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An Investigation of Plant-Microbe Interactions Under Cadmium Stress in Agar-Based Medium, Hydroponics, and Soil Studies

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Graduate Program in Biology
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy
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AN INVESTIGATION OF PLANT-MICROBE INTERACTIONS UNDER CADMIUM
STRESS IN AGAR-BASED MEDIUM, HYDROPONICS, AND SOIL STUDIES

(Thesis format: Integrated Article)

by

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Graduate Program in Biology with Environment and Sustainability

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

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Abstract

This thesis investigated plant-microbe-metal interactions at two scales: a single plant-microbe system and an agricultural rhizobacterial community. The first objective was to investigate the effectiveness of a plant growth-promoting rhizobacterium (PGPR) on mediating cadmium stress in a plant model system. *Arabidopsis thaliana* Col-0 was inoculated with *Pseudomonas putida* UW4, which in its wild type form has been reported to reduce plant stress by simultaneously metabolizing the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) with the enzyme ACC deaminase and stimulating plant growth through the production of indole-3-acetic acid (IAA). A mutant strain that lacks ACC deaminase and a no bacteria treatment were used as controls. When plants were grown on agar-based or hydroponic Murashige and Skoog (MS) medium containing cadmium both strains of PGPR had deleterious effects on plant growth. Further investigation revealed that the PGPR were unable to survive in MS medium without the presence of a plant. Loss of plant growth-promotion was hypothesized to be due to the unfavourable environment for the bacterium. To test this, agricultural soil was maintained for 28 days with either MS medium or distilled water and the bacterial community profile was analyzed using terminal restriction fragment length polymorphism (TRFLP) analysis. A decrease in fragment richness was observed in the MS medium treatment, which lends further support to the theory that certain environmental conditions can be detrimental to rhizobacteria. The final objective was to determine if the rhizosphere microbial communities varied among two pairs of high and low metal-accumulating plants (two cultivars of *Triticum durum*, Kyle and Arcola, as well as *Brassica juncea* and *B. napus*). Plants were grown in agricultural soil containing cadmium and the microbial community profiles were analyzed using TRFLP. When the plant's metal-accumulating ability was well matched to the metal concentration in the soil a unique rhizobial community developed; when they were unmatched, the rhizobacteria did not differ from the bulk soil. As a whole, this thesis demonstrates the complex nature of plant-microbe-metal interactions and the need to continue to look at these systems. Knowledge gained will help in properly matching PGPR to field applications to increase the efficacy of bioremediation strategies, agricultural yields, and food safety.

Keywords

Rhizosphere, Plant-microbe interactions, Plant growth-promoting rhizobacteria (PGPR), Cadmium, Plant stress, Terminal restriction fragment length polymorphism (TRFLP)

Co-Authorship Statement

This thesis includes two manuscripts. The first manuscript is entitled “The message is in the medium: the putative PGPR *Pseudomonas putida* UW4 appears to become deleterious in the presence of MS medium” (Chapter 2) and is being submitted to Applied and Environmental Microbiology. This manuscript is co-authored with Dr. Gordon Southam and Dr. Sheila M. Macfie (supervisor). I designed and conducted the experiments, collected and analyzed all the data, and wrote the manuscript. Dr. Southam provided guidance for the microscopy component, including financial support, experimental design and interpretation. Dr. Macfie provided laboratory support and guidance in experimental design, data interpretation, and manuscript preparation. The second manuscript is entitled “It takes a community to raise a plant: TRFLP analysis of the rhizosphere microbial community of two pairs of high- and low metal-accumulating plants in two soil types” (Chapter 3) and is currently being prepared for publication. This manuscript is co-authored with Dr. Sheila M. Macfie (supervisor). I designed and conducted the experiment, collected and analyzed all the data, and wrote the manuscript. Dr. Macfie provided laboratory support and guidance in experimental design, data interpretation, and manuscript preparation. I am, or will be, the first author on both of these publications.

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List of Abbreviations

ACC – 1-aminocyclopropane-1-carboxylate

AcdS – ACC deaminase gene

CCME – Canadian Council of Ministers on the Environment

DF – Dworkin and Foster

IAA – Indole-3-acetic acid

MS – Murashige and Skoog

PGPR – Plant growth-promoting rhizobacteria

TRFLP – Terminal restriction fragment length polymorphism

TSA – Tryptic soy agar

TSB – Tryptic soy broth

US EPA – United States Environmental Protection Agency

Chapter 1

1 General Introduction

1.1 Overview

Often, when attempting to explain unexpected outcomes of agricultural or phytoremediation field trials, researchers use the catchall disclaimer that any number of unknown and complex interactions between plants, microbes, and the environment may be contributing to the result. Although many potential mechanisms have been proposed for the way plants and microbes interact, especially mechanisms of action for beneficial and deleterious microbes on plant health, growth and development, most of this research focuses on single pathways of interest under defined growth conditions and fails to address how these mechanisms may be altered by other environmental factors. Additionally, a lot of research has focused on the identification and isolation of potentially beneficial microbes from the rhizosphere of plants. However, very little has been done to characterize the role(s) of these microbes at the community level, their interactions with other microbes, or how the plants themselves, in turn, affect these microbes. This may be due to the fact that it has been difficult to characterize microbial communities in the past due to the limitations of culture-based methodology. However, with the development of new molecular techniques researchers can start to look at community interactions in a whole new way. Knowing the complexity of plant-microbe interactions, it is important that future research attempts to understand both small-scale plant-microbe-metal interactions, such as elucidating particular mechanisms of action for beneficial and deleterious microbes under different environmental conditions, as well as to examine plant-microbe-metal interactions as a whole ecosystem.

1.2 Cadmium as a Human Health and Environmental Issue

Cadmium (Cd) is a non-essential, potentially toxic, metal for almost all living things. Essentiality of elements required by plants was established by Arnon and Stout (1939), who deemed an element essential if: 1) the organism is unable to grow or complete its lifecycle in the absence of the element, 2) the element cannot be replaced

completely by another element, and 3) the element is involved in the organism's metabolism. Cadmium does not fit any of the above criteria, for any known organism except the marine diatom *Thalassiosira weissflogii*, which can substitute cadmium for zinc to maintain growth (Lane *et al.* 2005).

Cadmium exists naturally in soil and is also introduced anthropogenically through a variety of sources, such as atmospheric deposition, application of manure, fertilizing regimes, other soil amendments, and accidental contamination from industry (Alloway and Steinnes, 1999). Repeated application of phosphate fertilizer on agricultural fields is a major source of cadmium in agricultural systems and, consequently, human exposure (Sheppard *et al.* 2007; Grant *et al.* 2011). Phosphate fertilizer can contain up to 340 mg cadmium/kg fertilizer depending on the cadmium concentration in the phosphate rock used to manufacture it (Alloway and Steinnes, 1999). The accumulation of cadmium in food crops, especially food staples such as wheat, is of major concern to human health due to the potentially toxic effects. The United States Environmental Protection Agency (US EPA) has classified cadmium in Group B1, a probable human carcinogen, and lists both acute and chronic effects such as pulmonary irritation, kidney disease, and developmental abnormalities (US EPA, 2000).

In addition to human health effects, cadmium can have a negative impact on crop yields. Visible symptoms of cadmium toxicity in plants include browning of leaves, chlorosis, reddish veins and petioles, brown stunted roots, and severe reduction in growth (Kabata-Pendias and Pendias, 1994). Cadmium can interfere with photosynthesis by disrupting the function of the photosynthetic apparatus, including light harvesting complex II (Krupa, 1988), photosystems I and II (Siedlecka and Krupa, 1996), and chlorophyll content (Larsson *et al.* 1998). Cadmium also negatively affects water balance (Barceló *et al.* 1986), nutrient uptake (Yoshihara *et al.* 2006), and function of a variety of enzymes including rubisco, arguably the most important enzyme in plants since it is essential for carbon fixation (Siedlecka *et al.* 1997). However, plant sensitivity to cadmium varies widely among species and varieties.

The range of cadmium that naturally exists in soil is 0.01 – 1.0 mg/kg (Pais and Jones, 1997). The Canadian Council of Ministers of the Environment (CCME) set the maximum allowable concentration of cadmium in agricultural soil at 1.4 mg/kg (CCME, 2007). However, standards based on soil concentration fail to take into consideration the range of factors that may influence metal bioavailability such as pH (Naidu *et al.* 1997), organic matter content (Murray *et al.* 2011), and microbial processes. Microbes have been shown to both inhibit and stimulate the mobility of cadmium in soil. Organic substances produced by some microbes can chelate and mobilize cadmium (Bollag and Czaban, 1989) and microbial metabolism of organic matter, which normally binds cadmium, results in the release of cadmium from these complexes and increases its overall mobility (Cole, 1979). Conversely, microbial production of hydrogen sulphides results in the formation of stable, insoluble cadmium sulphides (Bollag and Czaban, 1989), which are unavailable for plant uptake.

Given the prevalence of cadmium in agricultural soils globally, and especially in Canada, and the significant impact that this has on human health and crop yields, cadmium is an important toxic metal to study.

1.3 Plant Responses to Toxic Metals

1.3.1 Ethylene Stress Response

The production of ethylene is the most common response of plants exposed to a variety of biotic and abiotic stresses. Ethylene is a gaseous plant hormone that, in low concentrations, is responsible for a wide range of processes including: developmental processes, such as formation of roots; flowering; sex determination; and acclimation processes, such as defense response to pathogens (Taiz and Zeiger, 2010). It is thought that low concentrations of ethylene exist in all plants and that the introduction of a stress results in an initial small peak in ethylene, which triggers protective responses that might ameliorate the stress. With continued exposure to the stress a second, larger peak of ethylene will then trigger stress-induced symptoms in the plant (reviewed in Glick, 2005), such as stunted growth and senescence (Gazzarrini and McCourt, 2003). Toxic metal

exposure, such as exposure to cadmium in concentrations above 1 μM , have been shown to stimulate stress ethylene production in leaf tissue (Fuhrer, 1982)

1.3.2 Plant Tolerance to Toxic Metals

Plants have evolved many mechanisms for coping with metal stress including different avoidance and tolerance strategies to limit the stress response. Avoidance strategies include excluding the metal from the plant tissue by immobilizing the metal in the soil. This can occur through the production of plant exudates, such as organic acids, that bind metal present in the soil making it less bioavailable to the plant (Costa *et al.* 1997) or by altering soil pH to reduce metal solubility (Yang, *et al.* 2001). Plants also have tolerance mechanisms to minimize the damage of non-essential metals when they enter the plant. Plants respond by binding metals with phytochelatins (Akhter *et al.* 2012) and/or organic acids (Sanita di Toppi and Gabbrielli, 1999) and compartmentalizing these complexes within metabolically inactive sites, such as the vacuole, where they are rendered inert (Salt and Rauser, 1995).

1.4 Plant-Microbe interactions

1.4.1 The Rhizosphere

The rhizosphere is the volume of soil surrounding plant roots that is directly influenced, chemically, physically, and biologically, by the plant root, leading to a favorable habitat for microorganisms (Sorenson, 1997). These microorganisms may in turn heavily influence the plant. Environmental and soil conditions have known effects on both plant and microbe development. While plant responses to metals are well documented (section 1.3), similar information on microbes is scarce. For example, it is known that exposure to toxic metals and other pollutants can reduce overall microbial biomass and diversity in soil (Giller *et al.* 1998). Beyond measures of biomass and metabolic rates there currently is no literature available on the response of microbes to toxic metal exposure. In addition to abiotic factors, biological interactions and the intimate relationship between plants and microbes in the rhizosphere can make it difficult to tease apart the degree and directionality of influence between these organisms.

1.4.2 Plant Influence on Microbes

The most fundamental way in which plants exert influence on the microbes in the rhizosphere is through the secretion of various exudates. Organic acids (Lugtenberg *et al.* 1999), photosynthetically-fixed carbon in the form of sugars (Marschner, 1995), and amino acids (Simons *et al.* 1997) have all been identified as major components of plant exudates. The increase in the availability of these nutrients stimulates proliferation of microorganisms and can alter the community structure by selecting for, or against, species that are most able to utilize those nutrients. Some exudates can specifically recruit beneficial microbes. When secreted from the roots of *Arabidopsis*, malic acid acts as a signal to recruit the beneficial bacterium *Bacillus subtilis* FB17 (Rudrappa *et al.* 2008). On the other hand, the secretion of defense proteins and other chemicals prevents the proliferation of some plant pathogens such as *Pseudomonas solanacearum*, *Pythium aphanidermatum*, *P. ultimum*, and *Rhizoctonia solani* (Flores *et al.* 1999).

1.4.3 Microbial Influence on Plants

Microbes found in the rhizosphere can have both beneficial and deleterious effects on plants. Many of the beneficial impacts are discussed below. Deleterious microbes include known plant pathogens, such as *Phytophthora cinnamomi* (Gotesson *et al.* 2002) and microbes that secrete phytotoxic metabolites, such as *Fusarium moniliforme*, which produces fusaric acid, a corn phytotoxin that interferes with seed germination and plant growth regulation. Additionally, microbes have the ability to make toxic metals more bioavailable to plants by chelation (Bollag and Czaban, 1989) and metabolism of organic-metal complexes that results in the release of metals (Cole, 1979). Microbes can also compete with plants for nutrients. In the case of iron, iron-chelating siderophores released by microbes bind to iron. Uptake of these iron-siderophore complexes requires a transporter that can be specific to the microbe that released it (Crosa, 1989), thereby limiting the availability of iron for the plants.

Most research on the microbial component of the rhizosphere has focused on characterizing the physiological function of microbial communities and on the identification and isolation of microbes of interest from the rhizosphere. For example,

past research has isolated cadmium-tolerant microbes from the roots of Indian mustard growing in contaminated soil (Belimov *et al.* 2005) and identified microbes that are capable of stimulating plant growth in stress conditions (Glick *et al.* 1998).

1.4.4 Plant Growth-Promoting Rhizobacteria

Plant growth-promoting rhizobacteria (PGPR) were first defined by Kloepper and Schroth (1978) as root-colonizing bacteria that exert beneficial effects on plant growth. Since being recognized as important for increasing seedling emergence, vigor, biomass, proliferation of root systems, and crop yield in many species, several studies have focused on identifying PGPR in natural systems and the development of these bacterial strains for commercial use (Podile and Kishore, 2006). Several direct and indirect mechanisms for growth-promotion have been documented. Direct mechanisms include nitrogen-fixation (Bashan *et al.* 2004), production of phytohormones such as the auxin indole-3-acetic acid (IAA), which stimulates cell growth and proliferation at low concentrations (Vessey, 2003), metabolism of the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) through the enzyme ACC deaminase (Glick *et al.* 1998), and increased availability of iron through bacterial production of siderophores (Kloepper *et al.* 1991). Indirect mechanisms include increased competition between PGPR and deleterious and pathogenic microorganisms for nutrients, a mechanism common in the nutritionally versatile pseudomonads (Walsh *et al.* 2001), and formation of biofilms that may help to exclude toxic metals from the plant (Stout *et al.* 2010).

One model for bacterial plant growth-promotion that has been thoroughly tested suggests two simultaneous mechanisms (Figure 1-1). First, bacterially produced IAA can stimulate root cell elongation and proliferation (Arshad and Frankenberger, 1991). Concurrently, the bacterial enzyme ACC deaminase acts as an extracellular sink for plant-produced ethylene precursor, ACC, by metabolizing it into the inert byproducts ammonia and α -ketobutyrate, reducing the amount of ACC available for conversion into ethylene and minimizing the stress response that is a result of increased ethylene concentration in the plant (reviewed in Gamalero *et al.* 2009).

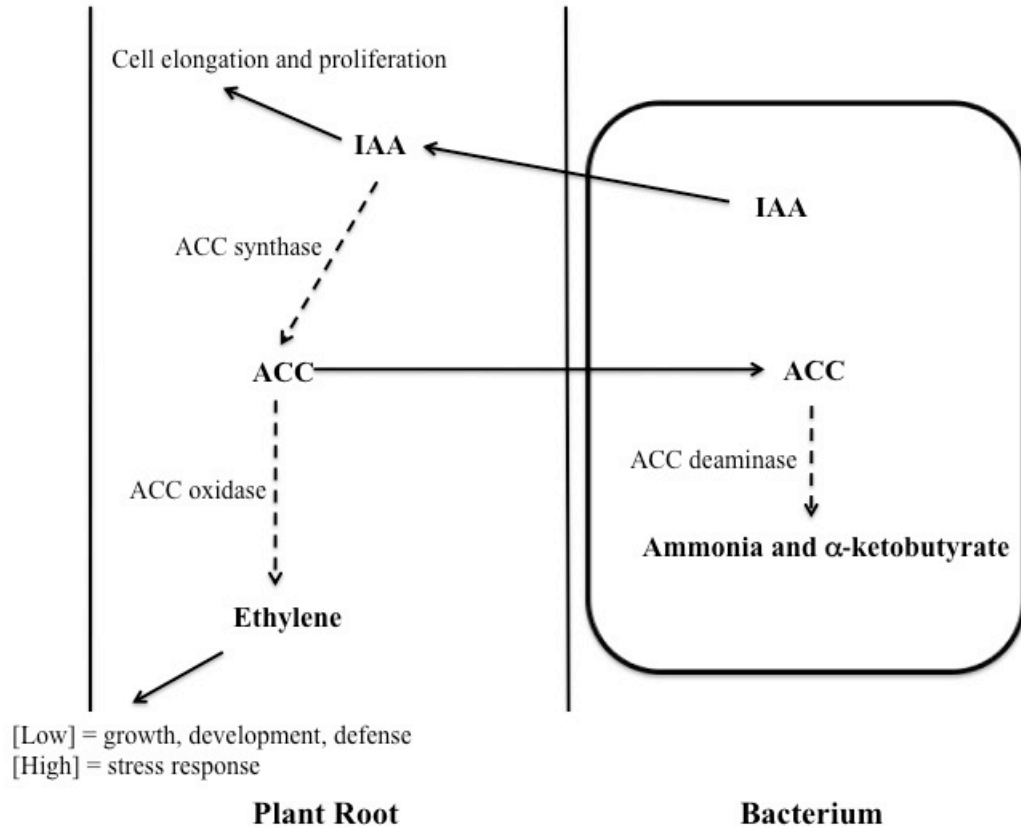


Figure 1-1: A model for plant growth-promotion by plant growth-promoting rhizobacteria (modified from Glick *et al.* 1998)

This figure illustrates the proposed influence of bacteria indole-3-acetic acid (IAA) and 1-aminocyclopropane-1-carboxylate (ACC) deaminase on plant production of ethylene. Bacterial IAA can either enhance root growth or increase the synthesis of ethylene. Bacterial ACC deaminase can divert ACC from the ethylene synthesis pathway and metabolize it into inert by-products. When kept at a low concentration ethylene is important for growth, development, and defense but at high concentrations it triggers a stress response in plants.

1.4.5 The PGPR *Pseudomonas putida* UW4

Pseudomonas putida is a gram-negative, aerobic, rod shaped bacterium in the family *Pseudomonadaceae* that is normally isolated from soil and water and has an optimum growth temperature between 25 – 30°C (Palleroni, 1984). *Pseudomonas putida* UW4 was isolated from the roots of common reeds (*Phragmites australis*) in Waterloo, Ontario, for its ability to utilize ACC as a sole nitrogen source (Glick *et al.* 1995). This bacterium was originally classified as *Enterobacter cloacae* based on fatty acid profiles (Shah *et al.* 1998) and was later reclassified as *P. putida* based on 16S rRNA gene sequencing with 98% sequence similarity in the GenBank database (Hontzeas *et al.* 2005). Research prior to this reclassification in 2005 named this organism *E. cloacae* UW4; subsequent work was done with the same lab strain under the name *P. putida* UW4. No further attempts have been made to identify this organism. Given the 2% 16S rRNA gene sequence dissimilarity there is a possibility that this organism will be reclassified at a later date.

This organism has garnered attention for its ability to promote plant growth by simultaneously 1) metabolizing the ethylene precursor ACC with the enzyme ACC deaminase and 2) stimulating plant growth through the production of the beneficial plant hormone IAA. A mutant of *P. putida* UW4 that can no longer produce the enzyme ACC deaminase, but continues to produce IAA, was created by the insertion of a tetracycline resistance gene into the coding region of the enzyme (Li *et al.* 2000). This mutant has since been used in ACC deaminase-containing PGPR studies to act as an ACC deaminase control. A large body of research has shown the ability of *P. putida* UW4 to promote plant growth and alleviate plant stress under a range of environmental stresses in many plant species. A summary of some of the stresses, plant species, and results that directly demonstrate the plant growth-promoting affects of this bacterium is presented in Table 1-1.

1.5 Characterizing Microbial Communities

Compared to determining the functional role(s) of microbial communities very little research has been done to characterize differences in microbial communities in

Table 1-1: Summary of past research utilizing the plant growth-promoting rhizobacterium *Pseudomonas putida* UW4

Bacterium	Plant Species	Stress	Results	Reference
<i>Enterobacter cloacae</i> UW4 ¹	Tomato (<i>Lycopersicon esculentum</i> cv. Heinz 902)	Flooding	Maintained root and shoot growth and increased chlorophyll content	Grichko and Glick (2001)
	Canola (<i>Brassica napus</i>)	Metal (As)	Increased biomass and metal accumulation	Nie <i>et al.</i> (2002)
	Common reed (<i>Phragmites australis</i>)	Metal (Co) Polycyclic Aromatic Hydrocarbons	Increased seed germination and plant size	Reed <i>et al.</i> (2005)
<i>Pseudomonas putida</i> UW4	Canola (<i>Brassica napus</i>)	Salt Temperature	Increased root and shoot biomass	Cheng <i>et al.</i> (2007)
	Canola (<i>Brassica napus</i>)	Flooding Metal (Ni)	Increased shoot biomass	Farwell <i>et al.</i> (2007)
	Cucumber (<i>Cucumis sativus</i>)	Salt	Promoted symbiosis with beneficial mycorrhizal fungi <i>Gigaspora rosea</i> resulting in increased biomass	Gamalero <i>et al.</i> (2008)
	Ryegrass (<i>Lolium perenne</i>) Barley (<i>Hordeum vulgare</i>) Tall fescue (<i>Festuca arundinacea</i> var. Inferno) Fall rye (<i>Secale cereale</i>)	Petroleum Hydrocarbons	Increased biomass, particularly in roots and ground cover	Gurska <i>et al.</i> (2009)
	Canola (<i>Brassica napus</i>)	Metal (Cd)	Increased root elongation	Belimov <i>et al.</i> 2001

¹ Before 2005, *P. putida* UW4 was classified as *E. cloacae* UW4 (Hontzeas *et al.* 2005)

² This is believed to be UW4, although not stated in the paper, based on other publications from this group

different environmental conditions and under different stresses. However, there has been a movement towards research of this nature. For example, one study used a combination of culture-dependent and molecular techniques to document differences in microbial strains in the soil surrounding two different clones of poplar trees (Gamalero *et al.* 2012). Another study used the molecular technique of terminal restriction fragment length polymorphism analysis to determine that metal contamination in soil reduced microbial richness and diversity (Tipayno *et al.* 2012).

