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An Investigation of The Plant Growth Promoting Abilities of Pseudomonas Fluorescens UW4 Under Toxic Metal Stress

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Abstract

I investigated plant-microbe-metal interactions under metal stress. In theory, plant growth promoting rhizobacteria (PGPR) promote plant growth by reducing stress ethylene and synthesizing indole-3-acetic acid (IAA). The PGPR *Pseudomonas fluorescens* UW4 and a mutant strain that lacked an enzyme critical to the reduction of plant ethylene were studied to determine if they could promote *Arabidopsis thaliana* growth under cadmium and copper stress conditions. Both strains of *P. fluorescens* UW4 adhered to roots and synthesized IAA, and the wild-type lowered metal stress-induced ethylene in *Arabidopsis*, but neither strain enhanced plant growth. Wildtype *P. fluorescens* UW4 and its mutant had no effect on altering the concentrations of other plant stress hormones with the exception of salicylic acid under copper stress. More work is needed to determine why *P. fluorescens* UW4 did not promote growth under metal stress conditions before it can be utilized in agricultural settings.

Keywords

Arabidopsis, Pseudomonas fluorescens UW4, Plant-microbe interactions, Plant growth-promoting rhizobacteria (PGPR), Cadmium, Copper, Ethylene, IAA

Co-Authorship Statement

This thesis includes four manuscripts. The manuscripts entitled “An analysis of the adhesiveness of *Pseudomonas fluorescens* UW4 to different substrates.” (Chapter 2) and "An investigation into the IAA hypothesis: Can the IAA produced by the PGPR *Pseudomonas fluorescens* UW4 increase plant growth?" (Chapter 4) will be submitted as notes to the *Canadian Journal of Microbiology*. The manuscripts entitled " How do we grow: Can *Pseudomonas fluorescens* UW4 increase the growth of *Arabidopsis* under cadmium stress in agar, hydroponics and Promix-BX?" (Chapter 3) and " The ethylene hypothesis: Can *Pseudomonas fluorescens* UW4 promote plant growth by reducing stress ethylene and influence other plant hormones in *Arabidopsis* under cadmium and copper metal stress?" (Chapter 5) will be submitted to *Plant Methods* and *Canadian Journal of Microbiology* respectively.

I will be the first author on all four of these publications, which will be co-authored with Dr. Sheila M. Macfie (supervisor). I designed and conducted the experiments, collected and analyzed all of the data, and will write the manuscripts. Dr. Macfie provided laboratory support, guidance in experimental design and data interpretation, and she will assist with manuscript preparation. Shagana Balasubramaniam will also be a co-author on the manuscript arising from Chapter 2, as she helped take confocal images and generate bacterial cell counts based on those images. Dr. Mamdouh Abou-Zaid will also be a co-author on the manuscripts arising from Chapters 4 and 5 because he helped to generate and interpret all of the HPLC data.

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List of Abbreviations

αKB – Alpha-ketobutarate

ABA – Abscisic acid

abu – Arbitrary unity

ACC – 1-aminocyclopropane-1-carboxylic acid

acdS – ACC deaminase enzyme

acdS – ACC deaminase gene

acdS^{-} - Nonfunctional ACC deaminase enzyme

AcN – Acetonitrile

ANOVA – Analysis of variance

ATP – Adenosine triphosphate

bdl – Below detection limit

ΔΔCq – Difference in expression of two genes (control - target gene) based on their cycle thresholds

cDNA – Complementary deoxyribonucleic acid

Col-0 – Columbia ecotype of *Arabidopsis*

d – Day

D0 – Inoculation as a seed (i.e on day 0)

D5 – Inoculation as a 5 day old seedling

DF – Dworkin and Foster

dNTP – Deoxynucleotide triphosphate

ESI – Electrospray Ionization

F – Forward primer

GC-FID – Gas chromatography-flame ionization detector

h – Hour

HCD – High energy collisional dissociation

HPLC-MS – High performance liquid chromatography- mass spectrometry

IAA – Indole-3-acetic acid

ICP-MS – Inductively coupled plasma- mass spectrometry

I.D. – Internal diameter

JA – Jasmonic acid

min – Minute

MS – Murashige and Skoog

m/z – Mass divided by charge number

OD_{600} – Optical density measured at a wavelength of 600 nm

PGPR – Plant growth promoting rhizobacteria

ppm – Parts per million

qPCR – Quantitative polymerase chain reaction

R – Reverse primer

RNA – Ribonucleic acid

rpm – Revolutions per minute

RT-PCR – Reverse transcription polymerase chain reaction

SA – Salicylic acid

SAM – S-adenosylmethionine

TET – Tetracycline

TSB – Tryptic soy broth

Chapter 1

1 General Introduction

1.1 Overview

Environmental degradation, climate change, extreme weather events and human population growth have and will continue to put a strain on global food production and the environment. With the human population currently surpassing 7 billion people and with projections of 10 billion people in the next 50 years (United Nations Dept. of Economics and Social Affairs, 2015), our current agricultural output may not be enough to sustain this population size . Providing sufficient food as well as cleaning up the environment in which crops grow is not an easy task to accomplish and achieving it will take time, money and new approaches. In the short term, food production can be increased by the use of fertilizers, pesticides, herbicides and selection of high-yielding cultivars. However, many of these options are not environmentally friendly as they can introduce or increase the concentration of toxic substances, including cadmium and copper, in the soil. Toxins can be taken up by plants, leading to reduced growth and the potential for these toxic substances to enter the food chain (Seenivasan *et al*., 2016).

A more sustainable and environmentally friendly solution to the problem of increasing plant growth and crop yields could be the expanded use of plant growth-promoting microorganisms. The use of plant growth-promoting microorganisms in agriculture, as well as in phytoremediation (the use of plants to take up toxic compounds from the environment), has become a very attractive technology and area of research as it has the potential for increasing plant growth and food production without the environmental impacts of current agronomic practices. We have an excellent understanding of some ways microbes interact with and benefit plants; for example, providing plants a usable source of nitrogen (ammonia) through nitrogen fixation by *Rhizobium* spp. bacteria (Pagan *et al*., 1975) and increasing nutrient availability to the plant by mycorrhizae (Marschner and Dell, 1994). However, fundamental mechanisms

used by other microbes that improve plant growth such as altering the concentrations of plant stress hormones are not as well understood (Glick, 2012). Additional understanding of the mechanisms microbes use to increase plant growth will likely hasten the acceptance of these organisms as suitable and effective components of agricultural practice. Therefore, it is imperative that researchers elucidate how these microbes promote plant growth, how these microbes affect plant physiology, whether or not these plant growth-promoting microbes can increase plant growth under stress conditions and how these microbes impact the environment and soil microflora.

1.2 Cadmium and copper pollution as a human health and environmental issue

Some metals are required by living organisms in small concentrations. Metals such as iron, copper and zinc are utilized by enzymes (often referred to as metalloenzymes) as cofactors, which can serve as electron donors or acceptors, structural regulators, and can help catalyze enzymatic reactions (Riordan, 1977; Gamalero *et al*., 2009). However, at high concentrations these same metals, as well as other non-essential metals such as lead, mercury and cadmium, can be detrimental to the health of organisms. When concentrations of toxic metals increase within the organism, they disrupt many physiological and biochemical processes such as altering enzyme function and generating reactive oxygen species which can cause DNA damage and disrupted cell membranes (Mithofer *et al*., 2004; Khan *et al*., 2009; Han *et al*., 2014; Jakovljević *et al.*, 2014).

Two metals of current environmental concern are cadmium, a non-essential toxic metal, and copper, an essential micronutrient that is a component of many proteins (Demirevska-Kepova *et al*., 2004). Excess cadmium and copper can reduce plant growth, potentially leading to decreased agricultural yields (Bankaji *et al*., 2014). Both of these metals are released from industry, mining and combustion of fossil fuels as well as through agricultural production inputs in the form of fertilizers and herbicides (Das *et al*., 1997; Sheppard *et al,* 2007; Atafar *et al*., 2010; Grant *et al.*, 2011; Shaltout *et al*., 2015).

For example, phosphate fertilizers can contain up to 340 mg/kg of cadmium (Alloway and Steinnes, 1999) or 182 mg/kg (de López Camelo *et al*., 1997) of copper, depending on the source of the phosphate rock used.

In addition to the negative effects on agriculture and the environment, the potential for the accumulation of cadmium and copper within food crops can pose a risk to public health. Cadmium is a carcinogen and acute or chronic exposure can lead to pulmonary irritation, kidney disease, and developmental abnormalities (US EPA, 2000). Chronic exposure to high concentrations of copper can lead to brain and kidney damage (Brewer, 2011). Given current agricultural practices and the potential impact these two metals have on human health, cadmium and copper are two very important toxic metals to study.

1.3 Plant response to metal stress

1.3.1 Effects of cadmium and copper on plant physiology

The toxic effects of cadmium on biological systems are numerous, species-specific and can be seen at very low concentrations (Das *et al.*, 1997; Clemens, 2006). The toxic form of cadmium is the Cd^{+2} ion. Plants grown in the presence of cadmium often exhibit chlorosis, leaf rolling, growth reduction and necrosis (Xue *et al*., 2013). In plants, cadmium disrupts the uptake and transport of iron, potassium, calcium, and phosphorus, leading to deficiencies in these nutrients and resulting in stunted growth (Das *et al.*, 1997). Cadmium is a potent competitive inhibitor of enzyme cofactors, such as zinc, in both the mitochondria and in the cytosol, thus decreasing the efficiency of energetic pathways and leading to decreased growth (Silverberg, 1976; Das *et al*., 1997). Further, cadmium disrupts mitosis by causing DNA damage and altering chromosomal structures, thereby preventing proper mitotic segregation (Rosas *et al*., 1984; Das *et al*., 1997). Cadmium also reduces and disrupts photosynthesis by inhibiting chlorophyll biosynthesis (Baryla *et al*., 2001), Rubisco activity, and the enzymes of the Calvin Cycle (Krupa *et al*., 1993; di Cagno *et al*., 1999).

The essential micronutrient copper plays an important role in carbon fixation and ATP synthesis. Copper is an essential component of plastocyanin in the photosynthetic system and cytochrome oxidase in the respiratory electron transport chain (Demirevska-Kepova *et al*., 2004). Although copper is an essential nutrient in plants, high concentrations of the Cu^{+2} ion can induce plant stress leading to reduced plant growth and crop yields. Lewis *et al*. (2001) showed that plants grown the presence of toxic copper concentrations exhibited leaf chlorosis as well as reduced growth. Furthermore, excess copper within plant tissues can lead to increased generation of reactive oxygen species through Haber-Weiss reactions and this oxidative stress can lead to damage to proteins, lipids and nucleic acids (Stadtman and Oliver, 1991; Hegedus *et al*., 2001). Excess amounts of copper have also been shown to affect photosynthesis by interacting with photosystem II reaction centres, thereby preventing the reduction of plastoquinone (Cid *et al*., 1994; Kupper *et al*., 2002). Toxic concentrations of copper have also been linked to decreases in ATP production as well as damage to the structure and function of chlorophyll (Cid *et al*., 1994; Kupper *et al*.; 2002).

It is clear that studying how to reduce the effects that cadmium and copper have on plant physiology is imperative in order to maximize plant growth potential.

1.3.2 Ethylene stress response

The production of ethylene is a common response seen in 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 the defense response to pathogens (Taiz and Zeiger, 2010). When plants are grown in stressed environments, higher concentrations of ethylene are produced by the oxidation of 1-amino-cyclopropane-1-carboxylate (ACC) by the enzyme ACC oxidase (Glick *et al*., 1998; Gamalero *et al*., 2009; Glick, 2014). This pathway is discussed in more detail in section 1.4.3. The ethylene stress response occurs in two phases. The first phase produces a small amount of ethylene, which is considered to be a protective response that triggers

the expressions of defense genes and synthesis of molecules combating the stress such as antioxidative enzymes and phytochelatins (Ciardi *et al*., 2000; Robison *et al*., 2001; Stearns and Glick, 2003; Jakovljević *et al*., 2014). If stress signals are still being transmitted a few hours to a few days after the initial ethylene response, a second much larger peak occurs, due to both increased ACC synthase and ACC oxidase induction, resulting in an accumulation of ethylene (Robison *et al*., 2001; Stearns and Glick, 2003). This accumulation of ethylene initiates processes such as senescence and chlorosis, which are inhibitory to plant survival (Robison *et al*., 2001; Stearns and Glick, 2003). Toxic metal exposure, such as exposure to cadmium in concentrations above 1 μM, have been shown to stimulate stress ethylene production in *Phaseolus vulgaris* leaf tissue (Fuhrer, 1982). A 15- to 30-fold increase in ethylene production has also been documented in *Spirodela oligorrhiza* when grown in the presence of 20 μM copper (Matto *et al*., 1986).

1.3.3 Plant tolerance to cadmium and copper stress

Physical barriers in the root are the first line of defense to toxic metals in (Figure 1.1A). Thick cuticles and plant cell walls, can also act as physical barriers to toxic metal stress. Plant trichomes, tiny hair-like structures on the leaf surface, can store toxic metals away from metabolic activity as well as produce secondary metabolites for detoxification (Hauser, 2014). For example, plants grown in excess cadmium or copper have been shown to have higher concentrations of cadmium and copper within cell wall complexes when compared to the cytoplasm (Iwasaki *et al*., 1990; Parrotta *et al*., 2015). Furthermore, plants can also immobilize toxic metals in soils by exuding compounds, such as organic acids, which bind to the metals making them less biologically available (Costa *et al.* 1997; Figure 1.1A). These and other exudates can also increase soil pH, which reduces metal solubility (Yang, *et al.* 2001). Once metals get past the plant's physical and external chemical defenses and enter into cells, biosynthesis of a suite of metal detoxifying and tolerance molecules is induced (Figure 1.1B). Induction of metallochaperones such as nicotianamide or glutathione, which form chelate complexes with metal ions, can prevent metals from interacting with enzymes and generating reactive oxygen species (Foyer and Noctor, 2005; Rausch *et al*., 2007). Metal

accumulation within plant tissues also has been shown to induce the hormones salicylic acid, jasmonic acid, and ethylene, which in turn stimulate the production of other metal-binding proteins such as metallothioneins and phytochelatins, thus altering plant physiology to combat the metal stress (reviewed in Vienweger, 2014, Figure 1.1B). Plants also can store chelated toxic metals into non-metabolically active sites, such as the vacuole, where they are no longer a threat to plant physiology and thus growth (Salt and Rauser, 1995).

1.4 Plant-microbe interactions

The rhizosphere is the zone of soil that is under the influence of plant root exudates, which can generate favorable conditions for the growth of microorganisms (Egamberdieva, *et al*., 2008). It has been well documented that rhizospheric microorganisms can dramatically impact the growth and survival of plants (Glick, 2012; 2014). Environmental and soil conditions will dictate the extent of microbial diversity in the rhizosphere as well as how plants will interact with these microbes.

1.4.1 Plant influence on rhizobacteria

In general, carbon sources within soils are relatively low leading to restricting the density of microbes. However, plants are able to exude up to 40% of their photosynthates into the surrounding soil (rhizosphere), which results in increased microbial growth (Bais, *et al*., 2006). Furthermore, the organic acids exuded by plants that impact metal availability in the rhizosphere can also, in combination with exuded amino acids, help to promote bacterial growth and diversity within the rhizosphere. For example, Rudrappa *et al.* (2008) demonstrated that *Arabidopsis* can exude malic acid to recruit and promote the growth of the beneficial bacterium *Bacillus subtilis*. Furthermore Kamilova *et al*. (2006) determined that *Solanum lycopersicum* (tomato) exuded citric acid, succinic acid and malic acid, which directly alter the bacterial community around its roots. Taken together, this indicates that plants have a profound influence on the makeup and growth of bacterial communities within the rhizosphere.

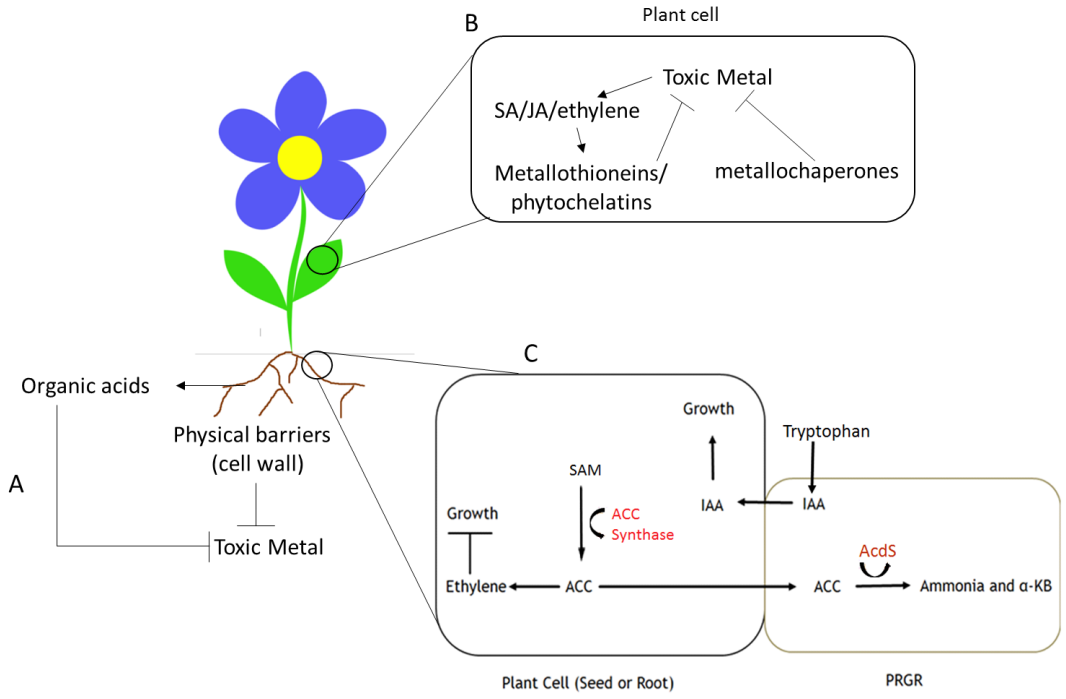

Figure 1.1 Plant metal defense and PGPR model. Plant primary defenses against toxic metal stress includes the use of physical barriers such as cell walls and trichomes and or the exudation of organic acids to bind to the toxic metal preventing plant uptake (A). Plant secondary defenses against toxic metal stress include chelation of intracellular toxic metal ions via metallochaperones or metallothioneins, preventing the toxic metal ion from causing cellular damage (B). External defense as explained by a model for how PGPR (plant growth-promoting rhizobacteria) could directly stimulate plant growth under stress conditions by producing IAA and/or reduce the plant stress response by metabolizing plant ACC using ACC deaminase and preventing the build-up of ethylene (C). Based on Glick *et al.* (1998); Rausch *et al.* (2007); Hauser (2014); Vienweger (2014); Parrotta *et al.* (2015).

1.4.2 Rhizobacterial influence on plants

There are three types of bacteria that can associate with plants: deleterious (pathogenic), beneficial, and neutral. The easiest of the three groups of bacteria to understand are the neutral bacteria as these do not impact plant growth or induce any deleterious effects and are thus not widely reported within the literature. Deleterious bacteria such as *Phytophthora cinnamomi* (Gotesson *et al.* 2002) and *Pseudomonas syringae* (Bashan and de-Bashan, 2002) can secrete phytotoxic metabolites that inhibit seed germination and reduce plant growth. Bacteria also have the ability to produce

chelating molecules and degrade organic acids released by plants; this makes metals more readily available for plants to take up and thus reduce growth (Cole, 1979; Bollag and Czaban, 1989; Glick, 2014). Furthermore, under nutrient limiting conditions, soil bacteria can directly compete with plants for nutrients resulting in a potential decrease in plant growth and crop yields (Whipps, 2001). While some bacteria can have negative impacts on plants, there are a variety of beneficial impacts that bacteria can have on plant growth under both stress and non-stress conditions. The impacts that beneficial bacteria have on plant growth will be discussed in the following section (1.4.3).

1.4.3 Plant growth-promoting rhizobacteria

Kloepper and Schroth (1978) first defined plant growth-promoting rhizobacteria (PGPR) as beneficial root-colonizing bacteria that enhance the growth of plants. Since then, many PGPRs have been isolated from soils and plant roots and have been shown to improve plant vigor, growth and crop yield (Ahemad and Kibret, 2014). However, as with plants, microbes including PGPRs are also susceptible to metal toxicity. In rhizospheric microbes, toxic metals such as cadmium and copper can cause protein denaturation, inhibit cell division, disrupt cell membranes, inhibit enzyme activity, cause DNA damage, as well as inhibit transcription (reviewed in Khan *et al*., 2009). Moreover, Giller *et al.* (1998) have shown that the presence of toxic metals reduces microbial growth and diversity within the rhizosphere.

Some of the ways in which PGPRs are able to promote plant growth include 1) fixing atmospheric nitrogen by converting diatomic nitrogen into ammonia and exuding it into the rhizosphere, which provides plants with a usable nitrogen source (Glick, 2012); 2) producing siderophores, which increase iron absorption and availability to both plants and bacteria when iron is limiting (Kloepper *et al*. 1991); 3) synthesizing and exuding the plant growth-promoting phytohormone indole-3-acetic acid (IAA; Glick *et al*., 1998); and 4) synthesizing the enzyme ACC deaminase, which is thought to play a role in the reduction of the stress response by reducing stress ethylene within plants by metabolizing the ethylene precursor ACC (Glick *et al*., 1998). PGPRs are also thought to promote plant

growth indirectly via out-competing pathogenic microbes for nutrients (Walsh *et al.* 2001) as well as by the formation of biofilms that can prevent pathogen and toxic metal entry into root cells (Stout *et al.* 2010).

Glick *et al*. (1998) proposed a model to explain how PGPR might reduce plant stress by producing both IAA and the enzyme ACC deaminase, and how these two molecules might interact to reduce plant ethylene under stress conditions (Figure 1.1C). The current hypothesis is that PGPRs synthesize IAA from tryptophan and exude IAA into the rhizosphere, which can be imported into the plant root where it either stimulates ACC synthase to convert S-adenosylmethionine to ACC or directly stimulates plant growth (Glick *et al*., 1998). The increased ACC produced from both the perceived stress and IAA-stimulated ACC synthase is removed from the plant to the bacteria where it is metabolized by the bacterial enzyme ACC deaminase into alpha-ketobutyrate and ammonia, the latter of which is used as a nitrogen source (Honma and Shimomura, 1978; Glick *et al*., 1998; Penrose and Glick, 2003). In effect, the PGPRs' ACC deaminase acts as an external sink for plant-produced ACC, thereby reducing the amount of ACC available to be converted into ethylene within the plant and reducing ethylene concentrations in stressed plants to near basal levels (Glick *et al*., 1998; Stearns and Glick, 2003).

Once stress ethylene is reduced, plants are able to continue growth as if the stress was not present. The ability of PGPRs to increase growth of plants under stress conditions has been well established. For example, cadmium-stressed *Brassica napus* (rapeseed) treated with an IAA-producing bacteria had increased root growth by as much as 97%, suggesting that bacterial-produced IAA can stimulate growth and increase plant tolerance to cadmium (Dell'Amico *et al.*, 2008). It has also been shown that ACC deaminase plays a role in increasing plant growth. *Escherichia coli* cells that expressed a cloned *Enterobacter cloacae* ACC deaminase coding gene increased root elongation in canola (*Brassica* sp.) by 31% in non-stressed conditions (Shah *et al*., 1998). Hontzeas *et al.* (2004) showed that canola treated with an ACC deaminase-producing PGPR had an 82% increased root length when compared with either the negative control or an ACC deaminase knockout mutant bacterium.

1.4.4 The PGPR *Pseudomonas fluorescens* UW4

Since the discovery of the enzyme ACC deaminase in soil bacteria by Honma and Shimomura (1978), many more PGPR with ACC deaminase activity have been identified and tested to determine if they reduce plant stress responses (Glick, 2005; Glick *et al*., 2007; Khan *et al*., 2009). One ACC deaminase- and IAA-producing PGPR is the UW4 strain of *Pseudomonas fluorescens*, which has been studied for its ability to reduce plant stress and has been used to determine a link between ACC deaminase and ethylene production (Grichko and Glick, 2001; Reed *et al*., 2005; Farwell *et al.*, 2007; Grurska *et al*., 2009).

This bacterium was classified as *Pseudomonas putida* in 2005 based on 16S rRNA gene sequencing **(**Hontzeas *et al.* 2005) and *Enterobacter cloacae* before that based on fatty acid profiles (Shah *et al.* 1998). In 2013, *P. putida* was reclassified as *P. fluorescens* based on whole-genome comparisons and four "housekeeping" gene-based phylogenies (Duan *et al.*, 2013).

Pseudomonas fluorescens is a gram-negative, aerobic, rod shaped fluorescent (when iron is limiting) bacterium in the Pseudomonadaceae that is normally isolated from soil and water and has an optimum growth temperature between 25 – 30ºC (Meyer and Abdallah, 1978; Palleroni, 1984). *P. fluorescens* UW4 was isolated near Waterloo, Ontario (Hontzeas *et al.*, 2005). A mutant strain of *P. fluorescens* UW4 has been created to test the impacts of bacterial ACC deaminase and IAA on plant growth and ethylene production in stressed environments. This mutant strain was produced by an insertion of a tetracycline (TET) resistance gene within the coding region of the ACC deaminase gene rendering the enzyme non-functional, but not affecting the production of IAA (Li *et al*., 2000).

1.5 Rationale and research objectives

Understanding how PGPRs increase plant growth and reduce plant stress will not only help increase our knowledge of the physiology and biochemistry of plant-microbe

interactions, but also help increase the efficacy and use of PGPRs for bioremediation and agricultural practices. Advancements in this field could help us understand what growth-promoting properties of PGPRs are more essential for plant growth as well as tease apart the complexity of how PGPRs alter plants at the biochemical, physiological, and ecological levels.

The primary goal of this research is to understand the relative roles of bacterial ACC deaminase and IAA in maintaining plant growth during metal stress. Both cadmium and copper will be used to induce plant stress because they are anthropogenic pollutants that decrease plant growth and induce ethylene production (Guerra *et al*., 2009; Zheng *et al.*, 2013). I chose the PGPR *P. fluorescens* UW4 because it can synthesize IAA from tryptophan and it contains the enzyme ACC deaminase. Moreover, there is a mutant strain with a non-functional ACC deaminase gene that retains IAA production (Li *et al*., 2000). By comparing plants inoculated with the mutant strain to those with the wildtype strain, I can evaluate the independent impacts of bacterial ACC deaminase and IAA on plant growth and ethylene production. *Arabidopsis thaliana* ecotype Columbia (Col-0) was chosen as a plant model because the roles of ethylene and IAA on growth have been well studied within this species and there are a wide variety of ethylene and IAA mutants available. *A. thaliana* is a member of the Brassicaceae (mustard) family, and was the first plant for which the complete genome was sequenced.

In Chapter 2, I will determine if bacteria are physically interacting with plant roots and whether or not this adherence is based on the intrinsic qualities of the root. I will also test whether cadmium has any effect on bacterial adherence.

In Chapter 3, I will test various parameters to determine the best conditions in which to test plant-microbe-metal interactions. To do this, I will determine the concentration of plant medium needed for plants to have healthy growth when placed in medium alone, but reduced growth when placed in cadmium-contaminated medium. I will also look at the best protocol to inoculate and grow *Arabidopsis* with *P. fluorescens* UW4 in order to have healthy controls and see growth promotion in the presence of cadmium. Finally I

will assess the growth promoting capacities of *P. fluorescens* UW4 in agar, hydroponics and Promix-BX.

In Chapter 4, I will test the IAA mechanism proposed in Figure 1.1C by first determining if *Arabidopsis* can exude tryptophan under metal-stressed and non-stressed conditions. I will also determine if *P. fluorescens* UW4 can synthesize IAA from tryptophan levels similar to those exuded by *Arabidopsis*. Finally, I will test whether the amount of IAA synthesized by the bacteria is enough to promote plant growth by supplementing *Arabidopsis* with an IAA concentration similar to the amount the bacteria synthesize.

In Chapter 5, I will test the ethylene mechanism in Figure 1.1C by confirming that *P. fluorescens* UW4 can express the ACC deaminase gene. I will also test whether this bacteria can reduce cadmium and copper induced stress ethylene as well as promote growth in *Arabidopsis*. To further test the potential ethylene reducing capabilities of this bacteria, I will determine whether *P. fluorescens* UW4 can reduce ethylene in ethylene over-producing *Arabidopsis* mutants. Lastly, I will look at whether *P. fluorescens* UW4 increases or decreases the concentration of other plant hormones, such as salicylic acid, which may allow me to expand the model proposed by Glick *et al.* (1998).

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Chapter 2

2 An analysis of the adhesiveness of *Pseudomonas fluorescens* UW4 to different substrates.

Within this chapter, the adherence of the PGPR *Pseudomonas fluorescens* UW4 to various substrates, including roots, will be examined. I will also test whether the toxic metal cadmium affects this adherence. My initial goal for these experiments was to determine whether or not the bacteria are adhering to plant roots due to something the plant is exuding or whether it is an intrinsic property of the root (or substrate).

