Phytodegradation	TPHs	Xin *et al.*, (2008)
Bean (*Phaseolous vulgaris* L.)	Rhizofiltration	U	Lee and Yang, (2010)
Brassica campestris	Phytodegradation	TPHs	Park *et al.*, (2011)
Brassica juncea	Phytoextraction	Ni/Cr	Kumar *et al.*, (2009)
Brassica juncea	Phytovolatilization	Se	Bañuelos *et al.*, (1997)
Brassica juncea	Rhizofiltration	U	Eapen *et al.*, (2003)
Canola (*Brassica napus*)	Phytovolatilization	Se	Bañuelos *et al.*, (1997)
Carex pendula	Rhizofiltration	Pb	Yadav *et al.*, (2011)
Chenopodium amaranticolor	Rhizofiltration	U	Eapen *et al.*, (2003)
Duckweed (*Lemna minor* L.)	Rhizofiltration	Pb	Yadav *et al.*, (2011)
Festuca arundinacea	Phytodegradation	TPHs	Park *et al.*, (2011)
Festuca rubra	Phytostabilization	Pb, Zn, Cu	Smith and Bradshaw, (1972)
Grasses	Phytostabilization	Cu	Salt *et al.*, (1995a)
Helianthus annuus	Phytodegradation	TPHs	Park *et al.*, (2011)
Maize (*Zea mays* L.)	Phytoextraction	Cd	Murakami *et al.*, (2007)
Mirabilis jalapa	Phytodegradation	TPHs	Peng *et al.*, (2009)
Nicotiana tabaccum L.	Phytovolatilization	Hg	Rugh *et al.*, (1998)
Poplars	Phytostabilization	As	Pierzynski *et al.*, (2002)
Pteris vittata	Phytovolatilization	As	Sakakibara *et al.*, (2007)
Rice (*Oryza sativa* L.)	Phytoextraction	Cd	Murakami *et al.*, (2007)
Sedum alfredii	Phytoextraction	Cd/Zn	Ye *et al.*, (2003)
		Pb/Zn	Li *et al.*, (2005)
Soybean (*Glycine max* L.)	Phytoextraction	Cd	Murakami *et al.*, (2007)
Sunflower (*Helianthus annuus* L.)	Rhizofiltration	U	Lee and Yang, (2010)
Switchgrass (*Panicum virgatum* L.)	Phytoextraction	Cd	Chen *et al.*, (2011a)

1.8 CAM and stress tolerance

Plants with the specialised form of carbon assimilation known as Crassulacean acid metabolism (CAM) which enables uptake of CO_2 at night have low rates of transpiration, so this would suggests a low rate of Cd transport into shoots via the transpiration stream. CAM plants are also known to be more tolerant than C3 and C4 plants to other stresses such as high light, high temperature, drought etc. (Table 1.8). It has recently been shown that *Sedum alfredii* (*S. alfredii*; belongs to Crassulaceae family) has an ability to tolerate and hyperaccumulate Cd (Yang *et al*. 2004; Zhou and Qiu 2005). However, no previous reports have studied the effects of heavy metals in general or Cd in particular in pineapple. One of the aims of this thesis is to test the hypothesis that pineapple, as a CAM plant, will be relatively tolerant to Cd.

Table 1.9 Growth characteristics of CAM plants suitable for cultivation as alternative crop in Cd-contamination area. Examples taken from Borland *et al.*, (2009), Nobel, (1988), Nobel, (1994), Day, (1993) and Winter and Smith, (1996).

Advantage	Example	Comment
High water-use efficiency	5-16 mmol CO_2 per mol H_2O on an annual basis	Typically 4- to 10-fold higher than C_3 plants
High drought tolerance	Can grow in areas with as little as 25 mm $year^{-1}$ precipitation	Tissues can tolerant up to 90% loss of water content (cacti)
Tolerance of high temperature	Up to 70 °C, based on 50% loss of cell viability after 1 h; can survive exposure to 74 °C	Typically upper limit of 50-55 °C in C_3 plants
Tolerance of high PPFD	Can tolerate > 1000 μmol $m^{-2}s^{-1}$ (or > 40 mol m^{-2} d^{-1}) without photoinhibition	Generally more tolerant of high PPFD than agronomically important C_3 plant
Tolerance of UV-B radiation	Only 1% incident UV-B transmitted through epidermis of *Yucca filamentosa* (Agavaceae)	Generally thick epidermis and high foliar concentrations of phenolics in CAM plants
Entire shoot surface typically photosynthetic	Whole shoot photosynthetic in both leaf- and stem-succulent species; limited bark formation even on stems of arborescent cacti	Many C3 species deciduous (shedding photosynthetic organs for part of year) or woody (limited stem photosynthesis)
High shoot:root ratio and harvest index	Shoot:root ratio as high as 10:1; above-ground biomass readily harvested	
High resistance to herbivores	Effective physical defences (stem succulents) and chemical defences (leaf succulents)	
High content of non-structural carbohydrate	Especially monocotyledons (~20% dry weight); ready convert to soluble sugars	
Low lignin content	Weak secondary thickening and lack of true wood formation	

1.9 *Ananas comosus*

Pineapple (*Ananas comosus* L.*)* is the most important representative of the Bromeliaceae family and is cultivated in tropical and subtropical regions for local consumption and international export. Pineapple ranks third in world tropical fruit production only preceded by banana and citrus (Uriza-Ávila, 2005). World pineapple production reached 15.5 million tons in 2004, with Asia contributing 50% and America contributing 31.6%. Thailand is the leader in production (approximately 1,700,000 tons per annum), and has 80,000 hectares under pineapple cultivation (FAO, 2005). Sales of pineapples by Thailand to the world market were worth USD 2.6 million in 2005. Pineapple is exported in its fresh state or as processed product (Pathaveerat *et al.*, 2008).

1.9.1 Plant morphology

Pineapple is a herbaceous plant approximately 1-2 metres tall and wide. The plant has apical morphology due to the arrangement of the leaves. The stem is club-shaped approximately 25-50 cm long, 2-5 cm wide at the base, 5-8 cm wide at the top and contains nodes and internodes (Collins, 1960; Purseglove, 1972; Bartholomew *et al.*, 2003). A fully grown pineapple plant has around 68-82 leaves arranged in the form of a dense compact rosette. The older leaves are located at the base of the plant and the younger ones in the centre. Leaves are usually sword shaped except for the ones at the tip. The margins may or may not contain spines. The upper and lower surfaces of the leaf are covered with hairs that are more pronounced on the lower surface (Purseglove, 1972; Morton, 1987; Bartholomew *et al.*, 2003; Coppens D'Eeckenbrugge and Leal, 2003). The leaves enclose the stem up to two thirds of its circumference, are wide at the base and form a sheath around the stem. This leaf arrangement leads to collection of water at the base, potentially providing aerial roots with water and nutrients (Bartholomew *et al.*, 2003). The upper and lower leaf surfaces are covered by trichomes which have many functions including reduction of water loss from the underlying stomata, increasing the reflectance of an individual leaf and absorption of water and nutrients (Batholomew and Kadzimin, 1977). Stomata are only found on the lower leaf surface, in furrows running along the length of the leaf. The low density of stomata and their small pore size compared with other plants suggest this as a morphological adaptation to conserve water (Batholomew and Kadzimin, 1977; Bartholomew and Malézieux, 1994). The D-leaves, which are the youngest physically mature whorl of leaves are often used as an indicator of the nutritional and moisture status of the plant and to assess environmental effects on the

plants. The D-leaf weight correlates strongly with the weight of the whole plant, which in turn correlates with fruit weight (Bartholomew *et al.*, 2003). The plant can flower after producing 70-80 leaves (Purseglove, 1972; Coppens D'Eeckenbrugge and Leal, 2003). Primary roots are found in very young seedlings. These roots die soon after germination and are replaced by the adventitious roots (Coppens D'Eeckenbrugge and Leal, 2003). The roots may spread up to 1-2 m laterally and 0.85 m in depth under optimal conditions (Purseglove, 1972). The number of roots produced after planting is correlated with shoot weight. Adventitious roots are present on the stem and grow in a distorted manner around the stem between the leaves, forming a tuft of fibrous roots near the base of the stem.

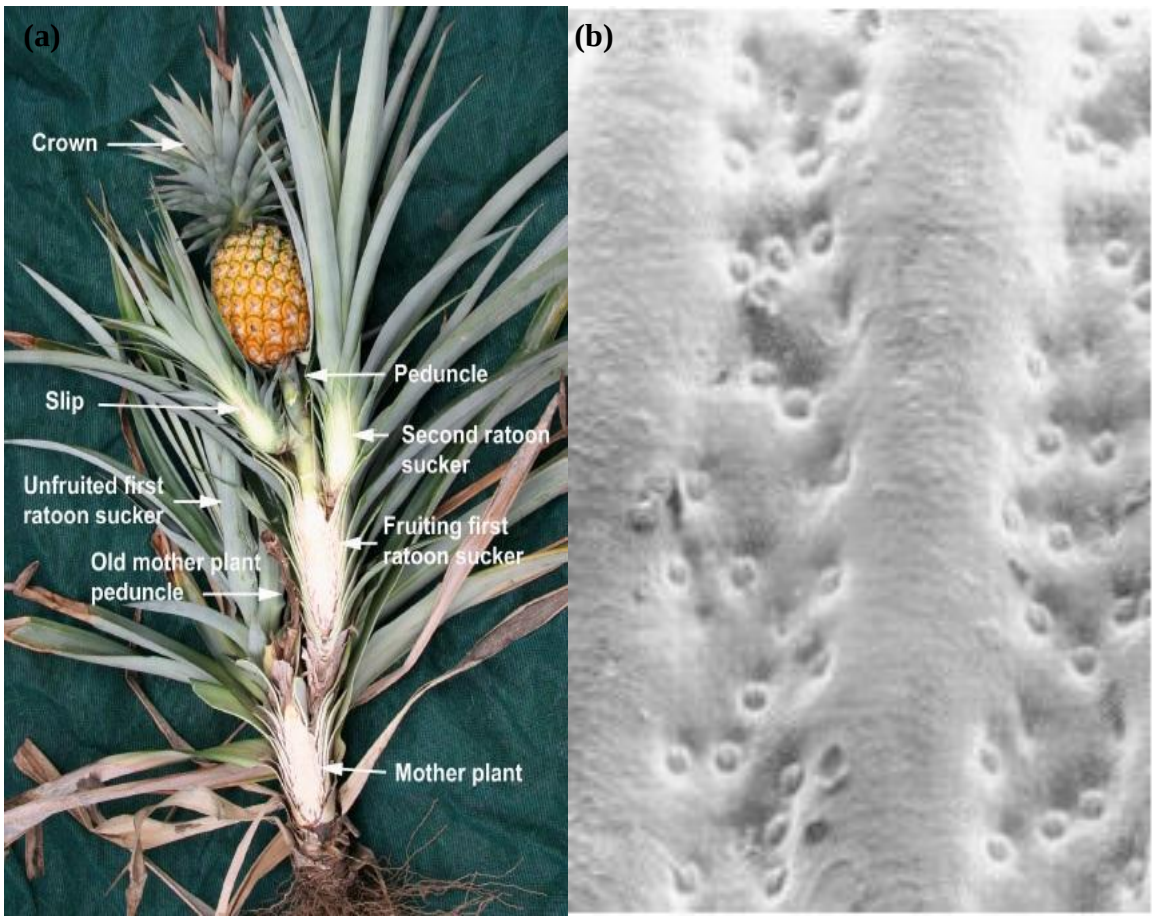

Figure 1.7 Morphology of pineapple (a); (b) lower surface with the trichomes rubbed off revealing the location of the stomata (leaf pores) in the furrows. The pictures are taken from Malézieux *et al.*, (2003).

1.9.2 CAM photosynthesis

The pineapple has a special photosynthetic pathway called Crassulacean Acid Metabolism (CAM). This metabolic adaptation, found in approximately 7% of all plant species, allows for high productivity under limited water availability (Cushman, 2005). Most plants must absorb carbon dioxide whilst the sun is shining for photosynthesis to take place and produce starch and sugars; therefore their stomata are open in the warmest, driest part of the 24 hour cycle and consequently much moisture escapes from the plant through the open stomata. CAM plants, on the other hand, have the unique ability to store carbon dioxide within the plant (as malic acid), this allows them to keep their stomata closed during the day but to open them at night when the atmosphere is cooler and evaporation is reduced (Nobel, 1991). When the sun comes out the next day the stomata close but carbon dioxide is released into the plant cells from the stored malic acid allowing photosynthesis to take place. Four phases of CAM metabolism can be described as shown in Figure 1.8 (Osmond, 1978).

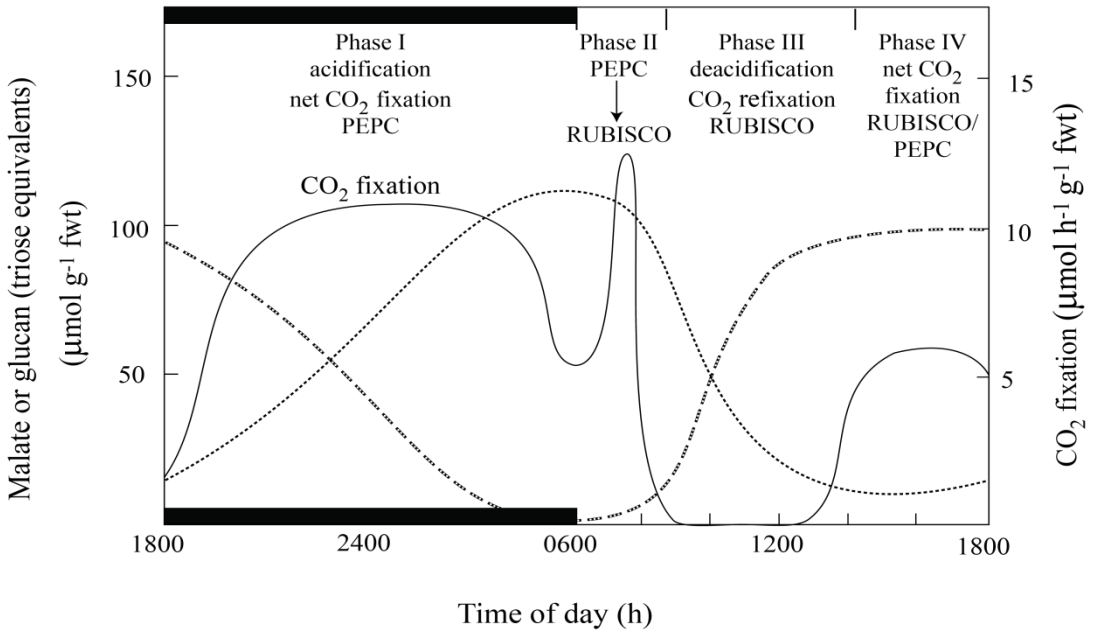

Figure 1.8 Day and night cycle of CO_2 uptake and malic acid accumulation in CAM. Phase I comprises night-time PEPC-driven CO_2 uptake and acid accumulation; phase II is a transitional phase with an accelerated burst of CO_2 uptake due to both PEPC-mediated and Rubisco mediated CO2 uptake; in phase III CO_2 uptake is diminished and decarboxylation of stored malic acid generates internal CO_2 for Rubisco-mediated CO_2 fixation; phase IV may include CO_2 uptake that is fixed directly by Rubisco. The diagram is taken from Leegood *et al.,* (1997).

Phase I The stomata open at night when evapotranspiration rates are low and atmospheric or respiratory-derived CO_2 is fixed as HCO_3^- by phosphoenolpyruvate carboxylase (PEPC). The PEPC is activated at night by a dedicated phosphoenolpyruvate carboxy kinase (PEPCK) which is regulated by the circadian clock (Hartwell *et al.*, 1996; Hartwell *et al.*, 1999). The oxaloacetate (OAA) formed during carboxylation is reduced to malate by malate dehydrogenase and transported into the vacuole via a malate-selective ion channel providing charge balance for a tonoplast-bound H^+-ATPase and⁄or H^+-Pyrophosphatase (H^+-PPiase) (Smith and Bryce, 1992; Bartholomew *et al.*, 1996; Smith *et al.*, 1996; Tsiantis *et al.*, 1996). Glycolytic breakdown of storage carbohydrate (starch and soluble sugar) provides the 3-C substrate phosphoenolpyruvate (PEP) for malate synthesis.

Phase II At the beginning of the day, the internal CO_2 partial pressure gradually increase and stomatal conductance declines. The degradation of PEPCK results in the dephosphorylation of PEPC in phase II, rendering the enzyme more sensitive to inhibition by malate (Borland *et al.*, 1999). In this phase both PEPC and Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) are active and malate is released from the vacuole (Cushman and Bohnert, 1999).

Phase III The decarboxylation of malate results in a high internal CO_2 partial pressure which results in low stomatal conductance, which limits transpiration during this phase. When stomata are closed, malate decarboxylation commences as the released CO_2 is refixed by Rubisco. The enzymes catalysing decarboxylation are either PEPCK or the malic enzyme cytosolic NAD^+-malic enzyme (NAD-ME; EC 1.1.1.40) and mitochondria NAD^+-malic enzyme (NAD-ME; EC 1.1.1.39). In PEPCK-type CAM plants such as pineapple, the OAA formed from malate by malate dehydrogenase (MDH) is converted to PEP and CO_2 by PEPCK in the cytosol. The PEP can be used to form 3-phophoglycerate (PGA), then triose phosphates, and to replenish the CAM carbohydrate.

Phase IV begins when malate reserves are eventually depleted, causing a reduction in intracellular CO_2 availability. This results in stomatal opening and atmospheric CO_2 is fixed in the Calvin cycle via Rubisco. This phase may involve both C_3 and C_4 carboxylation processes if PEPC become active before the end of the light period (Griffiths *et al.*, 1990). The carbohydrates that will provide substrates for the nocturnal

reactions are retained either in the chloroplast as starch or as sucrose/hexoses within the vacuole, depending on species (Christopher and Holtum, 1996; Borland *et al.*, 2001).

1.10 CAM biochemistry

During the dark period, storage carbohydrate is mobilised to form phosphoenolpyruvate (PEP), this may be through glycolytic breakdown of starch stored in the chloroplast or passive export of soluble sugars stored in the vacuole. In CAM species such as pineapple, fixation of CO_2 to PEP by PEPC leads to formation of oxaloacetate which is then converted to malate by malate dehydrogenase. Malate is transported to the vacuole through a voltage-gated, inward rectifying vacuolar malate channel, driven energetically by an H^+ transporting ATPase of the tonoplast which pumps $2H^+$ per malate (Holtum *et al.*, 2005). In the light period malic acid is released to the cytoplasm and transported to the chloroplasts to be decarboxylated by PEPCK and produce pyruvate and CO_2, the latter product enters the Calvin cycle where triosephosphate and carbohydrates are produced. Carbohydrates are broken down at night to produce PEP again during the following night. Pyruvate produced from the decarboxylation of malic acid with the intervention of pyruvate phosphate dikinase, is degraded in the mitochondria during the citric acid cycle, and pyruvate is metabolised to recover PEP. The pathway of CAM as described above is shown in Figure 1.9.

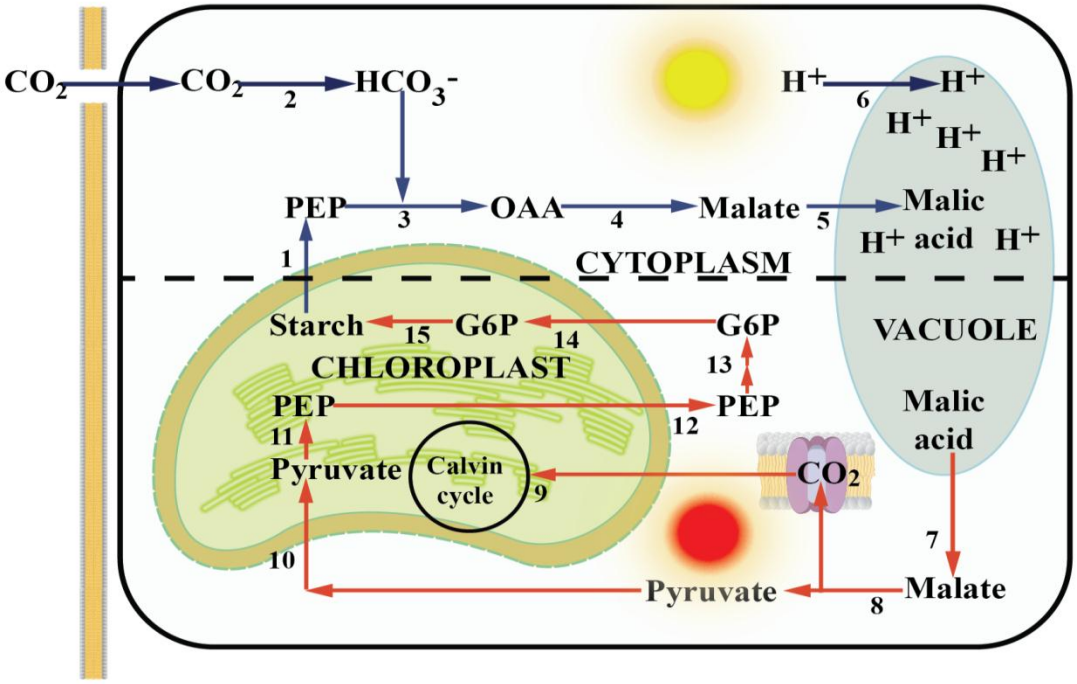

Figure 1.9 The pathway of crassulacean acid metabolism in a mesophyll cell of pineapple (*A. comosus*). Blue arrows represent dark reactions and red arrows represent light reactions. The dashed line running across the centre separates dark at the top from light at the bottom. The yellow-filled boxes on the left of the diagram represent the leaf epidermis, with the gap representing a stomatal pore. The enzymes that catalyse the reactions are as follows: (1) nocturnal starch breakdown possibly via chloroplastic starch phosphorylase and the export of G6P to PEP which is then used by phosphoenolpyruvate carboxylase (PEPC) as a substrate for CO_2 fixation; (2) carbonic anhydrase; (3) PEPC; (4) malate dehydrogenase; (5) voltage-gated malate channel, possibly a tonoplast membrane-targeted aluminium-activated malate transporter; (6) vacuolar H^+ ATPase; (7) unknown protein proposed to mediate malate efflux, possibly the tonoplast dicarboxylate transporter; (8) PEPCK; (9) ribulose bisphosphate carboxylase/oxygenase; (10) unknown pyruvate transporter on the inner envelope membrane of the chloroplast;(11) pyruvate orthophosphate dikinase; (12) phosphoenolpyruvate phosphate translocator; (13) gluconeogenesis; (14) GPT; (15) starch synthesis beginning with ADP-glucose pyrophosphorylase. The diagram is modified from Borland *et al.*, (2009).

1.11 Microbial inoculants and plant growth

Plant growth in agricultural soils is influenced by many abiotic and biotic factors. There is a thin layer of soil immediately surrounding plant roots that is an extremely important and active area for root activity and metabolism which is known as rhizosphere (Hiltner, 1904). A large number of microorganisms such as bacteria, fungi, protozoa and algae coexist in the rhizosphere. Bacteria are the most abundant among them (Lynch, 1990). Rhizobacteria inhabit plant roots and exert a positive effect ranging from direct influence mechanisms to an indirect effect. So, the bacteria inhabiting the rhizosphere and beneficial to plants are termed plant growth promoting rhizobacteria or PGPR (Kloepper *et al.*, 1980). In recent years, the number of PGPR that have been identified has seen a great increase, mainly because the role of the rhizosphere as an ecosystem has gained recognition in the functioning of the biosphere. Various species of bacteria have been used as inoculants to enhance plant growth and bacterial inoculation has a much better stimulatory effect on plant growth in nutrient deficient soil than in nutrient rich soil (Egamberdiyeva, 2007). PGPR are reported to influence plant growth, yield, and nutrient uptake by an array of mechanisms. Some bacterial strains directly regulate plant physiology by mimicking synthesis of plant hormones, whereas others increase mineral and nitrogen availability in the soil as a way to augment growth. Each bacterial isolate may exhibit two or three PGP traits, which may promote plant growth directly or indirectly or synergistically (Joseph *et al.*, 2007; Yasmin *et al.*, 2007b).

As mentioned above PGPR may induce plant growth promotion by direct or indirect modes of action (Beauchamp, 1993; Kloepper *et al.*, 1993; Kapulnik, 1996; Lazarovits and Nowak, 1997). Direct mechanisms include the production of stimulatory bacterial volatiles and phytohormones, lowering of the ethylene level in plants, improvement of the plant nutrient status and stimulation of disease-resistance mechanisms. Indirect effects originate for example when PGPR act like biocontrol agents reducing diseases, when they stimulate other beneficial symbioses, or when they protect the plant by degrading inhibitory xenobiotics in contaminated soils (Jacobsen, 1997). Based on their activities, Somers *et al.*, (2004) classified PGPR as biofertilizers (increasing the availability of nutrients to plants), phytostimulators (plant growth promoting, usually by the production of phytohormones), rhizoremediators (degrading organic pollutants) and biopesticides (controlling diseases, mainly by the production of antibiotics and antifungal metabolites).

1.11.1 Mechanisms of rhizosphere bacteria promoting plant growth

1.11.1.1 Ability to produce phytohormones

Recent studies confirmed that, a number of bacterial species mostly associated with the plant rhizosphere are found to be beneficial for plant growth, yield and crop quality. The plant promoting effect of the PGPR is mostly explained by the release of metabolites directly stimulating growth. The mechanisms of PGPR promote plant growth by producing phytohormones are shown in Table 1.9.

Table 1.10 Production of plant growth regulators by PGPR.

PGPR species	Mechanisms	Reference
Arthrobacter mysoren	IAA, ethylene	Pishchik *et al.*, (2002)
Arthrobacter simplex	IAA	Egamberdiyeva, (2005)
Azospirillum sp.	Giberellins	Lucangeli and Bottini, (1997)
Azotobacter chroococcum	IAA	Verma *et al.*, (2010)
Azotobacter sp.	IAA	Zahir *et al.*, (1998), Zahir *et al.*, (2000), Khalid *et al.*, (2001)
Bacillus amyloliquefaciens	IAA	Egamberdiyeva, (2005)
Bacillus firmus	IAA	Khan and Patel, (2007)
Bacillus licheniformis	Giberellins	Gutiérrez-Mañero *et al.*, (2001)
Bacillus pumilus	Giberellins	Gutiérrez-Mañero *et al.*, (2001)
Bacillus subtilis	Ethylene	Mansouri and Bunch, (1989)
Burkholderia sp.	ACC-deaminase	Madhaiyan *et al.*, (2007)
	IAA	Jiang *et al.*, (2008)
Enterobacter aerogenes	IAA, ACC-deaminase	Kumar *et al.*, (2009)
Flavobacterium sp.	IAA, ethylene	Pishchik *et al.*, (2002)
Klepsiella CIAM880	IAA, ethylene	Pishchik *et al.*, (2002)
Klepsiella sp.	IAA	Khan and Patel, (2007)
Kluyvera ascorbata	ACC-deaminase	Burd *et al.*, (1998)
Methylobacterium oryzae	ACC-deaminase	Madhaiyan *et al.*, (2007)
Microbacterium sp.	IAA, ACC-deaminase	Dell' Amico *et al.*, (2008)
Pseudomonas aeruginosa	Ethylene	Mansouri and Bunch, (1989)
Pseudomonas fluorescences	Cytokinin	García de Salamone *et al.*, (2001)
	IAA, ACC-deaminase	Dell' Amico *et al.*, (2008)
Pseudomonas putida	IAA	Khan and Patel, (2007)
Pseudomonas syringae	Ethylene	Sato *et al.*, (1997)
		Nagahama *et al.*, (1994)
Pseudomonas tabaci	Ethylene	Huang *et al.*, (2005)
Pseudomonas talaasii	IAA	Dell' Amico *et al.*, (2008)
Rahnella aquatilis	IAA, ACC-deaminase	Kumar *et al.*, (2009)
Streptomyces avermitilis	IAA	Dimkpa *et al.*, (2008)
Streptomyces coelicolor		
Streptomyces tanashiensis		

1.11.1.2 Increasing the availability of nutrients in the rhizosphere

Microorganisms have mechanisms that facilitate nutrient uptake or increase nutrient availability or stimulate plant growth. There are some PGPR that can fix nitrogen, solubilise mineral nutrients and also are able to provide plant with sufficient iron in iron-limited soil (Singh and Singh, 1993; Wang *et al.*, 1993; Glick *et al.*, 1999). The evidence of PGPR by increasing the availability of nutrients for the plant mineral are summarised in Table 1.10.

Table 1.11 The potential of PGPR to increase availability of nutrients in the rhizosphere.

PGPR species	Mechanisms	Reference
Arthrobacter sp.	Siderophore production	Kalinowski *et al.*, (2000)
Arthrobacter ureafaciens	P-solubilisation	Chen *et al.*, (2006)
Azospirillum	Nitrogen fixation	Bashan and de-Bashan, (2010)
Bacillus sp.	P-solubilisation	Prasanna *et al.*, (2011)
Bacillus firmus	P-solubilisation, Siderophore production	Khan and Patel, (2007)
Bacillus megaterium	P-solubilisation	Han *et al.*, (2006b)
Bacillus mucilaginosus	K-solubilisation P-solubilisation	Han *et al.*, (2006b)
Bradyrhizobium	Nitrogen fixation	Zahran, (2001) Zakhia and De Lajudie, (2001)
Burkholderia	Nitrogen fixation	Estrada-de los Santos *et al.*, (2001)
	P-solubilisation, Siderophore production	Jiang *et al.*, (2008)
Enterobacter aerogenes	P-solubilisation, Siderophore production	Kumar *et al.*, (2009)
Gluconacetobacter	Nitrogen fixation	Fuentes-Ramírez *et al.*, (2001)
Klebsiella sp.	P-solubilisation, Siderophore production	Khan and Patel, (2007)
Kluyvera ascorbata	Siderophore production P-solubilisation	Burd *et al.*, (1998) Prasanna *et al.*, (2011)
Mesorhizobium	Nitrogen fixation	Zahran, (2001)
Micrococcus sp.	P-solubilisation	Prasanna *et al.*, (2011)
Phyllobacterium myrsinacearum	P-solubilisation	Chen *et al.*, (2006)
Pseudomonas	Nitrogen fixation	Mirza *et al.*, (2006)
Pseudomonas fluorescens	Siderophore production	Dell' Amico *et al.*, (2008)
Pseudomonas putida	P-solubilisation, Siderophore production	Khan and Patel, (2007)
Rhizobium	Nitrogen fixation	Zahran, (2001)
Rhodococcus erythropolis	P-solubilisation	Chen *et al.*, (2006)
Sinorhizobium	Nitrogen fixation	Zahran, (2001)
Rahnella aquatilis	P-solubilisation, Siderophore production	Kumar *et al.*, (2009)
Streptomyces avermitilis	Siderophore production	Dimkpa *et al.*, (2008)
Streptomyces tendae	Siderophore production	Dimkpa *et al.*, (2009)

1.11.1.3 Inhibition of plant pathogen

The indirect mechanism of plant growth occurs when PGPR lessen or prevent the deleterious effect of plant pathogens by production of inhibitory substances or by increasing the natural resistance of the host. Control of phytopathogenic microorganism by releasing siderophores (Kloepper *et al.*, 1980; Lemanceau *et al.*, 1992), competitive for nutrients and space (Elad and Baker, 1985; Elad and Chet, 1987), antibiosis by producing antibiotics (Pierson and Thomashow, 1992), β-1,3-glucanase, chitinases, antibiotics, fluorescent pigment and cyanide (Lim *et al.*, 1991; Fridlender *et al.*, 1993) and degradation of toxin produced by pathogen (Borowitz *et al.*, 1992; Duffy and Défago, 1997). Some important plant disease controlling bacteria have been reported in Table 1.12.

Table 1.12 Potential of PGPR against certain diseases, pathogens and insects in different crops.

Crop	Disease/pathogen/insect	PGPR	Reference
Barley	Powdery mildew	*Bacillus subtilis*	Schönbeck *et al.*, (1980)
Beans	Halo blight	*Pseudomonas fluorescens*	Alstrom, (1991)
	Sclerotium rolfsii	*Pseudomonas cepacia*	Fridlender *et al.*, (1993)
Carnation	*Fusarium* wilt	*Pseudomonas* sp.	Van Peer *et al.*, (1991)
Cotton	*Meloidogyne incognita*	*B. subtilis*	Sikora *et al.*, (1998)
	Rhizoctonia solani	*P. cepacia*	Fridlender *et al.*, (1993)
	Helicoverpa armigera	*Pseudomonas gladioli*	Quingwen *et al.*, (1998)
Cucumber	*Cucumber anthracnose*	*Pseudomonas putida* *Serratia marcescens*	Wei *et al.*, (1991), Wei *et al.*, (1996)
	Pythium ultimum	*P. cepacia*	Fridlender *et al.*, (1993)
	Bacterial wilt	*P. putida, S. marcescens*	Kloepper, (1993)
	Cucumber mosaic virus	*P. putida, S. marcescens*	Raupach *et al.*, (1996)
	Striped Cucumber beetle	*P. putida, Flavimonas oryzihabitans*	Zehnder *et al.*, (1997)
Mung bean	*Aspergillus* sp., *Fusarium oxysporum*, *Rhizoctonia solani*	*Pseudomonas* sp.	Sindhu *et al.*, (1999)
Mung bean	Root rot, root knot	*P. aeruginosa, B. subtilis*	Siddiqui and Mahmood, (2001), Siddiqui and Ehteshamul-Haque, (2001)
Rice	Rice sheath blight	*Streptomyces* spp., *Bacillus cereus*, *P. fluorescens*, *Burkholderia*	Sung and Chung, (1997)
	Rhizoctonia solani (sheath blight pathogen)	*P. fluorescens* strains Pf1 and Fp7	Vidhyasekaran and Muthamilan, (1999)
	Blue mold	*S. marcescens*, *B. pumilus*, *P. fluorescens*, *B. pasteurii*	Zhang *et al.*, (2002)
Tomato	Tomato mottle virus	*B. subtilis*, *B. amyloliquefaciens*	Murphy *et al.*, (2000)
Wheat	Take all disease	*Bacillus, Pseudomonas, Rhodococcus*	Renwick *et al.*, (1991)

1.12 Project aims

Due to the high level of Cd contamination in some areas of Thailand that have led to prohibition of rice growth and consumption there is a need to examine other commercially useful plants that could be grown in such soils. Although many studies have considered the effect of Cd on a variety of plants no-one has previously examined the effect of Cd on pineapple. I choose pineapple (*A. comosus*), a CAM plant, as my model plant as it is one of the most commercially important plants in Thailand and can be, grown under a wide range of environmental conditions. I also wanted to test the hypothesis that the low transpiration rates conferred by CAM might render pineapple relatively tolerant to Cd in the soil. The specific aims of the thesis were to:

a) Examine the effect of Cd on growth, photosynthetic performance and CAM biochemistry (Chapter II).
b) Examine the accumulation of Cd in different parts of the pineapple plant to assess if the negative impacts of Cd on net CO_2 uptake and plant growth were related to Cd accumulation in particular plant tissues (Chapter III).
c) Examine the effect of Cd on pineapple rhizosphere soil microbial populations using a combination of traditional techniques (plate counting) and more advanced molecular techniques (DGGE) (Chapter IV).
d) Isolate Cd-resistant bacteria from Cd-contaminated soil and assess the Cd uptake ability of the isolates (Chapter V).
e) Examine the interaction between Cd-resistant bacteria and pineapple plants to promote plant growth and decrease metal toxicity in pineapple grown in Cd-contaminated soil (Chapter VI).

Chapter Two

The effect of Cd on plant growth, photosynthetic performance and CAM biochemistry

2 The effect of Cd on plant growth, photosynthetic performance and CAM biochemistry

2.1 Introduction

Physiological, photosynthetic properties and growth of plants in cadmium (Cd) contaminated soil have been studied by several researchers (Merakchiiska and Yordanov, 1983; Baszynski, 1986; Stiborová *et al.*, 1986b; Greger and Ögren, 1991; Siedlecka and Baszyńaski, 1993). An increase in Cd content in the environment results in a lower growth rate and changed intensity and direction of many metabolic processes in plant cells (Foy *et al.*, 1978; Seregin and Ivanov, 2001). It is often found that the photosynthetic rate of plants grown in Cd contaminated soil is lower than those of plants grown in uncontaminated soil (Vassilev *et al.*, 1993) and low productivity of plants grown in Cd-contaminated soil has been attributed to a combination of Cd-induced physiological stress and low photosynthetic activities (Padmaja *et al.*, 1990; Sanità Di Toppi and Gabbrielli, 1999; Burzyński and Kłobus, 2004; Azevedo *et al.*, 2005; Kučera *et al.*, 2008; Shukla *et al.*, 2008). Even at relatively low concentrations, Cd can exert strong toxic effects on crops (Seregin and Ivanov, 1998) and has been shown to inhibit chlorophyll synthesis, alter plant water and ion metabolism, and may even induce plant death (Sanità Di Toppi and Gabbrielli, 1999; Zhang *et al.*, 2003; Hassan *et al.*, 2005a; Wójcik *et al.*, 2005; Burzyński and Zurek, 2007; Ebbs and Uchil, 2008; He *et al.*, 2008). Cd induces oxidative stress by producing excessive reactive oxygen species (ROS) (Zhang *et al.*, 2010b), causing damage to membrane systems, cell organelles and DNA (Laspina *et al.*, 2005; Geissler *et al.*, 2009). Ultimately, exposure to Cd in soil can reduce plant growth and yield (Ekmekçi *et al.*, 2008)

Pineapple (*Ananas comosus* L.) is the most economically important representative of the Bromeliaceae family and is cultivated worldwide around the tropical and subtropical regions for local consumption and international export. Pineapple holds the third rank in world tropical fruit production only preceded by banana and citrus (Uriza-Ávila, 2005). World pineapple production reached 15.5 million tons in 2004, with Asia contributing 50% and America contributing 31.6%. Thailand is the leader in production (approximately 1,700,000 tons per annum), and has 80,000 hectares under pineapple cultivation (FAO, 2005). Sales of pineapples by Thailand to the world market were worth USD 2.6 million in 2005 and it is exported in its fresh state or as processed product (Pathaveerat *et al.*,

2008). There are no published studies for pineapple grown in Cd-contaminated soil; however there has been a study on the use of pineapple as an indicator of lead (Pb) toxicity in Pb-contaminated soil. It was found that pineapple has a high tolerance to Pb only showing visual symptoms of toxicity at 2,500 mg Pb kg^{-1} (Pengta, 2010)

Pineapple shows very high water use efficiency due to the fact that it operates using a specialized mode of photosynthesis known as Crassulacean acid metabolism (CAM) in which CO_2 is taken up at night. The CAM pathway allows stomata to remain shut during the day, reducing evaporation and potentially curtailing transport of metal contaminants from soil to shoot in the transpiration stream. CAM is especially common in plants adapted to arid conditions (Nobel, 1991). CAM is characterized by nocturnal CO_2 fixation via the cytosolic enzyme PEP carboxylase (PEPC). The substrate, PEP is produced via the glycolytic breakdown of carbohydrates and the product of PEPC-mediated carboxylation, malic acid is accumulated overnight in the vacuole. In the daytime, decarboxylation of malate via PEP carboxykinase (PEPCK) releases CO_2 which is re-assimilated via ribulose-1,5-bisphosphate carboxylase (Rubisco) behind closed stomata and storage carbohydrates are regenerated from pyruvate and/or PEP by gluconeogenesis (Cushman and Bohnert, 1999). CAM plants are generally very tolerant to a range of environmental stressors that include high temperature, high light and extreme drought and it is hypothesised that CAM plants may also be tolerant of heavy metal contaminants in soils.

Many studies have investigated the impact of Cd on plant growth and photosynthetic parameters. In general, a decrease in photosynthetic rates of plants grown in Cd-contaminated soil is often associated with development of visual symptoms on the leaves such as leaf necrosis and chlorosis (Greger and Lindberg, 1987; Vázquez *et al.*, 1989; Dong *et al.*, 2005). To my knowledge, there has been no study to examine the effects of Cd on plant growth and photosynthesis in CAM plants such as pineapple. A particular interest was to establish how Cd-contamination might influence photosynthetic performance and acclimation to contrasting light regimes in pineapple. It was hypothesised that Cd will suppress light use efficiency, curtail acclimation to contrasting light regimes and lead to reduced growth of pineapple. In order to test this hypothesis, measurements were made of leaf chlorophyll fluorescence, net CO_2 uptake and CAM

activity, the amounts of the key CAM enzymes PEPC, PEPCK and Rubisco in pineapple leaf tissue and overall plant biomass. Chlorophyll fluorescence is widely used to analyse light use efficiency of the photosynthetic apparatus and is often used as a non-invasive measure of how various abiotic and biotic factors might impact on the growth and physiology of plants (Sayed, 2003). To understanding the impact of Cd-contaminated soil on the ability of plants to acclimate to contrasting light regimes, leaf chlorophyll fluorescence and net CO_2 uptake were monitored in plants grown under low light intensity (250 µmol m^{-2} s^{-1}) and after transfer to high light intensity (800 µmol m^{-2} s^{-1}). To examine which biochemical aspects of photosynthetic metabolism may be particularly vulnerable to Cd-toxicity, measurements were made of titratable acidity, starch and sugar contents and the amount of protein such PEPC, PEPCK and Rubisco. This suite of measurements would provide indicators for the possible biochemical mechanisms underpinning any detrimental effects of Cd on CAM expression, photosynthetic performance and plant growth in *A. comosus*.

Overall, the aim of the chapter was to examine the effect of Cd on growth, photosynthetic performance and CAM biochemistry.

Individual objectives were to:

a) examine the effect of Cd on plant biomass by measurements of dry weight of different ages of leaves and of root tissue.
b) examine the effect of Cd on photosynthetic performance by measurements of leaf chlorophyll fluorescence and 24 hour patterns of net CO_2 assimilation.
c) examine the effect of Cd on CAM biochemistry by measuring day/night changes in leaf titratable acidity, starch and sugar contents and the abundance of key enzymes (PEPC, PEPCK and Rubisco) involved in the uptake and processing of CO_2.

2.2 Materials and Methods

2.2.1 Plant material and growth conditions

Soil was made up in pots containing a mixture of sand (East Riding Horticulture Ltd, UK) and John Innes No. 2 (JI no. 2) compost in the ratio of 1:1. The soil was thoroughly mixed with $Cd(NO_3)_2$ (Sigma-Aldrich) solution to obtain 0, 50, 100, and 150 mg Cd kg^{-1} soil and was incubated (aged) for four weeks without plants before being remixed and used as a growing medium for pineapple plants. Suckers of pineapple (*Ananas comosus* L.) approximately 1.0 cm diameter were potted up in 10 cm plastic pots in JI no. 2 plus sand (1:1) until the roots became exposed at the base of the pot. Plants were removed and potted up in 14 cm plastic pots with the following treatments: control, 50, 100, and 150 mg Cd kg^{-1} soil as $Cd(NO_3)_2$. The plants were grown in a controlled growth chamber at 27 ºC (day) and 19 ºC (night) under a 12 hour photoperiod with photon flux density of 250 µmol m^{-2} s^{-1} at leaf height for six months. After six months, plants were then moved to an intermediate light intensity at 550 µmol m^{-2} s^{-1} at leaf height for one month and subsequently were moved to the highest light intensity of 800 µmol m^{-2} s^{-1} at leaf height for two months before harvesting. The water content of the soil was maintained at 60% of its holding capacity by watering with tap water twice a week. Plants were destructively harvested at four months and a further batch was harvested nine months after transplantation for data collection.

