Soybean root exudates increase the physiological diversity of bacteria in cadmium-treated soil

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Abstract

Three soybean cultivars with contrasting retention of cadmium (Cd) in the root were grown in Cd-spiked nutrient solution and used to determine that symplastic compartmentalization of Cd in roots is probably responsible for retention of Cd in roots. Roots of the low Cd-accumulator AC Hime treated with 30 µM Cd exuded up to 10-fold higher concentrations of citric, succinic, fumaric and malic acids into the hydroponic solution when compared to control; concentrations of the same organic acids from the high Cd-accumulator Westag 97 increased by up to 3-fold. The same cultivars were grown in Cd-spiked soil and the physiological profiles of the rhizosphere bacteria were assessed using Biolog® EcoPlates™ to test the hypothesis that bacterial community diversity increases in response to organic acids exuded by the plant. Bacteria in the rhizosphere of AC Hime, the cultivar with the highest Cd-induced exudation of organic acids, had a distinct carbon utilization pattern, illustrating important interactions among rhizosphere bacteria, plants and toxic metals as a result of metal contamination.

Key words: Bacterial community diversity, Cadmium, Compartmentalization, Organic acids, Root exudates, Soybean
Summary for Lay Audience

Cadmium contamination in agricultural soils is increasing through anthropogenic activities such as mining, smelting and the disposal of municipal wastes. Crops grown on cadmium-contaminated soil may have reduced yield if they suffer from cadmium toxicity. Crops that can tolerate cadmium may pose a health risk if the cadmium reaches the edible parts. It is important to understand how plants can reduce the uptake and movement of cadmium from roots to aboveground parts, including leaves and seeds. This project studied soybean, whose seeds are used to make animal feed, tofu, oil and other soy products.

In this study, three cultivars (types) of soybean that vary in the amount of cadmium found in the seeds were used to determine what controls the movement of cadmium up from the roots. I discovered that the cultivar that kept more cadmium in the roots stored more cadmium inside the root’s cells (a region called the symplast). In all three cultivars, exposure to cadmium was associated with the production of molecules (called organic acids) that bind with cadmium. These molecules were released from roots into the surrounding environment, a region called the rhizosphere where many beneficial bacteria live. The cultivar with more cadmium stored in the root produced more organic acids and had a lower level of activity in the community of bacteria in its rhizosphere. However, the cultivar with less cadmium in the root had a stronger response to cadmium (a greater stimulation of organic acid production) and was associated with a higher level of activity in its community of bacteria. These results could help plant breeders to develop new cultivars with reduced cadmium uptake and movement. It also provides information about the relationship between plants and bacteria in contaminated soils.
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Co-Authorship Statement

A manuscript arising from this thesis will be co-authored with Dr. Greg Thorn, Dr. Mark Bernards and Dr. Sheila M. Macfie (supervisor). I designed and conducted the experiments, collected and analyzed all the data, and will write the manuscript. Dr. Thorn provided guidance, including training, experimental design and interpretation. Dr. Bernards provided training and guidance during protocol development for the analysis of organic acids. Dr. Macfie provided laboratory support and guidance in experimental design, data interpretation, and manuscript preparation.
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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABC</td>
<td>ATP-Binding Cassette</td>
</tr>
<tr>
<td>ACCD</td>
<td>1-Aminocyclopropane-1-Carboxylate Deaminase</td>
</tr>
<tr>
<td>ACS</td>
<td>1-Aminocyclopropane-1-Carboxylate Synthase</td>
</tr>
<tr>
<td>As</td>
<td>Arsenic</td>
</tr>
<tr>
<td>AWCD</td>
<td>Average Well Color Development</td>
</tr>
<tr>
<td>Ca</td>
<td>Calcium</td>
</tr>
<tr>
<td>Cd</td>
<td>Cadmium</td>
</tr>
<tr>
<td>CdCl₂</td>
<td>Cadmium Chloride</td>
</tr>
<tr>
<td>CEC</td>
<td>Cation Exchange Capacity</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony-Forming Unit</td>
</tr>
<tr>
<td>Cu</td>
<td>Copper</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Deionized Water</td>
</tr>
<tr>
<td>DW</td>
<td>Dry Weight</td>
</tr>
<tr>
<td>E</td>
<td>Evenness</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>Fe</td>
<td>Iron</td>
</tr>
<tr>
<td>H’</td>
<td>Shannon Diversity Index</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen Peroxide</td>
</tr>
<tr>
<td>Hg</td>
<td>Mercury</td>
</tr>
<tr>
<td>IAA</td>
<td>Indole-3-Acetic Acid</td>
</tr>
</tbody>
</table>
ICP-MS ................................................... Inductively Coupled Plasma – Mass Spectroscopy
K ................................................................. Potassium
LC–ESI–TOFMS ........................................................... Liquid Chromatography-Electrospray Ionization - Time of Flight Mass Spectrometer
LMWOA ......................................................... Low Molecular Weight Organic Acid
MT ................................................................. Metallothionein
OH ................................................................. Hydroxyl
P ................................................................. Phosphorus
Pb ................................................................. Lead
PC ................................................................. Phytochelatin
PCA .......................................................... Principal Component Analysis
PGPR ........................................................ Plant Growth-Promoting Rhizobacteria
pi .............................................................. Proportional Colour Development
R ................................................................. Richness
ROS ........................................................ Reactive Oxygen Species
S ................................................................. Sulfur
SEM ........................................................ Scanning Electron Microscopy
TCA ........................................................ Tricarboxylic Acid
ZIP .................................................. Zn-regulated transporter, Iron-regulated transporter-like Protein
Zn ................................................................. Zinc
CHAPTER ONE: INTRODUCTION

1.1 Overview

Cadmium (Cd) contamination in soils has become a major concern in recent years, due to its toxicity toward all living organisms (Rascio and Navari-Izzo, 2011). Anthropogenic activities, including agriculture, mining, metallurgy and manufacturing, can raise Cd concentrations to toxic levels (Cordero et al., 2004). The main source of Cd in agriculture arises from using fertilizer produced from Cd-containing phosphate rock; sources of Cd-free phosphate are limited and removal of Cd from the fertilizer is not economically favourable (Cupit et al., 2002). Crops grown on Cd-contaminated soil may accumulate high concentrations of Cd in their edible parts, which could directly or indirectly harm human health (Jegadeesan et al., 2010). Cadmium accumulation in humans can damage kidneys and cause calcium (Ca) deficiency (Kanzantzis, 2004). Thus, understanding the mechanisms of Cd accumulation in plant tissues is important to ensuring the consumer’s health.

While plants have some physiological control over metal uptake and translocation, rhizospheric bacteria play a crucial role in plant tolerance and bioavailability of toxic metals, including Cd, because they can accumulate, transform, or detoxify metal ions (Mishra et al., 2017). Understanding the mechanism of plant Cd uptake, translocation and distribution as well as the relationship between bacterial community diversity and plant-induced changes in the rhizosphere could help plant breeders to develop new cultivars to reduce Cd bioavailability and uptake in crops at reasonable costs.
1.2 Cadmium in the Soil Environment

Soil generally contains low concentrations of nonessential metals such as Cd, lead (Pb), mercury (Hg), and arsenic (As). The concentration ranges of these elements worldwide in agriculture soils vary: 0.95 to 214 mg kg\(^{-1}\) (Pb), 0.05 to 13.5 mg kg\(^{-1}\) (Cd), 0.05 to 0.73 mg kg\(^{-1}\) (Hg), and 0.78 to 92.7 mg kg\(^{-1}\) (As) (Rascio and Navari-Izzo, 2011). Among these metal pollutants, Cd is considered to be one of the most phytotoxic because the Cd\(^{2+}\) ion has high solubility in water and can be easily taken up by plants (Root et al., 1974). Cadmium is released into the environment either naturally by erosion of parent rocks, volcanic activity and forest fires, or through anthropogenic activities, including agriculture, mining, metallurgy and manufacturing (Rehman et al., 2018). Environment Canada (1994) estimates that 159 tons of cadmium are released annually to the Canadian environment as a result of domestic anthropogenic activities. Worldwide, around 30,000 tons of Cd are released into the environment, of which 13,000 tons result from human activity (Environment Canada, 1994). As the amount of Cd released into the environment rises, concentrations of Cd in soil may rise to toxic levels and endanger plant life (Cordero et al., 2004).

1.2.1 Effect of soil properties on metal bioavailability

Soil properties not only affect the total content or chemical forms of metals in soil but also affect their bioavailability through several mechanisms:

**Soil pH** is the main factor that affects metal bioavailability in soil since bioavailability is mostly dependent on the partitioning of the metals between the minerals and the soil solution. Overall, the solubility of metals in soil increases with increasing acidity (Ono et
In acidic soil, the amount of metals in soil solution is greater than the amount bound to or in soil particles, due to dissolution of metals from solid phases. Consequently, more metal ions enter the soil solution and the bioavailability of metals increases (Rieuwert et al., 1998). Wang et al. (2006) reported that decreasing the pH from 6.88 to 4.74 increased the bioavailability of Cd and zinc (Zn) to the roots of *Thlaspi caerulescens* and enhanced metal uptake by 36%.

**The organic matter** of soil can immobilize metal ions and decrease their bioavailability. The high affinity of ligands, especially carboxylate groups, in soil organic matter favours binding with metal ions and influences metal mobility in soil (Sheoran et al., 2016). The overall order of affinity for metal ions chelated by soil organic matter is as follows: Cu$^{2+}$ > Cd$^{2+}$ > Fe$^{2+}$ > Pb$^{2+}$ > Ni$^{2+}$ > Co$^{2+}$ > Mn$^{2+}$ > Zn$^{2+}$ (Adriano, 2001).

**Soil texture** is another factor that affects metal bioavailability. The higher the porosity of the soil, the less solution will be held against gravity; therefore, higher amounts of metals in soil solutions can be held in clay or clay loam soil, while coarse texture soils such as sand hold less (Sheoran et al., 2010; Scokart et al., 1983).

**The cation exchange capacity (CEC)** of soil is the capacity of soil particles to bind metal ions. In general, CEC is proportional to the clay content in soil. Immobilization of metal ions due to high CEC decreases the bioavailability of metals in soil; while a lower CEC in soil is associated with greater bioavailability of metals (Sheoran et al., 2016; Ono et al., 2019; Rieuwert et al., 1998; Eriksson, 1989).

### 1.3 Effect of Cd Toxicity on Plants

Typically, visible symptoms of Cd toxicity are easily noticeable when the total concentration of Cd in soil exceeds 8 mg kg$^{-1}$, or the bioavailable Cd concentration
becomes > 0.001 mg kg\(^{-1}\), or the Cd concentration in plant tissue reaches 3–30 mg kg\(^{-1}\) (He et al., 2017). The exceptions are for Cd-hyperaccumulating plants, such as *Solanum photeinocarpum* (also called *Solanum americanum*), in which tissue concentrations can reach as high as 200 mg kg\(^{-1}\) without causing visible symptoms of stress (Zhang et al., 2011).

In most environments, roots are the first tissue to experience Cd-induced damage. Cadmium can be sequestered in the epidermal and cortical cells of the root; however, Cd\(^{2+}\) ions are highly water soluble and can move symplastically toward the stele and be released into the xylem (Clemens et al., 2002). Cadmium ions can also be translocated into different plant parts via the phloem (Reid et al., 2003).

Cadmium toxicity inhibits many physiological processes in plants, resulting in chlorosis as well as decreased plant growth, reproduction, water uptake, nutrient uptake, and photosynthesis (Aidid and Okamoto, 1992 and 1993). The most commonly reported symptom of Cd toxicity is reduced plant growth rate. For example, Huang et al. (2015) studied Cd toxicity in two cultivars of hot pepper (*Capsicum annuum*) with different patterns of Cd accumulation. They reported that exposure to 10 \(\mu\)M Cd in hydroponic solution led to a 25-48% decrease in the root length, surface area, and number of root tips of both cultivars compared to the control with 0 \(\mu\)M Cd (Huang et al., 2015). In another report, by Hassan et al. (2016), potato (*Solanum tuberosum*) seedlings exposed to 60 mg Cd kg\(^{-1}\) soil were 73% and 90% shorter in shoots and roots than the 0 mg Cd kg\(^{-1}\) control seedlings respectively. Similarly, their results indicated that the dry biomass of the treated roots and shoots with 60 mg Cd kg\(^{-1}\) soil decreased 90% and 98% compared to the 0 mg Cd kg\(^{-1}\) control seedlings respectively.
Cadmium toxicity can inhibit mineral nutrient uptake through both direct and indirect effects. Direct effects include competition for shared membrane transporters. For example, Cd\(^{2+}\) can compete for Ca\(^{2+}\) transporters (Chen et al., 2018). Indirect effects include reduced root mass and surface area. Mineral deficiency or imbalance due to Cd-induced effects can reduce fruit production of plants. For example, Hediji et al. (2015) investigated long-term (90 days) Cd stress on tomato (*Solanum lycopersicum*) fruting in hydroponic culture. They showed that 100 \(\mu\)M Cd (high stress) reduced Zn and Cu concentration in shoots of tomato by up to 74%, and the plants did not produce fruit. Hediji et al. (2015) also found that concentrations of some macronutrients were altered. Specifically, they reported that when tomato was exposed to 100 \(\mu\)M Cd, the Ca concentration in roots was increased 1.6-fold while it was decreased by 52% in stems and 49% in leaves. Also, the high Cd (100 \(\mu\)M) treatment led to a 75% reduction of K and Mg concentration in all plant tissues. Additionally, Zn concentration in roots was increased 1.8-fold compared to the 0 \(\mu\)M Cd control plants; while Zn in leaves was decreased by up to 73%. Similarly, Carvalho Bertoli et al. (2012) reported that Cd-stressed tomato had 13% decreased K concentration in fruit and 77% less Mn concentration in roots compared to control plants, and Jinadasa et al. (2016) showed that Cd-stressed cabbage (*Brassica oleracea*) had altered Mn, Zn, Cu, iron (Fe), and Ca concentrations in the leaves, stem, and roots of cabbage. They reported under high (500 \(\mu\)g L\(^{-1}\)) Cd treatment of cabbage, the Zn concentration decreased 43% in leaves 29% in stems and 33% in roots. Moreover, Cd stress decreased Cu and Ca concentration in leaves and stems of cabbage by up to 15%, while there was not a significance difference in their concentrations in roots. Additionally, Mn concentrations were decreased 23% in leaves, 35% in stems and 54% in roots.
Chlorosis due to Cd toxicity appears to be due to Fe deficiency and disturbed Fe homeostasis. Lešková et al. (2017) studied Fe deficiency in *Arabidopsis thaliana* under Cd stress (0, 5, 20, 40 µM Cd). *Arabidopsis* showed visible chlorosis in young leaves at 40 µM Cd. The chlorosis in leaves of *Arabidopsis* was associated with a 70% decrease in chlorophyll levels compared to the 0 µM Cd control plants. Additionally, Hediji et al. (2010) showed that at high (100 µM) Cd stress, the content of chlorophyll (a+b) and carotenoids decreased 26% and 31%, respectively, compared to the 0 mM Cd control plants. The authors suggested that high (100 mM) Cd stress impaired the electron transport rates of photosynthesis system I and photosynthesis system II, by increasing the concentration of free oxygen radicals in plant cells, which caused reductions in carotenoid and chlorophyll levels (Hediji et al., 2010).