2.1 Introduction

It has been well established that soil microbes can interact with and promote the growth of plants (Glick *et al.*, 1998; Grichko and Glick, 2001; Shim *et al.*, 2015). Of the different types of soil microbes, bacteria with the ability to promote plant growth and reduce plant stress (e.g., drought, salt, toxic metal) have been of great interest. In particular, studying bacteria that can mitigate toxic metal stress, such as cadmium, in plants is imperative since toxic metals are released from industry and can accumulate in agricultural soils, reducing crop growth and yield (Das *et al*., 1997; Bankaji *et al*. 2014). Various mechanisms have been proposed to explain how bacteria are able to promote plant growth. These include increasing nutrient availability to plants (Dixon and Kahn, 2004), producing plant growth hormones (Costa *et al*., 2014), or reducing plant stress hormone levels by metabolizing stress hormone precursors (Glick *et al*., 2007). However, how these plant growth-promoting bacteria find and adhere to plant roots is still hotly debated.

Most soil bacteria are free-living and it has been shown that plant roots release compounds into the surrounding medium to help establish a hospitable environment for bacterial growth and colonization. Plants are known to exude sugars, amino acids, organic acids and hormones into the rhizosphere; with some studies indicating that up to

40% of photosynthates are exuded by roots (Bertin *et al.*, 2003; Bais *et al*., 2006). The exudation of these compounds may provide a chemical gradient extending from roots, which bacteria utilize through chemotaxis to locate and colonize the rhizosphere (Sood, 2003). Moreover, plant exudates may initiate bacterial colonization of roots by inducing the expression of substrate-adherence proteins such as adhesin or agglutinin (Buell and Anderson, 1992; O'Gara and Humphreys, 2001; Gotz, 2002). This may enable the bacteria to adhere to roots or soil particle surfaces in order to utilize the exuded carbon and nitrogen resources and establish a colony.

There are many physical and biological theories about how bacteria interact and adhere to plant surfaces; however, no one theory universally explains how bacterial adherence occurs. Some examples of theoretical mechanisms behind bacterial-substrate interactions include: a net balance between electrostatic attractive and repulsive forces between the cell and the substrate (Katsikogianni *et al*., 2004), a negative surface free energy of the bacterial-substrate interaction causing a spontaneous bacterial interaction with the substrate (Morra and Cassinelli, 1997), and bacterial-substrate hydrophobic and hydrophilic interactions (Jucker *et al.*, 1998; Hermansson, 1999). Furthermore, substrate composition (Silver, 2003; Whitehead *et al.*, 2004), roughness and configuration (Scheuerman *et al.*, 1998) play vital roles in how bacteria can interact and adhere to the substrate. Lastly, the characteristics of the bacteria such as bacterial surface charges (Katsikogianni *et al*., 2004) and hydrophobicity (Vacheethasanee *et al.*, 1998), influence whether or not a given bacterium will be able to interact with a given substrate.

Some biological theories to explain how bacteria adhere to plant roots and other substrates have been put forth. One of these theories proposes that lectins present on the root, leaf or seed surface act as a receptor for bacterial polysaccharides (Hirsch, 1999). It is also been determined that calcium plays a role in bacteria-substrate binding and has been demonstrated to be important in bacterial adherence to pea roots and glass (Smit *et al*., 1987). In particular, the calcium-binding protein rhicadhesin is secreted by bacteria and utilizes calcium ions to anchor itself to the bacterial cell surface and enable bacteria to adhere to surfaces (Smit *et al*., 1987). It is also been postulated that root tips may

contain receptors that recognize and bind to specific carbohydrate structures that are present on the bacterial surface (Matthysse and Kijne, 1998). These three main biological mechanisms of bacterial adherence, plant lectins, rhicadhesin, and plant receptors, are thought to be required for the initial steps needed for bacterial adherence to plant tissue and potentially other substrates. It has also been proposed that, after the initial adherence has occurred, the bacteria need to produce a more permanent association with the material and most likely will start the synthesis of pili or fimbria to achieve this (Vesper and Bauer, 1986; Smit, 1987). It is also been shown that some bacteria in the rhizosphere will produce bacterial cellulose fibrils to allow for permanent association with the plant (Robertson et. al., 1988).

Clearly, there are many variables involved in bacterial-substrate interactions; however, more work is needed in order to understand how stress conditions such as toxic cadmium stress will impact putative plant growth-promoting rhizobacteria (PGPR) adherence to roots as well as determine which potential mechanisms each PGPR utilizes to adhere to roots before they can be approved for agricultural use. This study tested whether adherence of the PGPR *Pseudomonas fluorescens* UW4 to the roots of *Arabidopsis thaliana* ecotype Columbia (Col-0) requires something produced (exuded) by living plant roots or if the bacteria adhere to inert substrates with physical characteristics similar to roots. To determine this, four different substrates (root, cotton, polyester and nylon) were inoculated with *P. fluorescens* UW4, or a mutant bacterium that has a non-functional 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzyme (plant ethylene reducing enzyme). After a period of incubation, confocal microscopy was used in order to count them. This study determined whether the toxic metal cadmium had any effect on the binding of *P. fluorescens* UW4 to the various substrates by having each inoculated substrate placed in cadmium-laced or cadmium-free medium. I hypothesized that *P. fluorescens* UW4 adherence to plant roots is independent of plant exudates and depends on the physical characteristics of the root itself. I also predicted that 1) *P. fluorescens* UW4 would adhere, to some extent, to all substrates, 2) that the presence of cadmium would have no effect on the ability of the bacterium to adhere to root since this

bacterium has been shown to promote growth in plants grown in cadmium contaminated media (Dell'Amico *et al*., 2008).

2.2 Methods

2.2.1 Bacterial strains and culture maintenance

Both bacterial strains, *P. fluorescens* UW4 and its ACC deaminase (acdS) mutant *P. fluorescens* UW4-*acdS*$^{-}$, were provided by Dr. Bernard Glick from the University of Waterloo. The ACC deaminase mutant was generated by an insertion of a tetracycline resistance gene within the coding region of the ACC deaminase gene rendering the enzyme non-functional (Li *et al*., 2000). This bacteria was initially classified as *Enterobacter cloacae* then reclassified as *P. putida* **(**Hontzeas *et al.*, 2005) and reclassified again as *P. fluorescens* based on whole-genome comparisons (Duan *et al.*, 2013).

Bacterial cultures were stored in 15% glycerol at -80°C Bacterial cultures were maintained according to Penrose and Glick (2003). Cultures were taken out of storage and streaked onto agar plates that contained tryptic soy broth (TSB; VWR Canada) and 1% bacteriological grade agar (BioShop Canada). Due to the tetracycline resistance gene in the mutant, it is necessary to grow the mutant in the presence of tetracycline (Li *et al*., 2000), thus 15 µg/mL tetracycline (BioShop Canada) was added to the TSB-agar medium for the mutant *P. fluorescens* UW4. Plates were incubated at 30°C for 24 h. A streak of bacterial colonies from each plate was transferred into 5 mL sterile TSB (with tetracycline 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. Liquid TSB cultures were then centrifuged at 2550 × g for 10 min at 4°C and washed twice with 5 mL DF salt minimal medium without nitrogen (Dworkin and Foster 1958). Bacterial cells were then re-suspended in 7.5 mL of DF salt minimal media without nitrogen, placed in an incubated shaker at 30°C and 200 rpm for 24 h. Overnight cultures were centrifuged at 2550 × g for 10 min at 4°C and the supernatant 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 0.1 (based on preliminary test; Figure B1) before inoculating plant seeds or other substrates.

2.2.2 Inoculation of materials

Arabidopsis thaliana (ecotype Col-0) seeds and 2 cm pieces of cotton, polyester and nylon thread were surface-sterilized by first soaking in 70% ethanol for 5 min (min), then in 1.5% sodium hypochlorite for 10 min followed by 3 rinses in sterile deionized water. Seeds were then stored at 4°C in the dark for 3 d to force synchronized germination prior to planting. The other materials were similarly treated. Seeds and threads were then inoculated with either *P. fluorescens* UW4, mutant *P. fluorescens* UW4 or 0.03 M $MgSO_4$ (control) for 1 h. Then seeds and threads were transferred onto 0.8% agar plates (one substrate per plate) containing 80% Murashige-Skoog (MS) medium with 1% sucrose, adjusted to pH of 5.8, with 0 or 20 µM cadmium dichloride ($CdCl_2$) (based on a preliminary study to determine the amount of $CdCl_2$ needed to induce stress in *Arabidopsis*; n= 3, Figure B2A). Sterile 2.5 cm^2 pieces of fiberglass window screen (Easy Screen, RCR International Inc, Quebec) were placed on the agar before the seeds or threads were added to act as a support matrix. Plates were sealed with Parafilm® and placed in a growth chamber (16:8 h light: dark cycle maintained at 22°C and 60% relative humidity) and for 7 d or until the plant shoots were larger than the holes in the screen. Autoclaved forceps were then used to transfer the screens holding the seedlings or threads into sterile glass jars containing 10 mL 80% MS medium, 1% sucrose, pH 5.8 and 0 or 20 µM $CdCl_2$. The openings of the jars were sealed with a foam stopper to allow for gas exchange as well as to prevent contamination. The plants and bacteria were allowed to continue to grow for another 7 d, at which time the materials were harvested and prepared for microscopy.

2.2.3 Confocal microscopy and bacterial adherence

Inoculated 14 d old plants and cotton, polyester and nylon threads in media containing either 20 µL cadmium chloride or no cadmium were stained using the

Live/Dead® BacLightTM Bacterial Viability Kit (Invitrogen, Cat# L7012), which uses the green fluorescent stain SYTO® – 9 (to stain living bacteria) and the red fluorescent stain propidium iodide (to stain dead bacteria). The staining kit was used following the manufacturer's instructions with slight modifications. In a sterile microcentrifuge tube, 3 µL each of SYTO® – 9 and propidium iodide were mixed into 1 mL of sterile reverse osmosis (RO) water and vortexed. After incubation in the dark at room temperature for 5 min, 200 µL the staining solution was added to microcentrifuge tubes containing a substrate 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 roots and threads were dipped in 3 successive rinses of sterile RO water in order to avoid removing loosely bound bacteria. Roots and threads were then mounted on glass microscope slides in approximately 50 µL ProLong® Gold antifade reagent (Invitrogen, Burlington, ON) and covered with a 0.17 mm thick cover slip. Roots and threads were imaged using a Zeiss LSM 510 Meta laser scanning confocal microscope (Carl Zeiss Inc, Germany) at 40× magnification. Living and dead cells were counted for each confocal micrograph using ImageJ (https://imagej.nih.gov/ij/download.html). Bacterial survival was calculated as a percentage of living cells relative to the total number of cells.

2.2.4 Statistical analysis

Two-way analysis of variance (ANOVA) and the Holm-Sidak post hoc test were performed using SigmaPlot version 13.0 to detect treatment effects and significant differences ($p < 0.05$) among treatment means. The Holm-Sidak test is among the more conservative post-hoc tests and is less likely to detect false differences among means.

2.3 Results

Confocal micrographs of *P. fluorescens* UW4 revealed that bacteria adhered to all substrates with nylon generally having the fewest bacteria (Figure 2.1). Based on the images, it is clear that nylon was not conducive to bacterial adherence. Furthermore, there appeared to be no difference between the *acdS*$^{-}$ mutant bacterium and the wildtype in terms of their ability to adhere to either plant root or the cotton, polyester or nylon

threads (Figure 2.1). There was also no effect of cadmium on the ability of either type of *P. fluorescens* UW4 to adhere to substrates (images not shown). It was noted that all materials fluoresced. To better detect bacteria, filters were used to screen-out the auto-fluorescence of roots and synthetic materials.

Based on ImageJ counts, cotton and polyester threads had a 3 to 4-fold higher *P. fluorescens* UW4 count compared to plant roots, while bacterial counts on nylon were 5 to 15-fold lower than those on control (no cadmium) plant roots and cotton or polyester threads (Figure 2.2A).Furthermore, bacterial strains did not differ in adhesion within a substrate type, except cotton threads inoculated with the wildtype bacterium. . Furthermore, cadmium had no effect on *P. fluorescens* UW4 bacterial counts on any of the 4 substrates (Figure 2.2A). Lastly, bacteria on plants roots had between a 50-90% higher survival in both cadmium-contaminated or control media than those on cotton, polyester or nylon (Figure 2.2B).

2.4 Discussion

As indicated by the confocal micrographs and the bacterial cell counts, was evident that the bacterium *P. fluorescens* UW4 was capable of adhering to plant roots as well as to cotton, polyester, and nylon threads, supporting my hypothesis that *P. fluorescens* UW4 adherence to plant roots, to some extent, is dependent on the physical characteristics of the root itself. However, I did not expect cotton and polyester threads to have up to 3-fold higher bacterial counts than those on plant roots since both materials physically resembled plant roots with similar roughness. This in part maybe due to the way in which the polyester and cotton were treated during the manufacturing process as this can increase the roughness of the threads (Tripp *et al*., 1957). I was less surprised to see that nylon thread had the least number of bacteria adhering to it, as the nylon surface is smooth whereas the other materials were rough. It was clear that *P. fluorescens* UW4 adheres best to cotton and polyester followed by *Arabidopsis* roots and then nylon, which suggests that *P. fluorescens* UW4 adherence to various substrates is, in part, dependent on the intrinsic characteristics or properties of a given substrate and may not be

influenced by plant exudates. It is also evident, however, that plant roots maintain higher bacterial survivability than do the other substrates; this is most likely due to plant exudates supplementing the nutritional needs of the bacteria.

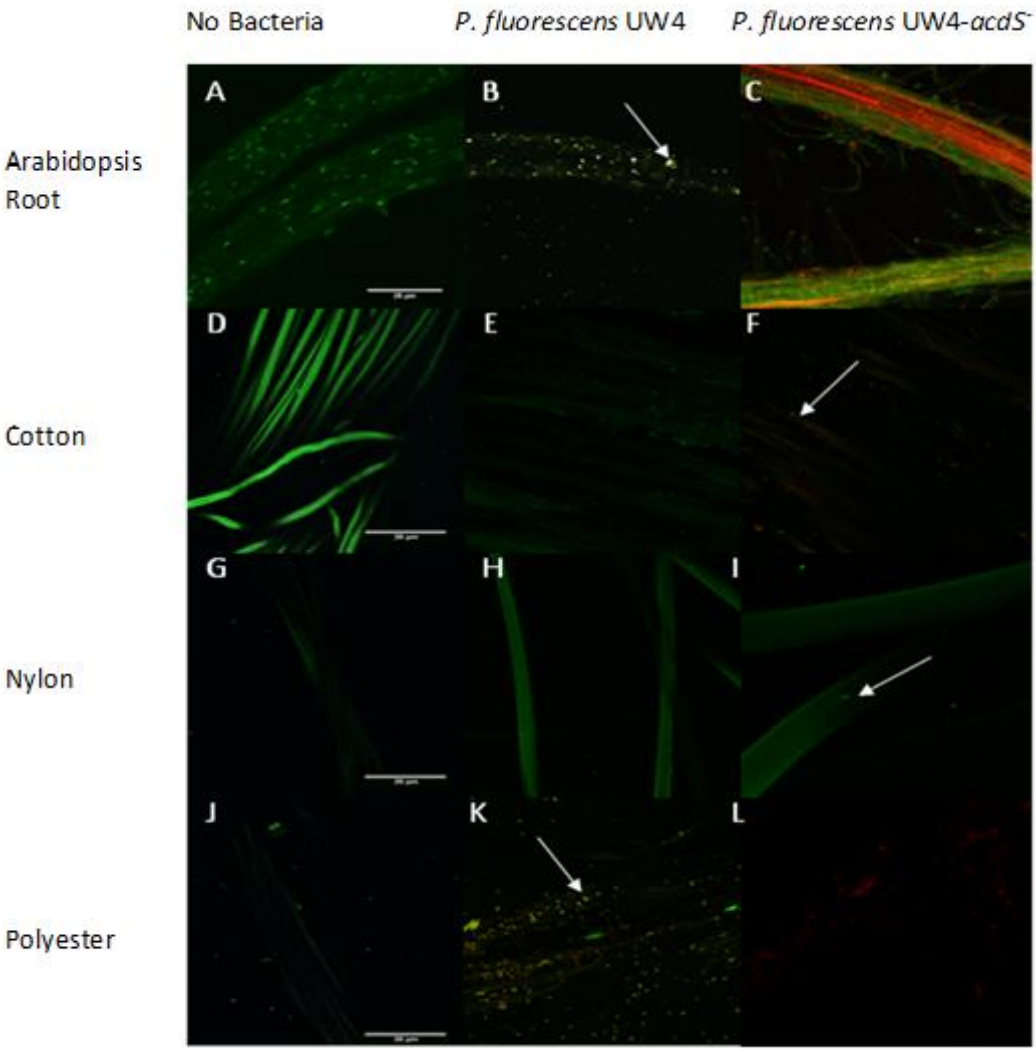

Figure 2.1 Confocal micrographs of roots and cotton, nylon and polyester thread inoculated with *P. fluorescens* UW4. Plants (row 1), cotton (row 2), nylon (row 3) and polyester (row 4) were inoculated with no bacteria (A,D,G,J), *P. fluorescens* UW4 (B,E,H,K) or mutant *P. fluorescens* UW4 (C,F,I,L) and images were taken after 14 d. Green fluorescence indicates live bacteria, red fluorescence indicates dead bacteria and yellow fluorescence indicates overlap of live/dead bacteria. The white arrows indicate examples of a bacterium or bacterial colony. Substrates, especially cotton and nylon, auto-fluoresced.

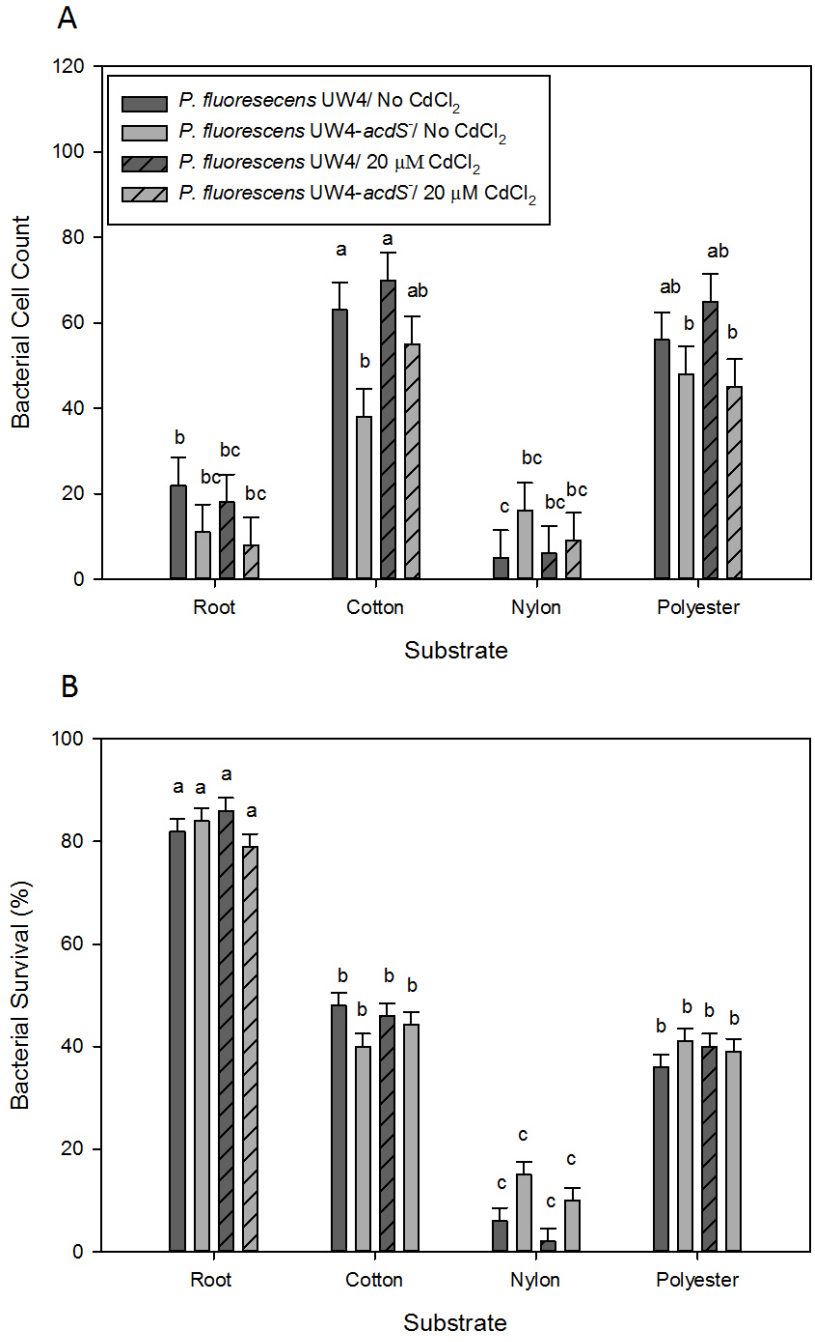

Figure 2.2. Bacterial adherence to substrates. Bacterial cell count (A) and survival (B) on various materials. Bacterial counts (live + dead) and survival (% living) were determined by counting the number of live and dead bacteria on roots and threads (n = 3) as seen on confocal micrographs via ImageJ. Vertical error bars represent standard error. Means not sharing a common letter are significantly different (two-way ANOVA followed by Holm-Sidak post-hoc test, $p < 0.05$).

Results obtained in this study were similar to those of other studies that tested different species of bacteria on the same types of materials that I use here. Both Sugarman and Masher (1981) who used *Enterobacteriaceae* (a PGPR) and *Staphylococcus aureus* (a pathogen)and Katz *et al.* (1980) who used *Staphylococcus aureus*, *Escherichia coli* (potential PGPR), *Bacteroides fragilis*, *Serratia marcescens* and *Shigella dysenteriae*, concluded that of the various types of materials tested, nylon always had the lowest bacterial counts and thus adhesion. Moreover, Hsieh and Merry (1986) determined that gram negative *E. coli* adhered to cotton and polyester equally well, whereas gram positive *Staphylococcus aureus* and *Staphylococcus epidermidis* adhered better to polyester than cotton. My study confirms that the gram negative *P. fluorescens* UW4 was able to adhere to both materials equally, suggesting that the lipopolysaccharides found on the outer membrane layer of gram negative bacteria do not discriminate against adherence to polyester or cotton. The relative ineffectiveness of cadmium to affect bacterial adherence and survivability corroborated reports from McEldowney (1994) and Manara *et al.* (2012), respectively. Manara *et al.* (2012) determined that *P. fluorescens* is tolerant of cadmium, as indicated by its ability to survive exposure to 250 μM $CdSO_4$. Moreover, McEldowney (1994) found a positive correlation between adhesion and cadmium concentration in the attachment of *Pseudomonas putida* H2 to glass. In my study, although not statistically significant, in some instances such as nylon, there was a trend of higher bacterial counts in cadmium-contaminated media; this may warrant further investigation.

2.5 Conclusion

Based on the confocal micrographs and bacterial counts I determined that *P. fluorescens* UW4 can adhere to most substrates and that the adhesion appears to be independent of plant-based interactions. My results, therefore, support the proposed physical models of bacteria-substrate interactions, at least in terms of adhesion. However, bacteria adhered to plant roots had greater survival than those adhered to non-root substrates, indicating that once the bacteria are on a root they may need to interact (e.g., get carbon or nitrogen sources) with the substrate or surrounding medium in order to stay

alive. Lastly, the level of cadmium used (20 μM) had no effect on the ability of the bacteria to adhere to a substrate or survive in the media, indicating that *P. fluorescens* UW4 and its mutant are tolerant of the amount of cadmium used.

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Chapter 3

3 How do we grow: Can *Pseudomonas fluorescens* UW4 increase the growth of *Arabidopsis* under cadmium stress in agar, hydroponics and Promix-BX?

In Chapter 2, we saw that the PGPR *Pseudomonas fluorescens* UW4 adhered to root surfaces under both cadmium-stressed and control conditions. Once adhered, can *P. fluorescens* UW4 promote plant growth under cadmium stress? In this chapter, I answered this question by using *Arabidopsis* inoculated with the PGPR *P. fluorescens* UW4. A secondary question was also addressed in this chapter: what is the best method for fostering PGPR-plant interactions in order to see maximum growth promotion? For example, I sought to find the best timing for inoculating plants with PGPRs in order to see the greatest growth promotion. Furthermore, I tested 3 plant media (Murashige and Skoog (MS) agar, MS hydroponics and Promix-BX) in which to grow *Arabidopsis* inoculated with *P. fluorescens* UW4 to determine whether or not the medium in which the plant-microbe interaction occurs affects the ability of PGPR to elicit plant growth promotion.

3.1 Introduction

Toxic metal pollution is a threat to both human and environmental health. Many metals such as iron, copper and zinc are required in small doses and are important for proper enzyme function and organismal development (Gamalero *et al*., 2009). However, at high concentrations these same metals, as well as other non-essential metals such as lead, mercury and cadmium, can be detrimental to the health of organisms. Many toxic metals cannot be eliminated from plants and thus accumulate within tissues, where they disrupt physiological processes and cellular biochemistry leading to decreases in growth, smaller yields and senescence (Khan *et al*., 2009; Han *et al*., 2014; Jakovljević *et al.*, 2014). One metal of current environmental concern is cadmium, which is released from industry, agriculture, mining and combustion of fossil fuels (Das *et al*., 1997; Clemens,

2006). Cadmium is a significant environmental pollutant since it is highly toxic to most organisms at small doses. In plants, cadmium inhibits root and shoot growth, as well as nutrient uptake (Sanita di Toppi and Gabrielli, 1999). When environmental cadmium concentrations reach as little as 10 ppm (John *et al*., 2007) stress responses can be induced, which include increased ethylene production (Guerra *et al*., 2009; Zheng *et al.*, 2013), antioxidant synthesis, and accumulation of metal-binding proteins (Devoto and Turner, 2003; Jakovljević *et al*., 2014).

Plant growth-promoting rhizobacteria (PGPR) can prevent reduced plant growth in stress conditions (Glick *et al.*, 2007). Some PGPR are able to synthesize the growth-promoting plant hormone indole-3-acetic acid (IAA) and have the ethylene-metabolizing enzyme ACC deaminase, which together are thought to play a role in reducing plant stress by increasing plant growth (Glick *et al*., 1998; Khan *et al*., 2009). The ability of PGPR to increase plant growth under stress conditions has been well established. For example, cadmium-stressed plants treated with IAA-producing bacteria had increased root growth suggesting that bacterial IAA can stimulate growth and increase plant tolerance to cadmium (Patten and Glick, 1996; Pishchik *et al*., 2002; Sheng and Xia, 2006). Hontzeas *et al*. (2004) showed that canola treated with an ACC deaminase-producing PGPR had 61% greater root length when compared with non-inoculated controls. More recently, PGPR have been shown to increase the growth of rice (Bal *et al*., 2013), and wheat (Nadeem *et al*., 2013) under salt stress, revealing that PGPR can reduce multiple types of stresses in various plant species.

One such PGPR is *Pseudomonas fluorescens* strain UW4, which has been studied for its ability to reduce plant stress and has been used to determine a link between bacterial ACC deaminase and ethylene production (Farwell *et al.*, 2007). *P. fluorescens* UW4 is a naturally occurring soil microorganism that was isolated near Waterloo, ON (Hontzeas *et al.*, 2005). An ACC deaminase mutant strain (*acdS*$^{-}$) of *P. fluorescens* was created to test the relative importance of ACC deaminase and IAA in the amelioration of biotic and abiotic stresses. This mutant strain was engineered by the insertion of a tetracycline

resistance gene within the coding region of the ACC deaminase gene, rendering the enzyme non-functional but not affecting the production of IAA (Li *et al*., 2000).

This investigation addresseswhether or not *P. fluorescens* UW4 can promote plant growth under cadmium stress and/or control conditions. It has been documented that *P. fluorescens* UW4 can increase plant growth in liquid medium (Hontzeas *et al*., 2004; Dell'Amico *et al*., 2008) and in soil (Cheng *et al*. 2007); however, in agar medium *P. fluorescens* UW4 was not able to promote plant growth (Columbus, 2013). Therefore, this study will also evaluate and optimize the medium (agar, hydroponics or Promix-BX) in which a PGPR-plant interaction occurs to determine which medium is best in order to see the greatest plant growth promotion by *P. fluorescens* UW4. This will be achieved by using *Arabidopsis thaliana* (ecotype Col-0) as the PGPR host plant. Lastly, in order to help future users of PGPRs, I set out to determine 1) the concentration of Murashige-Skoog (MS)-agar medium needed in order to have healthy control plants, but also show reduced growth in cadmium-contaminated medium, 2) whether bacteria need to be in contact with the root in order to promote growth, 3) whether tryptic soy broth (TSB), a bacterial medium, is required to support PGPR-plant interactions within agar medium, and 4) the impact on plant growth of inoculation of seeds *vs.* seedlings. I hypothesized that if PGPR's like *P. fluorescens* UW4 can promote plant growth in stressed conditions, and I inoculated *Arabidopsis* with *P. fluorescens* UW4 or an ACC deaminase mutant *P. fluorescens* UW4 grown in the presence of cadmium stress, then plants inoculated with *P. fluorescens* UW4 wildtype will show increased growth promotion. Moreover, I predict that the medium in which *P. fluorescens* UW4 and *Arabidopsis* are grown in should have no impact on the ability of the bacterium to increase plant growth under cadmium stress since the bacteria are in direct contact with the plant seed or root during the inoculation process.