2.2.2 Plant sampling strategy

The photosynthetic measurements were made on D-leaves, the youngest fully mature leaves on a pineapple plant. The weight and approximate area of D-leaf correlates well with whole plant growth (Malézieux *et al.*, 2003). Leaf gas exchange and chlorophyll fluorescence measurements were made on the second quarter of the leaf, measured from the tip of the leaf. After nine months of transplantation, leaf tissue samples for measurements of CAM biochemistry were taken from D-leaves. The plant leaves were wiped clean with a damp paper towel and the leaf samples were taken by using a metal leaf disc cutter. The leaf samples were then wrapped immediately in foil and snap frozen in liquid nitrogen and stored at -80 ºC until analysis.

2.2.3 Measurement of potential quantum yield of photosystem II

The maximum quantum yield of PSII (F_v/F_m) was measured using the Plant Efficiency Analyser (PEA; Hansatech Instruments Ltd, King's Lynn, Norfolk). The instrument analyses chlorophyll fluorescence induction, the time dependent changes in fluorescence that occur when a dark-adapted leaf is exposed to light. Leaf samples were dark-adapted for 30 minutes using manufacturer-supplied leaf clips, prior to measurement of F_v/F_m Genty *et al.,* (1989). Measurements were made four hours into the photoperiod, during phase III of the CAM cycle.

$$\frac{F_v}{F_m} = \frac{F_m - F_o}{F_m} \qquad [2.1]$$

Where F_m = maximum fluorescence yield of a dark-adapted leaf

F_o = minimum fluorescence yield of a dark-adapted leaf

F_v = variable fluorescence yield of a dark-adapted leaf

2.2.4 Leaf gas exchange

Measurement of net CO_2 uptake provided a direct, non-destructive method of measuring instantaneous and daily carbon gain. One advantage of measuring CO_2 uptake directly was the ability to identify the relative contribution of the four phases of the CAM cycle to total carbon gain. In this thesis net CO_2 uptake was measured on D-leaves, the youngest fully mature leaves on a pineapple plant using the bench-top Walz CMS-400 Compact Minicuvette System (Heinz Walz, Effeltrich, Germany), with an integral BINOS-100 infra-red gas analyzer (IRGA). The top part of the leaf was enclosed in a broad leaf chamber and the system was operated in open (differential) mode, with no addition of CO_2 to the flow of air through the system to compensate for CO_2 taken up during photosynthesis. Air was collected from outside the building and passed through a buffering chamber to reduce fluctuations in CO_2 concentration. The air was then saturated with water vapor, before passing through the input humidity control unit (Walz Cold Trap KF-18/2). Cooling the input gas to predetermined dewpoint temperature, using the Peltier effect, controlled humidity. This temperature was set manually at dawn and dusk, to a level lower than the growth room temperature, to avoid condensation within the apparatus. The air flow was then passed into the central unit, where it was split between two parallel paths, the measuring gas path and the reference gas path, each having a pump, mass flow

meter and electronic flow rate control. Maintaining the gas flow at approximately 400 ml min^{-1} allowed sufficient air to pass over the leaf surface to prevent a buildup of humidity in the leaf chamber, and thus maintain adequate boundary layer conductance, which might affect calculations of assimilation (Long and Hällgren, 1993). Gas exchange data were collected over a 24 hour period at 15 minute intervals. Data was downloaded onto and IBM compatible computer and analysed using DIAGAS software supplied by Walz.

2.2.5 Titratable acidity

Titratable acidity is one of the simplest methods to access the extent of CAM activity by extracting the leaf-sap and measuring the change in titratable acidity between dawn and dusk, resulting from malic acid synthesis and accumulation. The method is modified as described by (Cushman *et al.*, 2008). Leaf disc samples were taken from pineapple leaves at the start and end of the photoperiod, wrapped in aluminium foil then immediately placed in liquid nitrogen and stored at -80 °C. Four frozen leaf discs were placed in 5 ml of 80% (v/v) methanol preheated to 80 °C for 40 minutes. One milliliter of extract was diluted with 2 ml of deionised water and titrated against 0.005 M NaOH to neutrality, using phenolphthalein as an indicator.

Number of moles (Z) H^+ in 5 ml extract was calculated using the following equations.

$$Z \text{ (moles } H^+) = \text{NaOH titre x } 0.005/1000 \text{ x } 5 \qquad [2.2]$$

To express on a fresh weight basis

$$Z/\text{fresh weight (moles } H^+ g^{-1} \text{ fresh weight)} \qquad [2.3]$$

To express on an area basis

$$Zx10000/\text{area of four discs in } cm^2 \text{ (moles } H^+ m^{-2}) \qquad [2.4]$$

2.2.6 Leaf carbohydrate determination

The remaining plant tissue from methanol extraction (Section 2.2.5) was removed from 80% (v/v) methanol and washed with deionised water then blotted dry and ground in 1.2 ml acetate buffer (43 mM sodium acetate, 57 mM acetic acid, pH 4.5). Exactly 0.2 ml enzyme cocktail (3 units of amyloglucosidase; Sigma-Aldrich, USA and 0.25 units of α-amylase; Sigma-Aldrich, USA) was then added to this acetate buffer extraction and incubated overnight at 45 °C in order to digest starch from the leaf material. A control was prepared using 1.2 ml buffer and 0.2 ml enzyme solution, in order to adjust the results for any background changes in soluble sugar content due to the addition of the enzymes. Extracts were then centrifuged at 13,000 rpm (17,300 xg) for 5 minutes and 200 µl supernatant was analysed for glucose content using the method described below for the determination of total soluble sugar.

2.2.7 Soluble sugar determination

Total soluble sugars were measured in the same methanol extracts used for titratable acidity measurements and in the acetate buffer extract using the phenol-sulphuric acid colorimetric assay (Dubois *et al.,* 1956). One millilitre of 5% (v/v) phenol solution was added to 200 µl soluble plant extract and 800 µl deionised water followed by 5 ml of concentrated sulphuric acid. The solutions were mixed and allowed to cool for 15 minutes before absorbance was measured at 483 nm using a spectrophotometer (GENESYS 10 VIS, UK). The total soluble sugar content of the plant extract was quantified using a calibration curve established from a glucose solution of known concentration from 0 to 200 µg. Results were expressed as mmol glucose equivalent per unit leaf area.

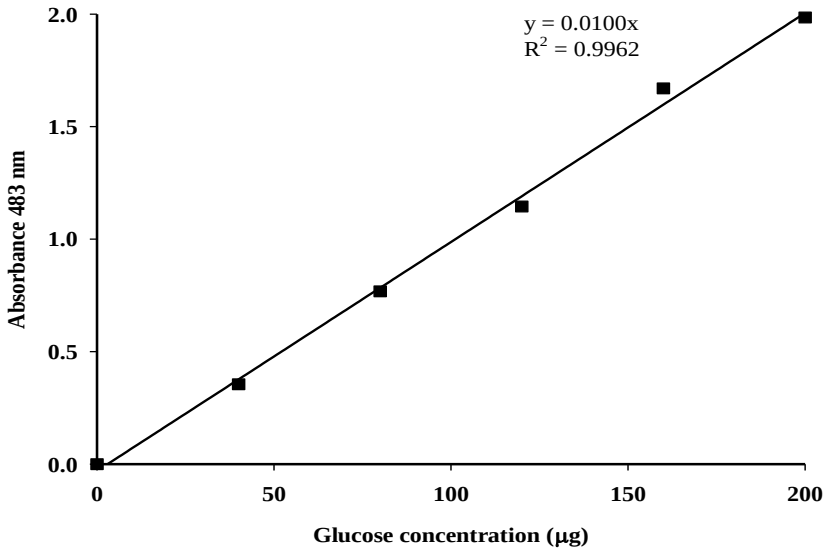

Figure 2.1 Typical linear calibration curve for determining leaf total soluble sugar content.

2.2.8 Western blotting for Rubisco, PEPC and PEPCK

2.2.8.1 Sample preparation

Samples were prepared from frozen leaf tissue as described by Borland *et al.,* (1998). Samples were prepared only from the dusk time points. Samples were ground to powder by adding liquid nitrogen in a pestle and mortar. The samples were weighed to give exactly 250 mg in a 2 ml eppendorf tube and then 280 µl chilled extraction buffer was added (300 mM Tris pH 8.3 and 100 mM NaCl with 50 µl DTT, 10 µl PMSF, 40 µl E-64, 40 µl Leupeptin, 40 µl Protease inhibitor cocktail, 40 µl EDTA). The ground plant tissue was left for 1 minute before the eppendorf was inverted and shaken. Samples were then centrifuged for 10 minutes at 13000 rpm (17,300 xg) at 4 ºC using a microcentrifuge (Eppendorf 5417R). The supernatant was removed to a new eppendorf tube then 10% (v/v) glycerol was added and mixed well. The samples were snap frozen in liquid nitrogen and stored at -80 ºC.

2.2.8.2 Protein estimation

Protein contents of the plant extracts were determined as described by (Bradford, 1976). This assay is a colorimetric assay for measuring total protein concentration resulting from the binding of Coomassie Brilliant blue (Bradford reagent) to protein. Bradford reagent was prepared by dissolving 100 mg of Coomassie Brilliant Blue (Sigma-Aldrich, USA) in 50 ml of 95% (v/v) ethanol and 100 ml 85% (v/v) orthophosphoric acid. Distilled water was used to make a final volume of one litre. The reagent was stored in a brown bottle to protect from sunlight and was shaken well before use. Exactly 20 µl of extracted samples were added into a cuvette and brought up to 100 µl with water. Coomassie Brilliant Blue G reagent 4 ml was added to each sample then mixed thoroughly. The samples were allowed to equilibrate for 15 minutes and then analysed with a spectrophotometer to determine their absorbance at 595 nm. Exactly 100 µl of deionised water and 4 ml of Bradford reagent were used as a blank. A standard curve was prepared with bovine serum albumin (BSA) in a range from 0-140 µg protein per millilitre for all experiments.

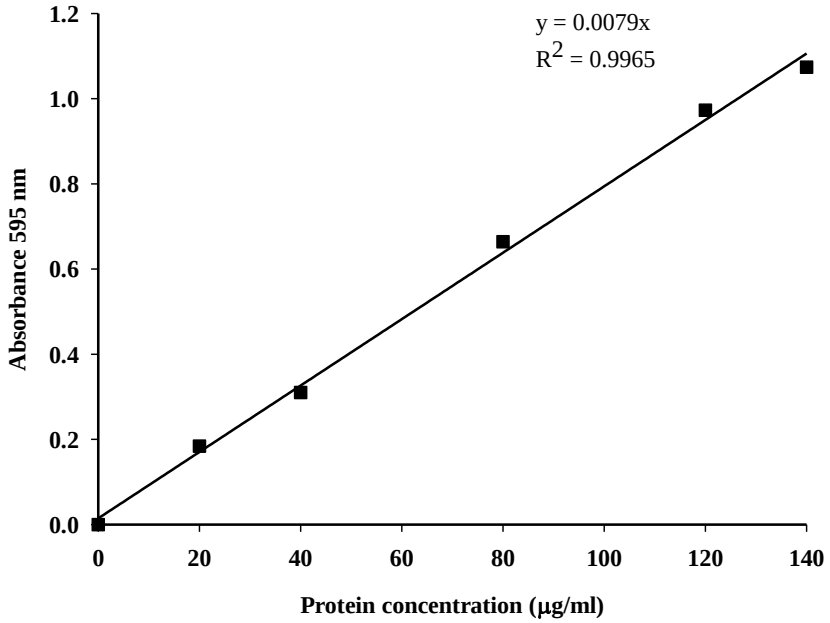

Figure 2.2 Typical linear calibration curve for the Bradford method of determining total soluble protein.

2.2.8.3 Preparation of gels for protein separation

Glass plates for gel preparation were cleaned with 95% (v/v) ethanol. The 12% separating gel was prepared in a glass beaker, using quantities set out in Table 2.1. The gel mixture was immediately transferred into the glass plates within the gel apparatus. The separating gel was overlaid with isopropanol (200 µl) to avoid inhibition of polymerisation by oxygen. After the separating gel had set (about 30 minutes) the isopropanol was poured off. The stacking gel was prepared as in Table 2.1 and then immediately poured on the top of the separating gel. A teflon comb was inserted to make the loading wells and the stacking gel was allowed to set for 30 minutes. The comb was removed and gels were placed in an electrophoresis tank which was filled with reservoir buffer (25 mM Tris-HCl pH 8.3, 200 mM glycine, 1% (w/v) SDS).

Table 2.1 Composition of separating gel and stacking gel for SDS-PAGE.

	12% Separating gel	4% Staking gel
30% acrylamide solution	7.25 ml	1.2 ml
Deionised water	6.25 ml	5.6 ml
LWGB buffer	4.50 ml	-
UPGB buffer	-	2.25 ml
10% (w/v) Ammonium persulphate, made up fresh before use	100 µl	54 µl
N,N,N',N'-tetra-methyl-ethylenediamine (TEMED)	20 µl	10 µl

Note: LWGB buffer - 1.5 M Tris-HCl pH 8.0, 0.4% (w/v) SDS

UPGB buffer - 0.5 M Tris-HCl pH 6.8, 0.4% (w/v) SDS

2.2.8.4 Sample loading, protein separation and visualisation

Protein samples were mixed with 1x SDS-PAGE loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 0.0025% (w/v) bromophenol blue). The samples were then boiled in a water bath for 10 minutes. Equal amounts of protein extract (15 µg) were loaded into each well. The first lane was used to run pre-stained protein molecular markers in the size range 10-170 kDa (Fermentas, UK). Samples were run at 75 V until the samples reached the top of the resolving gel (approximately 30 minutes), then run at 150V until the pre-stained standard and samples reached to the end of the gel (approximately 45 minutes).

An extra gel was run and stained to confirm that equal amounts of protein were loaded for each sample. The gel was removed from the apparatus and placed in fixative solution (80% (v/v) methanol and 14% (v/v) glacial acetic acid) for 3 minutes then the fixative solution was poured off. Coomassie® blue stain solution (12 ml Coomassie Blue® G-250 (Bio-Rad, USA) and 3 ml of methanol) was added to the gel and left overnight on a rocking shaker. The gel was then de-stained in a mixture of 30% methanol and 10% glacial acetic acid three times and then the image of the stained gel was captured using a digital camera.

2.2.8.5 Western blotting

After electrophoresis, the protein gel was immersed in blot transfer buffer (48 mM Tris-HCl, pH 9.2, 39 mM glycine, 10% (w/v) SDS, 20% (v/v) methanol) for 15 minutes. Blotting paper and Immobilin-P membrane (Millipore, USA) were cut to the same size as the gel and dipped in blot transfer buffer for a few minutes, the membrane having first been immersed in 100% (v/v) methanol for a few minutes. Proteins were transferred to the membrane using a Trans-Blot® SD Semi-dry transfer cell (Bio-Rad, USA). The transfer was conducted at 15 volts with a maximum current setting of 0.35A per gel for 45 minutes. The transferred protein on the membrane in the size range of Rubisco, PEPC and PEPCK was confirmed visually by staining the membrane with 0.1% Ponceau-S stain in 5% acetic acid (Sigma-Aldrich, USA) for 1 hour. The pre-stained protein on the membrane confirmed a successful protein transfer. Membranes were washed with Tris-buffered saline solution (TBS; 20 mM Tris-HCl, pH 7.3, 137 mM NaCl, 0.38% (v/v) 1 N HCl) buffer for 2 minutes and then stored in TBS overnight. Next day, the membrane was blocked with 5% skimmed milk in 1x TBS for 1 hour by shaking slowly at room

temperature. After blocking, the membranes were incubated with primary antibody (Rubisco, PEPC or PEPCK) in 5% skimmed milk in 1x TBS at the concentration 1:3000 for 1 hour on the rocking shaker. After incubation in the primary antibody, the membrane was washed two times with 1x TBST (0.1% (v/v) Tween 20 in TBS) for 10 minutes and then washed in TBS for 10 minutes. The membrane was incubated with secondary antibody (15 µl of goat anti-rabbit IgG; Sigma-Aldrich, USA in 15 ml skimmed milk solution) for one hour. After the secondary incubation, the membrane was then washed three times with TBST. Proteins were visualized by enhanced chemiluminescence (ECL). The membrane was soaked for 30 seconds per side in 3 ml ECL1 and ECL2 reagent (GE Health Suppliers, UK) mixed immediately then wrapped in cling film and placed in a film cassette. The film (Kodak Biomax-XAR) was placed on the membrane under darkness in a film cassette for 30 seconds to 5 minutes. Film was developed using Kodak developer and fixer reagents.

2.2.9 Plant biomass

Four and nine months after growth in Cd-contaminated soil, plants were carefully removed from the soil and separated into shoots and roots. The shoots and roots were washed thoroughly with tap water and then with distilled water. To remove surface adsorbed metal ions from the root, the roots were washed extensively with 10 mM $CaCl_2$ and then with distilled water. Leaves were separated into different ages as older leaves (lower leaves), intermediate leaves (middle leaves) and younger leaves (upper leaves). To determine the dry weight, shoots and roots were oven-dried separately at 70 °C for 72 hours to constant weight.

2.2.10 Statistical analyses

All data presented are the mean values of four replicate. Values are expressed as means of four replicates ± standard error (S.E.) in each group. All statistical analyses were performed using the statistical analysis package SPSS 17.0. One-way ANOVA was used to examine the significance and effect of Cd on plant growth, photosynthetic performance and CAM biochemistry. TWO-way ANOVA was used to determine the effect of Cd and light on net CO_2 uptake. Variance analysis was performed on all experimental data and significant differences ($P < 0.05$) between individual means (four replicates) was analysed using a post hoc Least Significant Difference test.

2.3 Results

2.3.1 The effect of Cd on potential quantum yield of photosynthesis in pineapple and acclimation to contrasting light intensities

As an indication of overall plant health under Cd, leaf chlorophyll fluorescence which is an indication of light use efficiency was determined (Figure 2.3). The results showed that the maximum quantum yield of PSII (F_v/F_m) did not differ significantly between control and the Cd treatments at the start of the experiment. However, as the experiment progressed, control plants showed a higher maximum quantum yield of PSII than the plants grown in different Cd concentrations. A large decrease in quantum yield of PSII was observed when plants were moved to higher light intensity of 500 µmol m^{-2} s^{-1}. After 2 weeks under this light regime, the F_v/F_m recovered in the control plants to a much greater extent than in Cd-treated plants. There was a second fall in F_v/F_m when plants were moved to an even higher light intensity (800 µmol m^{-2} s^{-1}). Only control plants showed a recovery in F_v/F_m under these high-light conditions. Plants in Cd were unable to fully acclimate to the increase in light intensity and F_v/F_m showed a gradual decline in Cd treated plants over the last 2-4 weeks of the experiment.

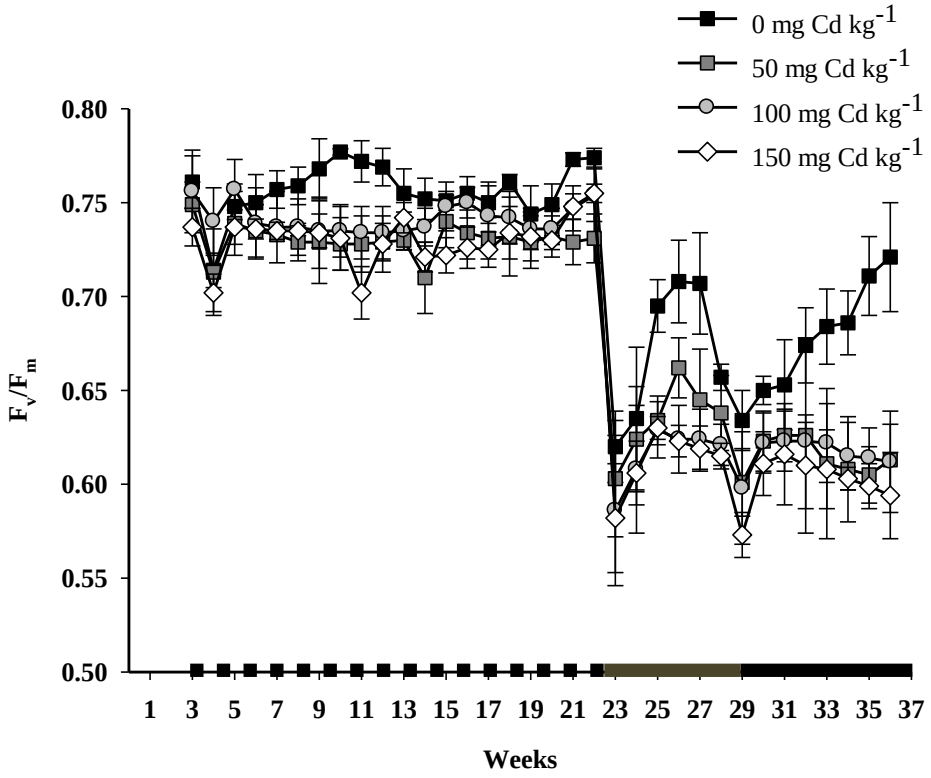

Figure 2.3 The maximum quantum yield of photosystem II (F_v/F_m) for D-leaves of *A. comosus* at different Cd concentrations. Values are the means of four replicates ± standard error. The dotted, grey and black bar on the x axis represent the contrasting light regimes of 250 µmol m^{-2} s^{-1}, 500 µmol m^{-2} s^{-1} and 800 µmol m^{-2} s^{-1}, respectively.

2.3.2 The effect of Cd on net CO_2 uptake in pineapple

Diel gas exchange patterns were monitored in control and treated plants after four months under light intensity 250 µmol m^{-2} s^{-1} (Figure 2.4A) and at the end of the experimental period under high light intensity 800 µmol m^{-2} s^{-1} (Figure 2.4B). The control plants had greater rates of nocturnal net CO_2 uptake than plants grown in presence of Cd. The results show that with increasing Cd concentrations there was a significant depression in dark CO_2 uptake.

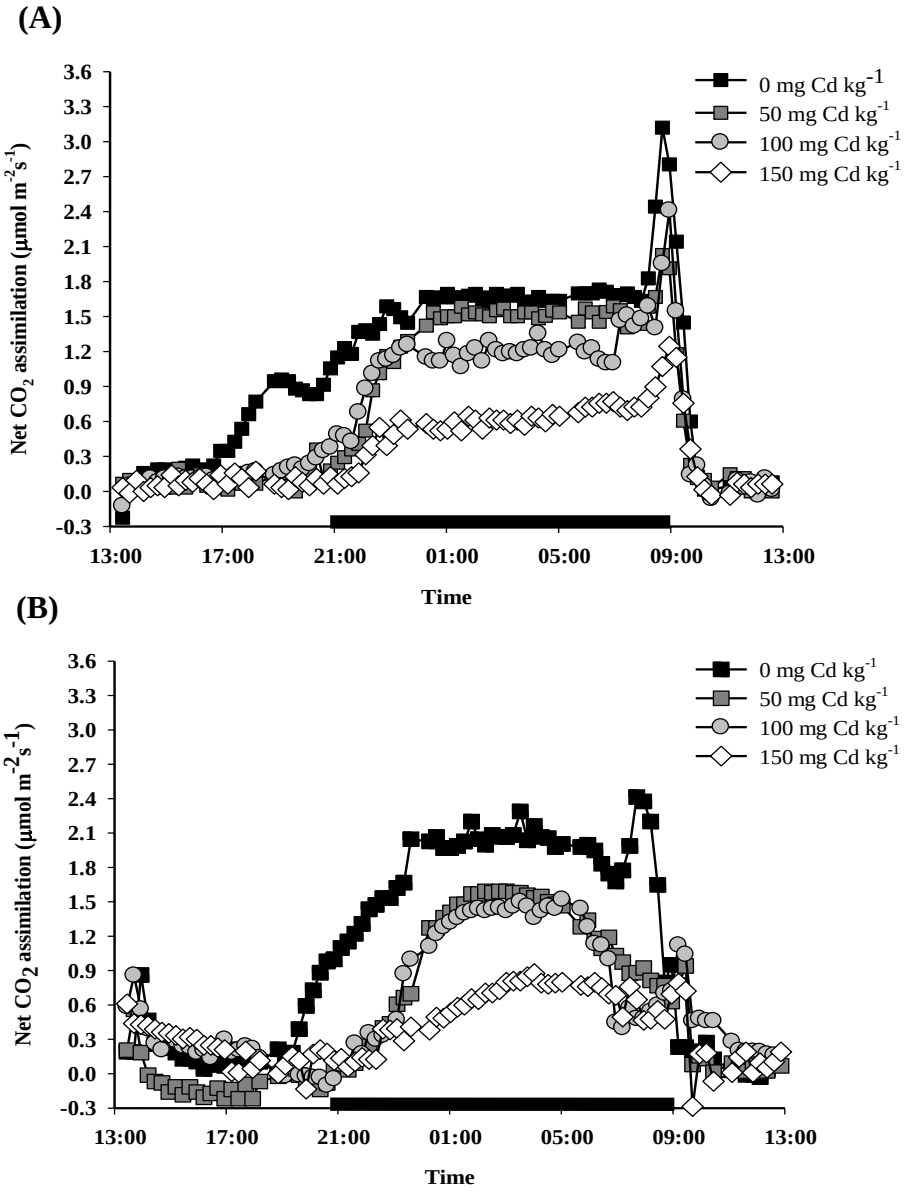

Figure 2.4 Rate of net CO_2 uptake from leaves of *A. comosus* after 4 months under light intensity of 250 µmol m^{-2} s^{-1} (A) and after 8 weeks under high light intensity 800 µmol m^{-2} s^{-1} (B) and subjected to 4 soil treatments of Cd. The shape of the assimilation curve, recorded over 24 hours, illustrated the day/night pattern of CAM activity at different cadmium concentrations. The black bar on the x axis represents the period of darkness. Each gas exchange curve is representative of that from four separate plants.

The integrated net CO_2 uptake calculated over 24 hours for pineapple grown in Cd-contaminated soil compared with control (without Cd) was calculated. The results revealed that Cd significantly decreased the net CO_2 uptake in pineapple leaves compared to control (Table 2.2). Moving plants from low to high light did not significantly alter net CO_2 uptake in any of the treatments.

Table 2.2 Net CO_2 uptake (mmol CO_2 m^{-2}) of pineapple grown in different concentrations of Cd under different light regimes.

Cd concentration (mg Cd kg^{-1})	**Low light intensity (250 µmol m^{-2} s^{-1})**	**High light intensity (800 µmol m^{-2} s^{-1})**
0	77.07 ± 7.29^{a}	66.88 ± 7.33^{a}
50	53.80 ± 8.93^{b}	47.38 ± 5.39^{b}
100	44.29 ± 2.10^{c}	39.35 ± 2.51^{b}
150	40.75 ± 3.67^{c}	34.64 ± 6.23^{c}

Different letters in columns and rows denote a significant difference between groups, such that groups not sharing a similar letter are significantly different from each other ($P < 0.05$). The effect of light and Cd on net CO_2 uptake were tested with two-way ANOVA. Each value represents means ± standard error of four replicates determinations.

2.3.3 The effect of Cd on CAM biochemistry

2.3.3.1 The effect of Cd on leaf titratable acidity, starch and soluble sugar

Figure 2.5 shows leaf titratable acidity expressed on a leaf area basis at the beginning (dawn) and end (dusk) of the photoperiod for control (soil without Cd) and plants grown in different concentrations of Cd. Plants were sampled after 9 months in Cd. The results showed a difference in titratable acidity between beginning and ending of photoperiod, indicating an overnight accumulation of acidity which is a diagnostic feature of CAM. The CAM activity (measured as overnight acid accumulation) was successively decreased as the concentration of Cd in soil increased and supports the trends noted with the impact of Cd on nocturnal CO_2 uptake (Fig 2.4, Table 2.2).

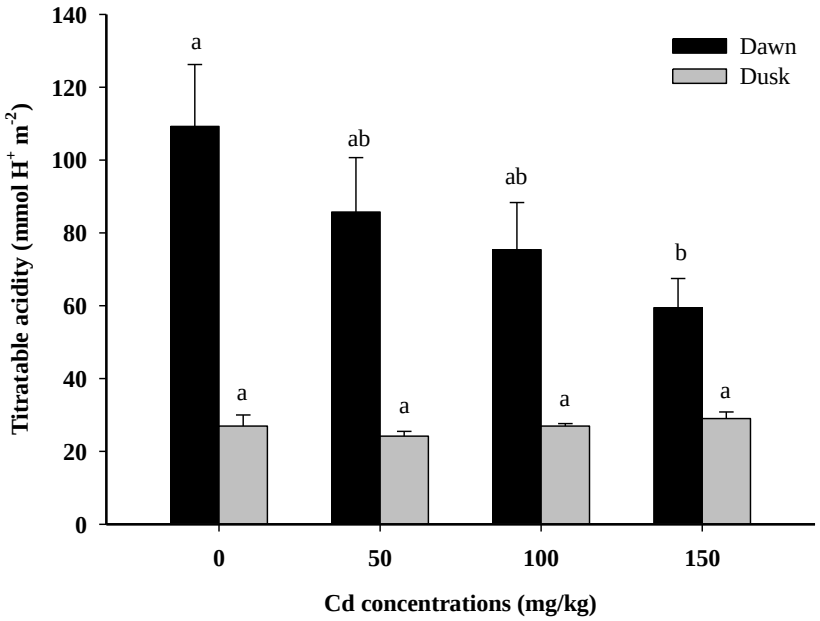

Figure 2.5 Titratable acidity from leaves of pineapple grown in different concentrations of Cd (0, 50, 100 and 150 mg Cd kg^{-1}). Plant leaf samples were taken at dawn and dusk. Data represent the means of four replicates with standard error. Different letters in the same photoperiod (Dawn or Dusk) indicate a significant difference between treatments at the level of $P < 0.05$.

In all treatments, the leaf starch content (Figure 2.6A) was lower than the soluble sugar content (Figure 2.6B). No significant difference in leaf starch content was observed between pineapple grown in Cd-contaminated soil and control plants. The results showed that leaf soluble sugar content of control plants was higher than Cd-treated plants. Leaf soluble sugar of control plants was significantly higher than treated plants especially at highest concentration of Cd (150 mg Cd kg^{-1}).

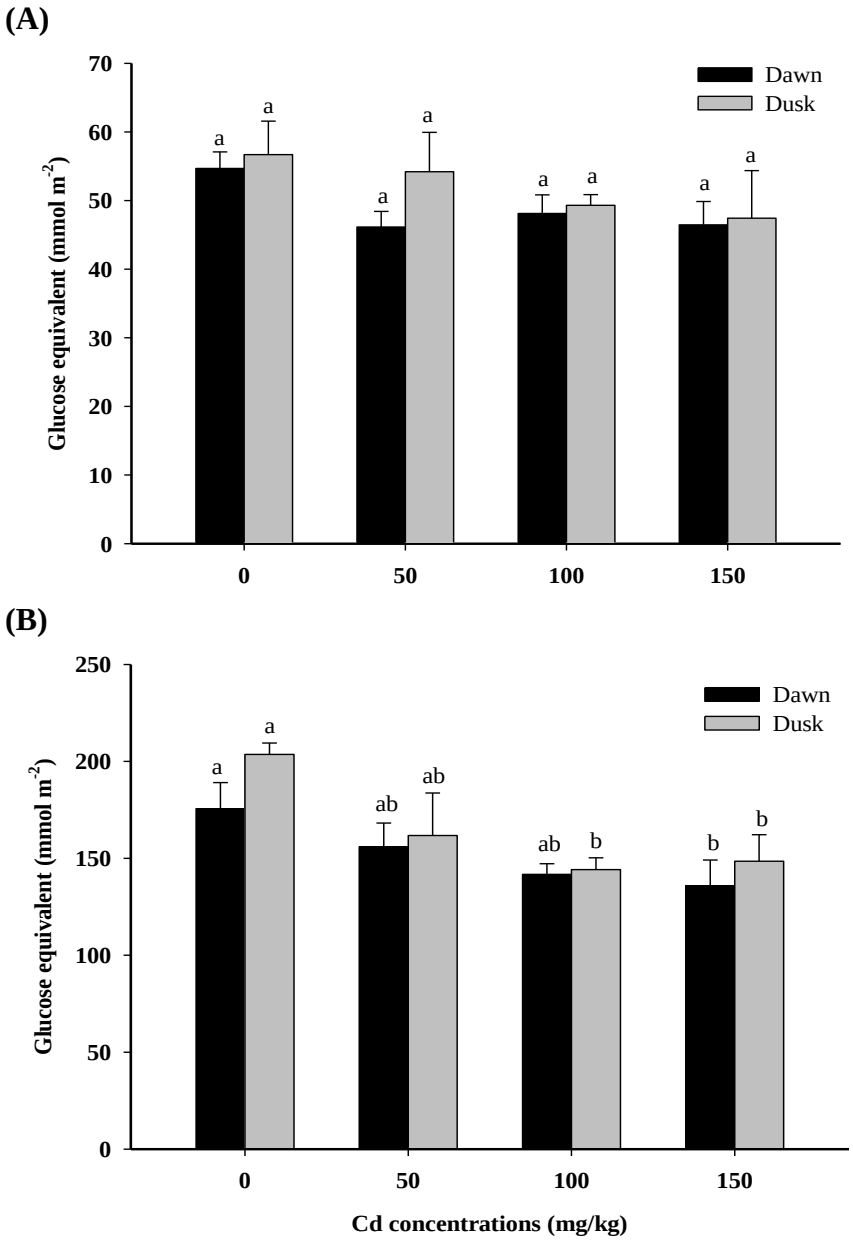

Figure 2.6 Starch (A) and soluble sugar contents (B) from leaves of pineapple grown in different concentrations of Cd (0, 50, 100 and 150 mg Cd kg^{-1}). Plant leaf samples were taken at dawn and dusk. Data represent means of four replicates with standard error. Different letters in the same photoperiod (Dawn or Dusk) indicate a significant difference between treatments at the level of $P < 0.05$.

2.3.3.2 The effect of Cd on PEPC, PEPCK and Rubisco protein contents

Comparing the amount of total soluble protein per unit leaf area in the pineapple grown in different Cd concentrations, it was found that plants grown in soil without Cd (control) had significantly higher protein content than plants grown in highest concentration of Cd (150 mg Cd kg^{-1}). For plants grown in Cd contaminated soil, protein content per unit leaf area was decreased as the Cd concentration increased (Figure 2.7).

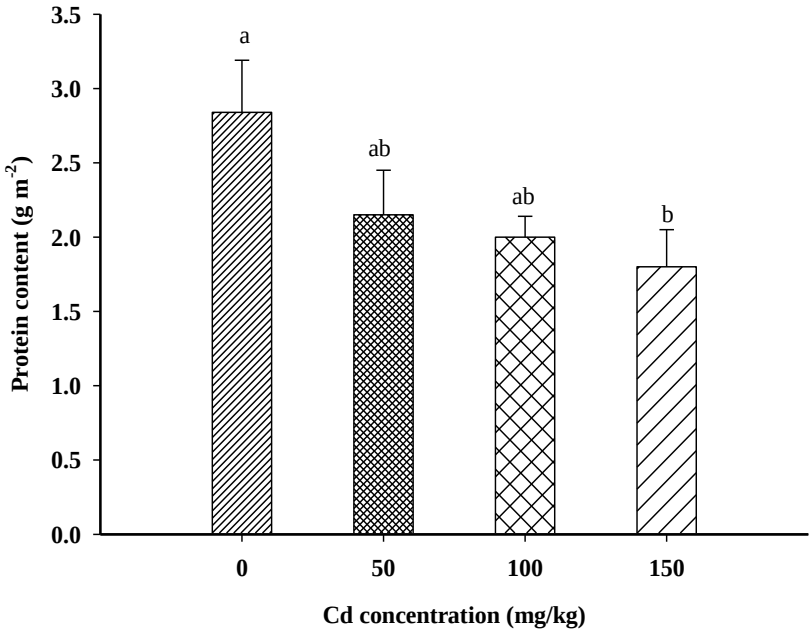

Figure 2.7 Amount of total soluble protein per unit leaf area for leaf tissues of *A. comosus* grown in different concentrations of Cd (0, 50, 100 and 150 mg Cd kg^{-1}) and acclimated to high light intensity (800 µmol m^{-2} s^{-1}). Values are the means of four replicates ± one standard error.

The impact of Cd on protein abundance of the key photosynthetic enzymes PEPC, Rubisco and the decarboxylase PEPCK was investigated using Western blotting (Figure 2.8). The abundance of PEPC protein was similar between control and treated plants. In contrast, PEPCK protein abundance decreased strongly in treated plants when compared with control. There also appeared to be more than one band of PEPCK protein in control plants and in plants grown in highest concentration of Cd (150 mg Cd kg^{-1}), suggesting the presence of different isoforms of PEPCK. Rubisco was more abundant in plants grown in soil without Cd than in treated plants. There was no correlation between PEPC

and the amount of malate accumulated overnight. However, there was a good correlation between PEPCK and Rubisco abundance and CAM activity.

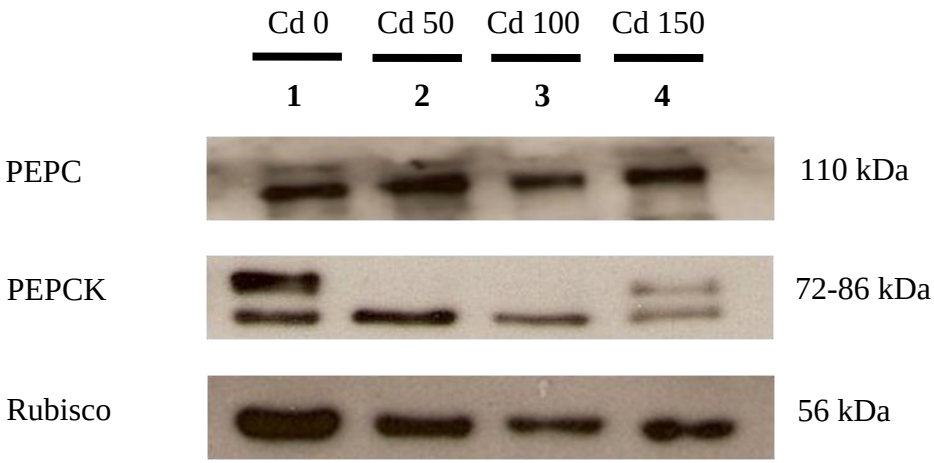

Figure 2.8 Western blots showing the relative abundance of Rubisco, PEPC and PEPCK proteins in leaf tissue of *A. comosus* grown in soil with different concentrations of Cd and samples after 8 weeks under high light intensity (800 µmol m^{-2} s^{-1}). The results are representative of four replicates. Lane 1 is control, Lane 2 is 50 mg Cd kg^{-1}, Lane 3 is 100 mg Cd kg^{-1} and Lane 4 is 150 mg Cd kg^{-1}. The molecular masses of the protein sub-units are indicated on the right hand side of the gel.

2.3.4 The effect of Cd on plant biomass

No plants showed any obvious visible symptoms of Cd toxicity even at 150 mg Cd kg^{-1} soil. The plants were harvested after four months under light intensity 250 µmol m^{-2} s^{-1} and at nine months under the higher light intensity of 800 µmol m^{-2} s^{-1}. The leaves selected were younger leaves (upper leaves), intermediate leaves (middle leaves), and older leaves (lower leaves). In general, plant biomass was decreased under the increasing concentrations of Cd (Figure 2.9). No significant effects of Cd were found for the older leaves (lower leaves) but significant effects of Cd on growth of intermediate leaves (middle leaves) was observed, especially under 150 mg Cd kg^{-1} soil when compared with control. In addition, significant effects of Cd on growth of younger leaves (upper leaves) and root were observed after 9 months exposure to Cd-contaminated soil.

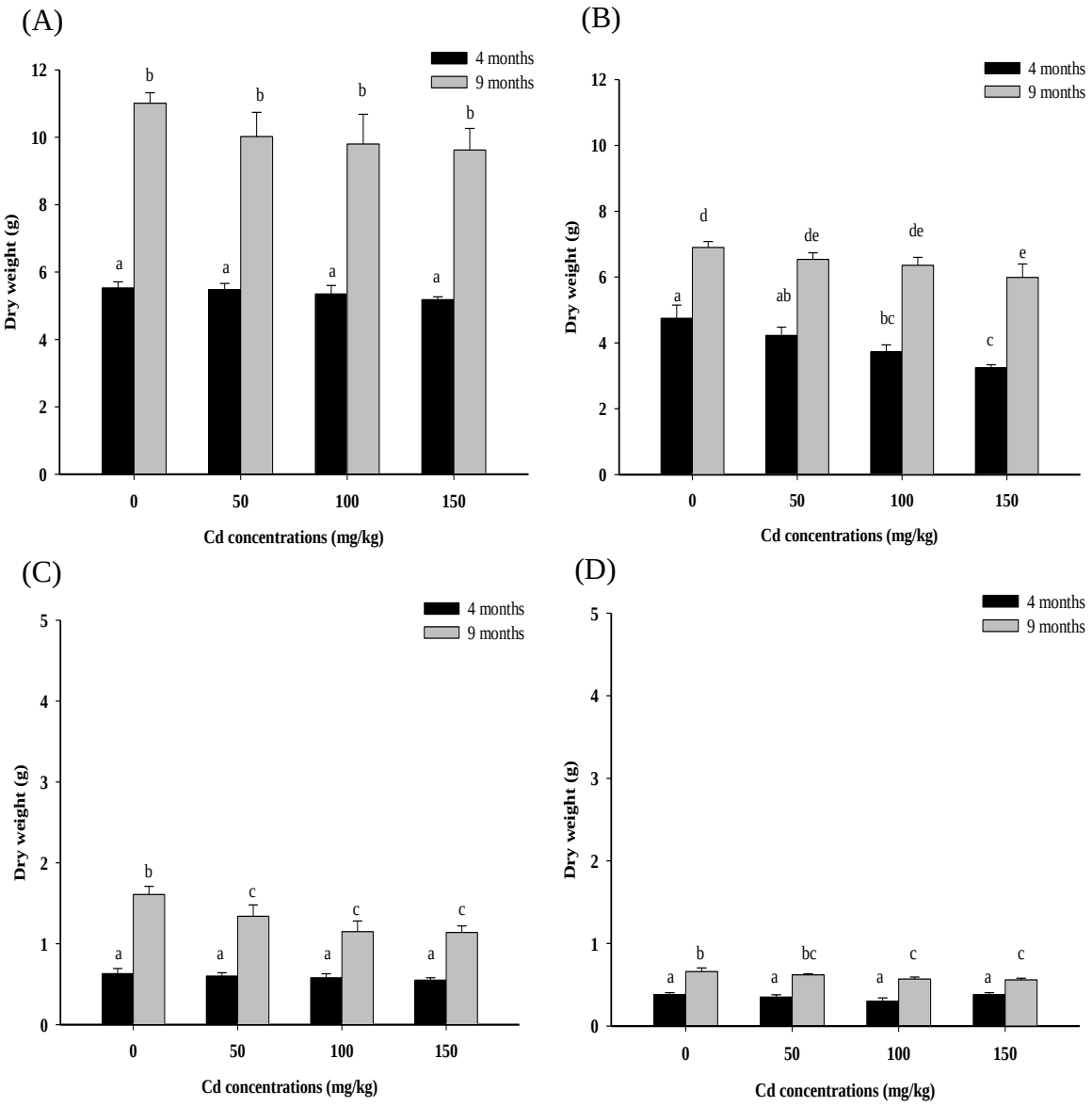

Figure 2.9 Plant biomass of pineapple after harvests at 4 months and 9 months under different Cd treatments. The samples were taken from older leaves (lower leaves; A), intermediate leaves (middle leaves; B), younger leaves (upper leaves; C) and roots (D). Pineapple plants treated with 0, 50, 100 and 150 mg Cd kg^{-1} soil as $Cd(NO_3)_2$. Values shown are the means of four replicates. Different letters above bars indicate significant differences ($P < 0.05$, Least Significant Difference test).