In onion (*Allium cepa*) root tip cells, Cd caused chromosomal irregularities (Liu et al., 1995). Huang et al. (2004) studied Cd-induced stress in tall fescue (*Festuca arundinacea*). In addition to reduced biomass and chlorophyll content, they reported Cd-induced production of reactive oxygen species (ROS), including superoxide anion radicals (O$_2^-$), hydroxyl radicals (·OH) and hydrogen peroxide (H$_2$O$_2$), and lipid peroxidation, resulting in necrosis and apoptosis in the roots. Cadmium does not have a redox-active role in plant cellular mechanisms but has the potential to replace redox-active metals in proteins and increase the production of ROS. This increase in reactive radicals could damage proteins, DNA, and membrane lipids (Xue et al., 2014).
1.4 Cadmium Tolerance Mechanism in Plants

Cadmium accumulates in many crops, in some cases without reducing plant yield (Jamali et al., 2007) and, in general, plants have four (not mutually exclusive) strategies to minimize metal-induced stress:

(1) **Immobilization** of metal ions in the rhizosphere or apoplast. This is a plant’s first barrier against Cd toxicity. Oxidation and/or precipitation of Cd in the rhizosphere and immobilization of Cd by binding to root cell walls (Nishizono et al., 1989) each prevent Cd$^{2+}$ ions from entering the root cells. Nishizono et al. (1989) showed that 90% of the Cu$^{2+}$, Zn$^{2+}$ and Cd$^{2+}$ ions in Polygonum cuspidatum plants were located in the root cell walls. The content and monomeric composition of lignin in soybean (Glycine max) roots was examined by Finger-Teixeira et al. (2010) in hydroponic condition. Lignin is a complex organic polymer that consists of phenolic heteropolymers covalently bound to both polysaccharides and protein in plant cell walls, especially in secondary wall layers. Finger-Teixeira et al. (2010) showed that when soybean was exposed to 100 µM Cd, lignin content was increased by up to 131% compared to the 0 µM Cd control plants. Additionally, the concentration of two lignin monomers, hydroxyphenyl and syringyl, in plants given a 100 µM Cd treatment were increased 39% and 46%, respectively, compared to the 0 µM Cd control; while there was not a significant difference in the lignin monomer guaiacyl in plants from the 100 µM Cd treatment. This study suggested that enhancing lignin polymerization can reduce the transport of Cd to the shoots. Additionally, lignin has the ability to bind with Cd and restrict its diffusion into cells. The high amount of lignin in secondary cell walls increases plant tolerance and reduces Cd toxicity (Parrotta et al., 2015).
Cadmium may also bind to other components of the root cell walls of plants. Wang et al. (2018) reported Cd binding to pectin and cellulose in root cell walls of soybean, although the proportion of Cd bound to each component varied among soybean genotypes.

(2) **Altered transport** of metal ions across plasma membranes. Due to the similarity in physicochemical properties between Cd and some essential elements such as Ca, Zn, Mn, Fe, and magnesium (Mg), transporters of these essential elements mediate Cd uptake into the cytosol (Nazar et al., 2012). Many transporter families contribute to Cd resistance. The most important of these are a family of ATP-binding cassette (ABC) transporters (Higgins et al., 1992). ABC transporters transfer substrate molecules across cellular membranes at the expense of ATP hydrolyzing. Kim et al. (2007) reported that the ABC transporter AtPDR8 has a major role in the efflux of Cd\(^{2+}\) or Cd-conjugates across the plasma membrane of root epidermal cells.

The ZIP (Zn-regulated transporter, Iron-regulated transporter-like Protein) family of transporters also mediates metal efflux in plant cells. Liu et al. (2019) suggested that the ZIP transporter OsZIP1 limited Cd accumulation in rice (*Oryza sativa*) tissues. However, the details of the mechanism remain unknown. Mills et al. (2003) studied a transporter from the P-type ATPase gene family, AtHMA4, in *Arabidopsis*. They suggested this transporter contributed to Zn homeostasis and Cd detoxification in plant cells. Uragushi and Fujiware (2013) reported that OsNramp5 is the main transporter for Cd and Mn uptake into inner root cells in rice.

(3) **Compartmentalization.** Typically, this involves transport across the tonoplast and retention of metal ions or metal-chelate complexes in the vacuole, which reduces the concentration of Cd\(^{2+}\) ions in the cytosol (Sanita di Toppi et al., 1999). Cadmium can move
from the cytosol into the vacuole through an H\(^+\)/Cd\(^{2+}\) antiport transporter or an ATP-dependent phytochelatin transporter (Salt et al., 1998). Park et al. (2012) studied two lines of *Arabidopsis* under hydroponic Cd stress: wild-type and transgenic plants. They strongly suggested the two ABC transporters, AtABCC1 and AtABCC2, are involved in vacuolar sequestration of Cd. Additionally, Gaillard et al. (2008) revealed that the ABC transporter AtABCC6 is responsible for transporting Cd\(^{2+}\) from the cytosol into the vacuole of *Arabidopsis* seedlings.

(4) **Synthesis of chelators** by plants plays a major role in Cd\(^{2+}\) detoxification. Organic acids and two types of peptides, phytochelatins (PCs) and metallothioneins (MTs), are the most important metal-chelators in plants. Phytochelatins provide a major Cd-detoxification system in plants; they are found in both shoots and roots (Clemens et al., 2003; Clemens, 2001; Clemens, 2006). Phytochelatins are enzymatically synthesized, cysteine-rich peptides with the general structure (\(\gamma\)-Glu-Cys)\(_n\)-Gly \((n = 2–11)\). Glutathione (GSH; \(\gamma\)-Glu-Cys-Gly) is the substrate for the synthesis of PCs by the enzyme phytochelatin synthase, which is activated by Cd, among other metals (Clemens et al., 2006). In general, PCs sequester Cd due to their potential to bind Cd by thiolate coordination. When Cd is bound with PCs, the Cd-PC complex is actively transported from the cytosol into vacuoles through either metal/H\(^+\) antiporters or ATP-dependent ABC transporters in the tonoplast (Mishra and Dubey 2006). Xin and Huang (2014) investigated the subcellular Cd distribution in roots of high- and low-Cd accumulator cultivars of hot pepper. Their results showed that 87% of all Cd content was stored in root vacuoles. Also, they investigated subcellular distribution of Cd in fruit and leaves of hot pepper and found that in the low-Cd accumulator cultivar, the amount of Cd in fruit cell walls was 51% higher than in high-
Cd accumulator cultivar, which in turn had 63% compared to the control with 0 mg kg\(^{-1}\). However, a greater proportion of Cd in roots was located in vacuoles; 82% for low-Cd accumulator and 87% the high Cd accumulator. Their results corroborate Cd-induced PCs synthesis in the cytosol, then the transfer of PC-Cd complexes into vacuoles and subsequent Cd detoxification. Also, Herbette et al. (2006) revealed that under Cd stress, the GSH content of *Arabidopsis* decreased while the PC content increased. These results provided strong evidence of PCs role in Cd sequestration.

Unlike PCs, MTs are constitutively expressed in plants and regulate the availability of essential metal cations (Clemens et al., 2006). Metallothioneins are cysteine-rich polypeptides that are produced by a family of genes. Lee et al. (2004) studied the Cd-response of metallothionein genes *AtMT1* and *AtMT2* in *Arabidopsis*. They found a 1.4-fold increase in expression of *AtMT2a* and *AtMT3* in response to Cd stress, which indicated these MTs could play a role in Cd detoxification; however, they determined that the MTs were not involved in Cd sequestration into vacuoles or other organelles. Zhigang et al. (2006) also measured increased expression of an MT-related gene (*BjMT2*) in response to Cd stress in *Arabidopsis*; however, this was accompanied by inhibited root growth.

While PCs and MTs chelate Cd\(^{2+}\) ions within plant cells, organic acids are important for both internal and external chelation and detoxification. Organic acids have high affinity for Cd\(^{2+}\) ions due to the reactivity of Cd\(^{2+}\) with thionyl and carbonyl groups (Wójcik et al., 2005). Ueno et al. (2005) reported that organic acids, especially citric and malic acids, can bind Cd\(^{2+}\) in vacuoles. They reported that the presence of Cd did not induce malate synthesis in *Thlaspi caerulescens*. They suggested that since the Cd-malate complex is unstable, the metal-malate complex forms inside the vacuoles as a result of an efficient
tonoplast transport of Cd and a constitutively high concentration of malate in the vacuoles. Additionally, by using nuclear magnetic resonance spectroscopy, they reported that Cd was complexed mainly with malic acid in leaves of *T. caerulescens*.

In addition to organic acids sequestrating Cd in vacuoles, organic acids can also sequester Cd through extracellular chelation. In a study by Zhu et al. (2011), Cd treatment (50 µM Cd) of high- and low-Cd accumulator cultivars of tomato stimulated exudation of organic acids by both cultivars; however, oxalic acid was the only organic acid exuded by roots of both cultivars of tomato under Cd stress. Their results showed that the amount of oxalic acid exuded by the low-Cd accumulator cultivar was 3.5-fold greater than the high Cd accumulator in plants from the 50 µM Cd treatment. Moreover, they suggested that oxalic acid has the potential to chelate and immobilize Cd, preventing its uptake by plant roots.

The role of organic acids in Cd tolerance is, however, complex because chelation of metal ions in the soil can sometimes increase the mobility and availability of metal ions for uptake by plants (Rajkumar et al., 2012). This apparent contradiction depends on two factors.

**Metal-organic acid complex solubility.** The solubility of a metal-organic acid complex depends on the type of organic acid chelator and type of metal. In general, organic acids have strong affinity to bind with metals; however, based on physicochemical properties, the contribution of organic acids to these complexations follow this sequence: citrate > tartrate ≈ malate > oxalate (Violante et al., 2010). Additionally, the type of metal plays an important role in solubility and bioavailability of the chelation complex; correlated to the first hydrolysis equilibrium constant, the solubility of metals is according to this sequence:
Pb > Cu > Zn > Cd (Ding et al., 2014). Despite the general relative orders of complexation and stability, the specific metal-organic acid combination controls bioavailability. For example, Yang et al. (2000) showed that the complex formed by oxalic acid with Pb is insoluble, and oxalic acid exuded by plant roots reduced the bioavailability of Pb in soil; in contrast, while Cd-oxalate is more soluble than Pb-oxalate, exuded oxalate also decreased Cd bioavailability in the solution and its uptake by roots.

The culture medium. Organic acids might perform differently in hydroponic solution compared to in soil (Zhu et al., 2011). In hydroponic culture, Cd is readily available for plant root uptake but binding with organic acids can reduce Cd activity and its bioavailability in solution. On the other hand, organic acids exuded by roots into soil may be directly involved in a number of processes, consequently, the bioavailability of a Cd-organic acid anion complex in soil will be affected by many interdependent factors, such as solid phase sorption/desorption reactions, leaching, growth conditions and microbial degradation (Zhu et al., 2011).

1.5 Role of Organic Acids in Cd Stress

Understanding the function of organic acids in physicochemical processes such as metal sequestration relies on understanding the origins and dynamics of organic acids in the soils. Plant roots, microorganisms and organic decomposition are the sources of organic acids in the soil (Adeleke et al., 2017).
1.5.1 Organic acid exudates from plant roots

In general, plant root exudates contain a variety of simple and complex organic compounds, such as enzymes, sugars, amino acids, phenolics, vitamins, purines, nucleosides, proteins, flavonoids and organic acids. Organic acids in soil are classified in two large groups, high molecular weight organic acids (a few hundred to a million daltons) and low molecular weight organic acids (46 to a few hundred daltons). Low molecular weight organic acids have one to three carboxylic acids groups, and include citric, oxalic, malic, succinic, lactic, acetic, fumaric, aconitic, isocitric, malonic, and maleic acids. Some of these organic acids, such as citrate, malate, fumarate, and aconitate, are intermediate molecules in the tricarboxylic acid (TCA) cycle (Igamberdiev and Eprintsev, 2016). Organic acids with lower mass are more soluble in water (Bolan et al., 1994) and their carboxylic acid groups easily chelate metal ions (Bala et al., 2007). For the purposes of this thesis, the term ‘organic acids’ will be used henceforth to mean low molecular weight organic acids (LMWOA).

Organic acids may assist Cd sequestration in plants through two strategies (Osmolovskaya et al., 2018). The first strategy is restricting Cd uptake by plant cells, due to exuded organic acids by plant roots (Zhu et al., 2011). Carboxyl groups of organic acids have strong affinity for Cd$^{2+}$ ions (Ismael et al., 2019), when these complexes are too large to cross membranes easily, their formation can prevent Cd influx into the root cells. The second strategy is associated with intracellular mechanisms of metal sequestration. After Cd enters a plant cells, the organic acids that are present in the cytoplasm chelate Cd and neutralize its toxicity; this leads to compartmentalization of free ions and complexes in vacuoles (Hall, 2002).
1.5.2 Organic acids derived from microbes

Soil microorganisms, such as bacteria and fungi, produce and release organic acids naturally and in response to biotic and abiotic stresses. As in plants, the TCA cycle is the primary pathway for generating low molecular weight organic acids in microorganisms. Also, like plants, microbes can release these organic acids into the environment. For example, fungi belonging to the Ascomycota and the Basidiomycota excrete organic acid intermediates from the TCA cycle, even under non-stressed conditions (Liaud et al., 2014). Also, Johnston et al. (1993) determined that a filamentous fungus, *Aspergillus niger*, produces and releases significant quantities of citric, gluconic and oxalic acids, along with other organic acids, during the fixation of carbon dioxide.

Microorganisms also release organic acids in response to abiotic stresses. For example, in response to deficiency of phosphate, microbes excrete organic acids to dissolve the insoluble inorganic phosphorus sources in soil (Adeleke et al., 2017). *Streptomyces* bacteria respond to deficiency of Fe by lowering the pH of their local environment through the production of organic acids and ammonia, which increases Fe bioavailability (Liermann et al., 2000).