3.2 Methods

3.2.1 Bacterial strains and culture maintenance

The two bacterial strains used in this investigation are *Pseudomonas fluorescens* UW4 wildtype and an ACC deaminase mutant *P. fluorescens* UW4-*acdS*$^{-}$, which has no measurable ACC deaminase activity (Li *et al*., 2000). Complete bacterial strain information and culture maintenance protocols can be found in section 2.2.1, with one modification: during the re-suspension of bacterial cells in 7.5 mL of DF salt minimal medium without nitrogen, 45 µL of 0.5 M ACC solution was added to each liquid culture, bringing the final ACC concentration to 3.0 mM. This ACC solution was added to induce ACC deaminase expression and thus temper the bacteria for plant inoculation.

3.2.2 Plant inoculation and growth conditions

Arabidopsis seed surface sterilization, inoculation with *Pseudomonas fluorescens* UW4 or *P fluorescens* UW4-*acdS*$^{-}$, agar/hydroponic media preparation, and growth conditions were outlined in section 2.2.2. The cadmium treatments chosen for most experiments were 0, 10 or 20 µM cadmium chloride, which was based on an analysis of a preliminary dose response analysis (Figure B2A).

3.2.3 MS concentration in growth media

The purpose of this experiment was to determine the MS salt concentration required in agar and hydroponic media to have healthy controls but also have decreased growth in the presence of cadmium. This was achieved by growing *Arabidopsis* in agar with either 50, 75, 80, 85, or 90% full strength MS salt in the presence of 0 or 20 µM cadmium chloride (n= 5). Plants were then grown for 14 d in a growth chamber (for growth chamber conditions see 2.2.2), at which time fresh weight and rosette (plant leaves in a circular arrangement) diameter were measured.

3.2.4 Influence of remote inoculation on plant growth

This study was conducted to determine whether or not *P. fluorescens* UW4 or *P fluorescens* UW4-*acdS*$^{-}$ was able to promote plant growth without being in contact with the roots. Since it has been established that PGPR such as *P. fluorescens* UW4 can synthesize and release hormones such as IAA or exude chelating agents to increase nutrient availability, I wanted to determine whether *P. fluorescens* UW4 or its mutant exuded compounds into the surrounding medium that indirectly promoted plant growth. To test this, *Arabidopsis* seeds were surface-sterilized and placed on MS-agar medium containing 0, 10 or 20 µM cadmium chloride (n= 5). Then, 5 µL of bacterial suspension was placed 5 cm from each seed. Petri plates were then wrapped in Parafilm® and put in a growth chamber to let the seedlings grow for 10 d.

3.2.5 Amount of TSB, time of inoculation, and growth in agar

This experiment was carried out to determine 1) whether the bacteria require TSB, a bacterial growth medium, to stay alive and provide growth promotion in agar, 2) whether it was best to inoculate plants as seeds or seedlings and 3) determine if *P. fluorescens* UW4 or its mutant could promote plant growth in agar. To test whether the bacteria need TSB in the medium, 1.5 g of TSB was added to 1 L of prepared MS-agar medium following the protocol in sections 2.2.1 and 2.2.2. TSB contains pancreatic digest of casein (amino acids), soy peptone, dextrose, sodium chloride and dipotassium phosphate. To determine the best way to inoculate *Arabidopsis* seedlings, bacteria were prepared following the protocols outlined in section 2.2.1. Then, either seeds were inoculated following protocols in section 2.2.2 or 5 µL of bacterial suspension was placed at the base of the 5 d old seedlings such that the bacteria were in contact with the roots (n= 5).

3.2.6 Growth in hydroponics

To determine whether *P. fluorescens* UW4 or its mutant were able to promote growth of *Arabidopsis* grown hydroponically, protocols outlined in sections 2.2.1 and 2.2.2 were

followed. To determine whether time of inoculation affected growth in plants grown hydroponically, the same protocol as in section 3.2.5 was followed (n = 4).

3.2.7 Growth in Promix-BX

This study was conducted to determine whether or not *P. fluorescens* UW4 or *P. fluorescens* UW4-*acdS*$^-$ could promote *Arabidopsis* growth in Promix-BX. Approximately 3 seeds (either inoculated or not) were planted in a 10 cm diameter pot containing autoclaved Promix-BX spiked with either 0, 10 or 20 µM cadmium chloride (n= 4). Promix-BX was contaminated by saturating it with either autoclaved water (control), or one of the cadmium concentrations. Plants were then placed in the growth chamber and watered when needed (25 mL, enough to saturate the Promix-BX but not cause leaching of cadmium). Due to the poor nutrient quality of Promix-BX, 25 mL half-strength Hoagland's solution was added to each pot once a week. Timing of inoculation was also tested in plants grown in Promix-BX to determine whether or not it affected plant growth, following the same protocols as outlined in section 3.2.5.

3.2.8 Long-term effects of PGPR on plant growth

This experiment determined whether *P. fluorescens* UW4 or its mutant could promote growth past the seedling stage (14 d) in *Arabidopsis*. For this experiment I tested the long-term effects of *P. fluorescens* UW4 in 100 mL glass jars containing 30 mL of MS-agar supplemented with either 0, 10 or 20 µM cadmium chloride (n= 4). To maintain a sterile growing environment, the tops of the jars were capped with autoclaved foam plugs, which also allowed for gas exchange. Plants were grown for 5 weeks in a growth chamber. Aboveground area and fresh weight (see section 3.2.9.1/4) were measured to determine whether or not *P. fluorescens* UW4 had any long-term beneficial effect on *Arabidopsis* growth.

3.2.9 Analysis of plant growth

3.2.9.1 Aboveground area

Plants were grown on horizontal agar plates as described in section 2.2.2 for 14 d. Five seeds were placed no less than 2 cm apart and away from the plate edge on each of 4 plates. Aboveground area was measured by taking images with a Canon EOS Rebel T5 18.0MP camera and images were analyzed using ImageJ (https://imagejnih.gov,ij/download.html).

3.2.9.2 Rosette diameter

Plants were grown on horizontal agar plates as outlined in section 3.2.9.1. The maximum rosette diameter for each plant was measured using digital calipers and recorded.

3.2.9.3 Root elongation

Plants were grown on vertical agar plates until the roots were close to, but not touching, the bottom of the plate (approximately 12-14 d depending on treatment). Five seeds were placed along a straight line across the diameter of the plate, 2 cm away from the edge. Primary root length for each plant was measured using digital calipers and recorded.

3.2.9.4 Biomass

When plants grown in agar, hydroponics and Promix-BX were harvested, as much agar, Promix-BX or excess water was removed from the roots as possible. Plant fresh weight (FW) was recorded using a Mettler Toledo™ MS-TS analytical balance.

3.2.10 Statistical analysis

Two-way analyses of variance (ANOVA) and Holm-Sidak post hoc tests were performed using SigmaPlot version 13.0 to detect treatment effects and significant differences among treatment means, respectively ($p < 0.05$).

3.3 Results

3.3.1 MS concentration

In determining the concentration of Murashige-Skoog (MS) required for healthy control plant growth based on a visual inspection, it was clear that 50% MS agar was not sufficient. *Arabidopsis* grown in 50% MS had yellow leaves and looked stressed. However, plants grown in the other four MS concentrations were green and looked relatively healthy. Plants that were grown in 75% and 80% MS medium contaminated with cadmium had yellowing leaves and appeared stressed. Plants grown in 90% MS were on average 30% heavier than plants from the other MS-treatments (Figure 3.1A). However, plants grown in 90% MS did not experience a decrease in fresh weight under cadmium stress. The only plants that decreased in fresh weight in response to cadmium, were those grown in 80% MS; these plants were 60% lighter when compared to their control. Similarly, the rosette diameters of plants grown in 85% and 90% MS were the same as their corresponding controls (Figure 3.1B). However, plants grown in 50% to 80% MS had 33% smaller rosette diameters when compared to control plants.

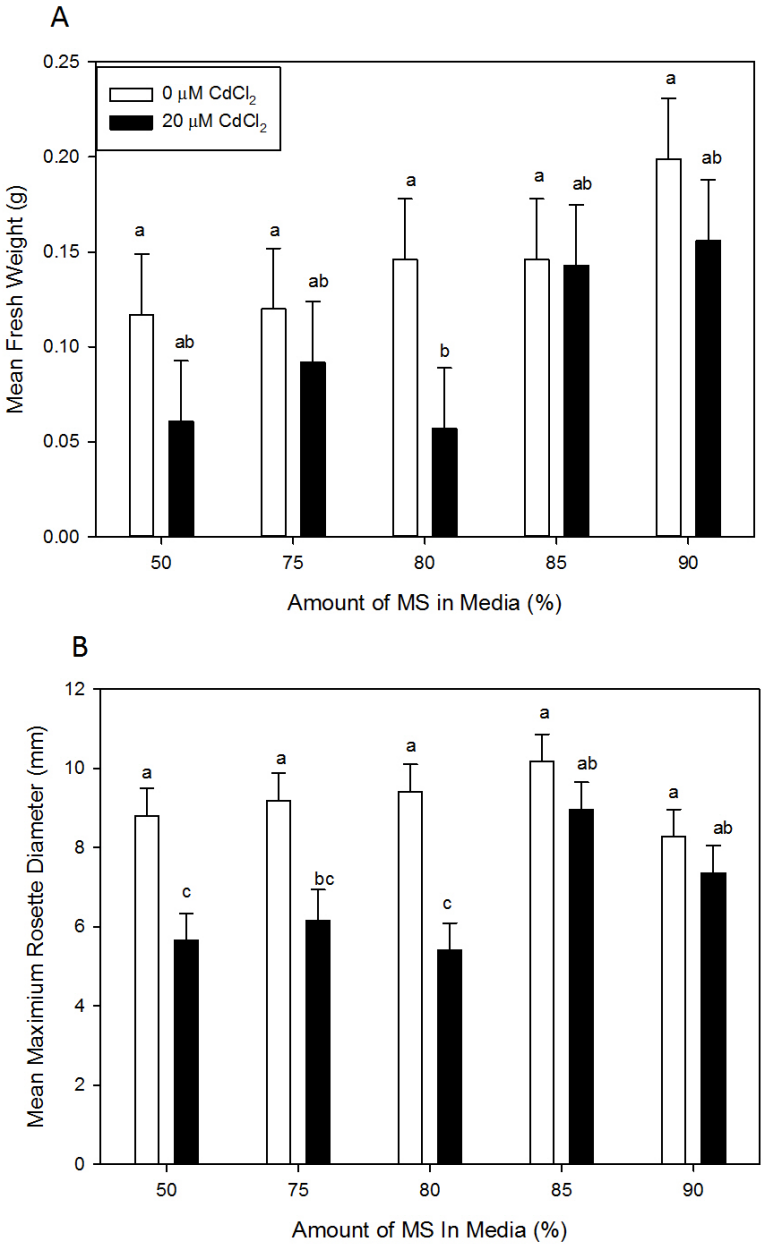

Figure 3.1. Growth in MS-agar medium. *Arabidopsis* was grown on agar containing 50, 75, 80, 85, or 90% MS medium with either 0 (white bars) or 20 μM (black bars) cadmium chloride. Plant growth was measured using fresh weight (A) and rosette diameter (B). Means within each figure panel not sharing a common letter are significantly different (two-way ANOVA followed by Holm-Sidak post-hoc test, $p < 0.05$, $n = 5$).

3.3.2 Influence of remote inoculation on plant growth

These experiments were performed to determine if bacteria needed to be in direct contact with plant roots to have an effect on growth. Plants grown near *P. fluorescens* UW4 had greater mass, of up to 250% and 71% when grown with 10 or 20 µM cadmium, respectively, when compared with plants inoculated with the mutant *P. fluorescens* UW4-*acdS*$^{-}$ and non-inoculated controls (Figure 3.2A). Remote inoculation had no effect on plant fresh weight at lower cadmium treatments, nor did it affect aboveground area (Figure 3.2B) or primary root length (Figure 3.2C) under any cadmium treatment. It is also worth noting that remote inoculation resulted in no obvious pathogenicity of the bacterium.

3.3.3 Amount of TSB and time of inoculation in agar

When bacteria are grown on their own, they are usually grown in a bacteria-specific medium such as TSB. Cultured plants, on the other hand, are usually grown in MS-agar. The experiment was done to determine the optimal mixture of TSB and MS-agar for combined plant and bacterial growth. Based on *Arabidopsis* growth in agar in the absence of bacteria, it appears that TSB has no consistent detrimental effects on plant fresh weight (Figure 3.3A) or aboveground area with the one exception for plants that underwent a mock inoculation on day zero (D0), which were 45 to 73% smaller than control plants when grown in 0 or 10 µM cadmium (Figure 3.3D). This unusual result could be due to extreme plant variation or some unknown effect induced by the mock inoculation of sterile 0.03 $MgSO_4$. However, it is clear that TSB inhibits primary root growth, as seen by 42%, 70%, and 25% reductions in primary root lengths for plants grown in 0, 10, 20 µM cadmium, respectively, when compared to plants grown in the absence of TSB (Figure 3.3G). Primary root length measurements for plants in the D0 treatment, with or without bacteria, could not be measured due to roots being shorter than 1 mm.

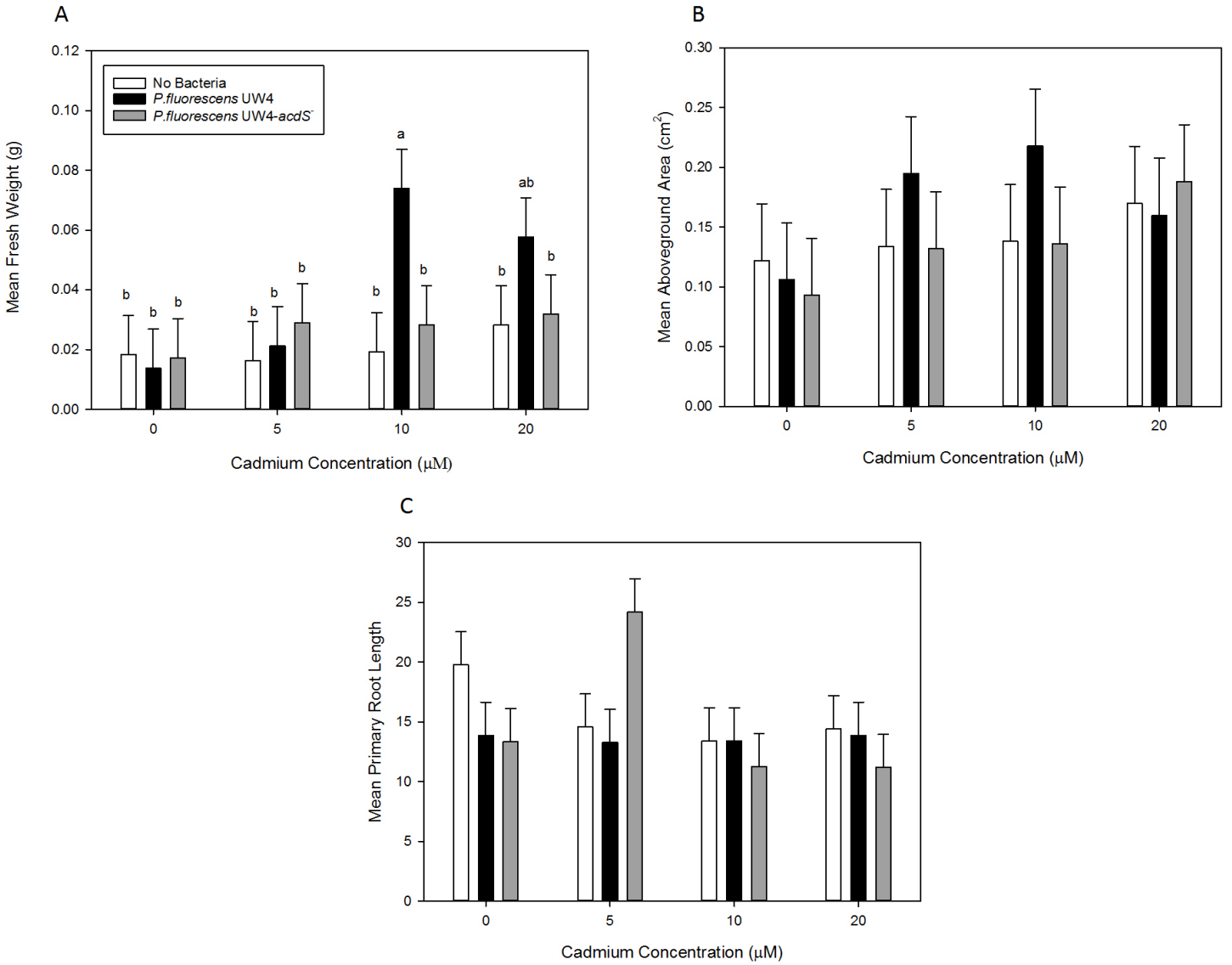
A
No Bacteria
P.fluorescens UW4
P.fluorescens UW4-acdS-
Mean Fresh Weight (g)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
b
b
b
b
b
b
b
a
b
b
ab
b
0
5
10
20
Cadmium Concentration (µM)
B
Mean Aboveground Area (cm2)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
5
10
20
Cadmium Concentration (µM)
C
Mean Primary Root Length
30
25
20
15
10
5
0
0
5
10
20
Cadmium Concentration (µM)

Figure 3.2. Effect of remote bacteria on plant growth. Influence of remote bacteria on *Arabidopsis* fresh weight (A), aboveground area (B), and primary root length (C) when treated with 0, 5, 10 or 20 µM cadmium chloride. Means within each figure panel not sharing a common letter are significantly different (two-way ANOVA followed by Holm-Sidak post-hoc test, $p < 0.05$, n=5).

When inoculated with bacteria, the absence or presence of TSB did not have an overall net effect on plant fresh weight or aboveground area. As for non-inoculated plants, there were unusual inconsistencies among the results, which again suggest that there could have been a lot of variance among individual plant responses. However, just like with non-inoculated plants, TSB in combination with bacterial inoculation inhibited root length most likely due to both the inhibitory effects of TBS on plant roots and bacterial overgrowth from having abundant food sources provided by TSB.

In all cases, plants inoculated with either bacterium weighed 50 to 90% less than non-inoculated controls, regardless of the presence of cadmium, TSB, or the timing of inoculation (Figure 3.3A,B,C). Moreover, plants inoculated with bacteria had 25-50% smaller primary root lengths than non-inoculated plants within the same treatment (Figure 3.3G,H,I). Nonetheless, it is clear that when growing *Arabidopsis* in agar, it is best to inoculate plants at the seedling stage rather than inoculating the seed itself. For example, *Arabidopsis* inoculated with the wildtype bacterium on D0 under no cadmium stress had 78% less fresh weight when compared to plants inoculated on D5, with or without TSB (Figure 3.3B). Moreover, plants inoculated with the mutant bacterium on D0 had a 23-73% smaller aboveground area for plants grown in 20 µM of cadmium (Figure 3.3E).

3.3.4 Growth and time of inoculation in hydroponics

In general, hydroponically grown *Arabidopsis* inoculated with wildtype *P. fluorescens* UW4 under both cadmium-stress and control conditions, showed no growth promotion and in some instances were smaller than non-inoculated controls (Figure 3.4). For example, control *Arabidopsis* inoculated on D0 with either bacterium had as much as 50% less fresh weight. Furthermore, plants inoculated with either bacterium on D5 had as much as 45% less aboveground area, especially for plants grown in the absence of toxic metal stress. For plants grown hydroponically, inoculating plants with bacteria at either inoculation times resulted in no growth promotion and in some instances a reduction in growth (Figure 3.4).

3.3.5 Growth and time of inoculation in Promix-BX

For *Arabidopsis* grown in Promix-BX, the time of inoculation made a significant difference in terms of plant growth (Figure 3.5). Plants inoculated with bacteria on D5 (Figure 3.5C,D) had a 33 to 55-fold larger aboveground area and a 1.3 to 2.2-fold greater fresh weight when compared to plants inoculated with bacteria on D0 (Figure 3.5A,B) within the same treatment group. Inoculation with *P. fluorescens* UW4 did promote plant growth in Promix-BX. Plants grown in 0 or 20 µM cadmium chloride inoculated with the wildtype bacterium were 315% and 400% larger in aboveground area when compared to plants inoculated with the mutant bacterium and non-inoculated plants (Figure 3.5C). Although the trends are the same as for aboveground area, fresh weight was not affected by bacterial inoculation (Figure 3.5D).

3.3.6 Long term effects of PGPR on plant growth

To determine if PGPR's association with the plant over a longer time span could elicit growth promotion, I inoculated plants with *P. fluorescens* UW4 or *P. fluorescens* UW4-*acdS*$^{-}$ and let them grow for five weeks. Inoculation with either bacterial strain appeared to be detrimental to plant growth (Figure 3.6). Plants inoculated with either bacterial strain had 50- 60% less aboveground area, with the exception of plants inoculated with *P. fluorescens* UW4 grown in the presence of 20 µM cadmium; these plants were 136% larger than control plants (Figure 3.6A). Furthermore, plants inoculated by either bacterium had 40%, 82% and 65% less fresh weight for plants grown in 0, 10, and 20 µM of cadmium, respectively, when compared to non-inoculated controls (Figure 3.6B).

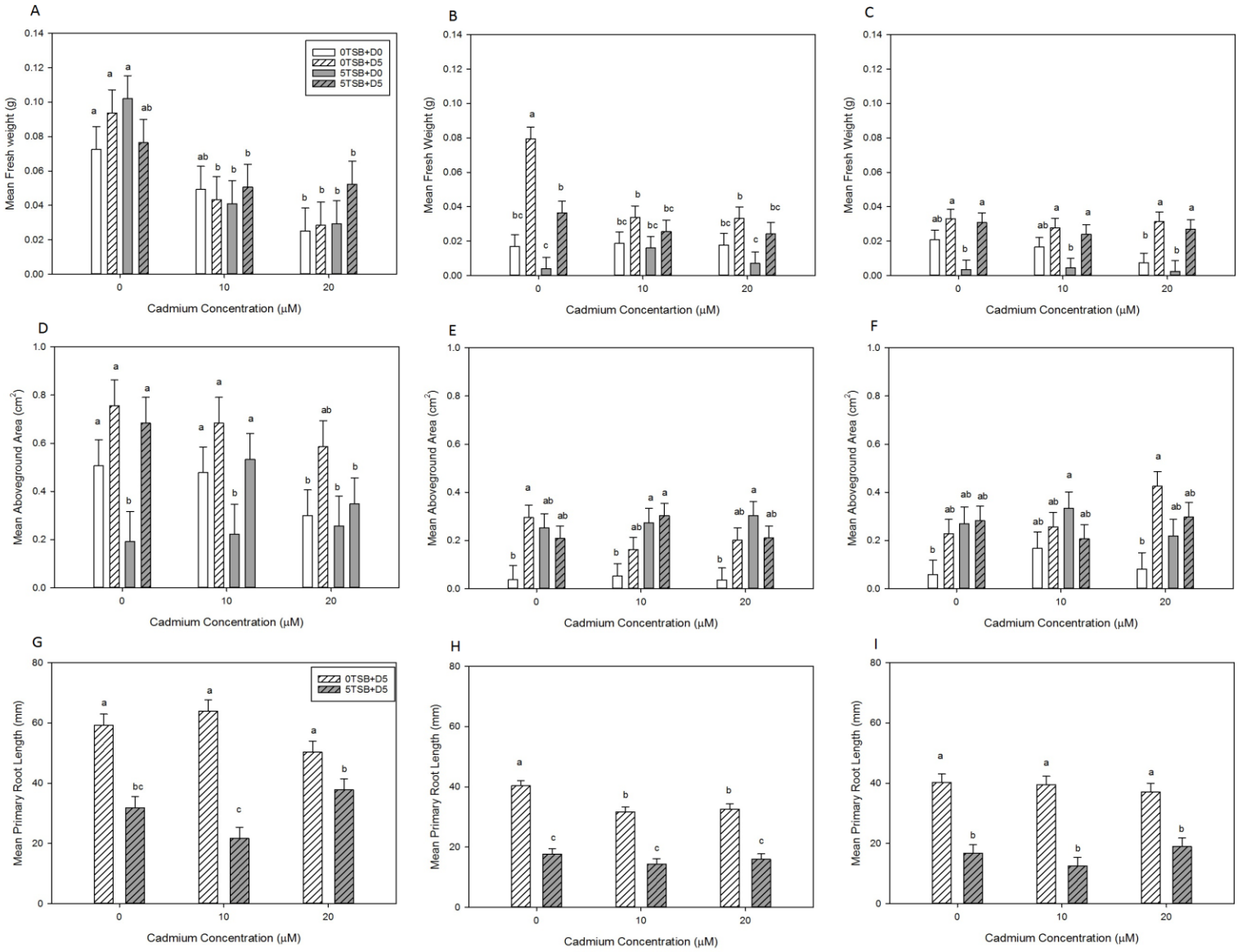
A
B
C
D
E
F
G
H
I
0TSB+D0
0TSB+D5
5TSB+D0
5TSB+D5
Mean Fresh weight (g)
Mean Fresh Weight (g)
Mean Aboveground Area (cm²)
Mean Primary Root Length (mm)
Cadmium Concentration (µM)
Cadmium Concentartion (µM)
0
10
20

Figure 3.3. Does TSB and timing of inoculation affect plant growth? *Arabidopsis* inoculated on day zero (D0, no-hashed bars) or day five (D5, hashed bars) by *Pseudomonas fluorescens* UW4 grown in cadmium-contaminated agar medium containing 5% TSB (grey bars) or no TSB (white bars). Plants were inoculated with either no bacteria (A, D, G) , *P. fluorescens* UW4 (B, E, H), or *P. fluorescens* UW4-*acdS*$^{-}$ (C, F, I) grown in agar for 14 d. Fresh weight (A-C), aboveground area (D-F), and primary root length (G-I) were measured to determine the effects of TSB and time of inoculation had on growth. Means within each figure panel not sharing a common letter are significantly different (two-way ANOVA followed by Holm-Sidak post-hoc test, $p < 0.05$, $n = 5$).

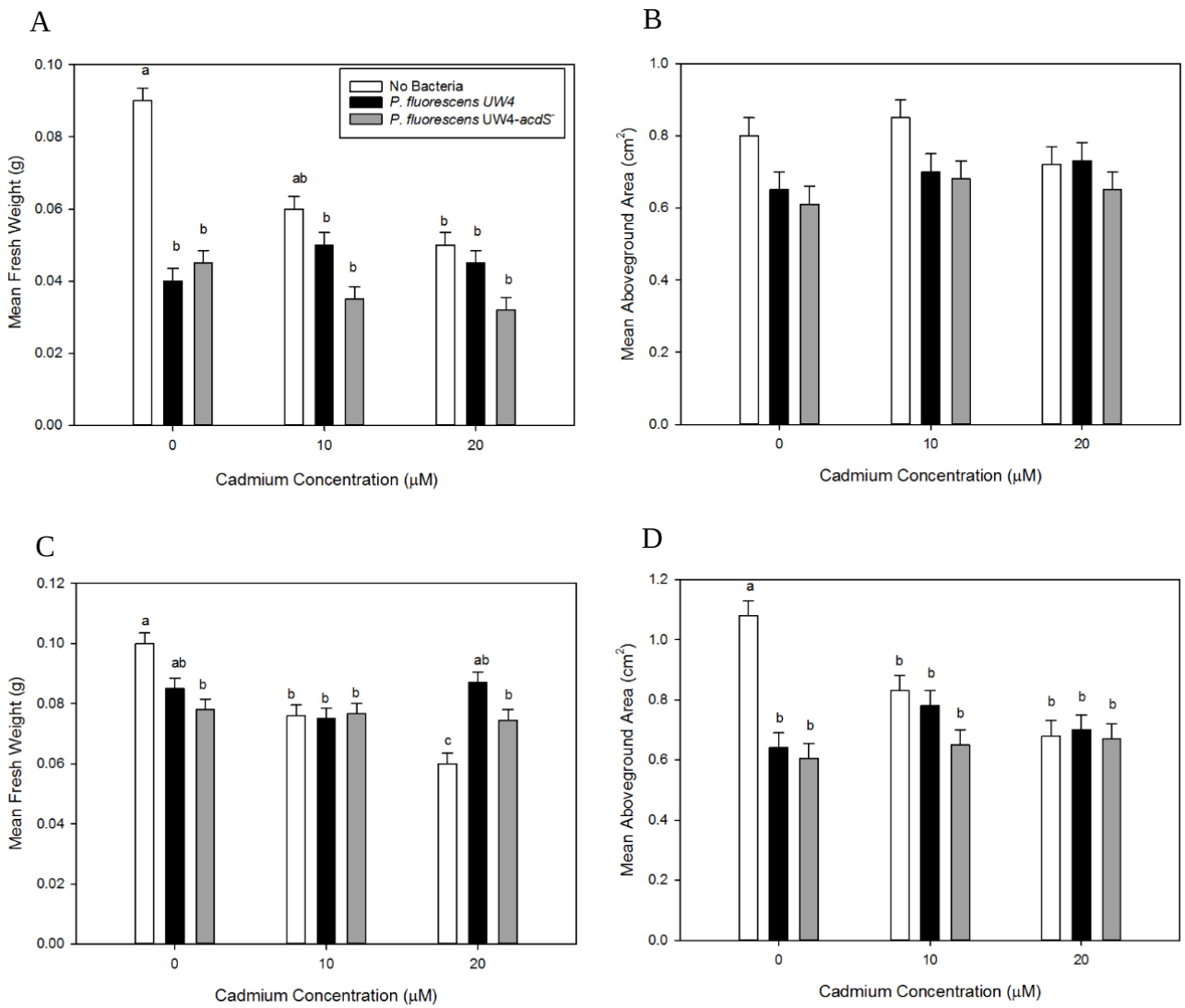
A
B
C
D
No Bacteria
P. fluorescens UW4
P. fluorescens UW4-acdS⁻
Mean Fresh Weight (g)
Mean Aboveground Area (cm²)
Cadmium Concentration (µM)
0
10
20
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.0
0.2
0.4
0.6
0.8
1.0
1.2
a
ab
b
c

Figure 3.4. Timing of inoculation on plant growth in hydroponics. Growth promotion of *Arabidopsis* by *P. fluorescens* UW4 or *P. fluorescens* UW4-*acdS*$^{-}$ inoculated on D0 (A, B) or D5 (C,D) grown hydroponically in the presence of cadmium. *Arabidopsis* was grown for 14 d and had mean fresh weight (A,C) and mean aboveground area (B,D) measuredMeans within each figure panel not sharing a common letter are significantly different (two-way ANOVA followed by Holm-Sidak post-hoc test, $p < 0.05$, $n = 4$).