Complex soil microbial communities can be difficult to characterize using traditional culture-based methods such as plate counts and metabolic profiling. This is in equal parts due to that fact that a large number of organisms that exist in nature cannot be cultured in laboratory conditions (Ward *et al.* 1990) and that any departure from the original environmental parameters during cultivation will alter the community structure by imposing new selective conditions (Dunbar *et al.* 1997). Modern molecular methods can overcome the problems associated with culture-based methods and allow for rapid, high-throughput processing of environmental samples. One quantitative molecular technique for rapid analysis of complex microbial communities using polymerase chain reaction (PCR) amplification with fluorescently labeled primers to detect terminal restriction fragment length polymorphisms (TRFLP) was first developed by Liu *et al.* (1997). This technique has since undergone many advancements to become one of the most commonly used, culture-independent techniques for rapid analysis of microbial community diversity because it has been shown to be highly reproducible and yields a higher number of operational taxonomic units than many other PCR-based methods (Osborn *et al.* 2000). Methods such as TRFLP analysis can allow researchers to “fingerprint” the microbial community at a given time point and allows for comparison of communities between samples. Analysis of TRFLP has been successfully used to analyze microbial populations isolated from a range of substrates including biofilms (Wuertz *et al.* 2004), water (Dorigo *et al.* 2005), and soil (Leckie, 2005) as well as for monitoring changes in communities in response to environmental changes such as phytoremediation processes (Tipayno *et al.* 2012).

The most common technique for TRFLP analysis involves amplifying small subunit (16S or 18S) rRNA gene amplicons from total genomic DNA isolated from an environmental sample using PCR where both primers are fluorescently labeled. The amplicons are then digested with one or more restriction enzymes, and the size and abundance of the resulting fragments are determined using a DNA sequencer. Since the size of the fragment reflects differences (polymorphisms) in the 16S rRNA genes, phylogenetically distinct populations can be determined and the pattern of fragments can be used to profile (“fingerprint”) the dominant contributors to the community (reviewed in Schutte *et al.* 2008). When combined with phylogenetic information and statistics software researchers are able to detect differences in the composition of microbial communities and determine plausible members of these communities.

Choice of primers can alter the TRFLP profiles that are generated. Once considered a ‘universal’ bacterial primer, 8fm has since been shown to amplify as low as 76% of 16S rRNA gene sequences that are available (Marsh *et al.* 2000). However, new primers are constantly being developed that can overcome these shortcomings. Therefore, primers should be selected based on the type of analysis that will be carried out and with the acknowledgement that some groups may be missed in the analysis.

Run-to-run variability, generally 0.5 – 1 bp, can result in size discrepancies among fragments from the same bacterial population (Schutte *et al.* 2008). However, since this is a known variable researchers can apply methods such as rounding to the nearest integer and manual “binning,” which when coupled with experimental duplicates and common sense allows for populations within the community to be properly grouped.

Finally, variability between samples may be masked by analytical variability related to DNA purification, efficiency, and pipetting error; therefore, care should be taken at all steps of the analysis to reduce this error. Also, this source of analytical noise can be reduced by transforming the data output into a binary presence/absence matrix to eliminate variability in abundance due to these errors (Culman *et al.* 2008).

Most of the limitations discussed above can be overcome with consistent and careful technique. With knowledge of the limitations and careful analysis of the data

researchers can quickly and repeatedly compare entire microbial communities and monitor changes over time.

It is possible to use TRFLP data to identify probable members of a microbial community by comparing detected fragment lengths to fragments predicted by databases of 16S rRNA gene sequences. However, this should be done with caution since the length of fragments measured by the DNA sequencer can be influenced by differences in electrophoretic mobility of DNA caused by the use of different fluorophores to label DNA fragments (Tu *et al.* 1998) and sequence composition, especially purine content (Kaplan and Kitts, 2003). Also, comparison to DNA databases is limited by the facts that not all isolates have been sequenced and there are many novel and unknown microbes yet to be identified, let alone sequenced (Blackwood and Buyer, 2007).

The repeatability, ease of use, and range of potential applications possible with TRFLP analysis makes it a good tool for to study diversity and changes in complex microbial community structure in a wide range of environmental samples.

1.6 Rationale and Research Objectives

Improvements in our understanding of plant-microbe interactions under a variety of environmental stresses on all scales, from single-pathway biochemistry to the whole ecosystem, is essential for understanding factors that affect the efficiency of both bioremediation processes and use of PGPR for agricultural purposes. Advancements in this field could help us to understand why field trials often fall short of the expected outcomes predicted from laboratory studies and make improvements for future field applications.

It has been generally accepted that plants and microbes influence each other as well as their environment, but the specifics of these interactions, especially under varying environmental conditions are often lacking. This could be due to the fact that it has been nearly impossible to include all environmental factors into lab testing or to test microbial communities in natural settings due to the limitations of culture-based techniques. With

increasingly complex lab systems and advancements in molecular techniques we can now start to get a glimpse of these complex systems.

The studies presented in this thesis were designed to investigate plant-microbe interactions under toxic metal stress starting from the small-scale, single bacterium system utilizing a known biochemical pathway, and moving up to investigating the plant-microbe interaction as a whole ecosystem.

The specific objectives of the studies reported in this thesis were to:

1) Investigate the efficiency of the putative PGPR *Pseudomonas putida* UW4 at ameliorating cadmium stress in *Arabidopsis thaliana* grown in the common plant nutrient MS medium (Chapter 2)

2) Determine whether MS medium would have a negative impact on the composition of native bacterial communities in agricultural soils (Appendix B)

3) Investigate the impact of high- and low metal-accumulating plants on the composition of the microbial community in their rhizosphere when grown in agricultural soils varying in cadmium concentration (Chapter 3)

The organisms used in each chapter were carefully selected based on the corresponding research objective. In Chapter 2, the plant model species *Arabidopsis thaliana* was used due to the fact that it has well characterized physiological and morphological responses to the two hormones of interest, ethylene and IAA (Le *et al.* 2001; Ruzicka *et al.* 2007; Pitts *et al.* 1998; Rahman *et al.* 2002), and that mutant lines were readily available. The documented and predictable detailed responses of *A. thaliana* to these hormones, would allow me to draw conclusions about the relative roles of bacterial IAA production and ethylene metabolism on mitigating plant stress. *Pseudomonas putida* UW4 had been well characterized and studied. There is a lot of evidence for its mechanisms of action (Figure 1-1) and it had been shown to be universally effective as a plant growth-promoter (Table 1-1). Additionally, an AcdS⁻ mutant of *P. putida* UW4 exists that can produce IAA but has no measureable ACC deaminase activity (Li *et al.* 2000). Using these two bacterial strains could allow for

distinctions to be made between the relative roles of these two pathways in plant growth promotion. Together, these two organisms were an ideal system for studying small-scale beneficial plant-microbe interactions.

To confirm that the results of Chapter 2 were not an artifact of using an agar-based medium or hydroponics I performed a side-project (Appendix B) to test the effect of MS medium on the native bacterial communities in soil. These communities were compared to those from soil watered with distilled water based on the fact that many PGPR studies that report positive results maintained their systems in this way.

In Chapter 3, I chose two pairs of high- and low-accumulating plants. Metal uptake in durum wheat (*Triticum durum*) is intensively studied due to the agricultural and economic significance of this crop. Natural variation in metal accumulation exists in the different cultivars of this crop (Garret *et al.* 1998). The cultivars Kyle and Arcola are known to be high- and low-accumulators, respectively (Chan and Hale, 2004). *Brassica juncea*, better known as Indian mustard, is the most popular metal hyperaccumulator used in phytoremediation, while *B. napus* is commonly used as a non-hyperaccumulating control (McGrath *et al.* 2001). Individually, each of these pairs of plants offers a good system to study metal-uptake and distribution in plants, as well as plant-microbe interactions arising from the plant's inherent metal accumulating abilities. The use of both pairs will allow for additional insight into these mechanisms at the genus level.

All of these studies use cadmium as a metal of interest for the reasons outlined in section 1.2.

1.7 References

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Chapter 2

2 The message is in the medium: the putative PGPR

Pseudomonas putida UW4 appears to become deleterious in the presence of MS medium

This chapter contains a series of experiments that were designed to evaluate the effectiveness of the putative plant growth-promoting bacterium *P. putida* UW4 under conditions of cadmium stress. My initial goal was to use the model plant *Arabidopsis thaliana* grown in agar and hydroponic conditions to determine the relative roles of two pathways responsible for bacterial promotion of plant growth. This project took an interesting turn, however, when I discovered that bacteria considered beneficial to plant growth under certain conditions could become deleterious.

2.1 Introduction

Naturally occurring soil bacteria that grow in close association with plants and are beneficial to plant growth are often referred to as plant growth-promoting rhizobacteria (PGPR). These PGPR have the ability to increase agricultural crop yields (Glick *et al.* 1997; Sziderics *et al.* 2007), act as biocontrol agents (Schroth and Hancock, 1982; Silva *et al.* 2004) and alleviate a variety of biotic and abiotic stresses (reviewed in Saravanakumar, 2012). It is also possible to use PGPR to enhance the efficacy of phytoremediation processes by maintaining plant growth and reducing the plant stress response in the presence of soil contaminants. Many studies have examined the ability of various PGPR to promote plant growth under stress conditions. However, the magnitude of the plant response in the presence of the bacteria, and the variables that were reported as indicators of plant growth promotion, has not been consistent among studies.

One of the most common responses of plants exposed to both biotic and abiotic stress is the increased production of ethylene. Ethylene production and subsequent ethylene-dependent signaling are involved in both stress-induced responses, such as

stunted growth and senescence, as well as in acclimation processes that can aid in plant performance and survival (Gazzarrini and McCourt, 2003). It is thought that in stressed plant tissues there is an initial small peak of ethylene that initiates a protective response followed by a much larger peak which initiates processes such as senescence (reviewed in Glick, 2005). Many PGPR are capable of producing the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase (AcdS), which acts as an extracellular sink for the plant-produced ethylene precursor ACC, metabolizing it into ammonia and α -ketobutyrate thereby reducing the amount of ACC available for conversion into ethylene and reducing the ethylene concentration in plant tissues (reviewed in Gamalero *et al.* 2009).

The reduction of ethylene by ACC deaminase is not the only contributor in promoting plant growth under stress conditions. Bacterial production of other plant hormones, such as the auxin indole-3-acetic acid (IAA), has also been implicated in influencing plant growth (Arshad and Frankenberger, 1991). One model for lowering plant ethylene concentrations using PGPR has been proposed that includes bacterial production of IAA to stimulate root cell elongation and proliferation while simultaneously reducing the amount of ethylene produced by ACC deaminase (Glick *et al.* 1998; Figure 1-1). Thus, the combination of the bacterial metabolism of the plant stress hormone ethylene and the bacterial production of IAA could explain plant growth promotion under stress conditions (Patten and Glick, 1996; reviewed in Gamalero *et al.* 2009).

The PGPR *Pseudomonas putida* UW4 has garnered a lot of attention for its ability to promote plant growth and root elongation effectively under salt, drought, flooding, heat, and metal stress in a variety of plant species such as canola, tomato, *Brassica* spp, cucumber, and peppers (reviewed in Saravanakumar, 2012). Isolated from roots of common reeds in Waterloo, Ontario, and originally classified as *Enterobacter cloacae* UW4, *P. putida* UW4 has been shown to contain the enzyme ACC deaminase, as well as to produce the beneficial auxin IAA (Glick *et al.* 1995). An AcdS⁻ mutant of *P. putida* UW4 was created that can produce IAA but has no measureable ACC deaminase activity

(Li *et al.* 2000). Using these two bacterial strains could allow for distinctions to be made between the relative roles of these two pathways in plant growth promotion.

The plant model *Arabidopsis thaliana* and its mutants are commonly used to identify genes involved in hormone signal transduction and hormone signaling during plant growth and development (Gazzarrini and McCourt, 2003). More specifically, the cellular organization of roots (Dolan *et al.* 1993; Carol and Dolan, 2002) and the roles of both ethylene and IAA in root development and plant growth have been extensively studied in *A. thaliana* (Le *et al.* 2001; Ruzicka *et al.* 2007; Pitts *et al.* 1998; Rahman *et al.* 2002). Given the documented and predictable detailed responses of *A. thaliana* to these hormones, the use of this plant is ideal for investigations of the relative roles of bacterial IAA production and ethylene metabolism on mitigating plant stress.

The objective of this study was to evaluate the relative effectiveness of the wild-type *P. putida* UW4 in mitigating cadmium-stress in *A. thaliana* grown in agar and hydroponic conditions. Nutrient agar and hydroponic media were used to control bioavailability of cadmium to the plants and to enable non-damaging harvest of intact plant roots.

2.2 Methods

2.2.1 Bacterial Strains

Wild type *P. putida* UW4 (AcdS⁺), and the ACC deaminase minus mutant (AcdS⁻), were provided by Dr. Bernard Glick (University of Waterloo). Bacterial cultures were stored in 15% glycerol at -80°C when not in use. The genome sequence of *P. putida* UW4 is available in the GenBank database (<http://www.ncbi.nlm.nih.gov/nuccore/CP003880.1>).

2.2.2 Bacterial Culture Maintenance

Bacterial cultures were maintained according to Penrose and Glick (2003). Cultures were taken out of storage and streaked onto tryptic soy agar (TSA) plates containing

approximately 20 mL of tryptic soy broth (TSB) (VWR Canada), 1.5% bacteriological grade agar (BioShop Canada), and 15 µg/mL tetracycline (TET, BioShop Canada) when necessary. Due to the presence of the tetracycline resistance gene in the AccS^- mutant it is necessary to grow the mutant in the presence of TET (Li *et al.* 2000). Plates were incubated at 30°C for 24 h or until late log phase. A streak of bacterial colonies from each plate was transferred into 5 mL sterile TSB (with TET when necessary) and allowed to grow in an incubating mini-shaker (VWR, Radnor, PA, USA) set to 30°C and 200 rpm for 24 h. To induce ACC deaminase activity, overnight cultures were centrifuged at 2550 g for 10 min at 4°C using a Sorvall Biofuge PrimoR bench top centrifuge (Thermo Scientific Co, Asheville, NC, USA) and the supernatant was discarded. Cells were washed twice with 5 mL DF salts minimal medium without nitrogen (Dworkin and Foster, 1958) and then re-suspended in 7.5 mL DF salts minimal medium with 45 µL 0.5 M ACC (Calbiochem) to obtain a final ACC concentration of 3.0 mM. The ACC solution acts as the sole nitrogen source for the cultures. Cultures were then incubated for 24 h at 30°C and 200 rpm. Overnight cultures were centrifuged at 2550 g for 10 min at 4°C and the supernatant was discarded. Cells were washed twice with 5 mL of 0.03 M MgSO_4 to ensure the cells were free of the bacterial growth medium and re-suspended in 0.03 M MgSO_4 to a final OD_{600} of 0.15 before inoculating plant seeds.

2.2.3 Plant Growth Conditions

Arabidopsis thaliana (Col-0) seeds were surface-sterilized in a sterile microcentrifuge tube by adding 1 mL 70% ethanol for 5 min, replacing the ethanol with a 30% bleach solution for 10 min, and rinsing three times with sterile distilled water (dH_2O). A small volume of dH_2O was left in the tube to avoid desiccation and the seeds were stored at 4°C in the dark for 3 days to synchronize germination (modified from Hetu *et al.* 2005). Seeds were inoculated with the appropriate bacterial strain, or 0.03 M MgSO_4 as a control, and then transferred to Petri dishes containing approximately 20 mL half strength MS medium, 0.8% agar, 1% sucrose, pH 5.8 and either 0, 0.1, 1.0, 5.0, or 10.0 µM CdCl_2 (Murashige and Skoog, 1962). This range of CdCl_2 concentrations was selected based on a dose response study that showed seeds had visible symptoms of Cd-stress at 10 µM CdCl_2 , including reduced size and root elongation, but failed to germinate on plates

containing 25 μM CdCl_2 (Appendix A). Plates were sealed with Parafilm and placed in a growth chamber with a 16:8 h light: dark cycle maintained at 22°C and 60% relative humidity (RH). Light intensity was determined to be $230 \pm 5.7 \mu\text{mol}/\text{m}^2/\text{s}$ as repeatedly measured using a Fieldscout Quantum Light Meter (Spectrum[®] Technologies Inc, IL, USA). Plates were placed in either a horizontal or vertical orientation depending on the data to be collected and grown for 7 or 14 d (Boyes *et al.* 2001). Plate position in the growth chamber was rotated daily to control for position-dependent variation.

For hydroponic studies *A. thaliana* (Col-0) plants were grown according to Hetu *et al.* (2005). Briefly, seeds were surface-sterilized and cold-synchronized as previously described and inoculated with the appropriate bacterial strain or control. Seeds were transferred onto agar plates as described above; however, sterile 2.5 cm^2 pieces of fiberglass window screen (Easy Screen, RCR International Inc, Quebec) were placed on the agar before the seeds were added to act as a support matrix for the roots. Seeds were placed in a growth chamber and allowed to grow for 7 d or until the shoots were larger than the holes in the screen. At this point, ethanol-flamed forceps were used to transfer the screens holding the seedlings into sterile glass jars containing 10 mL 0.5 MS medium, 1% sucrose, pH 5.8 and one of the experimental concentrations of CdCl_2 . The openings of the jars were covered in aluminum foil to prevent contamination. After 7 d the screens were transferred into 20 mL of fresh solution seedlings were allowed to grow for a total of 21 d from the time of plating. Hydroponic jars were maintained in a growth chamber with a 16:8 h light: dark cycle at 22°C and 60% RH on a rotary shaker set to 60 rpm. All manipulations took place in a sterile laminar flow hood using aseptic techniques.

The nutrient solution MS medium is common in plant studies, especially those with *Arabidopsis*. This medium was used in both of the methodological papers for growing and measuring morphological features of *A. thaliana* used in this experiment (Boyes *et al.* 2001; Hetu, *et al.* 2005). Additionally, it has been observed that without certain nutritional inputs, such as sucrose, it can be difficult to germinate *Arabidopsis* seeds reliably (personal observation). For these reasons, MS medium was chosen as the plant medium in this study. The following recipe for MS medium was diluted by 50% for all experiments: 1650 mg/L ammonium nitrate, 6.2 mg/L boric acid, 332.3 mg/L anhydrous

calcium chloride, 0.025 mg/L tribasic calcium phosphate – 6H₂O, 0.025 mg/L cupric sulfate - 5H₂O, 37.26 mg/L Na₂-EDTA, 27.8 mg/L ferrous sulfate - 7H₂O, 180.7 mg/L magnesium sulfate, 16.9 mg/L manganese sulfate - H₂O, 0.25 mg/L molybdic acid - 2H₂O, 0.83 mg/L potassium iodide, 1900 mg/L potassium nitrate, 170 mg/L monobasic potassium phosphate, 8.6 mg/L zinc sulfate - 7H₂O, 2.0 mg/L glycine, 100 mg/L myo-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine – HCl, and 0.1 mg/L thiamine – HCl.

2.2.4 Analysis of Plant Health and Growth

2.2.4.1 Maximum Photochemical Efficiency of Photosystem II

Fluorescence measurements used to calculate photosystem II (PSII) efficiency were made using an Imaging PAM Chlorophyll Fluorometer (Heinz Walz, Germany). Following dark adaptation for 1 h at room temperature plant shoots were exposed to a short (800 ms) pulse of saturating blue light ($\lambda = 470$ nm; 6000 $\mu\text{M photons/m}^2/\text{s}$) provided by the Imaging PAM photodiode (IMAG-L; Heinz Walz). The maximum photochemical efficiency of PS II was calculated as Fv/Fm (Maxwell and Johnson, 2000).

2.2.4.2 Chlorophyll Content

Plants were grown for 14 days on horizontal agar plates as described above. The shoot tissue was then harvested and chlorophyll was isolated from the tissue according to Pocock *et al.* (2004). Tissue was ground with liquid nitrogen in a chilled mortar and pestle. Chlorophyll was then extracted from a subsample of 0.1 g of shoot tissue by adding 1 mL of 80% acetone solution buffered with 2.5 mM sodium phosphate to a pH of 7.8 to minimize the conversion of chlorophylls to phaeophytins. The samples were then centrifuged for 5 min at 3024 g at 4°C to clarify the samples and remove whole chloroplasts. A volume of 0.05 mL of the pigment extract was then added to 0.95 mL of the acetone solution. A 200 μL aliquot of each sample was then added to a 96 well plate and absorbance was measured at 664, 647, and 750 nm using a Spectramax M2

Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). Chlorophyll content and chlorophyll a:b ratio were calculated (Porra *et al.* 1989).

2.2.4.3 Rosette Diameter

Plants were grown on horizontal agar plates as described above for 14 d. Five plants were placed on each of 4 plates no less than 2 cm apart and away from the plate edge. The maximum rosette diameter for each plant was measured using digital calipers and recorded.

2.2.4.4 Root Morphology and Elongation

Plants were grown on vertical agar plates as described above until the roots were close to, but not touching the bottom of the plate (approximately 7 d). An image was taken of each plate on a black background using a mounted Nikon D2X camera. Primary root length, number of lateral roots, and length of lateral roots were determined and recorded using ImageJ 1.44o (Rasband, 2012).

2.2.4.5 Biomass

Plants were grown hydroponically as described above for 21 d and the number of seeds per plate was recorded. On day 21 plants were separated into roots and shoots and the fresh weight (FW) was recorded. Root and shoot tissue were then placed into individual envelopes made out of aluminum foil and placed in a drying oven set to 60°C for 4 d or until a constant weight was reached. The root and shoot dry weight (DW) was recorded.