2.4 Discussion

This study investigated the effect of Cd on the photosynthetic performance, CAM activity/biochemistry and growth of pineapple. The influence of Cd on photosynthetic acclimation to contrasting light responses was examined in terms of monitoring light use efficiency and patterns of net CO_2 uptake. The hypothesis was tested that Cd can suppress light utilization efficiency and CAM activity and thus lead to reduced growth of pineapple.

2.4.1 The impact of Cd on plant growth

Reduction of leaf and root growth in pineapple grown in Cd-contaminated soil was observed in this study. Chen and Huerta, (1997) suggested that Cd-induced growth inhibition is due to an inhibition of photosynthesis. Many publications have revealed that Cd is an effective inhibitor of photosynthesis (Greger *et al.*, 1993; Chugh and Sawhney, 1999; Vassilev *et al.*, 2005; Ekmekçi *et al.*, 2008). Numerous studies have demonstrated that Cd-induced decreases in photosynthetic rate might result from reduced chlorophyll and carotenoid contents (Mobin and Khan, 2007; Shi *et al.*, 2010), obstructed electron transport (Baszynski *et al.*, 1980; Sigfridsson *et al.*, 2004), reduced enzymatic activity involved in CO_2 fixation (Malik *et al.*, 1992; Krantev *et al.*, 2008) and through CO_2 deficiency due to stomatal closure (Poschenrieder *et al.*, 1989; Shi and Cai, 2008). However, different plant species vary in their tolerance of Cd. Foyer and Noctor, (2005) showed that Cd toxicity in plants is due to alterations in the levels of reactive oxygen species (ROS). Cd enhances lipid peroxidation and generates oxidative stress in plants leading to growth inhibition and even plant death (Somashekaraiah *et al.*, 1992). The reduction in growth could be a consequence of Cd^{2+} interference with a number of metabolic processes associated with normal development, especially synthesis of proteins (Stiborová *et al.*, 1987), the activity of some important enzymes by binding to free amino carboxylate or side groups and replacement of some important metal ions associated with such groups (Van Assche *et al.*, 1988; Van Assche and Clijsters, 1990; Alia and Saradhi, 1991). Sheoran *et al.*, (1990) demonstrated that Cd^{2+} interferes with the formation of chlorophyll by interfering with photochlorophyllide reduction and the synthesis of aminolevulinic acid. Therefore, it is suggested that Cd^{2+} stress causes the recast of chlorophyll content, reduction of biomass, and inhibits plant growth.

In *Brassica napus*, Cd at low concentration (1 mg Cd kg^{-1}) reduced plant growth, photochemical quantum yield of photosynthesis, chlorophyll content and led to stomatal

closure (Larsson *et al.*, 1998; Baryla *et al.*, 2001). From previously published data, damaging effects of Cd at concentrations lower than 4 mg Cd kg^{-1} have been reported for several Cd-sensitive plants, such as sugar beet (Greger *et al.*, 1991; Greger and Ögren, 1991), tomato (Baszynski *et al.*, 1980; Dudka *et al.*, 1983), bean (Vazquez *et al.*, 1992), and spruce (Godbold, 1991). Moreover, in a Cd-tolerant clone of the grass *Holcus lanatus*, metal tolerance was found at 18 mg Cd kg^{-1} (Hendry *et al.*, 1992). It was found that tomato exposed to 2 mg Cd kg^{-1} showed decreased plant biomass and those plants exposed to 0.2 mg Cd kg^{-1} showed visual symptoms of Cd toxicity which become more severe with increasing Cd level and extended time exposure. Ghani, (2010) showed that mung bean had a significant reduction in plant biomass at highest concentration of Cd supplied (12 mg Cd kg^{-1}). Shukla *et al.*, (2002) demonstrated that wheat treated with 0.5, 1.0, 2.5, and 5.0 mg Cd kg^{-1} showed symptoms of Cd toxicity as observed by various morphological parameters which were recorded with the growth of plants. Rascio *et al.*, (1993) demonstrated that the maize plants grown on culture media applied with 45 mg Cd kg^{-1} Cd showed a symptom of Cd toxicity such as length reduction, leaf bleaching, chloroplast ultrastructural alterations, and inhibition of photosynthetic activity. There are a few studies in CAM species regarding Cd tolerance. Zhou and Qiu, (2005) reported that Cd could suppress cell expansion and induce senescence in the CAM species *Sedum alfredii*. Root elongation and leaf expansion of *S. alfredii* were inhibited at 183 mg kg^{-1}. Moreover, the root color became brown/black and the older leaves were found to fall off readily at high Cd concentrations. Interestingly, the typical visible symptoms of Cd toxicity such as leaf necrosis, chlorosis, reddish-brown discoloration of the leaf blades and browning of root system was not observed in pineapple plants even at the highest concentrations of Cd (150 mg Cd kg^{-1}). At this highest Cd concentration, the growth of the whole pineapple plant was not strongly inhibited. This suggests that pineapple is a relatively Cd tolerant species.

The results from the present study showed that Cd had a greater effect on intermediate leaves (middle leaves) compared to the older leaves (lower leaves) and younger leaves (upper leaves). Intermediate leaves, which are the youngest physically mature whorl of leaves, the D-leaves, are often used as an indicator of the nutritional and moisture status of the plant and to assess environmental effects on the plant. In particular, the D-leaf weight correlates strongly with the weight of the whole plant (Batholomew and Kadzimin, 1977). This is related to the results of leaf gas exchange analysis presented here that showed the effect of Cd on the intermediate leaves by suppressing net CO_2 uptake which

may in turn result in growth reduction.

2.4.2 The impact of Cd on light use efficiency

It is well-known that Cd is potentially detrimental to several photosynthetic parameters in plants (Mobin and Khan, 2007; Krantev *et al.*, 2008; Shi *et al.*, 2009; Shi *et al.*, 2010). In this chapter, the impacts of Cd on both the apparent quantum yield (light-use efficiency) of photosynthesis and on net CO_2 uptake were assessed. Results demonstrated that the maximum quantum yield of PSII (F_v/F_m) in Cd-treated plants was generally lower than that of control. Moreover, F_v/F_m in Cd-treated plants was suppressive decreased compared with control when plants were moved to high light intensity. The chlorophyll fluorescence parameter ratio of variable fluorescence to maximal fluorescence (F_v/F_m) has been frequently used to detect stress-induced effects on light utilization efficiency of the photosynthetic machinery (Maxwell and Johnson, 2000; Roháček, 2002). Atal *et al.*, (1991) also showed a reduction in F_m and F_v in wheat seedlings (*Triticum aestivum* L.) due to Cd treatment. Ralph and Burchett, (1998) suggested that the Cd-induced decline in F_m observed in seagrass (*Halophila ovalis*) was due to a change in the ultrastructure of the thylakoid membrane, negatively affecting the electron transport rate. Vassilev and Manolov, (1999) suggested that Cd reduced the value of F_v/F_m in barley plants by inducing a down regulation of PSII in order to avoid an over-reduction of primary electron acceptor (Q_A), thus reducing the load on the electron transport chain. Additionally, a reduction in F_v/F_m ratio, especially under stress conditions, is often an indicator of photoinhibition or other kind of injury to PSII components (Roháček, 2002). From the results on pineapple where the ratio of variable fluorescence to maximum fluorescence (F_v/F_m) decreased under Cd stress, this indicated inhibition of electron transfer reactions in PSII (Sigfridsson *et al.*, 2004; Azevedo *et al.*, 2005; Küpper *et al.*, 2007). Krupa, (1988) found that Zea mays exposed to 0.2 mM of Cd for 10 minutes under dark incubation showed inhibition of PSII electron flow in chloroplasts. Photosystem II was highly sensitive to the harmful effect of Cd and its functioning was inhibited to a much greater extent than that in photosystem I. Similar results have been obtained in other plants under Cd toxicity such as maize (Ekmekçi *et al.*, 2008), peanut (Shi and Cai, 2008) and safflower (Shi *et al.*, 2010).

Cd also curtailed the ability of pineapple to acclimate to higher light intensities. This was demonstrated by the slower recovery of F_v/F_m in Cd-treated plants compared to control when moved from 250 µmol m^{-2} s^{-1} to 500 µmol m^{-2} s^{-1} and then onto 800 µmol m^{-2} s^{-1}.

Plants typically experience a drop in F_v/F_m when transferred from low to high light intensities (Oquist *et al.*, 1992; Dias and Marenco, 2007). This is attributed to photoinhibition of photosynthesis (Long *et al.*, 1994; Tsonev *et al.*, 1999). Maxwell and Johnson, (2000) suggested that a reduction in F_v/F_m indicated an increase in photoinhibition, which could be due both to photoprotective processes and damage to photosynthetic apparatus. The injuries in the photosynthetic apparatus provoked by high light are connected mainly with PSII and lead to inactivation of electron transport following oxidative injuries in the reaction centres of PSII, especially D1 protein (Krause, 1988). Generally, sun-acclimated plants tend to have a greater maximum rate of photosynthesis than shade plants, and an increased capacity for photoprotection (Boardman, 1977; Demmig-Adams and Adams III, 1992). In contrast, many bromeliads, including *A. comosus*, *Aechmea magdalenae* and *Bromelia humilis* have a photosynthetic capacity independent of photon flux density (PFD), with high rates of PSII electron transport and high potential for the dissipation of excess light energy (Fetene *et al.*, 1990; Skillman and Winter, 1997). However, there may also be chronic photoinhibition when bromeliads are grown under high light, as observed in *A. magdalenae* (Skillman and Winter, 1997). The recovery in F_v/F_m after transfer from low to high light intensity depends on the PSII efficiency recovery after photoinhibition or the turnover of the D1 protein associated with the reaction centres (Osmond, 1978; Demmig-Adams and Adams III, 1992; Thiele *et al.*, 1998). Prasil *et al.*, (1992) found that recovery the photoinhibited reaction centre is repaired by replacing the damaged D1 protein with its newly synthesized copy. The reduced ability of Cd-treated pineapple to acclimate to higher light intensities might suggest that the heavy metal contamination curtailed repair of the D1 protein. The study of Delahunty, (2003) found that pineapple acclimated to low light had a higher maximum quantum yield of PSII than plants acclimated to high light. The absorption of light is closely related to chlorophyll content (Evans, 1996). For a given amount of nitrogen, there is a trade-off between the amount invested in pigment-protein complexes (light capture) and the amount invested in soluble proteins per unit leaf area (Evans, 1996). Thus the pineapple that was grown under Cd-stress in low light may have invested more resources in pigment-protein complexes, and could thus absorb a greater percentage of incident light, compared to the Cd-treated plants acclimated under high light and hence operate with greater photosynthetic efficiency under low light intensity (Salisbury and Ross, 1992).

2.4.3 Net CO_2 uptake and CAM activity in pineapple under Cd-stress

A decline in F_v/F_m in Cd treated plants could be attributed to a reduction in the rate of net CO_2 uptake. If CO_2 uptake is reduced under Cd-stress there will be less demand for ATP and reductant to be generated via the electron transport chain and F_v/F_m will decline (Saccardy *et al.*, 1998; Georgieva *et al.*, 2000).When 24 hour gas exchange patterns were compared for pineapple control and Cd-treated plants, control plants had higher rates of nocturnal net CO_2 uptake when compared with treated plants. The impact of Cd on nocturnal net CO_2 uptake was even more apparent in plants under the higher light intensity. It is well known that Cd is an effective inhibitor of C3 photosynthesis (Greger *et al.*, 1993; Chugh and Sawhney, 1999; Vassilev *et al.*, 2005). Hermle *et al.*, (2007) found that Cd reduced both net CO_2 uptake and stomatal conductance. Cd-induced decreases in water-use efficiency have been reported as a result of a greater decline in photosynthesis in response to the heavy metal treatment than in stomatal conductance. Thus, the heavy metal treatment appeared to cause a reduction in carboxylation efficiency, which limited CO_2 gain, as shown by Kitao *et al.*, (1997).

Several studies have shown that Cd-induced decrease in the rate of net CO_2 uptake can be attributed to stomatal closure which may be linked to the water-stress that can result from exposure to Cd (Bazzaz *et al.*, 1974; Lamoreaux and Chaney, 1977; Schlegel *et al.*, 1987). Barcelo *et al.*, (1988) and Marchiol *et al.*, (1996a) reported that root hydraulic conductivity into xylem vessels decreased from two to four times depending on the applied Cd stress and characteristics species. Barcelo *et al.*, (1988) considered that the reasons for reduced water movement were the decreased vessel radius and number of vessels due to Cd-induced inhibition of division, elongation and differentiation of cambium cells. These authors hypothesised that this is a consequence from disturbed hormonal balance, but until now, there are no data supporting the suggestion. According to Fuhrer, (1982) another reason for decreased water movement could be the structural disorders in some vessels because of the accumulation of lignin-like insoluble phenols and depositions of calcium oxalate (Van Balen *et al.*, 1980). Although CAM plants like pineapple demonstrate higher water use efficiency than C4 and C3 plants (Cushman and Bohnert, 1997). Zhou and Qiu, (2005) found that the water content of *S. alfredii* leaves (a CAM species) was significantly affected at high Cd concentrations. This suggests Cd could disturb plant-water relationships in pineapple with reduced stomatal conductance in Cd-treated plants perhaps accounting for the lower net CO_2 uptake reported in this thesis.

2.4.4 The impact of Cd on CAM biochemistry of pineapple

The present study revealed that net CO_2 uptake was a sensitive measure of the impact of Cd-toxicity in the CAM species pineapple. The impact of Cd in reducing net dark CO_2 uptake and CAM activity in pineapple was supported by measurements of the overnight accumulation of leaf titratable acidity which successively declined as Cd concentration increased. A reduction in CAM activity could be due to a reduction in the nocturnal provision of PEP from carbohydrates that were built up during the previous day and/or a reduction in the amount of key enzymes involved in the uptake and processing of CO_2 across the 24 hour CAM cycle. From results of the impact of Cd on the abundance of key proteins involved in the uptake and processing of CO_2 in CAM, PEPCK abundance was found to decrease strongly in treated plants when compared with control. PEPCK is believed to be the major decarboxylase in pineapple (Walker and Leegood, 1996) and a reduction in abundance of this protein could limit day-time breakdown of malic acid and the provision of CO_2 for Rubisco. The multiple protein bands observed for PEPCK in control and Cd-treated plants at 150 mg Cd kg^{-1} suggested the presence of different PEPCK isoforms that were not present in Cd-treated plants at 50 mg Cd kg^{-1} and 100 mg Cd kg^{-1}. In the C4 monocot *Urchloa panicoides*, PEPCK is encoded by four differentially expressed genes, two transcripts being expressed predominantly in leaves, and two in the roots (Finnegan *et al.*, 1999). In *Arabidopsis thaliana*, there are three possible PEPCK gene sequences (Finnegan *et al.*, 1999). In contrast, relatively little work has been done on PEPCK expression in CAM plant, so the number of isoforms present in pineapple is not known.

Cd also had a negative impact on the abundance of Rubisco protein in pineapple as illustrated by the blots for the large sub-unit of this protein. The results are consistent with those of Wang *et al.*, (2009a) who showed that Cd- elicited a decrease in Calvin cycle activity in maize as indicated by a reduction in Rubisco activity. According to Krupa *et al.*, (1993) in Cd-treated bean plants the decrease in energy consumption by a reduction in Calvin cycle activity could help account for a down regulation of PSII and light use efficiency (i.e. decline in F_v/F_m). It has been reported elsewhere that the inhibitory influences of Cd stress on the activity of Rubisco occurred before changes were manifest on PSII electron transfer. Di Cagno *et al.*, (2001) stated that F_v/F_m did not change in sunflower plants exposed to Cd treatments for 15 days, but Rubisco activity was reduced, suggesting that the Calvin cycle components of photosynthesis are more vulnerable to Cd than the light reactions. It has been suggested that Cd^{2+} ions lower the activity of Rubisco

and damage the structure of Rubisco by substituting for Mg^{2+} ions and may also shift Rubisco activity towards oxygenation reactions (Siedlecka *et al.*, 1998). The results of Malik *et al.*, (1992) showed that Cd caused an irreversible dissociation of the large and small subunits of Rubisco, which lead to total inhibition of the enzyme. Hajduch *et al.*, (2001) observed drastic reductions and fragmentation of Rubisco in Cd-stressed rice leaves.

The negative impacts of Cd on PEPCK and Rubisco abundance could account for the lower carbohydrate contents of leaves of Cd-treated pineapple plants. Soluble sugars were the predominant form of storage carbohydrate in pineapple leaves and were 3-4 times higher than leaf starch content. Whilst there were only slightly decrease in starch content in Cd-treated plant, the decrease of soluble sugar in Cd-treted plants were significantly reduced that could be limited dark CO_2 uptake. In contrast to the reduced abundances of PEPCK and Rubisco elicted by Cd in pineapple leaves, the abundance of PEPC protein was similar in control and Cd-treated plants. Thus, the reduction of net nocturnal CO_2 uptake in Cd-treated plants could not be attributed to altered PEPC abundance. It is well documented in CAM plants that PEPC is regulated via post-translation modification that is catalysed by a dedicated kinase which phosphorylates/activates PEPC at night (Nimmo, 1993; Vance, 1997; Nimmo, 2000). A decreased phosphorylation state of PEPC in pineapple grown under Cd-stress could result in lower rates of net CO_2 uptake at night. The phosphorylation status of PEPC in CAM plants is believed to be under circadian control (Nimmo *et al.*, 1987; Hartwell *et al.*, 1996; Hartwell *et al.*, 1999) but is also influenced by cytosolic malate concentration (Borland *et al.*, 1999). If malate sequestration into the vacuole is curtailed, this could feedback negatively on the activity of PEPCK and reduce nocturnal CO_2 uptake. The reduced CAM expression in Cd-treated leaf tissue of pineapple plants, compared with control plants, could be attributed to a reduced supply of PEP for organic acid synthesis. Raveh *et al.*, (1998) demonstrated that in the CAM spcies *Agave americana*, where the variegated green and yellow leaf tissue had similar activities of PEPC but a three-fold difference in malate accumulation, the lower sugar content in the yellow areas dictated the level of CAM activity. Although as discussed above, Cd resulted in only a marginal decrease in soluble sugar content in leaves of pineapples, it is possible that Cd curtailed the breakdown of sugars to provide PEP. Soluble sugars are stored in the vacuole in pineapple leaves (Kenyon *et al.*, 1985) so again, transport processes across the tonoplast membrane could be a key target for the negative impacts of Cd.

2.5 Conclusions

Physiological processes such as photosynthesis have been shown to be very sensitive to Cd in plants. In the CAM plant pineapple, a decrease in F_v/F_m, reduction in net CO_2 uptake, reduced plant biomass and depressed CAM activity accompanied exposure of plants to Cd. However, the plants grown in Cd contaminated soil did not show any visual toxicity symptoms and biomass reductions were only 14% of control at the highest Cd concentration used. These results indicate the potential of pineapple to resist Cd toxicity. Further work is needed to establish the mechanisms that underpin the reduction in nocturnal net CO_2 uptake in pineapple exposed to Cd.

Chapter III

Cadmium accumulation in pineapple (*Ananas comosus* L.)

3 Cadmium accumulation in pineapple (*Ananas comosus* L.)

3.1 Introduction

Concern over the possible health and ecosystem effects of heavy metals in soils and accumulation in plants has increased in recent years. Among the various toxic metals, Cd causes many problems for human health and ecosystems in general (Izadiyar and Yargholi, 2010). Although plants do not require Cd for growth or reproduction (Kabata-Pendias and Pendias, 2001). Cd is readily absorbed and accumulated in vegetation, thus increasing the potential for contamination of the food chain (Galal-Gorchev, 1993). In Thailand, soil contamination problems with Cd are still unsolved especially in high risk zones such as industrial and mining areas (Unhalekhaka and Kositanont, 2008). The biggest Zn production in Thailand (Cd is associated with Zn) is located in the Mae Sot district in Tak province and the estimated mine production capacity is 50000 metric tons (Padaeng Industry Public Company Limited, 2008). Mining actions such as drilling, explosions, material transfer, mine tailings disposal and drainage may cause Cd to be widely distributed throughout such areas, as mentioned in the research by Soil Analysis Division, Land Development Department, Thailand. The average Cd level in the sediment which came from the collapsed of cinders around the zinc mining area at Mae Sot was 228.5 mg/kg soil (Tunmanee and Thongmarg, 1994). Previous research revealed that Cd accumulation in rice grains from plants grown in Cd-contaminated areas in Thailand was higher than recommended by EU Maximum Permissible level and the Thai government has prohibited rice cultivation in Tak Province (Simmons *et al.*, 2005).

Due to Cd contamination in soils and crops cultivated in the Mae Sot district in Tak province, the Thai government is attempting to find alternative plants to grow in that area. Pineapple is the most economically important fruit in Thailand and has 80,000 hectares under pineapple cultivation (FAO, 2005). The export volume in 2005 was 30.61% compared with other major fruits in Thailand (Chomchalow *et al.*, 2008). In addition, pineapple is a CAM plant that has high tolerance to drought and stress conditions (Bartholomew and Malézieux, 1994). Therefore, the study in Chapter II examined if pineapple could grow in Cd-contaminated soil. The data presented in Chapter II indicated that Cd had an affect on the rates of net CO_2 uptake, photosynthetic performance, CAM biochemistry and plant growth, but no visible toxicity symptoms such as leaf chlorosis or necrosis was observed. However, more specific information is needed in relation to the

absorption and transfer of Cd from the root region to different parts of pineapple, especially the levels of accumulation of Cd in pineapple.

Understanding the degree of Cd accumulation in different parts of the pineapple plant is required for informing the potential risks to human health of the people in Thailand who grow pineapple in Cd-contaminated soil area. Such information is also valuable for rice farmers in that area who might want to consider cultivating pineapple in substitution of rice and using the high-sugar containing leaves that remain after fruit harvesting as a source of bioethanol.

Several factors can influence Cd uptake and accumulation in plants. Cd extractability in soil is a key factor influencing Cd uptake and accumulation in plants and may be more critical than the total concentration of Cd in soil (Sarwar *et al.*, 2010). Estimates of metal extractability in soil indicate specific bioavailability, reactivity, mobility and potential uptake of the metal ion by plants (Mcbride, 1994; Luo and Christie, 1998). Thus, in any study investigating heavy metal contamination in soil, both the total concentration as well as the extractable concentration of metal ions should be measured. A number of factors affect Cd extractability in soil, including soil pH, organic matter, presence of other ions, root exudates, types and cultivars of crop plants, and plant age (Harter and Naidu, 2001; Jung, 2008). All of these factors influence the solubility of Cd-containing compounds and the release of Cd into the soil solution and can also affect the ability of plants to take up Cd from soil (McBride, 1989). Some of these factors have positive effects while others have negative effects on Cd extractability. In general, among soil factors which affect Cd extractability, soil pH is the most important (Marschner, 1995; McBride *et al.*, 1997a). Under acidic conditions, H^+ ions displace metal cations from the cation exchange capacity (CEC) of soil components and cause metals to be released from sesquioxides and variable-charged clays to which they have been chemisorbed (i.e. specific adsorption; (Mcbride, 1994). The retention of metals to soil organic matter is also weaker at low pH, resulting in more extractable metal in the soil solution for root absorption. Many metal cations are more soluble and extractable in the soil solution at low pH below 5.5 including Cd, Cu, Hg, Ni, Pb and Zn (Mcbride, 1994; Blaylock and Huang, 2000). Numerous studies have shown an indirect linear relationship between soil pH and Cd extractability such that with a decrease in soil pH, metal uptake by plants increases (Kirkham, 2006; Kim *et al.*, 2009).

Overall, the aim of the chapter was to examine the influence of Cd on soil pH, extractable Cd, total Cd in soil and the magnitude of Cd accumulation of different tissue of pineapple plants grown in Cd-contaminated soil. Bulk soils and rhizosphere soils were compared to investigate the distribution of Cd within the soil and to assess the effect of plant roots on Cd uptake. Different ages of leaves and roots were analysed separately to the degree of Cd translocation from the below ground to above ground tissues of pineapple plants.

Specific objectives were to:

a) examine the influence of Cd on soil pH, extractable Cd and total Cd in bulk soil and rhizosphere soil.
b) examine the effect of Cd on Cd accumulation in different plant parts of pineapple.

3.2 Materials and Methods

3.2.1 Plant material and growth conditions

Soil was made up in pots containing a mixture of sand (East Riding Horticulture Ltd., UK) and John Innes No. 2 compost (John Innes Manufacturers Association; UK) in the ratio of 1:1. The soil was thoroughly mixed with $Cd(NO_3)_2$ (Sigma-Aldrich) solution to obtain 0, 50, 100, and 150 mg Cd kg^{-1} soil and was incubated (aged) for four weeks without plants before being remixed and used as a growing medium for pineapple plants. Suckers of pineapple (*Ananas comosus* L.) approximately 1.0 cm diameter were potted up in 10 cm plastic pots containing a mixture of sand and John Innes No. 2 compost in the ratio of 1:1 without Cd application until the roots became exposed at the base of the pot. Plants were removed and potted up in 14 cm plastic pots with the following treatments: 0, 50, 100, and 150 mg Cd kg^{-1} soil as $Cd(NO_3)_2$. The plants were grown in a controlled growth chamber at 27 ºC (day) and 19 ºC (night) under a 12 hour photoperiod with photon flux density (PFD) of 250 µmol m^{-2} s^{-1} at leaf height for six months. After six months, plants were then moved to an intermediate light intensity at 550 µmol m^{-2} s^{-1} at leaf height for one month and subsequently were moved to the highest light intensity of 800 µmol m^{-2} s^{-1} at leaf height for two months before harvesting. The water content of the soil was maintained at 60% of its holding capacity by watering with tap water twice a week. Plants were destructively harvested after four months growth in Cd-contaminated soil and a further batch were harvested after nine months growth in Cd-contaminated soil.

3.2.2 Soil analysis

3.2.2.1 Soil sampling strategy

Soil samples were collected from pots every three weeks and after each of the two harvests. Every three weeks, soil samples were taken from around the inside edge of each pot (bulk soil) and near the root zone (rhizosphere soil) using a cork borer 1.5 cm in diameter. Soil samples were air-dried at room temperature and were ground to pass a 2 mm sieve, homogenized and stored in plastic bags at room temperature until analysis of extractable Cd and pH. For harvesting plant samples, plants were removed from the pots and bulk soil was separated from rhizosphere soil by hand to remove soil that did not adhere to the roots. This soil was then put in a plastic container. The soil which was still attached to the roots was carefully removed from the root by shaking very gently and this was put in a plastic container. Soil samples were prepared as described above and stored

at room temperature for a few days before analyse extractable Cd, pH and total Cd.

3.2.2.2 pH measurement

Soil pH was determined using 1:2.5 of soil: distilled water (w/v) following two hours equilibrium with shaking on a reciprocal shaker for 30 minutes. The solution was allowed to stand for 30 minutes before measurement using a pH meter (JENWAY 3020 MODEL, UK).

3.2.2.3 Extractable Cd in soil

Extractable Cd was determined as described by Si *et al.*, (2006). Soil samples (1.0 g) were added to 50 ml centrifuge tubes and 20.0 ml of 0.1 M $Ca(NO_{3)2}$ was added. The samples were shaken on a reciprocal shaker for 2 hours, followed by centrifugation at 6000 rpm for 10 minutes. The centrifuged samples were filtered through Whatman No. 40 filter paper and the filtrate was collected in scintillation vials. All the samples were then stored at 4 ºC until the analysis of metal was conducted by using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES; PerkinElmer, USA).

3.2.2.4 Measurement of total Cd in soil

The Aqua Regia Digest method used was adapted from Fortune *et al.*, (2000). Approximately 0.25 g of ground fresh soil was weighed into a digestion tube and 4 ml of concentrated hydrochloric (HCl) and 1 ml of concentrated nitric acid (HNO_3) were added. The samples were vortexed and left to digest overnight in a fume cupboard. The following morning, the digestion tubes were placed in a heating block with scrubber and heated using the programme as outline in Table 3.1.

Table 3.1 Heating programme of Aqua Regia Digest method for soil sample.

Temperature (ºC)	Time (h)
25	2
60	3
105	1
125	2

The following morning, 5 ml of 25% HCl was added to each tube, vortexed and heated for 1 hour at 80 °C. Finally, 18 ml of distilled water was added and the tube was heated for a further 30 minutes. Once cool, the sample was filtered through Whatman No. 40 filter paper and made up to 25 ml in a volumetric flask with distilled water. The filtered sample was analysed using ICP-OES.

3.2.3 Plant analysis

3.2.3.1 Plant sampling strategy

Four and nine months after growth in Cd-contaminated soil, plants were carefully removed from the soil and separated into shoots and roots. The shoots and roots were washed thoroughly with tap water and then with distilled water. To remove surface adsorbed metal ions from the roots, the roots were washed extensively with 10 mM $CaCl_2$ and then with distilled water. Leaves were separated into different age categories as older leaves (lower leaves), intermediate leaves (middle leaves) and younger leaves (upper leaves).

3.2.3.2 Total Cd uptake by pineapple

The nitric-perchloric acid digest method used was based on Zhao *et al.*, (1994) and described in Fortune *et al.*, (2000). Oven-dried root and shoot samples were ground in a stainless steel mill to pass through a 0.5 mm sieve. Sub-samples of shoots (0.50 g) and roots (0.25 g) were weighed into a digestion tube and 5 ml of $HNO_3/HClO_4$ acid was added (85% HNO_3 and 15% $HClO_4$). The sample was mixed using a vortex mixer and left

to digest overnight in a fume cupboard. The following morning, the digestion tubes were placed on a heating block with scrubber and heated using the program outlined in Table 3.2. The following morning, 5 ml of 25% HCl was added to each tube, mixed and heated for 1 hour at 80 °C. Finally, 18 ml of distilled water was added and the tube was heated for a further 30 minutes. Once cool, the sample was filtered through Whatman No. 40 filter paper and made up to 25 ml in a volumetric flask with distilled water. The filtered sample was analysed using ICP-OES.

Table 3.2 Heating programme of Aqua Regia Digest method for plant samples.

Temperature (ºC)	Time (h)
60	3
100	1
120	50 mins
200	2 hrs 30 mins

3.2.4 Statistical analyses

All data presented are the mean values of four replicates. Values are expressed as means ± S.E. in each group. All statistical analyses were performed using the statistical analysis package SPSS 17.0. The statistical data variance of total Cd in soil as well as total Cd uptake by pineapple was analyzed by Analysis of Variance (ANOVA) statistical models, at the 95% confidence level. The data variance was compared with those of means using a post hoc Least Significant Difference test.

3.3 Results

3.3.1 The impact of Cd on soil pH

Figure 3.1 shows the effect of Cd concentrations on soil solution pH in both bulk soil and rhizosphere soil compared with control (unplanted). Generally, the pH decreased from the start of the experimental period and then increased slightly at the end of experimental period. The results demonstrated that the pH of Cd contaminated soil, especially at 150 mg Cd kg^{-1} soil was lower than the pH of soil without added Cd. Plants reduced the pH of soil compared to that in the unplanted pots and the pH from bulk soil was significantly higher than that of the rhizosphere soil in all four Cd treatments.

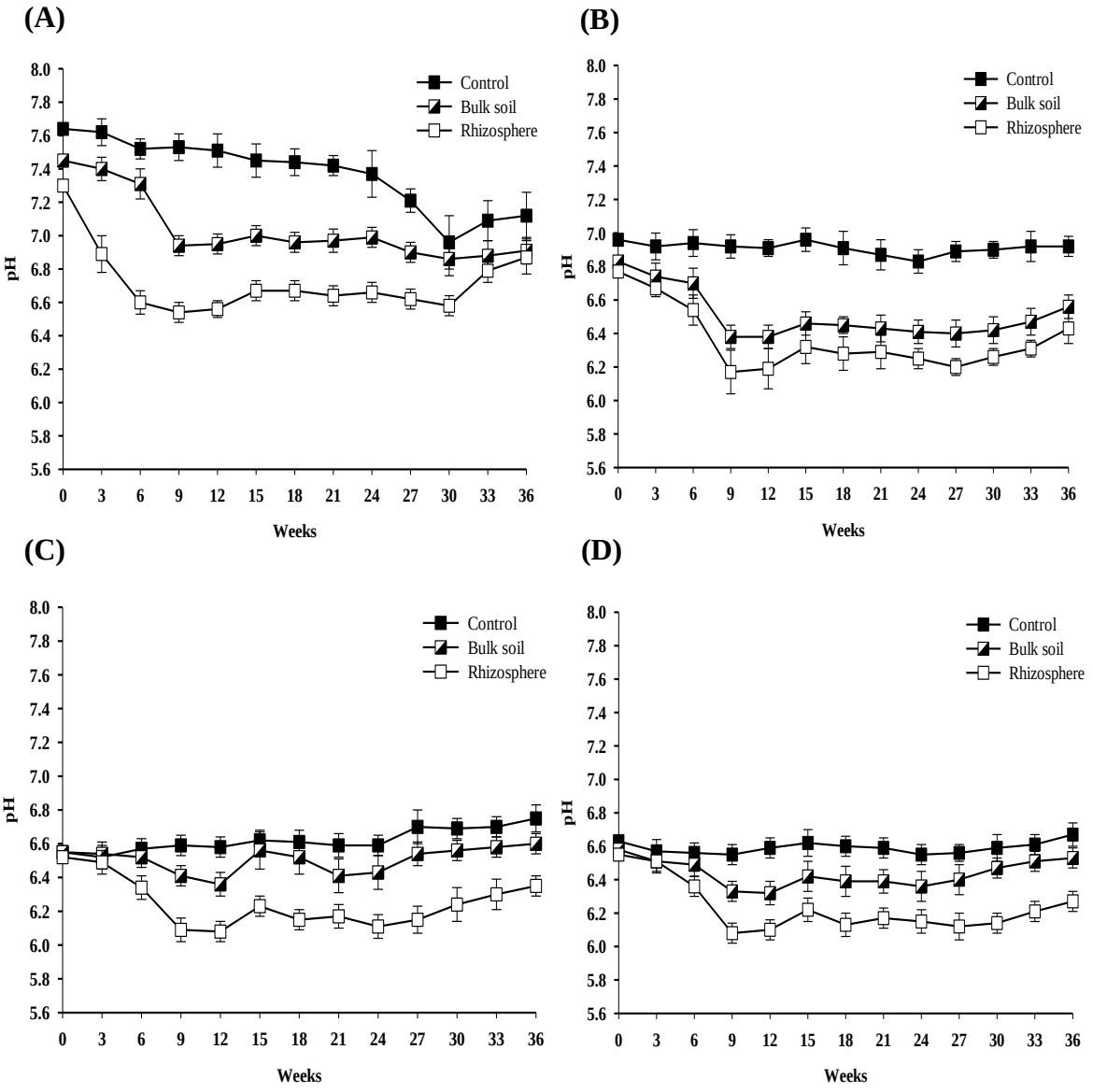

Figure 3.1 pH of bulk soil with and without (control) pineapple plants and rhizosphere soil at different soil Cd concentrations: (A) 0 mg/kg; (B) 50 mg/kg; (C) 100 mg/kg; (D) 150 mg/kg. Values are the means of four replicates ± standard error.

3.3.2 Extractable Cd

The extractable Cd (as measured by calcium nitrate extraction and ICP-OES) varied among the different treatments and in different soil fractions (Figure 3.2). Cd extractability in soil increased with increasing Cd amendment. Extractable Cd increased significantly ($P < 0.05$) after 3 weeks of plantation. Reduction in extractable Cd was observed at all levels of Cd concentrations from weeks 3-36. The results revealed that the extractability of Cd in control soil (without plants) was higher than that observed in either bulk or rhizosphere soil at the highest concentration of Cd (150 mg Cd kg^{-1}).

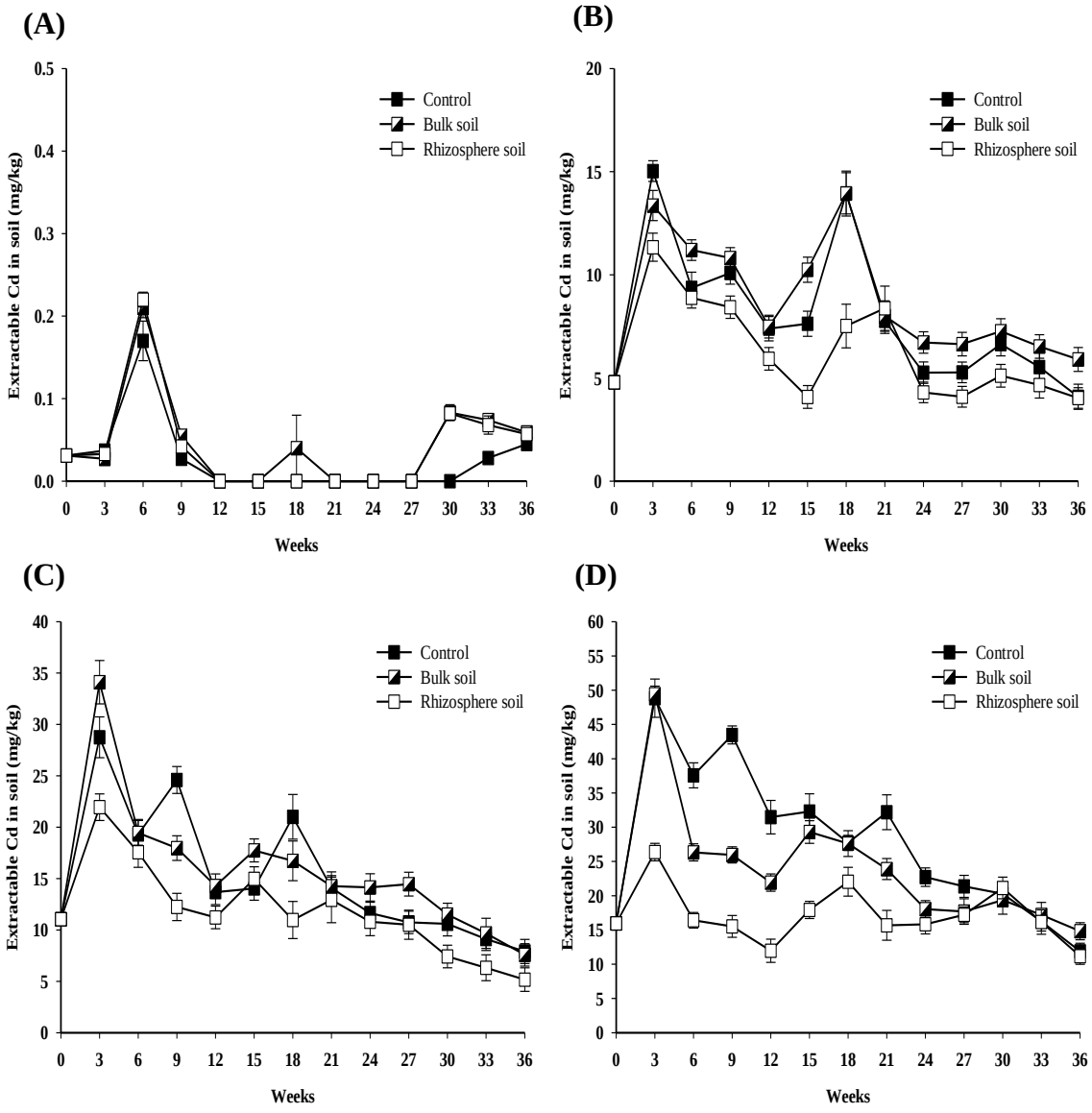

Figure 3.2 Extractable Cd in control (unplanted) and in bulk and rhizosphere soils (with pineapple plants) at different soil Cd concentrations: (A) 0 mg/kg; (B) 50 mg/kg; (C) 100 mg/kg; (D) 150 mg/kg. Values are the means of four replicates ± one standard error.

3.3.3 Partitioning of total Cd in soil

The total Cd concentrations in soil increased with increasing Cd amendments. The results demonstrated that total Cd levels in bulk soil were significantly higher than in the rhizosphere soil. At the end of the experiment, Cd levels in the rhizosphere soil of all treatments were almost half of those measured at the start of the experiment (i.e. in comparison to total Cd in pots that were unplanted).

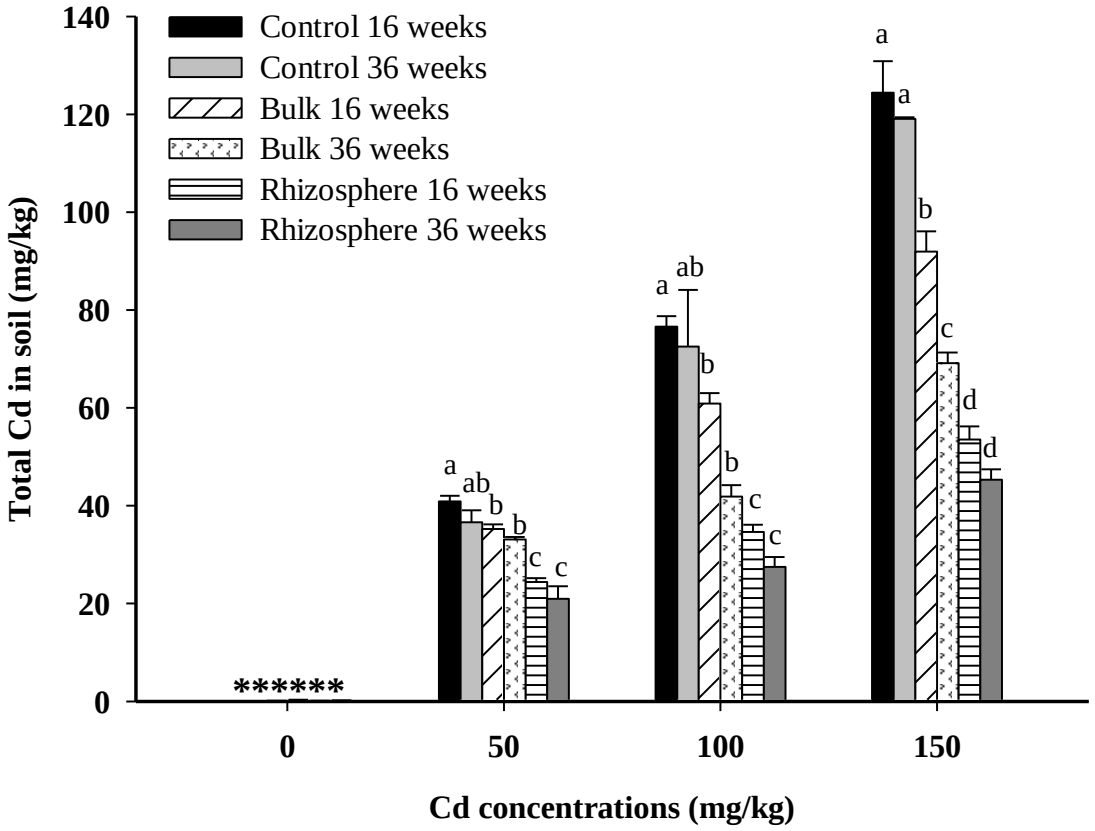

Figure 3.3 Cd concentrations in the soil solution with and without pineapple plants in the presence or absence of 50, 100 and 150 mg Cd kg^{-1}. Samples were taken from control soil (without plant), bulk and rhizosphere soil. Values not followed by the same letters in each concentration are significantly different at the 5% level according to Least Significant Difference test. * denoted not detected Cd.