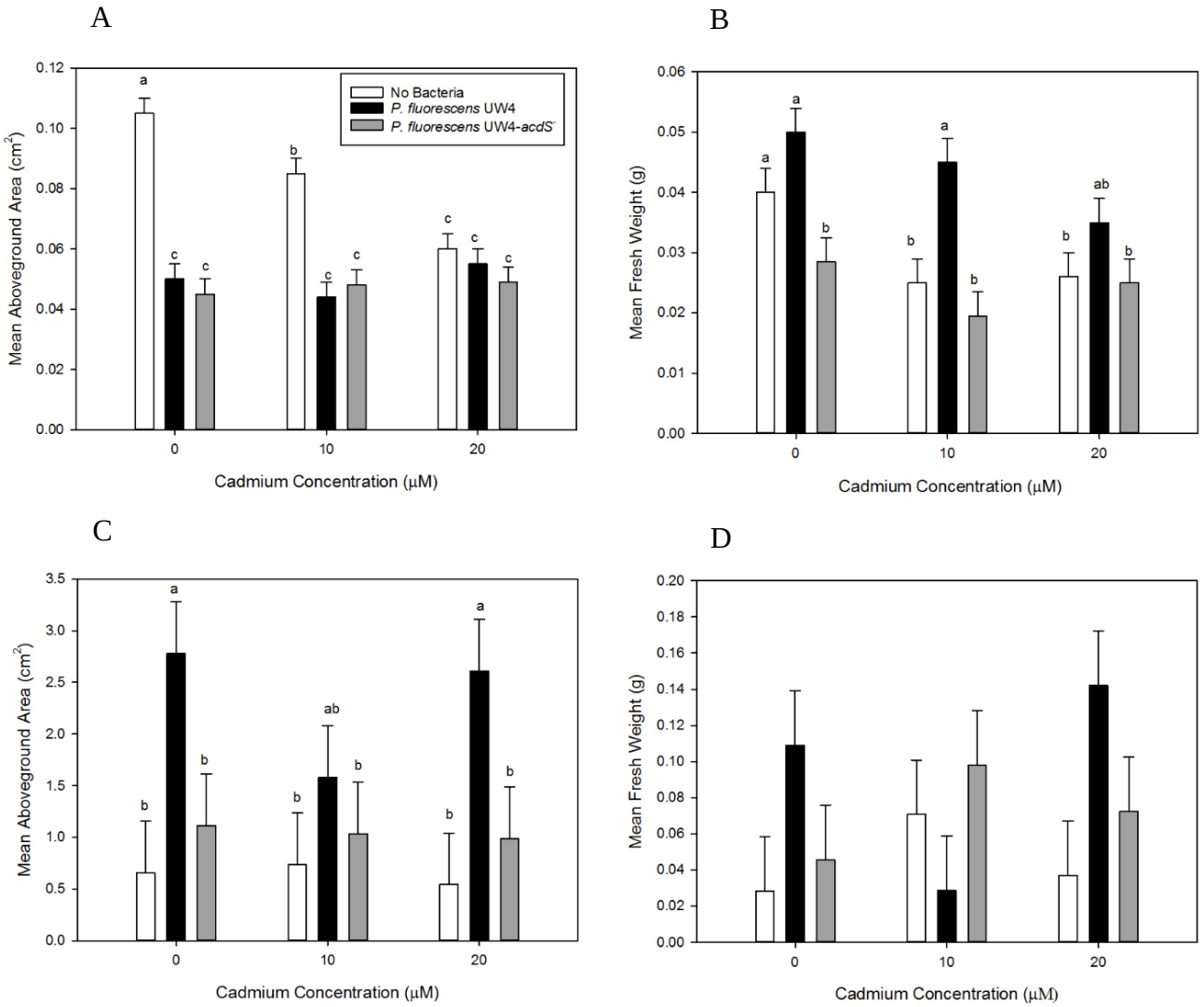
A
B
C
D
No Bacteria
P. fluorescens UW4
P. fluorescens UW4-acdS⁻
Mean Aboveground Area (cm²)
Mean Fresh Weight (g)
Cadmium Concentration (μM)
0
10
20

Figure 3.5. Effects of inoculation timing on plant growth in Promix-BX. Mean aboveground area (A,C) and fresh weight(B,D) of *Arabidopsis* when inoculated with *P. fluorescens* UW4 or *P. fluorescens* UW4-*acdS*$^{-}$ inoculated on D0 (A,B) or D5 (C,D) grown in cadmium contaminated Promix-BX. Plants that were inoculated with *P. fluorescens* UW4 on D5 had between 55-70% larger aboveground areas than control and *P. fluorescens* UW4-*acdS*$^{-}$ inoculated plants.. Means within each figure panel not sharing a common letter are significantly different (two-way ANOVA followed by Holm-Sidak post-hoc test, $p < 0.05$, $n = 4$.

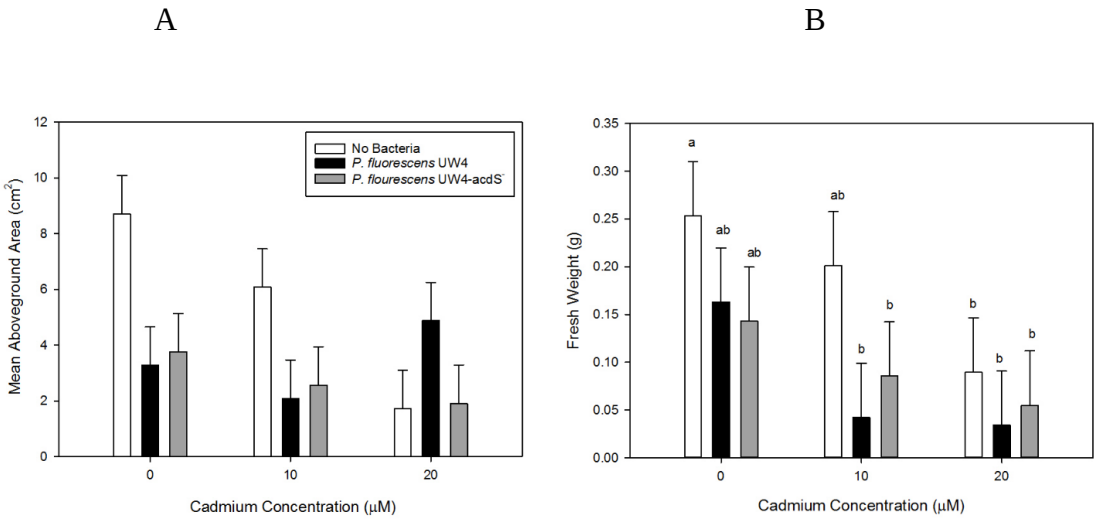

Figure 3.6. Effects of long term association with PGPR on plant growth. Long term (5 weeks) growth promotion of inoculated *Arabidopsis* by *P. fluorescens* UW4 grown in cadmium- contaminated agar. Mean aboveground area and fresh weight were measured to determine the effects of long-term association with *P. fluorescens* UW4. Means within each figure panel not sharing a common letter are significantly different (two-way ANOVA followed by Holm-Sidak post-hoc test, $p < 0.05$, $n = 4$).

3.4 Discussion

Understanding how to grow and facilitate a PGPR-plant interaction is just as important as determining whether or not a PGPR can promote plant growth. As demonstrated within this chapter, if a PGPR-plant interaction is not optimized, this can result in decreases in plant growth and potential yield. In an attempt to help future users of PGPR, I set out to determine the optimal conditions for testing PGPR-plant growth promotion in *Arabidopsis* under cadmium stress by looking at, 1) the amount of MS plant medium needed in agar such that control plants are healthy and still exhibit cadmium stress, 2) whether PGPRs can enhance plant growth without being in direct contact with plant roots, 3) whether the bacterial medium TSB was required to support PGPR-plant interactions within agar medium, 4) whether the timing of plant inoculation, as a seed or seedling, had any impacts on plant growth, and 5) whether the medium (agar, hydroponics, or Promix-BX) in which a PGPR-plant interaction occurs in made a difference in terms of the ability of a PGPR to promote plant growth. Furthermore, the results from these experiments also allow for the determination of whether or not *P. fluorescens* UW4 promotes plant growth under cadmium stress, both short term (2 weeks) and long-term (5 weeks).

3.4.1 MS concentration in the media

When growing plants in either agar or hydroponic medium, it is important that the amount of nutrients present is sufficient to have healthy plants. For *Arabidopsis*, having a medium that contains at least 75% MS is required in order to have healthy plants. However, a lesson learned from this experiment is that a too rich medium (more nutrients) can mitigate the effects of a metal stress. For example, plants that were grown in 85 or 90% MS did not differ in terms of aboveground area or rosette diameter when grown in the presence of cadmium, when compared to non-metal stressed controls.

This is of importance when studying PGPR-plant interactions as it demonstrates nutrient-rich media have the potential to act as a confounding variable when looking at PGPR growth enhancement under stress conditions. This also suggests that plants that have an abundance of nutrients available, at least with mild cadmium stress, can achieve normal growth. This result of high nutrients within a plant's growing medium alleviating abiotic stress has also been seen with salinity stress in *Triticum aestivum* (wheat; Tahir *et al*., 2011) and *Pisum sativum* (pea) under nickel stress (Shahid *et al*., 2014). Therefore, a medium that is not too rich or too poor in nutrients needs to be selected in order to observe metal toxicity and not act as a confounding variable in assessing PGPR plant growth. To study PGPR effects on *Arabidopsis*, I recommend using an agar medium that contains 80% MS as this not only allows for healthy control plants but also enables metal-stress to reduce growth.

3.4.2 Influence of remote inoculation on plant growth

It has been well documented that PGPRs, once associated with plant roots, can promote growth in a variety of plant species (Pishchik *et al*., 2002; Hontzeas *et al*., 2004; Sheng and Xia, 2006; Bal *et al*., 2013; Nadeem *et al*., 2013). However, can these same PGPRs promote plant growth by just being close in proximity to the root? Based on my results, the remote influence of *P. fluorescens* UW4 was sufficient to increase plant fresh weight by up to 250%, but no changes in aboveground area or root length were seen. Furthermore, remote influence of *P. fluorescens* UW4-*acdS*$^{-}$ had no effect on any plant growth measures indicating that ACC deaminase maybe an important property for *P. fluorescens* to increase plant mass from a distance. Even though *P. fluorescens* UW4 and its mutant are able to synthesize and exude IAA (Li *et al*., 2000), a known plant growth-promoting hormone, it might only do so when in association with plant roots. Moreover bacterial IAA is synthesized from tryptophan, often exuded by plant roots (Kamilova *et al*., 2006), thus I speculate that *P. fluorescens* UW4 did not have adequate tryptophan to synthesize enough IAA to promote significant plant growth under these conditions since plants inoculated with the mutant did not see increased mass. These results suggests that

P. fluorescens UW4 and perhaps other PGPRs do not need to be in direct contact with roots in order for the plant to reap their benefits. However, the type of growth promotion wanted, whether that be larger tubers or bigger leaves needs to be considered. Based on my results, I would put forth to potential farmers or researchers who grow agricultural products such as lettuce or leafy greens to inoculate the soil near the plant roots such that the aboveground growth benefits from PGPR presence within the soil. However, I would not recommend this technique to farmers who grow root vegetables, as I did not see any root growth promotion when the bacteria were grown away from the roots. Instead perhaps, these types of farmers should inoculate their root growing vegetables directly with a PGPR to see increased growth in tubers and or roots.

3.4.3 TSB in agar

In media containing TSB, aboveground leaf area was equal to that of control plants; however, TSB did inhibit root growth by up to 70%. To my knowledge, no study has been conducted on the toxicity of TSB to plants. However, Street *et al*. (1960) have shown that many of the amino acids released (L-glutamic acid, L-proline, L-leacine, L-lycine, etc.) after casein digestion inhibit the growth of excised tomato roots by up to 50%. I think that this may be the main reason why TSB significantly inhibits root growth. Moreover, root length inhibition was more severe in plants grown in the presence of TSB and bacteria. I did notice that plants inoculated with *P. fluorescens* UW4 or *P. fluorescens* UW4-*acdS*$^{-}$ and grown in the presence of TSB had bacterial pooling near the site of inoculation, indicating substantial bacterial growth. This increase in bacterial growth could overwhelm the plant's defenses and/or deprive the roots of nutrients, resulting in smaller roots. Although this decrease in root length did not affect aboveground growth, I think that if I had allowed the experiment to continue past 14 d I would have seen a difference in terms of fresh weight and aboveground area when compared to plants not grown in TSB. This notion is supported by Yang *et al*. (1998), who found that when bacteria, including PGPR, exceed the microbial carrying capacity (plants providing photosynthates, amino acids, etc.) of plant roots, plant growth and health were negatively affected. The bacteria are most likely able to survive on plant roots in the absence of TSB as indirectly evident by plants

inoculated with bacteria in no TSB having similar aboveground area and fresh weight as those inoculated plants grown in the presence of TSB. From this experiment I can recommend that *Arabidopsis* should not be grown in media containing TSB and that, if possible, PGPR should be grown in the absence of TSB so as not to induce bacterial overpopulation resulting in reduced plant growth.

3.4.4 Time of inoculation

In the literature, inoculating seeds seems to be widely accepted as the best method of inoculating plants (e.g., Li *et al*., 2000; Dell' Amico *et al*., 2008; Nadeem *et al*., 2013), but is it really the best method? My results in agar and Promix-BX indicate that it is actually better to inoculate plants as seedlings (D5) rather than as seeds (D0), at least for the combination of plant and bacteria that I used. For plants grown in agar, inoculating plants with bacteria on D5 rather than D0 resulted in a 500% increase in plant growth. Similar results were seen in Promix-BX, where plants inoculated on D5 were 510% larger than control plants. This dramatic difference in growth suggests that the day of plant inoculation of a putative PGPR is crucial in achieving an effective symbiosis. Given that it takes at least two days for *Arabidopsis* to emerge from the seed coat, based on my observations, inoculation of seeds would give the bacteria a two day head start in terms of growth. The freshly emerged root could be growing into bacterial concentrations that surpass the microbial carrying capacity of the seedling. Giving the plant a chance to establish before inoculating with a PGPR, especially in Promix-BX, would allow for maximum growth potential as well as potential PGPR plant growth promotion.

3.4.5 Growth in agar (short and long term), hydroponics and Promix-BX

The putative PGPR *P. fluorescens* UW4 has been shown to increase plant growth in a variety of species (Patten and Glick, 1996; Li *et al*., 2000; Gamalero *et al*., 2009). However, my results indicate that for *Arabidopsis* grown in agar or hydroponic solution, no matter for how long, *P. fluorescens* UW4 or its ACC deaminase mutant do not

promote plant growth and in many instances can be detrimental to the plant. For plants grown on agar there was up to 90% loss in aboveground growth and a 50% loss and root length when plants were grown with either bacterial type in both metal-stress and control conditions. A similar trend was also seen in the hydroponic studies: there was 50% less plant growth when inoculated with ether *P. fluorescens* UW4 strain. This suggests that ACC deaminase in the wildtype *P. fluorescens* UW4 may not be induced enough to reduce ACC concentrations within stressed plant tissue to have a beneficial impact on plant growth under cadmium stress. However, in Promix-BX, the result was quite interesting. Plants inoculated with wildtype *P. fluorescens* UW4 did have up to 525% growth promotion in terms of aboveground area and the plants were 266% heavier, at least in the short term.

My data are partially consistent with what others have found when growing plants in association with PGPR. For example, Cheng *et al*. (2007) reported an increase of 700% in fresh weight in canola inoculated with *P. fluorescens* UW4 when grown in soil under salt stress. Although the growth promotion seen in my plant's fresh weight was not as high as Cheng *et al*.'s (2007), it is nonetheless a significant promotion in plant growth. Where my results disagree with the literature is in terms of agar and hydroponic studies. Canola grown hydroponically in association with PGPR had an increase in both aboveground measures and root lengths (Dell'Amico *et al*., 2008). Although there were few studies of PGPR-plant interactions done in agar, Kurepin *et al*. (2015) found that plants inoculated with PGPR had up to 80% increases in growth. To explain discrepancies between my results and the literature, it is possible that *Arabidopsis* and *P. fluorescens* UW4 do not form a symbiotic relationship but rather form a slightly pathogenic one, resulting in growth inhibition. It has been documented that when *Arabidopsis* is grown in the presence of pathogenic bacterial flagellin (main protein in bacterial flagellum), fresh weight decreased by up to 78% (Gomez-Gomez *et al*., 1999) and this loss in mass is similar to what I saw generally in *Arabidopsis* inoculated with *P. fluorescens* UW4. Furthermore, 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 (Hopkins and Huner, 2004). It is

possible that under my experimental conditions, the two bacterial strains over-produce IAA, leading to the suppression of plant growth. This notion has also been proposed by Kremer (2006) who showed that deleterious rhizobacteria may over-produced IAA resulting in suppressed plant growth. Moreover, Xie *et al.* (1996), who grew canola plants with IAA-overproducing mutants of *P. fluorescens* GR12-2, also reported inhibited root elongation. Taken together, it is clear that the PGPR *P. fluorescens* UW4 may promote growth under the right conditions with the right species in stress conditions, although in my studies growth promotion was not generally seen.

3.5 Conclusion

When growing *Arabidopsis* inoculated with *P. fluorescens* UW4, the results vary with the growth medium used. In order to have healthy *Arabidopsis* controls grown in agar that also show a cadmium-induced reduction in plant growth, it is recommended to have an MS strength of 80%. Also, it is very clear that inoculating seedlings on D5 results in better plant growth than inoculation on D0. It was also revealed that, in general, no matter the medium (agar, hydroponics, Promix-BX) in which the *Arabidopsis-P. fluorescens* UW4 interaction occurs, *P. fluorescens* UW4 does not increase *Arabidopsis* growth under cadmium stress. I address the question of whether or not the amount of IAA synthesized and exuded by *P. fluorescens* UW4 may inhibit plant growth in the next chapter.

3.6 References

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Chapter 4

4 An investigation into the IAA hypothesis: Can the IAA produced by the PGPR *Pseudomonas fluorescens* UW4 increase plant growth?

In the light of not seeing plant growth promotion by *Pseudomonas fluorescens* UW4 in Chapter 3, I decided to investigate whether the plant growth promoting mechanisms (namely, indole-3-acetic acid (IAA) and bacterial acdS) of *P. fluorescens* UW4 were valid for this bacterium. Bacterial acdS will be explored in Chapter 5. In this chapter I will explore IAA by determining whether the amount of IAA produced by *P. fluorescens* UW4 is stimulatory or deleterious to plant growth, as I postulated in section 3.4.5. To understand the role of bacterial IAA on plant growth, the various components of the tryptophan/IAA portion of Glick *et al.*'s (1998) model (Figure 1.1) were tested. First, the amount of tryptophan exuded by *Arabidopsis* roots was determined. Then the amount of exuded tryptophan was added to the growth medium of *P. fluorescens* UW4 and its mutant to determine the amount of IAA synthesized. Lastly, the amount of IAA synthesized by *P. fluorescens* UW4 was then added to the *Arabidopsis* agar growth medium where the effects of IAA were measured in terms of growth. I expected that the amount of IAA produced by *P. fluorescens* UW4 would promote plant growth.

4.1 Introduction

Improving plant health and growth may be one of the most important areas of research in the 21st century. As the human population continues to increase, so too will the demand for increased agricultural products, along with increased food sustainability and security (Baldos and Herte, 2014). With the implications of climate change as well as environmental degradation, current agricultural practices may not be enough to meet our current and future demands for agricultural products (Wheeler and von Braun, 2013). It has been proposed that the use of plant growth-promoting microbes may increase plant growth (Shah *et al.*., 1998; Glick *et al*., 2007), increase agricultural yields (Glick *et al*.,

1997; Sziderics *et al*., 2007), and mitigate plant stress (Sheng and Xia, 2006; Khan *et al.*, 2009; Shim *et al*.., 2015).

Of the various types of plant growth-promoting microbes that have the potential to improve plant growth, bacteria that interact with plant roots seem to be promising. These particular bacteria, dubbed plant growth-promoting rhizobacteria (PGPR), inhabit the rhizosphere where they can interact with plant exudates—carbohydrates, organic acids, amino acids and secondary metabolites—as well as physically interact with the plant root (Bertin *et al.*, 2003; Bais *et al.*, 2006).

Mechanisms by which PGPR are able to promote plant growth were discussed in more detail in section 1.4.3. Of relevance to this chapter, synthesis and exudation of IAA was proposed by Glick *et al*. (1998) to be one of the mechanisms utilized by some PGPRs to improve plant growth. It is thought the bacteria synthesize the plant growth hormone IAA through a tryptophan-dependent indole-3-pyruvic acid pathway (Spaepen *et al.*, 2007), although some bacteria can synthesize IAA through a tryptophan-independent pathway (Prinsen *et al.*, 1993). Based on the hypothesis proposed by Glick *et al*. (1998), host plants exude the amino acid tryptophan into the rhizosphere, which is then taken up by PGPRs and used as a substrate to synthesize IAA. IAA is then exported back into the rhizosphere, where it is available for plant uptake, potentially increasing plant growth (Glick *et al*., 1998).

The ability to synthesize IAA has been well documented for many putative PGPR species such as *Azospirillum brasilense* (Baudoin *et al*., 2010), *Escherichia sp. (Costa et al., 2014), Bacillus* sp. JH 2-2 (Shim *et al*., 2015*),* and *Pseudomonas putida* (Glick *et al*., 2005). Moreover, IAA-producing PGPRs are able to stimulate and/or increase plant growth. For example, the IAA-producing PGPR *Bacillus* sp. JH 2-2 improved *Brassica juncea* L. growth by 365-735 % (Shim *et al*., 2015*)*. Patten and Glick (2002) also demonstrated that IAA-producing *Pseudomonas fluorescens* UW4 (formerly called *P. putida*), can improve root length by 35% as well as overall biomass of *Brassica napus*. Moreover, Lin and Xu (2013) revealed that inoculation of *Arabidopsis* with an IAA-producing *Streptomyces* sp. resulted in 34% increased plant biomass as compared to the

untreated control or plants inoculated with a *Streptomyces* mutant that did not have a functional IAA biosynthetic pathway. However, many of the studies mentioned above, did not directly test whether or not the growth promotion seen by their respective PGPRs was indeed due to the synthesis and uptake of bacterial IAA by plants or if it was instead due to another known PGPR plant growth mechanism such as increasing nutrient availability to the plant or lowering plant ethylene via the bacterial enzyme ACC deaminase.

This investigation will seek to answer whether the IAA produced by PGPRs is sufficient to stimulate or inhibit plant growth. Such information is important to determine before wide spread use of PGPRs can be incorporated into agricultural practices. To determine if bacterial IAA will affect plant growth, the host plant *Arabidopsis thaliana* (ecotype Col-0) will be grown hydroponically under metal and non-metal stressed conditions as it has been reported that plants may exude more tryptophan (IAA precursor) into the rhizoshere under stressed conditions (Horii *et al*., 2009). The PGPR *Pseudomonas fluorescens* strain UW4, which can produce IAA and has a functioning ACC deaminase enzyme (lowers plant ethylene), and a mutant, *P. fluorescens* UW4-*acdS*$^{-}$, which has a non-functional ACC deaminase enzyme but retains the ability to synthesize IAA (Li *et al*., 2000), will be utilized in this study. It is hypothesized that if the PGPR *P. fluorescens* UW4 and its mutant synthesize IAA from tryptophan and the IAA synthesized by them can promote plant growth, then the concentration of IAA produced by *P. fluorescens* UW4 and its mutant will be able to promote *Arabidopsis* growth.

4.2 Methods

4.2.1 Culture maintenance and plant growth conditions

Refer to section 2.2.1 and 2.2.2 for procedures on bacterial culture maintenance and plant growth conditions, respectively. In this study plants were grown in hydroponics in the presence of 0 or 20 µM cadmium chloride ($CdCl_2$) or copper sulfate ($CuSO_4$). Metal concentrations were based on a preliminary dose response study (Figure B2A,B)

4.2.2 Plant tryptophan: isolation and measurement

Arabidopsis thaliana seeds were surface-sterilized, synchronized and grown as described in section 2.2.2. After 7 d of growth, autoclaved forceps were used to transfer the screens (and the seedlings) into sterile glass jars containing 25 mL 80% MS medium, 1% sucrose, pH 5.8 and 0 or 20 µM $CdCl_2$ with 3 biological replicates per treatment. The openings of the jars were covered with a foam stopper to allow for gas exchange as well as to prevent contamination. The plants were then allowed to grow for another 9 d, with 1 mL of medium being sampled from each jar every other day; samples were stored at -20°C in 1.5 mL Eppendorf tubes until analysis. To prepare for analysis, the samples were freeze-dried for 24 hours and then re-suspended in 1 mL in high performance liquid chromatography (HPLC) grade acetonitrile. After 5 min of vortexing, samples were then filtered (0.22 µm) to remove any particulate matter. HPLC-MS (mass spectroscopy) was performed using a Thermo Scientific LTQ Orbitrap Discovery (MS 2.5.5) equipped with an Autosampler Accela AS 2.2.1, and pump 1.04.05. The instrument was equipped with a CORTECS C18+ column (Waters), 50 mm length, 2.1mm I.D., and 1.6 µm particle size, that was operating at room temperature. Injection volume was 10 µL. A solvent gradient was employed in this study with a flow rate of 0.4 mL/min. Solvent A was composed of AcN acidified with 0.1 vol% of formic acid whereas solvent B was composed of water acidified with 0.1 vol% of formic acid. The gradient was programmed as follows: solvent A 2 vol%, increased to 10 vol% at 2 min, increased to 25 vol% at 6 min, increased to 50 vol% at 10 min, increased to 75 vol% at 14 min, increased to 95 vol% at 18 min, decreased to 2 vol% at 20 min, followed by 2 min of isocratic elution with 2% of solvent A (total elution time 22min). The LTQ Orbitrap MS was equipped with an electrospray ionization (ESI) source operating in positive ionization mode using the following operating parameters: electrospray voltage of 3.1 kV, sheath gas flow rate of 8 abu (arbitrary unity), auxiliary gas flow rate of 1 abu, capillary temperature of 270°C, capillary voltage set to 49.00 V, and tube lens offset at −148.43 V. Instrument calibration was performed externally prior to each run sequence, employing the Thermo Scientific Pierce LTQ Velos ESI positive ion calibration solutions. Accurate mass spectra of $[MM+H]^+$ ions were recorded from 100 to 1000 m/z, the mass resolution power of the

mass analyzer was set to 30,000 (m/m) at m/z 400. Nitrogen gas (purity 99.95%) was used both as sheath gas and auxiliary gas to serve as the co-collision gas in the HCD cell and the bath gas in the C-trap. The retention time of tryptophan (MM+H = 205.2 m/z) was 0.4-0.8 min as determined by running a 1mg/ mL tryptophan standard in acetonitrile. In attempt to improve resolution, a duplicate set of samples was similarly processed using a C-18 Discovery HS F5 column (Sigma-Aldrich) with a 50 mm length, 2.1 mm I.D., and 3 µm particle size.

4.2.3 Bacterial IAA: isolation and measurement

Both bacterial strains were grown as described in section 2.2.1. After inoculating bacterial colonies into 7.5 mL of TSB, bacterial concentration was measured using a spectrophotometer and adjusted to an OD_{600} of 0.2. After this, 0, 62.5, 125, 250 or 500 µg/ mL of tryptophan was supplemented into the bacterial growth medium (n = 3 for each treatment) to stimulate IAA synthesis based on work conducted by Duca (2013). After 48 hs of growth in a incubating mini-shaker set to 30°C and 200 rpm, 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 1 mL of spent growth medium was transferred into a 1.5 mL Eppendorf tube and stored at -20° C. Samples were prepared and analyzed using HPLC-MS following the protocol in section 4.2.2. The retention time of IAA (MM+H = 176.2 m/z) was from 4.5-8 min as determined by running a 1mg/mL IAA standard in acetonitrile.

