Mechanisms of plant growth promotion of *Pseudomonas chlororaphis* PA23 and potential effects on soil microbial communities

by

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Abstract

Plant growth-promoting bacteria (PGPB) are a heterogeneous group of soil microbes that confer beneficial effects to plants. Recent interest in sustainable and organic agriculture has highlighted the potential uses of PGPB as agricultural adjuncts. Currently, PGPB are used to increase crop yield and productivity. Substantial work has been done on the active metabolites secreted by PGPB that drive plant growth. However, little is known about their effects on the global transcriptome of the host plant. *P. chlororaphis* PA23 is a gram-negative bacterium that has shown great potential as a plant growth promoting agent. Using a combination of next-generation RNA sequencing and bioinformatic analyses, the mechanisms that underlie *P. chlororaphis* PA23 induced growth in *Brassica napus* seedlings were investigated. The results showed that *P. chlororaphis* modulates plant phytohormone signaling systems resulting in elevated expression levels of genes associated with photosynthesis and root hair development. Next, the scalability of *P. chlororaphis* PA23 as an agricultural adjunct with a *Glycine max* field study was investigated. An increase in soybean pod set following *P. chlororaphis* PA23 treatment was observed, suggesting plant growth-promoting activity under field conditions. To determine the effects of *P. chlororaphis* PA23 on the microbial communities found in the soil, a combination of marker-based sequencing and bioinformatic analyses were utilized. Here I report that the addition of *P. chlororaphis* PA23 does not alter the bacterial or fungal species diversity. Furthermore, I report that the community composition within the rhizosphere does not change in response to *P. chlororaphis* PA23. This research provides the necessary information for the development of *P. chlororaphis* PA23 as an agricultural adjunct and lays the foundation for multi-year field studies that integrate the utilization of *P. chlororaphis* PA23 as a PGPB.

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Non-common Abbreviations Used

PGPB	Plant growth promoting bacteria
IAA	Auxin
CK	Cytokinin
GA	Gibberellic Acid
FPKM	Fragments per kilobase of exon per million mapped reads
FPD	Faith's phylogenetic diversity
LB	Lysogeny broth
ITS	Intertranscribed spacer
GO	Gene Ontology

Contributions of Authors

Chapter 2 will be submitted to Nature Scientific Reports and includes, Michael Becker, Ayooluwa Bolaji, Philip L. Walker, Teresa de Kievit, Dilantha Fernando, and Mark F. Belmonte as authors. I performed all experiments and techniques completed for the paper with training and assistance from the following people: Michael Becker (bioinformatics analyses), Ayooluwa Bolaji (*Arabidopsis thaliana* screening), Phillip Walker (bioinformatics analyses). Teresa de Kievit and Dilantha Fernando provided supervision and guidance. Mark F. Belmonte provided guidance, edited the document, and was the primary supervisor.

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CHAPTER 1: BACKGROUND AND RELEVANCE

Microorganisms, fungi, and bacteria can interact and colonize plant surfaces and tissues (Compant et al. 2019). These microorganisms can provide beneficial and life-supporting effects through various methods that include the secretion of signalling molecules, nutrient mobilization, and inhibition of plant pathogens (Pérez-Jaramillo et al. 2018). Recently, there has been a surge of interest in biome research and this has highlighted the potential for the manipulation of the plant microbiome and identification of beneficial microorganisms for agricultural use (Bashan et al. 2014; Berg et al. 2014). Together, these research studies may lead to solutions for current and future issues in global food and crop production (Chaparro et al. 2012b).

1.1 The Rhizosphere and Plant-Soil Interaction

The root is the functional organ that anchors the plant to the soil and acts as the primary site for nutrient and water uptake(Ryan et al. 2016). This organ also plays a critical role in the establishment and maintenance of the rhizosphere. The rhizosphere is the microbiome, the community of microorganisms in the soil that interacts with the root (Andreote and Pereira e Silva 2017). This microbiome can be divided into three zones: 1) endorhizosphere, 2) rhizoplane, 3) ectorhizosphere, and are categorized based on distance from the plant root (York et al. 2016). The endorhizosphere consists of the apoplastic, non-living spaces, that exist in the cortex and endodermal tissue layers of the root. Microbes can inhabit these areas, either as free-living organisms or as complex symbionts, providing beneficial effects to the host plant (Compant et al. 2010). The rhizoplane is the zone of soil between the root epidermis and mucilage - a mixture of glycoproteins and exopolysaccharides. The microbial population in this area is heavily influenced by root exudates (Wieland et al. 2001). Lastly, the ectorhizosphere is the distal area that extends from the rhizoplane into the surrounding bulk soil (Bakker et al. 2013). Plant