1.6 Plant-Microbe-Metal Interactions

Plants can increase access to nutrients and/or reduce metal toxicity by changing the pH or redox conditions in the rhizosphere (the portion of soil that is under the influence of root exudates) or by exuding chelators (Kamnev et al., 2008). These chemical changes can increase microbial growth in the rhizosphere compared to the bulk soil, and the diversity of bacteria in this region is generally 10 to 100 times higher than that in the bulk soil (Weller
& Thomashow, 1994). The rhizosphere microbes that have received the most attention include Rhizobia (N-fixing bacteria), mycorrhizal fungi, and plant growth-promoting rhizobacteria (PGPR) (Kamnev et al., 2008; McNear Jr, 2013). Although the following four mechanisms serve to protect the bacteria from metal toxicity, they can have a secondary effect of protecting nearby plants by reducing the bioavailability of Cd in the soil.

**1) Altering metal solubility in the rhizosphere.** The rhizosphere bacteria can alter soil pH, induce oxidation/reduction reactions, and release chelators (McNear, 2013). Under nutrient-deficient conditions, water-soluble bacterial siderophores (organic molecules that preferentially chelate iron but can also form stable complexes with Cd and other metals) can increase the bioavailability of metals in soil (Lebeau et al., 2008; Dimkpa et al., 2008; Ma et al., 2009). Similarly, Lia et al., (2012) discovered that rhizosphere bacteria associated with a Cd/Zn hyperaccumulating plant (*Sedum alfredii*) increased metal mobilization with the increased production of five organic acids: formic, acetic, tartaric, succinic, and oxalic acids.

Additionally, sulfate-reducing bacteria (*Desulfovibrio desulfuricans*) can reduce the bioavailability of toxic metals such as Cd (White and Gadd, 1998) by formation of insoluble metal sulfide compounds. The solubility of metal sulfates is very low, which can cause precipitation of metals from soil solution and decrease metal uptake by plants. During the processes of sulfate reduction by bacteria, the chemical reduction of metals utilizes protons and increases environment pH, further reducing metal solubility (White et al., 1997). In another study, Olaniran et al. (2013) showed that solubility of metal sulfides decreased by increasing the pH of environments. In a lower pH environment, more protons are available...
to saturate metal binding sites in soil; therefore, acidity generates more free ionic metal species, increase their solubility and bioavailability for plant uptake.

(2) Making insoluble Cd-sulfide complexes. Sulfur-reducing bacteria can immobilize Cd\(^{2+}\) in the soil either by producing H\(_2\)S through their normal metabolic processes or by excreting H\(_2\)S in response to Cd stress. Due to the resulting redox reaction, Cd\(^{2+}\) precipitates as CdS, which is less bioavailable to the plant and to bacteria. For example, in Klebsiella pneumoniae precipitation of Cd\(^{2+}\) to CdS on the cell surface occurs during Cd stress (Belliveau et al., 1987, Sharma et al., 2000).

(3) Producing organic chelators. Rhizosphere bacteria can excrete organic molecules that chelate Cd\(^{2+}\), making it less available to them and to plants. Organic molecules with functional groups such as sulfhydryl, carboxyl, hydroxyl, sulfonate, amine, and amides can bind Cd\(^{2+}\) and reduce bioavailability of Cd. Pseudomonas putida, for example, secretes extracellular polymeric substances that contain carboxyl and phosphate groups, which can bind Cd\(^{2+}\) and reduce bioavailability of Cd to the plant root and bacteria (Gupta and Diwan, 2017).

(4) Binding/sequestration of Cd to the cell wall and mucous layer. Negatively charged functional groups on the cell surface of the microorganisms, and on exuded proteins and extracellular polymers, can bind metal ions (Dong et al., 2007). In addition, Surowitz et al. (1984) reported that bacteria in the Bacillus genus decrease the Cd\(^{2+}\) bioavailability to plant by accumulating Cd inside their cells.
1.6.1 Effect of PGPR on Cd tolerance of plants

Common PGPR include members of the following genera: *Pseudomonas, Azotobacter, Burkholderia, Bacillus, Klebsiella, Enterobacter*, and *Azospirillum* (Verma et al., 2019). These bacteria can directly or indirectly affect plant growth and crop yield. Besides, these bacteria have crucial roles in phytostimulation, phytoremediation, and biofertilization (Saharan and Nehra, 2011). Li et al. (2012) described the role of PGPR in mobilization and phytoextraction of Cd from soil. Although Li et al. (2012) reported that PGPR increased Cd bioavailability and plant uptake by facilitating phytostabilization, Sharma and Archana (2016) described the effects of PGPR on decreasing bioavailability of Cd for plants.

Certain PGPR can also decrease Cd-induced stress using a variety of mechanisms that either (1) improve overall plant health (such as nitrogen fixation, siderophore production, phosphate solubilization, and/or the production of plant growth hormones such as indole-3-acetic acid (IAA)), (2) reduce the production of stress-induced ethylene (e.g., by producing 1-aminocyclopropane-1-carboxylatedeaminase (ACCD)) or (3) chelate Cd\(^{2+}\) ions by producing organic acids (Khanna et al., 2019).

1.6.1.1 Phosphate solubilization

Phosphorus (P) is a macronutrient that plays a role in photosynthesis, respiration, energy storage and transfer, cell division, cell enlargement and several other processes in plants (Malhotra et al., 2018). It also has an important role in reducing the bioavailability of Cd due to its high affinity for Cd\(^{2+}\) ions. Jiang et al. (2007) applied excess P to Cd-treated corn and reported that the amount of chlorophyll was increased 1.2-fold compared with the
plants that did not receive phosphorus treatment. Additionally, by using scanning electron microscopy (SEM), they found that most of the phosphate deposits with Cd were on the surface of the roots and bound to cell walls, preventing the transportation of Cd to the shoot. Many PGPR have the ability to solubilise mineral phosphate complexes in soil (Gyaneshwar et al., 2002). This results in increasing the concentrations of P in soil solution and producing insoluble Cd-P complexes. In turn, the uptake and translocation of Cd in plants is decreased and Cd\(^{2+}\) detoxification occurs in the soil (Sharma and Archana, 2016).

1.6.1.2 Nitrogen fixation

Some PGPR have the ability to fix atmospheric nitrogen and increase nitrogen supply to plants. Nitrogen-fixing PGPR can also alleviate Cd toxicity. For example, Guo et al. (2014) studied the effect on Cd stress by inoculating soybean (*Glycine max*) with two nitrogen-fixing PGPR bacteria: *Rhizobium* and *Bradyrhizobium*; 23.8% less Cd accumulated in inoculated soybean compared to uninoculated control. This result may be due to increased health and resilience of the nodulated plants.

The relationship between nitrogen supply and Cd tolerance is, however, complex. Jalloh et al. (2009) investigated the effect of four nitrogen fertilizer treatments on Cd stress in rice. They used CO(NH\(_2\))\(_2\) (urea), Ca(NO\(_3\))\(_2\) (calcium nitrate), (NH\(_4\))\(_2\)SO\(_4\) (ammonium sulphate) and organic fertilizer. The hydrolysis of NH\(_4^+\) and NO\(_3^-\) produces H\(^+\) and OH\(^-\) ions, respectively, and one would predict that NH\(_4^+\) treatment would reduce the rhizosphere pH and increase the solubility and bioavailability of Cd for plants. In addition, NO\(_3^-\) supply has been shown to increase organic acid production, which could increase chelation of Cd\(^{2+}\) (Hassan et al., 2005). However, under Cd stress, the NH\(_4^+\)-treated plants had 1.4-fold higher
grain yield compared to plants given NO$_3^-$ treatment and the Cd content of plants given NH$_4^+$ was 1/6 the amount of plants given NO$_3^-$, indicating a protective effect of NH$_4^+$. The reason for this result is not clear. In another study, Du et al. (2009) revealed that a nitrogen-efficient genotype of rice accumulated less Cd and exhibited higher Cd tolerance than a nitrogen-inefficient rice genotype.

1.6.1.3 Sufficiency of other minerals

As mentioned in section 1.2, toxic metals such as Cd can interfere with the uptake and transportation of vital nutrients in plants. Since Cd$^{2+}$ has similar chemical properties to Ca$^{2+}$, Mg$^{2+}$, Zn$^{2+}$, and Fe$^{2+}$, excess Cd can induce deficiency of these nutrients in plants. Belimov and Dietz (2000) investigated the influence of rhizobacterium inoculation and Cd stress on mineral nutrient content of barley. Their study revealed that inoculation of barley with different types of rhizobacteria increased by 8-16% the concentrations of P, Mg, Ca, S, Fe and Mn in roots and shoots, indicating that bacteria under Cd stress can either stimulate the uptake, or increase the bioavailability, of various minerals.

1.6.1.4 Siderophore production

Iron bioavailability in soils with neutral to basic pH is much lower than the nutritional requirements for plants and microorganisms, due to very low solubility of the Fe$^{3+}$ ion. Siderophores are small molecular weight compounds secreted by microorganisms and plants; they have high affinity to bind with Fe$^{3+}$, creating soluble Fe complexes in soil that can be actively taken up by plant roots and microorganisms (Crowley et al., 1991). However, previous studies have indicated that inoculation of plants with siderophore-
producing bacteria has two distinctive outcomes: inoculation may either promote or reduce toxic metal uptake, depending on the combination of plant, bacterium and metal. Sinha and Mukherjee (2008) reported reduced Cd uptake in mustard and pumpkin seeds that had been inoculated with *Pseudomonas aeruginosa*. Their results indicated that the siderophore-producing bacterium, *P. aeruginosa*, stimulated the growth of mustard and pumpkin plants and decreased Cd uptake in by 52% in roots and 37% in shoots. In contrast, Dimkpa et al. (2009) found that inoculation of sunflower (*Helianthus annuus*) seeds by the siderophore-producing bacterium *Streptomyces tendae* F4 enhanced Cd toxicity due to a 55% increase in Cd uptake by the plants. They discovered that *S. tendae* F4 produced three siderophores (desferrioxamine B, desferrioxamine E and coelichelin) that chelated with Cd$^{2+}$ and improved its bioavailability to plants.

### 1.6.1.5 Enzyme production

Under Cd stress, ethylene biosynthesis increases in various plant species (Sharma and Archana, 2016). Cadmium stress stimulates ethylene production; peak ethylene concentrations are found within 5-10 hr, then ethylene slowly drops to control levels (Bhattacharjee, 1997). This eventual reduction of ethylene in plants under Cd stress could be correlated to Cd sequestration in plant vacuoles (Sanita di Toppi et al., 1999).

Ethylene plays a crucial role in reducing Cd toxicity by stimulating glutathione (GSH) synthesis (Ovecka and Takác, 2014) because GSH reduces bioavailability of Cd by formation Cd-GSH complexes and GSH is a precursor to PCs (Mah and Jalilehvand, 2009). In mustard plants under Cd stress each of ethylene synthesis and 1-aminocyclopropane-1-carboxylic acid synthase (ACS) activity were increased up to 8.4-fold and 6.2-fold,
respectively, compared to control plants with no Cd treatment (Masood et al., 2012). ACS is a key enzyme in the plant’s ethylene biosynthesis pathway; it can be hydrolysed by bacterial ACCD when plants are under Cd stress (Sharma and Archana, 2016). Hydrolyses of ACC by ACCD by bacteria such as Enterobacter cloaceae was reported to increase Cd-stressed tomato growth by 3.8-fold (Grichko et al., 2000). In pea plants under Cd stress, inoculation with Pseudomonas brassicacearum, P. marginalis and Rhodococcus sp. increased plant biomass by 29%, 13%, and 11%, respectively, compared with non-inoculated plants. Additionally, the Rhodococcus sp. increased the content of K, Ca and S by 11%, 13%, and 29%, respectively, in Cd-treated plants (Safronova et al., 2006).

1.6.1.6 Hormone production

Indole-3-acetic acid is an auxin phytohormone, which is involved in root imitation, cell division and cell enlargement (Teale et al., 2006). Additionally, when this hormone is synthesized by PGPR, it promotes plant growth, root length, root tip number and root surface area (Chen et al., 2014). For instance, Sphingomonas sp. produced IAA and promoted 37% greater shoot growth in tomato (Khan et al., 2014). The content of IAA is reduced under Cd toxicity in plants. Arabidopsis seedlings under Cd stress had 36% less IAA after 72 hr of Cd treatment due to Cd disruption in the maintenance of auxin homeostasis (Hu et al., 2013). In another study, Cd decreased the endogenous auxin level in Arabidopsis seedlings to 53% of that of the untreated control after 7 days (Zhu et al., 2013). When under Cd stress, PGPR bacteria such as Azospirillum lipoferum, Arthrobacter myscrens, Agrobacterium radiobacter and Flavobacterium sp. produce sufficient IAA to increase the biomass of barley by up to 12% (Belimov and Dietz, 2000).
1.6.2 Effect of Cd on soil microbial communities

Cadmium contamination can also alter the composition, diversity and activity of soil microbial communities (Watt et al., 2006), which directly or indirectly affects plant-microbe interactions in the rhizosphere. Cadmium can be transported into bacterial cells via same transport systems used by essential divalent cations (Nies, 1999). Similar to plants, Cd$^{2+}$ causes toxicity in bacteria through numerous ways such as interacting with nucleic acids and binding with essential respiratory proteins (Pan and Yu, 2011). Also, Cd$^{2+}$ exposure can increase the production and accumulation of reactive oxygen species, which might inhibit microbial enzymatic activities (Li et al., 2018).

To inhibit toxicity, Cd must be rapidly and efficiently either removed from any cell or converted into a biologically inactive form. In general, bacteria have two basic mechanisms of Cd resistance: intracellular or extracellular; although, the second one is the main mechanism used by prokaryotes. Bacteria sequester Cd through biosorption of Cd$^{2+}$ on the bacterial cell wall, binding of Cd$^{2+}$ on the extracellular polymeric substances and biosurfactants, complexation of Cd$^{2+}$ by sulphur and phosphate, and removal of toxic Cd$^{2+}$ by an efflux system (Sharma and Archana, 2016).

Chemical substances excreted by some rhizospheric bacteria contain functional groups such as sulfhydryl, carboxyl, hydroxyl, sulfonate, amine and amides (Sharma and Archana, 2016), which can bind with Cd$^{2+}$ and immobilize it and prevent its entry into the plant root. The conversion of bioavailable Cd$^{2+}$ into inert chemical species is a crucial key for reducing the uptake of Cd by plants.

Using two pairs of high- and low-Cd-accumulating plants (Brassica and Triticum), Columbus and Macfie (2015) showed that plants that accumulate more Cd had similar
rhizosphere microbial communities when the plants were grown in Cd-spiked soil, and these communities were different from those of plants that did not accumulate as much Cd and were grown in Cd-free soil. This difference was not related to soil type, time, or plant genus. They speculated that plant-induced changes in the rhizosphere in response to potential Cd toxicity influenced the composition of the bacterial community. My project was designed to investigate further this phenomenon by using three *Glycine max* (L.) Merr. (soybean) cultivars with different Cd accumulation patterns.