2.2.4.6 Cadmium Content

The concentration of cadmium in roots and shoots was determined using a modified version of the Environmental Protection Agency test method SW-846 (United States Environmental Protection Agency, 2005). Dried root and shoot tissue was ground in a mortar and pestle and subsamples were taken for analysis. A standard reference material from the National Institute of Standards and Technology (NIST 1570a, trace

elements in spinach leaves) and reagent blanks were also included in the analysis. For shoot samples, 0.025 g of tissue was placed in a 15 mL glass test tube and for root samples 0.010 g of tissue was used. All the test tubes were placed in a rack and 250 μ L of ultrapure nitric acid (OmniTrace[®], EM Science, USA) was added to the shoot samples and 200 μ L to the roots samples. Test tubes were covered with glass marbles to prevent evaporation while allowing pressure to escape. The samples were allowed to sit overnight to allow for partial digestion of the organic matter in the samples. On the following day, the test tube rack was placed in a tray filled with sand and heated to 90-100°C on a hot plate until the vapors became transparent. The sand helped to ensure even distribution of heat among the samples during digestion. The samples were allowed to cool to room temperature before being filtered using qualitative grade filter paper (VWR, qualitative grade 413). Reverse osmosis water was used to rinse the test tube and bring the final volume of sample to 5 mL. The samples were analyzed for cadmium content by inductivity-coupled plasma atomic emission spectrometry (ICP-AES).

2.2.4.7 Light Microscope Images of Plant Roots

To determine whether the presence of the bacteria resulted in visible damage to plant roots, 7 d-old plants grown vertically on agar plates were mounted on a standard microscope slide in RO water and imaged using an Olympus CX31 light microscope with an Infinity-1 Camera and Infinity Analyze version 5.0.5 software. Toluidine Blue O (TBO) stain made the images dark and unclear and was therefore not used. Images were taken at the first root hair from the root tip of each plant.

2.2.5 Bacterial Growth and Plant Root Colonization

2.2.5.1 Growth Rate

The bacterial growth rates in MS and TSB media were determined using a BioScreen C apparatus (Oy Growth Curves Ab Ltd, New Jersey, USA). A 250 μ L aliquot of the ACC deaminase-activated cultures of wild type and mutant strains, standardized to the same starting OD₆₀₀, was added to an equal volume of medium, brought to the desired CdCl₂ concentration, and loaded into the BioScreen microplate.

The plates were placed in the apparatus, which preventing settling of the cultures and maintained an incubating temperature of 30°C while taking OD₆₀₀ readings every 15 min for 24 h. These data points were then used to create growth curves. Growth curves were also generated for both bacterial strains grown in 0.5 × MS medium with 1, 2, 3, 4, and 5 % sucrose, glucose, fructose, or a combination of glucose and fructose (1:1 ratio to obtain the same total concentrations as listed above) to determine the growth rate of each bacterial strain in MS medium with varying carbon sources and concentrations provided.

2.2.5.2 Bacterial Viability in Media

To determine whether the bacteria used were viable in the different liquid media used and the CdCl₂ concentrations tested, a streak of bacterial colonies grown on TSA (as previously described) was added to 5 mL of TSB (plus TET when necessary) or 0.5× MS with 1% sucrose ranging in concentration from 0 to 10.0 μM CdCl₂ and incubated for 24 h at 30°C and 200 rpm. Using an ethanol-flamed metal loop, a sample of each culture was then plated onto TSA (plus TET when necessary) and incubated for 24 h at 30°C at which point the plates were checked for bacterial colonies. If bacterial colonies failed to form on this bacterial medium they were considered not viable in the tested medium and CdCl₂ concentration. If bacterial colonies were able to form they were considered viable in the tested medium and CdCl₂ concentration.

2.2.5.3 Bacterial Viability on Plant Roots

To determine whether the presence of plants affected bacterial survival in the different media and CdCl₂ concentrations used the bacteria were grown with plants on agar plates as previously described for 7 d. Plant roots were then vigorously vortexed in 1 mL TSB medium in a sterile microcentrifuge tube for 1 min. The entire volume was then plated onto TSA using an ethanol-flamed glass rod. Plates were incubated at 30°C for 24 h at which point the plates were checked for formation of bacterial colonies.

2.2.5.4 Bacterial Colonization and Survival on Plant Roots

Fluorescence staining combined with confocal microscopy was used to visualize the bacterial colonization of plant roots and to determine whether the bacteria present were living or dead. Plant roots from 21 d old plants were stained using the Live/Dead[®] BacLight[™] Bacterial Viability Kit (Invitrogen, Cat# L7012), which uses green fluorescent stain SYTO[®] – 9 and red fluorescent stain propidium iodide. SYTO[®] – 9 stains both living and dead cells while propidium iodide will only penetrate cells with damaged membranes. When imaged under a fluorescence microscope living cells appear green while dead cells appear red. The staining kit was used with slight modifications from the manufacturer's instructions. In a sterile microcentrifuge tube 3 μ L of each SYTO[®] – 9 and propidium iodide were mixed into 1 mL of sterile RO water and vortexed. After incubation at room temperature for 5 min in the dark 200 μ L was added to fresh microcentrifuge tubes containing a plant root sample. The sample was allowed to sit for 25 min at room temperature in the dark. Instead of adding water to the tube to remove the excess dye the plant roots were dipped in three successive rinses of sterile dH₂O in order to avoid removing loosely bound bacteria. Plant roots were then mounted on a glass microscope slide in approximately 50 μ L ProLong[®] Gold antifade reagent (Invitrogen, Burlington, ON) and covered with a 0.17 mm thick cover slip. Plant roots were imaged using a Zeiss LSM 510 Meta laser scanning confocal microscope (Carl Zeiss Inc, Germany) at 63 \times magnification and glycerol immersion. Roots were imaged in the X, Y, and Z planes so that the images could be stacked and living and dead colonies could be counted on the entire plant root surface. Variability in counts resulted in the Z plane stacks being discarded.

2.2.5.5 Statistical Analysis

Two-way analysis of variance (ANOVA) and Tukey's post hoc test were performed using SigmaPlot version 11.0 to detect treatment effects and significant differences among treatment means ($p < 0.05$). All experiments in section 2.2.4 were repeated at least twice with the same result. The data presented are the data collected on the final trial.

2.3 Results

2.3.1 Analysis of Plant Health and Growth

The photosynthetic health of *A. thaliana* plants inoculated with either *P. putida* UW4/AcdS⁺ or *P. putida* UW4/AcdS⁻ at a range of CdCl₂ concentrations is shown in Figure 2-1. The plants showed no visible response to the presence of either bacterial strain and appeared healthy in all treatments. Photosystem II efficiency decreased by as much as 10% with increasing CdCl₂ concentration (Figure 2-1 A); however, within a given concentration there was no difference in PSII efficiency among the bacterial treatments ($p > 0.05$, $n \geq 3$). Chlorophyll a and chlorophyll b content were reduced by an average of 23% at higher concentrations of CdCl₂ (Figure 2-1 B) but there was no difference in chlorophyll a:b ratio among the CdCl₂ treatments (Figure 2-1 C, $p > 0.05$, $n = 4$). As with PSII efficiency, neither chlorophyll content nor chlorophyll a:b ratio were affected by bacterial treatment.

Increasing the concentration of CdCl₂ decreased all aspects of plant size that were measured (Figure 2-2). Unexpectedly, plants that were inoculated with either of the putative plant growth-promoting bacteria were either the same size or smaller than the control plants. Plants inoculated with the mutant that lacked ACC deaminase, *P. putida* UW4/AcdS⁻, were often larger than those inoculated with wild type *P. putida* UW4/AcdS⁺, although this pattern was not significant at all concentrations of CdCl₂ ($p > 0.05$, $n \geq 3$). Mean rosette diameter of the plants was often lower in bacterial treatments than in controls when CdCl₂ was present (Figure 2-2 A) while primary root length (Figure 2-2 B), number of lateral roots (Figure 2-2 C) and mean length of lateral roots (Figure 2-2 D) usually decreased in the presence of bacteria even in the absence of CdCl₂.

Similarly, shoot biomass decreased with increasing CdCl₂ concentration and plants from both bacterial treatments often had reduced biomass compared to controls (Figure 2-3). At 0 and 0.1 μ M CdCl₂, plants inoculated with *P. putida* UW4/AcdS⁺ had reduced shoot biomass compared to plants inoculated with no bacteria or *P. putida* UW4/AcdS⁻. At CdCl₂ concentrations of 1 μ M or higher, both bacterial treatments induced

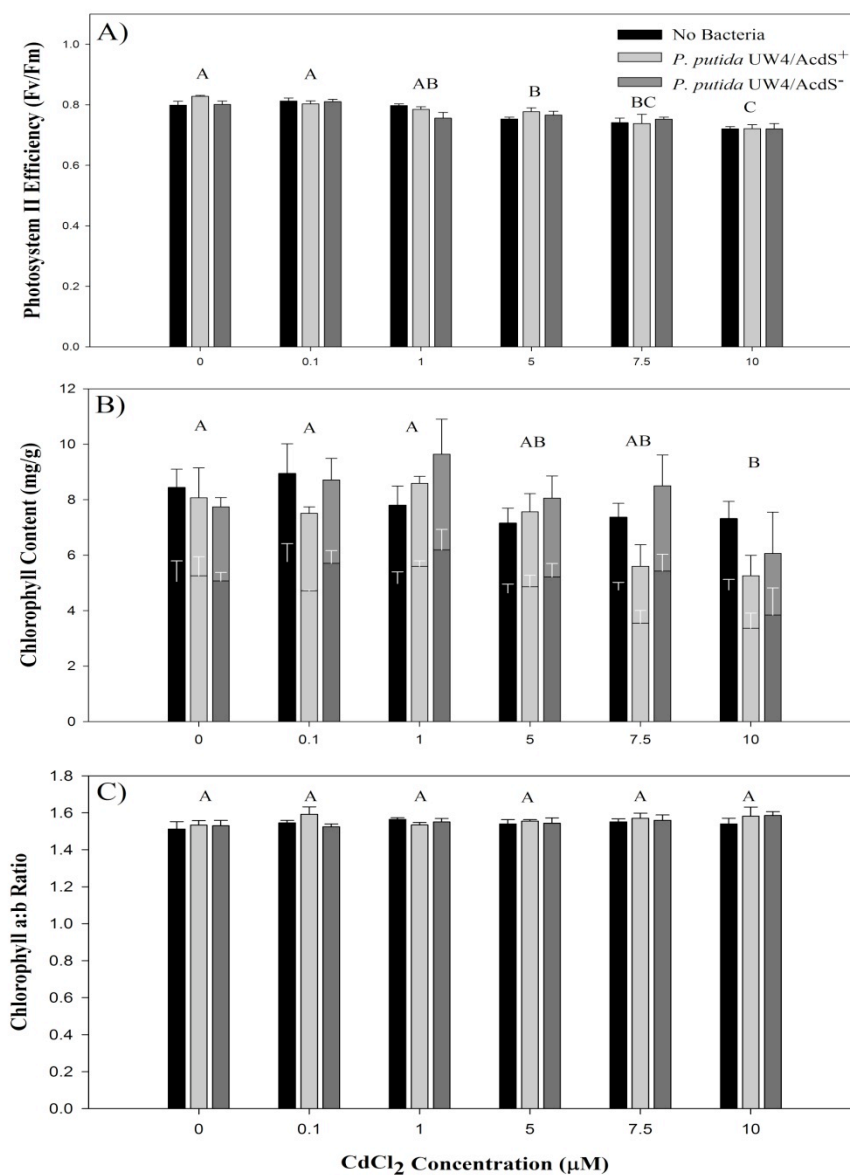


Figure 2-1: Photosynthetic health of plants in different bacteria and cadmium treatments

Measured as A) photosystem II efficiency B) chlorophyll content (Chl a: top bar, Chl b: bottom bar) and C) chlorophyll a:b ratio when plants were inoculated with no bacteria, *P. putida* UW4/AcdS⁺, or *P. putida* UW4/AcdS⁻ at a range of CdCl₂ concentrations.

Vertical error bars represent standard error. Treatments not sharing a common letter are significantly different (two-way ANOVA followed by Tukey post-hoc test, $p < 0.05$, $n = 3$).

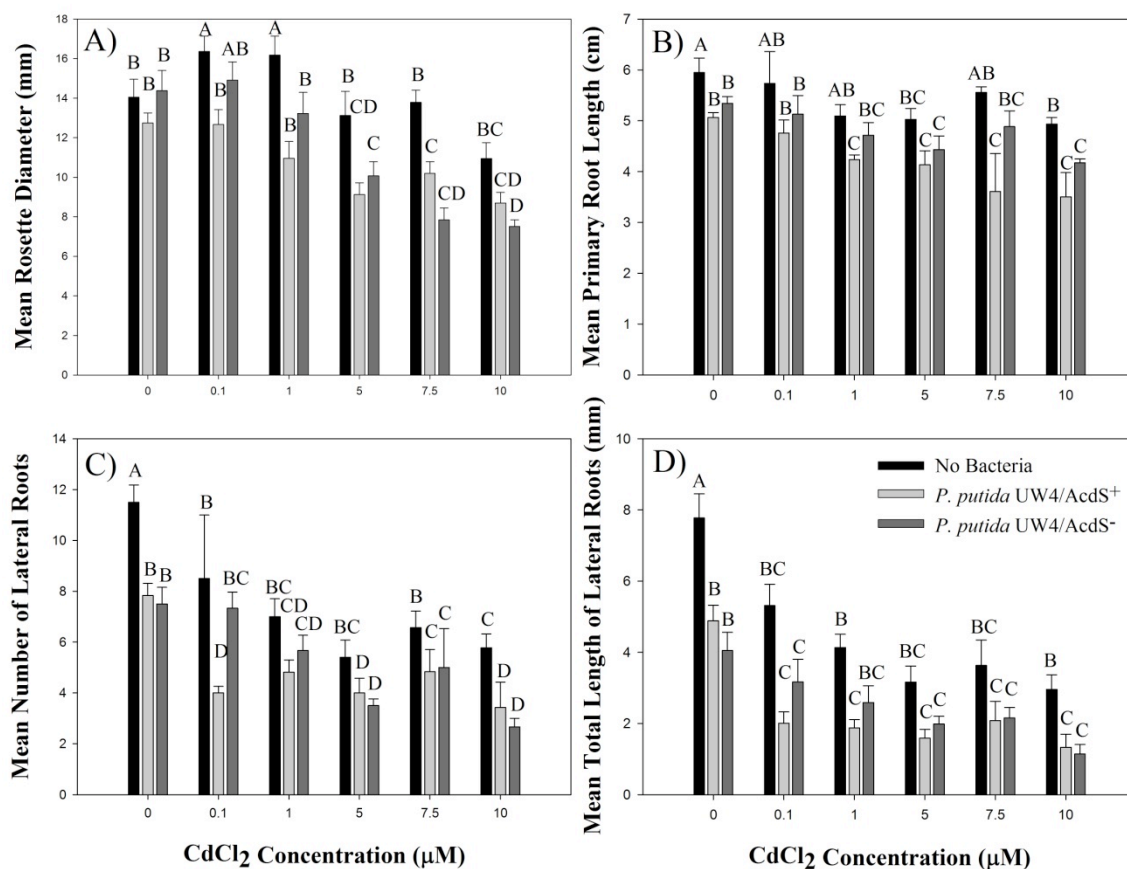


Figure 2-2: Plant size and growth of plants in different bacteria and cadmium treatments

Measured as mean A) rosette diameter B) primary root length C) number of lateral roots and D) total length of lateral roots of plants inoculated with no bacteria, *P. putida* UW4/AcdS⁺, or *P. putida* UW4/AcdS⁻ at a range of CdCl_2 concentrations. Vertical error bars represent standard error. Treatments not sharing a common letter are significantly different (two-way ANOVA followed by Tukey post-hoc test, $p < 0.05$, $n \geq 3$).

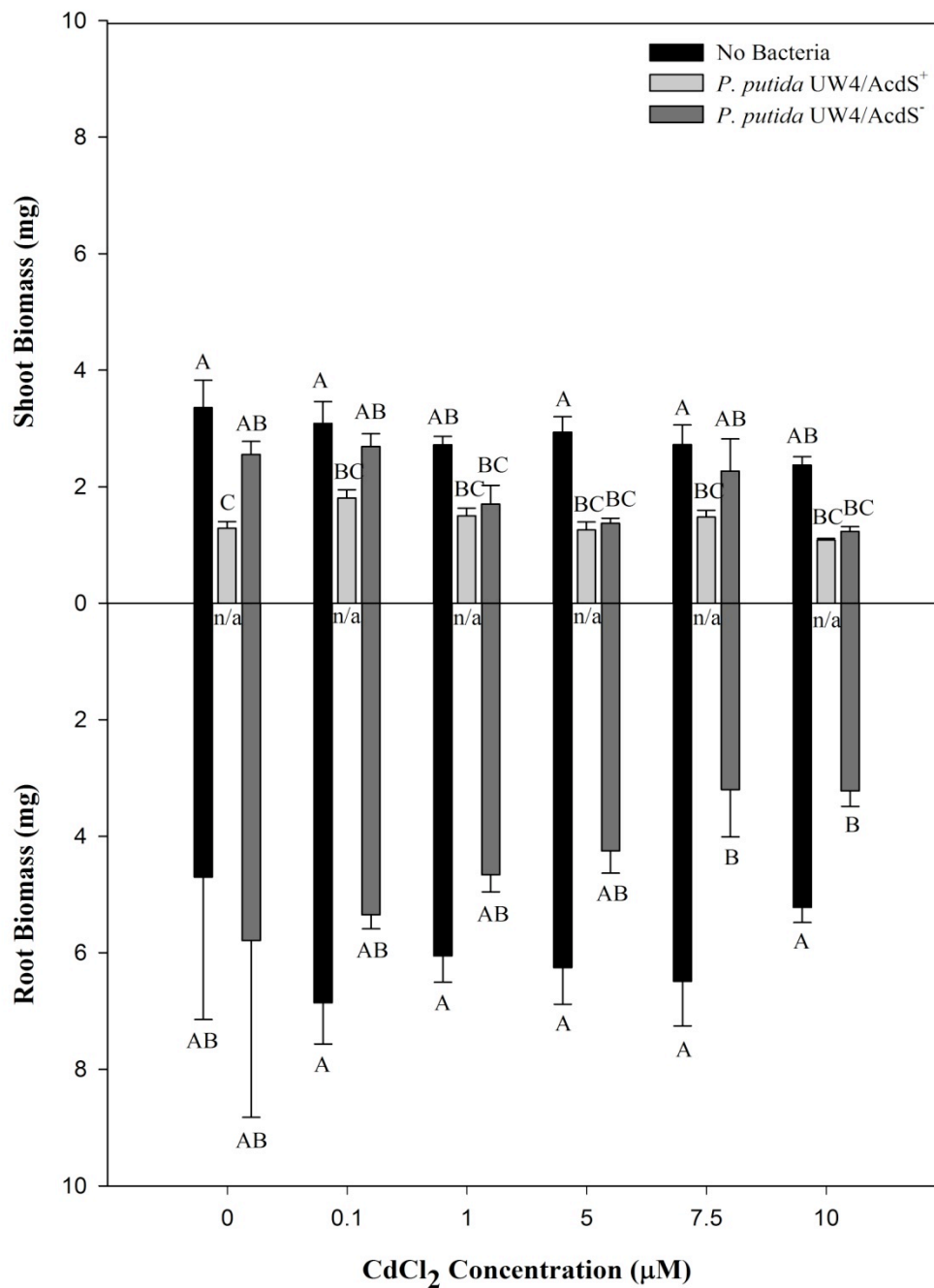


Figure 2-3: Dry Weight of shoots (top) and roots (bottom) from plants in different treatments

Vertical error bars represent standard error. Treatments not sharing a common letter are significantly different (two-way ANOVA followed by Tukey post-hoc test, $p < 0.05$, $n \geq 3$). Roots inoculated with *P. putida* UW4/AcdS⁺ were too small to weigh (n/a).

approximately a 50% reduction in shoot biomass. Within a bacterial treatment, root biomass did not vary with increasing CdCl₂ concentration; however, root biomass of plants inoculated with *P. putida* UW4/AcdS⁻ was 20 - 30% lower than those from the no bacteria treatment ($p < 0.05$, $n = 3$). Plants in the hydroponics studies had to be grown on sterile mesh, and a small portion of root biomass was lost in the mesh. The amounts of roots that could be harvested from plants inoculated with *P. putida* UW4/AcdS⁺ were below the amount that could be measured (i.e. < 0.1 mg/25 plants). Plants in the no bacteria treatment contained up to 40% more cadmium in the shoot tissue and up to 80% more cadmium in the root tissue than plants in either of the bacterial treatments (Figure 2-4). Since the biomass and cadmium content data were not collected in a paired manner (data were collected from independent experiments) statistical analysis was not possible.

The decreased size of plants grown in the presence of bacteria and CdCl₂ did not appear to be due to damage of the plant roots (Figure 2-5). In over 130 images taken there were only 5 with signs of damage. These did not come from any one experimental treatment, and this damage likely occurred during sample mounting.

When this phenomenon of PGPR inhibiting plant growth was first documented it was assumed that there was a contamination of the stock *P. putida* cultures. After confirming with the Glick lab that these strains were still able to promote plant growth, and confirming that the stock culture was a pure culture, the experiment was repeated with new stock culture with the same results.