influence on the ectorhizosphere community can vary widely and is governed by differences in root morphology, chemical, biological, and physical gradients that exist radially and longitudinal along the root axis (Hunter et al. 2014). These differences are accentuated by biotic and abiotic conditions that occur during the life cycle of the plant. Specifically, the presence of pathogens and varying levels of nutrient limitation can further alter root morphology (Pandey et al. 2017).

Root systems provide a unique ecological niche for microorganisms (Beckers et al. 2017). Various compounds, known as root exudates, including organic and amino acids, enzymes, growth factors, and sugars are secreted from the root; these substances act as substrates for microbial metabolism and influence growth dynamics (Canarini et al. 2019). Bacterial members of the rhizosphere are well defined and agricultural soil samples are dominated by Firmicutes, Acidobacteria, Verrucomicrobia, Bacteroidetes, and Proteobacteria (Liu et al. 2019). Fungal communities of the rhizosphere are less defined with all six fungal phyla being represented; however, the Ascomycota, Basidiomycota, and Zygomycota comprise a majority of detected species (Wang et al. 2017). Although root morphology and exudates play a role in the development and maintenance of the rhizosphere population; the initial microbial members are vertically and horizontally inherited from the seed and bulk soil respectively (Berg and Smalla 2009; Frank et al. 2017). Previous studies suggest that plant roots significantly alter the microbe composition in the rhizosphere. For example, denaturing gradient gel electrophoresis analysis of bulk soil and strawberry rhizosphere samples revealed similar detected bacterial species but with significant differences in their relative proportions. Furthermore, differences in the relative bacterial abundances in rhizosphere samples, when compared to bulk soil, were more pronounced in the second year of the study (Smalla et al. 2001). Together, these results suggest

that the initial composition of the rhizosphere is derived from the soil and active selection over time results in defined differences in the relative abundance of detected bacteria.

1.2 General Functions of The Rhizosphere

Organisms in the rhizosphere can have positive, neutral, or negative effects on the plant. Deleterious organisms can be classified as plant predators or pathogens and reduce overall plant health and growth. For instance, many fungal species are the causal agents of plant diseases; for example, root rots (*Rhizoctonia* spp complex), white stem mold (*Sclerotinia sclerotiorum*), and blackleg disease (*Leptosphaeria maculans*) (Bardin and Huang 2001; Erlacher et al. 2014; Becker et al. 2017b). Bacterial species also contribute to numerous plant disease complexes such as leaf spot (*Pseudomonas syringae*) and plant rot (*Xanthomonas* spp)(Ryan et al. 2011; Xin et al. 2018). Together, these organisms can reduce crop yield and direct control through pesticides or indirect control through other rhizosphere microbes can reduce disease symptoms and cause potential crop losses.

Rhizosphere microbes that enhance plant health and growth can be classified as plant growth promoting microbes; specifically, plant growth promoting rhizobacteria (PGPR) and plant growth promoting fungi (Lugtenberg and Kamilova 2009; Backer et al. 2018). These organisms influence plant growth through a range of mechanisms including biocontrol of pathogens, biofertilization, and directed growth stimulation. Biocontrol is the control of pathogens or pests by a living organism (Fernando et al. 2007; Duke et al. 2017b). Various members of the rhizosphere exhibit direct antagonism towards bacterial and fungal pathogens. Biocontrol activity is associated with volatile compounds such as hydrogen cyanide, lytic enzymes, lipopeptides, and siderophores which reduce pathogen population and alleviate disease symptoms (Selin et al. 2014; Nandi et al. 2015b; Chauhan et al. 2015). Direct plant growth

promotion is well documented for *Pseudomonas*, *Bacillus*, and *Trichoderma* (Ousley et al. 1993; Garcia et al. 2001; Tahir et al. 2017). These plant growth promoting bacteria and fungi alter the nutrient status of plants. For example, nitrogen-fixing bacteria and mycorrhizal fungi can increase the levels of usable nitrate in the soil for plants (Chen et al. 2018; Pankievicz et al. 2019). PGPRs can also influence plant growth by altering or inducing plant phytohormone signalling resulting in differences in gene expression, growth rates, and organ morphology (Kudoyarova et al. 2019).