3.3.4 Cd uptake by different parts of pineapple

In an effort to understand Cd accumulation in different parts of pineapple, pineapples were grown in soil containing 0, 50, 100 and 150 mg Cd kg^{-1} and harvested at four months and nine months. Plant tissues were analysed for Cd content. Cd uptake (µg Cd/g dry weight) as well as the allocation of Cd in different plant parts is shown as Figure 3.4. The results show that Cd concentrations in plant leaf and root tissues increased with an increase in Cd level applied, especially in root tissue (Figure 3.4). Cd concentrations in root and shoot were significantly different among the treatments. Compared with different parts of pineapple, roots showed higher Cd concentration (as measured by µg Cd/g dry weight) than in leaf tissues. Furthermore, the results of Cd accumulation in different parts of leaf tissue showed that Cd accumulation increased with leaf age as Cd content was highest in lower leaves (oldest leaves) followed by middle leaves and then upper leaves.

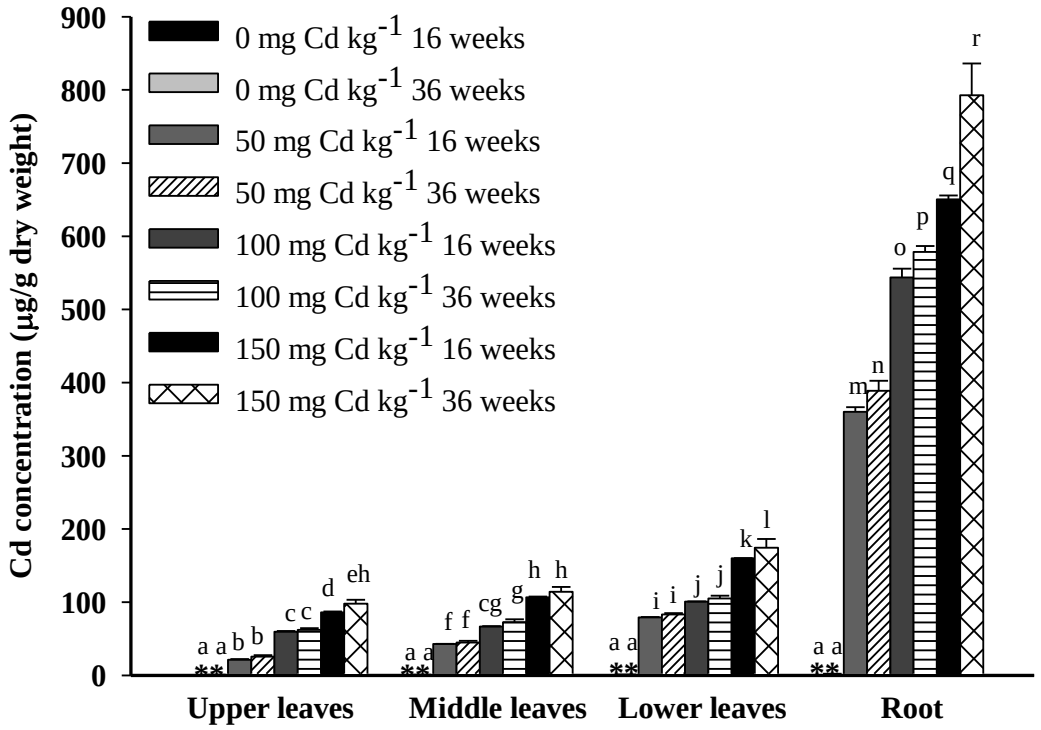

Figure 3.4 Cd accumulations in leaves and roots of pineapple after harvest at 4 months and 9 months. The samples were taken from younger leaves (upper leaves), intermediate leaves (middle leaves), and in older leaves (lower leaves). Pineapple plants treated with 0, 50, 100 and 100 mg Cd kg^{-1} soil as $Cd(NO_3)_2$. Values not followed by the same letters are significantly different at the 5% level between the four treatments of metal concentrations according to Least Significant Difference test. * denoted not detected Cd.

Total Cd uptake by pineapple plants: Although roots accumulated more Cd (measured per g dry weight), the total amounts of Cd taken up by roots and shoots (in terms of the whole plant) gave different results as the shoots constituted a much larger amount of plant biomass than the roots (see Table 3.3). After 4 months, shoot tissues had accumulated 92% of the total Cd taken up by the plant while, only 8% of the total Cd plant uptake was retained in root tissues. Similar results were found in pineapple exposed to Cd for 9 months (Table 3.3).

Table 3.3 Cd concentrations in the shoots and roots of pineapple grown in Cd-contaminated soil at different concentrations (0, 50, 100 and 150 mg Cd kg^{-1}) for 4 months and 9 months.

Treatment (mg Cd kg^{-1})	**First Harvest (4 months)**			
	Dry weight (g)	**Cd concentration (µg g^{-1} dry weight)**	**Total Cd uptake (µg $plant^{-1}$)**	**%Cd uptake**
Shoot				
0	10.90	0	0	0
50	10.30	143.21	1475.06	92.13
100	9.65	226.81	2188.72	93.06
150	8.98	352.36	3164.19	92.76
Root				
0	0.38	0	0	0
50	0.35	360.03	126.01	7.87
100	0.30	543.80	163.14	6.94
150	0.38	650.38	247.14	7.24
	Second Harvest (8 months)			
	Dry weight (g)	**Cd concentration (µg g^{-1} dry weight)**	**Total Cd uptake (µg $plant^{-1}$)**	**%Cd uptake**
Shoot				
0	19.53	0	0	0
50	17.90	153.69	2751.05	91.94
100	17.31	239.79	4150.76	92.64
150	16.75	386.72	6477.56	93.59
Root				
0	0.66	0	0	0
50	0.62	388.54	241.08	8.06
100	0.57	578.43	329.71	7.36
150	0.56	792.80	443.97	6.41

3.4 Discussion

The present investigation was undertaken to examine the influence of Cd on soil pH, extractable Cd, total Cd in soil and the magnitude of Cd accumulation in different tissues of pineapple plants grown in Cd-contaminated soil. Bulk soils and rhizosphere soils were compared to investigate the distribution of Cd within the soil and to assess the effect of plant roots on Cd uptake on soil Cd levels. Different ages of leaves and roots were analysed separately to the degree of Cd translocation from the below ground to above ground tissues of pineapple plants.

3.4.1 The impact of Cd on soil pH and extractable Cd

In the present study, soil pH decreased for up to 12 weeks of plant growth and then stabilized. At the end of the experimental period the pH was slightly increased (Figure 3.1). The acidification of soil pH might be caused by uptake of positive charge ion such as potassium or ammonium, which is necessary for plant nutrition (Marschner, 1995; Miller and Cramer, 2004; Bravin *et al.*, 2009). In addition, the pH from bulk soil was higher than rhizosphere soil. Many studies found that plant growth can alter the rhizosphere pH and subsequently the bulk soil pH (Page *et al.*, 1981; Xian, 1989; Haynes, 1990; Bernal and McGrath, 1994; Hattori, 1996). Osorio Vega, (2007) suggested that the rhizosphere pH is usually lower than the bulk soil. Changes in plant rhizosphere pH may result from; (a) an imbalance of ion uptake by plants (Riley and Barber, 1971; Nye, 1981; Marschner *et al.*, 1986; Bernal and McGrath, 1994; Hattori, 1996), (b) exudates such as organic acids released by plant roots which lower the pH (Chiang *et al.*, 2006), (c) production of CO_2 by respiration processes (Marschner, 1997) and (d) organic matter decomposition (Marschner, 1997). Many previous reports showed that in metal contaminated soil, pH in the rhizosphere of plants such as swiss chard, kenaf (*Hibiscus cannabinus*), and *Thlaspi caerulescens* was lower than in bulk soil (Page *et al.*, 1981; Hattori, 1996; Delorme *et al.*, 2001).

Soil pH is considered to be one of the most important factors controlling the extractability of heavy metals in soil (Bruemmer *et al.*, 1986; Sauvé *et al.*, 2000; Tsadilas, 2000). Generally, Cd extractability increases with reduced soil pH (Dániel *et al.*, 1997; Lehoczky *et al.*, 1998). Many studies found that extractable Cd was greatly increased with decreasing pH in plants such as rape (*Brassica napus*), *Thlaspi caerulescens*, lettuce and barley (Andersson and Nilsson, 1974; Lehoczky *et al.*, 1998; Wu and Zhang, 2002b;

Wang *et al.*, 2006a). Levels of extractable Cd in the rhizosphere were lower than observed in the bulk soil. It could be assumed that the lower pH of the rhizosphere soil would result in an increased in extractable Cd but this was not the case. There are potentially several reasons for the results observed e.g. Cd uptake by the plant reduced the extractable levels of Cd seen in the rhizosphere soil or that metal ions were bound to organic materials present in the rhizosphere (Abumaizar and Khan, 1996). In the present work assessment of extractable Cd in each of the 4 Cd treatments indicated that about 15% of the total Cd content in soil was extracted in 0.1 M $Ca(NO_3)_2$. The results demonstrated that there were continuous changes in extractable Cd within the rhizosphere of pineapple. Initially, the amount of extractable Cd in contaminated soils appeared to increase during the first 3 weeks of the experiment and decreased thereafter. It seems that the watering cycle had some effect on altering extractable Cd. Si *et al.*, (2006) showed that wet-dry cycles had the effect of altering Cd, Pb and Zn extractability. Extractable Cd was increased at the first cycle and then decreased at the second and third cycles of wetting/drying. In addition, the extractable Cd in the soil rhizosphere decreased over time and extractable Cd in rhizosphere soil was lower than bulk soil. The decrease of extractable Cd in rhizosphere soils with the long exposure to Cd may be due to; (a) the uptake of Cd by the plant (Lorenz *et al.*, 1994; Hamon *et al.*, 1995; Wang *et al.*, 2006b), (b) exudates and metabolites released by the roots and microbial metabolites in the rhizosphere soils (Marschner and Römheld, 1983; Leyval and Berthelin, 1993) may make the heavy metals change from a tight-bound to a loose-bond (Wang *et al.*, 2009b), (c) metals become more tightly bound with organic materials (McLaughlin, 2001). Previous studies found that the extractable of metal in soil during growth of radish in contaminated soil increased initially then dropped sharply afterwards (Lorenz *et al.*, 1994; Hamon *et al.*, 1995). Furthermore, many studies reported that extractable Cd in the rhizosphere of plant such as *Thlaspi caerulescens*, *Thlaspi ochroleucum* and *Sedum plumbizincicola* was lower than in the bulk soil (Knight *et al.*, 1997; McGrath *et al.*, 1997; Wang *et al.*, 2006b; Liu *et al.*, 2011).

3.4.2 Partitioning of total Cd between bulk soil and rhizosphere

Although various soil parameters as described above can affect the extractable Cd, the total amount of the element present in the soil and how this element is partitioned between bulk soil and rhizosphere soil are key factors affecting the Cd contents of plant (Alloway, 1990). In the present study, Cd concentration in the rhizosphere was most depleted compared with the bulk soil and non-planted soil especially at highest Cd concentrations (150 mg Cd kg^{-1}). Wang *et al.*, (2009b) suggested that rhizosphere soil is located in a zone around absorbing roots where metal ions are absorbed, thereby heavy metal contents are lower than those in the bulk soil. Many studies found that the Cd concentration in the rhizosphere soil of plants such as lettuce, cabbage, carrot, radish, barley, potato and wheat was decreased as Cd accumulation in plants increased (Chumbley and Unwin, 1982; Alloway, 1990; Kabatta and Pendias, 2001; Greger and Landberg, 2008).

3.4.3 Cd accumulation in pineapple plant

In the present study, the concentration of Cd in plant tissues increased in line with increasing Cd concentration in soil. Similar to earlier reports of Cd accumulation by plants (Jiang *et al.*, 2004; Shao *et al.*, 2007), the Cd concentration in Indian mustard (*Brassica juncea*) and rice (*Oryza sativa*) increased with increasing Cd concentration in soil. Cd was present in both shoots and roots of the pineapple showing that Cd was mobile within the plant. Most of the Cd taken up by the plant was transferred to shoots (approximately 92% of total plant Cd was in shoots) but the roots contained more Cd per g dry weight than shoots. Cd accumulation in shoots can be explained because: (a) Cd is a mobile element, easily absorbed by roots and transported to shoots (Sękara *et al.*, 2005), (b) root-to-shoot translocation of Cd probably occurs via the xylem and is driven by transpiration from the leaves (Salt *et al.*, 1995b; Hart *et al.*, 1998), so efficiency of xylem loading, therefore, may play an important role in high Cd accumulation in shoots. Many studies demonstrated that Cd can accumulate in shoots more than roots in plants such as *Sedum alfredii*, *Sesbania drummondii*, *Salix matsudana* (Yang *et al.*, 2004; Israr *et al.*, 2006; Ling *et al.*, 2011).

The older leaves of pineapple plants were observed to accumulate more Cd when expressed on a µg Cd per g dry weight compared to the intermediate and younger leaves. Cd accumulation in shoots is driven mainly by mass flow due to transpiration (Bazzaz *et al.*, 1974). Treatments that reduce transpiration, such as application of Cd to induce

stomatal closure (Poschenrieder *et al.*, 1989) may reduce translocation of Cd to the upper leaves. Many studies found that Cd accumulation in plants also appeared to be correlated with leaf age. The lowest accumulation of Cd was observed in the young leaves of lettuce, rye, tobacco and maize (*Zea mays* L.), while middle leaves and older leaves the level of accumulated Cd remained much higher (Wagner and Yeargan, 1986; Florijn and Van Beusichem, 1993a; Krupa and Moniak, 1998; Drązkiewicz *et al.*, 2003). The study of Drązkiewicz *et al.*, (2003) showed that oldest leaves of maize grown in the presence of Cd 70 mg Cd kg^{-1} accumulated more Cd than middle leaves and young leaves. The Cd content in older leaves, middle leave and young leaves of maize was 622, 457 and 399 µg g^{-1} dry weight, respectively. The age-dependent response of the pineapple leaves to Cd in three leaf age categories (older, mature and young leaves) can be explained as the long-term exposure of the older leaves to Cd. The amount of Cd^{2+} translocated from the roots to the leaves depends on the importance of binding and complexing at root level, and on transport efficiency through the xylem (Marchiol *et al.*, 1996b). Our results showed that Cd can accumulate in pineapple leaves and increases as the Cd concentration increase when compared with control. It is apparent that Cd is translocated to the leaves, therefore explaining the toxic effects of Cd, such as growth inhibition and inhibition of photosynthetic performance as observed in Chapter II.

3.5 Conclusions

Pineapple plants take up Cd from the soil, resulting in lower Cd concentrations in the rhizosphere soil compared with the bulk soil. Cd accumulation by pineapple increased as the Cd concentration in soil solution increased. The uptake of Cd by pineapple may be due to the release of Cd from soil particles under acidic conditions in rhizosphere soil. The present study revealed that Cd uptake levels varied in the roots and shoots of pineapple grown in Cd-contaminated soil: shoot tissues contained 92% of the total Cd taken up by the plant, and only 8% was retained in root tissues. However, the roots contained higher amounts of Cd per g dry weight than shoots. In addition, leaf age influenced Cd accumulation with older leaves showing higher Cd contents than younger leaves.

Chapter Four

Effect of Cd on pineapple rhizosphere soil microbial populations

4 Effect of Cd on pineapple rhizosphere soil microbes

4.1 Introduction

Heavy metal contamination of soils originating from agricultural fertilizers, sewage sludge or industrial activities such as metal mining and smelting is a major environmental problem in many parts of the world. The resulting damage is difficult to cure as metals cannot be chemically degraded (Salt *et al.,* 1995a). Heavy metal pollution could have long-term adverse affect on soil organisms and soil ecosystem processes, including microbial activities (Babich and Stotzky, 1985; Baath, 1989; Giller *et al.,* 1998). Cd also decreases the morphological diversity of soil micro-organisms and strongly inhibits cell growth and fermentation activities (Babich *et al.,* 1983; Duxbury and Bicknell, 1983). Microbial community structure has been measured to evaluate soil quality (Parr and Papendick, 1997) and used as a contamination indicator (Renella *et al.,* 2004). According to Brookes, (1995) microbiological parameters such as the number, weight and activity of micro-organisms can be good indicators of soil contamination with heavy metals, including Cd. As a rule, Cd has a negative effect on the growth of soil micro-organisms as it can greatly depress their numbers (Hattori, 1992; Hiroki, 1992; Nada *et al.,* 1997). Therefore, any alteration in the size and structure of the soil microbial community in polluted soils has been proposed to be a sensitive indicator of heavy metal effects on soil ecology (Moreno *et al.,* 2002; He *et al.,* 2005). Furthermore, changes in soil microbial structure can be studied to find which microbial groups and which concentrations of pollutant affect the structure of the most important microbial communities, since any related changes might be associated with soil function change and may be used as a tool in the search for new micro-organisms capable of concentrating or absorbing metals to facilitate their elimination or immobilization (Ros *et al.,* 2009).

Microbial community abundance and structure can be studied using cultivation-dependent or cultivation-independent techniques. Cultivation-independent techniques (e.g. DNA based approaches) can normally detect a much greater diversity and complexity of organisms than standard culture approaches (Torsvik *et al.,* 2002; Zhou *et al.,* 2004; Janssen, 2006). The development of culture-independent molecular techniques capable of characterizing the dynamics of microbial community structures, such as Denaturing Gradient Gel Electrophoresis (DGGE), has made it possible to monitor the responses of

soil microorganisms to heavy metals at the community level (Renella *et al.*, 2004; Frey *et al.*, 2006).

Since plants are the main suppliers of organic materials to the ecosystem, they indirectly affect soil microbial communities, as well as diverse ecosystem processes especially at the rhizosphere (Kowalchuk *et al.*, 2002). The rhizosphere is known to be an area of increased microbial numbers and activity (Kennedy, 1998; Barea *et al.*, 2005) but to our knowledge the pineapple rhizosphere has not been studied previously. Accordingly, more in depth work on the effect of Cd contamination on microbial community abundance in the pineapple rhizosphere is needed. For further information on microbiology of the rhizosphere the reader is directed towards recent reviews by Kent and Triplett, (2002) and Nihorimbere *et al.*, (2011).

Overall, the aim of the chapter was to examine the effect of Cd on pineapple rhizosphere soil microbial populations using a combination of traditional techniques (plate counting) and more advanced molecular techniques (DGGE).

Specific objectives were to:

a) examine changes in microbial community structure between bulk and rhizosphere soil of pineapple grown in presence or absence Cd using plate counting technique.

b) examine differences in microbial communities structure between bulk and rhizosphere soil of pineapple grown in presence or absence Cd using DGGE techniques.

4.2 Materials and Methods

4.2.1 Plant material and growth conditions

Soil was made up in pots containing a mixture of sand (East Riding Horticulture Ltd., UK) and John Innes No. 2 compost (John Innes Manufacturers Association; UK) in the ratio of 1:1. The soil was thoroughly mixed with $Cd(NO_3)_2$ (Sigma-Aldrich, USA) solution to obtain 0, 50, 100, and 150 mg Cd kg^{-1} soil and was incubated (aged) for four weeks without plants before being remixed and used as a growing medium for pineapple plants. Suckers of pineapple (*Ananas comosus* L.) approximately 1.0 cm diameter were potted up in 10 cm plastic pots containing a mixture of sand and John Innes No. 2 compost in the ratio of 1:1 without Cd application until the roots became exposed at the base of the pot. Plants were removed and potted up in 14 cm plastic pots with the following treatments: 0, 50, 100, and 150 mg Cd kg^{-1} soil as $Cd(NO_3)_2$. The plants were grown in a controlled growth chamber at 27 °C (day) and 19 °C (night) under a 12 hour photoperiod with photon flux density of 250 µmol m^{-2} s^{-1} at leaf height for six months. After six months, plants were then moved to an intermediate light intensity of 550 µmol m^{-2} s^{-1} at leaf height for one month and subsequently were moved to the highest light intensity of 800 µmol m^{-2} s^{-1} at leaf height for two months before harvesting. The water content of the soil was maintained at 60% of its holding capacity by watering with tap water twice a week. Plants were destructively harvested at four months and a further batch was harvested nine months after transplantation for data collection.

4.2.2 Soil sampling strategies

Every three weeks, bulk soil (away from plant roots) and rhizosphere soil samples (0-10 cm depth) were taken for total microbial counts (bacteria and fungi). Samples were taken using a cork borer 1.5 cm in diameter and the rhizosphere soil samples were taken as close to the plant stem as possible. After 36 weeks growth, all plants and soil were removed from pots and each plant was shaken carefully to remove the bulk soil (collected for analysis). The soil still adhering to the roots, defined as rhizosphere soil, was separated from the roots. Samples from unplanted pots were used as controls. Subsamples (5.0 g wet weight) were placed in polyethylene tubes and kept at -80 °C until further use for bacterial community structure using DGGE techniques. Bulk and rhizosphere soil of each Cd concentration (0, 50, 100 and 150 mg Cd kg^{-1}) were used for total viable counts.

For DGGE analysis, only bulk and rhizosphere soil at 0 mg and 150 mg Cd kg^{-1} were used to analyse the differences in microbial community structures.

4.2.3 Plate counting

The total number of culturable bacteria and fungi were enumerated using the plate counting technique as described by Khan *et al.*, (2010). Soil (1 g fresh weight) from each treatment was weighed into sterile Falcon tubes containing 9 ml of sterile Ringer's solution (Sigma-Aldrich, USA) and shaken for 15 minutes at 250 rpm. From each soil extract, 10-fold dilutions were prepared in test tubes containing 9 ml of sterile Ringer´s solution. The total number of culturable bacteria was estimated using plate counts on Nutrient agar (Sigma-Aldrich, USA) amended with 0.1 g/l cycloheximide. Colony-forming units of fungi were determined using plate counts made on Rose Bengal chloramphenical agar (Merck, Germany). The plates were inoculated with 100 µl soil dilutions and incubated at 26 °C for a week.

4.2.4 DNA extraction

DNA from frozen soil samples was extracted from four replicates samples using the PowerSoil® DNA Isolation Kit as described by the manufacturer (MoBio Laboratories, USA). Soil samples 0.25 g (wet weight) were added to PowerBead tubes then the tube was gently mixed using vortex mixer (Vortex-Genie 2, VWR, UK). The PowerBead tube contains a buffer that will (a) help disperse the soil particle, (b) begin to dissolve humic acids and (c) protect nucleic acids from degradation. C1 solution contains sodium dodecyl sulphate (SDS) and other disruption agents required for complete cell lysis (60 µl) was added to the extract and the tubes were briefly vortexed. The soil debris was removed by centrifugation at 10,000 x g for 30 seconds at room temperature and the supernatant transferred to a clean 2 ml microfuge tube. The solution C2 (250 µl), contains a reagent to precipitate non-DNA organic and inorganic material was added to the supernatant, mixed by vortexing for 5 seconds and incubated at 4 °C for 5 minutes. The solution was then centrifuged at room temperature for 1 minute at 10,000 x g. Approximately 600 µl of supernatant was transferred to a clean 2 ml microfuge tube, 200 µl of solution C3, contain a second reagent to precipitate additional non-DNA organic and inorganic material, added and the solution vortexed briefly before being incubated at 4 °C for 5 minutes. The aqueous phase containing nucleic acids was separated by centrifugation at room

temperature for 1 minute at 10,000 x g. The supernatant (750 µl) was transferred to a clean 2 ml microfuge tube and 1.2 ml of solution C4 that contains high concentration salt solution was added to the supernatant prior to vortexing for 5 seconds. 675 µl of this solution was loaded to a spin filter which collected the nucleic acid and centrifuged at 10,000 x g for 1 minute at room temperature. This step was repeated until no solution remained. Exactly 500 µl of solution C5 (ethanol) was added to the spin filter and the filter centrifuged (10,000 x g) at room temperature for 30 seconds. The filtrate was discarded and centrifugation repeated (at room temperature) for 1 minute at 10,000 x g to remove any remaining liquid. The spin filter was placed in a new 2 ml microfuge tube and solution C6 (50 µl of 10 mM Tris-EDTA (TE) buffer) was added to the centre of the white filter membrane. The tube was centrifuged at room temperature for 30 seconds at 10,000 x g. The spin filter was discarded and the filtrate containing nucleic acid was stored at -20 °C until used. The yield and quality of the extracted DNA was checked visually verified by 1.5% (w/v) agarose gel electrophoresis as described in section 4.2.5.

4.2.5 DNA agarose gel electrophoresis

4.2.5.1 Agarose gel preparation

Agarose (0.75 g) was dissolved in 50 ml Tris-Borate EDTA buffer (TBE buffer; 89 mM Tris-HCl, pH 8.2, 89 mM Boric acid, 2 mM EDTA) by heating in a microwave until dissolved. The solution was allowed to cool and ethidium bromide (Sigma-Aldrich, USA) was added (0.05 µg/ml). Gel (1.5%) were then poured in a 110 x 60 mm gel tank, any bubbles were removed with a pipette tip (gel comb was added) and the gel allowed to set for 30 minutes.

4.2.5.2 DNA detection and visualisation

For electrophoresis, DNA samples were mixed with 6x DNA loading dye (10 mM Tris-HCl pH 7.6, 60 mM EDTA, 60% (v/v) glycerol, 0.03% (w/v) bromophenol blue, 0.03% (w/v) xylene cyanol FF) in the ratio of 4:2 prior to loading. TBE buffer (89 mM Tris-HCl, pH 8.2, 89 mM Boric acid, 2 mM EDTA) was used as the running buffer for electrophoresis. Gels were run at 100 V for 40 minutes using Nucleic acid electrophoresis system (i-Myrun.NC, Cosmo Bio Co., Ltd., Japan). Gels were visualised under UV light using a gel documentation system (Flour-S MultiImager, Bio-Rad, USA).

4.2.6 PCR-DGGE bacterial community analysis

4.2.6.1 Soil samples

Samples of bulk and rhizosphere soil were collected from pineapple grown in Cd concentration 0 mg and 150 mg Cd kg^{-1} at harvesting period (36 weeks). Plants and soils were removed from the pots and separated as described in Section 4.2.2. Control pots (not planted with and without Cd) were used as control for each soil types. DNA was extracted from the soil as described in Section 4.2.4.

4.2.6.2 PCR amplification of bacterial 16S rRNA

Bacterial 16S rRNA was amplified using standard prokaryotic primers 357F (5´-CCTACGGGAGGCAGCAG-3´) and 518R (5´ATTACCGCGGCTGCTGG-3´) (Muyzer *et al.*, 1993). The GC-rich sequence (5´-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCAGCACGGGGGG-3´) was incorporated in the forward primer 357F at its 5´ end to prevent complete dissociation of the DNA fragments during DGGE. PCR reaction was performed on an iCycler Thermal Cycler (Bio-Rad, USA) in 0.2 ml tubes using 30 µl reaction volumes. The reaction mixture contained 3 µl of 10x PCR buffer containing Mg^{2+} (MP Biomedicals, UK), 3 µl of 2.0 mM dNTP (Bioline, UK), 0.75 µl of 10 mM of each primer, 0.75U Taq polymerase (Bioline, UK) and 0.5 µl of DNA template. Cycling conditions used to amplify the 16S rDNA gene fragment were 94 °C for 5 minutes, followed by 30 cycles of 94 °C for 30 second, 53 °C for 30 second, and 72 °C for 1 minute and a final extension at 72 °C for 10 minutes. 2 µl of the PCR products were checked by electrophoresis in 1.5% agarose gels stained with ethidium bromide (Section 4.2.5) prior to DGGE.

4.2.6.3 Preparation of gels

Two glass plates (16x20 cm and 20x20 cm), spacer, comb and rubber base were cleaned with Decon detergent (Decon Laboratories, UK) rinsed with distilled water and left to air-dry. The spacers and rubber base were greased with petroleum gel to prevent leakage. The glass plates were then sprayed with 70% (v/v) ethanol and wiped with blue roll, air dried then assembled. The spacer was arranged along the sides between the bigger and smaller plates and clamps attached to both sides to hold the plates firmly together so as to form a gel sandwich which was then placed on the rubber base and positioned onto the casting

stand. Denaturing solutions of 30% and 60% gel solutions were prepared as follows (Table 4.1):

Table 4.1 Composition of denaturing solution for DDGE techniques.

Composition	**30%**	**60%**
Urea	5.04 g	10.08 g
40% acrylamide	10.0 ml	10.0 ml
Formamide (deionised)	4.8 ml	9.6 ml
50x TAE buffer	0.8 ml	0.8 ml
10% (w/v) ammonium persulphate (APS), made up fresh before use	200 µl	200 µl
N,N,N´,N´-tetra-methyl-ethylenediamine (TEMED)	20 µl	20 µl

The 30% and 60% solution gels were prepared in separate glass beakers. Gel solution components (urea, 40% acrylamide, formamide and 50x TAE buffer) were mixed made up to 30 ml with deionised water and then filtered through a 0.45 µM nylon filter into a measuring cylinder before addition of the final required amount of water (final volume 40 ml) to dissolve any residual urea. The solutions were then transferred to small clean glass beakers prior to gel formation. Ammonium persulfate (200 µl) and TEMED (20 µl) were added to each solution prior to loading. The gel solution (30% and 60%) were immediately transferred into the glass plates within the gel apparatus to avoid disruption of the gradient and premature polymerisation by using a peristaltic pump (Mini-Pump variable flow MPP100, CBS Scientific, UK) at 4 rpm. This produced a gel with a range of urea increasing from 30% - 60% concentration. A teflon comb was inserted to make a wells and the gel was allowed to set for 2 hours before the comb was removed gently from the gels prior to run electrophoresis.

4.2.6.4 Sample loading and visualisation

The gel sandwich prepared as described in section 4.2.6.3 was then secured onto a frame and placed into a gel tank which contained 1xTAE buffer (40 mM Tris acetate, 1 mM EDTA pH 8.0) heated to 65 ºC. The wells were rinsed with buffer to clear away unpolymerised gel prior to loading the PCR samples. For DGGE analysis, PCR products from each sample were separated on 10% acrylamide gel that contained a denaturant gradient range from 30% to 60%. Exactly 20 µl of the PCR products were loaded into each well, then electrophoresis was performed in 1xTAE buffer at 60 °C, 50 V for 13 hour using the Bio-Rad DCode^{TM} Universal Mutation Detection System (Bio-Rad Laboratories, USA). After running, 9 µl SYBR® Green (Sigma-Aldrich, USA) in 50 µl 1xTAE buffer was poured onto the gel surface (to stain DNA) and the gels left in the dark for 20 minutes before being washed in 500 ml 1xTAE buffer for 30 minutes. After this, the gels were rinsed with distilled water. An image of the gels was taken by a gel documentation system (Flour-S MultiImager, Bio-Rad, USA) and electronic images stored for further analysis.

The gel banding patterns were examined and selections of DGGE bands were excised under UV trans-illumination (UVP, San Gabriel, USA) using a sterile pipette tip and kept in molecular grade water (Sigma-Aldrich, USA). Bands were selected for excision based on their presence or absence in different samples and if their intensity increased (indicating a potential enrichment of the species) in particular samples. Exactly 0.5 µl of DNA solution from excised bands was re-amplified with primer 357F and 518R with the following thermal program: 94 °C for 5 minute, followed by 30 cycles of 94 °C for 30 second, 53 °C for 30 second, and 72 °C for 1 minute and a final extension at 72 °C for 10 minutes. The PCR product was purified as described by Dugan *et al.,* (2002). ExoSAP-IT reagent (USB Corporation, USA) was used according to the manufacturer's recommended protocol (2 µl reagent per 5 µl amplified PCR product). The ExoSAP-IT reagent was added to an aliquot of the PCR product and incubated in a DNA Engine Thermal Cycler (PTC-200, BIO-RAD, USA) at 37 °C for 15 minutes followed by an additional incubation at 80 °C for 15 minutes. DNA sequencing was carried out at Genevision, Newcastle, United Kingdom. Two microliters of purified PCR product were added as template to 10 µl sequencing reactions containing the BigDye V3.1 sequencing chemistry (Life Technologies Applied, UK) and 0.4 µM of primer. DNA fragments were sequenced with an AB3730xl automated DNA sequencer (Applied Biosystems Inc.,

USA). Software (Applied Biosystems Inc., USA) was used to create sequence contigs from the fragments obtained. Similarity searches of the sequence obtained from the DGGE bands were performed using BLAST (www.ncbi.nlm.nih.gov/BLAST/).

4.2.6.5 Analysis of DGGE patterns

DGGE images were analyzed with BioNumerics 3.5 (Applied Maths Belgium). This software identifies the bands occupying the same position in the different lanes of the gel. The numbers of bands were used to indicate the level bacteria diversity of the bulk soil and rhizosphere communities of pineapple grown in with or without Cd compared with control (not planted).

4.2.7 Statistical analysis

Analysis of variance (ANOVA) and the post hoc Least Significant Difference test ($P < 0.05$) were used to compare treatment means. All the statistical analyses were carried out using SPSS 17.0. The diversity of the microbial communities was analyzed by Shannon index (H) according to the following equation: $H = -\sum P_i ln P_i$ (Hill *et al.*, 2003). Where, P_i is the importance of the bands in a lane. The Shannon index (H) was calculated on the basis of peak heights of the bands. The importance probability P_i was calculated $P_i = n_i/N$, where n_i is the height of peak and N is the sum of all peak heights in the lanes. The Simpson index of dominance (Simpson, 1949) was calculated using the following function $S = \sum P_i^2$ showed as ($1/S$).

4.3 Results

4.3.1 Microbial enumeration studies (total viable counts)

4.3.1.1 Bacterial viable counts

Throughout the pot experiment, the total bacterial viable count in soils at different Cd treatment levels (0, 50, 100 and 150 mg Cd kg^{-1} soil) was enumerated in both bulk and rhizosphere soil (See Figure 4.1 A-D) at 3 week intervals. The viable counts in rhizosphere soil were significantly higher than in bulk soil for pots containing plants only and the Cd level 100 mg Cd kg^{-1} soil. For example, after 36 weeks incubation, the total count for bacteria in the bulk soil for control pots (plants but no Cd) was 5.02 x 10^5 cfu/g dry soil while in the same control pots the viable count from rhizosphere soil was 6.75 x 10^5 cfu/g dry soil. As the Cd concentration increased to 150 mg Cd kg^{-1} soil then the bulk and rhizosphere viable counts became more similar and were not significantly different at most times during the experiment.

Typically, Cd at a level of 150 mg Cd kg^{-1} soil reduced viable bacterial counts by around 80-90% compared to the controls (plants with no Cd) e.g. after 36 weeks incubation rhizosphere soil from control pots contained 6.75 x 10^5 cfu/g dry soil while rhizosphere soil from test pots (150 mg Cd kg^{-1} soil) contained 8.63 x 10^4 cfu/g dry soil.

4.3.1.2 Fungal populations

Figure 4.2 shows the fungal total viable counts obtained in soil containing varying Cd levels (0, 50, 100, 150 mg Cd kg^{-1} soil). The number of fungi was significantly decreased ($P < 0.05$) in the Cd-amended samples compared to the control (without Cd) at the end of the experiment. This decrease in the number of fungi was markedly higher in soil samples amended with Cd at 150 mg Cd kg^{-1} soil than at the lower Cd levels. For example, in control rhizosphere samples after 36 weeks incubation the fungal count was 5.70 x 10^2 cfu/g dry soil whereas in Cd amended soil (150 mg Cd kg^{-1}) the rhizosphere contained 2.56 x 10^2 cfu/g dry soil. Overall, fungal viable counts were significantly greater ($P < 0.05$) in rhizosphere soil than in bulk soil but this effect was more observable as the duration of the experiment increased. In Cd contaminated soils, the fungal rhizosphere counts behaved slightly differently to the bacterial counts as fungal counts in the rhizosphere remained significantly higher than in the bulk soil samples at the end of the experiment.

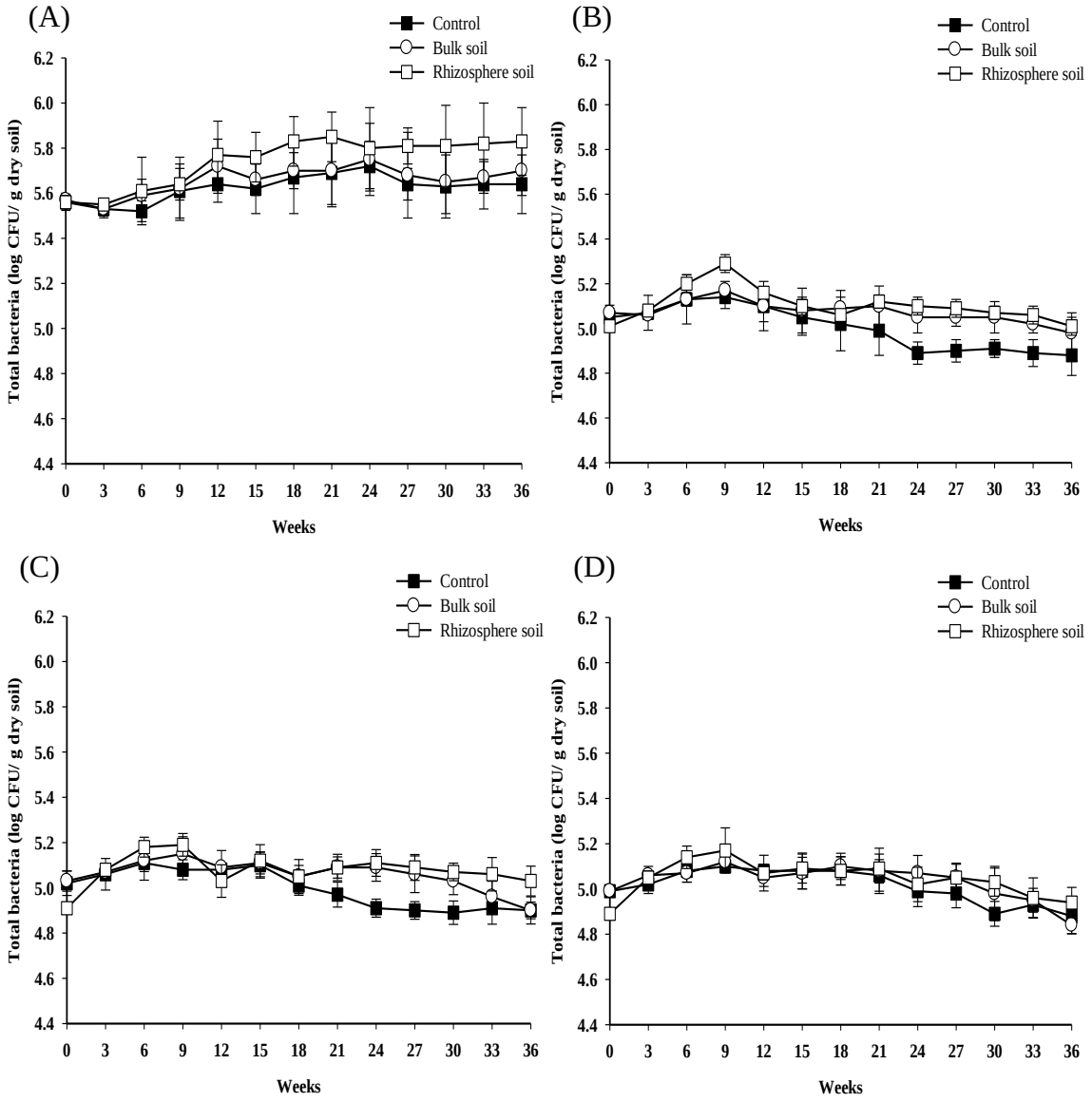

Figure 4.1 Total viable counts of bacteria in bulk, rhizosphere and control soils (without plants): (A) 0 mg/kg; (B) 50 mg/kg; (C) 100 mg/kg; (D) 150 mg/kg. Values are the means of three replicates ± one standard error.