4.2.4 Plant growth induced by IAA

Arabidopsis thaliana seeds were surface-sterilized and stratified as described in section 2.2.2. Seeds were sown on 0.8% agar plates containing 80% MS medium with 1% sucrose, adjusted to pH of 5.8. Upon cooling of molten MS-agar medium, 2.5 µg/ mL of IAA (based on results from section 4.2.3) was added to half of the plates with 3 biological replicates used per treatment. Each Petri plate contained 4 seedlings and measurements for plants within a plate were pooled to obtain a single value per plate prior to calculating the treatment averages. Plates were sealed with Parafilm® and placed

in the growth chamber either in a horizontal (aboveground growth) or vertical (below ground growth) orientation under the conditions previously mentioned in 2.2.2. After 14 days of growth, rosette diameter and primary root length were measured using digital calipers, fresh weight was measured, and aboveground area was determined from photographs using image J (https://imagej.nih.gov/ij/download.html.).

4.2.5 Statistical analysis

One-way analysis of variance (ANOVA) and the Tukey post hoc test were performed using SigmaPlot version 13.0 to detect treatment effects and significant differences among treatment means ($p < 0.05$) respectively.

4.3 Results

4.3.1 Plant-produced tryptophan

Tryptophan was not detected in the spent growth medium of *Arabidopsis* grown in the presence of cadmium, copper or no metal stress (lowest standard = 1 μg/mL). This was confirmed using both a C18 cortex column as well as a C18 Discovery HS F5 column. Samples were further concentrated 2-fold by allowing the acetonitrile to evaporate and then reconstituting solutes in 500 μL acetonitrile; however, no tryptophan was detected.

4.3.2 Bacterial IAA

Due to the inability to detect tryptophan in root exudates, the ability of bacteria to synthesize IAA from exogenous tryptophan was tested using concentrations based on previous work by Duca (2013). Wildtype P. *fluorescens* UW4 synthesized up to 2.5 ng/mL of IAA when supplemented with tryptophan (Figure 4.1). The amount of IAA produced did not increase with greater than 125 μg/mL of tryptophan in the medium. Surprisingly, however, the mutant P. *fluorescens* UW4-*acdS*$^{-}$ did not produce detectable

concentrations of IAA when supplemented with tryptophan, as was confirmed by a second run of the experiment.

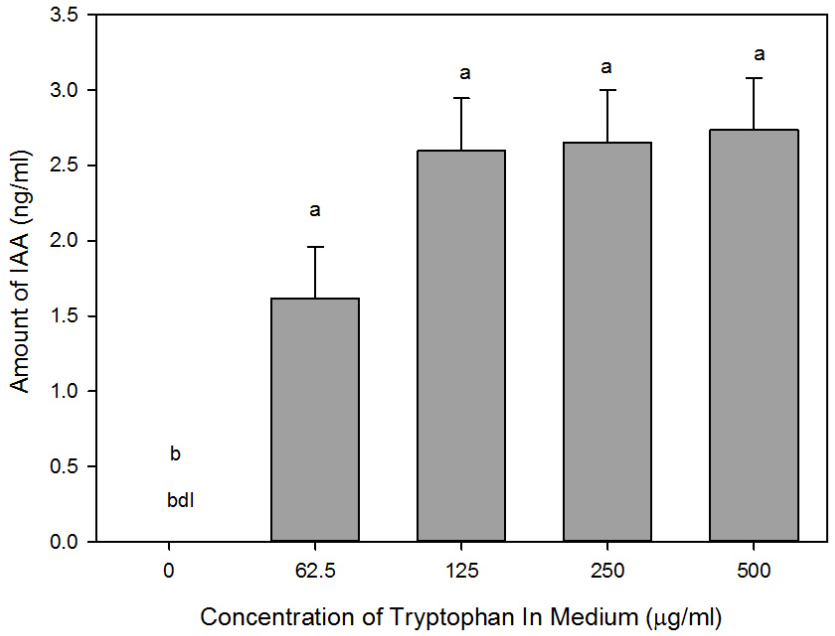

Figure 4.1. Concentration of IAA produced by wildtype *P. fluorescens* UW4. Bacterial strains, with an initial OD_{600} of 0.2, were grown in TSB supplemented with 0, 62.5, 125, 250 or 500 µg/ mL of tryptophan. After 48 hs of growth, cultures were centrifuged and 1 mL of spent growth medium was sampled. Samples were analyzed using HPLC-MS with a C18 Cortex column. Error bars represent standard error, n = 3. Means not sharing a common letter are significantly different (one-way ANOVA followed by Tukey post-hoc test, $p < 0.05$). bdl = below detection limit.

4.3.3 Plant growth induced by IAA

Based on the experiment in section 4.3.2, *Arabidopsis* was grown on MS-agar supplemented with 2.5 ng/mL IAA. Plants grown in the presence of 2.5 ng / mL IAA had increases in some aspects of growth as compared to controls: 50% larger aboveground area (Figure 4.2A) and 40% more fresh weight (Figure 4.2C). However, there was no effect of IAA on mean rosette diameter (Figure 4.2. B) or primary root length (Figure 4.2. D).

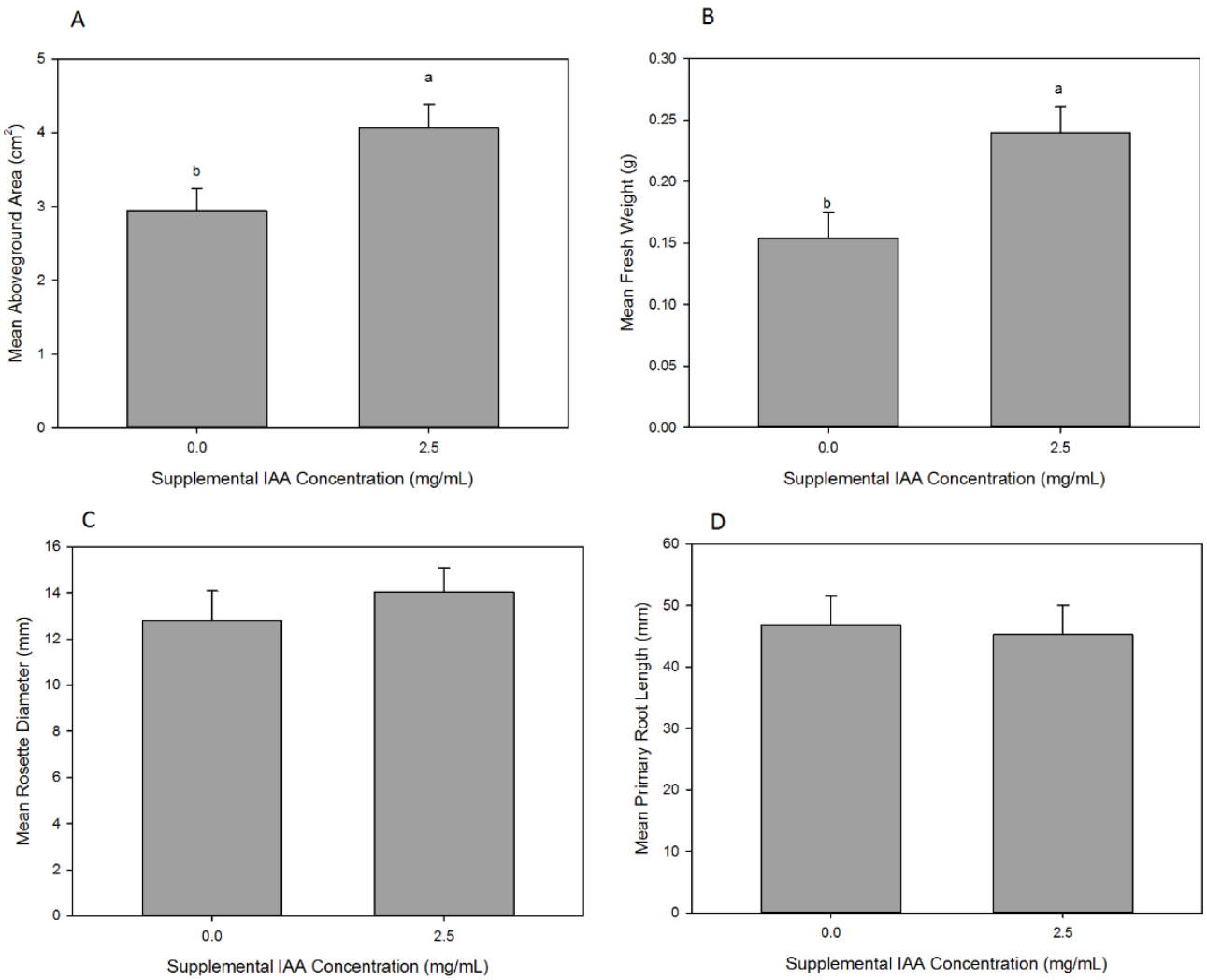
A
Mean Aboveground Area (cm²)
0
1
2
3
4
5
b
a
0.0
2.5
Supplemental IAA Concentration (mg/mL)
B
Mean Fresh Weight (g)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
b
a
0.0
2.5
Supplemental IAA Concentration (mg/mL)
C
Mean Rosette Diameter (mm)
0
2
4
6
8
10
12
14
16
0.0
2.5
Supplemental IAA Concentration (mg/mL)
D
Mean Primary Root Length (mm)
0
10
20
30
40
50
60
0.0
2.5
Supplemental IAA Concentration (mg/mL)

Figure 4.2. Effect of exogenous IAA on *Arabidopsis* growth. Plants were grown on 80% MS medium with our without exogenous IAA. Each of A) aboveground area B) rosette diameter C) fresh weight and D) primary root length were measured after 14 d (n= 3). Error bars represent standard error. Means not sharing a common letter are significantly different (one-way ANOVA followed by Tukey post-hoc test, $p < 0.05$).

4.4 Discussion

4.4.1 Plant-produced tryptophan

One aspect of the PGPR hypothesis proposed by Glick *et al*. (1998) is that PGPRs can synthesize the plant growth hormone IAA from tryptophan present in the rhizosphere. The release of tryptophan by plants is one of the ways thought to facilitate beneficial microbe interactions with the rhizosphere, and tryptophan exudation is assumed to occur at a basal level, even under non-stressed conditions (Glick *et al*. 1998; Malhotra and Srivastava, 2008). However, at least for *Arabidopsis*, this may not be true. Tryptophan was not detected in exudates of *Arabidopsis* grown in hydroponics under non-stressed conditions, even after the samples were concentrated 2-fold. Furthermore it has been proposed that under stress conditions, such as toxic metal stress, plants may exude more tryptophan, and other carbon compounds, into the rhizosphere to help mitigate the stress (Henry *et al*., 2007). As was found under control conditions, addition of cadmium or copper to the growth medium did not result in measureable tryptophan in the spent growth medium. Since the lowest tryptophan standard detected was 1 µg /mL, I cannot conclusively state that metal stress does not induce tryptophan exudation as values under this threshold would not have been detected. It is possible that *Arabidopsis* needs to be grown for longer than 16 d to bring the concentration of exuded tryptophan to above 1 µg /mL or take a larger sample volume and concentrate it. Based on work done by Strehmel *et al*. (2014) it is known that *Arabidopsis* can exude tryptophan into the surrounding medium. Moreover, Kravchenho *et al*. (2004) have shown a minimum concentration of tryptophan is needed to be exuded by plant roots in order for IAA-producing PGPR's to improve plant growth. Specifically, they reported that 2.8-5.3 ng/g exuded by tomato was insufficient for growth promotion while 290-390 ng/g exuded by radish was sufficient to promote growth. If the *Arabidopsis* used in my experiments exuded amounts of tryptophan comparable to the tomato seedlings then it is possible that the IAA-producing abilities of *P. fluorescens* UW4 would not have been detected

4.4.2 Bacterial IAA

Since I was not able to measure tryptophan exuded by *Arabidopsis*, I utilized tryptophan concentrations similar to those used by Duca (2013), a former student of Dr. Bernard Glick. I confirmed that *P. fluorescens* UW4 can synthesize the plant growth hormone IAA when supplemented with tryptophan. The amount of tryptophan produced in my experiment, 2.5 ng/ mL, was 20% lower than that found by Duca (2013), and 99% lower than Li *et al*. (2000) originally reported. Nonetheless, this indicates that wildtype *P. fluorescens* UW4, if given enough tryptophan, has the potential to produce IAA. However the mutant bacterium, *P. fluorescens* UW4-*acdS*$^{-}$, which had been previously demonstrated to synthesize IAA from tryptophan (Li *et al.* 2000), produced less than 1 µg/mL of IAA. It is possible that the mutant lab strain may have lost the ability to synthesize IAA from tryptophan. An experiment should be done to ensure that the mutant has a functional indole-3-pyruvic acid pathway, which is required for tryptophan-dependent IAA synthesis.

4.4.3 Plant growth induced by bacterial IAA

Upon determining that wildtype *P. fluorescens* UW4 produces a maximum of around 2.5 ng/mL of IAA, this amount was then added to the growth medium of *Arabidopsis* to determine its impacts on growth. Aboveground area and fresh weight increased by 50% and 40%, respectively. The increase in plant area and biomass observed in my study is similar to what other studies have seen with supplemental IAA or IAA-produced by PGPR (Lin and Xu, 2013; Patten and Glick, 2002). Most notable, however, is that many studies involving IAA-producing PGPRs report anywhere from 100 to 800% aboveground growth promotion (Glick *et al*., 2005; Shim *et al.*, 2015). These large increases in plant growth were not seen in my study and may indicate that other plant growth-promoting mechanisms such as increased nutrient acquisition or decreasing the amount of plant stress hormones may be how *P. fluorescens* UW4 increases plant growth.

It was surprising to find that exogenous IAA had no effect on primary root length. This runs counter to most experiments done with supplemental IAA or IAA-producing

PGPRs, which report enhanced root growth, root biomass and root length (Patten and Glick, 1996; Kravchenko *et al*., 2004; Shim *et al.* 2015). It is possible that supplying *Arabidopsis* with exogenous IAA stimulates aboveground growth to a greater extent than below ground growth. Kravchenko *et al*. (2004) also reported that IAA-producing bacteria stimulated shoot growth 2-fold more than root growth for radish. Moreover, the effects of supplemental IAA on root growth might take longer to manifest than shoot growth and thus 14 d may not be enough to see root growth promotion in *Arabidopsis*.

4.5 Conclusion

Based on the experiments conducted within this study, I can confidently say that *P. fluorescens* UW4 can produce IAA when given tryptophan and that there is the potential for increased growth promotion as a result of bacterial IAA synthesis and export. Moreover, *Arabidopsis* may exude tryptophan from its roots; however, the amount exuded was not detectable in my experiment and may explain why, in Chapter 3, plants inoculated with *P. fluorescens* UW4 did not experience growth promotion under control and metal stress conditions. I believe that some IAA-producing PGPRs will promote plant growth only if the host plant exudes enough tryptophan, rendering growth promotion only to certain plant species. Alternatively, supplementing tryptophan to low-exuding plants, such as tomato and possibly *Arabidopsis*, may allow associated PGPR's the opportunity to produce IAA and stimulate plant growth. Thus, the IAA aspect of the PGPR plant growth hypothesis appears to be valid under certain conditions (i.e., having enough tryptophan in the rhizosphere) and bacterial IAA can play a role in promoting aboveground plant growth.

4.6 References

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Chapter 5

5 The ethylene hypothesis: Can *Pseudomonas fluorescens* UW4 promote plant growth by reducing stress ethylene and influence other plant hormones in *Arabidopsis* under cadmium and copper metal stress?

In this chapter, I will explore and try to answer the key question of whether or not the PGPR *Pseudomonas fluorescens* UW4 is able to reduce plant stress ethylene under metal stress. I will also evaluate whether *P. fluorescens* UW4 can alter the levels of other plant hormones such as salicylic acid (SA), jasmonic acid (JA) and abscisic acid (ABA). I expected that wildtype *P. fluorescens* UW4 will be able to reduce ethylene since it contains the enzyme ACC deaminase; however, the reduction in ethylene may not be enough to confer growth promotion. An ACC deaminase mutant *P. fluorescens* UW4-*acdS*$^{-}$ will be used to determine whether any growth promotion seen in stressed plants inoculated with *P. fluorescens* UW4 is due to the bacteria reducing ethylene in stressed plant tissue. The results of Chapter 3 intrigued me; I wondered whether or not *P. fluorescens* UW4 did not promote growth because the bacteria altered the levels of other plant hormones and/or activated other hormone-induced stress pathways that limit plant growth.

5.1 Introduction

A stress is any condition that threatens an organism's homeostatic state (Taiz and Zeiger, 2010) and plants are exposed to many challenges and stresses that can be deleterious to growth and survival. Both biotic and abiotic stresses are sensed through the induction of signaling cascades that activate ion channels, which lead to an increase in the synthesis of stress hormones such as ABA, SA, JA, and ethylene (Fraire-Velazquez *et al.*, 2011).

Ethylene, a gaseous plant hormone, is particularly important to the abiotic stress response. Ethylene is produced at low levels under non-stressed conditions and contributes to growth regulation, fruit development and natural senescence (Gamalero *et al*., 2009). Stressed plants produce additional ethylene by the oxidation of 1-amino-cyclopropane-1-carboxylate (ACC) to ethylene by the enzyme ACC oxidase (Glick *et al*., 1998; Gamalero *et al*., 2009). In mildly stressed plants, a small amount of ethylene initiates a protective response; increased stress causes greater ethylene production, which initiates reduced growth and senescence (Stearns and Glick, 2003). In particular, toxic metal stress has been shown to initiate deleterious ethylene production (Fuhrer, 1982; Matto *et al*., 1986) and reduce plant growth (Bankaji *et al*. 2014).

The toxic metals cadmium and copper are readily released from industry (Das *et al*., 1997) and are found in fertilizers (de López Camelo *et al*. 1997; Alloway and Steinnes, 1999) and pesticides (Das *et al*., 1997). Applications of cadmium or copper contaminated agricultural products can increase their concentrations within agricultural fields resulting in reduced crop growth and the potential for these toxic metals to enter our food supply. However, plants are caninteract with beneficial organisms such as fungi and bacteria located within the rhizosphere, the volume of soil under the influence of the plant root, to help mitigate environmental stresses such as excess toxic metals (Sorensen, 1997).

Plant growth-promoting rhizobacteria (PGPR) are one such group of beneficial organisms in the rhizosphere. They form symbiotic relationships with their host and reduce plant stress (Glick, 2014). For example, some PGPR produce ACC deaminase, which modulates plant ethylene biosynthesis and is thought to help promote plant growth under stress conditions (Glick *et al*., 1998; Figure 1.1). The ability of PGPR to increase plant growth under stress conditions is well established. For example, cadmium-stressed barley treated with *Arthrobacter mysorens* 7 or *Flavobacterium* sp. L30 had larger roots than did non-inoculated plants (Pishchik *et al*., 2002). Similarly, sunflower had greater biomass and root/shoot length when grown in copper-contaminated soils, when also inoculated with *Acinetobacter* sp. CC30 (Rojas-Tapias *et al*., 2012). All of the PGPR mentioned above produce IAA and contain the ACC deaminase gene (*acdS*). PGPRs that

have ACC deaminase has also been shown to increase the growth of rice (Bal *et al*., 2013) and wheat (Nadeem *et al*., 2013) under salt and water stress. Moreover, it has been shown that some PGPRs associated with plant roots may be able to alter the levels of plant hormones such as SA, JA and ABA by either directly stimulating or inhibiting their production (Kurepin *et al*., 2015), potentially decreasing plant stress and increasing plant growth. PGPRs that synthesize and exude IAA and/or have ACC deaminase may also indirectly alter plant hormones via decreasing ethylene and increasing endogenous IAA concentrations within plant tissues resulting in potential changes in the concentrations of other plant hormones. For example, it is known that ethylene and IAA can alter the concentrations of hormones such as JA (Devoto and Turner, 2003), SA (Wang *et al*., 2002) and ABA (Wilson *et al*., 1990), thus a PGPR that can alter IAA and ethylene concentrations could also alter these hormones. Clearly, PGPR can increase plant growth under a variety of stresses. However, how ACC deaminase and/or bacterial IAA reduce plant stress is still unresolved.

Glick *et al*. (1998) were the first to propose mechanisms for how bacterial IAA and ACC deaminase could reduce plant stress (Figure 1.1C). First, if bacteria are in close contact with the root, bacterial IAA might be taken up by the plant where it could stimulate ACC synthase to convert S-adenosylmethionine to ACC and/or directly enhance root growth (Glick *et al*., 1998). Second, ACC exuded from the root could be taken up by the PGPR and metabolized into ammonia and α-ketobutyrate by the bacterial enzyme ACC deaminase (Honma and Shimomura, 1978; Glick *et al*., 1998). If the amount of ACC remaining in the root is low, ethylene concentrations might be maintained near basal levels thereby preventing stress-induced responses (Glick *et al*., 1998).

The PGPR *Pseudomonas fluorescens* UW4 has been shown to promote plant growth and root elongation 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). *P. fluorescens* UW4 was first isolated from roots of common reeds in Waterloo ON, and has been shown to contain the enzyme ACC deaminase, as

well as to produce the beneficial auxin IAA (Glick *et al.* 1995). An ACC deaminase-mutant of *P. fluorescens* UW4 (called UW4-*acdS*$^{-}$) was created that can produce IAA but has no measureable ACC deaminase activity (Li *et al.*, 2000). Comparing the effects of these two bacterial strains on plant growth allows for the relative roles of these two pathways on plant growth promotion to be assessed.

My investigation will answer if wildtype *P. fluorescens* UW4 can reduce toxic metal-induced ethylene accumulation and thereby increase plant growth. This study will also determine whether or not *P. fluorescens* UW4 can alter the concentrations of SA, JA and ABA *in planta*. To determine whether *P. fluorescens* UW4 can alter plant hormones, the host plant *Arabidopsis thaliana* (ecotype Col-0) will be grown in nutrient-agar contaminated with cadmium or copper and inoculated with *P. fluorescens* UW4 or its ACC deaminase mutant, *P. fluorescens* UW4-*acdS*$^{-}$. By comparing plants inoculated with the mutant strain to those with the wildtype strain, I can evaluate the independent impacts of bacterial ACC deaminase and IAA on plant growth, ethylene production, and concentrations of SA, JA and ABA. I hypothesized that if an ACC deaminase containing PGPR like *P. fluorescens* UW4 can reduce plant ethylene and promote growth in stressed conditions, then *Arabidopsis* inoculated with wildtype *P. fluorescens* UW4 grown in the presence of cadmium or copper stress, will have lower ethylene levels and greater growth promotion, than plants inoculated with *P. fluorescens* UW4-*acdS*$^{-}$ and non-inoculated controls. I also hypothesized that since PGPRs and other bacteria can induce SA synthesis within plant tissue (Métraux, 2001; Kurepin *et al*., 2015) then inoculation with *P. fluorescens* UW4 or *P. fluorescens* UW4-*acdS*$^{-}$ will increase SA within *Arabidopsis*. Lastly, I hypothesized that if PGPRs can alter plant hormone concentrations, then inoculation with *P. fluorescens* UW4 or *P. fluorescens* UW4-*acdS*$^{-}$ will alter JA and ABA levels within plant tissue due to crosstalk between these hormones with ethylene, SA and IAA.

5.2 Methods

5.2.1 Bacterial strains and culture maintenance

See sections 2.2.1 and 3.2.1 for full methods.

5.2.2 Plant variety and growth conditions

For this study, *Arabidopsis thaliana* (ecotype Col-0) and three ethylene over-producing *A. thaliana* mutants, *eto1-1*, *eto2*, and *eto3* were used. The *eto1-1*, *eto2* and *eto3* mutants were identified and characterized previously (Guzman and Ecker, 1990; Kieber *et al*., 1993; Roman *et al*., 1995) and have mutations in enzymes from the ethylene biosynthetic pathway such as ACC synthase resulting in the ethylene over-producing phenotype. All three mutants belong to the Columbia (Col) parental line. The *eto1-1* mutant line overproduces ethylene by 6.5 fold over wildtype and has smaller rosettes and grows more slowly (Woeste *et al*., 1999). Both *eto2* and *eto3* mutant lines overproduce ethylene by 18- (Vogel *et al*., 1998) and 13.5-fold (Woeste *et al*., 1999) respectively, as well as have smaller roots than wildtype (Kieber *et al*., 1993). Seed sterilization, inoculation with bacteria and growth conditions are the same as in section 3.2.2. Plants will be grown in the presence of 0, 10, or 20 μM of cadmium chloride or copper sulfate. These concentrations were chosen based on a dose response as seen in Figure B2A,B.

5.2.3 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 were prepared for staining following the methods in section 2.2.3.

5.2.4 Analysis of plant health and growth

Plant fresh weight (biomass), area, and primary root length were measured following the protocols in section 3.2.9.

5.2.5 Cadmium and copper content

Cadmium and copper concentrations in plants were measured to ensure the metals were being taken up and to determine whether bacterial inoculation affected metal uptake. The concentration of cadmium and copper 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 plant tissue was ground using a mortar and pestle and subsamples were taken for analysis. The amount of plant tissue added to a 15 mL glass test tube was 0.05 g. A standard reference material from the National Institute of Standards and Technology (NIST 1570a, spinach leaves) and reagent blanks were also included in the analysis. All of the test tubes were placed in a rack and 200 μL of ultrapure nitric acid (OmniTrace®, EM Science, USA) was added to each sample. Test tubes were covered with glass marbles to prevent evaporation while allowing pressure to escape. The samples were allowed to sit overnight at room temperature 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 (to ensure even heat distribution) and heated to 90-100°C on a hot plate until the vapors became transparent. 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 12.5 mL. The samples were filtered again (0.45 μm) immediately prior to being analyzed for cadmium and copper content by inductivity-coupled plasma atomic emission spectrometry (ICP-AES).

5.2.6 *AcdS* gene expression under cadmium and copper stress

Expression of the ACC deaminase gene (*acdS*) was measured to verify its presence in the wild type bacterium and absence in the mutant, and to determine if bacterial ACC deaminase expression is affected by metal stress. Bacteria were grown as described in sections 3.3.1 with 0 or 20 µM of cadmium chloride or copper sulfate. After 24 hr incubation, the bacteria were lysed with Trizol® and RNA extracted using chloroform followed by isopropyl alcohol to precipitate the RNA. Bacterial RNA quality was checked using a Nanodrop spectrophotometer. Then, all RNA was converted into cDNA using Pac DNA polymerase, dNTP's and a Qiagen QuantiTect Reverse Transcription Kit. The cDNA was stored at -20˚C or used immediately for RT-PCR or qPCR (RT-PCR and qPCR conditions are presented in Table C1). To see if cadmium or copper altered ACC deaminase gene expression, PCR products were run on 1% agarose gels in 0.5× TBE buffer for 45 min. qPCR was used with SYBR® Green fluorescence dye. To quantify expression, a ΔΔCq method ($p < 0.05$) was preformed using Bio-Rad CFX Manger 3.1 software (melt curve for qPCR is found in Figure C1). The primers used for both RT-PCR and qPCR were bacterial ACC deaminase for *acdS* gene expression and the ribosomal subunit16S for the control gene (Table C1).

5.2.7 Ethylene production

This series of experiments was done to determine if the wild type *P. fluorescens* UW4 reduced ethylene production in *A. thaliana* (Col-0) and three mutants that overproduce ethylene, under cadmium and copper stress. In the experiment, *A. thaliana* (Col-0) and its ethylene mutants were inoculated with wildtype *P. fluorescens* UW4 or *P. fluorescens* UW4-*acdS*$^-$, each at OD_{600} of 0.1 or with 0.03 M $MgSO_4$ (as a control).

5.2.7.1 Cadmium and copper induced ethylene stress

Arabidopsis thaliana (Col-0) was grown in 40 mL a borosilicate glass vial containing the same media compositions as outlined in section 3.3.2. Vials were covered with foam plugs for the first 10 d to allow ethylene, which would inhibit early growth, to escape; before the foam was replaced with screw-on low-bleed septa caps. After 14 d of growth, 1 mL of headspace from each vial was sampled (Abts *et al*., 2013) and the ethylene concentration was measured using gas chromatography with a flame ionization detector (GC-FID) following the protocol of Zheng *et al*. (2013). Ethylene measurements were normalized to plant size by dividing ethylene concentration by the aboveground area of each plant.

5.2.7.2 Ethylene mutant study

Inoculated *A. thaliana* (Col-0), and 3 ethylene over-producing mutants, *eto1-1*, *eto2*, and *eto3*, were grown in 5 mL vials containing 3 mL MS agar medium (without added cadmium or copper) and sealed with foam stoppers. After 12 d of growth, foam stoppers were replaced with rubber septa and the plants continued to grow for an additional 2 d. Ethylene concentrations were determine by following the methods described in section 5.2.7.1.

5.2.8 Plant hormone isolation and concentration

Arabidopsis thaliana (Col-0) seeds were grown on MS-agar plates containing 0, 10 or 20 μM of cadmium chloride or copper sulphate and grown and inoculated with *P. fluorescens* UW4 or its mutant as described in section 3.3.2. After 14 d of growth, plants were harvested and hormones extracted following a protocol modified from Forcat *et al.* (2008). Plants were frozen using liquid nitrogen, ground using a mortar and pestle, and 10 mg tissue (root plus shoot) was placed into a 1.7 mL Eppendorf tube. Then, 400 μL of 10% methanol containing 1% acetic acid was added to each Eppendorf tube to extract the plant hormones. Each treatment included an extraction control containing no plant

material. Samples were vortexed for 1 min and placed on ice for 30 min, followed by centrifugation at 13,000 g for 10 min at 4˚C. The supernatant was collected into a separate Eppendorf tube and the sample was re-extracted following the same procedure as stated above and the supernatants were pooled. Samples were then placed at -20˚C until ready to be analyzed. Samples and standards as well as HPLC-MS conditions were the same as described in section 4.2.2. The retention times for ABA (MM+H = 265.3 m/z), SA (MM+H = 139.1 m/z), and JA (MM+H = 211.2 m/z) were 4.8, 9.95, and 8.95 min, respectively, as determined by running a 1 mg/ mL standard of each compound in acetonitrile.