### 1.7 Rationale and Hypothesis

To study the relationship between bacterial community diversity and plant-induced changes in the rhizosphere, I chose three soybean cultivars that have contrasting patterns of Cd accumulation: AC Hime (Poysa and Buzzell, 2005), AC X790P (Poysa and Buzzell, 2001) and Westag 97 (Ablett et al., 1999). The concentrations of Cd in seeds of field-grown AC Hime, AC X790P and Westag-97 are 0.537 ± 0.046, 0.397 ± 0.03 and 0.170 ± 0.01 mg kg⁻¹, respectively (Jegadeesan et al., 2010). In addition, Wang et al. (2014) found that Westag 97 sequesters Cd in its roots and restricts Cd translocation to leaves and seeds, whereas AC Hime had less Cd accumulation in roots and translocated more Cd to stems and leaves. Due to this negative correlation between Cd accumulation in roots and the concentration of Cd in grain, roots of AC X790P are expected to have concentrations of Cd intermediate between AC Hime and Westag 97.

I hypothesize that Cd accumulation in the root positively correlates with the exudation of TCA-cycle derived organic acids and that bacterial diversity positively correlates with the amounts of organics acids exuded into the rhizosphere. Because the roots of these three soybean cultivars are expected to accumulate Cd in different amounts, and possibly in
different compartments (Ma et al., 2016), I predict that cultivar Westag 97 will accumulate more Cd in its root cell walls and will respond to this apolastic Cd by exuding more low molecular weight organic acids. Subsequently, the bacterial diversity is predicted to be highest in the rhizosphere of Westag 97. The cultivar AC Hime is predicted to have the lowest amount of Cd bound to cell walls, the lowest exudation of organic acids, and the lowest bacterial diversity in the rhizosphere. The cultivar AC X790P is predicted to be intermediate in these characteristics.

My first objective (1) is to determine the concentration of Cd that will induce mild Cd stress, which is defined as causing a 25-30% reduction in biomass when compared to control (no Cd) plants (Baker and Walker, 1989). This is important because it ensures that the plants are not experiencing so much Cd-induced stress that the TCA-cycle fails to function. The next three objectives will be used to test my hypothesis: (2) measure the proportion of Cd bound to the cell walls of the roots, (3) quantify organic acid exudation by plant roots, and (4) determine the diversity of the bacterial community in the rhizospheres.
CHAPTER TWO: MATERIALS AND METHODS

2.1 Seed Source, Germination and Growth Conditions

For this study, seeds of three soybean cultivars, Westag 97 (high root Cd), AC Hime, (low root Cd), and AC X790P (intermediate root Cd) were selected based on their differential Cd accumulation (Jegadeesan et al., 2010). This range of patterns of Cd retention in the roots provides a good model to study the relationship between Cd accumulation in the root and plant-induced changes in the rhizosphere. If there is a direct relationship, then the results for AC X790P’s organic acid exudates, microbial functional diversity and other variables will lie between those of the other two cultivars. The Westag 97 seeds were kindly supplied by Dr. Milad Eskandari (Ridgetown College, University of Guelph, Ontario) and Dr. Kangfu Yu generously supplied seeds for AC Hime and AC X790P (Agriculture and Agri-Food Canada, Harrow, Ontario).

All seeds were surface-sterilized with 5% sodium hypochlorite solution for 3 min and rinsed 3 times with sterilized deionized water (dH$_2$O) then placed in Petri dishes to germinate. After 2 days, seedlings of uniform size were selected for one of two culture conditions: hydroponics or soil. Due to the difficulty of harvesting roots and collecting root exudates from plants grown in soil, one set of seeds was grown in hydroponic culture to estimate the proportion of Cd bound to root cell walls and to quantify the organic acids exuded by roots. To characterize the bacterial community in the rhizosphere, a second set of plants were potted in soil from a soybean field at Environmental Sciences Western Field Station, provided by Peter Duenk (the University of Western Ontario, Canada, London).
Seedlings were grown in a growth chamber with a 16:8 hr light: dark cycle (124 ± 3 μmol m⁻² s⁻¹) and maintained at 22°C and 60% relative humidity.

2.2 Hydroponic Culture

2.2.1 Establishing the cadmium dosage for mild stress

This preliminary experiment was designed to determine the concentration of Cd that would cause mild stress for each soybean cultivar in hydroponic condition. After surface-sterilizing seeds, three seeds of each soybean cultivar were placed in the fold at the top of a transparent polyethylene rhizobag (CYG germination pouch, Mega International, Minneapolis USA) filled with 150 mL of quarter-strength Hoagland’s nutrient solution (Table 2-1) and 0, 5, 10, 20, 30, 50, 100 or 200 μM CdCl₂•4H₂O (with three replicates of each treatment). Since soybean seeds require dark for germination, the rhizobags were placed in a plastic rack in a cardboard box for the first 3 days of treatment. On days 7, 10 and 13 of experimental treatment, the nutrient solution of each rhizobag was replenished with 150 mL of half-strength Hoagland’s nutrient solution (Table 2-1) and 0-200 μM CdCl₂ as above.

On day 14 of experimental treatment, plants were harvested, and the roots blotted dry with paper towel. Seedlings were separated into roots and shoots using a razor blade and the fresh biomass of each tissue was weighed. Samples were then placed in paper towel and left in an oven at 60°C for 72 hr or until constant weight. The dry biomass of roots and shoots was recorded. The concentration of cadmium that caused a 25-30% reduction in biomass relative to control plants was used in future experiments because it caused only mild stress.
Table 2-1: Chemical composition of half-strength Hoagland’s nutrient solution (Hoagland and Arnon, 1950)

<table>
<thead>
<tr>
<th>Hoagland’s reagents</th>
<th>Concentration (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Macronutrients</strong></td>
<td></td>
</tr>
<tr>
<td>MgSO₄•7H₂O</td>
<td>1×10⁻³</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>2×10⁻³</td>
</tr>
<tr>
<td>KNO₃</td>
<td>5×10⁻³</td>
</tr>
<tr>
<td>Ca(NO₃)₂•4H₂O</td>
<td>5×10⁻³</td>
</tr>
<tr>
<td>Fe-EDTA</td>
<td>9×10⁻⁶</td>
</tr>
<tr>
<td><strong>Micronutrients</strong></td>
<td></td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>4.6×10⁻⁵</td>
</tr>
<tr>
<td>MnCl₂•4H₂O</td>
<td>0.9×10⁻⁵</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>0.8×10⁻⁶</td>
</tr>
<tr>
<td>CuSO₄•5H₂O</td>
<td>0.3×10⁻⁶</td>
</tr>
<tr>
<td>Na₂MoO₄•2H₂O</td>
<td>0.1×10⁻⁶</td>
</tr>
</tbody>
</table>

This preliminary experiment determined that 20 μM to 30 μM CdCl₂ caused a 25-30% reduction in biomass of AC Hime, AC X790P, and Westag 97 (see section 3.1.1). Therefore, both dosages were used in the experiment that was designed to quantify Cd bound to root cell walls and organic acid exudations.

2.2.2 Quantifying Cd bound to root cell walls and Cd in shoots

Seeds of each soybean cultivar were sown in rhizobags and grown in a growth chamber as described in section 2.2.1, except the experimental treatments were 0, 20 or 30 μM CdCl₂ and 4 seeds were sown into each bag (three bags for each experimental treatment). Plant tissues were harvested on day 14 of experimental treatment. To estimate the proportion of Cd bound to the root cell walls, roots from one half of the plants in each rhizobag (still attached to their shoots) were rinsed in dH₂O and soaked in 5.0 mM CaCl₂ at 0°C (ice water bath) for 30 min to desorb Cd from the cell walls (Buckley et al., 2010). The amount of Cd in these desorbed roots represented the amount in the symplast. The other half of the roots
in each bag were rinsed in dH$_2$O and used to measure total root Cd. The amount of Cd in the apoplast (cell walls) was calculated as apoplastic Cd = total Cd - symplastic Cd.

Roots and shoots were separated using a razor blade and fresh tissue biomass was weighed. Samples were then placed in an oven at 60°C until they reached a constant weight, when dry biomass was recorded.

### 2.2.2.1 Quantifying the amount of Cd in roots and shoots

The concentration of Cd in oven-dried desorbed roots, unprocessed roots and shoots was determined using the Environmental Protection Agency test method SW-846 (US EPA, 2007) as modified by Akhter and Macfie (2012). To determine the efficiency of the digestion procedure NIST SRM (National Institute of Standards and Technology Standard Reference Material 1570A, spinach leaves) samples were also dried and prepared for analysis.

Dried samples were hand-chopped to ~1 mm pieces and 0.1 g subsamples were weighed into acid-washed test tubes. Afterwards, 1 mL ultra-pure nitric acid (OmniTrace® Ultra™) was added to each tube and a marble was placed on top of the tube to prevent evaporation of acid and loss of sample during digestion. Blank nitric acid samples were also digested to verify that there was no contamination. Acid digestion proceeded at room temperature overnight, approximately 18 hr, then samples were heated at 90°C until the fumes were transparent. After cooling to room temperature, samples were filtered through a Grade 413 filter paper (VWR International). Deionized water was used to rinse the test tube contents into the final test tube and the samples were brought up to a final volume of 25 mL. Samples were sent to the Biotron Experimental Climate Change Research Centre at the University
of Western Ontario for ICP-MS (Inductively Coupled Plasma – Mass Spectroscopy) analysis for Cd concentration using an Agilent 7700 Series ICP-MS.

### 2.2.3 Quantification of exuded organic acids

The samples collected for this analysis came from the same experiment described in section 2.2.2. On day 14 of experimental treatment, 30 mL of the spent nutrient solution in each rhizobag were transferred to a 50 mL polyethylene tube and stored in a -80°C freezer to stop biological processes (e.g., enzyme activity and decomposition).

After thawing and filtering the samples using a Grade 413 filter paper (VWR International), 120 μL tartaric acid (0.2 mM) was added as an internal reference to each 10 mL subsample of spent nutrient solution. The mixture was centrifuged at 24,000 g for 5 min at 25°C. The supernatant was dried in a speed vacuum system (Virtis bench Top 3.5 Freeze Dryer and Eppendorf SpeedVac) set at 45°C for a period of 72 hr. Afterwards, 1 mL of pure methanol was added to the dried samples and the solutions were transferred to 1.5 mL microcentrifuge tubes, they were briefly vortexed and then the supernatants were dried using a speed vacuum. After drying, another 1 mL methanol was added to remove any mineral salts remaining from the nutrient solution, the tubes were vortexed, and the supernatants were dried using a speed vacuum.

To prepare samples for analysis, 120 μL of pure methanol was added to dried samples. To identify and quantify exudates in the growth medium, 20 μL of the sample was directly injected into an Agilent 6230 LC–ESI–TOFMS (liquid chromatography-electrospray ionization - time of flight mass spectrometer) through a Dual Spray ESI source with a gas temperature of 325°C flowing at 10 mL min⁻¹, and a nebulizer pressure of 40 psi. The
fragmentor voltage was set to 150 V with a capillary voltage of 4000 V and a skimmer voltage of 65 V. The instrument was set to negative ESI mode.

Calibration curves for the five organic acids detected in the spent media (citric acid, fumaric acid, malic acid, malonic acid, succinic acid) and tartaric acid (the internal reference) were generated using the LC–ESI–TOFMS system (see Appendix A and B). This analysis included three sets of serial dilutions from three independent concentrated mixtures containing all 6 organic acids in methanol.

2.3 Soil Culture

2.3.1 Establishing the cadmium dosage for mild stress

It was expected that the concentration of total Cd that would cause mild stress (25-30% reduction in biomass) would differ between the rhizobag and soil experiments because Cd$^{2+}$ will adsorb to organic matter and clay in the soil, making it less bioavailable. Each pot was filled with 1 kg soil that was thoroughly mixed with 0, 5, 25, 50, 75 or 100 mg CdCl$_2$ (with three replicates of each treatment). The pots were watered to 70% field capacity then covered and placed in the growth chamber for 21 days to allow the Cd to equilibrate in soil solution.

For each of the three soybean cultivars, one surface-sterilized seed was placed in each pot. The plants were grown for 30 days and watered every fourth day to maintain approximately 70% field capacity.

Plants were harvested on day 30 of treatment. The roots were gently removed from the soil to minimize damage. The plants were rinsed with dH$_2$O to remove all soil particles
then blotted dry with paper towel. Plants were separated into roots and shoots using a razor blade and fresh tissue biomass was weighed. Samples were then placed in paper towel and left in an oven at 60°C for 144 hr or until constant weight, and the dry biomass was recorded.

This preliminary experiment determined that 40 mg CdCl₂ per kg soil caused a 25-30% reduction in biomass for each of AC Hime, AC X790P, and Westag 97 (see section 3.2.1). Therefore, that dose was used in the experiment that was designed to describe bacterial diversity.

2.3.2 Diversity of the bacterial community in the rhizospheres of three soybean cultivars

Seeds of each soybean cultivar were surface-sterilized and grown in pre-treated potted soil in a growth chamber as described in section 2.3.1, except the experimental treatments were 0 or 40 mg kg⁻¹ CdCl₂ (n = 3 for each experimental treatment).

Plants were harvested on day 30 of experimental treatment. The stem and any roots that remained attached to the stem were gently removed from the soil to minimize the damage to the roots. The rhizosphere samples were taken by placing the roots in a plastic bag, gently shaking off the soil that adhered to roots, then the stem and roots were removed. Each rhizosphere sample was homogenized by shaking the plastic bag. To evaluate the density and functional diversity of bacteria in the rhizosphere, 2 g of freshly collected rhizosphere were used (see section 2.3.2.2).

Since it was too difficult to collect all of the roots from soil, only the shoots were harvested for biomass measurements; after separating plant tissues into roots and shoots,
shoot tissues were rinsed of soil with dH₂O, placed in paper towel and left in an oven at 60°C for 144 hr or to constant weight, then shoot dry biomass was recorded.

### 2.3.2.1 Cultivar-induced changes in soil pH

To determine if the three soybean cultivars had varied effects on soil pH, 25 g of the soil remaining in each pot after rhizosphere harvest was mixed with 40 mL of dH₂O, then each sample was stirred for 45 min then left to stand for 4 hr. The 4 hr equilibration period provides time for some slowly soluble constituents to approach solution equilibrium (Hanlon, 2015). Then, without stirring the sample, the solution was filtered through a Grade 413 filter paper (VWR International) and the extract was collected in a 50 mL polystyrene centrifuge falcon tube. The pH was measured using a Thermo Scientific Orion Star A211 pH Meter, with a 9107APMD AquaPro Triode Low Maintenance pH, probe (calibrated to pH 4, 7 and 10).