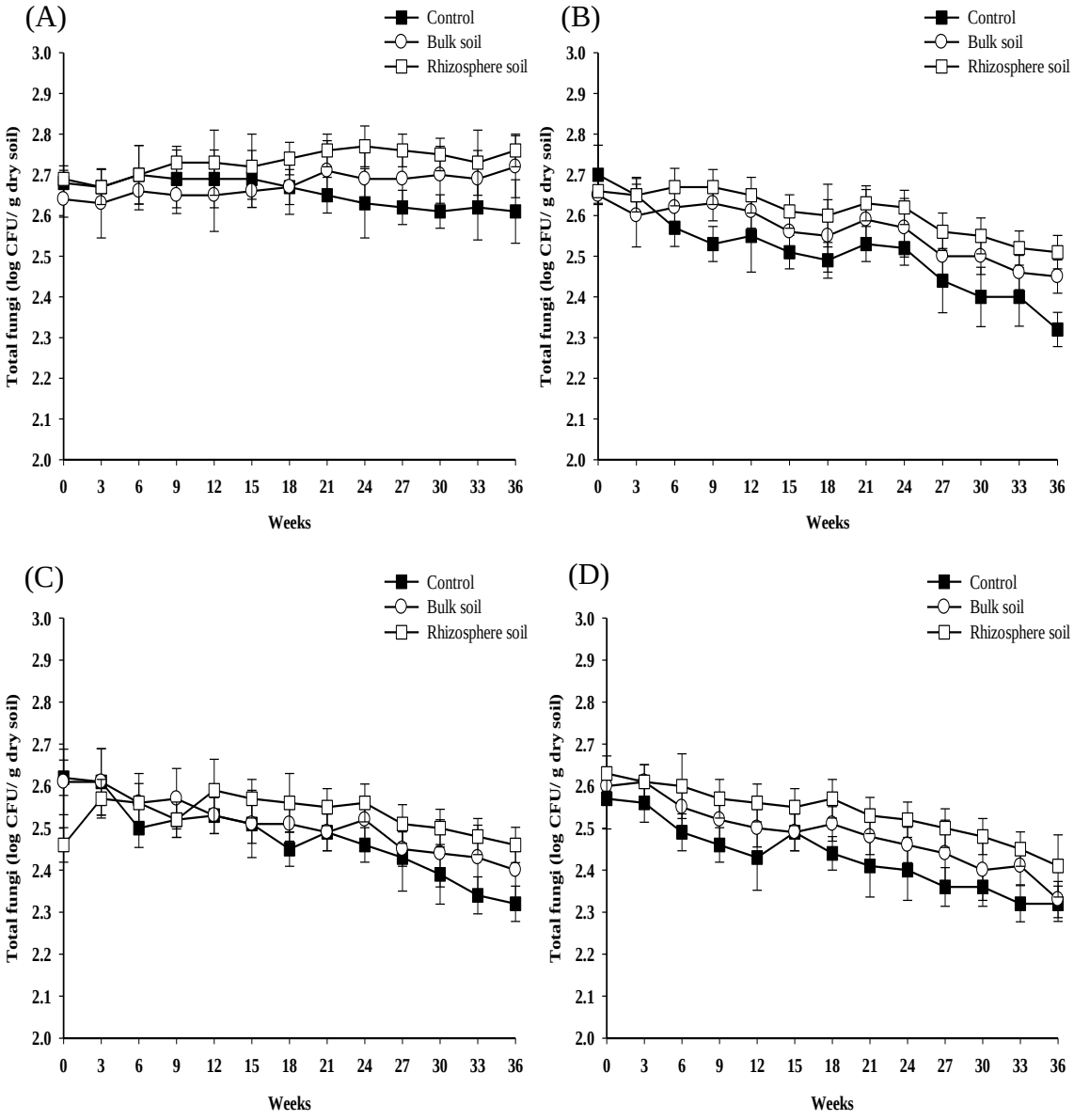

Figure 4.2 Total viable counts of fungi in bulk, rhizosphere and control soils (without plants): (A) 0 mg/kg; (B) 50 mg/kg; (C) 100 mg/kg; (D) 150 mg/kg. Values are the means of three replicates ± one standard error.

4.3.1.3 Morphology of bacterial and fungal colonies isolated during viable count studies (visual observations)

In the present investigation, typical bacterial colony types isolated from bulk soil without Cd were round, entire and creamy or white in colour while in the rhizosphere soil the bacterial colonies were round, white (filamentous edge) or flat, entire with a brown colour. Fungal colonies isolated from bulk soil without Cd were different to those from the rhizosphere soil (with larger brown colonies being present in rhizosphere samples). See Figure 4.3 for examples of colonies.

In Cd-contaminated soil, there appeared to be less morphological diversity of both bacterial and fungal colony types found compared to control soils (Figure 4.4) typical bacterial colony types isolated from bulk soil with Cd were small and white, (round entire raised and small round entire convex) while the white and brown colonies were found in rhizosphere soil. Fungal colony morphology was also different in bulk and rhizosphere samples in Cd contaminated soil.

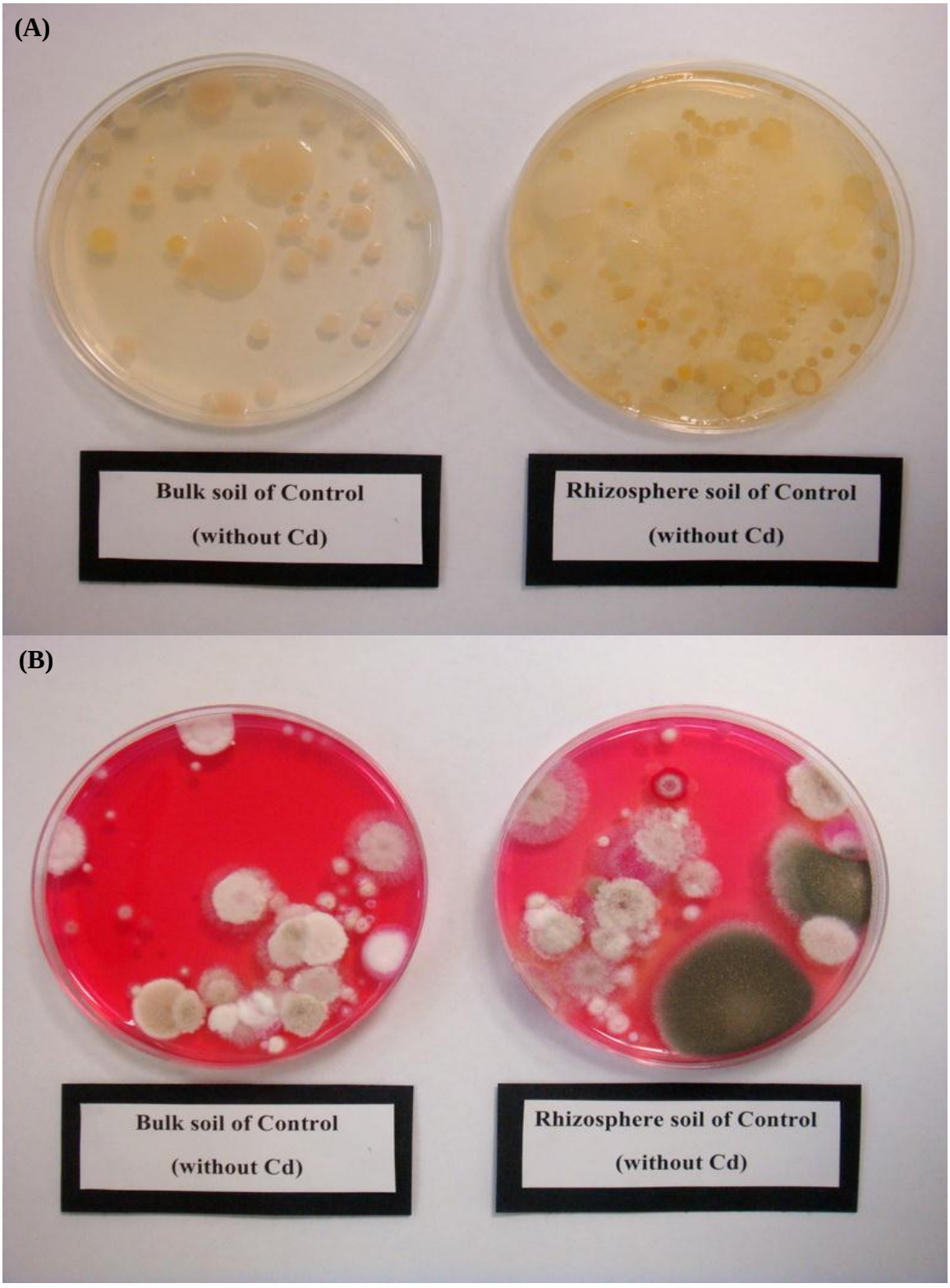

Figure 4.3 Morphology of colonies isolated from bulk and rhizosphere of pineapple grown in soil without Cd at 36 weeks. The soil sample dilutions were isolated on Nutrient agar (A) and Rose Bengal agar (B).

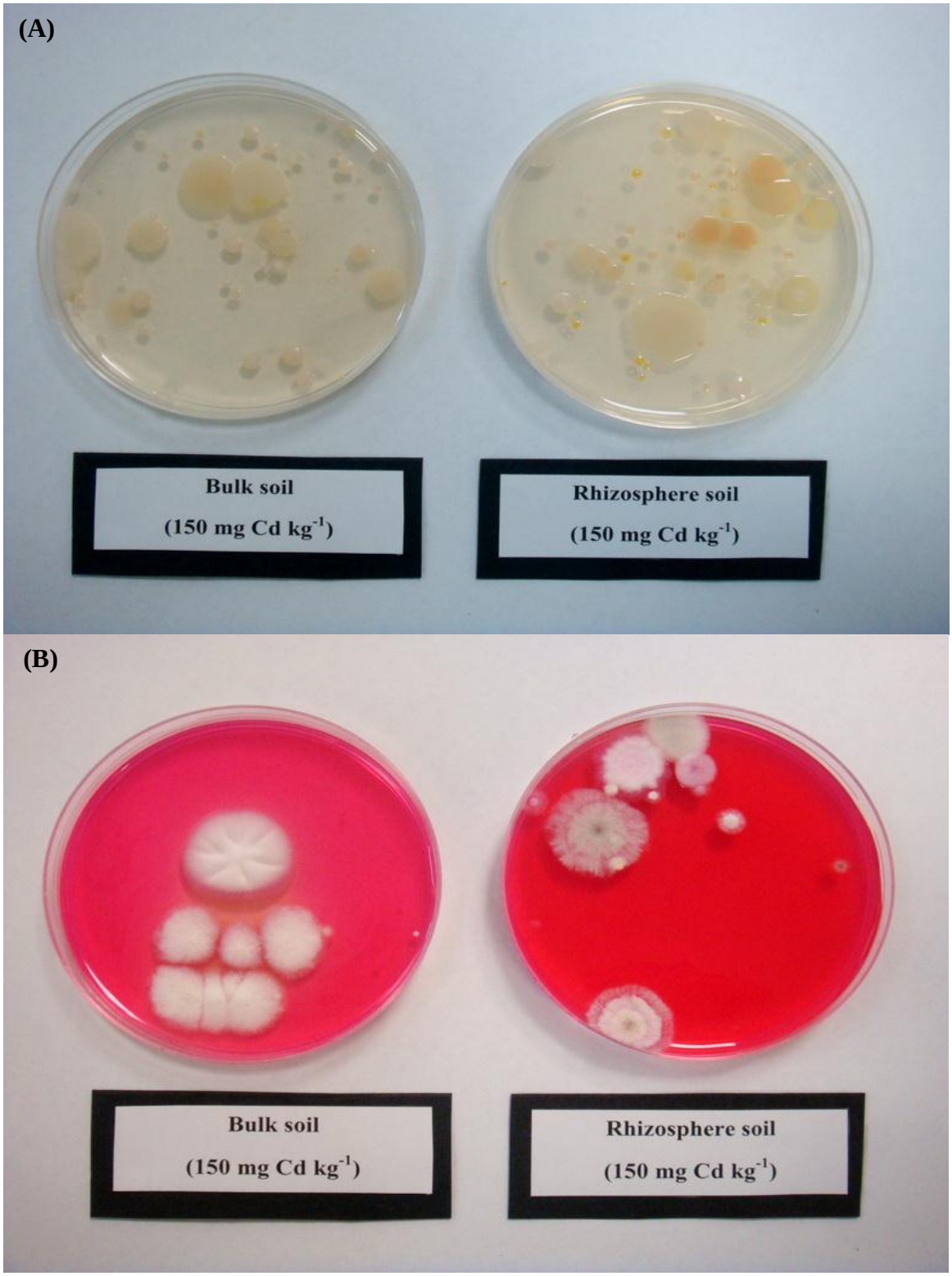

Figure 4.4 Morphology of colonies isolated from bulk and rhizosphere of pineapple grown in Cd-contaminated soil at 150 mg Cd kg^{-1} at 36 weeks. The soil sample dilutions were isolated on Nutrient agar (A) and Rose Bengal agar (B).

4.3.2 Molecular analysis of the soil bacterial diversity

The DGGE banding patterns generated from the universal bacterial primers (357F and 518R) were used to examine the effects of the Cd on soil microbial communities after growing *A. comosus* for 36 weeks, and allowed for the comparison of bacterial diversity in either planted or unplanted soils with and without 150 mg Cd kg^{-1} (Figure 4.5A and 4.5B). Each of the distinguishable bands in the separation pattern, in theory, represents an individual bacterial species and overall a large number of bands were observed in each sample. In the present study, the numbers of 16S rRNA bands were significantly higher ($P < 0.05$) in the rhizosphere soil than in the bulk soil, indicating the presence of a higher number of bacteria species (Table 4.2)

Table 4.2 Number of DNA bands in the PCR-DGGE profile of bulk and rhizosphere soil. The samples were taken from pineapple grown in presence and absence of Cd (150 mg Cd kg^{-1}) at 36 weeks.

Soil Types	Number of bands
Control (non-planted without Cd)	57 ± 1.67^{a}
Bulk soil without Cd	57 ± 1.00^{a}
Rhizosphere soil without Cd	62 ± 1.67^{b}
Control (non-planted with Cd)	48 ± 1.67^{c}
Bulk soil with Cd	49 ± 1.67^{c}
Rhizosphere soil with Cd	53 ± 0.33^{d}

Different letter indicates a significant difference between groups, such that groups not sharing a similar letter are significantly different from each other ($P < 0.05$) according to LSD test.

Visual inspection of the DGGE profiles (Figure 4.5A and B) appears to show that the soil samples with and without Cd are different to one another. A number of bands (a total of 40 bands were selected) that were either present or absent in the different profiles or clearly increased in intensity in one profile over another, were excised from the gel and subject to sequencing in order to ascertain if any particular genera or species were selected in the pineapple rhizosphere or selected by the presence of Cd in the soil.

The results of the partial sequence analysis of these bands and their tentative phylogenetic affiliation are shown in Table 4.3. A total of 8 phyla belonging to *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, *Acidobacteria*, *Verrucomicrobia*, *Firmicutes*, *Chloroflexi* and *Nitrospirae* were found overall. Four phyla such as *Bacteroidetes*, *Proteobacteria*, *Actinobacteria* and *Firmicutes* were found in both non-contaminated and Cd-contaminated soils. Other phyla such as *Nitrospirae*, *Verrucomicrobia*, *Acidobacteria* and *Chloroflexi* were also only found in Cd-contaminated soil. The two phyla found only in the rhizosphere soil of Cd-contaminated soil were *Verrucomicrobia* and *Nitrospirae*. The phylum found only in rhizosphere of non-contaminated soil was *Bacteroidetes* and these appeared to be increased in number of bands in the rhizosphere. The presence of Cd in soil appeared to select for species such as *Verrucomicrobia*, *Chloroflexi* and *Nitrospira* sp. as they were only found in Cd contaminated soil. Other species such as *Bacteroidetes*, *Flavobacteria*, *Arthrobacter* sp., *Sphingobacteriales* appeared to increase in number of bands due to Cd contamination.

In non-contaminated soil, the increased intensity of two DNA band was observed in the rhizosphere soil (1 and 5). The DNA sequences of bands 1 and 5 were identified as *Bacteroidetes* and *Flavobacteria*. Two predominant bands in bulk soil were observed (10 and 11) and identified as *Actinobacterium* and *Gamma-proteobacterium*. The most abundant group in non-planted soil was *Actinobacterium* (14 and 15). In Cd-contaminated soil, the sequence of two strong bulk soil bands (30, 31) compared to other treatments showed similarity to *Chitinophagaceae* and *Branchymonas* sp. Band 24 and 25 predominant bands in rhizosphere soil were identified as *Lacibacter* sp. and *Verrucomicrobia*. A specific band (18) found in the rhizosphere soil but not in bulk and non-planted soil had 100% similarity to *Arthrobacter* sp. Three dominant bands (32, 36, 37) were observed in non-planted soil and showed a high similarity to *Chloroflexi*, *Flavobacteria* and *Spingobacteriales*.

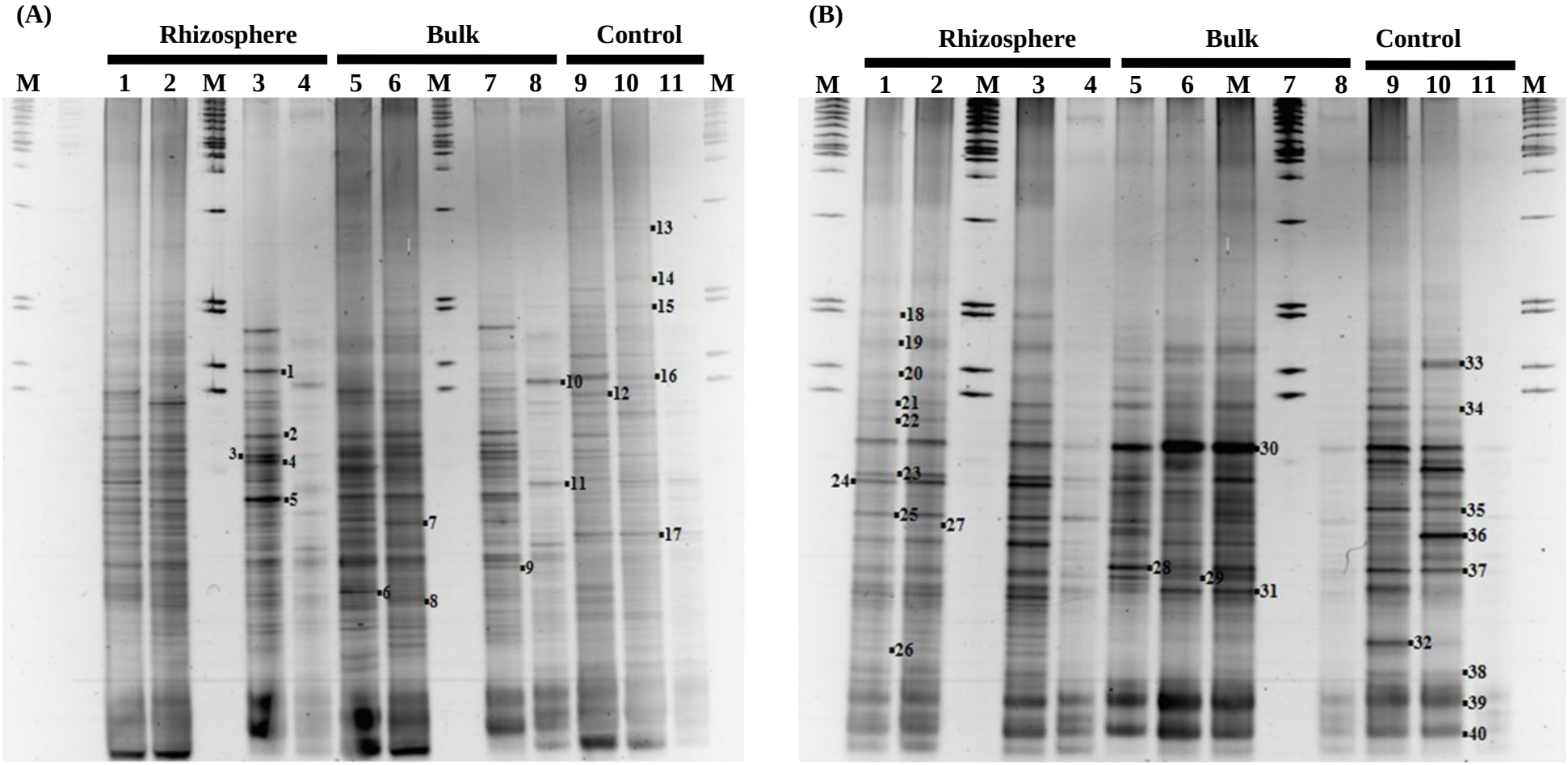

Figure 4.5 DGGE banding pattern of bacteria 16S rRNA PCR-DGGE profiles in bulk soil and rhizospere soil of pineapple grown under absence (A) and presence (B) of Cd (M marker; lane 1-4: rhizoshere soils; lane 5-8: bulk soil; lane 9-11: control). The numbers represent bands that were picked for DNA isolation, PCR and bacterial identification.

Table 4.3 Nucleotide sequence BLAST results of DGGE amplicons generated using bacterial 16S rRNA gene-specific primers.

Band no.	Accession number	Microorganisms	Group affiliation	Similarity (%)	Habitat	Reference	Sample type
1	JF990554.1	*Bacteroidetes*	*Bacteroidetes*	100	agricultural soil	Cui, (2011)	non-contamianted rhizosphere
2	JF803808.1	*Terrimonas* sp.	*Bacteroidetes*	98	farmland soil	Zhang *et al.*, (2011a)	non-contaminated rhizosphere
3	JF703575.1	*Chitinophagaceae*	*Bacteroidetes*	100	soil from lead-zinc mine tailings	Zhang *et al.*, (2012)	non-contaminated rhizosphere
4	BA000012.4	*Mesorhizobium loti*	*Proteobacteria*	100	nitrogen fixing bacteria	Kaneko *et al.*, (2000)	non-contaminated rhizosphere
5	SF423919.1	*Flavobacteria*	*Bacteroidetes*	100	activated sludge	Lee and Ang, (2011)	non-contaminated rhizosphere
6	FJ712870.1	*Rhizobium* sp.	*Proteobacteria*	96	rhizosphere soil of *Arabidopsis thaliana*	Micallef *et al.*, (2009)	non-contaminated bulk
7	HQ230896.1	*Camamonas* sp.	*Proteobacteria*	100	activated sludge	Chen, (2010)	non-contaminated bulk
8	EF437255.1	*Rhizobium* sp.	*Proteobacteria*	94	arid Tunisian soils	Mahdhi *et al.*, (2008)	non-contaminated bulk
9	GQ410532.1	*Pseudomonas sp.*	*Proteobacteria*	97	rhizosphere soil of *Archis hypogaea*	Haldar *et al.*, (2011)	non-contaminated bulk
10	GU127269.1	*Actinobacterium*	*Actinobacteria*	96	sediment	Silva *et al.*, (2009)	non-contaminated bulk
11	GU016261.1	*γ-proteobacterium*	*Protobacteria*	100	forest soil	Lin *et al.*, (2011)	non-contaminated bulk
12	JN000339.1	*Arthrobacter* sp.	*Actinobacteria*	100	soil from uranium mine	Islam *et al.*, (2011)	non-contaminated control soil
13	HQ132417.1	*Erythromicrobium* sp.	*Proteobacteria*	100	contaminated estuarine sediment	Zhang *et al.*, (2010a)	non-contaminated control soil
14	FJ916464.1	*Actinobacterium*	*Actinobacteria*	100	dissolved organic carbon	Jones *et al.*, (2009)	non-contaminated control soil
15	HM622569.1	*Actinobacterium*	*Actinobacteria*	98	tomato root	Tan *et al.*, (2011a)	non-contaminated control soil
16	EU999035.1	*Enterococcus faecium*	*Fermicutes*	100	resistant antibiotic bacteria	Depardieu *et al.*, (2009)	non-contaminated control soil
17	AY838324.1	*Cellulosimicrobium* sp.	*Actionobacteria*	100	activated sludge	Purohit *et al.*, (2004)	non-contaminated control soil
18	FR772113.1	*Arthrobacter* sp.	*Actinobacteria*	100	antarctic soil	Peeters *et al.*, (2011)	Cd-contaminated rhizosphere soil
19	JF683274.1	*Rhodococcus* sp.	*Actinobacteria*	95	rhizosphere soil	Compant *et al.*, (2011)	Cd-contaminated rhizosphere soil
20	FJ464979.1	*Aeromicrobium* sp.	*Actinobacteria*	98	antarctic soil	Tam and Wong, (2008)	Cd-contaminated rhizosphere soil
21	JF984284.1	*Nitrospira sp*	*Nitrospirae*	100	agricultural soil	Cui, (2011)	Cd-contaminated rhizosphere soil

Table 4.3 (Continued)

Band no.	Accession number	Microorganisms	Group affiliation	Similarity (%)	Habitat	Reference	Sample type
22	JF989639.1	*Bacteroidetes*	*Bacteroidetes*	100	agricultural soil	Cui, (2011)	Cd-contaminated rhizosphere soil
23	GU929377.1	*Flavobacteriales* sp.	*Bacteroidetes*	100	soil from wheat straw decomposition	Li *et al.*, (2010)	Cd-contaminated rhizosphere soil
24	JF703346.1	*Lacibacter* sp.	*Bacteroidetes*	100	soil from lead-zinc mine tailing site	Zhang *et al.*, (2012)	Cd-contaminated rhizosphere soil
25	HQ326120.1	*Verrucomicrobia*	*Verrucomicrobia*	100	rhizosphere soil of banana	He *et al.*, (2010)	Cd-contamianted rhizosphere soil
26	JF990091.1	*Acidobacteria*	*Acidobacteria*	94	agricultural soil	Cui, (2011)	Cd-contaminated rhizosphere soil
27	HM486314.1	*Microbacterium* sp.	*Acidobacteria*	92	wetland soil	Paixao *et al.*, (2010)	Cd-contaminated rhizosphere soil
28	JF987079.1	*Bacteroidetes*	*Bacteroidetes*	100	agricultural soil	Cui, (2011)	Cd-contaminated bulk soil
29	JF703594.1	*Sphingobacteriales*	*Bacteroidetes*	96	soil from lead-zinc mine tailing site	Zhang *et al.*, (2012)	Cd-contaminated bulk soil
30	JF703358.1	*Chitinophagaceae*	*Bacteroidetes*	96	soil from lead-zinc mine tailing site	Zhang *et al.*, (2012)	Cd-contaminaed bulk soil
31	JF77296.1	*Brancymonas* sp.	*Proteobacteria*	97	hydrocarbon contaminated sludge	Das *et al.*, (2011)	Cd-contaminated bulk soil
32	JF990161.1	*Chloroflexi*	*Chloroflexi*	100	agricultural soil	Cui, (2011)	Cd-contaminated control
33	HM755854.1	*Arenibacter* sp.	*Bacteroidetes*	100	bacterial biomass from natural gas production plant	Lim *et al.*, (2011)	Cd-contaminated control
34	JF990165.1	*Bacteroidetes*	*Bacteroidetes*	100	agricultural soil	Cui, (2011)	Cd-contamianted control
35	HQ144207.1	*Firmicutes*	*Firmucutes*	100	cow dung	Girija *et al.*, (2010)	Cd-contaminated control
36	JF947979.1	*Flavobacteria*	*Bacteroidetes*	100	biofilm formed in a desalination plant	Elifantz *et al.*, (2011)	Cd-contaminated control
37	AM935178.1	*Sphingobacteriales*	*Bacteroidetes*	97	aliphatic hydrocarbon-contamanated soil	Militon *et al.*, (2010)	Cd-contaminated control
38	AY823432.1	α-*proteobacterium*	*Proteobacteria*	96	hexachlorocyclohexane (HCH) contaminated soil	Neufeld *et al.*, (2006)	Cd-contaminated control
39	JF411325.1	*Arthrobacter* sp.	*Actinobacteria*	100	soil from ancient Bozkol village	Osman, (2011)	Cd-contaminated control
40	JF989848.1	*Acidobacteria*	*Actinobacteria*	98	agricultural soil	Cui, (2011)	Cd-contaminated control

To allow more accurate assessment of the differences in microbial diversity between bacteria populations in the various soil samples (bulk and rhizosphere soils with and without 150 mg Cd kg^{-1} cluster analysis using Bionumerics software was performed followed by estimations of diversity using the Shannon and Simpson Indices.

Cluster analysis of DGGE profiles of PCR-amplified soil DNA targeting 16S rRNA genes in soil with and without Cd at 36 weeks of growing pineapple are shown in Figure 4.6. The results showed that the microbial population in the rhizosphere soils are different to bulk soils. The non-contaminated rhizosphere bacterial population were different to the Cd-contaminated rhizosphere population.

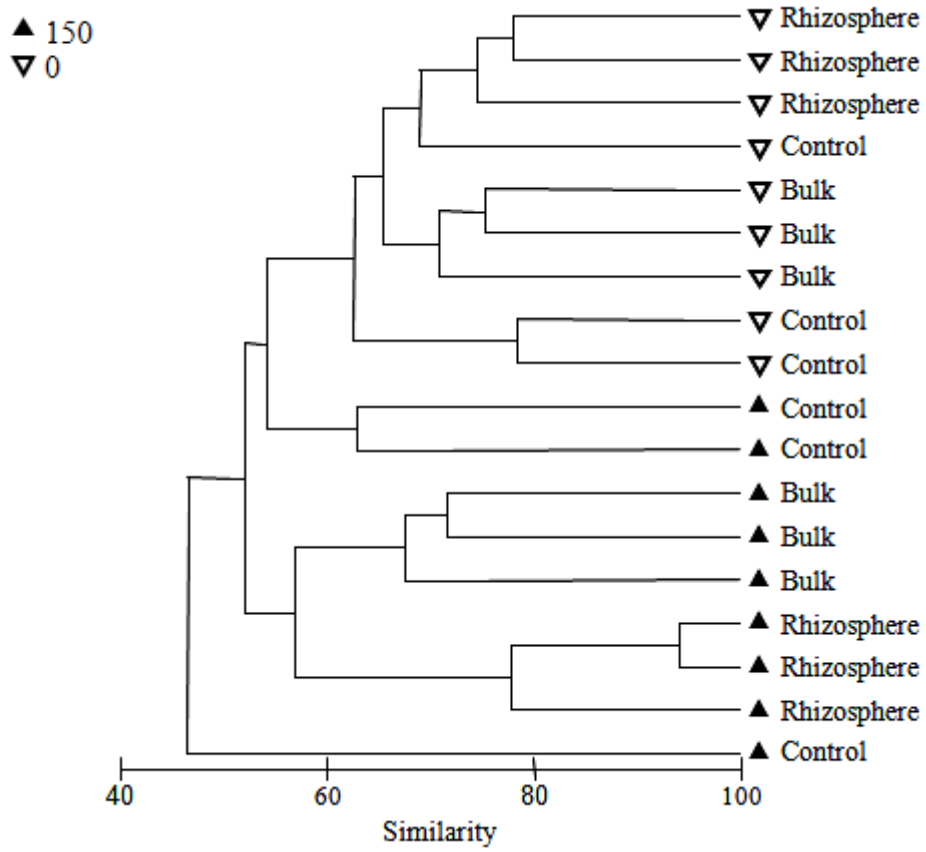

Figure 4.1 Cluster analysis of bacteria 16S rRNA PCR-DGGE profile in bulk soil and rhizosphere soil of pineapple grown under presence and absence of Cd. The white and black triangle represent soil sample without Cd and with Cd 150 mg Cd kg^{-1}, respectively.

Following cluster analysis the Shannon index of diversity (*H*) and the Simpson index of dominance (1/*S*) were calculated (Table 4.4). Both these indexes showed a number of interesting changes. For example, both the Shannon and Simpson indices of bacteria in rhizosphere soil were significantly higher than those of both the control (unplanted) and bulk soil. When Cd was present both indices were significantly reduced when comparing similar samples; e.g. Cd contaminated rhizosphere soil samples had a significantly lower Shannon index than non-contaminated rhizosphere samples.

Table 4.4 Diversity indices of soil bacteria communities in bulk soil and rhizosphere soil of pineapple grown in Cd-contaminated soil using DGGE banding pattern.

Soil type	***H***		**1/*S***	
	0 mg Cd kg^{-1}	**150 mg Cd kg^{-1}**	**0 mg Cd kg^{-1}**	**150 mg Cd kg^{-1}**
Control	3.55^{a}	3.24^{c}	8.89^{a}	7.13^{c}
Bulk	3.53^{a}	3.26^{c}	8.57^{a}	7.20^{c}
Rhizosphere	3.69^{b}	3.39^{d}	10.28^{b}	7.83^{c}

H Shannon diversity index, 1/*S* Simpson index. For each parameter, values followed by the same letter are not significantly different according to Least Significant Difference post hoc test ($P < 0.05$) between different treatments for each soil type group.

4.4 Discussion

Overview: Several methods were used to determine if pineapple plants influenced the numbers and diversity of the microbial communities in the rhizosphere of Cd-contaminated soil. By using more than one method to study the microbial population, a more complete account of the microbial population can be obtained (Kirk *et al.*, 2004). The results from plate counts and DGGE indicated that the pineapple produced a specific rhizosphere microbial population. Plant induced changes in microbial community composition and activity have long been reported (Borga *et al.*, 1994; Marschner *et al.*, 2001; Zak *et al.*, 2003; Carney *et al.*, 2004; Carney and Matson, 2005). In addition, the work found that Cd contamination (in all samples analysed) significantly altered microbial numbers (viable counts) and bacterial populations as analysed by DGGE.

4.4.1. Rhizosphere effect of pineapple on bacterial populations in non-contaminated soil

4.4.1.1 Viable counts

In the present study, I found that viable counts of bacteria from rhizosphere soil were higher than from bulk and unplanted soil. Previous studies found that the rhizosphere had an effect on the populations of bacteria and numbers of viable bacteria are generally higher in the rhizosphere soil than in bulk soil (Clark, 1949; Brown, 1975; Brimecombe *et al.*, 2001). The study of Christensen *et al.*, (1992) in wheat plants showed that total bacterial number in bulk, rhizosphere and unplanted soil was 7.5×10^6, 6.3×10^7 and 1.3×10^7, respectively. The higher bacterial number in the rhizosphere soil may be due to the root exudates that stimulate the growth of bacterial and fungal populations in the root zone (Rovira, 1965). Several studies have indicated that the structural and functional diversity of rhizosphere populations is affected by the plant through the release of root exudates and by providing surfaces for colonization (Sorensen, 1997; Jaeger *et al.*, 1999; Bais *et al.*, 2006).

My study demonstrated that the number of fungi reduced as time increased. The reduction in viable count could be from the effect of Cd on fungi growth (Arriagada *et al.*, 2004) and the dilution and plating favour the species that produce large numbers of spore and not the active fungi that are growing as hyphae (Curl and Truelove, 1986).

4.4.1.2 Microbial population (DGGE) studies (non-contaminated soils)

The degree to which bacterial populations are enriched in the rhizosphere of pineapple plants compared to the surrounding bulk soil, indicating shifts of relative abundance, was analysed. Results from DGGE analysis suggested the pineapple plants altered the composition of the microbial community. Moreover, the community structure of soil bacteria in the rhizosphere was significantly different from that in bulk soil. Four phyla such as *Bacteroidetes*, *Proteobacteria*, *Actinobacteria* and *Firmicutes* were found in non-contaminated soil. *Proteobacteria* was the dominant phylum (increase in number of bands) that was found in non-contaminated soil. The phylum that was only found in the rhizosphere of non-contaminated soil was *Bacteroidetes* and this phylum appeared to be increased in number of bands in the rhizosphere. In non-contaminated soil, the increased intensity of two DNA band was observed in the rhizosphere soil (1 and 5). The DNA sequence of band 1 and 5 was identified as *Bacteroidetes* and *Flavobacteria*. Culture-independent analyses performed with various plants often reveal that *Bacteroidetes* and *Flavobacteria* are dominant species found in the rhizosphere of many plant species such as *Brassica napus* (Kaiser *et al.*, 2001) and *Amaranthus hybridus* (Oyeyiola, 2010).

The microbial community diversity of the Shannon index (*H*) and the strength of the dominance or Simpson index (1/*S*) were calculated based on the number of bands and their relative intensities among the individual samples (Ponnusamy *et al.*, 2011). In this study, the diversity based on Shannon index was higher in the rhizosphere soil (3.69) than in bulk soil (3.53). A previous study found that Shannon index values in the rhizosphere and bulk soil of *Fritillaria thunbergii* was 3.16 and 2.95, respectively (Yuan *et al.*, 2010). Similar results was observed that Shannon index values in the rhizosphere soil and bulk soil of switchgrass (3.08 and 3.07) and jathropha (3.02 and 3.01) (Chaudhary *et al.*, 2012). The Simpson index in the rhizosphere soil (10.28) was higher than in the bulk soil and control (non-planted soil). A previous study showed that Simpson index in the rhizosphere soil of *Spartina alterniflora* was 14.0 (Wang *et al.*, 2007). For Clustering analysis, bulk soil samples apart from rhizosphere samples indicated that the microbial community in plant rhizosphere were different from bulk soil. It may be due to a rhizosphere effect where the environments surrounding the plant root contain higher densities of microbial populations compared to bulk soil (Kowalchuk *et al.*, 2002).

4.4.2 Effect of Cd contamination on microbial numbers and populations

4.4.2.1 Effect of Cd contamination on microbial viable counts

In this study, the numbers of culturable bacteria and fungi decreased in all Cd-treatments. The decrease in the number of bacteria and fungi was probably a result of Cd toxicity (Khan *et al.*, 2010). Many reports showed that Cd added to soil at a higher dose (30 and 40 mg Cd kg^{-1}) depressed the number of soil microorganisms (Hiroki, 1992; Nada *et al.*, 1997; Jiang *et al.*, 2000). Also Jiang *et al.*, (2000) found that the number of bacteria in soil contaminated with Cd applied with or without other heavy metals was significantly smaller than in the control (without Cd). The study of (Khan *et al.*, 2007) reported that Cd had an effect on bacterial populations in the rhizosphere soil of maize. The total viable count was 3.6×10^5 cfu/g and significantly less than that in the control (5.5×10^5 cfu/g).

4.4.2.2 Effect of Cd contamination on microbial populations (DGGE studies)

DGGE fingerprints of PCR-amplified 16S rDNA genes were used to monitor the effect of Cd on soil bacterial community structure in bulk soil and rhizosphere soil of pineapple plants. In this study, Cd-contamination could be seen to visually affect the intensity, number and types of bands (species) seen on DGGE gel profiles (Figure 4.5) in all soil samples analysed. Previous studies have revealed that both short-term and long-term exposure to heavy metals result in adverse effects on microbial activities and abundance, and altered microbial community structures (McGrath *et al.*, 2001; Tsezos, 2001; Lasat, 2002; Tsai *et al.*, 2005; Khan *et al.*, 2007; Chien *et al.*, 2008; Ros *et al.*, 2009). It could be possible that microorganisms from these soils were less adapted or inhibited by high level Cd concentratrions (Hattori, 1992; Ranjard *et al.*, 1997).

The specific two phyla found only the rhizosphere soil of Cd-contaminated soil were *Verrucomicrobia* and *Nitrospirae* while the *Actinobacteria* appeared to be predominant general in the rhizosphere soil of Cd-contaminated soil. The presence of Cd in soil appeared to select for species such as *Verrucomicrobia, Chloroflexi* and *Nitrospira* sp. These species were only found in Cd contaminated soil. Other species such as *Bacteroidetes, Flavobacteria, Arthrobacter* sp., *Sphingobacteriales* appeared to increase in number of bands due to Cd contamination. In this thesis, species such as *Rhizobium* sp., *Enterococcus faecium* and *Cellulosimicrobium* sp. were found to be metal sensitive

species and not present in Cd-contaminated soils. Many studies suggest that *Actinobacteria* is the most common of the dominant bacterial populations found in the rhizosphere of many different plant species (Singh *et al.*, 2007). Also most of the species (such as *Arthrobacter* sp. *Lacibacter* sp., *Nitrospira* sp., *Verrucomicrobia*, *Sphingobacteriales* and *Chlroflexi*) found in this study have previously been found in metal contaminated sites (Ellis *et al.*, 2003; Belimov *et al.*, 2005; Piotrowska-Seget *et al.*, 2005; Abou-Shanab *et al.*, 2007a; Militon *et al.*, 2010; Das *et al.*, 2011; Zhang *et al.*, 2012).

The present study showed a significant reduction in the number of bands in rhizosphere soil under Cd-stress compared with non-Cd contamination and this is supported by the lower Shannon index value observed. Both the Shannon and Simpson indices were reduced compared to non-contaminated soil. In addition, the Shannon index in the Cd rhizosphere soils (3.39) was higher than bulk soil and control (non-planted). Despite the high level of Cd contamination used in this experiment, (soil was incubated with Cd for 36 weeks) it still appeared that bacterial diversity remained relatively high indicating population resilience. A previous study by Wang *et al.,* (2007) reported that the Shannon diversity index for different heavy metal contaminated soils ranged between 2.20-2.81 compared to 3.24-3.39 found in this study. The differences in the microbial diversity were confirmed by the Simpson index values. The Simpson index of rhizosphere soils (7.83) was higher than bulk soil (7.20) and control (non-planted; 7.13). Previous study by Kim *et al.,* (2010) found that the Simpson index value in the rhizosphere soil of *Abutilon avicennae* (27.15) and *Echinochloa crus-galli* (20.98) grown in metal contaminated soil were higher than control (non-planted; 20.69). In Cd-contaminated soil, cluster analysis showed that microbial community in rhizosphere were different from bulk soil. Plant rhizosphere provides an important ecological niche for microorganism. In contrast to bulk soil, the soil associated with plant root has significantly higher densities of microorganisms (Lynch, 1990; Rangarajan *et al.*, 2002; Jones *et al.*, 2004; De Leij *et al.*, 2007; Hinsinger *et al.*, 2009; Doi *et al.*, 2011). The adaptation of bacteria in the rhizosphere of plants might be stimulated by the alterations of root exudates in response to the metal stress (Wang *et al.*, 2008b).

4.5 Conclusion

The results presented here have shown for the first time that the pineapple rhizosphere has an increased microbial (bacterial and fungal numbers) count and a distinct and more diverse bacterial population (as assessed by DGGE) when compared to the surrounding bulk soil. Shannon and Simpson indices were similar to those found in previous bacterial studies and are typically higher than those found for higher organisms (e.g. plants).

Cd pollution of soil reduced both microbial numbers and bacterial diversity of all soil samples analysed but the diversity of bacteria remained relatively high despite being incubated for 36 weeks with Cd at a very high level. Significant bacterial population changes were observed due to the presence of Cd. This indicates the adaptability of the bacterial population to metal contamination and suggests that the high bacterial diversity of soils has a protective effect on soil functions (not tested in this study). The next stages of this work attempted to isolate a selection of Cd resistant bacteria from soil and use them as inoculants to enhance the growth of pineapple in Cd-contaminated soil.

Chapter V

Cd tolerance and Cd-binding capacity of bacteria isolated from Cd-contaminated soil

5 Cd tolerance and Cd-binding capacity of bacteria isolated from Cd contaminated soil

5.1 Introduction

In heavy metal contaminated sites, soil bacteria are usually exposed to heavy metals resulting in the establishment of heavy metal-resistant bacterial populations (Ellis *et al.*, 2003; Piotrowska-Seget *et al.*, 2005). Heavy metals are able to induce increased resistance levels in soil bacteria and modify bacterial responses to environmental conditions either by inducing mutations or by altering physiological responses (Vattanaviboon *et al.*, 1999). Generally, exposure of bacteria to a low dose of one stress can induce a subsequent increase in resistance to the same (adaptive) or unrelated (cross protection) stress (Mongkolsuk *et al.*, 1997). Strains isolated from heavy metal polluted sites generally possess resistance to metals (Patra *et al.*, 2004).

Biomass of bacteria has been known to readily adsorb or accumulate metal ions (Volesky and Holan, 1995; Kratochvil and Volesky, 1998; Tsezos, 2001; Volesky, 2001) and binding of metal cations on the outer surface of bacterial cells has become an attractive mechanism for remediating metal-polluted environments (Doyle, 1989; Ehrlich, 1997). Being metabolism-independent, cell-surface binding can occur in either living or inactivated microorganisms, whereas the intracellular and extracellular accumulation of metals are usually energy-driven processes, thereby taking place only in living cells (Lu *et al.*, 2006). The bacterial cell wall is the first component that comes into contact with metal ions, where the solutes can be deposited on the surface or within the cell wall structure. Since the mode of solute uptake by dead or inactive cells is extracellular, the chemical functional groups of the cell wall play vital roles in the passive process of biosorption (Vijayaraghavan and Yun, 2008). Because the cell surface of bacteria carries a net negative charge due to the presence of carboxyl, amine, hydroxyl, phosphate and sulfhydryl groups (Tortora *et al.*, 2005), it can adsorb appreciable quantities of positively charged cationic metals such as Cd (Scott and Palmer, 1990). Furthermore, heavy metal resistance through production of binding proteins enhances the suitability of bacteria for biosorption of heavy metals dissolved in solution (Higham *et al.*, 1984).