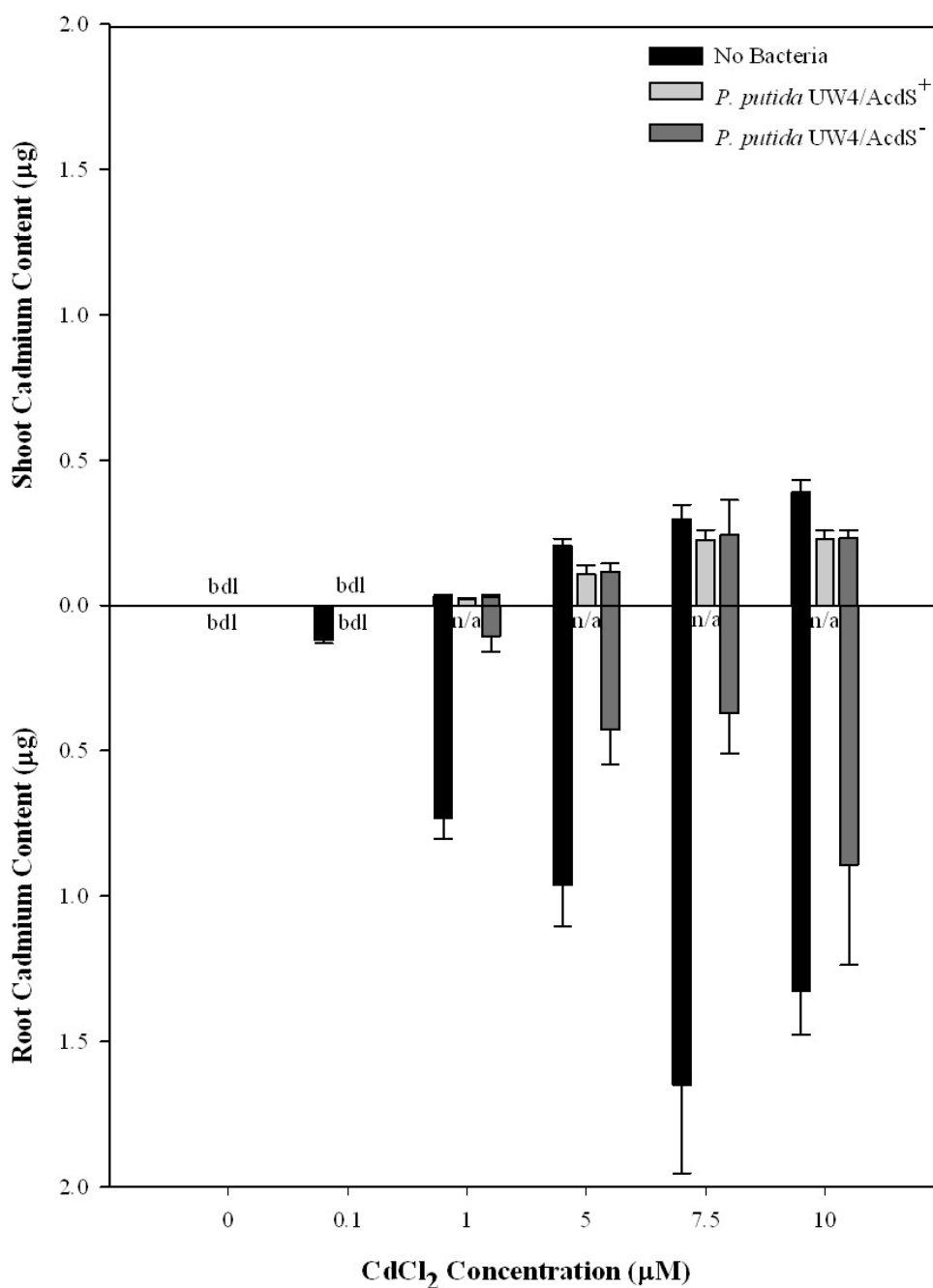


Figure 2-4: Total amount of cadmium in shoots (top) and roots (bottom) from plants in different treatments

Vertical error bars represent standard error. Roots inoculated with *P. putida* UW4/AcdS⁺ were too small to process for cadmium content (n/a); in some treatments, cadmium was below the detection limit (bdl). Due to the unpaired collection of data statistical analysis was not possible.

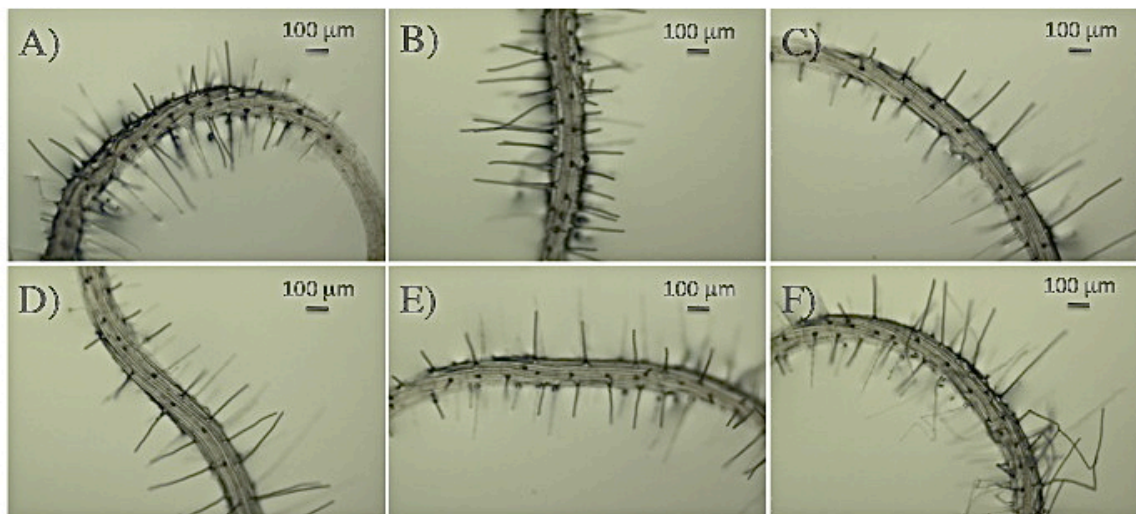


Figure 2-5: Light microscope images of plant roots

Plants were inoculated with A, D) no bacteria, B, E) *P. putida* UW4/AcdS⁺, or C, F) *P. putida* UW4/AcdS⁻ in media supplemented with 0 μM CdCl₂ (Top) or 10 μM CdCl₂ (Bottom).

2.3.2 Bacterial Growth and Plant Root Colonization

The growth curves of the two bacterial strains in bacterial broth (TSB) or the plant medium used in all of the above plant studies ($0.5 \times$ MS with 1% sucrose) with varying concentrations of CdCl_2 are plotted in Figure 2-6. There was no effect of CdCl_2 on either bacterial strain (one-way ANOVA at 24 h, $p > 0.05$ for all main effects, $n = 4$). Both bacterial strains grew to the same final OD_{600} at all the tested CdCl_2 concentrations in TSB. However, neither strain was able to grow in MS medium. Growth curves were also plotted for both strains in MS media with a range of 1 to 5% sucrose, glucose, fructose, or a combination of glucose and fructose; however, neither strain was able to grow in any of these conditions (data not shown).

To determine whether the bacteria were alive, despite the lack of growth in MS medium, samples from the growth curve study were plated onto TSA and allowed to grow for 24 h. Interestingly, although neither strain was able to grow on TSA after 24 h in liquid MS medium (Figure 2-7 A, D), both strains were able to grow when plated from culture in TSB (Figure 2-7 B, E), and could be repeatedly recovered from plant roots that were grown in MS medium (Figure 2-7 C, F).

Further evidence for bacterial growth on plant roots grown in MS medium comes from fluorescence micrographs that repeatedly showed a close association of both strains with plant roots grown in MS medium (Figure 2-8). Additionally, the majority of bacteria on the plant roots were alive at the point the micrographs were taken. However, there appeared to be fewer *P. putida* UW4/AcdS⁻ visible on the plant roots than *P. putida* UW4/AcdS⁺ despite being inoculated at the same OD_{600} . This can also be seen in the relative amount of bacteria growing on the plates in Figure 2-7. It was not possible to enumerate the bacteria visible on the plant roots in Figure 2-8. Background autofluorescence inhibited the use of counting software and duplicate manual counts were not consistent.

To determine if the failure to thrive on MS medium was an artifact of agar or hydroponic culture, changes between the bacterial community structure in two soil types maintained with either dH_2O or MS medium were assessed (Appendix B). The native

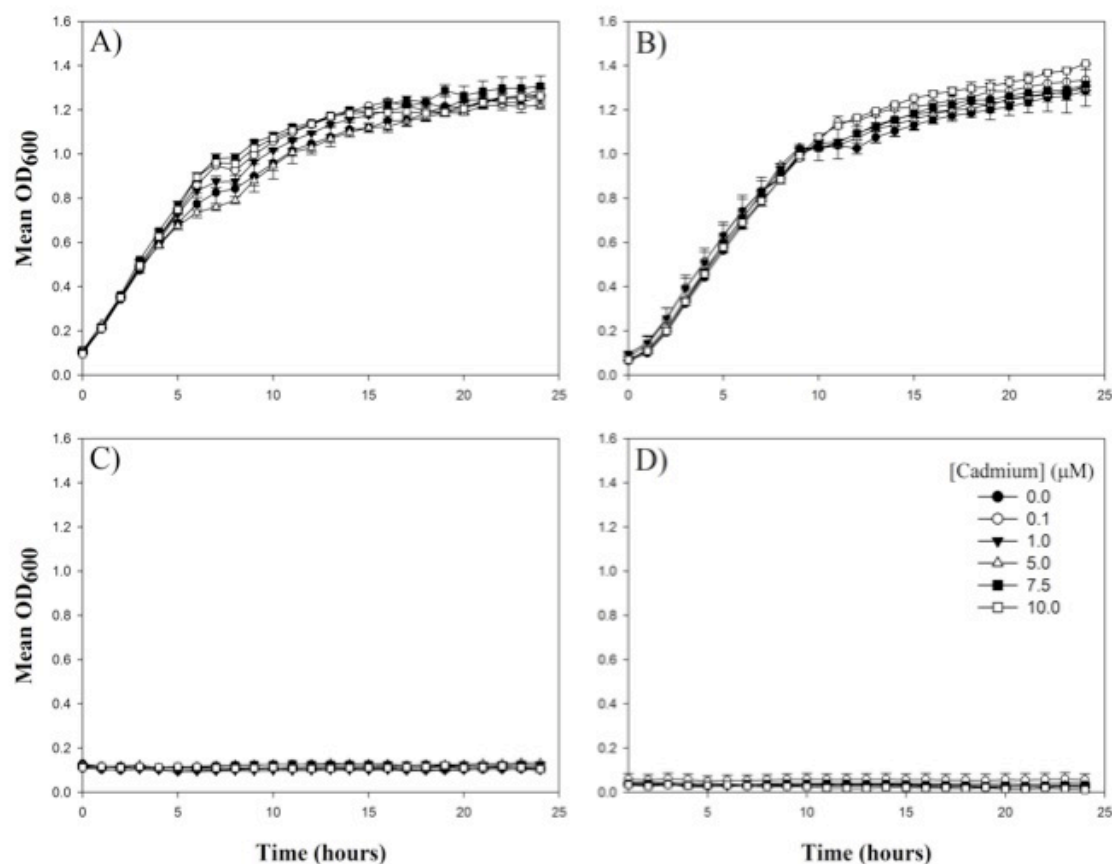


Figure 2-6: Bacterial growth curves in TSB and MS medium at a range of CdCl₂ concentrations

Growth curves were created for A) *P. putida* UW4/AcdS⁺ and B) *P. putida* UW4/AcdS⁻ in TSB, C) *P. putida* UW4/AcdS⁺ and D) *P. putida* UW4/AcdS⁻ in 0.5 × liquid MS medium with 1% sucrose grown in 0 to 10 μM CdCl₂ for 24 h. Vertical error bars represent standard error. One-way ANOVA at 24 h, $p > 0.05$ for all main effects, $n = 4$.

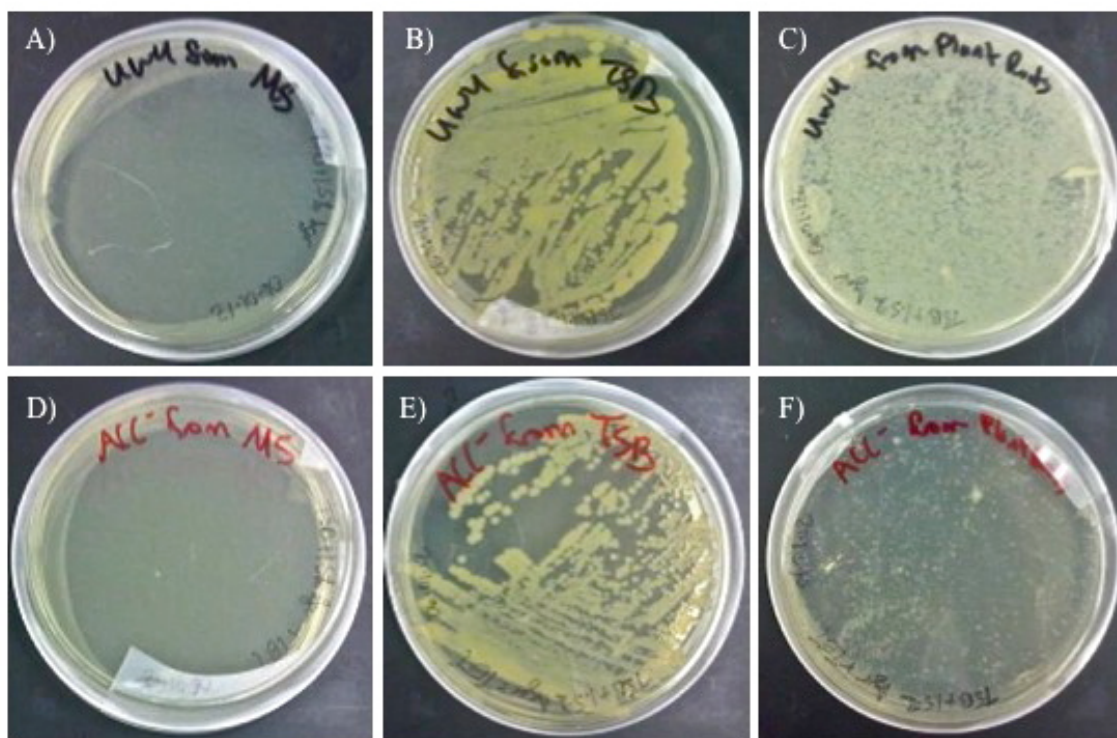


Figure 2-7: Inoculation of TSA with *P. putida* UW4/AcdS⁺ (top) and *P. putida* UW4/AcdS⁻ (bottom) from the cultures in Figure 2-6

Plates were inoculated with cultures from A, D) liquid MS, B, E) TSB, or C, F) plant roots grown on MS medium and then grown for 24 h.

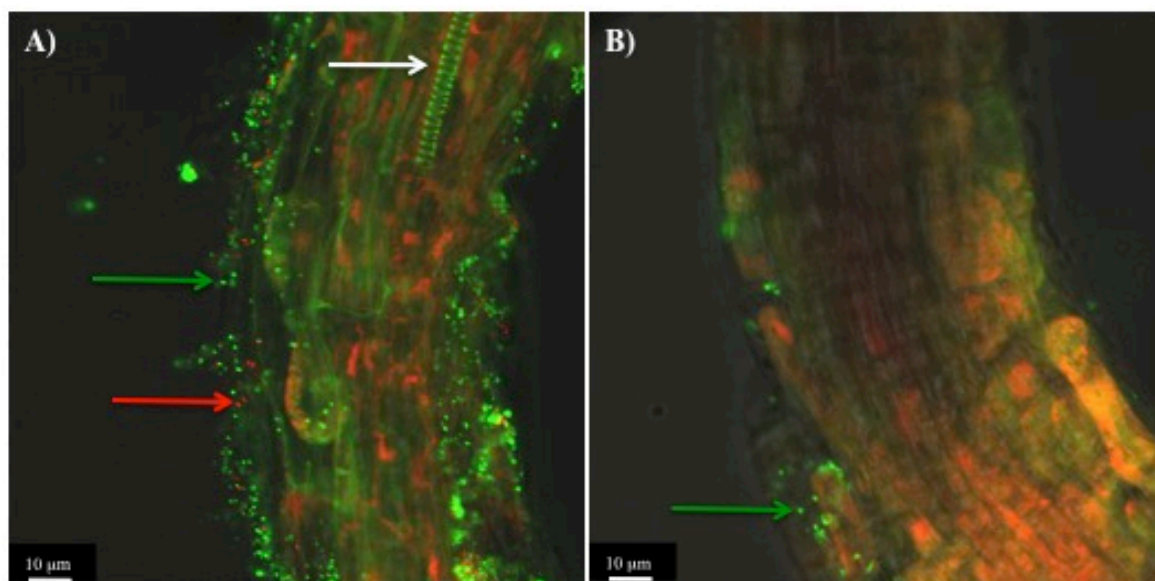


Figure 2-8: Fluorescence micrographs of plant roots with living and dead bacteria

Plant seeds were inoculated with A) *P. putida* UW4/AcdS⁺ or B) *P. putida* UW4/AcdS⁻ and grown hydroponically for 21 d. After staining, living bacteria are stained green and dead bacteria are stained red and appear as small areas of high intensity indicated by green and red arrows. Diffuse green is background autofluorescence of the plant root where the white arrow indicates the location of the xylem. Diffuse red and orange is indicative of background staining.

bacterial communities in the soil were impoverished, as seen in the reduction in fragment richness, when the soil was watered with MS medium but not when watered with dH₂O.

2.4 Discussion

2.4.1 Analysis of Plant Health and Growth

Glick *et al.* (1998) proposed a model to explain how two simultaneous mechanisms result in plant growth promotion under stress conditions (Figure 1-1). First, PGPR that are closely associated with a plant root are able to produce the plant auxin IAA which, when taken up by the plant, can stimulate plant cell proliferation and/or elongation or stimulate the activity of ACC synthase, which produces ACC, the precursor of the plant stress hormone ethylene. Secondly, the PGPR stimulate the exudation of ACC from the plant root where it is used as a nitrogen source by the bacterium and metabolized by the enzyme ACC deaminase. This should reduce the amount of ACC available to the plant for production of ethylene. This process could reduce the ethylene stress response of the plant allowing it to maintain plant growth and health under stress conditions. Specifically, plants that have been inoculated with a PGPR that produces ACC deaminase (*P. putida* UW4/AcdS⁺) should be photosynthetically healthier, have longer roots, and larger shoots than plants that have not been inoculated with bacteria or inoculated with a bacterium that cannot produce ACC deaminase (e.g., *P. putida* UW4/AcdS⁻), although some amount of growth promotion could still be seen due to the production of IAA. Support for this model has been repeatedly shown in the literature (reviewed by Saravanakumar, 2012).

To determine the extent to which the wild type and mutant strains of *P. putida* UW4 affect the growth and health of *Arabidopsis thaliana* (Col-0) in the presence of cadmium stress, a wide range of variables were measured in this study. The plants in all treatments showed no visible symptoms of cadmium-toxicity (e.g. chlorosis, necrosis, etc.) and the different bacterial treatments had no effect on the parameters related to photosynthetic health (Figure 2-1). An increase in all three of these parameters has been reported, for example when *P. putida* UW4 was used to ameliorate flooding stress in

tomatoes (Grichko and Glick, 2001), but neither chlorophyll content nor photosynthetic efficiency were reported in other studies using this strain (Mayak *et al.* 2004a; Mayak *et al.* 2004b), which suggests that these parameters were either not measured or were not reported due to a lack of response.

As expected, increasing concentrations of cadmium in the growth medium resulted in smaller plants, both aboveground and below ground (Figure 2-2). Unexpectedly, and contrary to all published experiments with these strains, I found that inoculation with either *P. putida* UW4/AcdS⁺ or *P. putida* UW4/AcdS⁻ decreased the size of the plants for all parameters measured. All previous reports on these strains of *P. putida* led me to expect that the bacterial treatments would have had a positive effect on plant size under stress conditions, and that plants inoculated with *P. putida* UW4/AcdS⁺ would have been larger than those inoculated with *P. putida* UW4/AcdS⁻ due to a lack of ACC deaminase production in the mutant strain. For example, Li *et al.* (2000) found that when canola was inoculated with *P. putida* UW4/AcdS⁻ root elongation was not observed and roots inoculated with the mutant were shorter than those inoculated with the wild type. However, the plants in my study were smaller in the presence of bacteria and were often larger when inoculated with *P. putida* UW4/AcdS⁻ than with the wild type strain (Figure 2-2 and Figure 2-3). The fact that there was a reduction in the size of the plants even in the control 0 μ M cadmium treatment suggests that this was not a response to the metal stress but was instead due to the plant medium used in the study.

Both wild type *P. putida* UW4/AcdS⁺ and the mutant *P. putida* UW4/AcdS⁻ have been shown to produce the principal plant auxin IAA (Li *et al.* 2000; Saleh and Glick, 2001). While it is well established that auxins are essential for stimulation of growth and development of plants, excessive exogenous IAA is known to have an inhibitory effect on root length and plant growth, a response that is generally attributed to an increased production of ethylene in the presence of IAA (Hopkins and Huner, 2004). Reduced or inhibited root development may also lead to overall nutrient deficiencies that would produce overall smaller plants due to a reduced root surface area for nutrient and water uptake. Kremer (2006) proposed the overproduction of IAA as a mechanism by which deleterious rhizobacteria may act to suppress plant growth. Additional proof for the

potential negative role of bacterially produced IAA comes from a study by Xie *et al.* (1996), who grew canola plants with IAA-overproducing mutants of *P. putida* GR12-2, a putative PGPR. A mutant that produced 4-times the IAA compared to the wild type inhibited root elongation. This was possibly due to a high degree of interaction between bacterially produced IAA and the plant enzyme ACC synthase, which quickly converts ACC to ethylene at a rate that cannot be effectively reduced by ACC deaminase. However, a mutant that produced 3 times as much IAA as wild type did not inhibit root elongation, suggesting that the threshold between IAA concentrations that can be considered beneficial versus detrimental may be small. It is possible that under my experimental conditions, the bacterial strains were over-producing IAA, leading to the suppression of plant growth.

The pathway by which microbes are able to synthesize IAA varies, is dependent on environmental conditions, and may be a deciding factor in whether a microbe is beneficial or pathogenic (reviewed in Patten and Glick, 1996). For example, while there are reported cases of IAA synthesis via the indoleacetamide pathway in plants, such as in the case of Japanese cherry (Saotome *et al.* 1993), it is thought to be a predominantly microbial process. It has been suggested that plants may lack the ability to regulate exogenous IAA from this pathway to the same extent that regulation of IAA from a shared pathway between plants and microbes occurs (Patten and Glick, 1996). Additional support for this theory comes from the fact that the primary IAA synthesis pathway for many phytopathogens is the indoleacetamide pathway. For example, pathogenic strains of *Erwinia herbicola* contain both indoleacetamide and indolepyruvic acid pathways, but when the indoleacetamide pathway was inactivated, pathogenicity was reduced (Manualis *et al.* 1991). In contrast, PGPR, such as *Enterobacter cloacae* (Koga *et al.* 1994) and *Pseudomonas putida* GR12-2 (Patten and Glick, 2002), mainly utilize the indolepyruvic acid pathway and have been shown to promote root elongation and lateral root formation. It is possible that, in my experiment, the non-ideal growth conditions in MS media (discussed below) caused the bacterial strains to shift IAA production to one of the bacteria-specific pathways that have been shown to be pathogenic to plants, resulting in an inability of the plants to regulate exogenous IAA, and that the surplus IAA resulted in reduced growth.