The synthesis and secretion of the three main phytohormones auxin (IAA), cytokinin (CK), and gibberellic acid (GA) have been reported in PGPRs (Liu et al. 2013; Kang et al. 2017; Patel and Saraf 2017). Bacterially synthesized auxin may have different effects on the plant and its action is dependent on both endogenous and exogenous auxin levels (Spaepen and Vanderleyden 2011). Previous studies show that exogenous auxin can alter the transcription rates of genes associated with plant signalling, cell wall development, and plant defense (Hong et al. 1991; Spaepen et al. 2014; Llorente et al. 2016). The mechanisms associated with PGPR synthesized CK and GA are not as well studied. Exogenous cytokinin may have potential effects on root exudate production and transcriptional rates of genes linked to photosynthesis (Ruzzi and Aroca 2015; Cortleven and Schmülling 2015). Exogenous gibberellic acid has been tied to increases in shoot growth and interacts with auxin to alter root growth and morphology (Vacheron et al. 2013).These previous studies suggest that phytohormones synthesized by PGPRs play a critical role in the mechanisms of plant growth promotion. However, due to differences in exogenous phytohormone synthesis rates, endogenous phytohormone concentrations, and microbe population densities, the extent of growth promotion may vary. Thus, more studies are required

to elucidate the specific mode of action for potential PGPR and their range of possible effects on economically important crop species.

1.3 Biocontrol and Plant Growth Promoting Activity of *Pseudomonas Chlororaphis* PA23

The Pseudomonads are a group of bacteria that are found in agricultural soils. Members of this genus, such as *Pseudomonas fluorescens* and *Pseudomonas chlororaphis*, have been reported to promote plant growth and suppress plant pathogens (Garcia et al. 2001; Gholami et al. 2009). *Pseudomonas chlororaphis* strain PA23 is a rod-shaped gram-negative bacterium that was originally isolated from the soybean rhizosphere (Fernando et al. 2005). Previous studies with this bacterium have identified potent biocontrol activity against fungal pathogens and parasitic nematodes (Ramarathnam et al. 2011; Klaponski et al. 2014; Nandi et al. 2015a; Duke et al. 2017b). These studies have also identified the active biocontrol molecules – pyrrolnitrin, phenazine, and hydrogen cyanide. Interestingly, in the absence of pathogens, the application of *P. chlororaphis* PA23 resulted in larger and healthier *Brassica napus* seedlings. Although the *P. chlororaphis* PA23 genome is sequenced and previous studies have identified active metabolites (Loewen et al. 2014), we have yet to investigate the potential affecters and underlying genetic mechanisms that drive enhanced plant growth. Thus, further studies on the metabolome of *P. chlororaphis* PA23 and RNA sequencing of the host plant may shed light on the mechanisms that drive the observed growth phenotype.

1.4 Implementation of PGPBs as an Agricultural Tool – Benefits and Challenges

Recent interest in sustainable and organic agriculture has highlighted the potential utility of plant growth promoting bacteria. PGPBs can reduce application rates and dependencies on chemical pesticides and fertilizers. However, there remain barriers to the widespread implementation of PGPBs for agricultural usage. These barriers include the formulation of a

stable product, reproducibility, and potential effects on the ecosystem (Bashan et al. 2014). The development of a shelf-stable PGPB formulation can lead to increased use. For example, the powdering of *B. subtilis* spores has led to the development of commercially available products (Radhakrishnan et al. 2017). Also, reproducibility is a substantial barrier in the development of novel PGPBs and is linked to poor knowledge in the underlying mechanisms for PGPB action. The introduction of next-generation sequencing has increased our understanding of these mechanisms and has led to improved PGPB-crop management strategies (Kumar et al. 2012). Lastly, the effects of an exogenous PGPB on the ecosystem is considered a barrier for the registration of a novel product in North America. Specifically, the effects of a PGPB on the microbial communities in the field are not well studied and changes may have lasting effects on soil health and crop productivity. The development of marker-based 16S rDNA and intertranscribed spacer region and next-generation sequencing has increased our ability to identify potential changes following PGPB application (Caporaso et al. 2012). Together, the development of formulations, increased understanding of plant growth promotion mechanisms, and risk management of potential ecosystem effects will lead to the wider acceptance and implementation of PGPBs in the agricultural sector.