The main aims of this chapter were to isolate Cd-resistant bacteria from Cd-contaminated soil and assess the Cd uptake ability of the isolates. The ultimate aim (see Chapter VI) was to examine the possibility of using these Cd-resistant bacteria to enhance the growth of pineapple in a Cd-contaminated soil. It was hypothesised that Cd-resistant bacteria could be used as soil inocula to reduce Cd uptake by the plant; the Cd-resistant bacteria being able to complex Cd by either passive or active uptake mechanisms while in the soil, hence reducing the amount of Cd available to the plant.

Specific objectives were to:

a) isolation of Cd-resistant bacteria from Cd contaminated soil.
b) identification of Cd-resistant bacteria by 16S rRNA sequence to ensure pathogens (plant or animal) were not isolated.
c) determination of Cd resistance of the various isolates.
d) determination of Cd uptake abilities of the most resistant isolates.

5.2 Materials and Methods

5.2.1 Soil sampling strategy

Soil samples were collected from pots containing pineapple grown in Cd-contaminated soil (150 mg Cd kg^{-1}) for nine months. Plants were removed from pots and each plant was shaken carefully to remove the bulk soil. The soil still adhering to the roots, defined as rhizosphere soil, was separated from the roots. Rhizosphere soil subsamples were stored in a polyethylene bag and kept at 4 ºC until they were used to isolate Cd-resistant bacteria by using plate count techniques.

5.2.2 Isolation of Cd-resistant bacteria

Cd-resistant bacteria were isolated according to the modified method described by Sinha and Mukherjee, (2008). Soil (10 g fresh weight) from each treatment was weighed into 250 ml Erlenmeyer flasks containing 90 ml of sterile Ringer's solution ($CaCl_2$ 0.12 g, KCl 0.105 g, $NaHCO_3$ 0.05 g, NaCl 2.25 g in 1 L water; Sigma-Aldrich, USA) and shaken for 15 minutes at 250 rpm at room temperature. Soil particles were allowed to settle for 1 hour and ten-fold dilutions were prepared in test tubes containing 9 ml of sterile Ringer's solution. Exactly 100 µl of soil dilutions were plated on Nutrient agar (NA) media containing 50 mg Cd l^{-1} as $Cd(NO_3)_2$. To prevent the growth of soil fungi, the media were supplemented with 10 mg l^{-1} cycloheximide (Sigma-Aldrich, USA) after autoclaving. Plates were incubated for 5 days at 26 ºC. Cd-resistant colonies were purified on the same media by streaking three times onto the fresh media. Nine bacterial isolates showing different morphological appearances on agar media were selected and stored at 4 ºC.

5.2.3 Identification and characterization of Cd-resistant bacteria

For the 16S rRNA analysis, genomic DNA was extracted using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, USA). Bacterial 16S rRNA gene primers (27F, 5'-AGAGTTTGATCMTGGCTCAG- 3' and 1525R, 5'-AAGGAGGTGATCCAGCC-3') were used to amplify a 16S rRNA gene fragment from genomic DNA (Morel *et al.*, 2009). The PCR mixture (50 µl) contained 1 µl of DNA template, 5.0 µl of 10x PCR buffer free of Mg^{2+} (Bioline, UK), 1.5 mM $MgCl_2$ (Bioline, UK), 0.2 mM dNTP (Fermentas, UK), 0.2 µM of each primers and 1.25 U of Taq DNA polymerase (Bioline, UK). The PCR was performed in a DNA Engine Thermal Cycler (PTC-200, BIO-RAD, USA) at 95 ºC for 1

minute, followed by 30 cycles of 95 ºC for 1 minute, 55 ºC for 1 minute and 72 ºC for 1 minute, followed by a final extension performed at 72 ºC for 10 minutes. Exactly 15 µl of PCR product were purified by adding ExoSAP-IT reagent (2 µl reagent per 5 µl amplified PCR product; USB Corporation, UK). Two microliters of purified PCR product were added as template to 10 µl sequencing reactions containing the BigDye V3.1 sequencing chemistry (Life Technologies Appplied, UK) and 0.4 µM of primer. DNA fragments were sequenced with an AB3730xl automated DNA sequencer (Applied Biosystems Inc., USA). Applied Biosystems DNA sequencing analysis 5.2 software (Applied Biosystems Inc., USA) was used to create sequence contigs from the fragments obtained. Similarity searches of the sequence obtained were performed using BLAST function at NCBI database (http://www.ncbi.nlm.nih.gov).

5.2.4 Cd tolerance of the bacterial strains

After the preliminary isolation of the Cd-resistant bacteria, all bacterial strains were tested to determine which was the most Cd-resistant using broth assay described by (Madhaiyan *et al.*, 2007). The bacterial strains were pre-cultured in Nutrient broth (NB) without heavy metal and used to inoculate into the Tris-buffered low-phosphate (TLP) media containing different concentrations (0-150 mg l^{-1}) of Cd. This medium is designed to avoid the precipitation of heavy metal (Burd *et al*. 1998) and contained (g l^{-1}) Trizma base 6.06 g, NaCl 4.68 g, KCl 1.49 g, NH_4Cl 1.07 g, Na_2SO_4 0.43 g, $MgCl_2.6H_2O$ 0.2 g, $CaCl_2.2H_2O$ 0.03 g, $Na_2HPO_4.12H_2O$ 0.23 g, Fe(III)(NH_4) citrate 0.005 g and supplemented with trace metals (1 ml of stock solution of trace metals per litre where the stock solution consists of $FeSO_4.7H_2O$ 0.2 g, $ZnSO_4.7H_2O$ 0.01 g, $MnCl_2.4H_2O$ 0.003 g, $CoCl_2.6H_2O$ 0.02 g, $CuCl_2.6H_2O$ 0.001 g, $NiCl_2.6H_2O$ 0.5 g, $Na_2MoO_4.2H_2O$ 0.5 g, H_3BO_3 0.03 g) and 0.2% sodium gluconate. Bacterial growth after 24 hours inoculation with shaking (150 rpm/min) at 26 ºC was recorded by measuring the absorbance at 600 nm. The two most resistant bacteria that had the highest cell growth were selected for use in all further experiments.

5.2.5 Cd biosorption by bacterial cells

Biosorption by bacterial cells was evaluated by allowing a known amount of bacterial biomass to interact with metal ions in solution. Bacterial pre-cultures were prepared by inoculating a loopful of bacteria into 50 ml Nutrient broth (NB; Sigma-Aldrich, USA) for biomass production and were incubated for 24 hours. Approximately 1 ml of pre-culture was inoculated into NB and cultures incubated on a rotary shaker (150 rpm/minute) at 26 ºC until the aliquots reached an optical density of 1.2. Bacterial cells were harvested by centrifugation for 10 min at 4600 x g and washed with phosphate buffer. Fresh microbial biomass (0.05 g) was re-suspended in 250 ml Erlenmeyer flasks, each containing 50 ml solution of 20, 50, 100 and 150 mg l^{-1} $Cd(NO_3)_2$ dissolved in Milli-Q water. The flasks were incubated on a rotary shaker (150 rpm/minute) at 26 ºC for different periods of contact (0-60 minutes). The cell solutions were centrifuged and supernatants were analyzed by ICP-OES (Model Vista-MPX, Varian Incorporated, Australia) for Cd. Blanks were examined throughout the sorption experiments to correct for any glassware sorption of metals. Percentage metal removal or biosorption and specific metal uptake (Q) were calculated (Volesky and May-Phillips 1995) using this equation:

$$Q\ (mg/g) = \left[\frac{C_i - C_e}{M}\right] \times V \qquad [5.1]$$

$$M = \left[\frac{C_i - C_e}{C_i}\right] \times 100 \qquad [5.2]$$

where Q is the specific metal biosorption (mg metal/ g biomass), M is the percentage metal removal, C_i and C_e are the initial and equilibrium metal concentration in solution (mg l^{-1}) respectively, V is the volume of metal solution (ml) and m is the mass of bacterial cell (mg).

5.2.6 Cd biosorption by bacterial cells in soil solution

Soil samples (2.5 g) containing 150 mg Cd kg^{-1} were added to 250 ml Erlenmeyer flasks (washed with 0.1 M HCl before the experiment). Exactly 50 ml of water was added to the soil samples. The samples were shaken on a shaker for 2 hours (to extract Cd and produce soil solution), followed by centrifugation at 6000 rpm for 10 minutes. The centrifuged

samples were filtered through Whatman No. 40 filter paper and the filtrate (soil solution plus Cd) collected in Erlenmeyer flasks. Fresh microbial biomass (0.05 g, produced as in Section 5.2.5) was added to Erlenmeyer flasks containing 50 ml of Cd solution extracted from Cd-contaminated soil. The flasks were incubated on a rotary shaker (150 rpm/minute) at 26 ºC for different periods of contact (0-60 minutes). The cell solutions were centrifuged and supernatant were analysed by ICP-OES for residual Cd. All the biosorption experiments were repeated three times.

5.2.7 Statistical analysis

All data presented are the mean values of three replicates. Values are expressed as means ± standard error (S.E.) in each group. All statistical analyses were performed using the statistical analysis package SPSS 17.0. One-way ANOVA was used to examine the significance of data. Variance analysis were performed on all experimental data and significant difference ($P < 0.05$) between individual means (three replicates) was analysed using a post hoc Least Significant Difference test.

5.3 Results

5.3.1 Isolation and identification of Cd-resistant bacteria

Nine morphologically distinct Cd-resistant bacterial isolates were identified based on the 16S rRNA gene sequence analysis as *Microbacterium esteraromaticum, Bacillus firmus, Microbacterium aoyamense, Arthrobacter humicola, Cellulomonas hominis, Micrococcus antarcticum, Bacillus cereus, Pseudomonas citronellolis* and *Sporosarcina globispora* (Table 5.1). The similarity of each strain with known bacterial 16S rRNA sequences present in the Genbank database is shown in Table 5.1.

Table 5.1 The identification of Cd-resistant bacteria isolated from rhizosphere soils of pineapples grown in Cd-contaminated soil.

Bacterial strain	% Similarity
Microbacterium esteraromaticum	99.380
Bacillus firmus	99.754
Microbacterium aoyamense	99.225
Arthrobacter humicola	99.720
Cellulomonas hominis	99.145
Micrococcus antarcticum	99.587
Bacillus cereus	99.866
Pseudomonas citronellolis	99.456
Sporosarcina globispora	98.691

5.3.2 Cd tolerance of the various bacterial isolates

Analysis of the Cd-resistance of the bacterial isolates was performed by a broth assay. An increasing concentration of $Cd(NO_3)_2$ from 0-150 mg Cd l^{-1} was used in liquid broth and the culture growth was determined by measuring absorbance at 600 nm at 24 hours. All strains were able to tolerate the highest concentrations of Cd added (150 mg l^{-1}), however *B. firmus* and *A. humicola* appeared to be more tolerant to Cd when compared with other bacterial strains (Table 5.2). From the potential of growing in high concentration of Cd solution, these two bacterial strains were chosen for further study of their Cd-binding capacity.

Table 5.2 Percentage of cell growth (as measured by OD) compared to control (without Cd) of Cd-resistant bacteria isolated from rhizosphere of pineapple grown in Cd-contaminated soil. Bacteria were exposed to different concentrations of Cd up to 150 mg Cd l^{-1} for 24 hours. The values presented are mean and standard error of triplicate samples.

Species	% Cell growth			
	Cd concentrations (mg Cd l^{-1})			
	20	50	100	150
C. hominis	55.83±3.63	31.00±0.76	12.33±1.20	3.75±0.43
B. cereus	38.06±0.56	27.74±1.34	13.44±1.24	4.09±0.39
M. aoyamenes	44.59±1.84	28.29±1.02	14.50±2.27	4.32±0.62
S. globispora	41.21±0.93	26.87±1.14	10.61±1.56	4.34±0.36
M. esteraromaticum	42.48±1.37	22.29±1.98	12.38±1.25	4.38±0.25
P. citronellolis	42.41±0.97	28.52±1.09	17.22±2.37	4.72±0.48
M. antarcticum	38.38±0.62	25.59±0.80	15.41±2.15	4.86±0.31
B. firmus	59.09±1.31	32.58±1.54	19.02±1.33	5.00±0.39
A. humicola	62.88±3.30	34.85±1.65	19.85±1.79	5.23±0.39

5.3.3 Cd biosorption by bacterial cells

In this study, two sets of biosorption data were gathered: specific metal uptake (Q) and percent biosorption (or percent metal removal). Specific metal uptake describes how much heavy metal was removed from solution without reference to how much was present initially in solution. Percent biosorption, however, describes how much heavy metal was removed from solution with reference to how much was present initially.

Calculation of Q in mg/g (metal/biomass) unit was based on the study of Al-Garni, (2005).

5.3.3.1 Effect of contact time on the biosorption process

Figure 5.1 shows Cd biosorption by the two bacterial isolates over time and shows that for both bacteria the major amount of metal uptake occurs within 10 minutes of contact. After that, the rate decreases until it reaches a constant value after 20 minutes. This result represents the equilibrium time at which an equilibrium metal ion concentration is presumed to have been attained. Similar results were obtained for the different Cd levels used.

5.3.3.2 Effect of metal concentration on Cd biosorption

The effect of initial metal concentration on Cd biosorption by living biomass of *B. firmus* and *A. humicola* was evaluated at 40 minutes as shown in Figure 5.2. The results indicated that biosorption (mg Cd per g biomass) increases with an increase in Cd concentration. *A. humicola* showed no significant difference in Cd uptake when compared with *B. firmus*; both isolates were able to take up approximately 40 mg Cd per g biomass when exposed to an initial level of 150 mg Cd per ml solution. The maximum percentages of Cd removal of *A. humicola* and *B. firmus* for Cd at the initial concentration of 150 mg l^{-1} were 32.88 and 31.63%, respectively (Figure 5.3). Furthermore, *A. humicola* showed significantly more Cd removal at the lowest concentration of Cd (20 mg l^{-1}) used when compared to *B. firmus* although the % removal difference was relatively small (approximately 45% removal with *B. firmus* compared to approximately 52% removal with *A. humicola*.

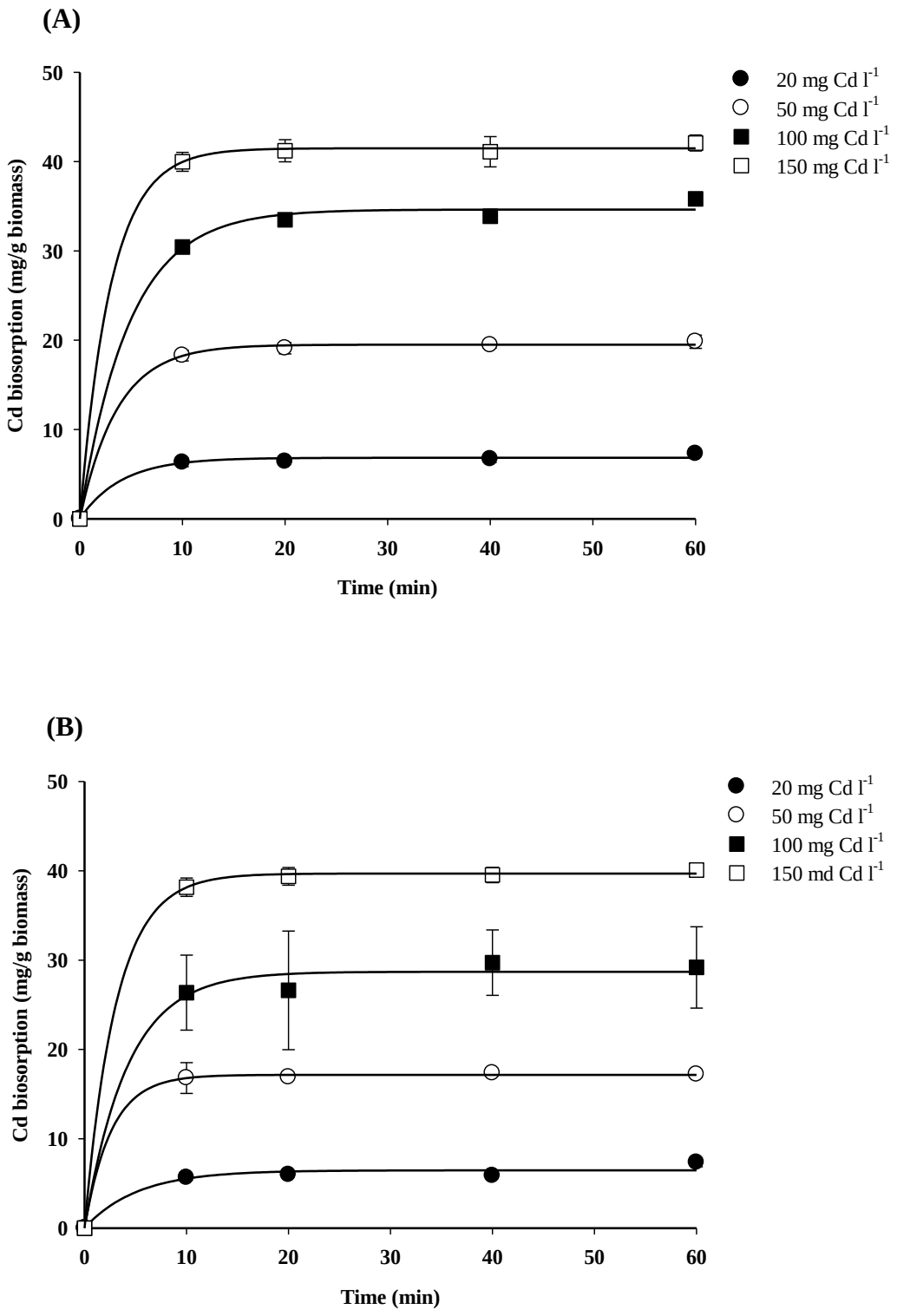

Figure 5.1 Biosorption of Cd by *A. humicola* (A) and *B. firmus* (B) over time at different initial concentrations of Cd (20, 50, 100 and 150 mg l^{-1}).

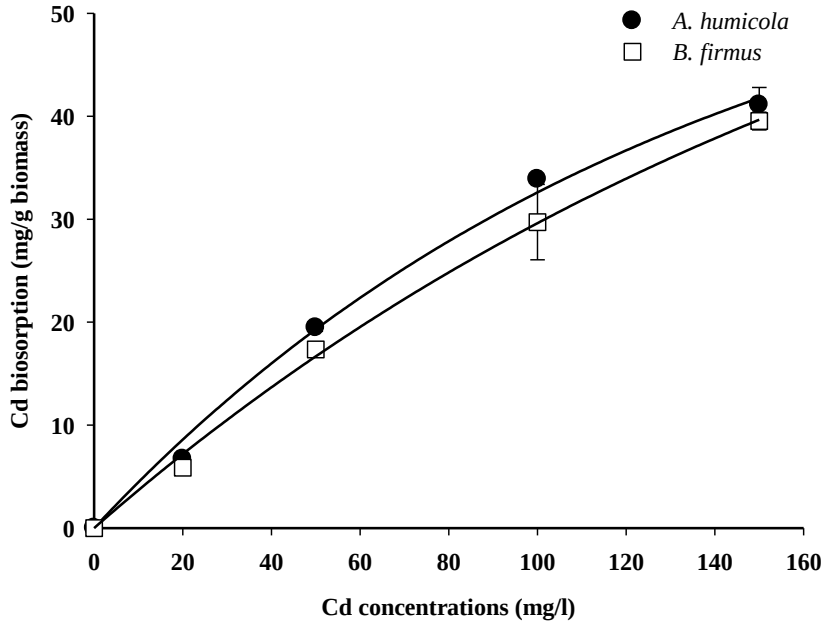

Figure 5.2 Effect of initial concentrations on Cd adsorption by *A. humicola* and *B. firmus* after 40 minutes of exposure to Cd.

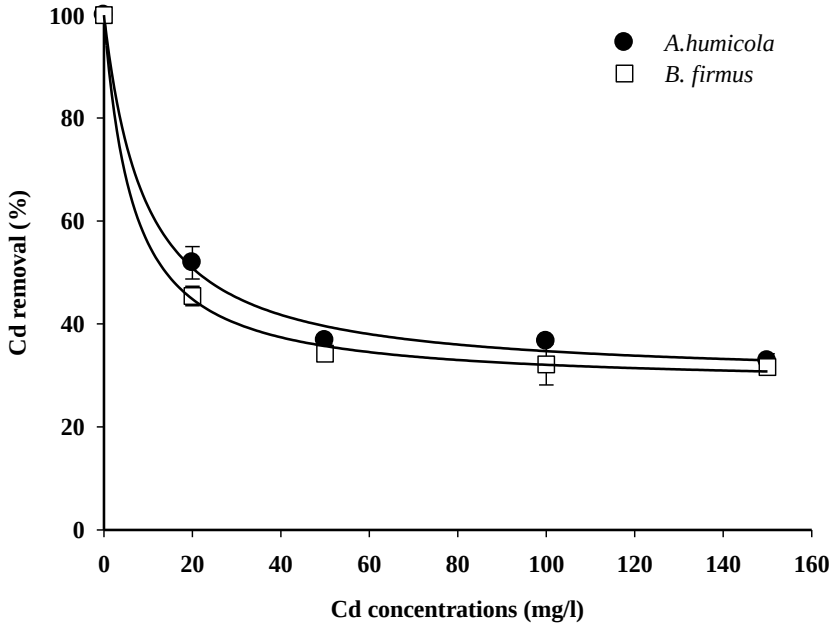

Figure 5.3 Effect of initial concentrations of Cd on percentage Cd removal by *A. humicola* and *B. firmus* after 40 minutes of Cd exposure.

5.3.3.3 Cd biosorption and removal ability by bacterial cells

In the present study, I wanted to assess Cd biosorption of *A. humicola* and *B. firmus* from the soil solution extracted from Cd contaminated soil at 150 mg Cd kg^{-1} (i.e. could the bacteria also be capable of taking up metal from soil solution?). For this purpose, Cd in soil solution was obtained by extracting Cd-contaminated soil with water. The bacterial biomass of *A. humicola* and *B. firmus* were then added to the soil solution that contained 12 mg Cd l^{-1}. The results from this study showed that both bacterial strain demonstrated ability to remove Cd from soil solution. Over time, Figure 5.4 shows that the rate of Cd uptake increased rapidly in the first part within 10 minutes of contact. After that, the rate decreased until it reached a constant value after 20 minutes. This result was similar to the result we obtained from Section 5.3.3.1. Biomass of *A. humicola* and *B. firmus* (at the initial concentration of 12 mg l^{-1} soil solution) were able to remove 51.88 and 45.43% Cd from solution, respectively (Figure 5.5).

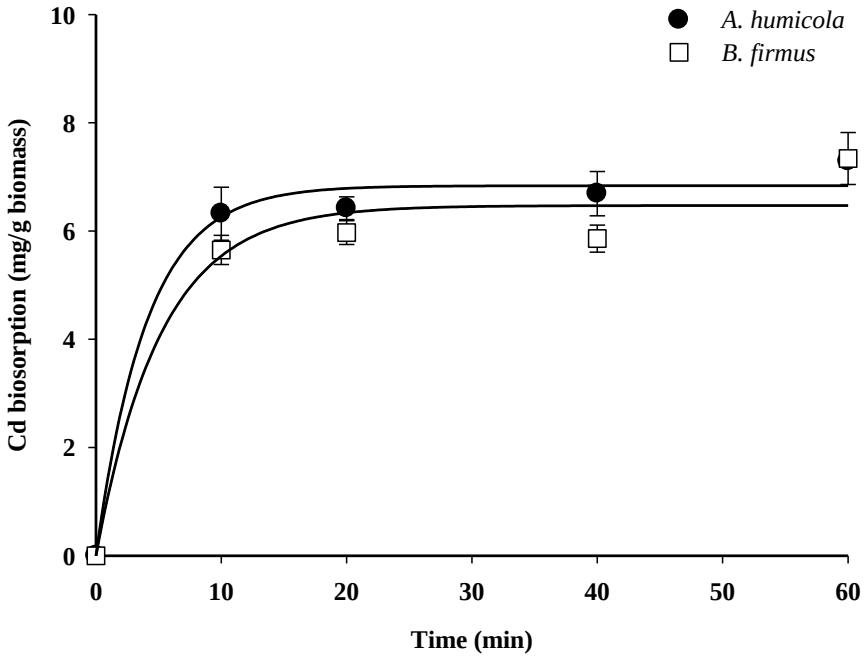

Figure 5.4 Biosorption of Cd extracted from soil solution obtained from Cd-contaminated soil by *A. humicola* and *B. firmus* over the reaction time at the intial concentration 12 mg Cd l^{-1}.

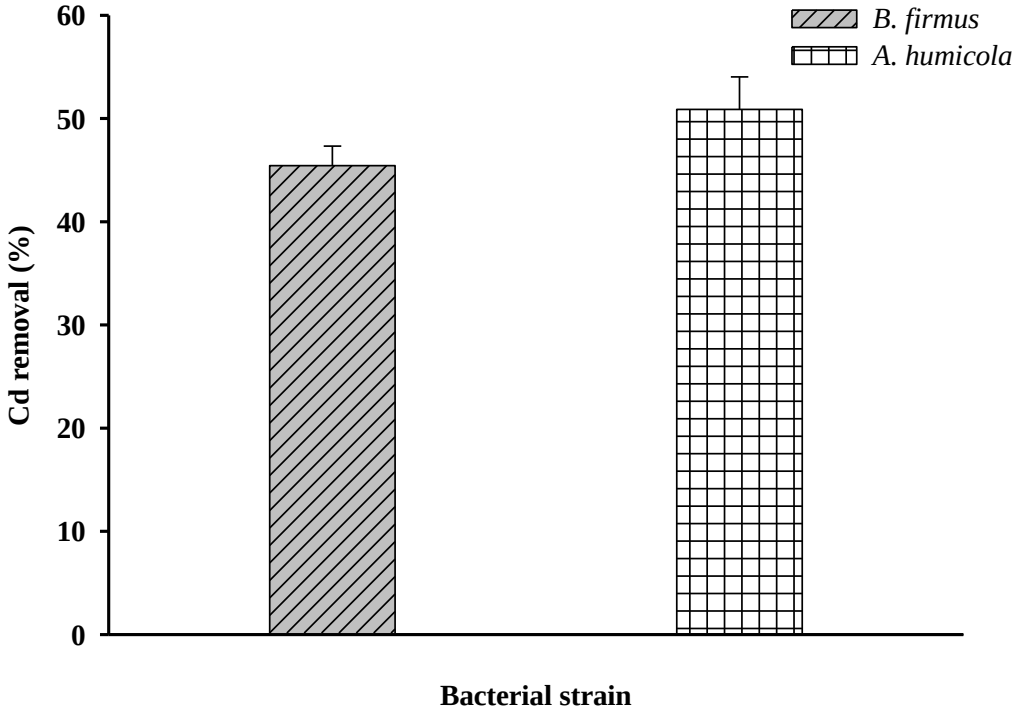

Figure 5.5 Percentage of Cd removal from soil solution by *B. firmus* and *A. humicola* over time at the initial concentration 12 mg Cd l^{-1}.

5.4 Discussion

5.4.1 Isolation of Cd-resistant bacteria and their resistance to Cd toxicity

A significant number of Cd-resistant bacteria were isolated from the rhizosphere soils of the pineapple plants grown in Cd-contaminated soil, suggesting that these bacterial populations had adapted to the presence of Cd. Adaptation of microorganisms to metal pollution is known from the literature (see Section 5.1 Introduction) but as control (non-contaminated soils) were not sampled in this work adaptation of the bacterial population to Cd cannot be confirmed in this study.

Altogether, nine morphologically distinct Cd-resistant bacteria were isolated and 16S rRNA gene sequence analysis showed that the bacteria covered six different genera: *Microbacterium, Bacillus, Arthrobacter, Cellulomonas, Micrococcus, Pseudomonas* and *Sporosarcina*. Previous studies have shown that the genera *Bacillus*, *Micrococcus*, *Arthrobacter*, *Sphingomonas*, and *Microbacterium* are common metal-tolerant Gram-negative and Gram-positive bacteria (Ellis *et al.*, 2003; Piotrowska-Seget *et al.*, 2005; Abou-Shanab *et al.*, 2007b). The results of Zouboulis *et al.*, (2004) showed that the dominant heavy metal resistant bacterium isolated from polluted soil is Gram-positive and in our work the majority of isolates were also Gram-positive. Nasrazadani *et al.*, (2011) suggested that Gram-positive bacteria have the highest resistance to heavy metal because of their ability to absorb and bind metals on the surface of their cell walls. This has been linked to the more complex cell wall structure of Gram-positive bacteria (compared to Gram-negative bacteria) which has been proposed to exert greater control over the absorption of external contaminants such as heavy metals (Beveridge and Murray, 1980; Graham and Beveridge, 1994; Beveridge, 1999). Long-term contact of bacteria with heavy metals can lead to more resistance and this is a major mechanism underpinning the tolerance of bacteria to heavy metals.

All isolated bacteria were tested to determine which was the most Cd-resistant by broth assay. All bacterial strains were able to grow with Cd at high concentration (150 mg l^{-1}) but growth was much reduced (approximately 95%). Two bacterial strains (*B. firmus* and *A. humicola*) were found to be the most resistant to Cd, with an ability to grow slightly better (few percent) in the presence of Cd than the other bacterial strains. Bae *et al.*, (2003) suggested that bacterial isolates are able to survive high Cd concentrations in both

liquid and solid media due to constitutive resistance mechanisms as well as resistance mechanisms that are induced in response to the presence of Cd. Percentage of cell growth at different concentrations of Cd suggest that the resistance against Cd was dependent on the isolates. The variation in metal tolerance might be due to the presence of one or more types of tolerance strategies or resistance mechanisms exhibited by different bacteria such as efflux or binding of toxic ions, enzymatic detoxification, extra/intracellular immobilisation and transformation of the toxic ion to a non-toxic species (Silver and Misra, 1988; Nies, 1992; Hassan *et al.*, 1999).

5.4.2 Metal uptake by bacterial cells

B. firmus and *A. humicola* (which were the most Cd-resistant bacteria) were selected for further study as it was assumed that higher Cd resistance infers a better capacity to retard metal diffusion to the inside of the bacterial cell by biosorbing heavy metals onto the cell surface, or by compartmentalizing the heavy metals (Nies, 1999; Ahalya *et al.*, 2003) i.e. these particular isolates may be useful as microbial inoculants to enhance growth of pineapple in Cd-contaminated soil. The selected isolates were studied with regard to their ability to bind Cd at concentrations varying from 20 to 150 mg Cd l^{-1}. Our study showed that the rate of Cd uptake by bacterial cells increased rapidly within 10 minutes of contact. After that, the rate decreased until it reached a constant value of Cd concentration. The extent of biosorption not only depends on the type of metal ions, but also on the bacterial genus, due to variations in cellular constituents. Very short contact times were generally sufficient to attain metal-bacterial biomass steady state. This is because biomass was either used in the form of fine powder or wet cells; where mass transfer resistances are usually negligible (Vijayaraghavan and Yun, 2008). The short time required for Cd biosorption in this experiment is in accordance with the results obtained by other authors (Volesky, 1990; Sar *et al.*, 1999; Zouboulis *et al.*, 2004). Gabr *et al.*, (2008) showed that the maximum biosorption of lead and nickel was reached after 30 minutes. The rapid rate of removal indicates that passive Cd biosorption to the cell surface was taking place. Mechanisms of cell surface sorption are independent of cell metabolism; they are based upon physicochemical interactions between metal and functional groups of the cell wall. Since this process is independent of the metabolism, binding of metals is very quick up (Kaduková and Virčíková, 2005; Gabr *et al.*, 2008). Active metal uptake mechanisms (i.e. those involving bioaccumulation) were not examined in this work.

The results described in this chapter also showed that the Cd biosorption (mg Cd per g biomass) increased with an increase in initial Cd concentration. The level of Cd taken up per g biomass by both bacteria in this study compare favourably with previous studies on Cd uptake. For example, *Bacillus thuringiensis* (El-Helow *et al.*, 2000) had a biosorption capacity of 11.72 mg Cd per g biomass while *A. humicola* and *B. firmus* had biosorption capacities of 19.83 and 17.21 (at an initial level of 50 mg Cd l^{-1}). In contrast, the percentage of Cd removal decreased with an increase in initial Cd concentration and *A. humicola* removed more Cd from the solution than *B. firmus*. The decrease in percentage of Cd removal at higher Cd levels may be attributed to a lack of sufficient free sites for Cd biosorption i.e. metal binding sites become saturated with Cd and the bacterial cell surface is not capable of binding any more Cd. At lower concentrations, Cd ions present in the solution could interact with the binding sites of bacterial cells and thus the percentage of Cd removal is higher than those observed at higher Cd concentrations. Similar results were obtained by other researchers (Pardo *et al.*, 2003; Kaduková and Virčíková, 2005; Lu *et al.*, 2006; Tunali *et al.*, 2006; Gabr *et al.*, 2008). These authors stated that the metal removal capacity of the biomass was decreased by increasing the initial concentration of heavy metals.

In the present work, the ability of the two isolates to remove metal from soil solution was also examined. Soil solution is known to contain many types of compounds known to complex metals e.g. organic ligands (Jones and Brassington, 1998; Violante *et al.*, 2010) and this complexation may reduce the amount of Cd taken up by the bacteria. If the bacteria were to be used in further work as soil inoculants to potentially reduce Cd uptake by pineapple plants grown in Cd-contaminated soil, it was important to show that they were both able to remove Cd from soil solution. The hypothesis assumes that addition of Cd complexing bacteria to soil should reduce Cd availability to the plant thereby reducing Cd effects on the plant. Accordingly a soil extract was made from contaminated soil (12 mg Cd l^{-1}) and bacteria were exposed to this extract. Both bacterial strains showed good ability to remove Cd from the soil solution demonstrating that they were able to either compete for Cd ions with soil complexing agents or were able to remove Cd complexed to ligands found in soil solution.

5.5 Conclusions

Nine Cd-tolerant bacterial strains were isolated from the root zone of pineapple (*A. comosus* L.) grown in Cd-contaminated soils. All bacterial strains showed tolerance to Cd at 150 mg Cd l^{-1}. The two most resistant strains, *B. firmus* and *A. humicola* were selected for further study of their Cd binding capacity. Both isolates showed excellent Cd biosorption capacity and were also able to remove Cd from soil solution. Based on these results these bacteria were used as soil inoculants in further studies (Chapter VI) to examine their ability to reduce the toxic effects of Cd on pineapple as observed in earlier work in this thesis.

Chapter VI

Use of bacterial inoculants to promote pineapple growth in Cd-contaminated soil

6 Use of bacterial inoculants to promote pineapple growth in Cd-contaminated soil

6.1 Introduction

Cd is a common heavy metal contaminant in the environment (Sheng *et al.*, 2008). It is a non-essential element in metabolic processes in plants and animals, and it can accumulate to levels that are toxic to organisms (Brune and Dietz, 1995). Previous work in this thesis found that Cd exposure in pineapple plants inhibited plant growth and had effects on CAM biochemistry such as reducing net CO_2 uptake, plant light-use efficiency and carbohydrate metabolism. Therefore, the potential use of bacterial inocula to improve pineapple growth under Cd-stressed conditions was investigated in this chapter.

Microbial activity strongly influences metal speciation and transport in the environment (Lors *et al.*, 2004). Soil bacteria have been used to enhance crop production for decades. Some of the main functions of these bacteria are nutrient supply to crops (e.g. nitrogen fixation), stimulation of plant growth by plant hormone production, control or inhibition of the activity of plant pathogens and improvement of soil structure (Brierley, 1985; Davison, 1988; Ehrlich, 1990). Improvement of the interactions between plants and beneficial rhizosphere microorganisms can enhance biomass production and tolerance of the plants to heavy metals (Wenzel *et al.*, 1999; Glick, 2003). Many soil bacteria in the genera *Arthrobacter*, *Klebsiella*, *Pseudomonas* and *Bacillus* have been reported to be resistant to Cd and other toxic metals and play important roles in mobilization or immobilization of heavy metals (Gadd, 1990; Roane and Pepper, 1999; Filali *et al.*, 2000; Sharma *et al.*, 2000; Idris *et al.*, 2004). Bacterial inocula have been reported to improve plant growth by producing the essential elements for plant growth under nutrient imbalance conditions, under stress and metal contamination (Egamberdiyeva and Höflich, 2004). Creus *et al.*, (2004) suggest that inoculation with bacteria can result in a significant change in various plants growth parameters, which may affect crop yield.

Many researchers have used rhizosphere microbes to reduce the toxicity of heavy metals to plants (Burd *et al.*, 1998; Rajkumar *et al.*, 2006; Vivas *et al.*, 2006). Belimov *et al.*, (2005) found that Cd-resistant bacterial inoculants isolated from the rhizosphere of *B. juncea* could improve growth and development of plants in the presence of toxic Cd concentrations. Moreover, the study of Belimov *et al.*, (2001) demonstrated that the application of Cd-resistant bacteria protected plants against the toxic effects of Cd and

effectively promoted the growth of *B. napus*. Sinha and Mukherjee, (2008) demonstrated that Cd-resistant bacteria improved plant growth and reduced Cd uptake by pumpkin and mustard plants grown in Cd-amended soil.

Research to determine the potential effects of Cd-resistant bacteria on the growth and Cd uptake of pineapple in Cd-contaminated soil has not been performed. The previous work in Chapter II revealed that although pineapple accumulates Cd, the plants can grow in Cd-contaminated soil. However, Cd is generally toxic to pineapple impairing metabolism and reducing plant growth in comparison to plants grown in Cd-free soil. In Chapter V, Cd-resistant bacteria (*B. firmus* and *A. humicola*) were isolated from the rhizosphere of pineapple grown in Cd-contaminated soil and were shown to have good Cd-binding capacity. Therefore, in this chapter we tested the hypotheses that the interaction between Cd-resistant bacteria and pineapple plants may promote plant growth and decrease metal toxicity in pineapple grown in Cd-amended soil.

Specific objectives were to:

a) examine the effect of bacterial inoculations on photosynthetic performance by measurements of leaf chlorophyll fluorescence and 24 hour patterns of net CO_2 assimilation.
b) examine the effect of bacterial inoculations on CAM biochemistry by measuring day/night change in leaf titratable acidity, starch and sugar contents.
c) examine the effect of bacterial inoculation on plant biomass by measurements of dry weight of different ages of leaves and of root tissue.
d) examine the effect of bacterial inoculation on soil and plant nutrients by measurements of the N, P, K contents of soil and plant tissues.
e) examine the effect of bacterial inoculation on Cd accumulation in pineapple by measurements of Cd contents of different ages of leaves and of root tissue.

6.2 Materials and Methods

6.2.1 Pot experiments

The effect of Cd-resistant bacteria on plant biomass, plant biochemistry, plant nutrient and Cd uptake by pineapple growing in Cd-contaminated soil was conducted via pot experiments. Soil was made up in pots containing a mixture of sand (East Riding Horticulture Ltd., UK) and John Innes No. 2 compost (John Innes Manufacturers Association; UK) in the ratio of 1:1. The soil was thoroughly mixed with $Cd(NO_3)_2$ (Sigma-Aldrich) solution to obtain 150 mg Cd kg^{-1} soil and was incubated (aged) for four weeks without plants before being remixed and used as a growing medium for pineapple plants. Suckers of pineapple (*Ananas comosus* L.) approximately 1.0 cm diameter were potted up in 10 cm plastic pots containing a mixture of sand and John Innes No. 2 compost in the ratio of 1:1 without Cd application until the roots became exposed at the base of the pot (approximately 4 weeks), plants were then remove to potted up in a bigger plastic pots (14 cm) containing a mixture of sand and John Innes No. 2 compost in the ratio of 1:1 without Cd application until the roots became exposed at the base of the pot (approximately 4 weeks). Plants were removed and potted up in 18 cm plastic pots contained Cd-contaminated soil (150 mg Cd kg^{-1}) with the following three treatments: (a) control (non-inoculated), (b) inoculated with *B. firmus* and (c) inoculated with *A. humicola*. Cd-contaminated soil (150 mg Cd kg^{-1}) from the initial incubation time was used as control to compare the reduction of Cd concentration in soil of inoculated plants and non-inoculated plants.

For inoculation, the Cd-resistant bacteria were grown in Nutrient broth (NB). Cells in the exponential phase were collected by centrifugation (Sorvall Regend RT, DJB Labcare, UK) at 4600 xg for 10 minutes at 4 ºC, washed with phosphate buffer (Sigma-Aldrich, USA) and centrifuged again. Bacterial inocula were prepared by re-suspending pelleted cells in sterile Ringer´s solution. The inoculum at the rate of 10 ml per pot (10^8 cfu ml^{-1}) was applied to the soil near root zone at the time of transplantation, and then applied at 15-day interval until harvesting. For non-inoculated controls, equal volume of sterile Ringer´s solution was added to soil near root zone. The plants were grown in a growth chamber room at 27 ºC (day) and 19 ºC (night) under a 12 hour light/dark photoperiod with photon flux density of 250 µmol m^{-2} s^{-1} at plant height. The water content of the soil was maintained at 60% of its holding capacity by watering twice a week. Plants were

acclimated to these conditions for 16 weeks before harvesting and collecting data. After harvesting, the remaining plants were acclimated under a light intensity of 250 µmol m^{-2} s^{-1} at plant height for 2 weeks and then moved to acclimate under a light intensity of 500 µmol $m^{-2}s^{-1}$ for 14 weeks prior to data collection.

6.2.2 Chlorophyll fluorescence

The maximum quantum yield of PSII (F_v/F_m) was determined as previously described (Section 2.2.3), using the Plant Efficiency Analyser (PEA; Hansatech Instruments Ltd, King's Lynn, Norfolk).

6.2.3 Net CO_2 uptake

Gas exchange was measured using an open infra-red gas exchange system (Heinz Walz, Effeltrich, Germany) as previously described (Section 2.2.4).

6.2.4 CAM biochemistry

Leaf titratable acidity was determined as described in Section 2.2.5. The determination of starch and soluble sugar contents have also been previously described (Section 2.2.6 and 2.2.7, respectively).

6.2.5 Soil sampling strategy

Every four weeks, soil samples from each pot were taken around the edges of the pot (bulk soil) and near the root zone (rhizosphere soil) by using cylindrical cores 1.5 cm in diameter. Soil samples were air-dried at room temperature and were ground to pass a 2 mm sieve, homogenized and stored for subsequent analysis of pH and extractable Cd in soil. At the end of the experiment, plants were removed from the pots and bulk soil was separated from rhizosphere soil by hand to remove soil that did not adhere to the root. The soil attached to the roots was carefully removed from the root by shaking very gently. Fresh bulk soil and rhizosphere soil were stored at 4 ºC until analysis (nitrogen determination). The remaining soil at the final harvesting period was air-dried at room temperature and ground to pass a 2 mm sieve, homogenized and used for analyses pH,

extractable Cd, available phosphorus (P), available potassium (K) and total Cd concentration in soil.

6.2.5.1 pH measurement

The soil pH was determined as previously described (Section 3.2.2.2).