The roles of both ethylene and IAA in root development of plants, especially *Arabidopsis*, have been extensively studied in stressed (Potters *et al.* 2007) and non-stressed plants (Overvoorde *et al.* 2011; Ruzicka *et al.* 2007). Although the interactions between these two plant hormones are less well understood, it has been established that both hormones are essential for promoting root hair initiation and elongation (Pitts *et al.* 1998). It has also been shown that ethylene signaling is responsible for the transport of auxin to root cells that is necessary for growth, or growth inhibition depending on concentration (Ruzicka *et al.* 2007). The fact that there was a complete lack of root development in plants inoculated with *P. putida* UW4 AcdS⁺ (Figure 2-3) suggests that the delicate balance between plant hormones was interrupted. Since this response was seen only in plants inoculated with the ACC deaminase-producing strain it was likely due to a reduction in available ethylene and not IAA. Bacterial metabolism of ACC may reduce the ACC pool available for conversion to ethylene, reducing the ethylene concentration in the plant to a level that is not sufficient for proper signaling of transport of auxins to the root tips to allow for elongation and growth.

The concentration of cadmium in the roots of plants inoculated with the *P. putida* UW4/AcdS⁺ could not be measured due to a lack of tissue; however, root cadmium concentration was higher with no bacteria inoculation compared to plants inoculated with *P. putida* UW4/AcdS⁻, while shoot tissue cadmium concentration was higher in plants inoculated with bacteria than those not inoculated with bacteria (Figure 2-4). This suggests that the presence of bacteria may cause the plant to store less cadmium in the roots and translocate more cadmium to the shoots. Increased accumulation of metals, including cadmium, in plants inoculated with different PGPR, has previously been reported (Safronova *et al.* 2006), which could be due to microbial changes in metal availability (Gao *et al.* 2010). Translocation from roots to shoots depends on water relations and xylem loading and transport (Uraguchi *et al.* 2009), factors that could be altered by the presence of microbes.

One way in which bacteria can suppress plant growth is through mechanical damage to the cell walls that can be caused by bacterial production of phytotoxins and

cell wall-degrading enzymes (Kremer, 2006). This was not likely the case in this study since there was no evidence of mechanical damage to the plant roots (Figure 2-5).

There are a few documented cases of putative PGPR that have suppressed plant growth, such as the above-mentioned case of the IAA over-expressing *P. putida* GR12-2 mutant (Xie *et al.* 1996). However, all of these reports used a mutant strain lacking an essential process (Li *et al.* 2000) or over-expressing a deleterious process (Xie *et al.* 1996) to achieve growth suppression. Occasionally, the deleterious effect could be linked to a lack of stress conditions. For instance, one PGPR that promotes growth in the presence of a deleterious plant fungus by producing phytotoxins that kill the fungi suppressed plant growth when the fungus was not present (Maurhofer *et al.* 1992). To my knowledge, my study is the first case of bacterial growth-limiting conditions causing a putative PGPR to become deleterious that will appear in the literature. Personal communication with other research groups (Dr. B. Glick at the University of Waterloo and Dr. G. Lazarovits at A&L Laboratories Canada) suggests that this phenomenon has been witnessed in the past but has not been well documented.

2.4.2 Bacterial Growth and Plant Root Colonization

To ensure that the two strains of bacteria were not negatively affected by the presence of cadmium, the growth curves of the bacteria in bacterial broth (TSB) and the plant medium used in all the above plant studies (0.5 MS with 1% sucrose) with varying concentrations of CdCl₂ were plotted (Figure 2-6). There was no evidence that either strain was affected by cadmium (One-way ANOVA at 24 h, $p > 0.05$ for all main effects, $n = 4$). However, neither strain of bacteria used in my study was able to grow in MS medium. After comparing the constituents of the plant growth medium and the bacterial broth used, it appeared that MS medium might be lacking the quantity and type of carbon sources required for bacterial growth. Sugars did not seem to be the limiting factor since the addition of 1 to 5% sucrose, glucose, fructose, or a combination of glucose and fructose (1:1 ratio to obtain final concentration) was unable to promote bacterial growth in MS medium. However, when tested for viability in MS medium in the presence of plants, it was clear that the bacteria thrived (Figure 2-7 and 2-8). These results suggest

that the plant must be providing an additional nutritional component that is lacking in the MS medium, such as organic acids (Lugtenberg *et al.* 1999) or amino acids (Simons *et al.* 1997) or may be protecting the bacteria from the toxic effects of MS.

In a study on bacterial growth in plant culture media, Leifert and Waites (1992) tested the ability of common bacterial contaminants of plant tissue cultures to grow in MS medium. Most of the tested species were not able to grow in the medium without the presence of plants even when supplied with additional growth factors, which provides further support that the plants must release additional nutrients required for bacterial growth. Interestingly, Leifert and Waites (1992) also reported a decrease in plant size when the bacterial species were present; however, the bacteria used were common cell culture media contaminants, including *Lactobacillus plantarum* and *Staphylococcus* species, which were known to reduce plant size. The fact that the bacteria used in my study are putative PGPR suggests that the dependence of the bacteria on the plants for nutrition may result in a reduction in plant size. This might be explained by an increase in plant cell wall permeability caused by bacterially produced IAA, which increases availability of nutrients to the bacteria (Nelles, 1977). The loss of these essential nutrients from the plant might reduce overall growth. Additionally, the bacterium may be competing with the plants for nutrients that are essential for growth.

Another explanation for the unfavourable bacterial growth conditions in MS medium is the high concentration of salts in the medium. However, *P. putida* UW4 has been successfully utilized in studies on growth promotion of tomato plants grown under salt stress, therefore, this possibility is not likely (Mayak *et al.* 2004a).

Other possible explanations for the lack of an effect of bacterial treatments on plant photosynthetic health, and a deleterious effect on plant size, are that the bacteria either did not form an association with the plant root or that the bacteria died when grown in MS medium and emitted a substance that was harmful to the plants. However, it is clear from both the plated growth study (Figure 2-7) as well as the confocal micrographs (Figure 2-8) that the bacteria were associated with the plant root and that most of the bacteria present are alive in the current system. It was also clear that *P. putida*

UW4/AcdS⁺ was able to colonize the plant root better than *P. putida* UW4/AcdS⁻ as seen by the increased relative number of colony forming units in the plated viability test and the relative number of visible bacteria in the confocal micrographs. The ability of each strain to colonize the plant root might help to explain the difference in the magnitude of the negative effect each strain had on the plant measures. The fact that the mutant strain had less of a negative effect than the wild type strain could be due to reduced bacterial colonization of the plant root. This is consistent with a study that showed the density of *P. putida* UW4/AcdS⁻ was one log order lower than that of the wild type (Gamalero *et al.* 2008). Differential colonization might be related to the mutant strain having a longer lag phase growth rate (Li *et al.* 2000).

Finally, the bacterium may have produced a phytotoxin that was deleterious to plant growth. The pseudomonads are well known for their ability to act as biocontrol agents through the production of various substances. Some of these may act as phytotoxins. For example, *Pseudomonas syringae* produces the phytotoxin coronatine, which is believed to act as an analogue for one or more of the growth regulating jasmonates, disrupting growth (Brooks *et al.* 2005). A follow-up experiment should be completed to determine whether *P. putida* UW4 is producing known phytotoxins when grown in MS medium.

2.5 Conclusions

In my opinion, the negative effect of the bacteria observed in my current system was likely due to the bacteria being dependent on the plant for survival. In the process of providing the protective mechanism for bacterial survival, the association between plant and bacteria that in other systems has been beneficial has become deleterious possibly due to an over-production of bacterially produced IAA. The increase in IAA could be brought on by a bacterial growth-limiting environment and result in 1) overall suppression of plant growth, 2) an imbalance between necessary root growth and development hormones, and 3) an increase in plant cell wall permeability that increases nutrient leaching, all of which would lead to smaller plants. Additionally, while the plants in this study visibly appeared healthy, and were healthy based on photosynthetic

measures, it was only after careful examination of multiple growth parameters that the deleterious effect of these bacterial strains was noted. Thus, when evaluating the efficacy of putative PGPR, it is important to take many measures of plant health and growth to ensure that the PGPR applied are in fact beneficial.

The complex nature of plant-microbe interactions makes it difficult to make any definitive statement regarding a specific bacterial strain's potential to be a PGPR. My results tell a cautionary tale that should be taken into consideration when attempting to use PGPR in field settings since it can be difficult to control for all nutritional factors. I have shown that in a growth-limiting environment, the putative PGPR *P. putida* UW4 can become deleterious to plant growth and size while having no impact on overall plant health. This means that when using this strain in a field setting there may be a negative overall response without any obvious signs. Researchers need to be aware that plants can appear healthy while actually having reduced size, as this may affect agricultural yield and reduce remediation efforts. I suggest that before field application of any PGPR strain the independent growth of that strain is confirmed at the field site and under the fertilizer/nutrient conditions that will be used.

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Chapter 3

3 It takes a community to raise a plant: TRFLP analysis of the rhizosphere microbial community of two pairs of high and low metal accumulating plants in two soil types

The results of Chapter 2 sparked my interest in further exploring how bacteria, and other potentially beneficial microbes, respond to different environmental conditions in the rhizosphere of different plants. To keep the system as natural as possible, I used soil collected from active, cadmium-contaminated agricultural fields and examined the responses of their constituent microbial communities in the presence of plants that vary in their ability to take up and accumulate cadmium. I expected the different plant types to alter their rhizosphere in different ways and that these changes would be reflected in microbial community structure.

3.1 Introduction

Accumulation of cadmium, a non-essential metal, in food staples, such as wheat, is of concern to human health due to the potentially toxic effects. This element occurs naturally in soil but also has anthropogenic sources such as the application of manure and fertilizers at agricultural sites (Alloway and Steinnes, 1999). Phosphate fertilizers are often contaminated with cadmium at concentrations as high as 340 mg/kg depending on the cadmium concentration in the phosphate rock used to manufacture it. Repeated application of these fertilizers is a major source of cadmium in agricultural systems (Sheppard *et al.* 2007; Grant *et al.* 2011) and is therefore an important toxic metal for study.

Cadmium uptake in durum wheat (*Triticum durum*) has been intensively studied due to the agricultural and economic importance of this crop. Durum wheat is a food staple worldwide and is a major agricultural crop in the Canadian prairies. Natural accumulation of cadmium in wheat grains grown in Canada varies from 0.1 – 0.5 mg/kg

grain depending on the cultivar (Garret *et al.* 1998). Natural variation in the uptake and distribution of toxic metals exists among plant species and among varieties within a species (reviewed in Grant *et al.* 2008). Chan and Hale (2004) examined the distribution of cadmium among plant parts in two cultivars of durum wheat, Arcola and Kyle, using stable isotope labeling of cadmium. In addition to confirming Kyle as a high-accumulator and Arcola as a low-accumulator, it was found that Arcola had a decrease in root-to-shoot translocation and enhanced shoot-to-root retranslocation of cadmium compared to Kyle, meaning that more cadmium is accumulated in the shoot tissue in Kyle compared to Arcola. Similarly, natural high- and low-metal accumulators exist in the genus *Brassica*. *Brassica juncea* is an established metal hyperaccumulator that is frequently used in phytoremediation studies, while *B. napus* is a non-hyperaccumulator (McGrath *et al.* 2001). Each of these pairs of plants offers a good system to study mechanisms of metal-uptake and distribution in plants. The use of both pairs will allow for additional insight into these mechanisms at the genus level.

Differences in the composition of the microbiome surrounding plants are often described as a potential mechanism for differential metal accumulation in plants. Research has often focused on how the microbes in the rhizosphere can aid in the plants response to toxic metals. Some bacteria have been shown to increase metal bioavailability in soil (Bollag and Czaban, 1989), while others have been shown to reduce the plant stress response to toxic metals (Hontzeas *et al.* 2004). Plants are known to have dramatic influence on the rhizosphere by supplying nutrients via plant exudates and changing the soil physical and chemical properties. These exudates may act as signals for potentially beneficial microbes to colonize the plant root under conditions of metal stress. Attempts are often made to isolate beneficial microbes from the rhizosphere of plants that are known to accumulate metals, including the hyperaccumulating *B. juncea* (Belimov *et al.* 2005) for potential use in phytoremediation. However, few studies have been done to characterize how the host plant's inherent metal tolerance may shape the rhizosphere community that surrounds it.

The present study examined the microbial community profile of the rhizosphere of two sets of high- and low-metal accumulating plant types grown in each of two soils

that had been contaminated with varying concentrations of cadmium to determine how a plant's inherent metal-accumulating ability might influence the microbial community profile of its rhizosphere. This was accomplished by generating bacterial and fungal community profiles using terminal restriction fragment length polymorphism (TRFLP) analysis. Use of TRFLP has been shown to allow reliable and repeatable quantitative comparison of microbial communities in a culture-independent manner (Blackwood *et al.* 2003).

3.2 Methods

3.2.1 Soil Collection

Soil was provided by Dr. Cynthia Grant at Agriculture and Agri-Food Canada (AAFC), Brandon, Manitoba. Fertilized (high cadmium) and unfertilized (low cadmium) soil was collected from the top 7.5 cm of an agricultural field in Brandon, Manitoba immediately following harvest of durum wheat in August 2012. Soil was immediately packaged in a semi-dry state and shipped in 20 L plastic pails to London, Ontario for experimentation. The fertilized treatment was created by applying 80 kg of 11-52-0 (nitrogen-phosphorus-potassium) fertilizer/ha/year from 2002 to 2009 inclusive (a total of 354 kg/ha). The fertilizer contained cadmium as a contaminant, and was chosen as a way to elevate cadmium concentrations in the soil. The unfertilized treatment did not receive any phosphate fertilizer during this time and thus contained only background concentrations of cadmium. Nitrogen concentration was kept equal by adding urea to the unfertilized treatment (personal communication with Dr. Cynthia Grant, AAFC).

3.2.2 Soil Characterization

After sieving to < 2 mm, 3 subsamples of each soil were sent to A&L Canada Laboratories Inc. (London, Ontario) for soil analysis including phosphorus, nitrogen, potassium, magnesium, calcium, sodium, zinc, manganese, iron, copper, boron, aluminum and organic matter (OM) content as well as pH and cation exchange capacity (CEC). Before beginning the experiment, phosphorus was added to the unfertilized soil as Triple Superphosphate (Plant Products Co. Ltd., Brampton, ON) to balance the

phosphorus concentration in the two soils. The amount of phosphorus required was determined based on the soil report provided by A&L Canada Laboratories Inc.

The concentration of cadmium in the soil was determined using inductively-coupled plasma atomic emission spectrometry (ICP-AES). Soil was prepared as described in Chapter 2 with modifications. A total of 1 g of soil was digested in 1 mL of ultrapure nitric acid (OmniTrace[®], EM Science, USA) for each treatment and a soil standard reference material from the National Institute of Standards and Technology (NIST 2711 – Montana Soil) was used.

Particle size analysis was conducted according to Diaz-Zorita *et al.* (2002). Briefly, 20 g of oven-dried soil was thoroughly mixed with 200 mL of 1% sodium pyrophosphate to disperse and separate soil aggregates. This mixture was then poured into a 250 mL graduated cylinder and covered in Parafilm. The depth of settled particles was measured after exactly 2 min (sand), 2 hours (silt), and 18 hours (clay). Soil texture was determined based on the percentage of sand, silt, and clay and using the soil triangle according to the United States Department of Agriculture (2013).

3.2.3 Seed Sources and Plant Growth Parameters

Two pairs of high- and low-cadmium accumulating plants were used. The high accumulating durum wheat (*Triticum durum*) variety Kyle and low accumulating variety Arcola were provided by the AAFC Research Farm Seed Increase Unit (Indian Head, Saskatchewan) and the Durum and CPS Wheat Breeding Program Crop Development Centre, University of Saskatchewan (Saskatoon, Saskatchewan), respectively. The hyperaccumulating *Brassica juncea* and the low accumulating *B. napus* were both provided by AAFC Saskatoon Research Centre (Saskatoon, Saskatchewan).

Seeds were surface-sterilized in a 30% bleach solution for 5 min followed by 70% ethanol for 10 min. Seeds were then rinsed 3 times with sterile distilled water (dH₂O) to remove any remaining bleach or ethanol. Following surface sterilization, seeds were germinated on moist filter paper in the dark for 36 h. When radicles had emerged, seeds were transferred to individual 8 × 6 × 6 cm pots containing one of the two experimental

soils and allowed to grow for 28 d from time of planting. All pots were kept in a growth chamber maintained at 21°C and 60% RH with a 16:8 hour light:dark cycle. Light intensity was $230 \pm 5.7 \mu\text{mol}/\text{m}^2/\text{s}$ as repeatedly measured using a Fieldscout Quantum Light Meter (Spectrum® Technologies Inc, Illinois, USA). One treatment in each soil type was left unplanted to represent bulk soil. Each treatment contained 4 replicates.

3.2.4 Plant Growth Measures

Plants were harvested after 28 d in the growth chamber. The height of each plant from the soil to the tip of the longest shoot and the total number of leaves on each plant were recorded. Shoot tissue was then harvested and fresh weight (FW) was recorded before placing the shoots into individual aluminum foil envelopes and drying in an oven set at 60°C for 4 d or until constant weight. The shoot dry weight (DW) was then recorded.

3.2.5 Plant Tissue Cadmium Content

Plant shoot and root tissue was digested for metal content analysis as described in Chapter 2 with the following modification. At harvest, the root tissue was placed in a 50 mL tube with 25 mL of 1 mM ethylenediaminetetraacetic acid (EDTA) for 30 min to remove any surface-bound cadmium and prevent measurement of surface cadmium in tissue analysis.

3.2.6 Preparation of Samples for DNA Extraction

3.2.6.1 Seeds

Seeds for all species used were surface-sterilized as described above. Approximately 10 seeds (*Triticum*) and 25 seeds (*Brassica*) were then pulverized in a Kleco tissue pulverizer for 30 sec (Kleco Laboratory Equipment, Vasalia, CA, USA). Samples were taken from this homogenized tissue. The process was repeated with new seeds for each sample. Any microorganisms detected after surface sterilization were considered to comprise the seed endophyte community.

3.2.6.2 Unplanted Bulk Soil

A representative soil sample was collected directly from each unplanted pot. Soil was briefly mixed with a spatula and subsamples were taken from at least three locations in the pot to ensure a representative sample.

3.2.6.3 Rhizosphere

To collect the rhizosphere soil, and avoid bulk soil, the plant roots were removed from the pots and shaken until all loose soil particles were removed. Plant roots were placed in a 50 mL tube with 25 mL of distilled water and vortexed to remove the remaining soil from the roots. Samples were then centrifuged ($10,000 \times g$ for 5 min) to pellet the soil. The soil pellet was then used for DNA extraction.

3.2.7 DNA Extraction

Total community DNA was extracted from 250 mg of dry bulk soil, rhizosphere soil, or seed tissue using a NORGEN Soil DNA Isolation Kit (Cat # 26500, Biotek Corporation, Broadview Illinois, USA). Each sample was added to a tube containing glass beads along with 750 μL of lysis solution and 100 μL of lysis additive. Tubes were thoroughly agitated in a FastPrep[®]-24 (MP Biomedicals Canada, Montreal, QC) at maximum speed for 1 min to lyse the bacterial cells. The lysate was then clarified by centrifugation ($14,000 \times g$ for 1 min at room temperature) and 400 μL of the supernatant was removed and placed in a new sterile 1.7 mL microcentrifuge tube and mixed with 100 μL binding solution by inverting the tubes. Tubes were then placed on ice for 5 min before being clarified by centrifugation ($14,000 \times g$ for 1 min at room temperature) then 400 μL of the supernatant was removed and placed in a new sterile 1.7 mL microcentrifuge tube. An equal volume of 70% ethanol was added to each tube and mixed by inverting. This solution was added to the spin column and centrifuged ($14,000 \times g$ for 1 min at room temperature) in two equal volumes, followed by washing with 500 μL of Wash Solution I, two washes of 500 μL Wash Solution II, and a dry spin to remove all remaining ethanol from the column. The spin column was then placed into a new tube

and 50 μL of elution buffer was added to the spin column. Columns went through slow centrifugation ($200 \times g$ for 2 min at room temperature) to adsorb elution buffer to the column before being centrifuged to complete the DNA elution ($14,000 \times g$ for 1 min at room temperature). The concentration of extracted DNA was determined using an Eppendorf[®] BioPhotometer (Eppendorf Canada, Mississauga, Ontario) and diluted to exactly 30 $\mu\text{g}/\text{mL}$ in filter-sterilized 10 mM Tris, pH 8.0.

3.2.8 Polymerase Chain Reaction (PCR) Amplification

Bacterial 16s rRNA genes and fungal internal transcribed spacer (ITS) regions were PCR-amplified from total genomic DNA extract using common primers that target a wide range of members within Bacteria and Eukarya using highly conserved sequences. The fluorophore-labeled primer sets 63F and 1389R (for bacteria), and ITS1F and ITS4 (for fungi) were used (Table 3.1). The PCR reaction mix contained 37.5 μL DNA-free water, 5 μL 10 \times buffer, 2 μL 50 mM MgCl_2 , 1.25 μL 20 mg/ml BSA, 1 μL 4 mM dNTP, 1 μL 20 pmol forward primer, 1 μL 20 pmol reverse primer, 0.25 μL 5 U/ μL Taq polymerase, and 1 μL 30 $\mu\text{g}/\text{mL}$ template per reaction (bacteria), or 31 μL DNA-free water, 5 μL 10 \times buffer, 4 μL 50 mM MgCl_2 , 1.25 μL 20 mg/ml BSA, 2.5 μL 4 mM dNTP, 2.5 μL 20 pmol forward primer, 2.5 μL 20 pmol reverse primer, 0.25 μL 5 U/ μL Taq polymerase, and 1 μL 30 $\mu\text{g}/\text{mL}$ template per reaction (fungi). The PCR reaction took place in 0.2 mL reaction tubes and an Eppendorf Mastercycler gradient thermocycler (Eppendorf Canada, Mississauga, ON) with the following program parameters: an initial denaturation for 2 min at 94°C followed by 30 cycles of 1 min at 94°C, 1 min at 56°C and 2 min at 72°C and a final elongation at 72°C for 10 min (bacteria), or initial denaturation for 5 min at 94°C followed by 30 cycles of 30 sec at 94°C, 30 sec at 55°C and 30 sec at 72°C and a final elongation at 72°C for 10 min (fungi). To confirm that the PCR reaction was successful 3 μL of the reaction solution was mixed with 2 μL loading dye and subjected to gel electrophoresis on a 1% agarose gel stained with GelRed Nucleic Acid Stain (Biotium Inc, Hayward CA, USA). Positive controls of purified genomic bacterial and fungal DNA and negative controls of reaction mixture without template were used.