1.5 OBJECTIVES OF RESEARCH

Given that the global population is expected to exceed 9.5 billion by the year 2050 there is growing pressure to increase crop and food production. Currently, we rely on chemical additives, pesticides and fertilizers, to increase crop production. However, these chemicals can have deleterious effects on the ecosystem such as off-targets and algal blooms. Thus, the development of novel agricultural adjuncts and crop management strategies are critical for securing global food security in the coming decades. Plant growth promoting microbes are a

potential tool to increase agricultural output while reducing the dependence on chemical inputs. Although some commercial products are available, further studies are still required to identify additional PGPRs and to elucidate their modes of action. The objective of my study is to investigate the global gene expression response of *B. napus* following *P. chlororaphis* PA23 treatment and to assess the effects of *P. chlororaphis* PA23 on endogenous microbial communities in agricultural soils. By combining RNA sequencing, marker-based sequencing, and bioinformatics analyses my study aims to provide the necessary information for the commercialization of a PGPR and to contribute to the growing body of knowledge of PGPR-induced plant growth.

1.5.1 Transcriptomic Response of *B. napus* to *P. Chlororaphis* PA23 Treatment

Questions: What are the major transcriptomic changes responsible for increased growth rates of P. chlororaphis PA23 treated B. napus?

Hypotheses:

I hypothesize that the expression level of a large number of genes will change in response to *P. chlororaphis* PA23 treatment. Phenotypically I observed increased shoot and root growth suggesting that there are changes in the signalling systems that govern plant growth and development. Thus, it is likely that *P. chlororaphis* PA23 secretes phytohormones that alter plant signalling and expression rates of downstream effector genes. Furthermore, greener and darker foliage was observed, suggesting elevated levels of chlorophyll α and chlorophyll β. I hypothesize that *P. chlororaphis* PA23 alters the expression rates of genes associated with photosynthesis resulting in higher pigment concentration and photosynthetic rates.

Relevance:

The mechanisms that govern the *P. chlororaphis* PA23 – *B. napus* interaction have yet to be identified. The results from this study will provide insight into the mode of action for a potential agricultural adjunct and lay the framework for the commercialization of *P. chlororaphis* PA23.

1.5.2 Effects of *P. Chlororaphis* PA23 on Bacterial and Fungal Communities In Agricultural Soils

Questions: Does the application of P. chlororaphis PA23 alter pre-existing microbial populations in the field? If so, what are the shifts in these populations and are they detrimental or beneficial to soil health?

Hypotheses:

Based on the secretion of the active compounds phenazine, pyrrolnitrin, and hydrogen cyanide by *P. chlororaphis* PA23, I hypothesize that there will be small shifts in some bacterial and fungal taxa in both the rhizosphere and bulk soil of treated soybean (*Glycine max*); further, I expect that the overall microbial diversities of *P. chlororaphis* PA23 treated and mock-treated seeds will not differ significantly. Lastly, I predict that the microbial populations will differ based on timepoint and sample type (bulk soil versus rhizosphere).

Relevance

Currently, not much is known about the effects of PGPRs on the endogenous microbial population. By profiling the microbiome population of the *G. max* rhizosphere and bulk soil overtime and following *P. chlororaphis* PA23 treatment, I will be able to identify any composition shifts that may impact soil health. Additionally, the results from this study will provide insight into the rhizosphere and bulk soil composition of *G. max* agriculture in central

Canada and lay the necessary groundwork for the development and commercialization of *P. chlororaphis* PA23 as an agricultural adjunct.