6.2.5.2 Extractable Cd in soil

The soil samples were extracted with 0.1 M $Ca(NO_3)_2$ (Sigma-Aldrich, USA) as previously described (Section 3.2.2.3). The filtrates were then analysed using ICP-OES.

6.2.5.3 Total Cd in soil

The soil samples were extracted by using the Aqua Regia Digest method as previously described (Section 3.2.2.4). The filtrates were then analysed using ICP-OES.

6.2.5.4 Ammonium and nitrate determination in soil

Ammonium and nitrate levels in soil were determined as described by Keeney and Bremner, (1966). Ten grams of moist soil that had passed a 2 mm sieve were placed into 100 ml polypropylene bottles. Exactly 50 ml of 2 M KCl solution (Fisher Scientific, UK) was added to the polypropylene bottles. Samples were shaken on a horizontal shaker for 1 hour and soil extracts were then filtered through a Whatman No. 40 filter paper to produce a clear extract. The filtrates were analysed using Autoanalyser 3 (Bran+Luebbe, Australia).

6.2.5.5 Available potassium (K) in soil

Available K was determined according to Ministry of Agriculture, Fisheries and Food (1973). Soil (10 g and sieved to 2 mm mesh sieve) was added to a flask and 50 ml of 1 M ammonium acetate added. The soil samples were then shaken at 200 rpm on a horizontal shaker for 30 minutes and filtered through a Whatman No. 2 filter paper. The filtered extract was used for the determination of K by using a flame photometer (Model PEP7, JENWAY, UK). Standard K in a rage from 0-25 mg K per litre was prepared from K

standard solution 1000 mg K per litre (Sigma-Aldrich, USA). The instrument for K analysis was set up as detailed in the instruction manual (JENWAY, USA). The instrument was adjusted to read zero using deionised water then the instrument was set to read 200 using the highest standard concentration (25 mg K l^{-1}) and successively measured the 5, 10, 15 and 20 mg K l^{-1} solution three times each. The mean reading of each standard concentration was constructed a calibration graph. The samples were analysed the concentration of K in the soil sample solution by referring to the calibration graph.

6.2.5.6 Available phosphorus (P) in soil

Available P was determined according to Ministry of Agriculture, Fisheries and Food (1973). An air dried soil sample was ground to pass a 2 mm mesh sieve and then soil (5.0 g) was added to a polypropylene bottle. Sodium bicarbonate (50 ml) was added to the bottle and the mixture shaken at 200 rpm on a horizontal shaker for 30 minutes. Extract solutions were filtered through a Whatman No. 2 filter paper and the filtrate used for the determination of phosphate. Exactly 5 ml of each soil extract was added to a 100 ml Erlenmeyer flask. 0.15% (w/v) ammonium molybdate (20 ml) (Fisher Scientific, UK) and 5 ml of 1.5% ascorbic acid solution (Fisher Scientific, USA) were added and the resulting solution allowed to stand for 30 minutes for complete colour development. The samples were then analysed with spectrophotometer (Biochrom Libra S11 Visible Spectrophotometer, UK) at 712 nm.

6.2.6 Plant sampling strategy

After growing pineapple plants in Cd-contaminated soil for 16 weeks and 32 weeks, plants were carefully removed from the soil and separated into shoots and roots. The shoots and roots were washed thoroughly with tap water and then with distilled water. To remove surface adsorbed metal ions from the roots, the roots were washed extensively with 10 mM $CaCl_2$ (Fisher Scientific, UK) and then with distilled water. Leaves were separated into different ages as older leaves (lower leaves), intermediate leaves (middle leaves) and younger leaves (upper leaves).

6.2.6.1 Plant biomass

Plant biomass was determined as previously described (Section 2.2.9).

6.2.6.2 Total Cd uptake by pineapple

Plant samples were digested using the nitric perchloric acid digest method as previously described (Section 3.2.3.2).

6.2.7 Plant nutrient analysis

6.2.7.1 Determination of total nitrogen (N) and carbon content (C) in plants

Plant samples (lower leaves, middle leaves, upper leaves and roots) were oven-dried at 70 ºC for 72 hours, ground in a pestle and mortar to pass a 1 mm mesh sieve. Dried plant leaf samples (100 mg) were weighed into a tin foil cup. The cup was carefully folded and squashed into a pellet to expel the air using a tool provided by ELEMENTAR. Nitrogen and carbon analysis was carried out on a Vario Macro Cube (ELEMENTAR Analysensysteme GmbH, Germany). Working temperature of the combustion tube was 960 ºC and that of the reduction tube was 830 ºC. Helium (99.996%) was used as the carrier gas (120-125 kPa) at the flow rate of 600 ml min^{-1} and oxygen (99.995%) (200.0 kPa) as oxidizing agent. Sulfanilamide (ELEMENTAR Analysensysteme GmbH, Germany) was used as the standard for C and N elemental analysis.

6.2.7.2 The preparation of plant material by dry combustion for P and K determination

Plant samples (lower leaves, middle leaves, upper leaves and roots) were oven-dried at 70 ºC for 72 hours, ground in a pestle and mortar to pass a 1 mm mesh sieve. Two grams of each sample was transferred into a crucible and placed in a muffle furnace (Model HRF7/45, Carbolite, UK). The temperature of the muffle furnace was increased to 450 ºC and maintained at this temperature until a whitish-grey ash remained. The crucible was removed from the muffle furnace, cooled and cover with a watch glass. 6 M HCl (10 ml) (Fisher Scientific, UK) was added and the mixture placed in a steam water bath to evaporate the solution to dryness. The crucible was also heated over a small flame until the residue was visibly dry. The residue was moistened with 2 ml of HCl (36% w/w HCl), covered with a watch glass and gently boiled for 2 minutes. Approximately 10 ml of

water was added and the mixture boiled again. The crucible was removed and the watch glass rinsed, collecting the washings in the crucible. The contents of the basin were transferred into a 50 ml graduated flask and diluted to 50 ml before being filtered through Whatman No. 2 filter paper.

6.2.7.3 Determination of K in pineapple

Solution (1.0 ml) prepared as described in Section 6.2.7.2 was added to a 100 ml graduated flask and diluted to 100 ml. The K content in the plant extract was determined using a flame photometer (Model PEP7, JENWAY, UK) as described in Section 6.2.5.5. Standard concentrations in a range 0-25 mg K per litre were prepared to construct a calibration curve for all of the samples.

6.2.7.4 Determination of P in pineapple

Plant extract solution (5 ml) prepared as described in Section 6.2.7.2 was added to a 100 ml of Erlenmeyer flask. Exactly 20 ml of 0.15% (w/v) ammonium molybdate (Fisher Scientific, UK) solution and 5 ml of 1.5% ascorbic acid (Fisher Scientific, UK) solution was added to the plant solution and allowed to stand for 30 minutes for complete colour development. The samples were then analysed with a spectrophotometer (Biochrom Libra S11 Visible Spectrophotometer, UK) at 712 nm by using P standards in the rage 0-25 mg P per litre.

6.2.8 Statistical analysis

All data presented are the mean values of three replicates. Values are expressed as means ± S.E. in each group. All statistical analyses were performed using the statistical analysis package SPSS 17.0. Variance analysis was performed on all experimental data and significant differences ($P < 0.05$) between individual means (three replicates) was analysed using a post hoc Least Significant Difference test.

6.3 Results

6.3.1 Effect of inoculation on photosynthetic performance

6.3.1.1 The effect of inoculation on potential quantum yield of photosynthesis in pineapple and acclimation to contrasting light intensities

Using chlorophyll fluorescence technique to analyse the maximum quantum yield of photosystem II (PSII) revealed significant differences in F_v/F_m between pineapple inoculated with bacterial strains and non-inoculated pineapple (control) (Figure 6.1). Inoculated plants (with both bacterial species used) had a higher maximum quantum yield of PSII than control. Plants inoculated with *B. firmus* showed higher F_v/F_m under light intensity 500 µmol m^{-2} s^{-1} compared to 250 µmol m^{-2} s^{-1}. Under high light intensity, inoculated plants were able to acclimate to the increase in light intensity, as indicated by the maintenance or indeed slight increase (plants inoculated with *B. firmus*) in F_v/F_m values compared to those measured at the lower light intensity. F_v/F_m showed a gradual decline in non-inoculated plants over the last 5 weeks of the experiment (Figure 6.1).

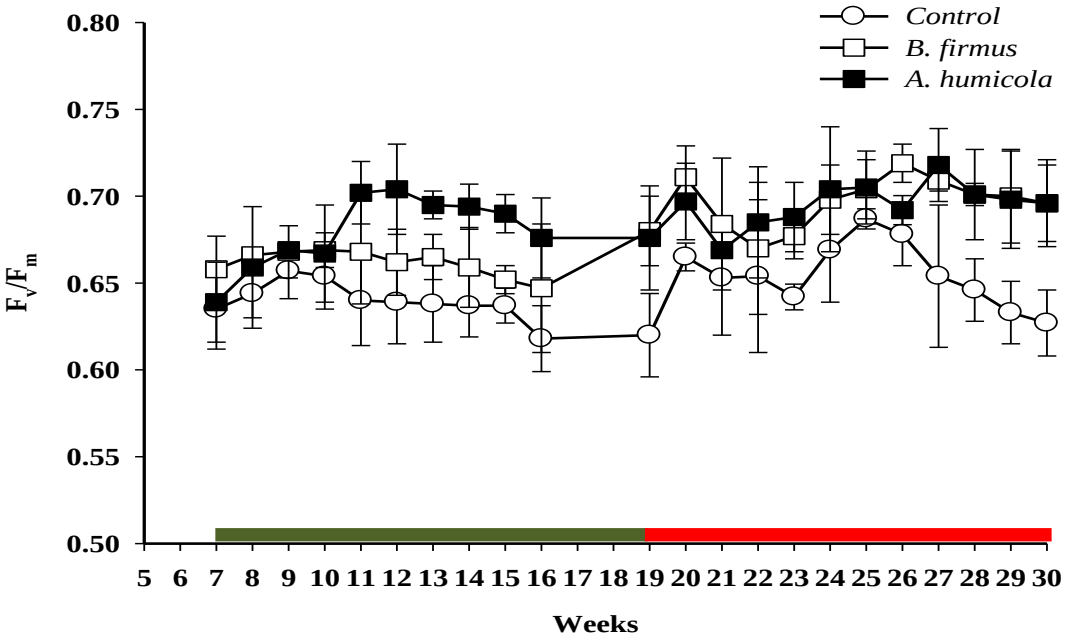

Figure 6.1 The maximium quantum yield of photosystem II (F_v/F_m) for D-leaves of *A. comosus* exposed to 150 mg Cd kg^{-1} soil inoculated with *A. humicola* and *B. firmus* compared with controls (non-inoculated). Values are the means of three replicates ± standard error. The green and red bars on the x axis represent the contrasting light regimes of 250 µmol m^{-2} s^{-1} and 500 µmol m^{-2} s^{-1}, respectively.

6.3.1.2 The effect of inoculation on net CO_2 uptake in pineapple grown in Cd contaminated soil

Net CO_2 assimilation was monitored in control and treated plants at the end of 16 weeks of the experimental period under light intensity 250 µmol m^{-2} s^{-1} and after a further 14 weeks under high light intensity of 500 µmol m^{-2} s^{-1} (Figure 6.2). A significant increase in net CO_2 uptake was found in pineapple grown in Cd-contaminated soil inoculated with *B. firmus* and *A. humicola* when compared with non-inoculated plants. The results show that plants inoculated with *A. humicola* had a greater net CO_2 uptake than plants inoculated with *B. firmus*. Under different light regimes, net CO_2 uptake of plants acclimated under high light intensity was significantly lower than plants acclimated under low light intensity.

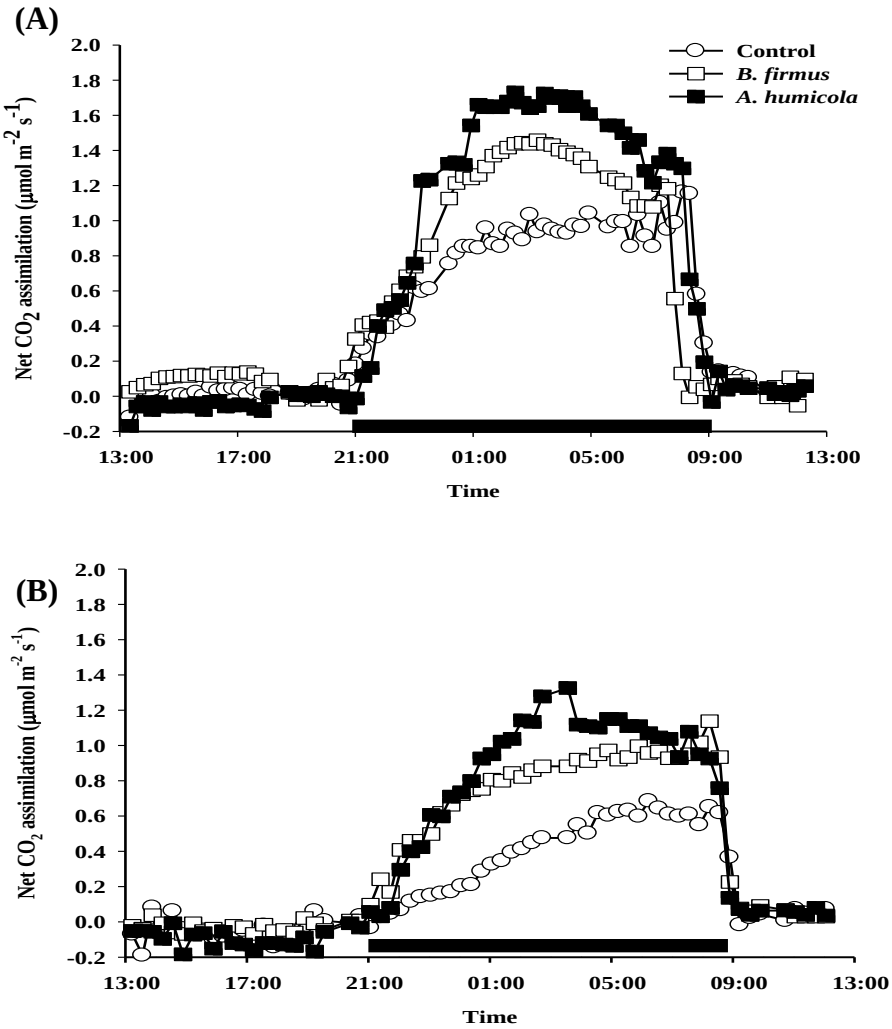

Figure 6.2 Rates of net CO_2 uptake from leaves of *A. comosus* grown in Cd-contaminated soil inoculated with *A. humicola* and *B. firmus* compared with control after 16 weeks under light intensity of 250 µmol m^{-2} s^{-1} (A) and after a further 14 weeks under high light intensity of 500 µmol m^{-2} s^{-1}. The shape of the assimilation curve, recorded over 24 hours, illustrated the day/night pattern of CAM activity at different Cd concentrations. The black bar on the x axis represents the period of darkness. Each gas exchange curve is representative of that from three separate plants.

The total net CO_2 uptake over 24 hours for pineapple grown in Cd-contaminated soil with or without bacterial inoculations was calculated (Table 6.1). The results revealed that bacterial inoculations significantly increased the net CO_2 uptake in pineapple leaves compared to control without inoculation. No significant difference in the total net CO_2 uptake was observed between pineapple inoculated with *B. firmus* and *A. humicola*. Under different light regimes, the net CO_2 uptake of pineapple leaves under high light intensity was significantly suppressed when compared with low light intensity. However, the bacterial inoculations increased the net CO_2 uptake compared to non-inoculated plants under the high light regime (Table 6.1).

Table 6.1 Net CO_2 uptake over 24 hours (mmol CO_2 m^{-2}) of pineapple grown in Cd-contaminated soil inoculated with bacterial strains *B. firmus* and *A. humicola* compared to control under different light regimes.

Treatment	Low light intensity (250 µmol m^{-2} s^{-1})	High light intensity (500 µmol m^{-2} s^{-1})
Control (non-inoculated)	49.37 ± 3.46^{a}	38.72 ± 3.05^{c}
B. firmus	71.77 ± 5.53^{b}	50.24 ± 1.78^{d}
A. humicola	84.03 ± 8.88^{b}	57.28 ± 2.79^{d}

Different letters in columns and rows denote a significant difference between groups, such that groups not sharing a similar letter are significantly different from each other ($P < 0.05$).

6.3.2 Effect of inoculation on CAM biochemistry

6.3.2.1 Leaf titratable acidity

Figure 6.3 shows leaf titratable acidity expressed on an area basis at the beginning (dawn) and end (dusk) of the photoperiod for control (Cd-contaminated soil without inoculation) and plants grown in contaminated soil inoculated with *B. firmus* and *A. humicola*. All plants showed a typical CAM pattern of changes in titratable acidity, with an increase in acid content during the dark period, followed by a progressive decrease during the light phase. The CAM activity (measured as overnight acid accumulation) was significantly increased in inoculated plants compared to the control (non-inoculated). However; no significant differences in overnight acidity accumulation was observed between pineapple inoculated with *B. firmus* and *A. humicola*. After transfer to the higher light intensity,

plants showed less CAM activity (i.e. less dawn/dusk difference in titratable acidities) compared to plants under the lower light. This is in agreement with the lower rates of dark CO_2 uptake noted for plants grown under higher PFD (Figure 6.2).

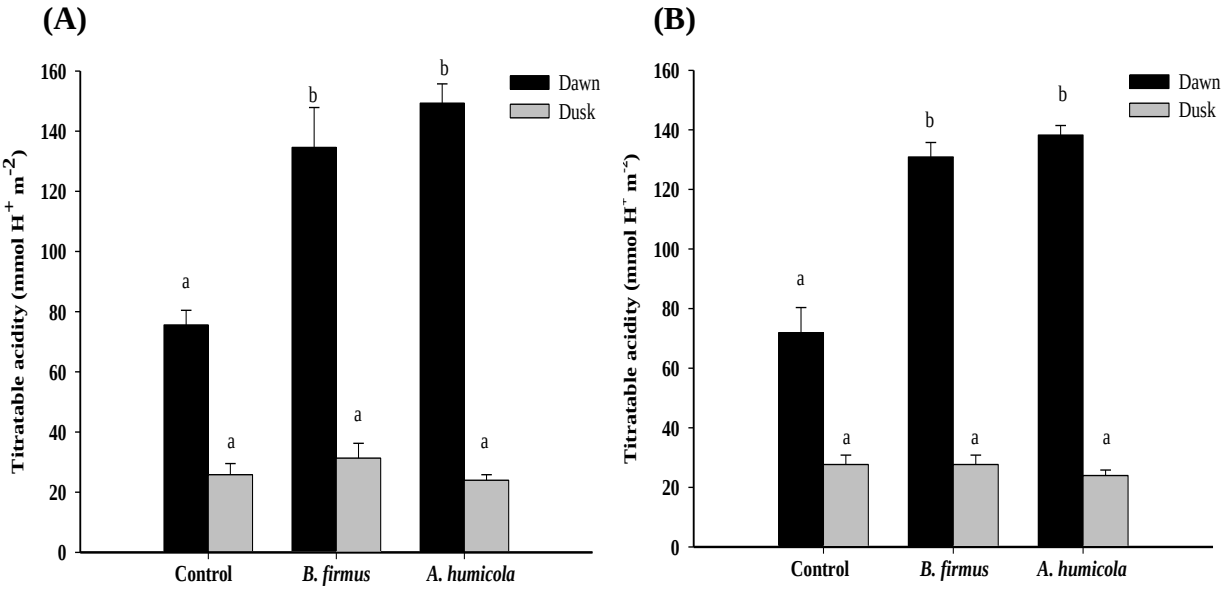

Figure 6.3 Influence of bacterial inoculation on leaf titratable acidity of pineapple plants grown in soil treated with 150 mg Cd Kg^{-1} for 16 weeks under light intensity of 250 µmol m^{-2} s^{-1} (A) and after a further 14 weeks under high light intensity of 500 µmol m^{-2} s^{-1} (B), expressed per unit leaf area. Plant leaf samples were taken at dawn and dusk. Data represent the means of three replicates with standard error. Different letters in the same photoperiod (Dawn or Dusk) indicate a significant difference between treatments at the level of $P < 0.05$.

6.3.2.2 Starch and soluble sugar content

Starch and soluble sugar contents were significantly increased in pineapple inoculated with bacterial strains compared to non-inoculated control plants (Figure 6.4A; 6.4B; 6.4C and 6.4D). However; no significant differences of starch and soluble sugar contents were found between plants inoculated with *B. firmus* and *A. humicola*. Under high light regimes, the total starch and soluble sugar contents were decreased when compared to those measured under low light intensity (Figure 6.4B; 6.4D).

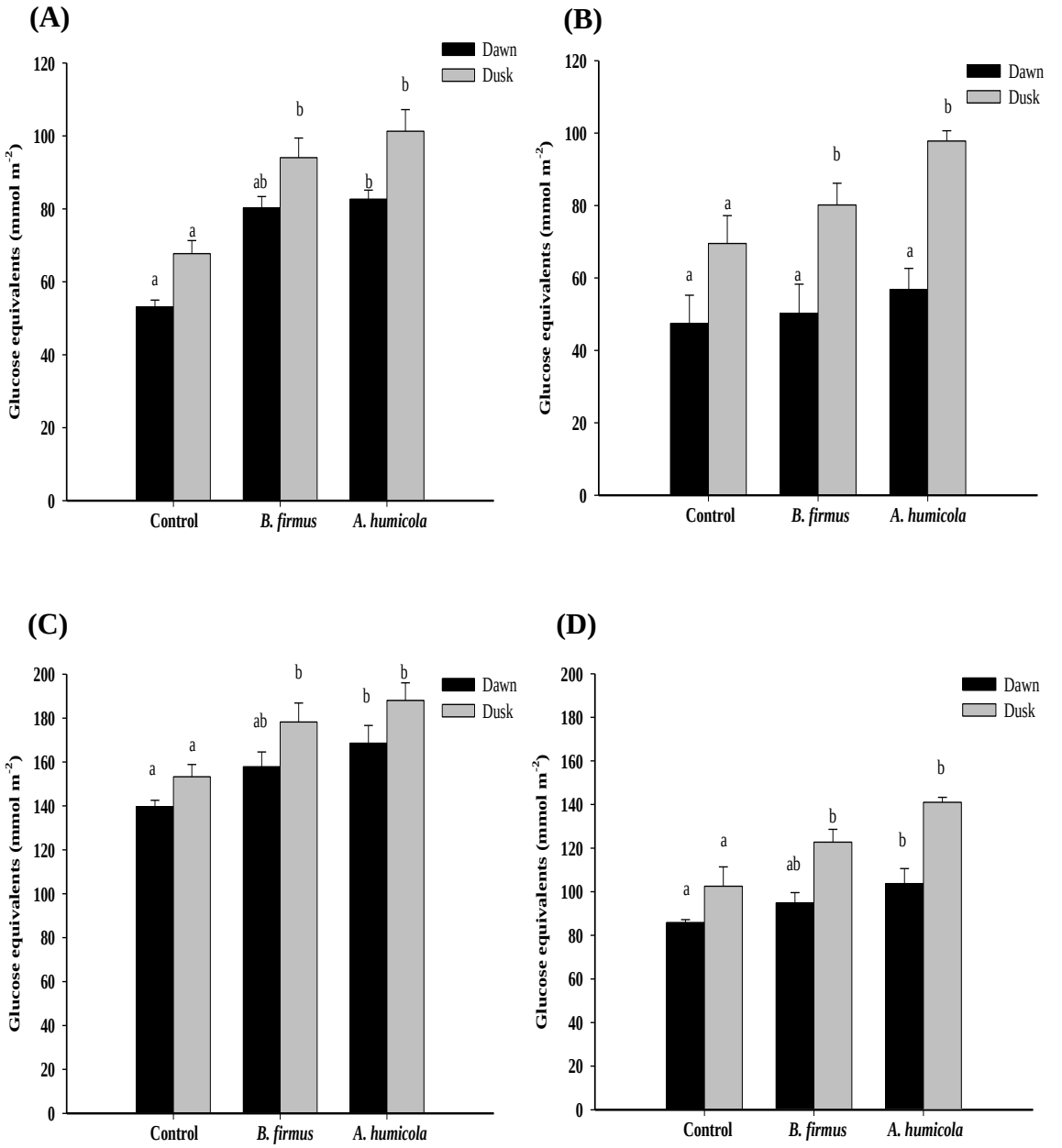

Figure 6.4 Influence of bacterial inoculation on starch (A, B) and soluble sugar content (C, D) of pineapple plants grown in soil treated with 150 mg Cd kg^{-1} for 16 weeks under light intensity of 250 µmol m^{-2} s^{-1} (A, C) and after a further 14 weeks under high light intensity of 500 µmol m^{-2} s^{-1} (B, D), expressed per unit leaf area. Plant leaf samples were taken at dawn and dusk. Data represent the means of three replicates with standard error. Different letters in the same photoperiod (Dawn or Dusk) indicate a significant difference between treatments at the level of $P < 0.05$.

6.3.3 Effect of inoculation on plant biomass

Inocula of *B. firmus* and *A. humicola* were applied to the pots in which pineapples were grown in Cd-contaminated soil at 150 mg Cd kg^{-1}. The plants were harvested at 16 weeks under light intensity of 250 µmol m^{-2} s^{-1} and after another 14 weeks under high light intensity of 500 µmol m^{-2} s^{-1}. Dry weights of leaves and roots were determined. No visible symptoms of Cd toxicity such as chlorosis and/or necrosis of leaves were observed in either inoculated plants or non-inoculated plants (Figure 6.5).

Figure 6.5 Pineapple grown in Cd contaminated soil (150 mg Cd kg^{-1}) incubated for 32 weeks with bacterial inoculants (*B. firmus* and *A. humicola*) compared to control (non-inoculated plants).

This study demonstrated that bacterial inoculation could promote pineapple growth in Cd exposed plants. For above ground tissue, significant increases in dry weight of lower leaves, middle leaves and upper leaves of pineapple plants were observed when the soil was incubated with *A. humicola* for 32 weeks compared to control. In addition, significant increases in dry weight of lower leaves and middle leaves were observed in plants incubated with *B. firmus* for 32 weeks compared with control. However, no significant difference in dry weight of upper leaves in plants incubated with *B. firmus* was observed compared to control. Significant increases ($P < 0.05$) in root dry weight were observed in pineapple inoculated with *A. humicola*. However no significant increases in root dry

weight of pineapple inoculated with *B. firmus* were observed compared with control (Figure 6.6).

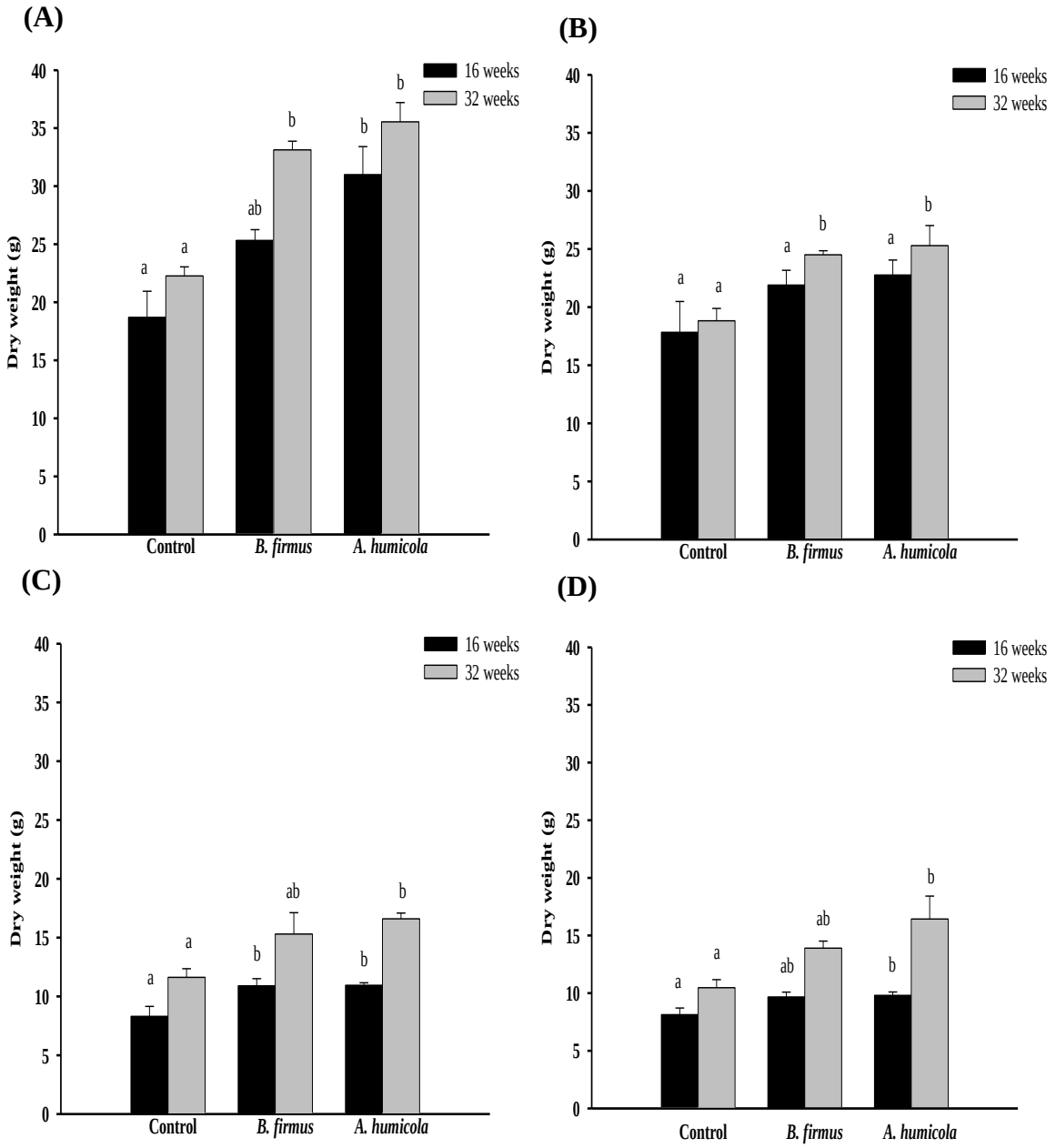

Figure 6.6 The influence of bacterial strain on lower leaves (A), middle leaves (B), upper leaves (C) and root dry weight (D) of pineapple on soil treated with 150 mg Cd kg^{-1} compared with control (uninoculated). The plants samples were harvested at 16 weeks under low light intensity of 250 µmol m^{-2} s^{-1} and after a further 14 weeks under high light intensity of 500 µmol m^{-2} s^{-1}. The leaves were taken from younger leaves (upper leaves), intermediate leaves (middle leaves), and older leaves (lower leaves). Values shown are the means of three replicates. Different letters in the same harvesting period (16 weeks or 32 weeks) indicate a significant difference between treatments at the level of ($P < 0.05$, Least Significant Difference test).

6.3.4 Effect of inoculation of soil pH

Soil solution was extracted from the bulk soil and rhizosphere soil of pineapple plants for pH analyses every 4 weeks. Figure 6.7 shows the effect of bacterial inoculations on soil solution pH in both bulk soil and rhizosphere soil compared with control (non-inoculated). Generally, the pH slightly decreased throughout the experimental period. Rhizosphere pH was decreased slightly in *B. firmus* inoculated soil at some time points.

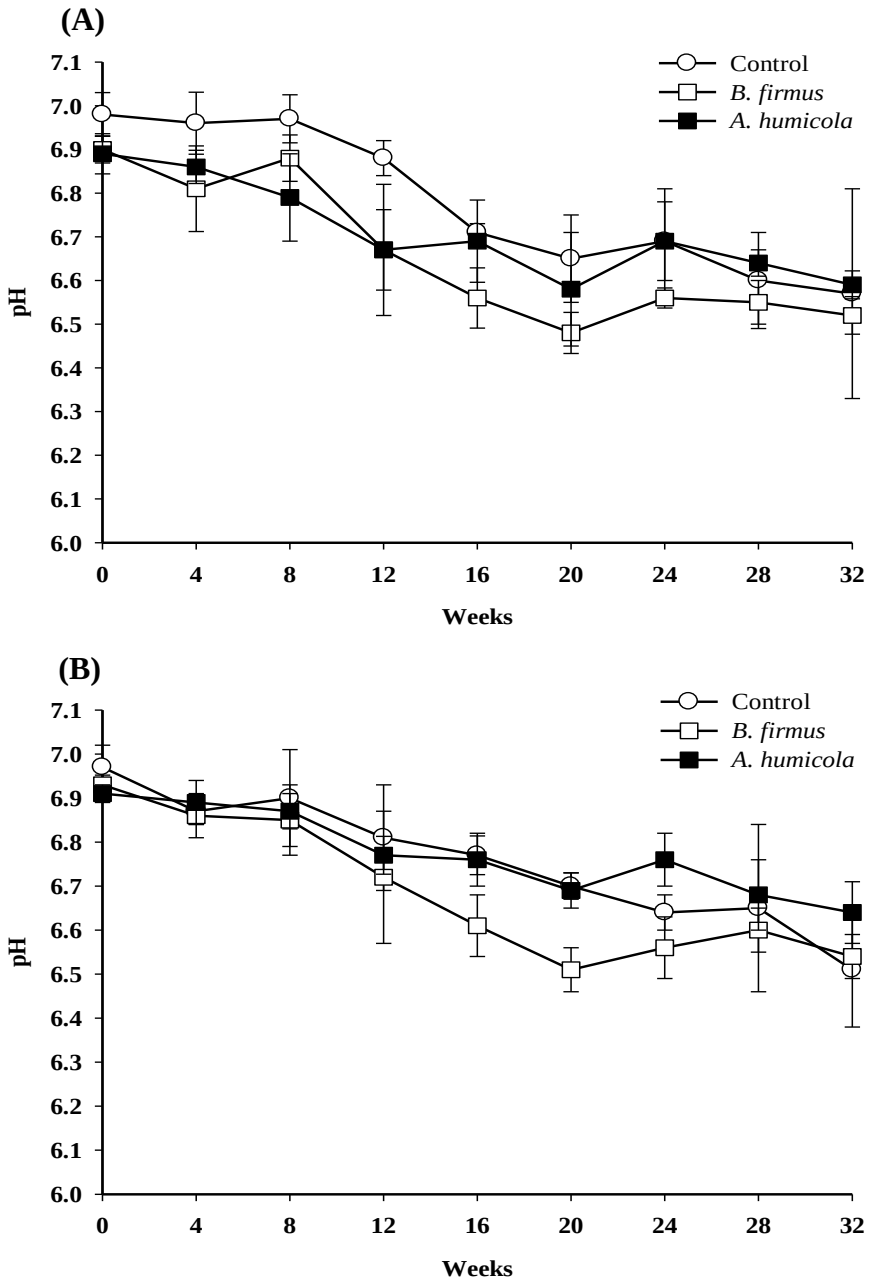

Figure 6.7 pH in the bulk soil and rhizosphere soil of pineapple grown in Cd-contaminated soil incubated with two strains of bacterial inoculations (*B. firmus* and *A. humicola*) compared with control (non-inoculated plants): (A) bulk soil (B) rhizosphere soil. Values are the means of three replicates ± one standard error.

6.3.5 Effect of bacterial inoculation on extractable Cd in soil

A reduction in extractable Cd was observed in all treatments of pineapple grown in Cd-contaminated soil with time. Levels of extractable Cd in bulk soil were higher than those observed in rhizosphere soil. At 16 week, significant difference in extractable Cd levels in rhizosphere soil of plants inoculated with *B. firmus* was observed compared with non-inoculated control.

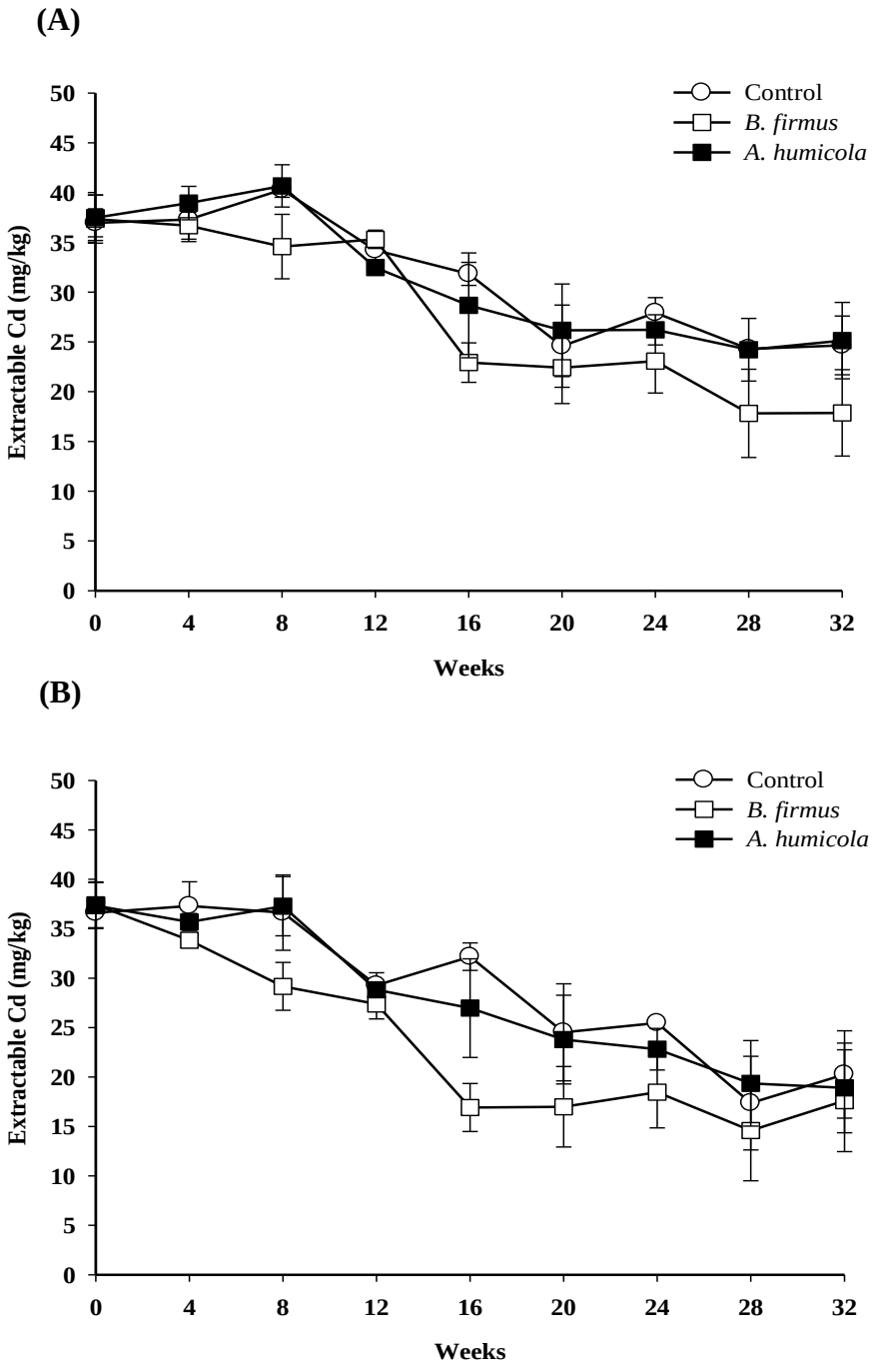

Figure 6.8 Extractable Cd in soil extracts from pineapple grown in Cd-contaminated soil incubated with two strains of bacterial inoculations (*B. firmus* and *A. humicola*) compared with control (non-inoculated plants): (A) bulk soil (B) rhizosphere soil. Values are the means of three replicates ± one standard error.

6.3.6 Effect of inoculation on total Cd in soil

The amount of Cd present in the soil at harvesting of pineapple was determined for different treatments. No significant differences in total Cd levels in soil were observed between inoculated plants and control (non-inoculated plants). After 32 weeks of incubation, control rhizosphere soils (non-inoculated) had Cd levels of 72.38 ± 11.96 mg Cd kg^{-1} while soils inoculated with *B. firmus* and *A. humicola* had Cd levels of 87.53 ± 15.47 and 89.84 ± 8.42 mg Cd kg^{-1} soil respectively. Our study found that the amount of Cd in bulk soil was higher than in rhizosphere soil (Figure 6.9).

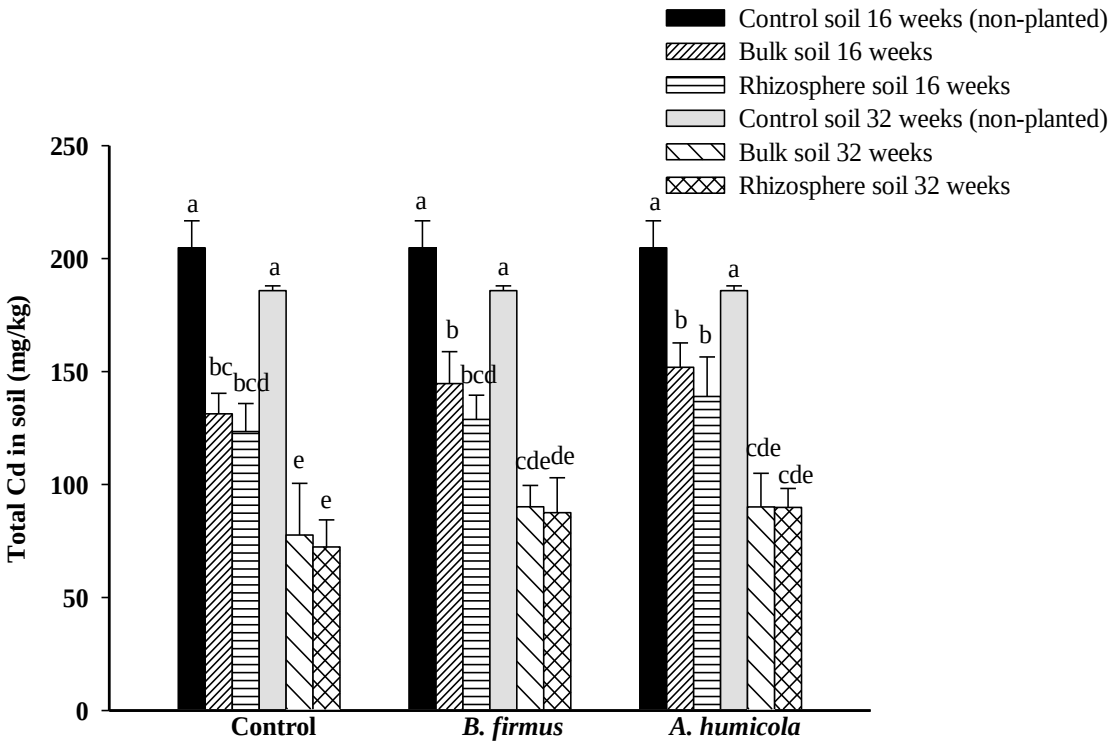

Figure 6.9 Total Cd concentrations in the soil after growing pineapple in the presence of Cd with or without bacterial inoculation. Samples were taken from bulk and rhizosphere soil at 16 weeks and 32 weeks after transplantation. Values not followed by the same letters in each treatment are significant at the 5% according to Least Significant Difference test.