5.2.9 Statistical analysis

Two-way analysis of variance (ANOVA) and Holm-Sidak post hoc test were performed using Sigma Plot version 13.0 to detect treatment effects and significant differences among treatment means, respectively ($p < 0.05$).

5.3 Results

5.3.1 Bacterial colonization and survival on plant roots

Both *P. fluorescens* UW4 and mutant *P. fluorescens* UW4-*acdS*$^-$ are able to adhere to *Arabidopsis* roots (Figure 5.1). Furthermore, there appeared to be no difference between the mutant bacterium and the wildtype in terms of their ability to adhere to plant roots. Neither the presence of 20 μM cadmium (Figure 5.1 D,E,F) or copper (Figure 5.1 J,K,L) affected the survival of the bacteria or its adherence to the roots.

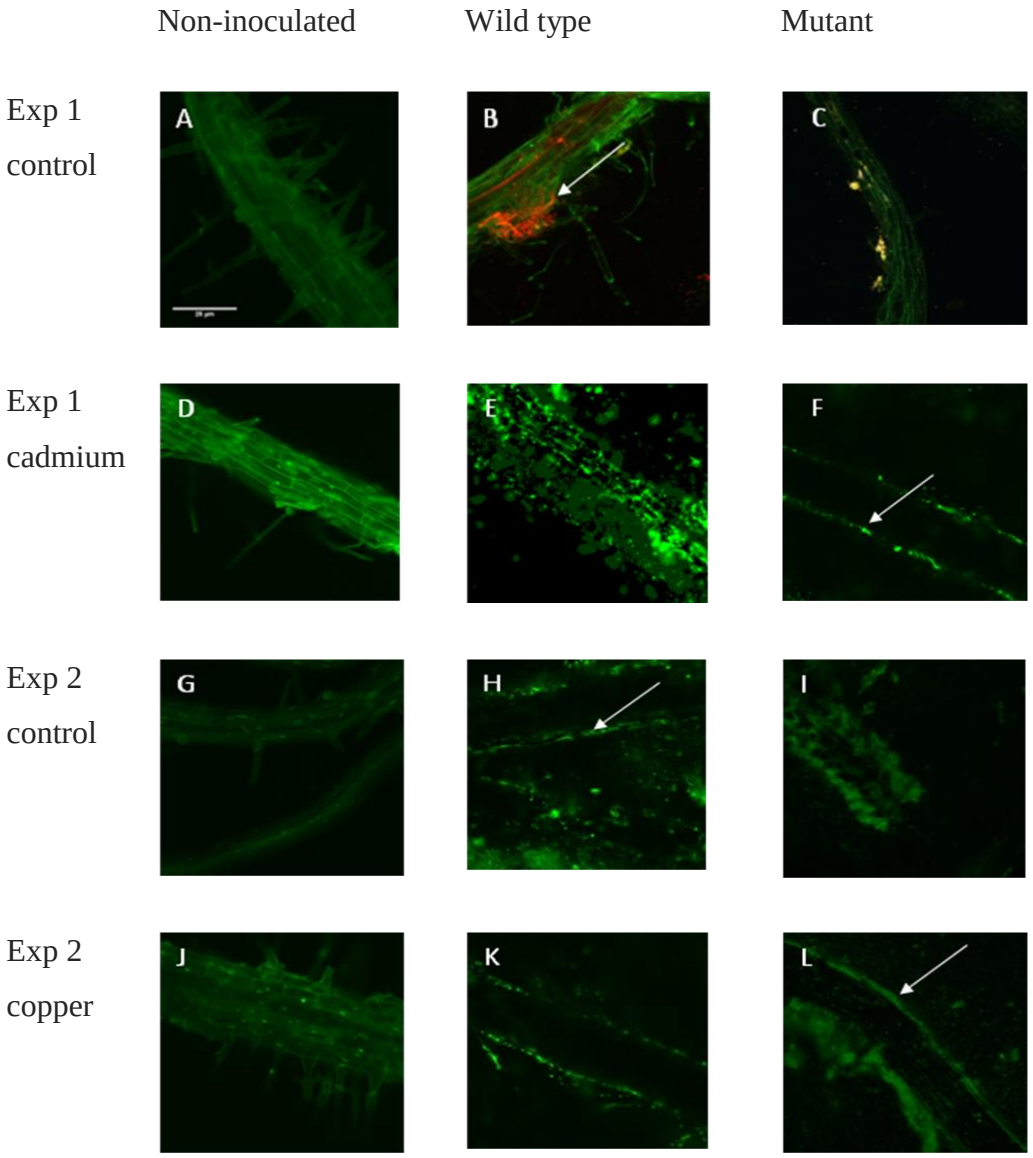

Figure 5.1. Confocal micrographs of roots inoculated with *P. fluorescens* UW4 grown under metal stress. Fluorescence confocal micrographs of bacterial colonization of *Arabidopsis* roots in the absence of cadmium (A, B, C) and copper (G, H, I) or 20 µM cadmium chloride (D, E, F) or 20 µM copper sulfate (J, K, L). Plants were inoculated with no bacteria (A, D, G, J), wildtype *P. fluorescens* UW4 (B, E, H, K), or mutant *P. fluorescens* UW4-*acdS*$^{-}$ (C, F, I, L). The white arrows indicate examples of a bacterium or bacterial colony. Green fluorescence indicates live bacteria, red fluorescence indicates dead bacteria and yellow indicates overlap of live/dead bacteria. Roots autofluoresced green.

5.3.2 Analysis of plant size

Plants inoculated with either bacterial strain were generally smaller than non-inoculated plants. The bacteria reduced both aboveground size (Figure 5.2A,B,C) as well as impaired root elongation (Figure 5.2D,E,F). In addition, plants inoculated with the bacteria (Figure 5.2B,C) were more chlorotic (yellowing of the leaves) than the non-inoculated control (Figure 5.2A).

This general trend of inoculated plants being smaller is quantified in Figure 5.3. Plants inoculated with *P. fluorescens* UW4 had equal or smaller size under cadmium and copper stress than non-inoculated controls or those inoculated with the mutant bacteria. For plants that were grown in the presence of cadmium (Figure 5.3A-C) inoculation with the wildtype or mutant bacteria resulted in a 47-73% smaller aboveground area when compared to non-inoculated controls. Inoculation of control plants resulted in a 40% lower fresh weight but under cadmium stress, inoculation did not result in further reductions in fresh weight (Figure 5.3B). Plant primary root length was reduced 25-46% by bacterial inoculation, with or without cadmium (Figure 5.3C). In contrast, for plants grown in 20 μM copper, wildtype *P. fluorescens* UW4 appeared to result in increased aboveground area and fresh weight, although the increase was not statistically significant (Figure 5.3D.E). Primary root length of plants inoculated with wildtype *P. fluorescens* UW4 had 35-53% smaller roots, compared to control plants grown in copper-contaminated medium (Figure 5.3F).

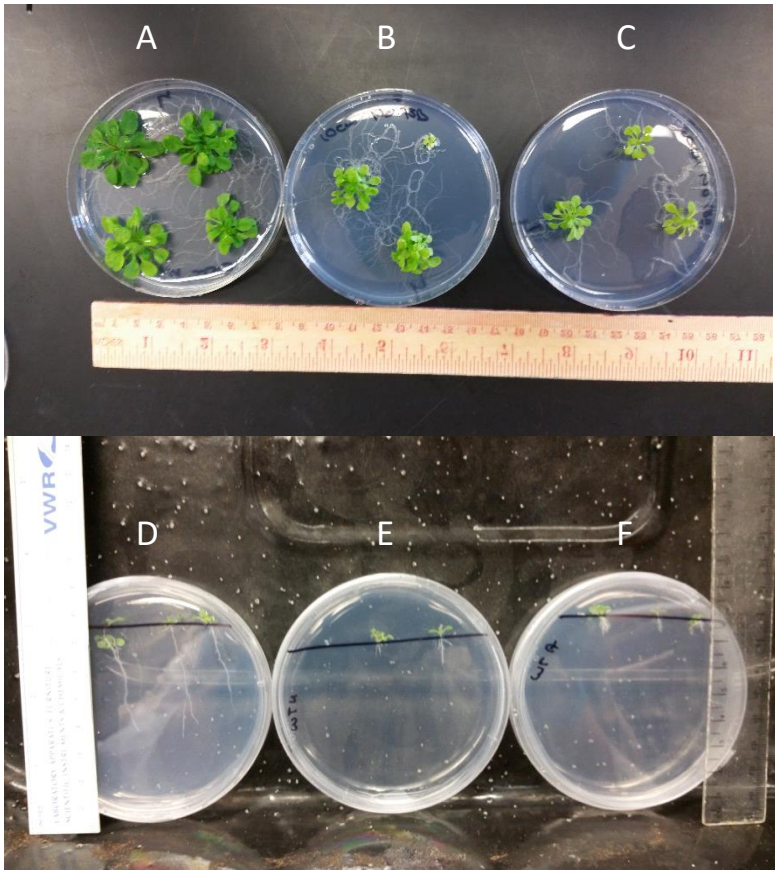

Figure 5.2. Images of *Arabidopsis* grown with and without cadmium stress. Shoots (A-C) and roots (D-F) are shown for plants grown for 14 d on MS-agar medium supplemented with 10 µM cadmium chloride Plants were non-inoculated (A,D), or inoculated with *P. fluorescens* UW4 (B,E), or *P. fluorescens* UW4-*acdS*$^{-}$ (C, F). Images were taken using a Canon EOS Rebel T5 18.0MP camera.

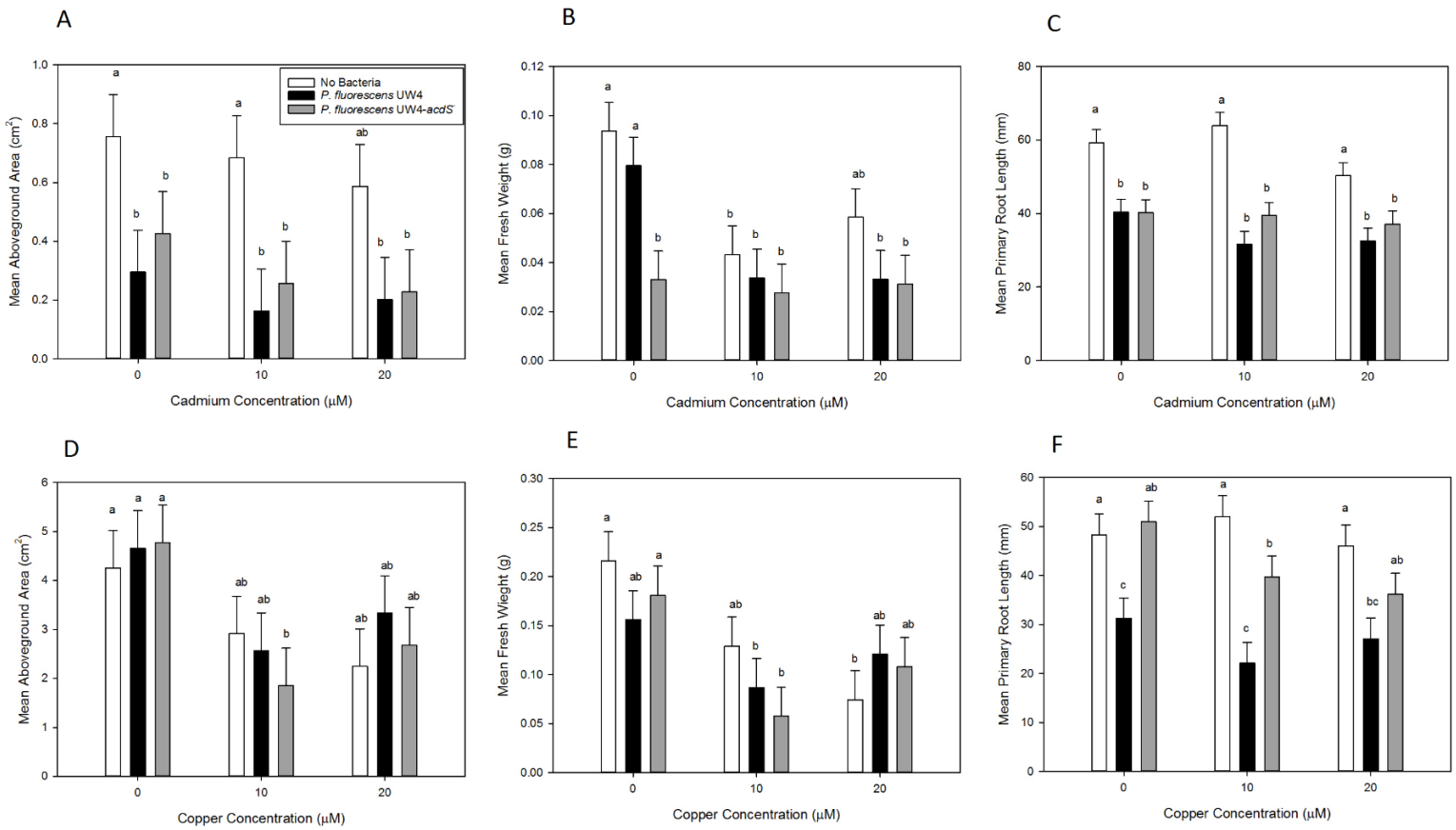

Figure 5.3. Growth of Arabidopsis inoculated with plant *P. fluorescens* UW4 grown under metal stress. Leaf area, plant fresh weight and primary root length were measured for 14 day old *Arabidopsis* grown in 80% agar and 1% sucrose supplemented with 0, 10 or 20 µM of either cadmium chloride (A-C) or copper sulphate (D-F). Leaf area was determined by taking images with a Canon EOS Rebel T5 18.0MP camera; images were analyzed using ImageJ. Error bars represent standard error. Means not sharing a common letter are significantly different (two-way ANOVA followed by Holm-Sidak post-hoc test, $p < 0.05$, n =5).

5.3.3 Cadmium and copper content

The concentration of cadmium within plant tissue increased with the dose in the growth medium (Figure 5.4A), indicating that plants take up cadmium, in general, proportional to its presence in the medium. Plants inoculated with both the wildtype or mutant bacterium had less cadmium when compared to non-inoculated controls, although this difference was not significant (Figure 5.4B). Although increasing copper in the growth medium appeared to increase copper in the plants, the differences were not significant, nor did inoculation affect the uptake of copper. In all cases, however, the large error bars in Figure 5.4 indicate that there was a lot of sample variance, which could be corrected by increasing the sample size or the sample mass that was collected for analysis.

5.3.4 *AcdS* gene expression under cadmium and copper stress

The phenotypes of the bacterial strains were confirmed. The wildtype bacterium, *P. fluorescens* UW4 did express the ACC deaminase gene (*acdS*) while the mutant did not (Figure 5.5A,B). Moreover, the *acdS* transcript was produced under both metal stress and potentially non-metal stressed environments (Figure 5.5A,B). In conjunction with standard RT-PCR, qPCR data revealed that relative *acdS* expression was not affected by 20 μM cadmium or copper (Figure 5.5C,D).

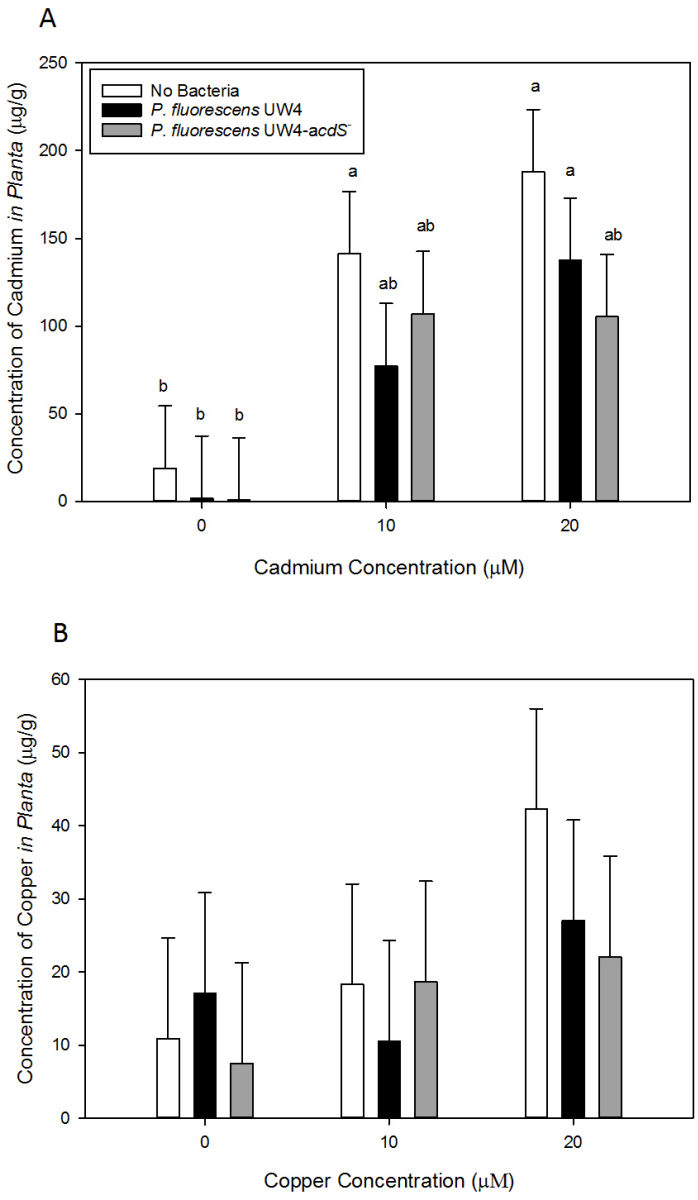
A
No Bacteria
P. fluorescens UW4
P. fluorescens UW4-acdS-
Concentration of Cadmium in Planta (µg/g)
250
200
150
100
50
0
b
b
b
a
ab
ab
a
a
ab
0
10
20
Cadmium Concentration (µM)
B
Concentration of Copper in Planta (µg/g)
60
50
40
30
20
10
0
0
10
20
Copper Concentration (µM)

Figure 5.4 Toxic metal concentrations in *Arabidopsis* inoculated with bacteria. Cadmium and copper content were measured in 14 day old *Arabidopsis* grown in 80% agar and 1% sucrose supplemented with 0, 10 or 20 μM of either cadmium chloride (A) or copper sulphate (B). Dried tissue samples (combined shoot and root) were acid digested then analyzed using ICP-MS. Error bars represent standard error. Means not sharing a common letter are significantly different (two-way ANOVA followed by Holm-Sidak post-hoc test, $p < 0.05$, $n = 3$).

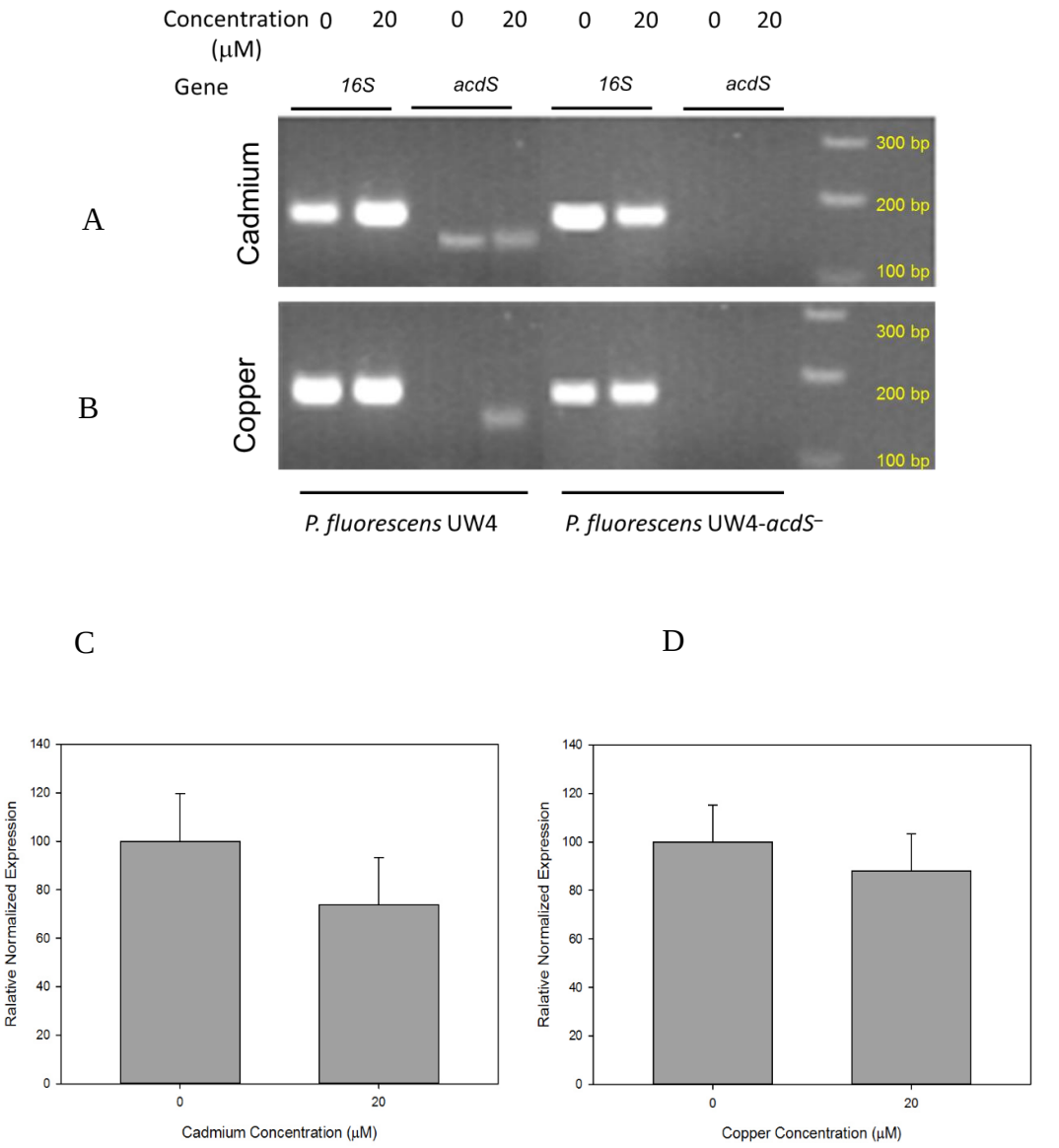

Figure 5.5. Expression of bacterial ACC deaminase under cadmium and copper stress. *Pseudomonas fluorescens* UW4 and *P. fluorescens UW4-acdS*$^{-}$ were grown for 24 h in TSB medium supplemented with 20 μM cadmium chloride (A,C) or 20 μM copper sulfate (B,D). Bacterial RNA was extracted, converted into cDNA, and analyzed using (A,B) RT-PCR or (D,C) qPCR($\Delta\Delta$Cq, $p > 0.05$, and error bars represent standard error).

5.3.5 Ethylene production

Non-inoculated metal-stressed plants produced up to 25-fold more ethylene than the controls, which produced negligible amounts of ethylene (Figure 5.6). Plants inoculated with wildtype *P. fluorescens* UW4 produced 72-100% less ethylene than did plants inoculated with *P. fluorescens* UW4-*acdS*$^{-}$ and non-inoculated controls under the highest doses of cadmium or copper in the growth medium (Figure 5.6). Plants inoculated with the *P. fluorescens* UW4-*acdS*$^{-}$ had similar ethylene levels to those of non-inoculated plants, as expected since the ACC deaminase gene was non-functional.

Although wildtype *P. fluorescens* UW4 reduced the production of metal-induced ethylene in *Arabidopsis* (Col-0) (Figure 5.6), it did not do the same to the *Arabidopsis* ethylene over-producing mutants (Figure 5.7). In the absence of metal stress, inoculation with bacteria increased the amount of ethylene produced by wildtype plants by 5-fold (Figure 5.7A). A similar pattern was seen for the ethylene over-producing *eto1-1* mutant for which ethylene increased by 40% and 3-fold with plants inoculated with the wildtype or mutant *P. fluorescens* UW4, respectively (Figure 5.7 A). For the *eto2* mutant the presence of the bacteria had no effect on ethylene levels when compared to non-inoculated *eto2* plants. Inoculation of *eto3* mutant roots with wildtype *P. fluorescens* UW4 resulted in a 37% decrease in plant ethylene and brought it to a concentration comparable to that of non-inoculated wild type *Arabidopsis* (Col-0). In terms of the ability of *P. fluorescens* UW4 to increase the growth of these mutants, it is clear that, in general, they have no net beneficial effects on promoting growth. In particular the presence of wildtype *P. fluorescens* UW4 reduced primary root length in the wild-type *Arabidopsis* by 77% (Figure C). However, inoculation with *P. fluorescens* UW4 on *Arabidopsis* ethylene over-producing mutants did not reduce root length, indicating a potential protective effect against ethylene-induced root inhibition.

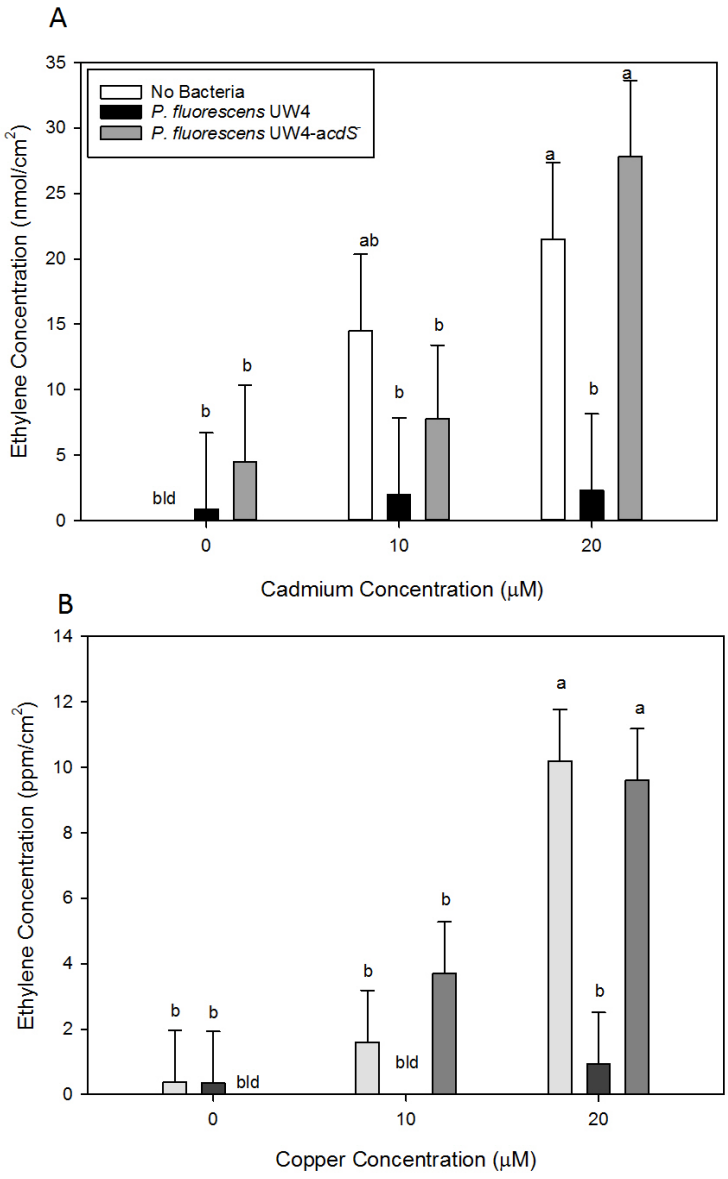

Figure 5.6. Cadmium- and copper-induced ethylene production. Ethylene was measured in 14 day old *Arabidopsis* grown in 80% agar and 1% sucrose supplemented with 0, 10 or 20 µM of either cadmium chloride (A) or copper sulphate (B). Control and inoculated *Arabidopsis* were grown in 40 mL glass vials containing MS-agar supplemented with up to 20 µM cadmium or copper. After 14 d, 1 mL of headspace was removed using a syringe and injected into a GC-FID. Error bars represent standard error. Means not sharing a common letter are significantly different (two-way ANOVA followed by Holm-Sidak post-hoc test, $p < 0.05$, $n = 3$). bdl = below detectable limits.