### 2.3.2.2 Bacterial density and functional diversity

To determine the density of cultivatable bacteria in the rhizosphere samples, suspensions were prepared by mixing 1 g of rhizosphere soil into 10 mL of sterile 0.85% (w/v) NaCl in 15 mL Falcon centrifuge tubes. After vortexing for 1 min, aliquots (1 mL) of the supernatant were transferred to create a ten-fold dilution series (up to 10⁻⁷), and a 0.1 mL aliquot of each dilution spread onto nutrient LB agar plates (in triplicate). Inverted plates were incubated at room temperature for 48 hr, and the number of colonies forming units (CFU) counted.
For 20 plates of LB medium, 5 g Bacto™ Tryptone (Fisher Scientific), 2.5 g yeast extract (Fisher Scientific), 5 g NaCl (Caledon) and 7.5 g Bacto™ agar (Fisher Scientific) were dissolved in 500 mL dH₂O. The solution was autoclaved for 20 min, poured into the plates, then allowed to cool at room temperature overnight before storing at 4°C.

To assess the physiological diversity of bacteria in the rhizosphere samples, Biolog® EcoPlates™ were used. This product is specifically designed for bacterial community analysis and ecological studies. The fundamental principle of the Biolog® technique is to use the varying ability of microbes to metabolize different carbon sources to characterize the diversity of community-level physiological profiles. The Biolog® EcoPlate™ contains 31 of the most useful carbon sources for soil organisms. The metabolism of each carbon source releases a water-soluble tetrazolium salt, which is reduced to a purple formazan (Figure 2-1). Formazan can be detected spectrophotometrically by absorption at 590 nm and the intensity of purple color indicates how much of each substrate has been utilized (Chojniak et al., 2015).

**Figure 2-1: Reduction of tetrazole to purple formazan.** Bacteria will convert MTT to formazan only if the carbon source in the Biolog® EcoPlate™ well was metabolized.
All solutions, transfer equipment, and glassware were sterilized with an autoclave at 121°C for 20 min prior to use. All work during plate preparation was done in a laminar flow hood to minimize the risk of contamination.

Cell suspensions were obtained by adding 1 g of each rhizosphere soil to 1 mL of sterile 0.85% (w/v) NaCl then vortexing for 2 min to help separate microorganisms from soil or/and root surfaces. The samples were then left for a few minutes to allow the large particles to settle out. Aliquots of 400 μL of supernatant were diluted in 19.6 mL of sterile 0.85% (w/v) NaCl. Afterwards, 150 μL of the cell suspension was inoculated into each well of a Biolog® EcoPlate™ using a sterile pipette. To ensure that the aliquots were representative of the bacterial community, 3 replicate plates were used for each rhizosphere sample.

All plates were placed in polyethylene bags to reduce desiccation, and then incubated in the dark at room temperature. After 72 hr and 168 hr incubation, color development in each well was measured as absorbance at 600 nm using a microplate reader (Turner BioSystems, Inc. Modulus™ II Microplate Reader).

The absorbance values from the control wells in each Biolog® EcoPlate™ were subtracted from the absorbance values for the corresponding wells containing the 31 carbon sources.

(Equation 2-1)

\[
\text{Absorbance value} = W - C
\]

where W is the absorbance value of one well and C is the mean value of the control well.
After this normalisation, the average absorbance value of each three replicate wells was calculated to obtain a single value for each carbon source within a Biolog® EcoPlate™. Wells showing very little colour response sometimes gave negative absorbance values after normalisation. Such negative numbers and absorbance values below 0.06 were set to zero (Gryta et al., 2014).

The average well colour development (AWCD) value across the whole plate for each rhizosphere sample was calculated (Preston-Mafham et al., 2002).

(Equation 2-2)

\[ AWCD = \frac{\sum (W - C)}{R} \]

where R is substrate richness, which here is the number of metabolised carbon substrates among the 31 carbon sources.

The proportional colour development (pi) is a measure of how a bacterial community utilized each individual carbon source relative to its utilization of all carbon sources; it was calculated using the following formula:

(Equation 2-3)

\[ pi = \frac{(W - C)}{\sum (W - C)} \]

where \((W - C)\) is the absorbance value in each well over the sum of total absorbance values.

The Shannon index \((H')\), also known as Shannon's diversity index, is a common ecological metric used to understand how many species are in a community and how evenly
each individual is represented in the community (Gryta et al., 2014). Within the context of a Biolog® EcoPlate™, H’ is used as a measure of the physiological diversity of the microbial community – not the diversity of species presents in the sample but rather the diversity within the community in terms of its members’ ability to metabolize different carbon sources.

(Equation 2-4)

\[ H' = -\Sigma pi \times \ln pi \]

where \( pi \) = proportional color development of the well over total color development of all wells of a plate.

The evenness (E) of a community varies from close to 0 to 1, where 1 represents a perfectly even community with an equal proportion of all individual species. In this context, E measures the homogeneity of bacterial community in terms of using particular carbon sources.

(Equation 2-5)

\[ E = H'/\ln C \]

where \( H' \) is the Shannon Diversity Index and \( C \) is the number of the carbon source on the Biolog® EcoPlate™ (C = 31).
2.4 Statistical Analyses

The mean, standard deviation and standard errors of mean were calculated for each data set using MS-Excel. SigmaPlot® v11 was used to evaluate differences among treatment groups for the data related to Cd toxicity, uptake and adsorption to root cell walls, as well as the organic acid data. The Shapiro-Wilk test was used to verify normality and homogeneity of variance prior to analysis of variance (ANOVA) tests. The main effects of cultivar and Cd treatment were determined using two-way ANOVA (P < 0.05) where no interaction among main effects was found. In cases where ANOVA detected significant main effects, Holm-Sidak pairwise multiple comparisons were performed to determine significant differences among means (P < 0.05). For the data collected using Biolog® EcoPlate™, the main effects of cultivar and Cd treatment on AWC, richness, evenness, and diversity were analyzed by the Shapiro-Wilk test followed by two-way ANOVA and the Holm-Sidak test using SigmaPlot® (P < 0.05). To visualize the relationship in carbon utilization patterns among the rhizosphere samples, Principal Component Analysis (PCA) was performed on the normalized AWCD data set using R version 3.6.0; the R packages used were precomp, devtools, and ggbiplot.
CHAPTER THREE: RESULTS AND DISCUSSION

For logistical reasons, plants were grown under each of two conditions (hydroponic culture and soil culture) to test the hypothesis that seedlings with more Cd adsorbed to root cell walls exude more organic acids into their rhizosphere and that the diversity of the microbial community in the rhizosphere is correlated with the amounts of organic acids. Collecting intact roots and exudates from hydroponic solution results in a higher recovery than similar collections from soil. On the other hand, the microbial community in the rhizosphere is best studied in plants grown in field-collected soil.

Two preliminary experiments to select experimental doses of Cd in hydroponic and soil culture, respectively, were essential to ensure that the seedlings were experiencing only mild Cd stress. Moderate to severe Cd toxicity disrupts DNA replication, gene expression, and cell division (Meng et al., 2017), enhances the production of ROS, which may cause damage to the photosynthetic system (Xue et al., 2014), and/or inhibits Calvin-Benson cycle enzymes activities, especially Rubisco, which are important in CO₂ fixation (Ashida et al., 2007). In order to ensure that the treated plants in my study still had the capacity to produce and exude organic acids, it was important to choose a dose of Cd that was enough to elicit a stress response but not so high as to prevent TCA cycle function.

In this chapter, the results for the hydroponic study are presented and discussed, then the results from the soil study. These sections are followed by some limitations and suggestions for future work, as well as a general discussion of the conclusions arising from the two studies combined.
3.1 Hydroponic Culture

3.1.1 Determining the Cd dosage for the experimental treatments

To determine the dosage of Cd that would induce mild Cd stress in three soybean cultivars (AC Hime, AC X790P, and Westag 97), seedlings were exposed to a range of Cd concentrations in hydroponic solution for 14 days. The extent of toxicity was assessed by measuring the dry weight of roots and shoots. Low concentrations of Cd (5 and 10 μM CdCl₂, and up to 20 μM in AC Hime) stimulated shoot (Figure 3-1A) and root (Figure 3-1B) growth compared to control (0 Cd) plants, while concentrations of Cd above 50 μM reduced dry mass to 50% or less of the control plants.

The positive low-dose response phenomenon is called hormesis – a term used by toxicologists to refer to any beneficial effect induced by low doses of a toxicant (Mattson, 2008). Although the three soybean cultivars responded similarly to high (> 50 μM) Cd concentrations, lower concentrations (20 μM or less) caused hormesis for these plants. Based on the dry weights of shoots and roots, Westag 97 and AC X790P had the highest hormesis at a Cd concentration of 10 μM, whereas AC Hime (shoot and root) had the highest hormesis at 20 μM Cd. The maximum Cd-induced hormetric increases in dry biomass for Westag 97 were 35% and 75% for shoots and roots, respectively, compared with control plants. For AC X790P shoot and root biomass increased by 31% and 35%, respectively, and for AC Hime shoot and root biomass increased by 33% and 30%, respectively.
(A) Shoot dry biomass

(B) Root dry biomass

Figure 3-1: Dose response curves for soybean cultivars Westag 97, AC X790P, and AC Hime. Plants were grown in hydroponics with 0 to 200 µM CdCl₂ for 14 days. Means of three replicates for shoots (A) and roots (B) are plotted; bars indicate standard errors of the mean; DW = dry weight.
Here, for the first time, the hormetic effect of a low dose of Cd on seedlings of soybean cultivars AC Hime, Westag 97 and AC X790P was measured. However, Sobkowiak and Deckert (2003) studied the effect of Cd on growth of soybean suspension-culture cells in the range of 1–11 μM Cd in hydroponic culture and showed that Cd stimulates (1–4 μM) and then inhibits (>6 μM) the growth of soybean cells. While Wang et al. (2016) studied Cd stress (0, 9, 23, 45, 90 μM) in five soybean cultivars for 18 days in hydroponic culture, they did not observed any hormetic effects; at each concentration of Cd, the shoot and root biomass of these cultivars decreased (32-93% and 28-91%, respectively) compared to control with 0 Cd.

Hormesis in response to Cd has been reported in other plants. For example, a hydroponic study on oilseed rape revealed that after 28 days with 5 μM Cd the shoot and root dry weights increased 14% and 37%, respectively, compared to control with 0 Cd respectively; and then dropped with >45 μM Cd (Durenne et al., 2018). The mechanism behind hormesis is related to altered nutrient uptake. For example, Zhouli et al. (2015) reported that a low concentration of soil Cd (10 mg kg\(^{-1}\)) had a beneficial effect on *Lonicera japonica* photosynthetic system and increased the concentration of chlorophyll (31%) and carotenoids (26%) due to a synergistic interaction between Cd and the accumulation and translocation of Fe. Zhou and Qiu (2005) reported that the amount of Fe was 10-fold higher in *L. japonica* roots due to a hormetic effect in plants with Cd treatment. Since Fe is essential to the metabolism of mitochondria and chloroplasts (Paul et al., 2018), exposure to a low dose of Cd caused an increase in plant growth rate. Additionally, Lin et al. (2007) reported that Cd (<3.3 mg kg\(^{-1}\)) increased wheat seedling fresh weight of shoot and roots by 68%
and 44%, respectively; they suggested the hormetic effects on plant growth is due to an increase in plant cell division rate (Sobkowiak and Deckert, 2003).

The competition between $\text{Zn}^{2+}$ and $\text{Cd}^{2+}$ for the same cellular binding sites can help explain the hormetic effect. Zinc is an essential nutrient for plants which allows multiple transcription factors to bind to the regulatory regions of genes (Hafeez et al., 2013). Zinc is also a component of enzymes that participate in DNA replication and RNA translation (Sluyser et al., 1993). Cadmium has a strong affinity to functionally substitute $\text{Zn}^{2+}$ (Hafeez et al., 2013) because of their similarity in physicochemical properties. Hormesis is believed to occur when the concentration of Cd is below the threshold for negative effects and if molecules that normally contain Zn can function with Cd. In this situation, low amounts of Cd can mimic the effect of adding Zn and stimulate the physiological process.

As expected, high concentrations of Cd inhibited the growth of all three soybean cultivars. The maximum reduction in dry weight of Westag 97 shoots (Figure 3-1A) and roots (Figure 3-1B) were 75% and 76%, respectively, compared with its control (0 Cd) when exposed to 200 $\mu$M Cd treatment. Each of AC X790P and AC Hime were more sensitive to Cd; they died when they were exposed to 200 $\mu$M (Figure 3-1).

Mild Cd stress, defined by having a 25-30% reduction in dry biomass relative to control plants (Baker and Walker, 1989), was found for Westag 97 and AC X790P shoots and roots grown in the 20 $\mu$M Cd treatment (Figure 3-1). For AC Hime, however, mild stress of shoots and roots was found at 30 $\mu$M Cd. Additional symptoms of mild Cd stress in these plants were rolled leaves, necrosis (brown spots), and altered root morphology. Increasing the Cd treatment resulted in thicker and shorter roots, and root tips became brownish
compared to control (0 Cd) plants, symptoms that are typical of Cd stress (Arduini et al., 1994). For comparison among cultivars, 0, 20 and 30 μM CdCl$_2$ were chosen as the experimental treatments for the subsequent hydroponic experiments.

3.1.2 Soybean growth in the experimental treatments

The results from the dose-response experiment described in part 3.1.1 were confirmed in this experiment; mild Cd stress was induced by 20 μM Cd in Westag 97 and AC X790P, and by 30 μM Cd in AC Hime (Figure 3-2). This is the first report of the relative responses of the three cultivars to low concentrations of Cd.

3.1.3 Concentration of Cd in soybean cultivars

As expected, increasing the concentration of Cd in solution resulted in elevated concentrations of Cd in shoots (Figure 3-3A) and roots (Figure 3-3B). Low concentrations of Cd in shoots and roots of control plants (0 Cd) were detectable in each cultivar, which may be explained by residual Cd in the seed because the parental soybean plants had been cultivated in agricultural fields (Jegadeesan et al., 2010; Peng et al., 2017; Godinho et al., 2018).