Table 3-1: PCR targets, primers, and sequences

Target	Primer	Sequence
Bacteria	1389R	5'-ACGGGCGGTGTGTACAAG-3'
	63F	5'-CAGGCCTAACACATGCAAGTC-3'
Fungi	ITS1F	5'-CTTGGTCATTTAGAGGAAGTAA-3'
	ITS4	5'-TCCTCCGCTTATTGATATGC-3'

Bacterial (Marchesi *et al.* 1998) and fungal (Manter and Vivanco, 2007) primers were chosen for the ability to reproducibly amplify diverse organisms with minimal bias from mixed template samples

3.2.9 Terminal Restriction Fragment Length Polymorphism (TRFLP) Analysis

3.2.9.1 DNA Purification

The amplified DNA was purified using DNA Clean and ConcentratorTM – 5 Kit (Zymo Research Corporation, Irvine CA, USA – cat # D4014). In a sterile 1.7 mL tube 250 μ L DNA binding buffer was added to the remaining amplified DNA sample. This mixture was then loaded into a Zymo-Spin Column placed in a 2 mL collection tube. Columns were centrifuged at $10,000 \times g$ for 30 sec at room temperature. After discarding the flow through, 200 μ L of wash buffer was added to the column and centrifuged. The wash step was repeated followed by a dry spin to remove all remaining solution. The column was then placed in a new 1.7 mL tube and 13 μ L of DNA elution buffer was added directly to the column. Centrifugation was repeated to elute the DNA.

3.2.9.2 Restriction Enzyme Digestion

The purified amplicons were digested with the restriction enzyme *HhaI*. According to the supplier this enzyme has the restriction site 5'-GCG[^]C-3'. The reaction contained 10 μ L purified DNA, 2.5 μ L 10 \times buffer C, 0.25 μ L acetylated BSA, 0.5 μ L *HhaI*, and 11.75 μ L DNA-free water per reaction. Reaction mixtures were incubated at 37°C for 3 h to allow for digestion and then placed in a 65°C water bath for 20 min to heat-kill the enzyme and stop the reaction. The samples were diluted in 75 μ L water for a final volume of 100 μ L for fragment analysis.

3.2.9.3 Fragment Analysis

Samples were sent to the Advanced Analysis Centre Genomic Facility (University of Guelph, Guelph, Ontario) for fragment analysis using the Applied BioSystems 3730 DNA Analyzer.

All steps from DNA extraction to fragment analysis were duplicated for a single pot from each treatment. These duplicates were used to ensure repeatability of the methodology.

3.2.10 Data and Statistical Analysis

Differences in each soil characteristic between fertilized and unfertilized soils were assessed using a Student's t-test. Within a genus, for each plant parameter, differences among the high and low accumulating plants in fertilized or unfertilized soil were calculated using a two-way ANOVA followed by Tukey's post-hoc test. Statistical analysis was performed in SigmaPlot 11.0.

The TRFLP fragments from each sample were analyzed using GeneMarker[®] AFLP/Genotyping Software version 2.2.0 to generate a raw data matrix based on relative peak intensity. Fragment lengths less than 30 bp were eliminated from all datasets to exclude the presence of primers from the analysis. A baseline threshold of 50 fluorescence units was used to distinguish 'true peaks' from background noise (Culman *et al.* 2008). To account for run-to-run variability each profile underwent alignment by placing each fragment size peak into a "bin" using nearest integer rounding and visual inspection of the peaks. The matrix generated by GeneMarker was then transferred to a Microsoft Office Excel v12.3.5 spreadsheet and turned into a presence/absence matrix. A binary presence/absence matrix eliminates variability related to factors such as DNA purification efficiency, pipetting errors, and community structure (Blackwood *et al.* 2003, Dunbar *et al.* 2001). Due to the binary nature of the data set, indices of community diversity could not be calculated since these calculations require information about the frequency of occurrence of individual fragments (relative abundance). A Bray-Curtis similarity matrix was then generated in Primer 5.2.4 and non-metric multidimensional scaling (NMDS) plots were generated using 10 iterations. For NMDS, a Kruskal's stress value of below 0.15 is considered good and below 0.1 is considered ideal (Clarke, 1993). Analysis of similarity (ANOSIM) was performed for all samples including all factors: time, soil type, genus, plant type, metal accumulating ability, and accumulating ability/soil type match. For this last parameter, high accumulating plants were assumed

to have a good match with the high cadmium soil, and low accumulating plants were assumed to have a good match with the low cadmium soil. Bacterial and fungal data were run separately.

Fragment richness was calculated from the binary matrix by treating each fragment length as an 'individual' species. Therefore, fragment richness is equal to the total number of unique fragment lengths present in each sample. Within a plant type, including bulk soil, differences among treatments was analyzed with a one-way ANOVA followed by Tukey's post-hoc test. For all statistical tests a p value of ≤ 0.05 was considered significant.

3.3 Results

3.3.1 Soil Characterization

The concentrations of various trace elements in the two experimental soils are given in Table 3-2. There was a statistical difference between the concentration of phosphorus, potassium, iron, zinc, and cadmium between the fertilized and unfertilized soils (t-test, $n = 3$, $p < 0.05$). However, cadmium was the only element that was present in a concentration above the CCME limits, or considered in excess, and it was above this threshold in both soil types. Organic matter content, pH, cation exchange capacity and soil texture are given in Table 3-3. These physical and chemical properties did not vary between the two soils (t-test, $n = 3$, $p > 0.05$).

Table 3-2: Trace element concentrations in unfertilized and fertilized soil (mean \pm SE)

Macronutrients							
Soil	Calcium (mg/kg)	Magnesium (mg/kg)	Nitrogen (NO ₃) (mg/kg)	Phosphorus (mg/kg)	Potassium ¹ (mg/kg)	Sodium (mg/kg)	
Unfertilized	3673.3 \pm 99.1	571.7 \pm 27.4	7.7 \pm 0.3	19.0 \pm 1.0*	225.7 \pm 8.8*	10.0 \pm 0.6	
Fertilized	3566.7 \pm 70.0	623.3 \pm 28.4	9.7 \pm 0.6	47.3 \pm 8.9	197.7 \pm 11.8	11.3 \pm 0.7	
Micronutrients						Non-essential	
Soil	Boron (mg/kg)	Copper (mg/kg)	Iron (mg/kg)	Manganese (mg/kg)	Zinc (mg/kg)	Aluminum (mg/kg)	Cadmium (mg/kg)
Unfertilized	1.4 \pm 0.0	1.7 \pm 0.0	40.3 \pm 0.3*	141.3 \pm 4.3	2.8 \pm 0.1*	420.3 \pm 12.5	2.38 \pm 0.17*
Fertilized	1.3 \pm 0.1	1.6 \pm 0.0	43.7 \pm 0.9	128.3 \pm 7.8	5.2 \pm 0.6	439.7 \pm 6.2	3.07 \pm 0.10
CCME Limits ²	n/a	63	n/a	n/a	200	n/a	1.4
Excess Soil ³ Concentration	> 3 - 5	> 17 - 25	n/a	> 150-200	>10	n/a	n/a

* represents significant difference ($p < 0.05$)

¹Concentration of potassium in typical agricultural soils is 212.75 \pm 81.66 (Gosling and Shepherd, 2005).

²Canadian Council of Ministers of the Environment (CCME, 2006)

³Excess soil concentrations source: Sillanpaa (1982)

Table 3-3: Physical and chemical properties of unfertilized and fertilized soil (mean \pm SE)

Soil	Organic Matter (%)	pH	Cation Exchange Capacity (meq/100 g)	Sand (%)	Silt (%)	Clay (%)	Texture
Unfertilized	5.3 \pm 0.1	8.0 \pm 0.0	23.7 \pm 0.7	69.1 \pm 3.2	25.8 \pm 1.7	5.0 \pm 1.5	Sandy Loam
Fertilized	5.2 \pm 0.1	7.8 \pm 0.0	23.6 \pm 0.6	66.5 \pm 5.5	24.9 \pm 3.5	4.1 \pm 2.2	Sandy Loam

No significant difference in variables was detected between fertilized and unfertilized soil (t-test, $p > 0.05$, $n = 3$)

3.3.2 Plant Growth Measures

Plant growth measures are presented in Table 3-4. The mean height within the *Triticum* cultivars was equal among all treatments. Kyle grown in unfertilized soil had the lowest number of leaves, followed by Kyle and Arcola grown in fertilized soil, while Arcola grown in unfertilized soil had the highest number of leaves. Shoot dry weight was lowest in Kyle grown in unfertilized soil compared to all other samples (two-way ANOVA with Tukey's post-hoc, $n = 4$). Among the *Brassica* species, there was no treatment effect on mean height or shoot dry weight; however, *B. napus* had consistently fewer leaves compared to *B. juncea* independent of soil treatment (two-way ANOVA with Tukey's post-hoc, $n = 4$).

3.3.3 Plant Tissue Cadmium Content

Plant tissue cadmium concentration and total content is given in Table 3-5. For durum wheat, both cadmium concentration and total cadmium content was higher in the shoots of the Kyle variety grown in fertilized soil compared to unfertilized soil. Cadmium was below the detection limit in shoots of the Arcola variety. However, root cadmium concentration was equal between durum wheat cultivars when grown in the same soil type and was lower in plants from unfertilized soil than from fertilized soil (two-way ANOVA, $n = 3$). The concentration and total amount of cadmium in the shoots did not vary between the *Brassica* species among soil treatments; however, root cadmium concentration ranged from below detection limit in the unfertilized soils to 1.659 – 2.940 $\mu\text{g/g}$ in the fertilized soil for both species.

Table 3-4: Plant height, number of leaves, and shoot dry weight for each plant type in unfertilized and fertilized soil (mean \pm SE)

Species	Expected Cadmium Accumulation	Soil	Height (cm)	Number of Leaves	Shoot Dry Weight (mg)
<i>Triticum durum</i> var. Kyle	High	Unfertilized	40.03 \pm 0.17 a	5.0 \pm 0.2 a	230 \pm 10 a
		Fertilized	44.65 \pm 0.25 a	7.5 \pm 0.5 b	358 \pm 42 b
<i>Triticum durum</i> var. Arcola	Low	Unfertilized	45.63 \pm 1.77 a	10.25 \pm 0.9 c	369 \pm 25 b
		Fertilized	44.15 \pm 2.45 a	8.0 \pm 0.8 b	340 \pm 25 b
<i>Brassica juncea</i>	High	Unfertilized	23.83 \pm 1.43 a	9.3 \pm 0.8 b	284 \pm 27 a
		Fertilized	23.58 \pm 4.78 a	8.8 \pm 0.8 b	349 \pm 57 a
<i>Brassica napus</i>	Low	Unfertilized	16.7 \pm 1.7 a	4.3 \pm 0.8 a	215 \pm 7 a
		Fertilized	17.4 \pm 0.96 a	4.5 \pm 0.5 a	361 \pm 61 a

For each variable within each genus values not sharing a common letter are significantly different ($p < 0.05$)

Table 3-5: Plant shoot and root cadmium content (mean \pm SE)

Species	Expected Cadmium Accumulation	Soil	Shoot Cadmium Concentration ($\mu\text{g/g}$)	Total Shoot Cadmium μg	Root Cadmium Concentration ($\mu\text{g/g}$)
<i>Triticum durum</i> var. Kyle	High	Unfertilized	0.410 \pm 0.093 b	0.121 \pm 0.055 b	1.992 \pm 0.290 a
		Fertilized	0.791 \pm 0.242 c	0.286 \pm 0.058 c	2.613 \pm 0.506 b
<i>Triticum durum</i> var. Arcola	Low	Unfertilized	bdl a	bdl a	2.169 \pm 0.344 a
		Fertilized	bdl a	bdl a	2.993 \pm 0.172 b
<i>Brassica juncea</i>	High	Unfertilized	0.705 \pm 0.242 a	0.228 \pm 0.076 a	bdl a
		Fertilized	0.907 \pm 0.205 a	0.305 \pm 0.067 a	2.940 \pm 1.798 b
<i>Brassica napus</i>	Low	Unfertilized	0.481 \pm 0.137 a	0.099 \pm 0.028 a	bdl a
		Fertilized	0.780 \pm 0.224 a	0.358 \pm 0.108 a	1.659 \pm 0.231 b

For each variable within each genus values not sharing a common letter are significantly different ($p < 0.05$)

bdl = below detection limit ($< 0.001 \mu\text{g/g}$)

High or low accumulation as predicted from Chan and Hale, 2004 (*Triticum*) and McGrath *et al.* 2001 (*Brassica*)

3.3.4 TRFLP Microbial Community Analysis of Similarity

3.3.4.1 Bacterial Communities

Fragment richness, calculated from TRFLP profiles, in bulk soil, rhizosphere, and endophyte bacteria communities are given in Table 3-6. Fragment richness of the endophyte communities and of unfertilized soil was low and fragment richness in the fertilized soil was about two times greater than that of the unfertilized soil. Within the rhizosphere samples fragment richness values were highest for matched and unmatched Kyle, unmatched Arcola, and matched *B. napus* (one-way ANOVA within plant type, including bulk soil, followed by Tukey's post hoc test, $p < 0.05$, $n = 3$). Fragment richness was the same in matched and unmatched rhizosphere of *B. juncea*.

The NMDS ordination for bacteria community profiles from TRFLP analysis are plotted in Figure 3-1 and Figure 3-2. These plots show relative (dis)similarity between bacterial communities, where greater distance between points is equivalent to greater dissimilarity. There was no difference between the bacterial community profiles between the two soil types (Figure 3-1 A) or time points (Figure 3-1 B) based on one-way ANOSIM ($p > 0.05$, $n =$ at least 6). Similarly, there was no difference between the rhizosphere communities when plant type, accumulation ability, or genus was plotted (data not shown). However, the seed endophyte bacteria (Figure 3-1 C) were different between the *Triticum* cultivars Kyle and Arcola and the *Brassica* species. Despite the lack of separation based on factors such as soil, time, plant type, and genus it was found that the rhizosphere bacteria communities were significantly different when considered as being matched for the plant's cadmium accumulating ability and the soil type (i.e. high accumulating plant in more contaminated soil or low accumulating plant in less contaminated soil) or unmatched (i.e. high accumulating plant in less contaminated soil or low accumulating plant in more contaminated soil) (Figure 3-1 D).

Table 3-6: Bacterial fragment richness in bulk soil, matched and unmatched rhizosphere, and seed endophytes (mean \pm SE)

Bulk Soil		Plant Type	Rhizosphere		Endophytes
Fertilized	Unfertilized		Matched	Unmatched	
150 \pm 9 b	86 \pm 35 a	<i>Triticum durum</i> var. Kyle	469 \pm 39 d	228 \pm 9 c	67 \pm 11a
		<i>Triticum durum</i> var. Arcola	309 \pm 131c	563 \pm 37 d	58 \pm 7 a
		<i>Brassica juncea</i>	173 \pm 20 b	300 \pm 103 c	47 \pm 6 a
		<i>Brassica napus</i>	391 \pm 177 c	109 \pm 10 b	45 \pm 7 a

Bacteria species richness for each plant type was compared among bulk soil, rhizosphere soil, and seed endophytes. Values not sharing a common letter are significantly different (one-way ANOVA, $p < 0.05$, $n = 3$). Where matched rhizosphere was sampled from a plant with high accumulating ability in more contaminated soil or low accumulating ability in less contaminated soil and unmatched rhizosphere was sampled from a plant with high accumulating ability in less contaminated soil or low accumulating ability in more contaminated soil.

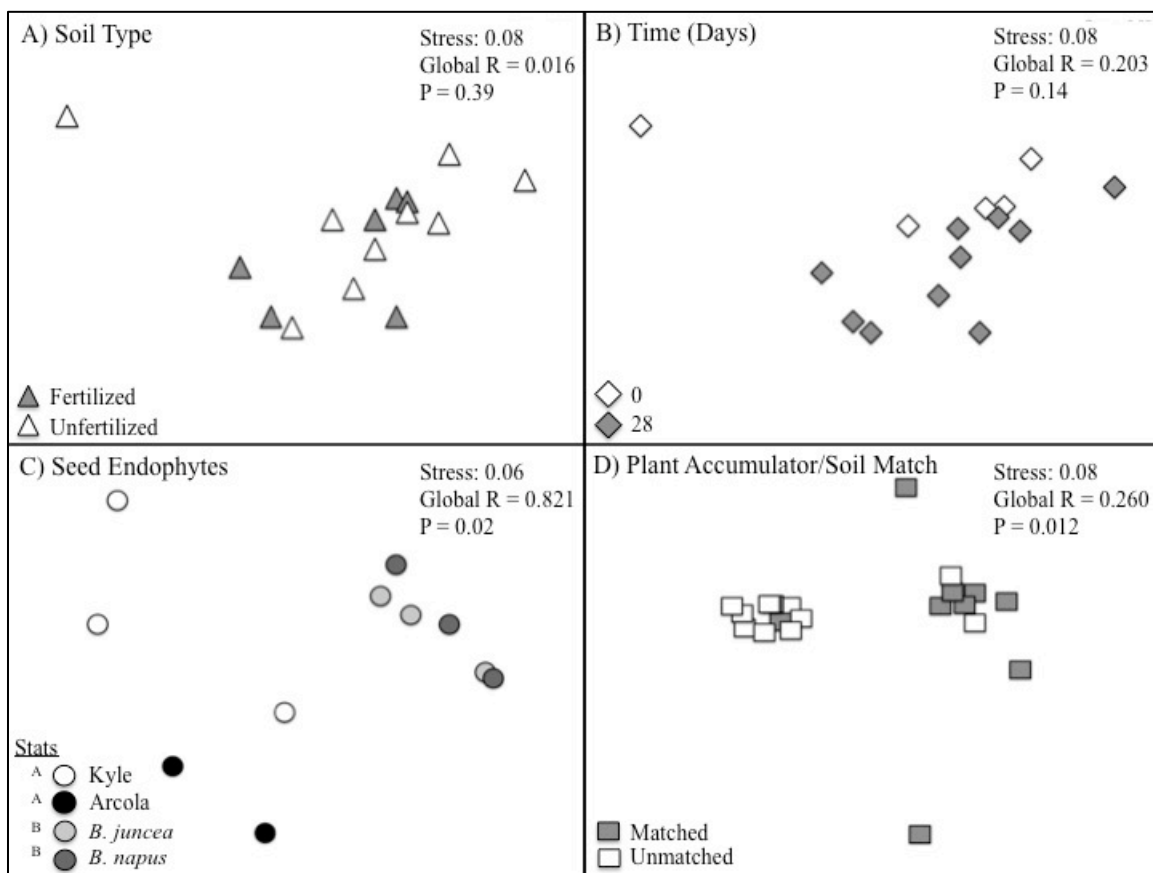


Figure 3-1: NMDS ordination based on Bray-Curtis similarities of soil bacteria community TRFLP profiles

Dis(similarity) is reflected in the distance between points for A) soil types B) time C) seed endophytes and D) plant accumulating ability/soil type match. Where matched rhizosphere was sampled from a plant with high accumulating ability in more contaminated soil or low accumulating ability in less contaminated soil and unmatched rhizosphere was sampled from a plant with high accumulating ability in less contaminated soil or low accumulating ability in more contaminated soil. Global R and P are based on the ANOSIM overall trend. Treatments not sharing a common letter in the stats column were significantly different (ANOSIM with pair wise post-hoc test for plant type, $p < 0.05$, $n = 3$).

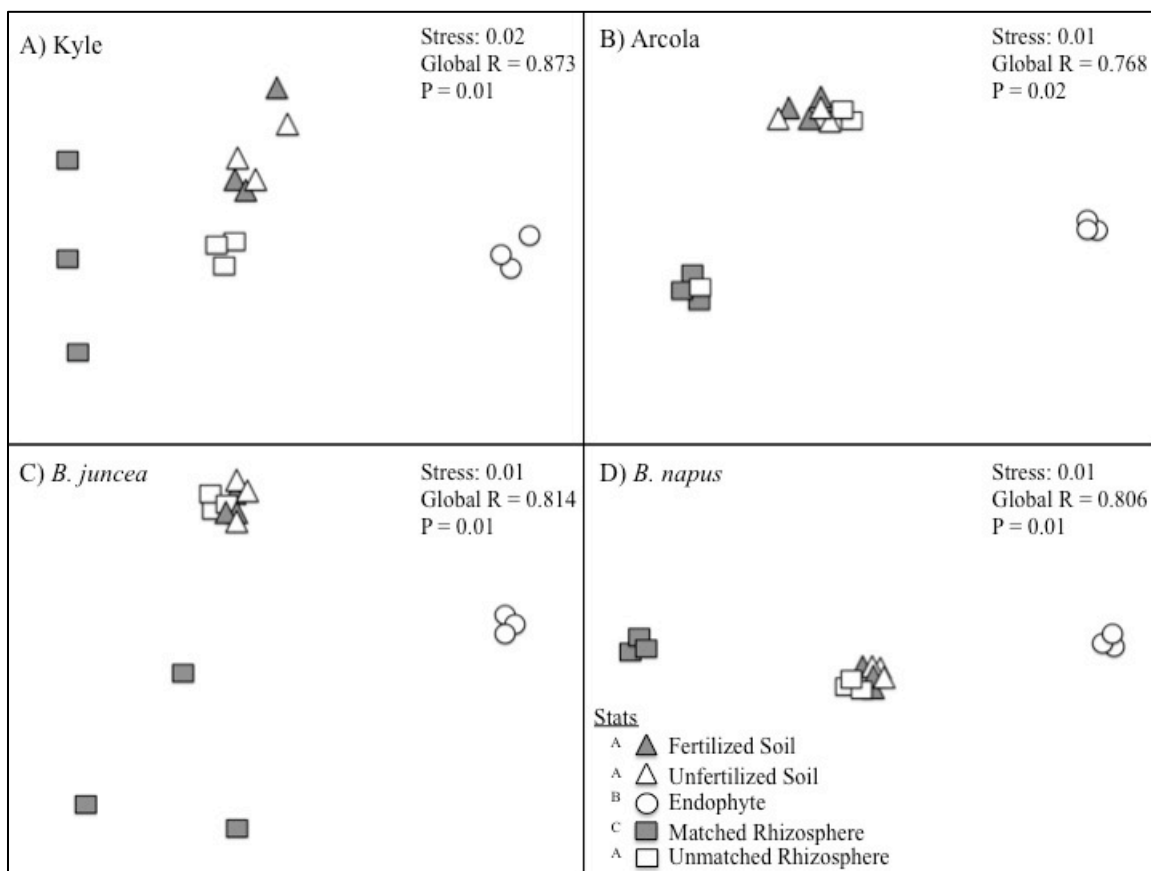


Figure 3-2: NMDS ordination based on Bray-Curtis similarities of bacteria community TRFLP profiles among bulk soil, endophytes, matched, and unmatched soil/plant communities in each plant type

Dis(similarity) is reflected in the distance between points for A) *Triticum durum* cv. Kyle B) *T. durum* cv. Arcola, C) *Brassica juncea* and D) *B. napus*. Where matched rhizosphere was sampled from a plant with high accumulating ability in more contaminated soil or low accumulating ability in less contaminated soil and unmatched rhizosphere was sampled from a plant with high accumulating ability in less contaminated soil or low accumulating ability in more contaminated soil. Global R and P are based on the overall trend. Treatments not sharing a common letter in the stats column were significantly different (ANOSIM followed by pair wise post-hoc test for each plant type, $p < 0.05$, $n = 3$).