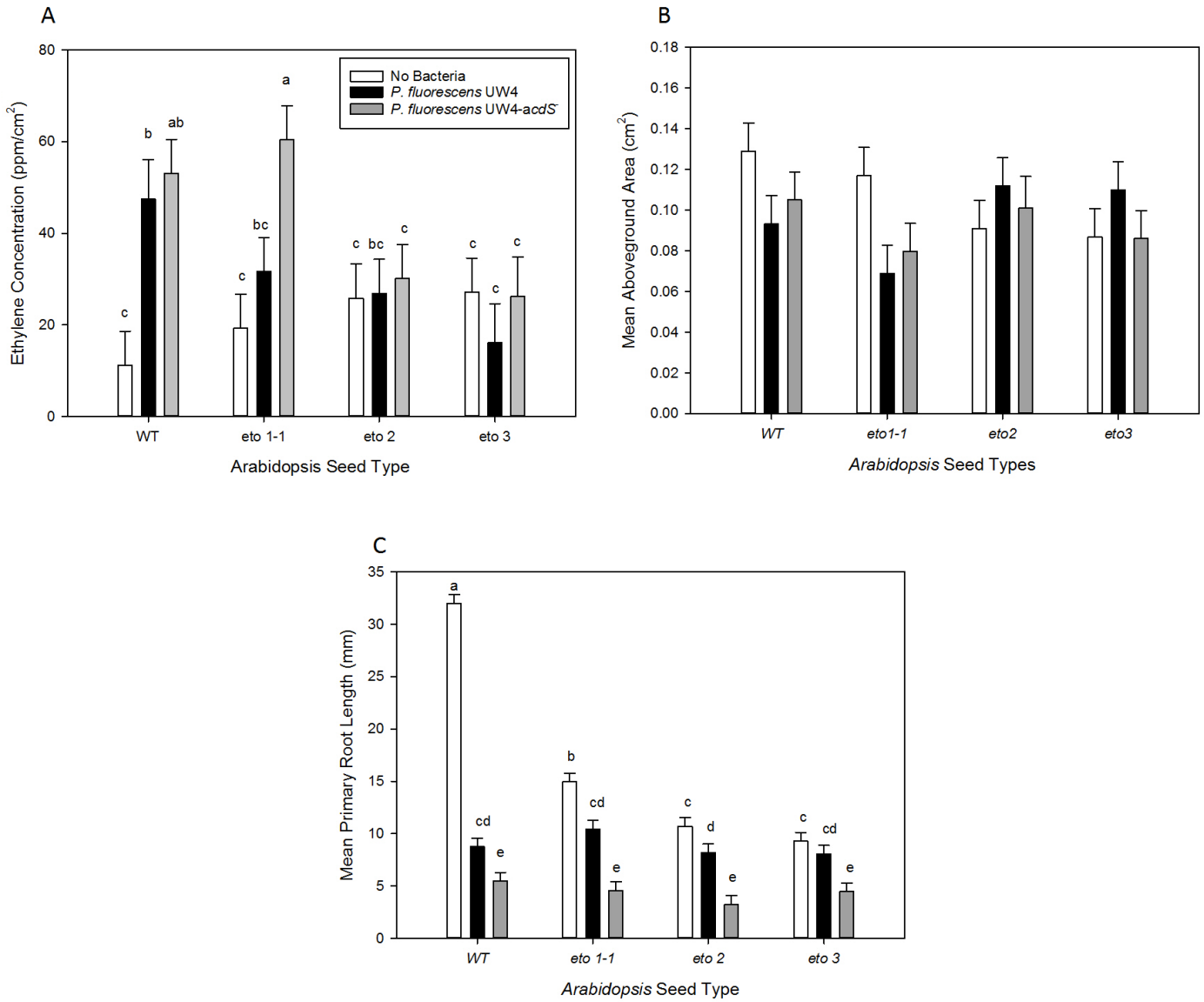
A
Ethylene Concentration (ppm/cm2)
80
60
40
20
0
No Bacteria
P. fluorescens UW4
P. fluorescens UW4-acdS-
c
b
ab
c
bc
a
c
bc
c
c
c
c
WT
eto 1-1
eto 2
eto 3
Arabidopsis Seed Type
B
Mean Aboveground Area (cm2)
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
WT
eto1-1
eto2
eto3
Arabidopsis Seed Types
C
Mean Primary Root Length (mm)
35
30
25
20
15
10
5
0
a
cd
e
b
cd
e
c
d
e
c
cd
e
WT
eto 1-1
eto 2
eto 3
Arabidopsis Seed Type

Figure 5.7. Inoculation to the rescue? Test of bacterial ACC deaminase on decreasing plant-produced ethylene in three ethylene over-producing 7 d old *Arabidopsis* mutants. Wildtype (WT), *eto1-1*, *eto2*, and *eto3* were inoculated with wildtype *P. fluorescens* UW4 or *P. fluorescens* UW4-*acdS*$^{-}$ and grown on MS agar medium in 5 mL glass vials. 1 mL of headspace was removed using a syringe and injected into a GC-MS. . Inoculation with wildtype *P. fluorescens* UW4 had a beneficial effect, lowering the amount of ethylene produced by *eto 3* by 37% (A). Inoculation with the mutant *P. fluorescens* UW4-*acdS*$^{-}$ also did not promote growth in most *Arabidopsis* mutants (B,C). Error bars represent standard error. Means not sharing a common letter are significantly different (two-way ANOVA followed by Holm-Sidak post-hoc test, $p < 0.05$, $n = 3$).

5.3.6 Plant hormone content

In general, the concentrations of the hormones ABA, SA and JA did not change between non- and metal-stressed conditions (Figure 5.8,D1-sample chromatogram). In all cases, the concentrations of these three hormones did not vary in response to inoculation with the mutant bacterium. While the concentrations of ABA, SA and JA seemed to increase in *Arabidopsis* inoculated with wildtype *P. fluorescens* UW4 there were no significant differences from the control, with the exception of SA, which increased by 30% in the 20 μM copper treatment (Figure 5.8D).

5.4 Discussion

5.4.1 Bacterial colonization and survival on plant roots

One of the criteria for increasing plant growth using PGPRs is that the bacteria are able to adhere to the plant root as well as stay alive. I show here that the bacteria are able to adhere to and survive on *Arabidopsis* roots (Figure 5.1). This result was not surprising given that *P. fluorescens* are known to adhere to plant seed and root surfaces (Hong *et al*., 1991). Furthermore, the ineffectiveness of cadmium or copper to affect the adherence and survival of *P. fluorescens* UW4 was also expected given that the bacterium can withstand up to 250 μM $CdSO_4$ (Manara et. al., 2012) and 3 mmol $CuSO_4$ (Chen et al., 2006), while still being able to adhere to surfaces (McEldowney, 1994). These results increase the likelihood that a plant-microbe interaction can occur between *Arabidopsis* and *P. fluorescens* UW4, which may increase plant growth and reduce metal-induced stress ethylene.

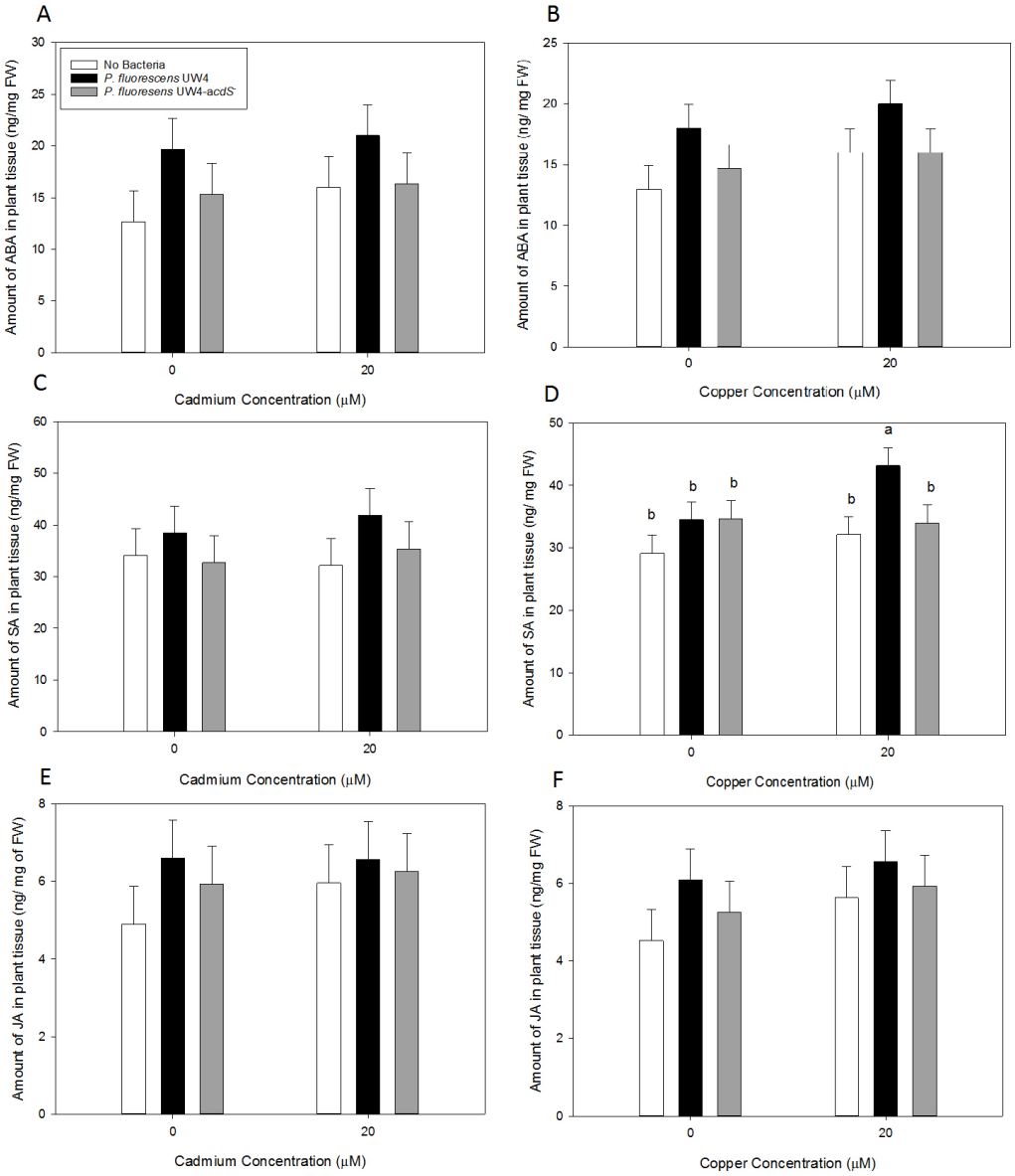

Figure 5.8. Can inoculation alter plant hormones? *In planta* concentration of ABA (A, B), SA (C, D) and JA (E, F) of were measured for 14 day old *Arabidopsis* grown in 80% agar and 1% sucrose supplemented with 0, 10 or 20 µM of either cadmium chloride (A, C, E) or copper sulphate (B, D, F). Plants were harvested and hormones were extracted for HPLC-MS analysis. Error bars represent standard error. Means not sharing a common letter are significantly different (two-way ANOVA followed by Holm-Sidak post-hoc test, $p < 0.05$, $n = 3$).

5.4.2 Analysis of plant growth

An important characteristic of a PGPR, as its name would imply, is to be able to promote plant growth. Based on my results, it is clear that *P. fluorescens* UW4 does not consistently promote *Arabidopsis* growth under metal and non-stress conditions (Figure 5.2, 5.3). Plants that were inoculated with wildtype *P. fluorescens* UW4 were up to 73%, 40%, and 46% smaller in aboveground area, fresh weight and primary root length, respectively, when compared to non-inoculated controls within the same cadmium treatment. Moreover, primary root length of *Arabidopsis* grown in copper contaminated medium inoculated with the wildtype bacterium were up to 53% smaller than plants inoculated with the mutant bacterium, which had roots of equal length to non-inoculated plants.

These results, as a whole, go against what has been reported in the literature about *P. fluorescens* UW4. All previous reports on the strains of *P. fluorescens* used here led me to expect that the bacterial treatments would result in increased plant size under stress conditions, and that plants inoculated with wildtype *P. fluorescens* UW4 would have been larger than those inoculated with mutant *P. fluorescens* UW4-*acdS*$^{-}$ due to a lack of ACC deaminase production in the mutant strain. For example Cheng *et al*. (2007) found that when canola plants were inoculated with wildtype *P. fluorescens* UW4 under salt stress, the canola fresh weight was 7-fold larger than non-inoculated plants in the same treatment. Moreover, Li *et al.* (2000) reported that when canola was inoculated with the mutant *P. fluorescens* UW4-*acdS*$^{-}$, root elongation was not observed and roots inoculated with the wildtype bacterium were 33% longer than those inoculated with the mutant or non-inoculated controls. In contrast, I observed no increase in growth, and instead observed a decrease in growth of *Arabidopsis* when inoculated with *P. fluorescens* UW4.

In my study, *P. fluorescens* UW4 appears to beslightly pathogenic, resulting in greater stress being added to the plant, thus reducing overall plant growth. My PGPR-plant interactions occurred in agar containing both sucrose and MS plant medium, which runs in stark contrast to the growth medium used in many studies that report positive PGPR-plant

interactions (Hontzeas *et al*., 2004; Dell'Amico *et al*., 2008). To further explain my negative PGPR-plant interaction, perhaps, the bacteria are competing with plants for nutrients such as ironresulting in reduced growth. It is also possible, since the bacteria cannot grow on MS medium alone (Figure A1A), which once adhered to plant roots, the bacteria become entirely dependent on the plant to survive. Thus the bacteria may start a pathogenic relationship with the plant, inducing greater plant exudation of photosynthates or amino acids to ensure their survival, at the expense of the plants. However, this is all speculation and should be tested to confirm or deny its validity.

5.4.3 Cadmium and copper uptake

A potential use of PGPRs is to assist in bioremediation of toxic metal-contaminated soils. When PGPRs are grown in association with plants there is the potential for increased metal uptake by plants as PGPRs can not only promote plant growth under metal stress conditions but they can also increase the bioavailability of metals through the production of siderophores and other chelating molecules (Huyer and Page, 1988). In my study, non-inoculated *Arabidopsis* grown in agar supplemented with cadmium or copper had increased concentrations of these metals within their tissues. However, plants that were inoculated with *P. fluorescens* UW4 had equal or less cadmium or copper within their tissues when they were grown in metal-supplemented media. This indicates that *P. fluorescens* UW4 would not be useful for increasing the phytoremediation capabilities of *Arabidopsis* and potentially other plant species. My result goes against Xu *et al*. (2015) and Kamran *et al*. (2015), who found that *P. fluorescens* increased copper and cadmium uptake in *Elsholtzia splendens*. However, Madhaiyan *et al*. (2007) reported a reduction in the accumulation of cadmium and nickel in the tissues of tomato plants that were inoculated with *Methylobacterium oryzae*. Taken together, these results suggest that the potential for PGPR to assist in the phytoremediation of metal-polluted media may be dependent on the species of PGPR used and host plant involved.

5.4.4 *AcdS* gene and ethylene reduction

Glick *et al*.'s (1998) model to explain how PGPR's increase plant growth under stress and non-stress conditions has two parts. One involves bacterial produced IAA and its effects on increasing plant growth, which I examined in Chapter 4. The second part of the model suggests that PGPR that contain the enzyme ACC deaminase can reduce stress-induced ethylene thereby preventing the deleterious effects that stress ethylene has on plant growth. To address this part of the model, I first needed to ensure that the putative PGPR *P. fluorescens* UW4 could actively transcribe the ACC deaminase gene as well as verify that the ACC deaminase mutant *P. fluorescens* UW4-*acdS*$^{-}$ did not. My RT-PCR results indicate that indeed the wildtype bacterium does contain a functional *acdS* gene while the mutant does not. My results are in agreement with what Li *et al*. (2000) originally found when they generated the *P. fluorescens* UW4-*acdS*$^{-}$ mutant. I also determined that *acdS* expression was not significantly reduced in wildtype bacteria by the highest cadmium or copper treatment utilized in my experiments. These results suggest that the wildtype bacteria have the potential to reduce plant stress ethylene.

When I grew *Arabidopsis* inoculated with wildtype *P. fluorescens* UW4 in the presence of 20 μM cadmium or copper, the wildtype bacterium was indeed able to reduce metal-induced ethylene. I also found that plants that were inoculated with the ACC deaminase mutant bacterium did not reduce metal-induced ethylene. The ability of ACC deaminase-containing PGPR, like *P. fluorescens* UW4, to decrease stress ethylene levels has also been documented in tomato (Ciardi *et al*., 2000; Robison *et al*., 2001) and *Trigonella* plants (Barnawal *et al*., 2013). Glick (2005) stated that a plant inoculated with an ACC deaminase-containing PGPR would see a 2-4 fold reduction in ethylene concentrations when grown in stressful environments. In my study, *Arabidopsis* inoculated with *P. fluorescens* UW4 had up to a 10-fold reduction in ethylene production in cadmium or copper contaminated media (Figure 5.6). This clearly indicates that the stress was sufficient to induce an ethylene response and that *P. fluorescens* UW4 can and will reduce stress-induced ethylene.

To further test the ethylene reduction component of Glick *et al*.'s (1998) model, I wanted to determine whether or not wildtype *P. fluorescens* UW4 could rescue ethylene over-producing *Arabidopsis* mutants by reducing the amount of ethylene produced to near wild-type levels. To test this, I used three ethylene over-producing mutants: *eto1-1, eto2* and *eto 3* (Guzman and Ecker, 1990; Kieber *et al*., 1993). While there was a trend towards overproduction of ethylene, the amount of ethylene produced by non-inoculated mutant *Arabidopsis* was not significantly different from that of non-inoculated wildtype (Figure 5.7), and thus there was not the expected over-production of ethylene in these mutants. This is in contrast to the report that the ethylene over-producing *eto3* mutant produces 13.5 times more ethylene than wildtype (Woeste *et al*., 1999). It is very likely that the increase ethylene seen in both wildtype and *eto 1-1*, as well as no decrease in ethylene seen in *eto2* mutants may be due to how the *Arabidopsis* was grown (5 mL vials with 3 mL of MS agar medium) adding additional stresses such as nutrient stress and or artificially concentrating the amount of ethylene being produced by only having 2 mL of headspace. Moreover, to potentially explain these differences, most of the ethylene values attributed to the ethylene over-producing mutants are from plants that were grown in the dark, while my plants were grown in the light in a growth chamber. Bassi and Spencer (1983) and Woeste *et al.* (1999) have shown that light can decrease the amount of ethylene produced by up to 50%, which may explain why the ethylene over-producing mutants may have produced significantly less ethylene than has been reported in the literature. Furthermore, as reported by Woeste *et al.* (1999), wild-type *Arabidopsis* grown in light produced 144% higher ethylene levels than those grown in the dark. This fact could explain why my wildtype *Arabidopsis* had higher levels of ethylene than the mutants.

It is clear that inoculation with *P. fluorescens* UW4 did not reduce the amount of ethylene generated by the three ethylene over-producing mutants. Only in the *eto3* mutant was there a 37% decrease in ethylene levels which made it more comparable to non-inoculated wildtype ethylene levels. Furthermore, wildtype and *eto1-1 Arabidopsis* had a 3- to 5-fold increase in ethylene after inoculation with the bacterium. This was surprising given that wildtype *P. fluorescens* UW4 significantly decreased cadmium- and copper-

induced ethylene in wildtype *Arabidopsis*. I also determined that inoculating the three ethylene over-producing mutants with *P. fluorescens* UW4 had no effect on growth promotion. This was expected given that I did not see growth promotion in metal-stressed plants and goes hand in hand with the inability of *P. fluorescens* UW4 to reduce ethylene levels in the *Arabidopsis* mutants. However a protective effect may have been seen in mutant *Arabidopsis* inoculated with the wildtype bacterium, which showed no decrease in primary root length when compared to wildtype *Arabidopsis* (Figure 5.7 C). This suggests a possible protective affect that *P. fluorescens* UW4 has on plant roots.

5.4.5 Plant hormone content

Based on Glick *et al*.'s (1998) model as well as results from my research, it is clear that PGPRs that contain ACC deaminase can directly alter the concentrations of ethylene produced by stressed plants. However, the reduction of ethylene, and potential increase of IAA in plant tissue from PGPR sources, does not seem to be enough to promote growth, at least with *Arabidopsis*. This prompted me to ask whether or not the bacteria are altering the concentrations of other plant hormones, such as SA, ABA, and JA, which may explain why I did not see plant growth promotion. For example, it has been well established that PGPR associations with plant roots increase SA concentrations within plant tissue (Kurepin *et al*. 2015), which can in turn reduce ethylene production (Wang *et al*., 2002, Figure 5.9). It has also been well established within the literature that the ethylene and JA biosynthetic and transduction pathways are coupled, indicating that a PGPR-induced ethylene reduction in plant tissue may also reduce JA concentrations (Devoto and Turner, 2003, Figure 5.9). Furthermore it has been documented that both ethylene and IAA can alter the concentrations of ABA within plant tissue (Wilson *et al*., 1990), and therefore, PGPRs that produce IAA and reduce ethylene may also increase ABA concentrations. I therefore decided to test whether or not the putative PGPR *P. fluorescens* UW4 could alter ABA, SA and JA concentrations within *Arabidopsis* tissue. That way I could potentially shed light as to why *P. fluorescens* UW4 did not promote growth in *Arabidopsis* and provide the opportunity to expand Glick *et al*.'s (1998) model.

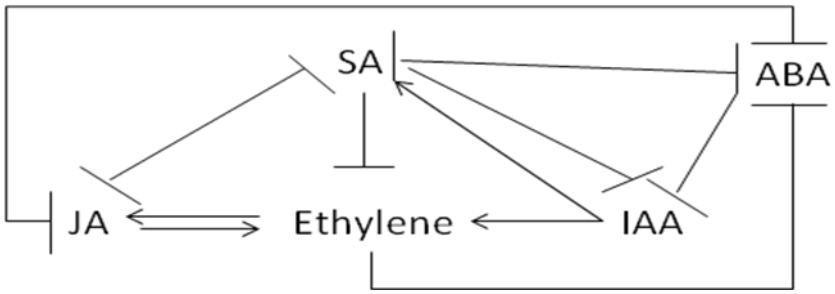

Figure 5.9 Hormone crosstalk. The interplay between ethylene, IAA, SA, JA and ABA suggests that PGPR reduced plant ethylene and increase IAA and SA levels in plants could indirectly influence the concentrations of other plant hormones. With a decrease in plant ethylene and increase in SA by PGPRs, JA concentration could decrease. Conversely, ABA levels could go up with less ethylene and JA to inhibit it or alternatively decrease depending on how much SA is induced under a PGPR interaction. Based on Wilson *et al*., (1990), Glick *et al.* (1998), Wang *et al*. (2002), Devoto and Turner (2003), Carvalho *et al. (*2015), Kurepin *et al*. (2015).

I determined that, in general, inoculation with *P. fluorescens* UW4 had no effect on ABA, SA and JA concentrations within either metal-stressed or control conditions. However, *Arabidopsis* inoculated with *P. fluorescens* UW4 had a trend towards increasing hormone concentrations and had a significant 35% increase in SA concentration in response to 20 μM copper. The increase in SA concentration was expected because other PGPRs increase SA within their host plant (Zhang *et al*., 2002). Although ABA and JA did not vary in concentrations, inoculation with wildtype *P. fluorescens* UW4 caused a trend towards higher ABA and JA concentrations under both metal-stressed and control conditions. These small increases, even though not statistically different from those of non-inoculated controls, may have biological significance in *Arabidopsis* and may explain, at least in part, why inoculation with wildtype *P. fluorescens* UW4 did not promote plant growth. I think that *P. fluorescens* UW4 associated with *Arabidopsis* roots have the potential to increase the concentrations of ABA, SA and JA within plant tissue. My results also support Kurepin *et al*. (2015), who

reported that PGPRs associated with plant roots can increase the concentrations of ABA, SA and JA by 30%, 70%, and 35% respectively.

5.5 Conclusion

I cannot definitively state that *P. fluorescens* UW4 is indeed a PGPR, at least with *Arabidopsis*. It is clear, that it is able to adhere to the plant root and does contain the enzyme ACC deaminase. Furthermore, it is able to reduce metal-induced stress ethylene in *Arabidopsis*, as suggested by Glick *et al*.'s (1998) model. However, *P. fluorescens* UW4 was not able reduce the amount of ethylene produced by the *Arabidopsis* mutants and it did not promote plant growth in cadmium- or copper-contaminated medium. This inability to promote plant growth may very well be due to the bacterium being slightly pathogenic or the bacterium being dependent on the plant for survival; it might be competing for nutrients or feeding on photosynthates from the plant, resulting in stunted plant growth. Furthermore, it appears that the bacterium does not alter SA, JA and ABA concentrations in *Arabidopsis*; however, a trend towards an increase in the concentrations of these hormones was seen when plants were inoculated with the wildtype bacterium. This may further help to explain why I did not see growth promotion in *Arabidopsis*, as the complex interplay between the various signaling cascades initiated by these hormones may have led to added stresses and thus reduced growth. Overall, *P. fluorescens* UW4 reduces stress-induced ethylene and may, given the right host and environment, promote plant growth under stressed and non-stressed conditions. In Chapter 6 I will present a revised PGPR-plant model, as well as elaborate on how bacteria-induced changes in ABA, SA and JA could affect plant growth.

5.6 References

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Chapter 6

6 General Discussion

6.1 Overview and future prospects

As the human population continues to increase, so too will the demand for food, fuels, plant-based products and the use of plants for phytoremediation. It is inevitable that PGPR and other rhizospheric microorganisms will be used alongside other advancements in plant/argri-technology to help meet these growing demands. Studies of plant-microbe interactions will not only help increase our understanding of how microbes can be used to increase plant growth, but we will also gain insight into how these interactions play into nutrient cycling, carbon sequestration, and ecosystem functioning. However, plant-microbe interactions are complex, biological systems that have many variables, some of which have yet to be fully understood, which makes studying them quite difficult. For example, the environmental conditions in which a plant-PGPR interaction occurs (e.g., pH of medium, nutrient availability, temperature, salinity and any other environmental stress), the plant system being used and the species of PGPR, can have profound impact on whether or not a given PGPR can promote plant growth. In an attempt to try to simplify plant-microbe interactions, researchers have proposed straightforward and relatively simple models to explain how PGPR can promote plant growth. However, the dangers in having simplified models, such as Figure 1.1, is the assumption that any plant growth promotion induced by a PGPR must be due to those simplified mechanisms and not some other unforeseen or confounding pathway or variable.

In this thesis, I set out to determine whether the PGPR *Pseudomonas fluorescens* UW4 could improve plant growth in cadmium- or copper- contaminated media and if it could, by what mechanism(s). I also tested the model proposed by Glick *et al*. (1998; Figure 1.1) to examine the relative roles that bacterial indole-3-acetic acid (IAA) and ACC deaminase have on plant growth under metal-stress and control conditions. Lastly, in an attempt to expand upon Glick's model, I looked at the impact that *P. fluorescens*

UW4 had on the concentration of three plant hormones, abscisic acid (ABA), salicylic acid (SA) and jasmonic acid (JA), and what this might mean in terms of plant growth promotion.

In Chapter 2, I measured adherence of the putative PGPR *P. fluorescens* UW4 and its ACC deaminase mutant *P. fluorescens* UW4-*acdS*$^{-}$ to varying substrates, including plant roots. Given that smooth materials such as nylon had fewer adhering bacteria than did more fibrous materials such as cotton or a plant root, adhesion seem to be correlated with a substrate's intrinsic physical characteristics (i.e., how rough its surface was). I also provided evidence to suggest that rendering the ACC deaminase gene non-functional had no effect on the ability of *P. fluorescens* to adhere to substrates. It has been well established that physical and chemical properties of a material play a role in the ability of bacteria, including that of PGPR, to adhere to its surface. It has also been well documented that *P. fluorescens* can adhere to various surfaces including roots and seeds (Hong *et al*., 1991); however, to my knowledge this is the first direct evidence that the *P. fluorescens* UW4 strain as well as its mutant can adhere to plant roots and other materials like cotton and polyester, independently of ACC deaminase. This may indicate that *P. fluorescens* UW4 has a substrate-binding protein such as rhicadhesin that enables the bacterium to adhere to a variety of substrates including plant roots. I also revealed that *P. fluorescens* can survive in media contaminated with 20 μM of cadmium and that the presence of cadmium does not affect *P. fluorescens*'s ability to adhere to substrates. This result corroboratesManara *et al*. (2012), who found that *P. fluorescens* can resist up to 250 μM which increases its efficacy to be utilized in phytoremediation of toxic metals. Lastly, I demonstrated that although *P. fluorescens* can adhere to many substrates, its survival in a nutrient-poor medium significantly increases when interacting with living plant roots. This piece of evidence indirectly supports the notion that, once established with the plant root, PGPR utilize root exudates, such as photosynthates and amino acids, to stay alive and provide potential growth-promoting benefits to plants when a stress is induced.

In Chapter 3, I set out to determine three things: 1) whether the environment (agar-with or without tryptic soy broth, hydroponics, and Promix-BX) in which a plant-PGPR

interaction occurs has any effect on its ability to promote plant growth, 2) identify the best method for inoculating plants with PGPRs, and 3) if ACC deaminase-containing PGPRs such as *P. fluorescens* could promote plant growth in *Arabidopsis* grown in cadmium-contaminated medium to a greater extent than PGPRs that do not contain the enzyme, such as the ACC deaminase mutant *P. fluorescens* UW4-*acdS*$^{-}$. It was often indirectly portrayed in the literature that the environment in which a PGPR interaction occurs does not matter in terms of its ability to promote plant growth (Hontzeas *et al*., 2004; Cheng *et al*. 2007; Dell'Amico *et al*., 2008; reviewed in Glick, 2014). I, however, found that the environment can play a role, and given the right environment PGPR can become deleterious to plant growth.