In shoots across all Cd treatments, the concentrations of Cd in AC Hime were 18-23 % higher than the concentrations in AC X790P, and the concentrations of Cd in AC X790P were 12-23% higher than in Westag 97 (Figure 3-3A), confirming the relative patterns of aboveground Cd accumulation in these cultivars (Wang et al., 2014; Wang et al., 2018).
Figure 3-2: Shoot and root dry biomass of soybean cultivars Westag 97, AC X790P, and AC Hime. Plants were grown in hydroponics with 0, 20 or 30 µM CdCl₂ for 14 days. Within each panel, different lower-case letters indicate significant differences in mean dry weight (Holm-Sidak test, P<0.05) of shoots (A) and roots (B). Means of three replicates are plotted; bars indicate standard errors of the mean; DW = dry weight.
(A) Cadmium concentration in shoots

![Graph A](image)

(B) Cadmium concentration in roots

![Graph B](image)

Figure 3-3: Total cadmium concentration in shoots and roots of soybean cultivars Westag 97, AC X790P, and AC Hime. Plants were grown in hydroponics with 0, 20 or 30 µM CdCl₂ for 14 days. Different lower-case letters indicate significant differences in mean cadmium concentration (Holm-Sidak test, P<0.001) in shoots (A) and roots (B). Means of three replicates are plotted; bars indicate standard errors of the mean; DW = dry weight.
Conversely in roots, the Cd concentrations in Westag 97 were 18-35% higher than the concentrations in AC X790P and Cd concentrations were lowest in AC Hime (Figure 3-3B), confirming previous reports about the relative Cd content in roots of these cultivars (Wang et al., 2014; Wang et al., 2018).

Grant et al. (1998) reported that the Cd concentration in shoots is determined by the capacity of roots to accumulate Cd; cultivars with a smaller capacity to accumulate Cd in roots would translocate and accumulate more Cd in shoots. My results corroborate this finding. Westag 97 accumulated more Cd in its roots, which seemingly prevented Cd from translocating into stems and leaves; conversely, AC Hime translocated more Cd to its shoots and accumulated less Cd in its roots.

One mechanism by which more (or less) Cd is preferentially retained in roots could be related to compartmentalization of Cd in endoplasmic reticulum (ER). Wang et al. (2018) found that in Westag 97 and AC Hime, Cd was mainly compartmentalized in the root ER through the Cd transporter GmHMA3w. They reported that the subcellular localization of GmHMA3w is the soybean root ER and overexpression of GmHMA3w in Westag 97 and AC Hime inhibited Cd translocation from the roots to the shoots but did not affect Cd accumulation. The amount of Cd translocation from root to shoot for AC Hime was higher than Westag 97, with 73% and 53% of the Cd being translocated, respectively.

3.1.4 Cadmium distribution pattern in roots

To understand the mechanism controlling differential accumulation of Cd in plant roots, three soybean cultivars were investigated for Cd segregation between apoplastic and symplastic. As the concentration of Cd in hydroponic culture increased, so did the
concentration of Cd in the apoplast (Figure 3-4A). Unlike the pattern for total root Cd (Figure 3-3B), there was less difference among cultivars and the pattern of Westag 97 > AC X790P > AC Hime was found only for control (0 Cd) plants, not for the Cd-treated plants. This suggests that a symplastic mechanism explains differential retention of Cd in the roots of soybean. Closer examination of the distribution of Cd within the roots (Figure 3-4B) reveals that the amount of Cd in the symplast of each Cd-treated cultivar was 1.6-fold to 4.2-fold higher than that the amount of Cd in their apoplast (Figure 3-4B). The amount of Cd in the symplast of Cd-treated Westag 97 roots was 69-81% of total Cd, AC Hime was 66-67% of total Cd and AC X790P was 62-69% of total Cd; the relative proportions of total Cd in symplast of these three soybean cultivars followed the order: Westag > AC X7890P ~ AC Hime. These results support the idea that symplastic mechanisms control differential retention of Cd in soybean roots.

For Cd toxicity to inhibit metabolism, symplastic pathways play a crucial role because apoplastic Cd is not available to inhibit enzyme activity (Li et al., 2017). Cadmium has the capacity to be transported to and stored in the roots and shoots through both apoplastic and symplastic pathways (Song et al., 2017). In the apoplastic pathway, Cd can move through extracellular fluid and gas spaces as well as between and within cell walls; while in the symplastic pathway, Cd moves from cell to cell through plasmodesmata (Song et al., 2017). The symplastic pathway is more complicated than the apoplastic pathway, due to involving many membrane transporters, including those in cellular organelles such as the vacuole and endoplasmic reticulum (ER) (Song et al., 2017; Benavides et al., 2005).
(A) Concentration of Cd in apoplast

(B) Distribution of Cd in apoplast and symplast

Figure 3-4: Cadmium in symplast and apoplast of the roots of soybean cultivars Westag 97, AC X790P, and AC Hime. Plants were grown in hydroponics with 0, 20 or 30 µM CdCl₂ for 14 days. (A) Concentration of Cd in apoplast was estimated by measuring the amount of Cd that was desorbed from the roots using 5 mM CaCl₂, and (B) Distribution of Cd in apoplast and symplast. Different lower-case letters indicate significant differences in mean cadmium concentration (Holm-Sidak test, P<0.001). Means of three replicates are plotted; bars indicate standard errors of the mean.
Wang et al. (2018) reported that, after two weeks in 80 μM Cd treatment, in transgenic Westag 97 and AC Hime (which lacked the GmHMA3w gene) 50% of the total amount of Cd in root accumulated in the soluble fraction including the vacuole. However, AC Hime accumulated 40% of the Cd in the root cell wall fraction and 10% in the organelle fraction while, Westag 97 accumulated 10% of the Cd in the root cell wall fraction and 40% in the organelle fraction. Comparing these results with non-transgenic soybean showed that the Cd concentration was reduced in the cell wall fraction, in the soluble fraction, and in the organelle fraction by 46%, 62%, and 83%, respectively, which indicated GmHMA3w transported intercellular Cd into the root ER and restricted translocation to the shoots (Wang et al., 2018). Based on the results of my study, increased compartmentalization of Cd in the symplast was found in Westag 97, the cultivar with less Cd in its shoots. These results confirm Wang et al.’s (2018) study. Additionally, my results could be interpreted as showing that the apoplastic pathway is important for the root to shoot translocation of Cd in soybean.

3.1.5 Organic acid exudates

Of the five organic acids that are frequently found in exudates of Cd-stressed soybean (malonic acid, malic acid, citric acid, succinic acid, and fumaric acid; Tawaraya et al., 2014), only malonic acid was not detected by the LC–ESI–TOFMS system in my experiment. Among these organic acids, the concentrations of succinic acid were 2-fold to 10-fold higher than the other organic acids in all cultivars and Cd treatments (Figure 3-5). Westag 97 had the highest concentrations of each organic acid, both in control conditions and when under mild Cd stress (Figure 3-5).
Figure 3-5: Concentrations of organic acids exuded by soybean cultivars Westag 97, AC X790P, and AC Hime. Plants were grown in hydroponics with 0, 20 or 30 µM CdCl2 for 14 days. (A) Citric acid (B) Succinic acid (C) Fumaric acid (D) Malic acid. Within each panel, different lower-case letters indicate significant differences in mean organic acid concentration (Holm-Sidak test, P<0.001). Means of three replicates are plotted; bars indicate standard errors of the mean.
In general, the concentrations of organic acids exuded by three cultivars of soybean increased with the amount of Cd in nutrient solution, especially for AC Hime in which citric acid increased 2-fold (Figure 3-6A), succinic acid increased 9-fold (Figure 3-6B), fumaric acid increased 11-fold (Figure 3-6C) and malic acid increased 7-fold (Figure 3-6D) relative to control values. The three exceptions to this general pattern were for AC X790P from the 20 μM Cd treatment, in which concentrations of succinic, fumaric and malic acids decreased by 21%, 24% and 50%, respectively. Nian et al. (2005) exposed 3-day-old seedling of soybean genotype LJ to 4 μM Cd for 24 hr; in root secretions they found both citrate and malate; where the secretion for both organic acids was < 50 nmol g⁻¹ root fresh weight and the amount of citrate was 2-fold higher than malate.

Studies of the other crops illustrate that the type and amount of organic acid root exudation varies with the plant species (reviewed in Lapie et al., 2019). For instance, Pinto et al. (2008) reported that roots of maize under Cd stress (5 mg L⁻¹) released only citrate, whereas sorghum under Cd stress (5 mg L⁻¹) released only malate. Moreover, their results showed that by increasing Cd concentration in solution, the amount of citrate in maize and malate in sorghum increased 4-fold and 20-fold, respectively, compared to control with 0 Cd. In another study, Zhu et al. (2011) showed that, when under Cd stress (25 and 50 μM), two wild tomato cultivars with low and high Cd accumulation in their roots, oxalate was the only organic acid exuded by their roots in response to Cd stress. Also, when the Cd concentration in solution increased, the amount of oxalate increased 0.8 to 1.5-fold in the high-Cd-accumulating cultivar and 1.5 to 2.3-fold in low-Cd-accumulating cultivar, which indicated that the amount of oxalate exuded by the low-Cd-accumulating cultivar roots was 1.5-fold higher than the high-Cd-accumulating cultivar.
Figure 3-6: Relative concentrations of organic acids exuded by soybean cultivars Westag 97, AC X790P, and AC Hime. Plants were grown in hydroponics with 0, 20 or 30 µM CdCl₂ for 14 days. (A) Citric acid (B) Succinic acid (C) Fumaric acid (D) Malic acid. Relative concentration was calculated in each mean value of treated plant divided by the corresponding control value. Within each panel. Means of three replicates are plotted; Since mean values were used to calculate concentrations as a percent of control, the stats were not performed on these data, bars indicate proportional error.
One plant strategy to detoxify Cd is to exude low molecular weight organic acids into the rhizosphere, which can chelate with Cd\(^{2+}\) to prevent its entrance into roots (Zhu et al., 2011). In my study, the relative increase in organic acid exudation in response to Cd was highest in AC Hime compared to Westag 97 and AC X790P (Figure 3-6). These results confirmed Zhu et al.’s (2011) study in which the cultivar with lower Cd contents in its roots exuded more organic acids to contribute to Cd resistance. Thus, AC Hime, by exuding more organic acids when under Cd stress, likely reduced the solubility and bioavailability of Cd for uptake by plant, through the formation of Cd-organic acid complexes (Adeleke et al., 2017).

**3.1.6 Summary and conclusions of the hydroponic experiments**

In terms of concentrations of Cd in roots and shoots of the three cultivars based on Wang et al.’s (2014) studies, my prediction that AC Hime would have the highest amount of Cd in its shoots, while Westag 97 would accumulate more Cd in its roots and cultivar AC X790P would be intermediate in these characteristics, was upheld. As expected, increasing the concentration of Cd in solution resulted in elevated concentrations of Cd in shoots and roots in all plants.

Additionally, I expected Westag 97 (with the highest amount of Cd in the roots) to have most of that Cd in the apoplast, and AC Hime (with the lowest amount of Cd in the root) to have less Cd in the apoplast. However, I found Westag 97 had less Cd in the apoplast compared to AC Hime and AC X790P; instead it had compartmentalized Cd in the symplast, likely associated with the root cell ER (Wang et al., 2018), which is an equally valid mechanism for retention of Cd in roots.
I expected Westag 97 (with highest amount of Cd in its roots) to exude more organic acids into the rhizosphere because it would produce more chelators to detoxify Cd and these would diffuse from the cell walls into the rhizosphere. This pattern was found. However, I discovered that AC Hime (with lowest amount of Cd in its root) had the highest Cd-induced increase in organic acid exudation into the rhizosphere compared to other cultivars in response to Cd.

Previous studies have shown that the organic acids secreted by plant roots not only play an important role in immobilization of Cd, they are also an important energy source for microorganisms (reviewed in Adeleke et al., 2017). On the other hand, microorganisms such as bacteria can also sequestrate Cd by producing organic acids and reducing Cd bioavailability through the formation of soluble or insoluble Cd complexes (Chellaiah, 2018). Sharma (2016) reported that mild Cd stress enhanced microbial activity in the rhizosphere due to the increased amount of organic acids secreted by plant roots; while Cd toxicity inhibited plant growth and decreased microbial enrichment in the rhizosphere. In my study using mild Cd stress, Westag 97 had the highest concentrations of Cd in its roots compared to AC X790P and AC Hime, but AC Hime had the strongest Cd-induced exudation response. In a study of two rice (*Oryza sativa*) cultivars with differential accumulation of Cd in their seeds, the rhizosphere bacterial community associated with the cultivar with high grain Cd was less diverse than the community associated with the cultivar that had low grain Cd; the authors suggested this was due to differential root exudates (Hou et al., 2018).

Based on the greater concentrations of organic acids exuded by Westag 97 compared to AC Hime and AC X790P, I predict that Westag 97 would be associated with higher
numbers, and perhaps diversity, of bacteria because organic acids can be used as an energy source by bacteria. Since bacteria do not grow well in hydroponic culture, the bacteria were studied the rhizosphere of soybean grown in soil.

3.2 Soil Culture

3.2.1 Determining the Cd dosage for the experimental treatments

To evaluate the dose required to induce mild Cd stress in soil for three soybean cultivars (AC Hime, AC X790P, and Westag 97), seedlings were sown in potted field soil treated with 0 to 100 mg Cd kg$^{-1}$ soil and grown for 30 days. Following harvest, the root and shoot dry weights were recorded. The patterns of results for plant biomass (Figure 3-7) are similar to those found in the hydroponic study (Figure 3-1). Specifically, hormesis was observed for shoots (Figure 3-7A) and roots (Figure 3-7B) grown with low doses of Cd (25 mg kg$^{-1}$ soil) and higher concentrations of Cd (50, 75, and 100 mg kg$^{-1}$ soil) resulted in very small plants. The physiological reasons for hormesis and Cd toxicity were explained in section 3.1.1.

The horneric effect for shoots and roots of all three cultivars (Westag 97, AC X790P and AC Hime) was found in the Cd treatment with 25 mg kg soil$^{-1}$ (Figure 3-7). The maximum hormetic increases in shoot dry weights of Westag 97, AC X790P, and AC Hime were 29%, 53% and 23%, respectively, compared with their controls (Figure 3-7A). The maximum hormetic increases in root dry weights of Westag 97, AC X790P, and AC Hime were 33%, 20% and 37%, respectively (Figure 3-7B). Similarly, Yang et al. (2016) reported a hormeric effect of Cd (3 mg kg$^{-1}$) on carrot in soil culture. They found that Cd
stimulated shoot and root of carrot by 47% and 12%, respectively, compared to control with 0 Cd.