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CHAPTER 2: GLOBAL RNA SEQUENCING OF *BRASSICA NAPUS* SEEDLING RESPONSE TO *PSEUDOMONAS CHLORORAPHIS* PA23

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2.1 ABSTRACT

Plant growth promoting bacteria (PGPB) are a growing subset of agricultural adjuncts which can be used to increase crop yield and plant productivity. Although, substantial research has been conducted on the metabolites and active molecules secreted by PGPBs; relatively little is known about their effects on the global transcriptome of the host plant. The present study was carried out to investigate changes in the gene expression landscape of early vegetative *Brassica napus* following treatment with *Pseudomonas chlororaphis* PA23. This newly identified PGPB was isolated from the soybean rhizosphere and has been extensively studied as a biocontrol agent. However, little is known about its effects on plant growth and development. Using a combination of RNA-sequencing and physiological analyses, we identified increased abundance of mRNA transcripts associated with photosynthesis and phytohormone response. Phenotypically we observed increased photosynthetic rates and larger root and shoot systems in *B. napus* following *P. chlororaphis* PA23 treatment. Lastly, we identified auxin production by *P. chlororaphis* PA23 which likely contributes to changes in gene expression and observed phenotypic differences in root and shoot structures. Together, the results of our study suggest that PA23 is a potent plant growth promoting agent with the potential for field applications as an agricultural adjunct.

2.2 INTRODUCTION

Plant associated soil bacteria are a group of microbes that reside within or on the surface of plant roots and can have profound effects on plant health. These microbes can further be divided into two groups: plant growth promoting bacteria (PGPB) which impact plant growth through direct interactions with the plant, or biocontrol bacteria (BCB) which indirectly enhance growth by inhibiting harmful pathogens(Backer et al. 2018; Köhl et al. 2019). PGPBs have been shown to affect gene expression, phytohormone signaling, and mobilize soil nutrients into the plant(du Jardin 2015). The Pseudomonads are a group of bacteria that are universally found in agricultural settings. Members of this genus have been studied extensively as both biocontrol and plant growth promoting agents(Garcia et al. 2001; Haas and Défago 2005; Gholami et al. 2009). For example, *Pseudomonas fluorescens* B16, promotes plant growth through the synthesis and secretion of the active molecule pyrroloquinoline which increased tomato plant height, flower number, and total fruit weight(Choi et al. 2008). Additionally, treatment of wheat with *Pseudomonas* -FAP2 and *Bacillus licheniformis* resulted in increased vegetative growth, chlorophyll content, transpiration rates, and net photosynthetic rates(Ansari and Ahmad 2019). Changes in vegetative growth, chlorophyll content, and photosynthesis has been associated with the production and secretion of bacterial metabolites that are analogous or similar to phytohormones(Kudoyarova et al. 2019). In addition, PGPBs are also capable of synthesizing plant growth promoting hormones; for example, bacterial auxin has been extensively studied as a plant growth promoting metabolite which modulates plant phytohormone signaling and alters root and shoot development(Spaepen and Vanderleyden 2011). Although the mechanisms of auxin induced growth are well understood, further work is required to determine the mode of

action for potential PGPB due to differences in auxin synthesis rates and the effects of varying auxin concentrations on plants(Zhang et al. 2019).

Other members of the Pseudomonads, including strains of *P. fluorescens* and *P. chlororaphis*, are potent biocontrol agents and directly inhibit the growth of fungal, bacterial, and eukaryotic pathogens through the action of secreted compounds (Fernando et al. 2005; Ramarathnam et al. 2011; Simonetti et al. 2012; Klaponski et al. 2014; Duke et al. 2017a). *P. chlororaphis* strain PA23, a microbe originally isolated from the soybean rhizosphere, is a potent biocontrol agent against fungal pathogens and parasitic nematodes(Selin et al. 2014; Nandi et al. 2015b). Given that the *P. chlororaphis* PA23 genome is sequenced, the secondary and secreted metabolites can be predicted. These predicated compounds include siderophores, hydrogen cyanide, and auxin, produced through the indole acetamide pathway, which may act to enhance plant growth while suppressing harmful pathogens(Loewen et al. 2014; Nandi et al. 2015b). Previous work has identified the mode of action for PA23 mediated biocontrol in the *Brassica napus-Sclerotinia sclerotiorum* pathosystem; specifically, PA23 primed plant pathogen defense systems resulting in enhanced tolerance to fungal infection(Duke et al. 2017b). However, little is known about *P. chlororaphis* plant growth promotion and few studies have investigated the interaction between *P. chlororaphis* and plant species, such as *B. napus*, that do not harbor a robust root microbiome.