It has been well established that environmental factors such as level of toxic metal pollution or pesticides can decrease microbial diversity and health (El Fantroussi *et al*., 1999; Sandaa *et al*., 2001). Not taking the environment in which a PGPR-plant interaction occurs may, in part, account for why successful laboratory or greenhouse tests on PGPR plant growth promotion do not achieve the same levels of success in field tests. For example, it has been extensively shown under laboratory conditions that some bacteria belonging to the *Pseudomonas* genus promote plant growth under a wide variety of stresses (Grichko and Glick, 2001; Farwell *et al.*, 2007; Gurska *et al*., 2009). However, when tested on winter wheat at two field sites, there was no difference in terms of growth between plants inoculated with *Pseudomonas* versus non-inoculated controls (de Freitas and Germida, 1992). Therefore, more work needs to be done before we can use PGPRs in agricultural situations. I think studies need to be done to determine how changing environmental factors such as changes in light intensity, moisture and nutrients will affect the PGPR-plant dynamics; in the real world, there are no controlled variables like there are in the laboratory. I also think more research needs to be done to determine how a PGPR inoculation will affect local microbial communities that are already established in the soil. Will the use of PGPRs reduced microbial diversity through competition or will other microbes reduce the beneficial effects that PGPRs offer? These questions need to be answered before we know the environmental efficacy of PGPRs as a technology to increase plant growth. I also think it is important to study and determine which PGPR interacts well with which plant species to produce maximum growth and yield benefit.

This way industry and agronomists can select specific PGPR to use to inoculate whichever plant species they are growing, and achieve maximum growth results.

In my study I showed that when PGPR have access to an abundance of nutrients (e.g., TSB and sucrose) the PGPR can lose their growth-promoting abilities and may become over-populated, which results in decreased plant growth. Furthermore, I also revealed that, in general, the type of medium in which the *Arabidopsis- P. fluorescens* interaction occurs (agar, hydroponics or Promix-BX) does not make a difference in the PGPR's inability to promote plant growth. I also was able to find an answer to the question: what is the best method for inoculating plants with PGPR? Most experiments to date inoculate the seeds of the plants rather than the seedlings (Li *et al*., 2000; Dell' Amico *et al*., 2008; Nadeem *et al*., 2013) and this may contribute to why field tests are not as successful as laboratory tests. Based on my results it is clear that, under laboratory conditions, plants should be inoculated at the seedling stage rather than as a seed to avoid excessive early bacterial growth, which may cause bacteria-plant competition for resources. Field experiments need to be done to determine if this result is upheld in agricultural soils. If my result holds true under field conditions, then farmers and/or manufacturers who may utilize PGPR, and want to maximize growth potential and yields, should allow the plant to establish before being inoculated with the PGPR. My results suggests that one can skip inoculating seeds and rather develop a liquid medium containing carbon and nitrogen sources to ensure bacterial survival, such that clients can spray or water the soil around young plants with the PGPR-inoculated liquid. This method of inoculating the soil around a young plant has been partially supported by my finding (Figure 3.2) that PGPRs can promote growth and are less pathogenic when not in contact with the roots. Future work is needed to test whether inoculation at a distance continues to promote growth past 10 d.

My studies (Chapters 3 and 5) are the first to report that inoculation of *Arabidopsis* with *P. fluorescens* UW4, grown under both cadmium-stress and control conditions, generally did not improve plant growth. I also believe this is the first time that this PGPR has been shown to be deleterious to plant growth. My studies were done using *Arabidopsis* and not a crop plant like those utilized in many PGPR studies, which could

be a reason as to why I did not see growth promotion. Perhaps *Arabidopsis* may not be a suitable model for these interactions as it may not form a beneficial symbiosis with *P. fluorescens* UW4. It is also possible that the deleterious effects of *P. fluorescens* UW4 on plant growth could be due to the bacteria increasing the concentrations of plant hormones, such as salicylic acid (SA; Figure 5.8E) , which are known to have an inhibitory effect on plant growth when levels are high. It was the result of not seeing plant growth promotion, which went against other studies of *P. fluorescens* UW4 (Li *et al*., 2000; Hontzeas *et al*., 2004; Cheng *et al*. 2007), which prompted me to test whether or not the bacteria were able to synthesize IAA from tryptophan, if they could reduce plant stress ethylene and whether or not they could alter plant hormones such as SA in order to explain this result.

In Chapter 4, I determined that *Arabidopsis* exuded less than1 mg/g of tryptophan into the surrounding medium. However, when supplemented with enough tryptophan *P. fluorescens* UW4 was able to synthesize IAA and that amount of IAA promoted aboveground plant growth. It has been well documented that other PGPR that synthesize IAA promoted plant growth (Patten and Glick, 2002; Shim *et al*., 2015). The relatively low amount of tryptophan exuded by *Arabidopsis* may help explain why *P. fluorescens* UW4 was unable to promote its growth in Chapters 3 and 5. Therefore, this may demonstrate that some IAA-producing PGPRs will promote plant growth only if the host plant exudes enough tryptophan, as shown the work done by Kravchenko *et al.* (2004) rendering growth promotion to only those plant species. Nevertheless, my results demonstrate that it is possible that IAA produced by PGPR may promote growth, which supports the IAA mechanism proposed by Glick *et al*. (1998).

In Chapter 5 as well as Chapter 2, I revealed that *P. fluorescens* UW4 can not only adhere to *Arabidopsis* roots but toxic metal treatment does not alter this adherence. I also demonstrated that *P. fluorescens* UW4 does reduce toxic metal-induced ethylene; however, *P. fluorescens* UW4 did not promote growth under cadmium or copper stress. It was also shown that *P. fluorescens* UW4 could increase the plant hormones salicylic acid (SA), abscisic acid (ABA) and jasmonic acid (JA) within *Arabidopsis* tissue. I proposed that PGPR that contain ACC deaminase, such as *P. fluorescens* UW4, could reduce plant

stress ethylene and my results support this idea. Moreover, my results also support Glick *et al*.'s (1998) model that PGPR that associate with plant roots under stress conditions are able to ameliorate stress ethylene. However, just like results in Chapter 3, the inability of *P. fluorescens* UW4 to promote *Arabidopsis* growth under both cadmium and copper stress suggests that there is more going on than what was proposed by Glick *et al*.'s (1998) model. My results, as well as results from studies like Kurepin *et al*. (2015), suggest that the model proposed by Glick *et al.* (1998) may be incomplete and needs to be revised.

Based on my results I propose that PGPR could affect other pathways to regulate ethylene production in *Arabidopsis*, such as the SA pathway (Figure 6.1). For example, PGPR interactions with plants induce SA production (Kurepin *et al*., 2015). Furthermore, this increase in endogenous SA can reduce ethylene production by down-regulating the enzyme ACC synthase (Leslie *et al*., 1988; Romani *et al*., 1989). Since ACC synthase is a rate-determining enzyme within the ethylene biosynthesis pathway, regulation by SA would reduce the ACC pool and thereby reduce the amount of ethylene that can be produced (Glick *et al*., 1998). It has also been shown that too much SA can have an inhibitory effect on plant growth, in particular on the roots (Kurepin *et al*., 2015). Furthermore, it is well established that JA and ethylene transduction pathways stimulate each other (Kim *et al*., 2015). Thus, it is possible that a reduction in ethylene could also reduce the amount of JA produced. Although, in my study this did not happen; instead, JA concentrations increased slightly in plants inoculated with *P. fluorescens*. This may be due to SA inhibiting ACC synthase, resulting in less JA binding to ACC to produce ACC-jasmonate, a possible JA storage molecule, as well as JA-dependent regulation of ethylene biosynthesis. With less ACC within plant cells, it is possible to see an increase in JA within plant tissues. It is also possible that the association between PGPR and plant roots could also increase the concentration of JA due to wounding of cell walls. Stawick *et al*. (1992) showed that increases in JA can have an inhibitory effect on plant growth.

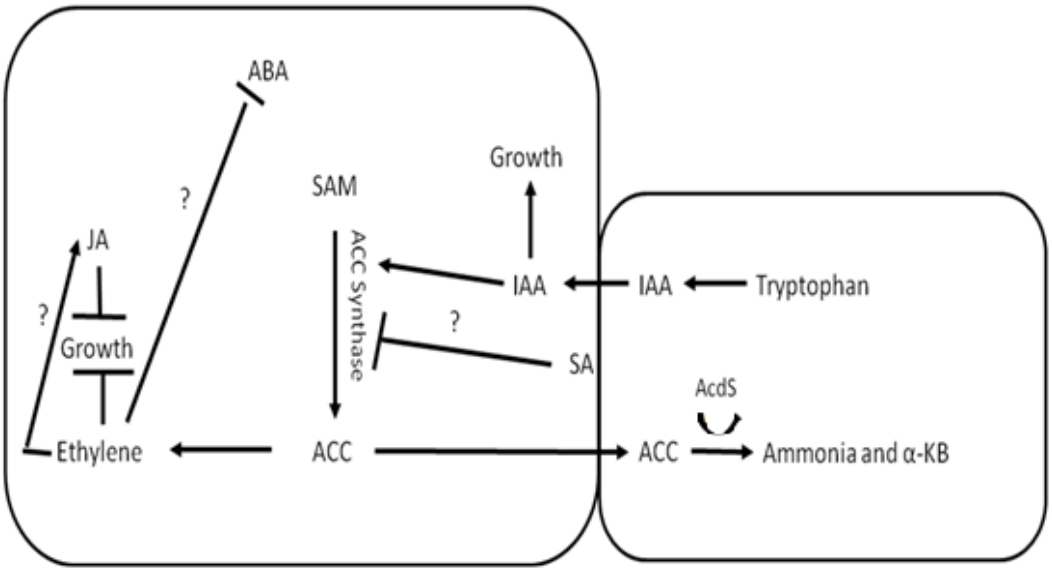

Figure 6.1. Revised model for how PGPR that contain the enzyme ACC deaminase and synthesize IAA could influence the concentrations of other plant hormones. Increases in endogenously produced SA, from PGPR interactions, can reduce ethylene production by down-regulating the enzyme ACC synthase, leading to reduced ethylene build-up under a stress and improving plant growth. Decreases in ethylene concentration whether by SA or *ACC deaminase*, could also reduce the amount of JA produced due to ethylene's positive influence on JA. A decrease in ethylene could reduce ethylene inhibition of ABA, resulting in an increase in the concentration of the growth hormone. However, this model is speculative and more research is needed to confirm the revised model. Based onWilson *et al*., (1990), Glick *et al.* (1998), Wang *et al*. (2002), Devoto and Turner (2003), Carvalho *et al. (*2015), Kurepin *et al*. (2015).

Lastly, it has been documented that ethylene is a negative regulator of ABA and thus reducing the amount of ethylene within plant tissue may lead to an increase in ABA (Ghassemian *et al*., 2000). Although most of these interactions that I propose in Figure 6.1 have not been directly tested, and thus are highly speculative, it nevertheless may spark interest and debate within the community such that more research is done to try and understand how PGPR affect hormone concentrations and what this means for plants in terms of growth and potential yield.

All of the results within this thesis clearly indicate that more work is needed to tease apart the complex dynamics and interactions between PGPR and plants. My work shows that not every PGPR can promote growth in all species under any condition. There are

limitations, whether they be environmental or biotic, species-specific PGPR interactions. Our job as researchers is to try to understand those limitations and try to find solutions to mitigate them. As technologies advance, so too will our insights into this incredibly complex system and evidence be gathered, for greater use and acceptance of PGPR in phytoremediation and agricultural practices.

In conclusion, I have shown that the PGPR *Pseudomonas flourescens* UW4 can synthesize IAA and reduce stress-induced ethylene in plants, giving rise to the possibility that, under the right conditions, it could promote plant growth. However, the same results that I obtained may not be achieved again under different conditions, with a different PGPR associating with a different plant species and/or a different stressor. With continued perseverance and experimentation we can understand and utilize PGPRs to enhance plant growth, provide a more stable environment, and increase our global food security.

6.2 References

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Appendices

Appendix A. Bacterial growth on agar

P. flourescens UW4 *P. flourescens* UW4-*acdS*$^{-}$

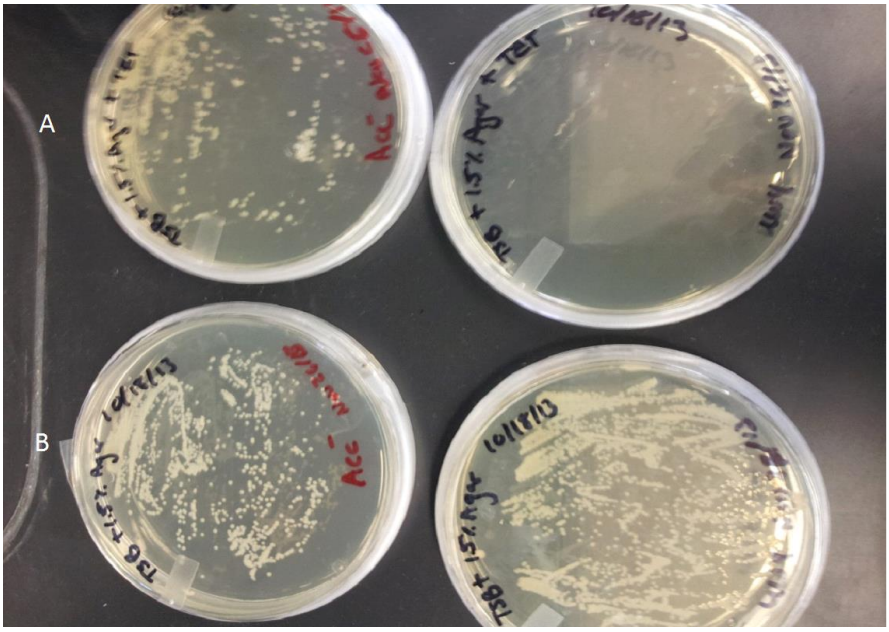

Figure A1.Bacterial growth on agar. Bacterial growth of *P. fluorescens* and its mutant on 0.8% agar medium containing A) tetracycline and B) no tetracycline after incubation at 30° C for 24 h. Only the mutant *P. fluorescens* UW4-*acdS*$^{-}$ was able to grow on plates containing tetracycline which indicates that the mutant does contain a tetracycline resistance gene and does not have a functional ACC deaminase while wildtype *P. fluorescens* UW4 does.

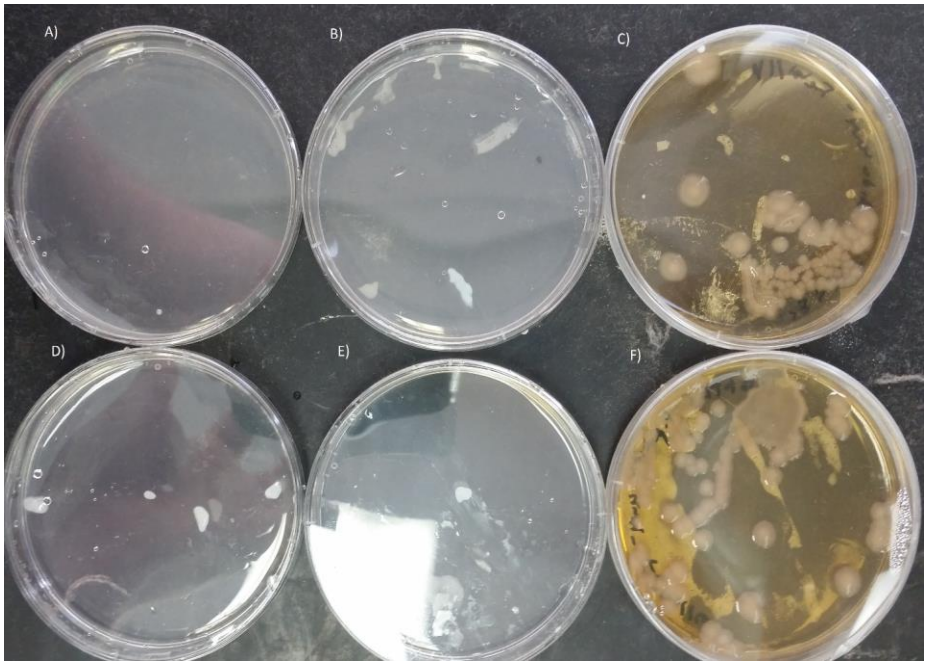

Figure A2. Bacteria growth on various agar media. Bacterial growth of *P. fluorescens* UW4 (A-C) and mutant *P. fluorescens* UW4-*acdS*$^{-}$ (D-F) on 0.8% agar medium containing 80% MS (A, D) or 80% MS + 1% sucrose (B, E) or 80% MS + 5% TSB +1% sucrose (C, F) incubated at 30° C for 24 h. Neither bacteria was able to grow on agar plates only containing MS or the 80% MS + 1% sucrose media. However, small bacterial colonies were visible on 80% MS + 5% TSB after 24 h of incubations.

Appendix B. Dose responses

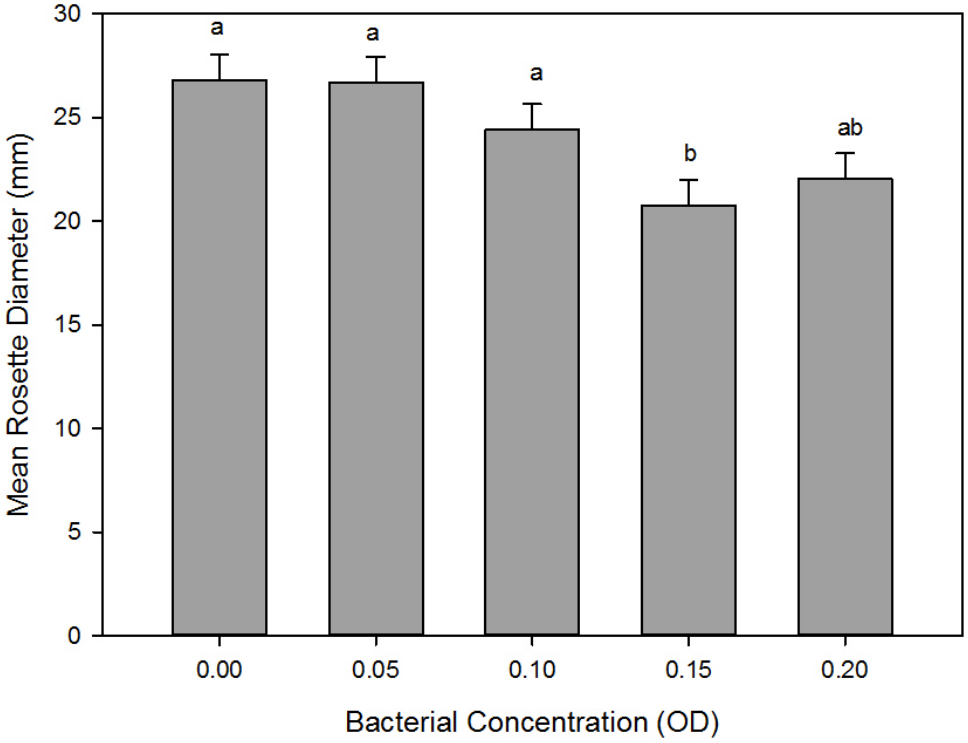

Figure B1. How much to inoculate? Mean rosette diameter of 14 d old *Arabidopsis* inoculated with wildtype *P. fluorescens* UW4. Inoculum densities were measured as optical density at 600 nm (OD_{600}). All experimental inoculation will occur at an OD_{600} of 0.1 as that inoculum did not harm plant growth. Vertical error bars represent standard error. Means not sharing a common letter are significantly different (one-way ANOVA followed by Tukey post-hoc test, $p < 0.05$, $n = 3$).

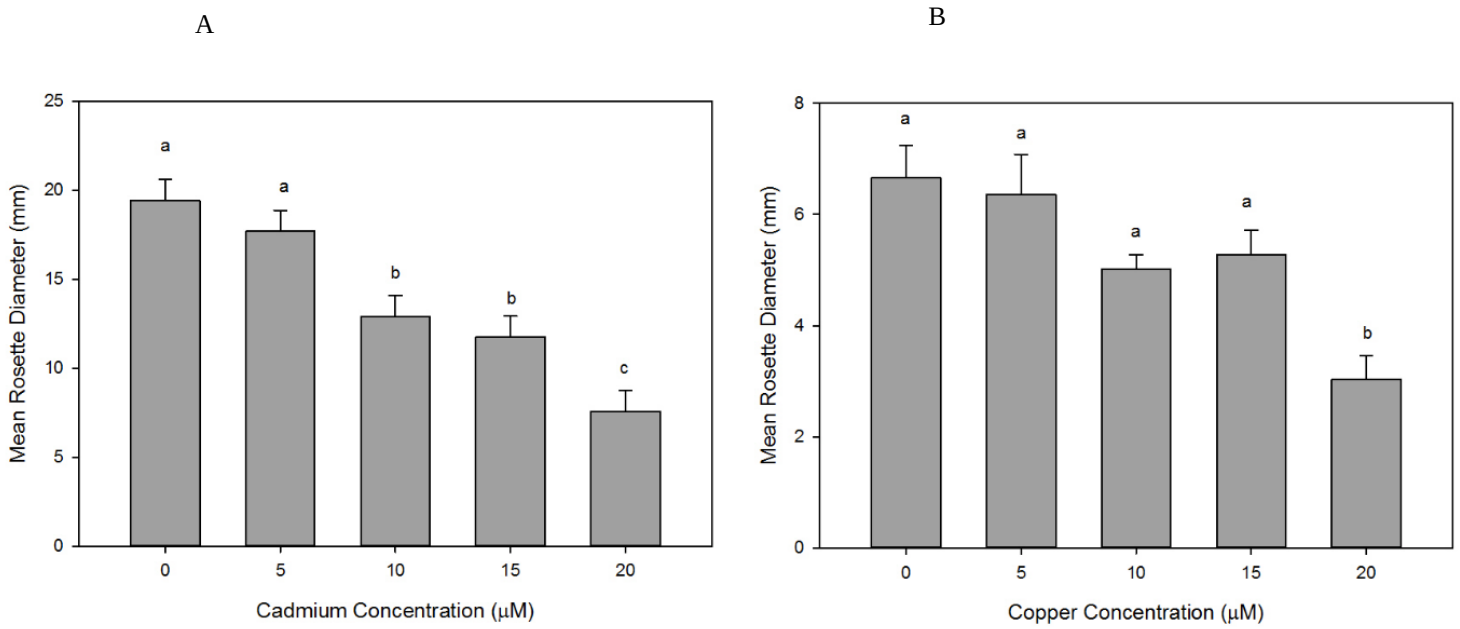

Figure B2. Toxic metal dose response. Mean rosette diameter and root length of *Arabidopsis* grown over a range of cadmium (A) and copper (B) concentrations. Concentrations of 0, 10 and 20 μM of cadmium or copper will be used in all future experiments as these concentrations elicited reduced growth. Vertical error bars represent standard error. Means not sharing a common letter are significantly different (two-way ANOVA followed by Tukey post-hoc test, $p < 0.05$, $n = 3$).

Appendix C. RT-PCR and qPCR primers and conditions

Table C1. Primer sequences and PCR conditions. Conditions* and primers with their sequences used for RT-PCR and qPCR. **F** denotes forward primer and **R** denotes reverse primers.

16S**F**	CTCGTAGTCCACGCCGTAAA
16S**R**	CGAATTAAACCACATGCTCCAC
AcdS**F**	GAAGTCGGCGTGAATCTGTG
AcdS**R**	CGCCAGTTCTTCGTCCTTGT

*Conditions used in both PCR and qPCR were; denaturation at 94℃ for 3 min, 32 cycles of amplification (94℃ for 30 sec, 58℃ for 30 sec, 72℃ for 90 sec) and final extension at 72℃ for 10 min.

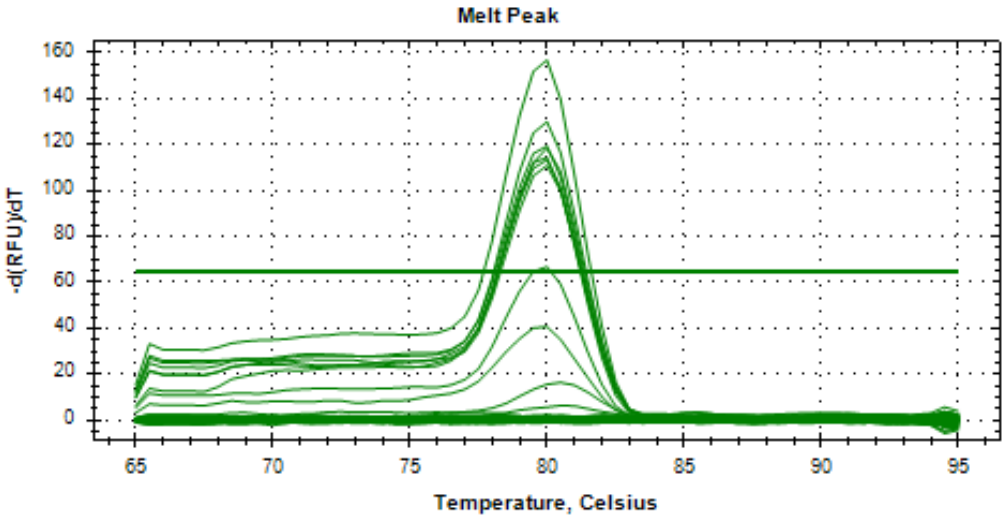

Figure C1. qPCR melt curve. A melt curve of qPRC primers *acdS* and *16s* with a unimodal peak indicating that each qPRC product had one amplicon.

Appendix D. Hormone chromatogram

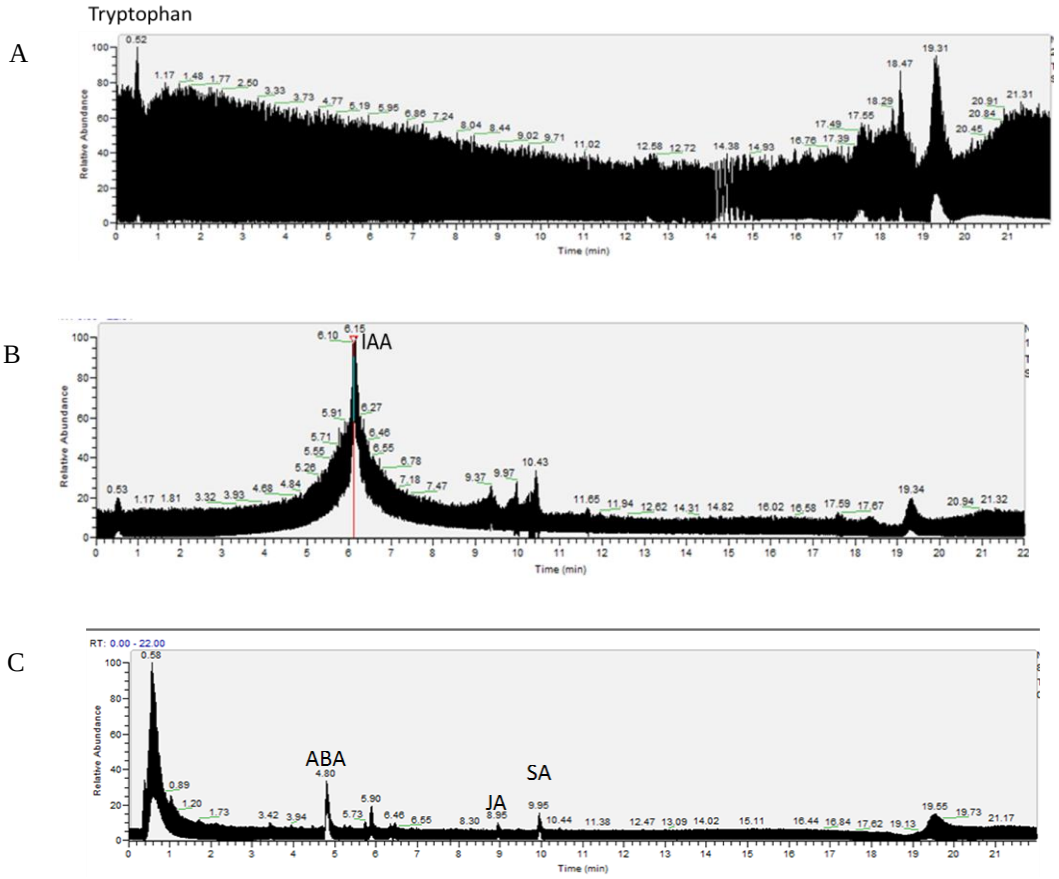

Figure D1. Sample HPLC-MS ion chromatograms. A) Chromatogram indicating the retention time of a tryptophan standard (1 mg/mL). B) Chromatogram indicating the retention time of an IAA standard (1 mg/mL). C) A sample chromatogram indicating the retention times of the plant hormones abscisic acid (ABA), jasmonic acid (JA), and salicylic acid (SA) using a C18 Cortex column, based on the retention times of their respective standards, of a non-metal treated and non-inoculated *Arabidopsis*.

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Frank, J.J., Macfie, S.M. 2015. *Do plant growth-promoting rhizobacteria increase plant growth and reduce the production of ethylene in Arabidopsis under cadmium and copper stress?* Canadian Society of Plant Biologists Eastern Regional Meeting. **(Poster presentation by J.J. Frank)**

Frank, J.J., Dhami, G., Macfie, S.M. 2014. *Do plant growth-promoting rhizobacteria reduce the production of ethylene in Arabidopsis under cadmium stress?* Canadian Society of Plant Biologists Eastern Regional Meeting. **(Poster presentation by J.J. Frank)**