(A) Shoots dry biomass

(B) Roots dry biomass

Figure 3-7: Shoot and root dry biomass of soybean cultivars Westag 97, AC X790P, and AC Hime. Plants were grown in Cd-spiked soil for 30 days. Means of three replicates for shoots (A) and roots (B) are plotted, bars indicate standard errors of the mean value; DW = dry weight.
Jia et al. (2015) also observed a hormetic effect on *Lonicera japonica*. They reported that Cd (10 mg kg\(^{-1}\)) treatment stimulated shoots biomass by 52%, while Cd stimulated roots biomass by 23% at 2.5 mg kg\(^{-1}\) Cd.

As expected, high concentrations of Cd inhibited plant growth. The maximum reduction in dry weight of Westag 97, AC X790P and AC Hime shoots (Figure 3-7A) were 93%, 91% and 81%, respectively, compared with their control (0 Cd) when plants were exposed to the 100 mg kg soil\(^{-1}\) Cd treatment. Conversely, the maximum reduction in dry weight of Westag 97, AC X790P and AC Hime roots (Figure 3-7B) were 91%, 86% and 91% respectively, compared with their control (0 Cd) when plants were exposed to the 100 mg kg soil\(^{-1}\) Cd treatment.

Shute and Macfie (2006) reported that in a 100 mg kg soil\(^{-1}\) Cd treatment of soybean, plant dry weight was reduced by 34% compared with control 0 Cd, while I found that a 100 mg kg soil\(^{-1}\) Cd treatment caused an 81-93% reduction in plant dry weight. This difference may be dependent on many factors including soil composition, pH, treatment length, and organic matter content. They reported that in their experiment the soil pH was 6.7, while in my study the average of the soil pH in bulk soils and rhizospheres was around 8 (Appendix C). The bioavailability of Cd in soil with pH 8 should be less than the soil with 6.7 (Ono et al., 2019), but other factors such as the amount of organic matter (Sheoran et al., 2016) and clay (Rieuwert et al., 1998) in soil can cause an increase in solubility of Cd in soil and therefore more Cd toxicity for plants. Additionally, they treated the soybean plants for 90 days with Cd while I treated the soybean plants for 30 days.
Mild Cd stress for each of Westag 97, AC X790P, and AC Hime was estimated to occur with 40 mg kg soil\(^{-1}\) Cd treatment (Figure 3-7); therefore, 0 and 40 mg kg soil\(^{-1}\) were chosen as the experimental treatments for the subsequent potted plant experiment.

### 3.2.2 Soybean growth under experimental treatments

The results from the dose-response experiment (described in section 3.2.1) indicated that mild Cd stress should occur with 40 mg kg soil\(^{-1}\). This prediction was confirmed, since dry biomass of Westag 97, AC X790P, and AC Hime shoots was decreased by 25% to 30% at this dose (Figure 3-8).

![Figure 3-8: Shoot dry biomass of soybean cultivars Westag 97, AC X790P, and AC Hime. Plants were grown in Cd-spiked soil for 30 days. Different lower-case letters indicate significant differences in mean shoot dry weight (Holm-Sidak test, P<0.001). Means of three replicates are plotted; bars indicate standard errors of the mean value; DW = dry weight.](image-url)
3.2.3 Quantifying bacterial communities in soil

Among the microorganisms in soil, bacteria are the most abundant. Approximately 4000 to 6000 different bacterial genomes can be detected, and more than 1000 million (10^9) viable bacterial cells are in a single gram (dry weight) of healthy soil (Dunbar et al., 2002). Variations in population numbers, activities and diversity (e.g., species and function) of soil organisms can be used as an indicator of the health and productivity of soil ecosystems (Hermans et al., 2017). In my study, while I did not identify microbial species on the plates, I saw many different colours and shapes of CFUs. The CFU density in the untreated bulk soil was about 20% higher than that of the Cd-treated bulk soil (brown bars in Figure 3-9). This decrease in CFU density is conceivably due to Cd toxicity. Šmejkalová et al. (2003) have reported similar results for soil contaminated with 100 mg kg^{-1} Cd; the total bacterial counts were three orders of magnitude below those in an untreated soil. Moreover, Al-Gaidi (2010) reported that soil contaminated with different concentrations of Cd (1.5, 3 and 6 mg kg^{-1}) had noticeably reduced total bacterial counts compared to soil contaminated with different concentrations of Pb (80 and 160 mg kg^{-1}).

Bacterial CFUs in the rhizospheres of control (0 Cd) plants were less than half of the values from control bulk soil (Figure 3-9). This phenomenon has been observed by others (for example Fernández-Gómez et al., 2019) and may indicate that the chemistry of the soil in the rhizosphere restricts the growth of some bacterial species and/or favours the growth of others. When comparing the bacteria in rhizospheres in control compared to Cd-treated soil, the only difference was found for AC Hime, for which the CFU counts of treated rhizosphere were 1.4-fold higher than untreated rhizospheres (Figure 3-9).
Figure 3-9: The mean of CFU density in rhizospheres of soybean cultivars Westag 97, AC X790P, and AC Hime. Plants were grown in Cd-spiked soil for 30 days. Different lower-case letters indicate significant differences in mean CFU (Holm-Sidak test, P<0.05). Means of three replicates are plotted; bars indicate standard errors of the mean value.

3.2.4 Microbial functional diversity in soybean rhizosphere

The metabolic functional diversity of soil bacterial communities was analyzed using Biolog® EcoPlates™ with 31 different carbon substrates. The carbon sources were assigned to five categories (guilds; Table 3-2): carboxylic acids, carbohydrates, amino acids, polymers, and amines/amides because different microbial communities have varying ability to utilize these carbon sources (Garland and Miles, 1991). In this study substrate utilization was monitored by measuring absorbance using a plate reader after 72 and 168
hr. Although the best resolution for microbial activity is typically obtained at the shortest incubation time (Frąc et al., 2012), absorbance data were collected at both time periods.

<table>
<thead>
<tr>
<th>Chemical guild</th>
<th>Substrate</th>
<th>Chemical formula</th>
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</thead>
<tbody>
<tr>
<td><strong>Amines/amides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenylethylamine</td>
<td>C₈H₁₁N</td>
<td></td>
</tr>
<tr>
<td>Putrescine</td>
<td>C₄H₁₂N₂</td>
<td></td>
</tr>
<tr>
<td>Glycyl-L-glutamic acid</td>
<td>C₇H₁₂N₂O₅</td>
<td></td>
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<tr>
<td>L-Arginine</td>
<td>C₆H₁₄N₄O₂</td>
<td></td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>C₄H₈N₂O₃</td>
<td></td>
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<tr>
<td>L-Phenylalanine</td>
<td>C₉H₁₁NO₂</td>
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<tr>
<td>L-Serine</td>
<td>C₃H₇NO₃</td>
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<tr>
<td>L-Threonine</td>
<td>C₄H₆NO₃</td>
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<tr>
<td><strong>Amino acids</strong></td>
<td></td>
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<tr>
<td>α-d-Lactose</td>
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<tr>
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<tr>
<td>N-Acetyl-d-glucosamine</td>
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<tr>
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<td>C₄H₆O₃</td>
<td></td>
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<tr>
<td>α-Keto butyric acid</td>
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<tr>
<td>D-Galacturonic acid</td>
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<tr>
<td>D-Malic acid</td>
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<tr>
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<tr>
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<td>C₃₆H₆₀O₃₀</td>
<td></td>
</tr>
<tr>
<td>Glycogen</td>
<td>(C₆H₁₀O₅)ₙ</td>
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<tr>
<td>Tween 40</td>
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<tr>
<td>Tween 80</td>
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<tr>
<td><strong>Polymers</strong></td>
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Average well color development (AWCD) for each plate is a measure of the microbial community’s ability to metabolize a variety of carbon sources. At 72 and 168 hr, the untreated (control) bulk soil sample had 1.3-fold and 1.4-fold higher AWCD values, respectively, than the Cd-treated bulk soil (brown bars in Figure 3-10A and B). This verifies that Cd inhibits the soil microbial community because less carbon was metabolized. Feng et al. (2018) also showed that Cd had capacity to change the microbial dynamic in soil and decrease the number of species of microbes in soil.

The AWCD at 72 hr of bulk soil in control conditions (0 Cd) was about 1.6-fold higher than the AWCD of rhizosphere soil from each of the cultivars (Figure 3-10A). This difference increased to 1.9-fold by 168 hr (Figure 3-10B), indicating that the activity level of the microbial community in the rhizosphere was slightly less than that of bulk soil. Fernández-Gómez et al. (2019) reported that the rhizospheres of *Calamagrostis crispa*, *Nassella nardoides*, *Jarava frigida*, and *Pycnophyllum bryoides* had lower values of richness and diversity index compare to the bulk soil. They suggested that due to biotic and abiotic factors, plants could recruit and conserve specific growth-promoting bacteria. For example, they found plants had more Alphaproteobacteria in the rhizospheres; some members of Alphaproteobacteria contain PGPR properties. Additionally, they suggested that changes in pH and moisture triggered the disappearances of Gemmatimonadetes and Planctomycetes in the rhizosphere community compared to bulk soil. Marilley et al. (1998) also found that taxonomic bacterial diversity of the rhizospheres of *Lolium perenne* and *Trifolium* was up to 10% lower compared to the bulk soil.
Figure 3-10: Biolog® EcoPlate™ average well color development (AWCD) and number (richness) of substrates metabolized by microbial communities from bulk soil and rhizospheres of soybean seedlings. Plants were grown in soil culture with 0 or 40 mg CdCl$_2$ kg$^{-1}$ soil for 30 days. Different lower-case letters indicate significant differences in mean values within each panel (Holm-Sidak test, P<0.001). Means of three replicates are plotted; bars indicate standard errors of the mean.
When soil was spiked with Cd, the AWCD values of both bulk and rhizosphere soil were similar to those of control (0 Cd) rhizosphere soil (Figure 3-10A and B), indicating that the plant offered some protection to microbes against Cd toxicity.

Richness is a measure of the variety of carbon sources that can be metabolized by a microbial community. Similar to the results for AWCD, richness was highest in control bulk soil and was about 30% lower at 72 hr (Figure 3-10C) and about 15% lower at 168 hr (Figure 3-10D) for control rhizosphere communities and all Cd-treated communities. In other words, I found no significant differences in AWCD or substrate richness among the microbial communities associated with the different soybean cultivars. Each of the substrate utilization evenness (Figure 3-11A and B) and Shannon diversity index (Figure 3-11C and D) were also generally not different between bulk or rhizosphere soil under either control conditions or with Cd treatment.

Xie et al. (2016) found that Cd decreased total bioactivity, richness, and diversity in soil microbes associated with Bermuda grass (Cynodon dactylon). I also found that the microbial community in the untreated bulk soil metabolized a greater number of substrates. Borymski et al. (2018) found similar results for the microbial metabolic activity of bulk soil exposed to Zn and Cd. Luo et al. (2019) had reported that increasing the amount of Cd in soils decreased the population size and overall activity of the soil microbial communities. Just as happens with plants, Cd can inhibit the growth and metabolism of soil microorganisms through functional disturbance, protein denaturation or the destruction of the integrity of cell membranes (Choi, 2009).
Figure 3-11: Evenness and functional diversity (Shannon diversity) of substrates metabolized in Biolog® EcoPlate™ by microbial communities from soil or rhizosphere of soybean seedlings. Plants were grown in soil culture with 0 or 40 mg CdCl$_2$ kg$^{-1}$ soil for 30 days. Different lower-case letters indicate significant differences in mean values within each panel except panel C, where there were none (Holm-Sidak test, P<0.001). Means of three replicates are plotted; bars indicate standard errors of the mean.
The microbial community of untreated (control) bulk soil metabolized all 31 of the carbon sources in the biochemical guilds (Figure 3-12), whereas the Cd-treated bulk soil community metabolized the polymers and carbohydrates (excluding D,L-α-Glycerol phosphate), amino acids (excluding L-Threonine, Glycyl-L-glutamic acid, and L-Phenylalanine), carboxylic acids (excluding 2-Hydroxy benzoic acid, α-Keto butyric acid) and amides (excluding Putrescine) (data not shown). In terms of chemical guild metabolism, however, there were no differences among Cd-treated soil and rhizosphere soil (Figure 3-12).

Overall, differences between rhizosphere communities as measured by AWCD (Figure 3-9A) and richness (Figure 3-9B) were not significant, nor were there differences in the utilization of carbon source from the different chemical guilds.

(A) 72hr   (B) 168hr

Figure 3-12: Patterns of substrate utilization in Biolog® EcoPlates™ by microbial communities from soil or rhizosphere of soybean seedlings. Plants were grown in soil culture with 0 or 40 mg CdCl₂ kg⁻¹ soil for 30 days. Bulk soil and rhizosphere suspensions were incubated on Biolog® EcoPlate™ and absorbances were read at 72 and 168 hr.
3.2.5 Principal Component Analysis

In order to visualize the carbon utilization patterns, and therefore the relationships among the microbial communities in soybean rhizospheres, Principal Component Analysis (PCA) of substrate utilization patterns was performed on the 72 hr Biolog® EcoPlate™ data. Principal Component Analysis is a dimensionality-reduction method, which simplifies a large data set by transforming variables into smaller data sets (principle components) while retaining all of the information in the larger data set (Garland and Miles, 1991). In general, PCA biplots are displayed on two axes, PC1 and PC2, with the first principle component axis (PC1) explaining a greater percentage of the variability in the data set than the second principle component axis (PC2).

I generated a PCA biplot using the AWCD data generated from the microbial community of rhizospheres and bulk soils. In the PCA biplot (Figure 3-13A), the first two principle components (PC1 and PC2) accounted for 73.2% and 9.7% of the variation, respectively, among all samples and distinguished the carbon utilization patterns in the two bulk soil samples (control and Cd-spiked) from each other and from the clusters for soybean rhizospheres. The carbon utilization patterns of the rhizosphere communities, whether exposed to Cd or not, were grouped together.

Each variable (carbon source) that went into the PCA analysis has an associated vector, and the arrow representing each vector on the biplot points in the direction of increasing values (increased metabolism) of that variable. In Figure 3-13A, all arrows point in the direction of the cluster of control bulk soil and away from other clusters.
(A) The relationship among the microbial communities of Cd-treated and untreated soybean rhizospheres and bulk soil.
(B) The relationship among the microbial communities of Cd-treated soybean rhizospheres

Figure 3-13: Ordination biplot showing the relationships among the microbial communities from all experimental treatments. A) The relationship among the microbial communities of Cd-treated and untreated soybean rhizospheres and bulk soil, B) The relationship among the microbial communities of Cd-treated bulk soil and Cd-treated soybean rhizospheres. The biplot was generated by principal components analysis (PCA) using the utilization of carbon substrate data collected from Biolog® EcoPlate™. The percentage variation in carbon utilization explained by each PC is indicated by the eigenvalue within the axis label. The colored ellipses enclose the results for the eight experimental treatments. The arrows indicate which carbon sources explain the variation among experimental treatments.
It was expected that less carbon utilization would be associated with the microbial communities in Cd-treated soils due to Cd toxicity towards microbes. The distinction between carbon utilization profiles of microbial communities in the Cd-treated bulk soil and in the rhizospheres in the biplot corroborates the AWCD and richness data (Figure 3-10). In the PCA biplot (Figure 3-13A) with the entire AWCD data set, carbon utilization patterns for rhizosphere microbes associated with the different soybean cultivars might have been undetected due to the strong effect of the bulk soil profile.