Despite recent advances in sequencing technologies, we have yet to fully understand the plant growth promoting effects of Pseudomonads and the effects of *P. chlororaphis* PA23 on early vegetative growth in *B. napus*. Given that plant growth is governed by multiple factors including gene activity, a holistic understanding of the gene expression landscape is required to fully understand the putative mechanism of *P. chlororaphis* PA23 plant growth promotion. In

our study, we used a combination of RNA-sequencing and physiological experiments to investigate the genetic and physiological mechanisms involved in *P. chlororaphis* PA23-mediated plant growth promotion. Specifically, we identified elevated expression levels of genes associated with photosynthesis, nutrient uptake, and phytohormone signaling which may contribute to increased vegetative growth rates of *B. napus* seedlings. Together, the results of our study provide a deeper understanding of the genetic mechanisms that govern *P. chlororaphis* plant growth promotion and contribute to the growing body of evidence for the use of PGPBs as an agricultural adjunct.

2.3 MATERIALS AND METHODS

2.3.1 Plant and Bacterial Materials:

B. napus cv. Westar seedlings were grown under controlled environment conditions with a 16-hour photo period (21^{o}C light period, 16^{o}C dark period, and 150 µE/m^2/s). Ten plants per treatment per time point were grown in Sunshine Mix (SunGro Horticulture, Agawam, MA) in plastic pots (8 cm x 12 cm x 6 cm). Each pot was treated with 25 mL of *P. chlororaphis* PA23 at a concentration of 1x10^9 cfu/mL or 25 mL of lysogeny broth (Difco Laboratories, Detroit, MI). Bacterial cultures were prepared from a frozen stock of PA23 stored in 10% skim milk solution (ThermoFisher, Waltham, MA). PA23 was grown on lysogeny broth agar plates (Difco Laboratories, Detroit, MI) at 28^{o}C for 24 hours. Isolated colonies were used to inoculate lysogeny broth and incubated for 24 hours in a rotary shaker at 28^{o}C and 250 rotations per minute. Cell pellets were formed through centrifugation at 5000 rpm for 10 minutes. Pellets were washed with sterile 0.9% saline, resuspended in lysogeny broth, and adjusted to a final concentration of 1x10^9 cfu/mL (OD_{600} = 1)(Duke et al. 2017a).

Arabidopsis thaliana Col-0 seeds were surface sterilized with ethanol and germinated on half-strength Murashige and Skoog agar media (2.2 g/L MS vitamins, 1% sucrose, 0.8% agar, pH 5.8). Agar plates were incubated vertically under controlled conditions with an 8-hour photo period (21^{o}C light period, 16^{o}C dark period, 150 µE/m^{2}/s). PA23 suspensions were prepared as previously described. Seven days after germination, Arabidopsis seedlings were treated with 2000 cfu of PA23 or mock-treated with 0.9% saline. Root architecture was visualized seven-days after bacterial exposure with the Leica M80 Dissecting microscope and images were captured with the Leica Application Suite.

We measured IAA production by *P. chlororaphis* PA23 using colorimetric spectrophotometry as described in Gordon and Weber(Gordon and Weber 1951) and Gang et al(Gang et al. 2019) . *P. chlororaphis* cultures were grown in 5 mL of nutrient broth in an incubating shaker at 28^{o}C and 180 rpm. This culture was used to inoculate nutrient broth amended with L-tryptophan (1 g/L); which was incubated in the dark at 28^{o}C and 180 rpm for 44 hours. After which, a 1.5 mL aliquot of the culture was centrifuged for 5 minutes at 16300 g to pellet cellular debris. The supernatant was removed and mixed with 1 mL of Salkowski's reagent(Szkop et al. 2012) The reaction was carried out for 30 minutes in the dark at 30^{o}C. Absorbance was measured at 546 nm and concentrations were determined with a linear regression against a standard curve containing indole-3-acetic acid.