To determine whether the seed endophytes or native soil bacteria could be contributing to the difference between the bacteria communities in matched or unmatched conditions, the community profiles for the endophytes, soil types, matched rhizosphere and unmatched rhizosphere, for each plant type were plotted in a NMDS ordination (Figure 3-2). It can be seen that for all plant types there was a significant difference between the seed endophytes and all the soil communities, the bacteria community in the unmatched rhizosphere is the same as the communities found in either soil type, and the communities in the matched rhizosphere are different than the communities found in any other treatment (one-way ANOSIM followed by pair wise post-hoc test, $p < 0.05$, $n = 3$). Also, the bacteria communities in the matched soil type appear to have the most variation among samples where as all other treatments form distinct clusters that reflect greater similarity among samples.

The dissimilarity between the matched rhizosphere versus the unmatched rhizosphere or bulk soil might be explained by an increase or decrease in unique fragments compared to the bulk soil. The numbers of unique and shared fragments identified by TRFLP analysis among treatments is represented in Figure 3-3. Approximately 40% of the fragments were found in all three treatments and 10% of the fragments were unique to a particular treatment. The bulk soil samples did not contain any unique fragments. The matched rhizosphere samples appear to be the most different, containing 150 unique fragments while the unmatched rhizosphere samples only contain 56 unique fragments. Similarly, the matched rhizosphere samples shared approximately 100 fewer common fragments with the bulk soil than the unmatched rhizosphere did.

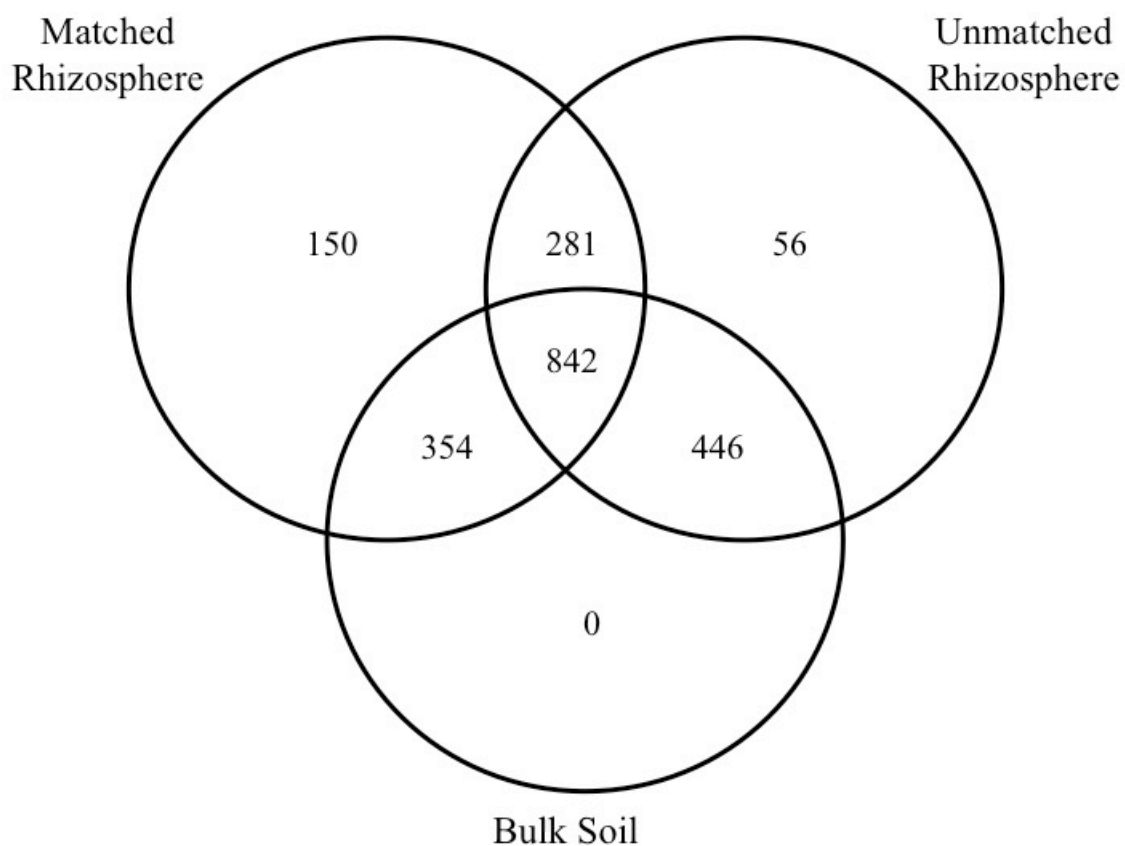


Figure 3-3: Schematic representation of the number of common fragments among matched rhizosphere, unmatched rhizosphere, and bulk soil samples

Number of fragments based on pooled TRFLP fragment presence/absence data. Within the matched rhizosphere treatment, samples from plants with high accumulating ability in more contaminated soil and those with low accumulating ability in less contaminated soil were pooled. Similarly, within the unmatched rhizosphere treatment, samples from plants with high accumulating ability in less contaminated soil and those with low accumulating ability in more contaminated soil were pooled. For the bulk soil treatment, the two soil types were also pooled. These pools were deemed acceptable since no significant differences were found among the components of each pool (Figures 3-1 and 3-2).

3.3.4.2 Fungal Communities

Fungal genomic DNA could not be repeatedly amplified from soil. After ruling out problems with the DNA extraction procedure, DNA inhibitors, and the primers, the results of the fungal analysis were omitted from this thesis due to the inconsistency. It is possible that the procedure used to extract DNA from the soil was not aggressive enough to lyse the fungal cells, therefore, there was little to no fungal DNA present for amplification. Future work should use different DNA extraction procedures to ensure isolation of fungal DNA from soil samples.

3.4 Discussion

3.4.1 Soil Characterization

Among the soil trace elements that were measured, the unfertilized and fertilized soils varied only in the concentrations of phosphorus, potassium, iron, zinc, and cadmium (Table 3-2). The initial difference in phosphorus, which was the result of applying phosphate fertilizer containing cadmium as a contaminant (personal communication, Dr. Cynthia Grant, AAFC), was eliminated prior to sowing the plants. However, it was determined that the difference between the two soils in the concentration of potassium, iron, and zinc, while statistically significant were not biologically significant. The typical concentration of potassium in agricultural soils is 212.75 ± 81.66 (Gosling and Shepherd, 2005). Both of the soils used were within this range and therefore, the difference between the two soils was likely not biologically significant. Similarly, the mean difference of 3.4 mg/kg iron observed between the two soil treatments would not have a biological impact since the concentration of iron in both soils is well above the 3.6 to 12.8 mg/kg range required by most crop species (Christ, 1974). Likewise, the concentration of zinc was an order of magnitude below CCME limits and half the concentration considered to be in excess in soil for both soil types. This indicates that the only known way in which the two soils varied was in the concentration of cadmium. The CCME sets thresholds for the concentrations of potentially toxic metals in agricultural

soils. The current CCME limit for cadmium is 1.4 mg/kg soil (CCME, 2006). Both the unfertilized and fertilized soils were above this threshold at 2.38 and 3.07 mg/kg, respectively. The unfertilized soil can therefore be considered a low cadmium treatment, while the fertilized soil can be considered a high cadmium treatment.

Many soil physical and chemical factors such as organic matter content, pH, cation exchange capacity, and soil texture can affect the bioavailability of metals in soil (Yang *et al.* 2001). It was therefore important to ensure that these factors did not vary between the soils used in this study to eliminate their effect on of cadmium bioavailability. The two soils did not vary among these measures. Given the 8-year history of different fertilizer regimes between the two soil types it is possible that the soils varied in other ways that were not measured in this study.

3.4.2 Plant Growth Measures and Plant Tissue Cadmium Content

Kyle and Arcola cultivars of durum wheat are regarded as being high- and low-cadmium accumulators, respectively (Chan and Hale, 2004), while *B. juncea* and *B. napus* are considered a metal hyperaccumulator and non-hyperaccumulator (McGrath *et al.* 2001). Variation in plant growth measures such as height, number of leaves, and shoot dry weight may occur within a plant genus when the plant is able to tolerate the contamination level of the soil in which it was grown. However, there was no variation in height or shoot dry weight among soil types and plant type within a genus, except for an unexpected reduction in dry weight when Kyle was grown in unfertilized soil (Table 3-3). The variation in the number of leaves between the two *Brassica* species may be due to phenotypic differences between the two species. Interestingly, the number of leaves among the *Triticum* cultivars appears to be related to whether the metal-accumulating ability of the plant type was matched to the contamination level of the soil type. The high-accumulating variety had an increase in the number of leaves when grown in the more contaminated fertilized soil while the low-accumulating variety had the highest number of leaves when grown in the less contaminated unfertilized soil. The tissue cadmium content data given in Table 3-4 is consistent with previous work documenting the metal-accumulating ability of these four plants, other than the failure of *B. juncea* to

accumulate more cadmium in the shoot tissue compared to *B. napus*. However, previous research has shown that some cultivars of *B. juncea* accumulate metals in the roots but fail to translocate these metals to the shoots (Kumar *et al.* 1995). It is also possible that 28 d was insufficient time for *B. juncea* to accumulate the expected higher amounts of cadmium in aboveground tissues.

3.4.3 TRFLP Microbial Community Analysis of Similarity

3.4.3.1 Bacterial Communities

Fragment richness was greater in the bacterial communities in fertilized compared to unfertilized soil, and seed endophytes and some rhizosphere soil had greater fragment richness than the bulk soil (Table 3-5). It is not surprising that the greatest fragment richness was in the rhizosphere communities. This could be due to the increase in nutrition sources, such as carbon, supplied by the plant to the soil environment. Between 5 and 21% of all photosynthetically fixed carbon is transferred to the rhizosphere through root exudates (Marschner, 1995). There does not appear to be any clear pattern in which plant types or plant-soil matches have affected fragment richness.

Assuming there was no major source of bacterial contamination in the growth chamber, all bacterial species found in the rhizosphere had to be present in either the bulk soil or endophyte communities. Therefore, increases in richness do not represent the appearance of new species, they are simply increases in fragment abundance above the fluorescence threshold used to differentiate ‘true peaks’ from background noise (Culman *et al.* 2008).

After plotting the TRFLP binary matrix as a series of NMDS ordinations it was determined that the following factors were not a source of the differences among bacteria communities found in the soil: soil type, time (Figure 3-1 A,B), genus, species, and cadmium-accumulating ability (not shown). The lack of difference between the soil types suggests that the concentration of cadmium in the fertilized soil was not high enough to select against any bacteria found in the unfertilized soil: however, one might expect a greater number of bacterial species in a non-contaminated soil. Any differences

that were seen in the experimental treatments must come from either seed endophytes or be the result of the interactions of plants with the soil.

The endophytic bacteria communities were different between the two plant genera (Figure 3-1 C). Ahlholm *et al.* (2002) found that host genetic variation can affect endophyte genetic variation in fungi and Manter *et al.* (2010) have shown cultivar-specific bacterial endophyte communities in potato. Additionally, it has been shown that the environmental conditions of the host plant, such as microclimate and biotic interactions, can influence the makeup of the endophyte community in corn (Pan *et al.* 2008). It is possible that if the parent plant were grown in contaminated soil the endophytes in the seed would be more likely to benefit the seedling if it germinated in a similarly contaminated environment, and *vice versa*. This could result in the greatest similarity in the rhizosphere bacterial communities being between the pairs of high- and low-accumulating plants. Kyle and *B. juncea* were more likely to have been previously grown in a contaminated soil, and might be expected to have similar endophytes. Similarly, the low-accumulating plants, *Arcola* and *B. napus*, might be expected to share endophytes characteristic of non-contaminated sites. However, when the endophyte community profile was plotted with bulk soil and matched and unmatched rhizosphere communities it was found that both matched and unmatched rhizosphere communities were significantly different than the endophytes for all plant types (Figure 3-2).

The bacteria communities in the rhizosphere of plant types that were grown in soil that matched their accumulating ability were different than those of plant types grown in unmatched soils (Figure 3-1 D). The dissimilarity between the matched and unmatched soil types suggests that the plant interaction with the soil is essential to forming the bacterial community in the rhizosphere.

The bacterial communities in the rhizosphere of the unmatched plants were the same as the communities in the bulk soil, while the bacterial communities in the rhizosphere of matched plants were significantly different (Figure 3-2) for all plant types. The plants grown in unmatched soil do not appear to influence the soil environment in a way that would allow new or different bacteria to colonize the rhizosphere. Additionally,

the communities are closely clustered in the NMDS plot suggesting very little variation in the bacteria community among samples.

The fact that the bacteria communities in the matched plant/soil rhizosphere are different than either the endophyte or bulk soil bacteria communities suggests that the matched plants have a greater ability to change their soil environment, which resulted in a shift in the bacterial community. This is also reflected in the number of unique fragments detected in the matched rhizosphere compared to either the unmatched rhizosphere or bulk soil samples (Figure 3-3). It is well established that plants exude a variety of compounds that may influence the composition of the soil microbial community by promoting symbiosis, inhibiting the growth of pathogens, and changing the chemical and physical properties of the soil. It has been shown that certain plant metabolites, when exuded into the environment, are capable of recruiting beneficial soil microbes. For example, malic acid secreted from the roots of *Arabidopsis* acts as a signal to selectively recruit the beneficial *Bacillus subtilis* FB17 in a dose-dependent manner (Rudrappa *et al.* 2008). Conversely, secretion of defense proteins and other chemicals can protect plant roots from pathogenic microorganisms during development (Flores *et al.* 1999). Any of these inputs may have lead to the proliferation of certain species to bring them above the TRFLP threshold and the subsequent increase in the number of fragments that were detected in the matched rhizosphere.

The bacterial communities in the rhizosphere of plants grown in matched soil had the greatest variation among the bacteria communities compared to all other treatments. This increase in variation suggests that the plants in this treatment were less selective when recruiting soil microbes than the unmatched plants, which cluster very closely together. It is possible that the beneficial input that lead to the increase in species does not put selective pressure on the community and allows a number of species to thrive.

It is important to note that none of the plants grown in any of the treatments showed signs of cadmium-induced stress, which suggests that the resulting changes in the rhizosphere of matched or unmatched plants do not reflect the plant's inability to alter the chemistry of their rhizosphere as a result of stress.

3.4.3.2 Fungal Communities

Fungal genomic DNA was difficult to amplify. Many samples lacked amplification, although positive and negative controls produced the expected results and bacterial genomic DNA isolated from the same soil sample amplified. Combined with the non-repeatability in the sequence results for those duplicate samples in which the DNA did amplify, the fungal communities have been left out of further analysis. However, I would expect the fungal community to respond in a similar way as the bacterial community given the close association between plants and fungi in the rhizosphere.

3.5 Conclusions and Future Directions

Further investigation into the significance of matching plant metal-accumulating ability to the contamination level of the soil is needed. Since the seed endophytes were ruled out as a source of this variation it is likely that the most important factor is related to plant exudation and recruitment of microbe colonization through changes in the soil immediately surrounding the plant root. It is easy to theorize about why a plant grown in a matched soil type would have the greatest ability to thrive and influence its environment. It is also understandable that a low-accumulating plant will not do well in a highly contaminated soil. However, it is unclear why the high-accumulating plants lose their ability to interact with and alter the rhizosphere environment in a less contaminated soil. It might help to test additional pairs of low- and high-accumulating plants within many other plant species to determine if this is a general phenomenon.

Future work should follow two paths. First, the fragments corresponding to the populations contributing the most to the differences between the communities should be identified. These TRFLPs could then be compared to phylogenic databases in an attempt to identify these bacteria. However, the fragments may not precisely match currently sequenced organisms since the detected fragment length can be influenced by the protocol (Tu *et al.* 1998) and sequence composition (Kaplan and Kitts, 2003), and these databases are limited by the small percentage of organisms that are currently sequenced

(Blackwood and Buyer, 2007). However, if the bacteria can be identified then the role of those bacteria in the plant-microbe-metal interactions could be elucidated and might help to explain differential metal tolerance, or metal uptake, in plants. Secondly, work needs to be done to elucidate the specific mechanism behind the influence of plants on the composition of the microbial community in the rhizosphere, especially the mechanisms that allow a plant to exert a high amount of influence over the microbes in a well matched soil versus an unmatched soil.

3.6 References

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Chapter 4

4 General Discussion

4.1 Overview and Future Prospects

The interaction between plants, microbes, and their environment continues to be proven a complex system to study. Trying to document the number of ways that these organisms interact with one another has been described as trying to open a window into a 'black box' of unknowns (Insam, 2001). At the same time, researchers who aim to clarify these unknowns, usually one at a time, have been criticized for looking at a single tree and thus overlooking the forest. Some have over-simplified the system by treating the entire soil microbial community as a single unit, allowing researchers to study the forest, rather than a tree (Stockdale and Brookes, 2006), but this approach fails to isolate subtleties behind the microbial interactions with other microbes and the environment. Failure to study the very complex and diverse interactions between plants, microbes, and the soil environment, on both the small scale and as a whole ecosystem, has not been due to lack of will or effort, but has been limited by the available methodology and technology.

Soil microbiology has experienced major changes in methods and scope in the last few decades. Early researchers used culture-based methods to enumerate bacterial and fungal biomass using serial dilutions on select media (reviewed in Stockdale and Brookes, 2006). However, simple methods that depend on extracting soils and counting the microorganisms fail to fully characterize the soil microbiota due to the fact that few microbes can be cultured. This type of community description also fails to identify and determine the relative importance of each microorganism's function in the soil. Scientists, therefore, needed to develop methodology to measure and monitor major soil processes, especially microbial influence on major geochemical cycles and plant growth. Methodology that enabled characterization of biochemical processes, such as enzyme activity and nutrient flux, allowed for more insight into the microbial role in ecosystem function but still lacked insight into the microbial impact on the community as a whole

(reviewed in Insam, 2001). More recently, molecular techniques have allowed for culture-independent analysis of the diversity of microorganisms in soil (reviewed in Hirsch *et al.* 2010). This type of methodology is getting closer to a system that allows for the study of the ‘whole forest’.

In the past, research on plant-microbe interactions has focused on characterizing the physiological function of microbial populations in soil and on the identification and isolation of microbes of interest from the rhizosphere, such as microbes that can fix atmospheric nitrogen (Bashan *et al.* 2004), produce phytohormones (Vessey, 2003), and aid pathogen defense (Walsh *et al.* 2001). Compared to determining the functional role(s) of microbial communities very little research has been done to characterize differences in microbial communities in different environmental conditions and under different stresses, likely due to the lack of methodology to do so. The lack of research at the ecosystem level may partially explain why microbes found to be beneficial in laboratory studies often fail to achieve the same level of success in field trials and why there is often high variability among field plot replicates. One of many examples includes a study of the ability of pseudomonad strains to promote growth and yield of winter wheat at two field sites. After 250 days there was no significant difference between the treatment and control plots despite promising lab studies (de Freitas and Germida, 1992).

In this thesis, I looked at plant-microbe interactions under cadmium stress at the small-scale, single bacterium (‘one tree’) level, as well as in a more complex ecosystem (‘whole forest’).

In Chapter 2, I showed that there are environmental conditions in which an otherwise universal PGPR can become deleterious to plant growth. When *Arabidopsis thaliana* was inoculated with the putative PGPR *Pseudomonas putida* UW4 in MS medium there was a negative impact on plant growth compared to a no bacteria treatment. I believe that this is the first time a seemingly universal PGPR has been shown to be deleterious. I have expanded upon the current model of plant-PGPR interactions by discovering that there are environmental conditions under which bacterial

interactions with the environment can alter the bacterial interaction with the plant (Figure 4-1 A). My work shows the importance of increasing the scope of PGPR research to include the ways in which the microbial-environment interactions might influence the plant growth-promoting abilities of a single bacterium.

It is well established that microbes are highly influenced by the environment and that environmental disturbances, such as the addition of toxic metals (Sandaa *et al.* 2001), pollutants (Ovreas and Torsvik, 1998), and pesticides (El Fantroussi *et al.* 1999), can decrease the number and diversity of microbial populations in soil. However, very little is known about how this change in the microbial community will, in turn, affect the plants. Also, in contrast to the deleterious impact of PGPR in MS medium documented in Chapter 2, there has been little to no investigation of whether these microbial changes will have a direct negative impact on plant growth. The reduction in microbial populations recorded as a result of the introduction of a plant nutrient solution (MS medium) documented in Appendix B suggests that even seemingly positive changes in the environment can have negative impacts on the microbial community, which can result in a negative impact on the plant.