Therefore, to examine the carbon utilization patterns and relationship among the microbial communities of Cd-treated bulk soil and the Cd-treated soybean rhizospheres, this subset of the AWCD data was plotted independent of the entire data set. In the PCA biplot (Figure 3-13B), PC1 and PC2 accounted for 42.2% and 25% of the variation, respectively, in carbon utilization and there was a clear separation along the PC1 axis between samples from Cd-treated bulk soil and the Cd-treated rhizospheres. Along the PC2 axis of Figure 3-13B, carbon utilization by microbes in the rhizospheres of AC Hime and Westag 97 had distinct patterns, and each was a subset of the profile for AC X790P. The difference between Westag 97 and AC Hime appeared to be increased metabolism of D-lactose by the microbes from the rhizosphere of Westag 97.

To better visualize the carbon utilization patterns and relationship among the microbial communities of Cd-treated soybean rhizospheres, this subset of the AWCD data was examined independently. In the PCA biplot (Figure 3-14), PC1 and PC2 accounted for 58.4% and 15.7% of the variation, respectively, in carbon utilization and there was a clear separation along the PC1 axis between samples from Cd-treated AC Hime rhizosphere and the Cd-treated Westag 97 rhizospheres and AC X790P is between the other two cultivar
Figure 3-14: Ordination biplot showing the carbon utilization patterns and relationship among the microbial communities of the Cd-treated soybean rhizospheres. The biplot was generated by principal components analysis (PCA) using a subset of the utilization of carbon substrate data collected from Biolog® EcoPlate™. The percentage variation in carbon utilization explained by each PC is indicated by the eigenvalue within the axis label. The colored ellipses enclose the results for the four treatments that received cadmium. The arrows indicate which carbon sources explain the variation among experimental treatments.
Figure 3-15: Ordination biplot showing the relationships among all control (0 cadmium) treatments. The biplot was generated by principal components analysis (PCA) using a subset of the utilization of carbon substrate data collected from Biolog® EcoPlate™. The percentage variation in carbon utilization explained by each PC is indicated by the eigenvalue within the axis label. The colored ellipses enclose the results for the four control treatments. The arrows indicate which carbon sources explain the variation among experimental treatments.
and using all carbon utilization in same rate. In Figure 3-14, all arrows points in the direction of the cluster of Cd-treated AC Hime rhizosphere and distinguishably away from the Cd-treated Westag 97 rhizospheres cluster. This indicates that lower rates of carbon utilization would be associated with the microbial communities in Cd-treated Westag 97 rhizospheres.

To determine if the difference between the rhizosphere communities of Cd-treated Westag 97 and AC Hime was due to cultivar-specific differences independent from the effects of Cd, another subset was examined: the bulk soil and the untreated rhizospheres. In the PCA biplot (Figure 3-15), PC1 and PC2 accounted for 86.6% and 4.2% of the variation, respectively. As with the PCA of the entire AWCD data set, this analysis distinguished clearly between the bulk soil and the rhizospheres, with little variation among the three soybean cultivars. This suggests competition between plants and bacterial communities to utilize carbon sources in soil because the extent of carbon utilization was highest for each carbon source in the bulk soil, which likely means that fewer microbes were present in the rhizosphere samples. This supports the results of Jiang et al. (2017), who found that the rhizosphere bacterial community of rabbit eye blueberry (Vaccinium ashei Reade) was less diverse than the bulk soil and suggested that plants select specific (presumably beneficial) microorganisms from the local microbial diversity in bulk soil.

Plants recruit bacteria to help them overcome Cd stress, and plants may influence the structure of the bacterial community through root secretions, especially organic acids (Feng et al., 2005) and by accumulating Cd in their tissues (Mohtadi et al., 2012). Pacwa-Płociniczak et al. (2018) found that Cd-stressed S. vulgaris increased the number of Cd-resistant bacteria in its rhizosphere by 20% compared to the bulk soil. Additionally, they
indicated that the presence of *S. vulgaris* has a higher impact on a soil bacterial community than did metal contamination. I found a distinct difference in carbon utilization patterns between Cd-treated AC Hime rhizosphere and the Cd-treated Westag 97 rhizosphere (Figure 3-14). The bacteria in the Cd-treated AC Hime rhizosphere community metabolized most of the biochemical guilds (excluding carboxylic acids: α-Keto butyric acid and 2-Hydroxy benzoic acid) whereas bacteria in the Cd-treated Westag 97 rhizosphere were separated from the others due to their increased D-lactose metabolism. However, PCA analysis of my data did not find clear differences in carbon utilization patterns between the rhizospheres of soybean cultivars without or with Cd toxicity (Figure 3-13 A).

### 3.2.6 Summary and conclusions from the soil experiments

The Biolog® EcoPlate™ is useful for characterizing the functional ability of microorganisms to utilize specific carbon substrates. In my study, Biolog® EcoPlate™ analyses showed that the untreated bulk soil sample had 60% to 90% higher AWCD values than the Cd-treated bulk soil (Figure 3-10A and B), which indicates that the untreated bulk soil had more physiologically active microbial communities. This increased activity could be due to healthier individuals or a higher microbial density. Chodak et al. (2013) found that metal toxicity caused a drastic decrease in microbial diversity through disrupting the soil composition and altering the structure of the microbial community. However, I did not find a significant difference in the diversity of carbon substrates utilized among samples after 72 hr incubation (Figure 3-11C). Additionally, PCA analysis of carbon utilization revealed a distinct difference between the untreated bulk soil and the Cd-treated bulk soil and treated or untreated rhizospheres (Figure 3-13A). Moreover, Hou et al. (2018) indicated that in the presence of plants and Cd the bacterial diversity in the rhizosphere of *Sedum*
*alfredii* decreased by 10%. They suggested that this reduction was due to a combination of reduced richness and evenness of the bacterial community. I also found that carbon utilization richness was 20% to 30% lower in the rhizosphere compared to bulk soil (Figure 3-10C and D) but evenness of carbon utilization in my study was similar between rhizosphere and bulk soil microbial communities (Figure 3-11A and B).

Although soil is assumed to be among the most diverse microbial habitats due to microsite niche heterogeneity (Leibold and McPeek, 2006), root exudates can alter soil carbon availability, pH, and water content, and reduce variances among soil microsites and caused decreases in richness of the rhizosphere bacterial community (Hou et al., 2018). Shi et al. (2015) reported that the presence of *Avena fatua* decreased bacterial diversity in the rhizosphere due to the plants creating conditions that select for certain bacterial species. Consequently, the richness of the microbial community could be reduced in the rhizosphere soil. Similarly, Shi et al. (2015) highlighted that the impacts of the root on carbon availability, pH, water, and soil air spaces could overwhelm and homogenize differences among soil microsites.

Due to these effects, the structure of the rhizosphere bacterial communities associated with a given plant cultivar is often unique and is correlated to the evolutionary (or breeding) history of the cultivars (Jiang et al., 2017). There is selective pressure for plants to boost populations of beneficial bacteria in the rhizosphere. This exclusivity would result in a rhizosphere community with less richness and more evenness. Because the combination of these factors acts to reduce richness and increase evenness, there is a reduction in the Shannon index (Hou et al., 2018) of utilized carbon substrates in the rhizosphere relative to the bulk soil.
While I found no differences among cultivars in terms of carbon utilization richness (Figure 3-10C and D), evenness (Figure 3-11A and B) or Shannon diversity (Figure 3-11C and D) in Cd-treated samples, my PCA analysis found a clear difference between the Cd-treated bulk soil and the Cd-treated rhizospheres based on utilization of carbon sources. This suggests that the plants under mild Cd stress influenced the bacterial community in their rhizospheres. In addition, PCA detected a difference in microbial carbon utilization in the rhizospheres of Cd-treated AC Hime and Westag 97 (Figure 3-14), which may be explained by the fact that AC Hime responded to Cd-treatment by increasing its relative exudation of organic acids relative to Westag 97 (Figure 3-6). I predicted that microbial diversity in the rhizosphere of Westag 97 would be higher than AC Hime, based on the observation that Westag 97 exuded higher concentrations of organic acids. While the diversity of the bacterial community was not directly evaluated, my experiment did find that the extent of carbon utilization was highest in the rhizosphere of the cultivar with the greatest exudation response.

Although organic acids exuded by roots are known to play a major role in plant-associated bacteria interactions (Canarini et al., 2019), other low molecular weight carbon substrates such as sugars and amino acids exuded by roots also fuel microbial metabolism (Canarini et al., 2019). In my study no single class of carbon source was identified as being responsible for a varied rhizosphere community based on the PCA biplots. Further chemical characterization of rhizosphere chemistry is needed before we fully understand plant-microbe interactions in the rhizosphere, especially when the plants are under mild Cd (or other metal) stress.
3.3 Limitations and Future Work

3.3.1 Microbial diversity

The objective of this study was to understand the cultivar-specific effects on the bacterial community of the rhizosphere under conditions of mild Cd stress. For characterizing the bacterial diversity Biolog® EcoPlate™ were used. However, this technique has limitations. First, it does not provide any information about the species of microbes that metabolized the carbon sources. While I found no evidence of fungal growth, both bacteria and archaea could have been in the samples. Secondly, not all microbes can be cultured and those that could metabolize the carbon substrates would be a subset of the actual microbes in the soil or rhizosphere samples. To overcome these limitations, the next-generation Illumina-based sequencing platform could be used to sequence thousands to millions of DNA molecules simultaneously and identify a rare species in low abundance in a bacterial community (Staley et al., 2013).

3.3.2 Alternative methods to collect the root exudate

One of the crucial steps in this study was collecting the root exudates, which can be collected from both soil and hydroponic systems, but each has limitations. First, in both cases, the microbial community in the growth medium could consume exudates prior to their collection. Second, exudates might adsorb to the solid phase in soil or (less likely) to the sides of the vessel used for hydroponic culture. Third, the presence of nutrient ions in the soil or hydroponic solution can interfere with instruments such as a liquid chromatograph (LC) to separate individual molecules and a mass spectrometer (MS) to
identify the molecules (Pitt, 2009). The first two problems are not easily overcome but ion interference can be solved by removing plants from their experimental treatments and placing them in sterile deionized water for 12 hr in the dark (Tawaraya et al., 2014) prior to exudate collection.

### 3.4 Overall Conclusions

Cadmium accumulation in edible parts of crops may cause potential risk to human health (Rascio and Navari-Izzo, 2011). Thus, minimizing Cd concentrations in edible plant products is necessary for protecting human health. Soybean easily accumulates high concentration of Cd in the seeds (Cupit et al., 2002) and it is important for us to understand the physiology and biology behind differential translocation of Cd from roots to shoots and seeds.

Cultivar selection is an interesting approach for reducing the potential health hazards associated with Cd concentration in crops. One aim would be to develop cultivars that accumulate Cd at a low enough level for safe consumption when grown in contaminated soils (Jegadeesan et al., 2010). Cadmium accumulates in seeds through translocation from root to shoot to seed, as discussed in section 3.1.4, and the symplastic compartmentalization of Cd in roots is probably responsible for reduced translocation of Cd to shoots and seeds for soybean cultivars. This information could help soybean breeders to develop a cultivar of soybean that stores more Cd through symplastic pathways (likely root cell ER, Wang et al., 2018) in roots and translocates less Cd to the edible parts. Moreover, my study showed that there could be a relationship between low shoot (and hence grain) Cd and less exudation of organic acids, so breeders could use the cultivar with less exudation of organic
acids. This might have an added benefit because organic acids have the potential to enhance metal bioavailability and uptake for plant by reducing soil pH and forming soluble complexes with metals.

Another strategy to limit Cd accumulation in edible plant parts is changing the soil environment. My study showed, (section 3.2.4), that the cultivar that exuded relatively more organic acids in response to Cd was associated with a higher extent of carbon utilization. However, while the presence of organic acids in soil may improve plant yield due to increased microbial activity, especially under deficiency of P (Hunter et al., 2014) and Fe (Dimkpa et al., 2008), reducing the soil pH by adding organic acids to the soil might enhance the bioavailability and mobility of metals that were not previously bioavailable (Mishra et al., 2017).

While changing the plant’s characteristic seems more practicable than changing the soil environment, breeding and testing a new cultivar is time-consuming because the low Cd characteristic must be included into a cultivar that has acceptable features for yield, agronomic suitability, quality and disease resistance. Besides, the chemical analysis required to select for low Cd accumulation is expensive (Jegadeesan et al., 2010).
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Appendices

Appendix –A: The following calibration curves were prepared in order to measure the concentrations of organic acids exuded by soybean cultivars Westag 97, and AC X790P, AC Hime grown in hydroponic culture.

(A) Citric acid

(B) Succinic acid
(C) Fumaric acid

\[ y = 666510x + 10345 \]
\[ R^2 = 0.9875 \]

(D) Malic acid

\[ y = 639041x + 16457 \]
\[ R^2 = 0.9779 \]
(E) Tartaric acid

(F) Malonic acid

Figure A-1 Calibration curves for different organic acid standards. Calibration curve for (A) Citric acid (B) Succinic acid (C) Fumaric acid (D) Malic acid (E) Tartaric acid and (F) Malonic acid, each dissolved in methanol.
Appendix–B: The following calibration curves were prepared in order to find the best solvent for measuring the concentration of organic acids exuded by soybean cultivars Westag 97, and AC X790P, AC Hime grown in hydroponic culture. I chose methanol for calibration curves due to the higher solubility of organic acids in methanol compared to water.

(A) Water

(B) Methanol
(C) Nutrient solution

Figure B-1 Calibration curves for different organic acid standards mixed in various solvents. Calibration curve for organic acids in (A) Water (B) Methanol and (C) Nutrient solution
Appendix–C: To determine if the three soybean cultivars had varied effects on soil pH, the pH of treated and untreated rhizospheres and bulk samples were examined. No differences were found.

Figure C-1: The mean pH of Cd-treated and untreated soybean rhizospheres and bulk soil samples. 25 g of treated and untreated rhizospheres and bulk samples were mixed with 40 mL of dH$_2$O, then each sample was stirred for 45 min then left to stand for 4 hr. After filtration, their pH was measured using pH Meter. There were no significant differences among treatments Means of three replicates are plotted; bars indicate standard errors of the mean.
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