2.3.2 cDNA Library Synthesis and Computational Analyses

Total RNA was collected from two biological replicates with each replicate consisting of at least 5-10 *B. napus* plants treated with PA23 or lysogeny broth at 24 hpt and 7 dpt. RNA was isolated with PureLink Plant RNA Reagent (Ambion, ThermoFisher, Waltham, MA) and DNA contamination was removed with the TURBO DNA *free*^{TM} (Ambion, ThermoFisher, Waltham,

MA) kit according to the manufacturer's recommendation. mRNA quality was determined with an RNA nanochip on the Agilent 2100 Bioanalyzer system (Agilent, Santa Clara, CA). cDNA libraries were prepared at Génome Quebec (Montreal, QC, Canada) with the Ultra II NEB DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA). Library quality and size distribution was determined on a high-sensitivity DNA chip on the Agilent 2100 bioanalyzer system. One hundred base pair single-end RNA sequencing was carried out on the Illumina HiSeq 2000 platform with a multiplex value of 16. Raw sequencing reads were trimmed, quality checked, and aligned according to the methods described in Becker et al 2017b. Data clustering was performed on averaged raw counts with the PVClust package of R-Studio. Differentially expressed genes were identified with CuffDiff and the output was used with Venny, an online venn-diagram tool (https://bioinfogp.cnb.csic.es/tools/venny/) , to identify treatment specific and co-expressed genes. Specific gene lists from Venny were used for GO term enrichment with SeqEnrich and terms were considered statistically enriched at $P < 0.05$(Becker et al. 2017a).

2.3.3 Targeted Real-Time Quantitative PCR

RNA was isolated from the same *B. napus* tissues as described above. Reverse transcription was carried out with the Maxima First Strand cDNA synthesis kit (ThermoFisher, Waltham, MA) according to the manufacturer's protocol. Directed quantitative PCR was carried out on the Bio-Rad CFX Real-Time System with SYBR Green SuperMix (BioRad, Hercules, CA) as per the manufacturer's instructions in a total reaction volume of 10 µL. Conditions for the reactions were as follows: 95^{o}C for 3 minutes, 44 cycles of 95^{o}C for 30 seconds, 58^{o}C for 30 seconds, and 72^{o}C for 30 seconds. Melt curves, 0.5^{o}C increments over a range of 55^{o}C to 95^{o}C, for each gene target was performed to identify splice variants, primer dimers, and off-target amplification. A list of primer sequences used in these experiments is given in Supplemental

Table T1. Relative transcript abundance was determined using the $\Delta\Delta C_t$ method, normalizing to the endogenous housekeeping gene β-*Actin* and using lysogeny broth treated *B. napus* as the reference sample(Livak and Schmittgen 2001). Results presented in Figure 2.6 are based on two experimental repeats of three biological replicates with each replicate consisting of at least five *B. napus* tissue systems. One-way ANOVA tests were performed with each gene to determine significant fold changes between treatment conditions ($P < 0.05$).

2.3.4 Extraction of Photosynthetic Pigments and Quantification of Photosynthetic Rates

B. napus leaf tissue was flash frozen and homogenized in liquid nitrogen. At least 10 mg of tissue was placed into vials containing 10 mL of 80% acetone (20% v/v 0.2M Tris-HCl pH 8.0). Vials were stored at -20^{o}C for 96 hours or until the pigments were cleared from the leaves. Pigment concentrations were determined with the Beer-Lambert Law and absorbance values at 663 nm and 645 nm(Pocock et al. 2004). Student's t-tests were performed to identify significant differences in chlorophyll α, chlorophyll β, and total pigment concentrations between the mock-treated and PA23-treated conditions. Infrared gas analysis (IRGA) equipment was used to determine relative photosynthetic rates. Ambient CO_2 levels were measured before the introduction of photosynthetic tissues and used as the zero value. *B. napus* leaves (7dpt) were placed into the leaf chamber (9 cm^2) at mid-day (12:00-14:00). Leaves were exposed to 200 $\mu E/m^2/s$ and fed ambient air at a rate of 0.003 L/s(Long et al. 1996). Readings were recorded after 15 minutes and ten replicates, each replicate consisting of an individual *B. napus* seedling, were used to determine statistical significance between the mock and PA23-treated conditions with a Student's t-test ($P < 0.05$)