In Chapter 3, I analyzed the microbial community profiles of the rhizosphere surrounding two pairs of high- and low-cadmium accumulating plants in an attempt to determine whether a plant's inherent metal-accumulating ability would influence the composition of the microbial community. Unexpectedly, I found that the composition of the microbial community did not vary with soil type, time, plant type, or metal-accumulating ability of the plant. However, when a plant's metal-accumulating ability was well matched to the level of metal contamination in the soil significant changes in the microbial community profile in the rhizosphere were seen. More specifically, well matched plants appeared to exert a high amount of influence on the composition of their rhizosphere, resulting in microbial populations that were different than the seed endophytes and those in the bulk soil, while unmatched plants were unable to alter the composition of the rhizosphere, resulting in microbial communities identical to that of bulk soil (Figure 4-1 B). This previously undocumented influence on the rhizosphere

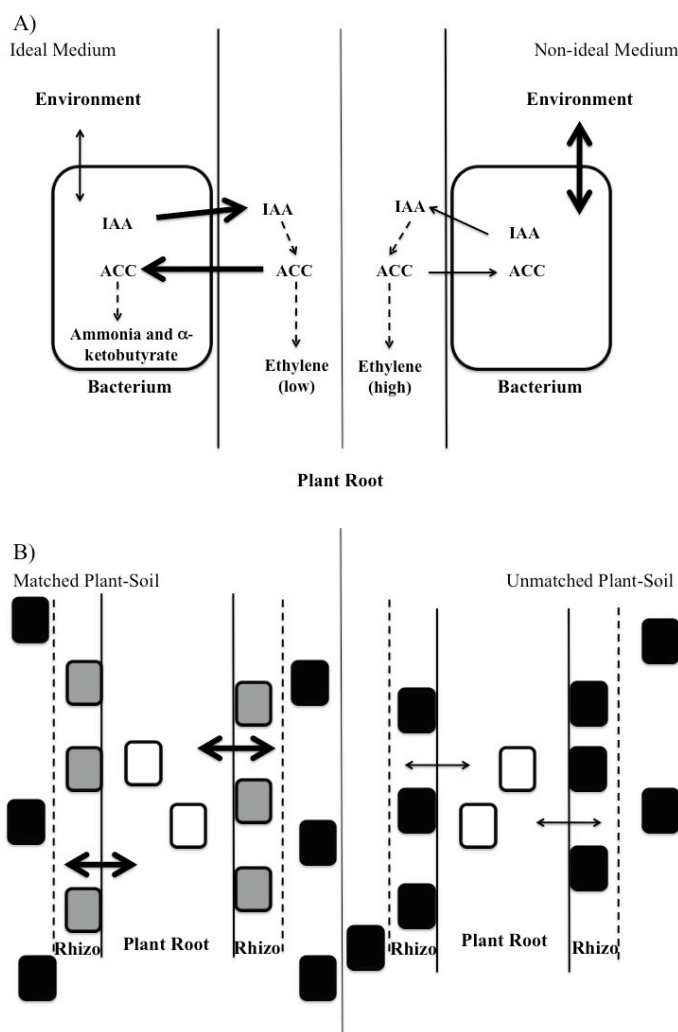


Figure 4-1: Proposed model for plant-microbe interactions influenced by environmental factors

A) In ideal growth conditions PGPR positively impact plant growth by producing IAA and metabolizing ACC with ACC deaminase requiring minimal microbial interaction with the environment, or at least minimal diversion of microbial resources away from those required to promote plant growth. However, under non-ideal conditions, the degree of microbial interaction with the environment increases, resulting in a reduction in the ability of the PGPR to mitigate plant stress and a subsequent reduction in plant growth. B) When grown in soil that is well matched to their inherent metal-accumulating ability, plants are able to exert a high degree of influence on the rhizosphere (Rhizo) resulting in microbial communities (grey) that are different from the seed endophytes (white) or those in bulk soil (black). However, when plants are grown in unmatched soil they are unable to establish a distinct rhizosphere and the resulting microbes are the same as those in bulk soil (black). Thicknesses of two-way arrows indicate the relative strength of the interaction. These figures are not to scale.

would not have been possible without the use of modern, molecular, culture-independent methodologies.

All of the results from this thesis show the need for more research in the field of plant-microbe interactions and the need to keep trying to look at the ‘whole forest’. Additionally, my work showcases how advancements in soil microbiological methods have allowed for new plant-microbe interactions to be identified. More specifically, all the results from this study show how environmental factors have a strong impact on plant-microbe interactions and that whether this impact will be positive or negative cannot be predicted.

Although the field of plant-microbe interactions is still limited by what we do not know, prospects for this field are bright, especially as techniques continue to be developed that will allow us to gain insights into this incredibly complex system. Developments will lead to important findings with applications in phytoremediation, agriculture, food safety, and climate change modeling of major nutrient cycles.

Perhaps the most marketable application of this type of research is the development of commercial cultures as inoculants for application at field sites to improve soil function, plant growth and establishment at phytoremediation sites, agricultural yield, crop health, and food safety. Some commercial cultures are already available (listed in Berg, 2009). However, there are often marked differences in efficiency between laboratory findings and field applications, and among field sites. Developing an understanding of the ways in which the soil environment might influence microbial impacts on the plant, such as the results presented in Chapter 2, might help to improve the efficiency of use of these cultures and develop new cultures that are better able to maintain growth-promotion under varying environmental stressors. Additionally, advancements in our understanding of how environmental conditions alter the composition of the microbial community (Appendix B) may help to maintain the function of these inoculants once they are in place.

By monitoring changes in the microbial community following environmental disturbance it might be possible to identify important contributors to ecosystem function

under the new environmental stressor. Using molecular approaches to monitoring combined with genome sequencing, Ranjard *et al.* (2000) were able to identify two organisms that increased in abundance with the addition of mercury (Hg(II)) to the soil. The results of Chapter 3 suggest that there is a need to continue to identify changes in microbial communities under different conditions since it is not possible to predict what impact different variables will have on community composition. As recently as 2002, few pollutants in soils had been examined for their effects on soil microflora in a culture-independent manner (Kent and Triplett, 2002). With the development of novel, high throughput, relatively inexpensive techniques, such as TRFLP, this trend has been changing. However, researchers need to start putting more emphasis on identifying those organisms that appear or disappear with a given treatment. Describing changes between communities should start to include probable identification of individual populations so that this information can be compared to the major changes in ecosystem functioning that correspond to the gain or loss of each organism. Comparison of TRFLP fragments to DNA sequence databases can lead to identification of major contributors to the microbial community, although these databases are currently limited by the relatively low number of organisms that have been sequenced (Blackwood and Buyer, 2007). Continuing the effort to identify and sequence novel strains will help to reduce this problem in the future.

In addition to monitoring changes in species abundance and community structure it is possible to apply molecular tools to identify microbial genes of interest. Some researchers have used TRFLP to detect functional genes of interest in microbial communities; for example, genes encoding for nitrogen fixation (Rosch and Bothe, 2004) and ammonia oxidation (Horz *et al.* 2000). Development of primer sets for other genes known to aid in plant-growth promotion, such as ACC deaminase, could lead to the discovery of novel PGPR and PGPR interactions. Given the fact that the nutrient solution I used in Chapter 2 and Appendix B had a strong negative impact on 1) plant growth in the presence of *P. putida* and 2) the microbial community in soil, it would be prudent to attempt to identify those bacterial species that were able to thrive in the MS medium and find other bacteria that contain beneficial genes, such as those that code for ACC deaminase, that may improve plant growth. These novel PGPR might be more appropriate for inoculation at field settings that regularly receive nutrient applications.

Climate change modeling emphasizes the importance of nutrient cycles in predicting an ecosystem's capacity to ameliorate excess atmospheric carbon dioxide. Most of these models emphasize the importance of terrestrial vegetation to sequester carbon dioxide. More recent models have shown that interactions between carbon cycling and nitrogen cycling can dramatically alter this predicted capacity (Thornton *et al.* 2007). What these models fail to consider is how climate change will impact the microbiome of these plants, and how the changes in the microbiome will also alter the plants' capacity to deal with climate change. Since nitrogen-fixing microbes have always been a priority for plant-microbe interaction studies I believe that there is a need to study climate change impacts, such as temperature and precipitation, on microbial communities in carbon sequestration zones, and how these impacts may alter the capacity to ameliorate climate change. Since I have shown that certain environmental stressors can result in detrimental impacts of microbes on plant growth, this reduction in sequestration capacity is a real threat.

It is my belief that any number of unknown factors can contribute to plant-microbe interactions and result in both beneficial and detrimental impacts on plant growth and health. Given that I have demonstrated that a PGPR can become deleterious in the presence of a plant nutrient medium, and that this medium can dramatically decrease the microbial fragment richness and diversity of native microbial populations in soil, I do not believe we can predict what environmental stressors will impact these interactions. Additionally, I do not believe we can definitively say whether a documented response would be repeated given a different stressor or in different plant species. I had originally hypothesized that a plant's inherent metal-accumulating ability would influence the composition of the microbial community in the rhizosphere. However, when I looked at the community profiles I found that the interaction between metal-accumulating ability and contamination level of the soil was more important than any other individual factor. This response suggests that the environmental interaction with plants and microbes can never be ignored. Only with thorough experimentation can we make definitive statements on plant-microbe-environment interactions.

4.2 References

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Appendices

Appendix A: *Arabidopsis thaliana* growth under cadmium stress

These photographs illustrate the protocol used to determine the range of CdCl₂ used to induce sub-lethal cadmium stress in Chapter 2. Maximum root length decreased with increasing CdCl₂ concentration and the radicles emerged, but failed to elongate at 25 μM CdCl₂ (Figure A-1). Shoots were stunted and chlorotic at 10 μM CdCl₂ and failed to develop at 25 μM CdCl₂ (Figure A-2).

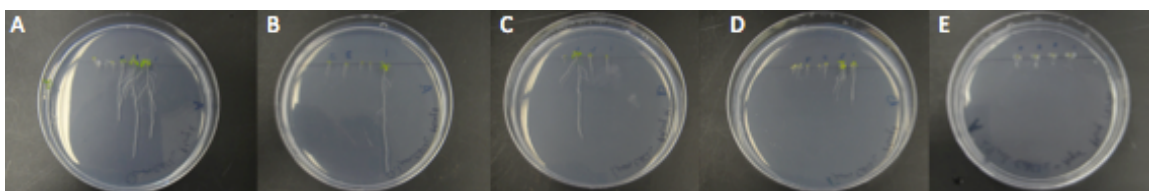


Figure A-1: *Arabidopsis thaliana* vertical root growth on agar plates with increasing CdCl₂ concentration

Images were taken after 14 d of growth at A) 0 B) 0.1 C) 1.0 D) 10 and E) 25 μM CdCl₂. The plate diameter was 9 cm.

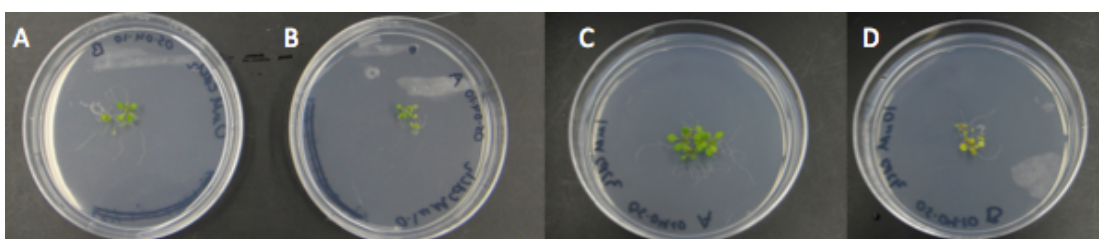


Figure A-2: *Arabidopsis thaliana* shoot growth on agar plates with increasing CdCl₂ concentration

Images were taken after 14 d of growth at A) 0 B) 0.1 C) 1.0 and D) 10 μM CdCl₂. The plate diameter was 9 cm.

Appendix B: A comparison of soil bacteria community profiles in soil maintained with distilled water or MS medium.

Rationale

This study was done to determine whether the MS medium-induced inhibition of bacterial growth observed in Chapter 2 would occur in a natural system that included soil and its constituent bacterial community. To accomplish this, soil was watered with either sterile distilled water (dH₂O) or MS medium and the soil bacteria community profile was assessed using TRFLP analysis.

Methods

The soil types are described in 3.2.1. Six pots were filled with the high cadmium soil and another six pots were filled with the low cadmium soil. Half of the pots within each soil type were watered with 10 mL dH₂O daily for 28 d, the other half were watered with 10 mL MS medium. Samples were taken at 0 d and 28 d to assess the bacterial communities. The TRFLP analysis followed the methods described in 3.2.6 through 2.3.9 and data and statistical analysis was performed according to 3.2.10.

Results

Fragment richness of bacterial communities, as assessed by TRFLP, are given in Table B-1. Initially (0 d), the high cadmium soil appeared to have greater richness when compared to the low cadmium soil. In both soil types, fragment richness increased from 0 to 28 d in soil maintained with dH₂O. In the high cadmium soil maintained with MS medium richness remained at 0 d values. In the low cadmium soil, the 28 d richness values did not differ between soil maintained with dH₂O or MS medium.

The NMDS analysis comparing overall similarity among bacterial populations based on TRFLP profiles is shown in Figure B-1. The distance between points reflects the relative (dis)similarity of bacteria communities among samples, with greater distance reflecting greater dissimilarity. Although there was no difference between communities

Table B-1: Bacteria fragment richness based on TRFLP presence/absence in high and low cadmium soil maintained for 28 days with either dH₂O or MS medium

Richness (S)	High Cd Soil			Low Cd Soil		
	Day 0	dH ₂ O Day 28	MS Day 28	Day 0	dH ₂ O Day 28	MS Day 28
	151 ± 9 a	466.5 ± 157 b	140 ± 25 a	91 ± 35 a	164 ± 46 b	159 ± 92 b

* within a soil type values not sharing a common letter are significantly different
(one-way ANOVA, p < 0.05, n = 3)

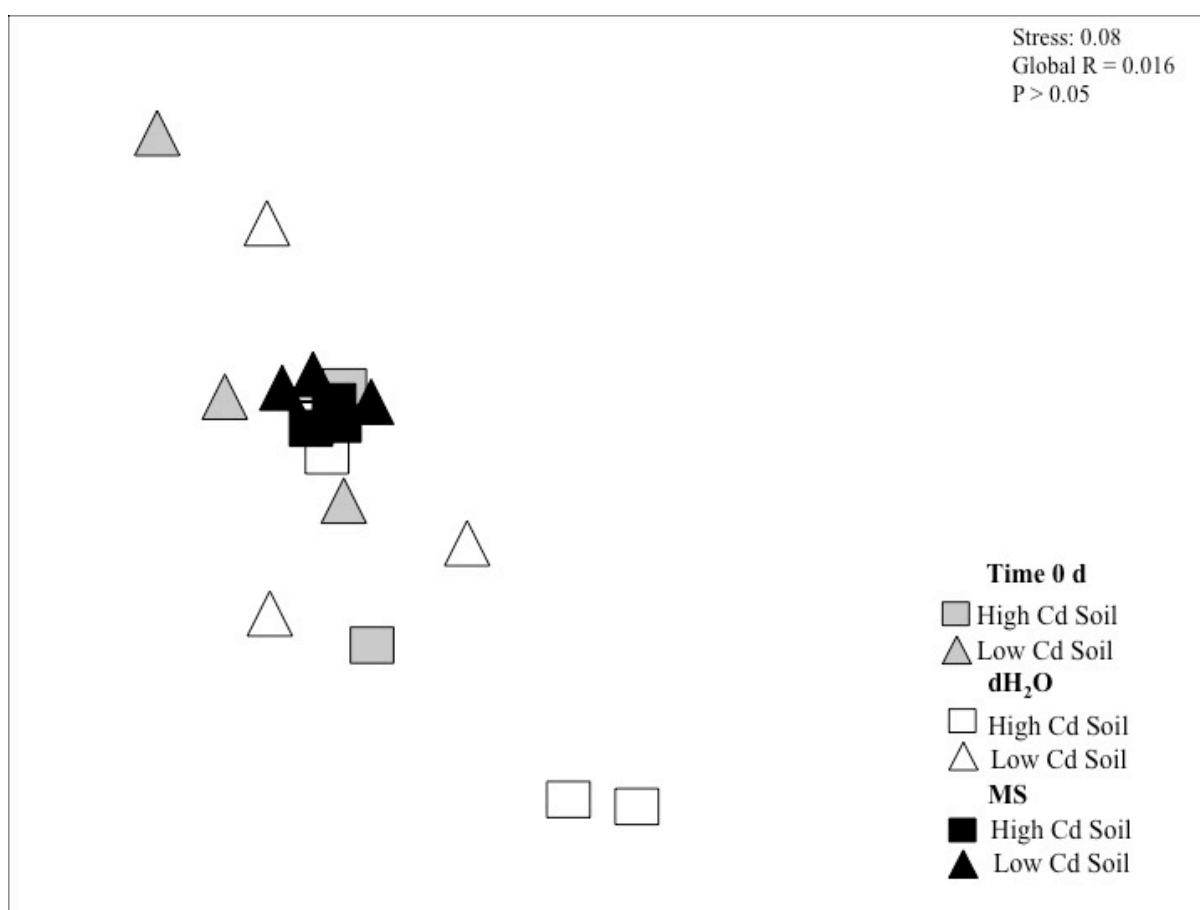


Figure B-1: NMDS ordination based on Bray-Curtis similarities of soil bacteria community TRFLP profiles between high and low cadmium soils at time 0 d or after 28 days maintained with either dH₂O or MS medium (ANOSIM, p > 0.05)

maintained with dH₂O and MS (ANOSIM, $p > 0.05$, $n = 3$), the communities in soil maintained with MS medium appear to be a nested subset of the contributors to the communities within soils given the dH₂O treatment. There was no difference between the two soil microbial community profiles at time 0 d.

Discussion

These results provide further evidence that MS medium is detrimental to bacteria growth and survival of some species of bacteria. In high cadmium soil, bacterial fragment richness was about three times higher after watering with RO water for a month compared to watering with MS medium. In addition, bacterial fragment richness was similar for both soil types watered with MS medium for 28 d. It is not clear why the bacterial richness in low cadmium soil at 28 d was not higher when given dH₂O but this may be related to the lower initial (0 d) bacterial richness in this environment. The concentration of phosphorus in the two soil types was balanced before the experiment. However, the difference in concentration of phosphorus in the two soils at the field site may have created an environment in which the low cadmium, and low phosphorus, soil had an impoverished microbial community compared to the soil in which phosphorus may have been limiting.

It is important to note that new species, or TRFLP fragments, as seen in the increase in fragment richness over time is not possible as no new species could be introduced to the system. However, increases in richness can be explained by the way fragments were analyzed. To differentiate ‘true peaks’ from background noise in the TRFLP profiles a minimum fluorescence threshold was set at 50 fluorescence units (Culman *et al.* 2008). It is possible that the environmental conditions in the growth chamber allowed for fragments from bacteria species that had a fluorescence signal below the threshold at 0 d to increase in abundance to a point where they produced a peak above this threshold at 28 d.

It can be seen in the NMDS plot that the bacteria communities maintained with MS medium appear to be a nested subset of the communities in the dH₂O treatments (Figure B-1). This most likely reflects the loss of species that are unable to grow in MS

medium and the corresponding loss of fragment peaks associated with those species. The greater similarity among samples maintained with MS medium is indicative of the decrease in bacterial species that were able to survive in this environment.

Recent research by Lau and Lennon (2011) has shown that soil with experimentally simplified microbial communities produced plants that are smaller, have reduced chlorophyll content, and produce fewer flowers compared to plants grown in soil with more complex microbial communities. This suggests that should there have been plants grown in the treatments described in this experiment, I would have seen reduced plant growth in the MS medium similar to that documented in Chapter 2.

Conclusions

It is obvious that the previously discussed phenomenon of MS medium reducing survival of *P. putida* UW4 (Chapter 2) was not due to agar or hydroponic effects and that a variety of other bacteria also appear to be negatively affected by this nutrient medium.

The fact that maintaining soil with MS medium results in changes to the bacteria community profile, and a reduction in the number of species able to grow in the soil, indicates that the conclusions from Chapter 2 are valid outside the system used.

References

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- Lau, JA, and Lennon, JT. 2011. Evolutionary ecology of plant-microbe interactions: soil microbial structure alters selection on plant traits. *New Phytologist* 192: 215-224

Curriculum Vitae

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Poster and Oral Presentations:

Columbus, M.P. and Macfie, S.M. 2012. *Plant Growth-Promoting Bacteria Appear to Become Deleterious in Growth-Limiting Conditions*. Canadian Society of Plant Biologists Eastern Regional Meeting. (**Oral presentation by M. Columbus – runner-up for President's Award**)

Columbus, M.P. and Macfie, S.M. 2012. *Plant morphological and physiological responses to toxic metal stress when grown in the presence of different plant growth-promoting bacteria*. Canadian Society of Plant Biologists Annual Meeting. (**Poster presentation by M. Columbus**)

Columbus, M.P., Macfie, S.M., and Southam, G. 2011. *The Use of Confocal Microscopy to Image and Quantify Bacteria-Root Associations*. Plant Canada 2011. (**Poster presentation by M. Columbus**)

Columbus, M.P., Macfie, S.M. and Southam, G. 2010. *The Use of Various Microscopy*

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Columbus, M.P., Silva, A.M., and Lefebvre, D.D. 2009. *The Use of Hybrid Poplar Trees in the Phytoremediation of a Contaminated Groundwater Plume Containing 1,4-Dioxane.* 19th Annual AEHS Meeting & West Coast Conference on Soils, Sediments, and Water. **(Oral presentation by M. Columbus)**

Columbus, M.P., Silva, A.M., and Lefebvre, D.D. 2009. *Phytoremediation of a Contaminated Groundwater Plume Containing 1,4-Dioxane and Other Organic Contaminants.* Ontario Centres of Excellence Centre for Earth and Environmental Technologies Student Symposium. **(Oral presentation by M. Columbus)**

Silva, A.M., **Columbus, M.P.**, and Lefebvre, D.D. 2008. *Phytoremediation of a Contaminated Groundwater Plume Containing 1,4-Dioxane and Other Organic Contaminants.* Eastern Regional Meeting of the Canadian Society of Plant Physiologists.

Columbus, M.P., Silva, A.M., and Lefebvre, D.D. 2007. *Characterization of Transpiration Rates of Poplar and Willow Trees.* Eastern Regional Meeting of the Canadian Society of Plant Physiologists. **(Poster presented by M. Columbus)**

Silva, A.M., **Columbus, M.P.**, and Lefebvre, D.D. 2007. *Phytoremediation of a Contaminated Groundwater Site.* Eastern Regional Meeting of the Canadian Society of Plant Physiologists.