6.3.7 The effect of soil inoculation with Cd-tolerant bacteria on Cd uptake by pineapple

The uptake of Cd by different parts of pineapple under inoculated and non-inoculated conditions in the presence of Cd was determined by ICP-OES analysis. In shoots, the Cd concentration was higher in the lower leaves than in middle or upper leaves. Inoculation by bacterial strains reduced significantly the Cd accumulated in root and shoots of pineapple with *A. humicola* performing better than *B. firmus* (Figure 6.10). These results indicated that the *A. humicola* and *B. firmus* decreased Cd concentration in pineapple shoots and roots conferring a protective (avoidance) effect in the presence of heavy metal.

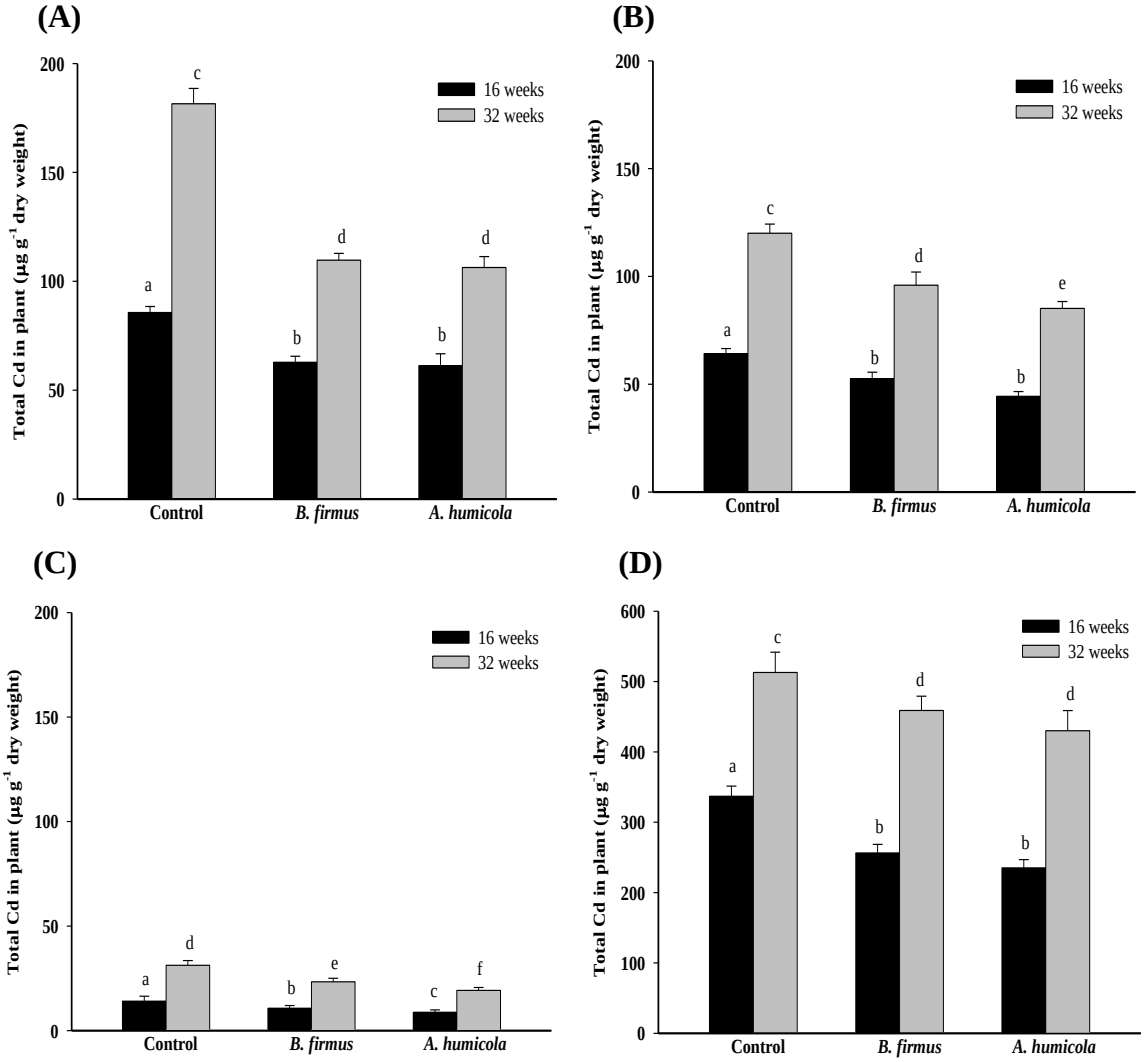

Figure 6.10 Cd accumulation in lower leaves (A), middle leaves (B), upper leaves (C) and root (D) of pineapple plants on soil treated with 150 mg Cd kg^{-1} compared with control (uninoculated). The plants samples were harvested at 16 weeks under low light intensity of 250 µmol m^{-2} s^{-1} and after a further 14 weeks under high light intensity of 500 µmol m^{-2} s^{-1}. Values shown are the means of three replicates. Different letters in the same harvesting period (16 weeks or 32 weeks) indicate a significant difference between treatments at the level of ($P < 0.05$, Least Significant Difference test).

Total Cd uptake by pineapple: Although roots accumulated more Cd per unit biomass the total amounts of Cd taken up by the root system in comparison to the shoot gave a different picture as the shoots constituted a much larger amount of plant biomass than the roots (see Table 6.2). At 4 months, shoot tissues had accumulated around 72-76% of the total Cd taken up by the plant while, only 24-28% of the total Cd plant uptake was retained in root tissues. Similar results were found in pineapple exposed to Cd for 8 months (Table 6.2). Thus, the Cd contents of the whole plants were similar across the 3 treatments.

Table 6.2 Cd concentration in the shoots and roots of pineapple grown in Cd-contaminated soil with bacterial inoculation compared control (non-inoculated plants).

Treatment (mg Cd kg^{-1})	**First Harvest (4 months)**			
	Dry weight (g)	**Cd concentration (µg g^{-1} dry weight)**	**Total Cd uptake (µg plant^{-1})**	**%Cd uptake**
Shoot				
Control	44.83	163.92	7348.53	72.85
B. firmus	58.13	126.05	7327.29	74.73
A. humicola	64.72	114.33	7399.44	76.26
Root				
Control	8.13	336.89	2738.92	27.15
B. firmus	9.67	256.27	2478.13	25.27
A. humicola	9.80	235.11	2304.08	23.74
	Second Harvest (8 months)			
	Dry weight (g)	**Cd concentration (µg g^{-1} dry weight)**	**Total Cd uptake (µg plant^{-1})**	**%Cd uptake**
Shoot				
Control	52.71	332.81	17542.42	76.56
B. firmus	72.93	228.96	16698.05	72.36
A. humicola	77.45	210.67	16316.39	69.78
Root				
Control	10.47	512.85	5369.54	23.44
B. firmus	13.90	458.89	6378.57	27.64
A. humicola	16.43	429.98	7064.57	30.22

6.3.8 Effects of bacterial inoculation on selected soil nutrients

Table 6.3 showed that bacterial inoculation significantly increased levels of soil nutrients (NH_4-N, NO_3-N and K) compared to control. No significant different in available P was observed between inoculated plants and control. In addition, the depletion of soil mineral in rhizosphere was observed when compared to bulk soil.

Table 6.3 Effect of bacterial inoculation on the amount of available soil mineral (N, P, K) in bulk and rhizosphere soil of pineapple grown in Cd-contaminated soil.

Treatment	NO_4^+-N (mg/kg)		NO_3^--N (mg/kg)		Available P (mg/kg)		Available K (mg/kg)	
	Bulk	Rhizos-phere	Bulk	Rhizos-phere	Bulk	Rhizos-phere	Bulk	Rhizos-phere
Control	8.46^{a}	5.05^{c}	10.02^{a}	7.40^{c}	23.07^{a}	18.29^{b}	169.24^{a}	150.04^{b}
B. firmus	10.59^{b}	6.80^{ac}	13.91^{b}	10.61^{a}	23.08^{a}	19.67^{bc}	193.52^{b}	177.38^{c}
A. humicola	11.73^{b}	8.15^{a}	15.22^{b}	11.38^{a}	24.42^{a}	21.95^{ac}	212.18^{b}	190.80^{c}

Different letters of each soil mineral indicate significant difference between means at $P < 0.05$ according to LSD test. Each value represent mean of triplicate determinations.

6.3.9 Effect of inoculation on plant macronutrient uptake

Total Nitrogen (N), Total Carbon (C), Phosphorus (P) and Potassium (K) in plant leaves and roots were differently affected by the microbial treatment applied in Cd-contaminated soil (Table 6.4 and Table 6.5). For above ground tissues, no significant different in N,P,K contents was observed between lower leaves, middle leaves and upper leaves. In addition, the application of *A. humicola* was more effective than *B. firmus* in increasing N, P and K concentration in shoots and roots. No significant difference of %C was observed between inoculated plants compared with control. Total NPK uptake by pineapple expressed as a dry weight basis showed that inoculated plants had improved NPK contents compared with control. In addition, shoot tissue NPK constituted around 93% of the total plant macronutrients while, only 7% of the total NPK was retained in root tissues (Table 6.6).

Table 6.4 Nitrogen, carbon contents and C/N ratio of different part of pineapple leaves and root.

Treatment	%N				%C				C/N ratio			
	Upper	**Middle**	**Lower**	**Root**	**Upper**	**Middle**	**Lower**	**Root**	**Upper**	**Middle**	**Lower**	**Root**
Control	1.14[a]	1.14[a]	1.18[a]	1.33[a]	43.31[a]	43.04[a]	42.79[a]	38.60[a]	37.89[a]	37.88[a]	36.39[a]	28.98[a]
B. firmus	1.20[a]	1.23[ab]	1.28[ab]	1.45[ab]	43.42[a]	43.40[a]	43.05[a]	40.55[a]	36.29[ab]	35.22[ab]	33.95[a]	27.93[ab]
A. humicola	1.30[b]	1.35[b]	1.41[b]	1.49[b]	44.07[a]	43.62[a]	43.71[a]	40.64[a]	34.05[b]	32.43[b]	31.07[b]	27.32[b]

Different letters in each column indicate significant difference between means at $P < 0.05$ according to LSD test. Each value represent mean of triplicate determinations

Table 6.5 Phosphorus and potassium contents of different part of pineapple leaves and root.

Treatment	%P				%K			
	Upper	Middle	Lower	Root	Upper	Middle	Lower	Root
Control	0.14[a]	0.17[a]	0.17[a]	0.19[a]	2.22[a]	2.38[a]	2.47[a]	2.69[a]
B. firmus	0.20[b]	0.22[ab]	0.22[b]	0.24[a]	2.56[ab]	2.61[a]	2.65[a]	2.76[a]
A. humicola	0.26[c]	0.26[b]	0.27[b]	0.31[b]	2.67[b]	3.29[b]	3.77[b]	3.93[b]

Different letters indicate in each column significant difference between means at $P < 0.05$ according to LSD test. Each value represent mean of triplicate determinations.

Table 6.6 NPK contents in shoots and roots of pineapple grown in Cd-contaminated soil at concentration 150 mg Cd kg^{-1} after 8 months incubation with *B. firmus* and *A. humicola* compared with control (non-inoculated plants).

Treatment	Shoot dry weight (g)	%N in shoot	Total N in shoot	Root dry weight (g)	%N in root	Total N in root	Total N per plant
Control	52.71	3.46	182.38	10.47	1.33	13.93	196.30
B. firmus	72.93	3.71	270.57	13.90	1.45	20.16	290.73
A. humicola	77.45	4.06	314.45	16.43	1.49	24.48	338.93
	Shoot dry weight (g)	**%P in shoot**	**Total P in shoot**	**Root dry weight (g)**	**%P in root**	**Total P in root**	**Total P per plant**
Control	52.71	0.48	25.30	10.47	0.19	1.99	27.29
B. firmus	72.93	0.64	46.68	13.90	0.24	3.34	50.02
A. humicola	77.45	0.79	61.19	16.43	0.31	5.09	66.28
	Shoot dry weight (g)	**%K in shoot**	**Total K in shoot**	**Root dry weight (g)**	**%K in root**	**Total K in root**	**Total K per plant**
Control	52.71	7.07	372.66	10.47	2.69	28.16	400.82
B. firmus	72.93	7.82	570.31	13.90	2.76	38.36	608.67
A. humicola	77.45	9.73	753.59	16.43	3.93	64.57	818.16

6.4 Discussion

Results from Chapter II demonstrated that pineapple was tolerant to Cd at the level of 150 mg Cd kg^{-1} in that no visible symptoms were observed but some physiological impacts of Cd toxicity were observed. One way to lessen the deleterious effects of Cd taken up from the environment might involve the use of Cd-resistant bacteria as soil inocula. In the present study, two rhizosphere bacterial strains were isolated in an attempt to reduce the Cd uptake by pineapple and promote plant growth under conditions of Cd contamination.

6.4.1 Effect of inoculation on soil properties

6.4.1.1 Effect of inoculation on soil pH

The results of several previous studies identified soil pH as an important property controlling sorption/desorption and solubility of native and added soil Cd (Gray *et al.*, 1998; Salam and Helmke, 1998; Gray *et al.*, 1999b) and therefore likely to play an important role influencing Cd extractability by plants (Gray *et al.*, 1999a). In the present work, the bacterial inoculants did not appear to change soil pH significantly and so any changes in Cd uptake observed in the plants were unlikely to be attributable to bacterial inoculation changing soil pH. These results contrast with other studies e.g. significant decreases of pH in rhizosphere soil were observed when the soil was inoculated with *Pseudomonas* sp. RJ10 or *Bacillus* sp. RJ16 (He *et al.*, 2009).

6.4.1.2 Effect of inoculation on extractable Cd levels in soil

The results in our study showed that soil-extractable Cd was not significantly reduced by the inoculation of *B. firmus* and *A. humicola* compared to non-inoculated control. The results in this work contrast with the previous observation that the presence of bacteria in the soil led to a reduction of mobile Cd in soil solution (Wu *et al.*, 2006; Jiang *et al.*, 2009). Madhaiyan *et al.*, (2007) demonstrated that the amount of Ni and Cd in the soil solution and heavy metal extractability was reduced by application of bacterial strains in heavy metal contaminated soil. Xu *et al.*, (2012) demonstrated that pakchoi (*Brassica chinensis* L.) inoculated with *Pseudomonas putida* (*P. putida*) significantly decreased extractable Cd in contaminated soil. Very high levels of Cd were used in the current work so it is possible that these levels were too high to produce any measurable effect of inoculation on extractable Cd levels.

6.4.1.3 Effect of inoculation on total Cd concentration in soil

Soil samples were analysed for total Cd present to determine if bacterial inoculation reduced Cd uptake by plants compared to non-inoculated controls. The results showed that Cd concentrations in soil were reduced from the initial levels present i.e. Cd was taken up by pineapple from soil. The total Cd levels that remained after 32 weeks in control bulk and rhizosphere soils (77.64 mg Cd kg^{-1} and 72.38 mg Cd kg^{-1}, respectively) were lower than those observed in inoculated soils (indicating a reduction in Cd uptake by plants) but the difference was not significant at the 5% level. Soil microorganisms such as bacteria are suggested to be the most active organic colloids in soil since their surfaces can interact strongly with metal ions in soil solution (Huang *et al.*, 2002). They could immobilise metals by binding metal in the soil onto their cell surfaces and lead to a reduction of metal uptake by plants (Leyval and Joner, 2000; Fein *et al.*, 2001; Zaidi and Musarrat, 2004). Support for such a mechanism The similar results has been reported by Zaidi *et al.*, (2006) who found that the high sorption capacity of the *Bacillus subtilis* strain SJ-101 for Ni was shown to protect *Brassica juncea* against Ni toxicity.

6.4.1.4 Effect of inoculation on Cd uptake by pineapple

The present study showed that the older leaves of pineapple plants accumulated more Cd (measured as µg Cd g^{-1} dry weight) than the middle or young leaves. Similar results were shown and described in Chapter III. On a whole plant basis, inoculated and non-inoculated plants accumulated Cd at approximately 3-fold higher Cd level in shoots than in roots. However, on a dry weight of tissue basis, Cd accumulated to highest concentrations in the roots. Application of *B. firmus* and *A. humicola* to soils contaminated with Cd reduced Cd uptake in roots and shoots of pineapple (expressed as µg Cd g^{-1} dry weight). Similar to our results, other studies have found that inoculation with bacterial strains reduced Cd uptake and promoted plant growth in barley, oat and tomato (Belimov and Dietz, 2000; Madhaiyan *et al.*, 2007; Pishchik *et al.*, 2009). From the previous study in Chapter V, the strains used in the present work were shown to bind Cd to their cells. Therefore, the reduced uptake in roots and shoots of inoculated plants might be attributed to the binding of Cd by the bacterial cell wall, rendering them unavailable for plant uptake (Zaidi and Musarrat, 2004; Wu *et al.*, 2006; Madhaiyan *et al.*, 2007; Sinha and Mukherjee, 2008; Dary *et al.*, 2010). Furthermore these bacterial strains

may also help the plant to acquire sufficient phytohormones and other nutrients for optimal plant growth in the presence of heavy metals (Zaidi *et al.*, 2006).

6.4.1.5 Effect of inoculation on selected soil nutrients

Soil inoculation with bacteria resulted in an increased content of available NH_4-N, available NO_3-N, available P and available K as compared to the control soil (non-inoculated plants). Many studies reported that bacterial inoculation increases available soil nutrients for plants such as cotton, rape, pepper, maize, bean, tomato, red pepper and rice (Hafeez *et al.*, 2004; Sheng, 2005; Han *et al.*, 2006a; Biari *et al.*, 2008; Elkoca *et al.*, 2010; Madhaiyan *et al.*, 2010).

The increased availability of soil nutrients could be due to (a) decomposition processes activated by bacteria that increased the release of nutrients from organic substances in the soil into available forms (Barker *et al.*, 1998; Bennett *et al.*, 1998; Kang *et al.*, 2002; Chen *et al.*, 2006; Pradhan and Sukla, 2006; Khan *et al.*, 2009) and/or (b) the bacteria die and release nutrients through mineralization. Through these mechanisms, the bacteria could help the plant to acquire sufficient nutrients for optimal plant growth and protect against Cd toxicity.

6.4.2 Effect of bacterial inoculation on photosynthetic performance of pineapple

6.4.2.1 Effect of inoculation on plant photosynthesis

In this study the maximum quantum yield of PSII (F_v/F_m), which characterises the maximal quantum yield of the primary photochemical reactions in dark-adapted leaves, was affected by Cd. Generally, the reduction of F_v/F_m due to Cd is based on damage to PSII as a result of stress (Rintamäki *et al.*, 1995; Pagliano *et al.*, 2006). It has been suggested that Cd can exert most of its effects on PSII (Bazzaz *et al.*, 1974; Van Duijvendijk Matteoli and Desmet, 1975; Nagel and Voigt, 1995; Voigt and Nagel, 2002). In addition, previous work in Chapter II demonstrated that the F_v/F_m ratio of pineapple grown in Cd-contaminated soil was decreased as the Cd concentrations increased compared to the control. This chapter showed that bacterial inoculation could improve the light-use efficiency of pineapple grown in Cd-contaminated soil resulting in higher F_v/F_m ratio than the control (non-inoculated). When plants were acclimated under high light intensity, inoculated plants were better able to acclimate to the increase in light intensity whilst F_v/F_m showed a gradual decline in the last 5 weeks in non-inoculated plants.

Inoculation with microbes could increase plant defence systems to withstand stresses which would help reduce Cd effects on photosystem II. Alternatively some bacterial strains directly regulate plant physiology by producing plant hormones, whereas others increase mineral and nitrogen availability in the soil as a way to enhance plant growth (Yasmin *et al.*, 2007a).

Bacterial inoculation increased net CO_2 uptake compared to non-inoculated plants. Inoculated plants under low light intensity attained a higher maximum rate of net CO_2 assimilation than those under high light intensity. Under Cd stress, CO_2 assimilation rate may be limited due to a disruption in PSII (discussed above) but also to a possible decrease in the amount and activity of Rubisco, either as the plants aged or in response to the higher light intensities (Stiborová, 1988). Chugh and Sawhney, (1999) pointed out that the deleterious effect of Cd on photosynthesis of plants could be due to a direct interference of metal on photosynthetic enzymes. Changes in CO_2 assimilation by the addition of Cd has been shown to be accompanied by a decrease in transpiration rates, indicating partial stomatal closure (Gouia *et al.*, 2003). The results from Chapter II revealed that Cd had an effect on the amount of the Rubisco enzyme of pineapple grown in Cd-contaminated soil. Decreased Rubisco activity was accompanied by a decline in the level of soluble sugars, suggesting that under Cd-stress sugar synthesis was reduced, relative to CO_2 fixation capacity of Rubisco (Leitao *et al.*, 2003). In this study we found that plants inoculated with *B. firmus* and *A. humicola* accumulated more soluble sugars compared to non-inoculated plants. This suggests that the bacterial inoculation reduced Cd toxicity to pineapple plants, resulting in an increased CO_2 fixation and improved Rubisco activity resulting in more accumulated soluble sugars than non-inoculated plants.

6.4.2.2 Effect of inoculation on CAM biochemistry

Results in this chapter showed that pineapple plants inoculated with bacterial strains under Cd-conditions had higher amounts of titratable acidity which can be attributed to improved carbohydrate status compared to control. Afef *et al.*, (2011) demonstrated that reduction of soluble sugar contents in leaves of tobacco was observed when plants were treated with Cd. In addition, the previous work in Chapter II revealed that Cd had an effect on the abundance of Rubisco protein of pineapple. Siedlecka *et al.*, (1997) showed that Cd had an effect on photosynthesis by inhibiting various reaction steps of the Calvin cycle. In many in vitro and in vivo experiments, disturbances in carbohydrate metabolism

are observed (Vassilev *et al.*, 1997) because of the inhibition of enzymes such as Rubisco (Stiborová, 1988; Mobin and Khan, 2007). The generally higher carbohydrates content in leaves of inoculated plants may potentially result in more PEP being available for nocturnal malate accumulation and a greater nocturnal uptake of atmosphere CO_2. Haider *et al.*, (2012) suggested that 20% of leaf biomass can be allocated to carbohydrates for PEP production in CAM plants. CAM expression and carbon assimilation requires the co-ordination of organic acid turnover and carbohydrate metabolism on a diel basis (Sutton, 1975; Whiting *et al.*, 1979). Graf *et al.*, (2010) suggested that plant growth is driven by photosynthetic carbon fixation during the day. Some photosynthate is accumulated as starch to support nocturnal metabolism and growth at night.

Under high light conditions, a reduction in carbohydrate contents was observed both in inoculated plants and non-inoculated plants compared to those under lower light and this may have contributed to the lower rates of dark CO_2 uptake and CAM activity in plants under the higher light intensity.

6.4.3 Effect of inoculation on plant nutrient levels

Inoculants of *B. firmus* and *A. humicola* increased essential plant mineral levels including N, P and K in pineapple grown in Cd-contaminated soil compared to non-inoculated plants. Similar results have been reported that inoculation with bacterial strains results in a significant improvement in plant growth and nutrient content through stimulation of biological N-fixation, production of phytohormones and increase in the uptake of N, P and K in soil in tomato, oil palm, eggplant, maize, *Brassica juncea*, wheat, cotton and rape (Lin *et al.*, 2002; Saubidet *et al.*, 2002; Amir *et al.*, 2005; Sheng, 2005; Wu *et al.*, 2005; Han *et al.*, 2006a; Wu *et al.*, 2006; Egamberdiyeva, 2007). Heavy metals including Cd may interfere with nutrient uptake by altering the plasma membrane permeability, and by affecting element transport processes across the membrane (Gussarsson, 1994). This chapter (Section 6.3.8) showed that bacterial inoculation increased soil mineral availability for pineapple grown in Cd-contaminated soil which may have increased nutrient uptake and plant growth (Omar, 1998; Wahid and Mehana, 2000; Lin *et al.*, 2002; Şahin *et al.*, 2004).

6.4.4 Effect of inoculation on plant biomass

The application of Cd-resistant bacterial inoculants to pineapple grown in Cd-contaminated soil seemed to be very effective in protecting plants from growth inhibition caused by Cd and this was strongly supported by the dry-weight biomass data. The biomass production of pineapple (both shoots and roots) was significantly reduced in the presence of Cd (as shown in Chapter II) and bacterial inoculation in Cd-contaminated soil significantly increased the biomass of pineapple in comparison to non-inoculated controls. Similar to our results, the inoculation of bacterial strains under Cd-stress had stimulatory effects on plant biomass in chickpea (*Cicer arietinum*), *Brassica juncea*; tomato, *Triticum aestivus*, canola (*Brassica campestris* cv. *Tobin*) (Hasnain and Sabri, 1996; Burd *et al.*, 1998; Gupta *et al.*, 2004; Wu *et al.*, 2006; Madhaiyan *et al.*, 2007). As discussed above the bacterial inoculants appear to reduce Cd toxicity by improving the nutrient (N,P,K) levels in the plant (Lin *et al.*, 2002; Sheng, 2005; Wu *et al.*, 2005; Wu *et al.*, 2006; Egamberdiyeva, 2007).

6.5 Conclusions

Inoculation of soils with microorganisms may reduce the toxicity of heavy metals to plants grown in contaminated soil (Madhaiyan *et al.*, 2007). This study demonstrated that the application of *B. firmus* and *A. humicola* to the rhizosphere soil improved the growth and photosynthetic metabolism of pineapple plants. Levels of available nutrients were higher in inoculated soils and this could lead to increased growth and protection against Cd toxicity. On a dry-weight basis (µg Cd g^{-1} dry weight), bacterial inoculation decreased the Cd uptake in plants most likely due to Cd binding by the Cd-resistant bacteria in the rhizosphere. The results clearly showed reduction in the accumulation of Cd in roots and shoots. *A. humicola* performed better than *B. firmus* in reducing the Cd uptake in plants.

Chapter VII

General Discussion

7 General discussion

7.1 Introduction

Cadmium (Cd) is a heavy metal pollutant of the environment. The Cd contamination of soils results from various agricultural, mining and industrial activities (Taylor, 1997; Singh, 2001; Kara *et al.*, 2004; Kovács *et al.*, 2006; Wang *et al.*, 2008a). Due to its high toxicity and solubility in water, Cd is considered a potentially phytotoxic element (Lockwood, 1976; Chaney *et al.*, 1977; Chaturvedi, 2004). In plants, general symptoms of Cd toxicity are stunted growth and chlorosis (Prasad, 1995; Dalla Vecchia *et al.*, 2005; Chaudhary and Sharma, 2009; Dinakar *et al.*, 2009). Cd has been shown to interfere with uptake, transport and use of several nutrient elements (Ca, Mg, P and K) and water by plants such as soybean, rice, pea, wheat and barley (Cataldo *et al.*, 1983; Barceló and Poschenrieder, 1990; Rubio *et al.*, 1994; Hernandez *et al.*, 1996; Sandalio *et al.*, 2001; Wu and Zhang, 2002a; Wu *et al.*, 2003; Dražić *et al.*, 2004). Cd can enter the food chain at concentrations that present potential risks to consumers (Chaney and Ryan, 1994). Extensive areas of paddy rice soils in Thailand have become so contaminated with Cd generated as a by-product of Zn mining that rice grown on the soils has caused human disease (Simmons *et al.*, 2005; Sampanpanish and Pongpaladisai, 2011). Such locations either require Cd remediation in order to continue growing rice for food or the adoption of alternative crops that are more Cd resistant than rice.

Pineapple (*Ananas comosus* L.) is the most popular and widely cultivated tropical fruit in Thailand and as a CAM plant which is tolerant to drought, high light and high temperatures, it was hypothesised that this species would be relatively tolerant to Cd. It has recently been shown that *Sedum alfredii* (*S. alfredii*; belongs to the Crassulaceae and show CAM) has an ability to tolerate and hyperaccumulate Cd (Yang *et al.*, 2004; Zhou and Qiu, 2005). However, no previous reports have studied the effects of heavy metals in general, or Cd in particular, in pineapple. Therefore, a better understanding of the impact of Cd on the growth and physiology of pineapple formed a key component of this thesis. The thesis also reported the isolation of metal resistant microbes that are able to detoxify Cd. These microbes were identified using molecular techniques and were added to metal contaminated soil to see if they were able to reduce the metal toxicity of Cd-contaminated soil in which pineapple was grown.

7.2 The effect of Cd on plant growth, photosynthetic performance and CAM biochemistry

Physiological processes such as photosynthesis have been shown to be very sensitive to Cd in plants and it has been suggested that depressed rates of photosynthesis may be largely responsible for reduced biomass production from plants grown on soils contaminated with heavy metals (Benavides *et al.*, 2005). In pineapple exposed to various concentrations of Cd, reduced rates of dark CO_2 uptake and growth were observed. In comparison to the control (Cd 0 mg kg^{-1}), maximum significant reduction in growth and photosynthesis were observed with 150 mg Cd kg^{-1} soil. The reduction of plant biomass of pineapple grown in Cd-contaminated soil compared with other plant species are shown in Table 7.1. Of the plant species considered, pineapple appears the most tolerant in terms of plant biomass production under Cd and it is possible that this tolerance is related to the presence of CAM, since the other CAM species on the list (*S. alfredii*) showed a similar response to that of pineapple in term of % reduction in dry mass at relatively high levels of Cd. CAM plants generally show lower rates of transpiration compared to C3 and C4 species (Borland *et al.*, 2009) so it is possible that the Cd tolerance of plants with this mode of photosynthesis may be related to reduced transfer of Cd from roots to shoots via the transpiration stream. However, the leaves of CAM plants also possess large central vacuoles, so tolerance to Cd could be a result of sequestration of the heavy metal in the vacuole. Patterns of Cd accumulation in pineapple is considered further in Section 7.3.

Table 7.1 Percentage of plant biomass reduction in different plant species those were grown in Cd-contaminated soil.

Cd concentration (mg Cd kg^{-1})	% Reduction in plant biomass	Plant species	Reference
9	36	Rice	Kibria *et al.*, (2006)
50	53	Corn	Chitra *et al.*, (2011)
50	65	Wheat	Chitra *et al.*, (2011)
50	49	Tobacco	Chitra *et al.*, (2011)
100	75	*Solanum photeinocarpum*	Zhang *et al.*, (2011b)
150	17	*Sedum alfredii*	Yang *et al.*, (2004)
150	14	Pineapple (*Ananas comosus* L.)	Chapter II

Data shown in the present study were in agreement with the idea that under Cd stress, CO_2 assimilation rate is limited due to a decrease in the amount and activity of Rubisco. Cd also resulted in impairment in carbohydrate metabolism in pineapple which would influence photosynthate partitioning and likely lead to impaired growth of pineapple in Cd-contaminated soil. In CAM plants, the primary enzyme responsible for CO_2 acquisition is PEPC yet the abundance of this protein was similar in control and Cd-treated plants. Thus, the reduction of net nocturnal CO_2 uptake in Cd-treated plants could not be attributed to altered PEPC abundance. However, Cd resulted in a decrease in soluble sugar content in leaves of pineapple, so it is possible that Cd curtailed the breakdown of sugar to provide PEP for CO_2 fixation which could account for the lower rates of dark CO_2 fixation that were observed.

A decrease in F_v/F_m was observed in Cd-treated pineapple plants, suggesting that Cd inhibited photoactivation of photosystem II by competitive binding to the essential Ca^{2+} site (Faller *et al.*, 2005). The decrease in CAM activity in Cd-treated plants noted during the transfer and acclimation to high light occurred with a drop in the potential quantum efficiency of photosystem II. The reduction in F_v/F_m indicated an increase in photoinhibition, which could be due both to photoprotective processes and damage to the photosynthetic apparatus (Maxwell and Johnson, 2000). Bromeliads such as pineapple have been shown to possess a photosynthetic capacity that is largely independent of photon flux density (PFD), with high rates of PSII electron transport and high potential for the dissipation of excess light energy (Fetene *et al.*, 1990; Skillman and Winter, 1997; Delahunty, 2003). However, it was found that *Aechmea magdalenae* (another bromeliad) showed symptoms of chronic photoinhibition when plants were grown under high light intensity. In the present study on pineapple, only control plants showed a recovery in F_v/F_m when transferred from low to high PFD and plants in Cd were unable to fully acclimate to the increase in light intensity. This suggest that there were differences in the activation status of PSII reaction centres or the turnover of the D1 protein associated with the reaction centres (Demmig-Adams and Adams III, 1992; Osmond, 1994; Thiele *et al.*, 1998) between control and Cd-treated plants. To test this hypothesis, the amount of D1 protein and the rates of D1 protein synthesis of pineapple grown in Cd-contaminated soil under different light regimes should be determined in future studies.

7.3 Cd-accumulation in pineapple (*Ananas comosus* L.)

The results from Chapter II showed that pineapple can grow in Cd-contaminated soil (albeit at a slow rate than that of control plants) and no visual toxicity symptoms were observed. The thesis examined the extent to which pineapple performance on Cd-treated soil was due to exclusion or accumulation of Cd in different tissues.

Soil pH is an important factor affecting Cd extractability in soil and in general, a decrease in pH decreases the sorption of Cd by soils and thus increases Cd extractability (Wang *et al.*, 2006b). The results in this study showed that levels of extractable Cd in the rhizosphere were lower than observed in the bulk soil. It could be assumed that the lower pH of the rhizosphere soil would result in an increase in extractable Cd but this was not the case. There are potentially several reasons for the results observed e.g. Cd uptake by the plant reduced the extractable levels of Cd seen in the rhizosphere soil or that metal ions were bound to organic materials present in the rhizosphere (Abumaizar and Khan, 1996). Cd accumulation by pineapple increased as the Cd concentration in soil solution increased. The uptake of Cd by pineapple may be due to the release of Cd from soil particles under acidic conditions in rhizosphere soil. Plants growing on Cd-contaminated soil showed Cd accumulation of the metal ion in all plant parts. Although Cd is a highly phytotoxic metal, it is easily taken up by plant roots growing on Cd contaminated soils and transported to above ground plant parts (Dalcorso *et al.*, 2010; Liu *et al.*, 2010; Lux *et al.*, 2011). The Cd concentrations in root and shoot tissues of pineapple compared with other plant species are shown in Table 7.2.

Table 7.2 Cd concentration in root and shoot tissues of different plant species compared with pineapple.

Treatment (mg Cd kg^{-1} soil)	Cd concentration (µg g^{-1} dry weight)		Plant species	Reference
	Shoot	Root		
9	54.42	367.19	Rice	Kibria *et al.*, (2006)
50	16.00	18.00	Corn	Chitra *et al.*, (2011)
50	20.00	25.00	Wheat	Chitra *et al.*, (2011)
50	45.00	35.00	Tobacco	Chitra *et al.*, (2011)
100	342.00	845.00	*Solanum photeinocarpum*	Zhang *et al.*, (2011b)
150	4000.00	6000.00	*Sedum alfredii*	Yang *et al.*, (2004)
150	386.72	792.80	Pineapple (*Ananas comosus* L.)	Chapter III

No previous studies have investigated the relationship between pH and pineapple metal uptake. It is hypothesised that the ability of pineapple to accumulate Cd will vary with soil texture, soil pH and concentration of Cd in soil. To test this hypothesis, further studies on the effect of different soil pH levels in different soil types on the extractable Cd and metal uptake in pineapple are needed to inform more effective methods of remediation of metal contaminants in soil.

7.4 Effect of Cd on pineapple rhizosphere soil microbial populations

Several methods were used to determine if pineapple plants influenced the abundance and/or diversity of the microbial communities in the rhizosphere of Cd-contaminated soil. The results from plate counts and DGGE indicated that a shift in the rhizosphere microbial populations occurred in the presence of pineapple roots. It is reported that Cd is toxic to soil microorganisms and might reduce soil microbial diversity (Kunito *et al.*, 1999). The results here showed that the pineapple rhizosphere has an increased number of bacteria and fungi and a distinct and more diverse bacterial population (as estimated by DGGE analysis) when compared to bulk soil. Certain bacteria in the phyla *Nitrospirae*, *Verrucomicrobia*, *Acidobacteria* and *Chloroflexi* were only present in Cd-contaminated soil.

There are some interesting implications from this study. The high bacterial diversity of soils may help protect soils from adverse effects of metals. A change in microbial populations due to the high Cd level was observed. Although it wasn't the aim of the present work to determine if soil functions were maintained at high Cd levels it is clear that a significant portion of the bacterial community were able to adapt to the Cd and persist even after 36 weeks exposure to high Cd levels. This suggests an important role for high bacterial diversity in soil in maintaining soil function. Interestingly, metals can have other effects on bacterial populations. Recent work in Newcastle University has indicated that metal stress can select for increased numbers of antibiotic resistant bacteria in soil (Knapp *et al.*, 2011). Therefore, future work should examine the effect of Cd on soil functions and also look for other indirect effects e.g. increases in antibiotic resistance.

7.5 Cd-tolerance and Cd-binding capacity of bacteria isolated from Cd-contaminated soil

Heavy metal contamination can result in the establishment of heavy metal-resistant bacterial populations (Ellis *et al.*, 2003; Piotrowska-Seget *et al.*, 2005). Many microorganisms have developed detoxification mechanism such as extracellular precipitation and exclusion, binding to the cell surface and intracellular sequestration to reduce the toxic effects of heavy metals (Silver and Phung, 1996). Nine Cd-resistant bacteria strains were isolated from the root zone of pineapple grown in Cd-contaminated soil. The isolated strains included *Microbacterium esteraromaticum, Bacillus firmus, Microbacterium aoyamense, Arthrobacter humicola, Cellulomonas hominis, Micrococcus antarcticum, Bacillus cereus, Pseudomonas citronellolis* and *Sporosarcina globispora*. Two bacterial strains (*B. firmus* and *A. humicola*) which were the most Cd-resistant bacteria were selected for binding-capacity as it was assumed that higher Cd-resistance infers a better capacity to retard metal diffusion to the inside of the bacterial cell by binding heavy metals onto the cell surface. The equilibrium time for biosorption of Cd was reached after 20 minutes at room temperature. The data showed that the percentage of Cd removal decreased with an increase in Cd concentrations. The decrease in percentage of biosorption may be due the saturation of adsoption sites (Lu *et al.*, 2006; Tunali *et al.*, 2006; Gabr *et al.*, 2008; Oh *et al.*, 2009).

It was also shown that *B. firmus* and *A. humicola* bound Cd from Cd and soil solution. This suggested that these bacteria could be used as soil inoculants to reduce Cd uptake by pineapple grown in Cd contaminated soil (see Section 7.6 below).

7.6 Use of bacterial inoculants to promote pineapple growth in Cd-contaminated soil

Studies have demonstrated that Cd-resistant bacteria can reduce toxicity of Cd towards plants (Belimov and Dietz, 2000; Pishchik *et al.*, 2002; Belimov *et al.*, 2005; Sinha and Mukherjee, 2008). Results presented in this thesis also showed that Cd uptake by pineapple (calculated as μg g^{-1} dry weight) was significantly reduced by soil inoculation with 2 species of Cd-resistant bacteria (*B. firmus* and *A. humicola*). Although a number of studies have demonstrated the importance of bacterial inoculation for plant growth and reduced heavy metal accumulation in heavy metal-polluted environments (Abou-Shanab *et al.*, 2003; Idris *et al.*, 2004; Khan, 2005; Sheng and Xia, 2006), to our knowledge, this is the first research report elucidating the role of Cd-resistant bacteria *B. firmus* and *A. humicola* to reduce Cd toxicity and promote plant growth of pineapple in Cd-contaminated soil. The results obtained here clearly indicate that inoculation of *B. firmus* and *A. humicola* not only protected pineapple from Cd toxicity but also promoted plant growth. Doelman, (1985) has reported that the efficiency of revegetation and phytoremediation of heavy metal-contaminated sites is closely related to the presence of metal resistant microbial populations in the soil, which likely conferred a better assimilation of nutrients as well as reduction in metal uptake by plants.

The use of bacterial inoculants to improve plant growth and reduce toxicity of metal in heavy metal contaminated soil is eco-friendly as it theoretically does not release toxic compounds into the environment (Gavrilescu, 2010). However, in large scale agriculture, the immediate response to soil inoculation with bacterial strains varies considerably depending on the bacteria, plant species, soil type, inoculants density and environmental condition. In general, shortly after the bacteria are introduced into the soil, the bacterial population declines progressively (Van Elsas *et al.*, 1986; Bashan and Levanony, 1988). This phenomenon may prevent the buildup of a sufficiently large bacterial population in the rhizosphere to obtain desired improvements in plant growth.

As the current work was carried out under greenhouse condition the stimulation of pineapple growth under actual Cd-polluted field conditions needs further study. It is possible that increased nutrient levels in soils as a result of bacterial inoculation (after

bacterial cell death) may reduce Cd toxicity. For example, simple phosphorus addition to soils may decrease plant Cd uptake due to Cd precipitation and the extra nutrients may provide pineapple protection against Cd. Many reports have demonstrated that phosphorus application reduced Cd phytotoxiciy to crop plants such as sudax (*Sorghum vulgare* L.), swiss chard (*Beta vulgaris* L.), indian mustard (*Brassica juncea)* and spinach (*Spinacia oleracea*) by limiting Cd extractability in the soil through immobilization, resulting in higher yield of plants (Hettiarachchi and Pierzynski, 2002; Bolan *et al.*, 2003b; Dheri *et al.*, 2007). Other simple chemical amendment could also be made e.g. lime or bone metal addition to reduce Cd toxicity to pineapple (Bolan *et al.*, 2003a; Sneddon *et al.*, 2006; Modin *et al.*, 2011; Tan *et al.*, 2011b). Future work is required to ascertain the exact effects of the bacterial inoculants on reducing Cd toxicity.

7.7 Conclusions

Cd had a negative effect on photosystem II and a key photosynthetic enzyme (Rubisco) in the CAM species pineapple. The uptake of Cd by pineapple may be due to the release of Cd from soil particles under acidic conditions in rhizosphere soil. The accumulation of Cd calculated as ug per g dry weight showed that roots contained higher amounts of Cd than shoots. In addition, leaf age influenced Cd accumulation with older leaves showing higher Cd contents than younger leaves. In Cd-contaminated soil, Cd exhibited toxicological effects on soil microbes which led to a decrease in microbial viable counts and bacterial diversity. DGGE banding patterns confirmed that the addition of metals had a significant impact on microbial community structure. It was shown that two bacterial species (*B. firmus* and *A. humicola*) isolated from Cd-contaminated soil were able to bind Cd from soil solution. The application of these two species as soil inoculants significantly enhanced pineapple biomass production on Cd-contaminated soils. Finally, the results and information in this study would be promoted to the farmer in Thailand especially in Tak province.

7.8 Future research

a) Investigate the effect of phosphate application on amelioration of Cd toxicity in pineapple (*Ananas comosus* L.)

b) Determine the effect of Cd on pineapple grown in different soil types contaminated with Cd.

c) Investigate the performance of pineapple and Cd uptake by roots, leaves and fruits under Cd-contaminated field conditions.

d) Evaluate the effect of lime amendments on Cd uptake and plant growth in pineapple grown in Cd-contaminated soil.

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