2.4 EXPERIMENTAL RESULTS

2.4.1 Phenotypic Differences in *B. Napus* Growth

First, we examined the effects of PA23 on *B. napus* seedling development at 24hpt (hours post treatment) and 7 dpt (days post-treatment). Plant size and dry weight of *B. napus* seedlings treated with PA23 were not significantly different compared to the mock-treated seedlings at 24 hpt (Figure 1A). However, at 7 dpt, the dry weight of PA23-treated seedlings increased by 56.8% ($p = 0.0001$) with a corresponding increase in the root/shoot ratio ($p < 0.015$) (Figure 2.1).

2.4.2 Global Comparisons of Gene Activity in the *B. napus – P. Chlororaphis* PA23 Interaction

To identify genes associated with increased growth in PA23-treated *B. napus* seedlings, we profiled the transcriptomes of the root and shoot tissue systems at 24 hpt and 7 dpt. First, hierarchical clustering analyses revealed relationships between tissue systems and bacterial treatments. Samples clustered first by organ system followed by developmental time point, and finally by PA23 treatment (Figure 2.2.A). Detected transcripts were then categorized into three expression levels: lowly (FPKM ≥1, <5), moderately (FPKM ≥5, <25), and highly (FPKM ≥ 25) expressed transcripts. We identified an even distribution of lowly (39%) and moderately (41%) expressed transcripts across all data points; however, we observed a 27% increase in highly expressed transcripts in PA23-treated shoots at 7 dpt. Cumulatively, our dataset detected 56,508 transcripts with an FPKM value ≥ 1 or 56% of the *B. napus* genome. To identify genes contributing to increased plant growth, we conducted differential gene expression analyses of PA23-treated *B. napus* against mock-treated controls. At 24 hpt, we detected 4,200 upregulated differentially expressed genes (uDEGs) in the shoot system and 3,267 uDEGs in the root system in response to PA23. The number of uDEGs decreased at 7dpt, 153 uDEGs were detected in the

shoot system and 3,534 uDEGs were detected in the root system (Figure 2.3). Using these co-expressed gene sets, we performed GO term enrichment to identify putative biological processes associated with PA23 treatment.

2.4.3 *P. Chlororaphis* PA23 Increases Expression Levels of Photosynthetic and Reactive Oxygen Scavenging Genes

Transcripts associated with the photosynthetic components were identified with the uDEGs in PA23-treated shoots at 24 hpt and 7 dpt. We also identified genes associated with reactive oxygen species (ROS) scavenging including superoxide dismutase activity and glutathione transferases. Specifically, we identified an upregulation of the photosystem gene, *LIGHT HARVEST COMPLEX PHOTOSYSTEM II SUBUNIT 6*, with an average increase of 28% and 39% in PA23-treated shoots at 24hptd and 7dpt respectively. We investigated additional transcripts associated with photosynthesis and observed an upregulation in *PHOTOSYSTEM II SUBUNIT Q-2* (25%), *LIGHT HARVESTING CHLOROPHYLL PROTEIN COMPLEX II SUBUNIT B1* (29%), *ATP SYNTHASE SUBUNIT* (27%), *PHOTOSYSTEM I REACTION CENTER SUBUNIT PSI-N* (27%), AND *PHOTOSYSTEM I SUBUNITS* (average of 23%) against the mock treatment at 24hpt. Differential upregulation of photosynthetic genes shifted to pigment binding proteins at 7dpt; here we detected a 36% increase in transcript abundance associated with *CHLOROPHYLL A/B BINDING PROTEIN* in PA23-treated *B. napus.*

Consistent with increases in the activity of photosynthetic genes, we observed an enrichment for catalase activity and superoxide dismutase activity, which are both associated with ROS scavenging in PA23-treated shoots at 24hpt. To further characterize genes responsible for ROS quenching at 24hpt, we investigated the levels of several transcripts including *COPPER/ZINC SUPEROXIDE DISMUTASE* which increased by 26% in the shoots of PA23-

treated *B. napus*. We observed an increase of *COPPER CHAPERONE* transcripts in PA23-treated shoots at both 24hpt (75% increase) and 7dpt (68% increase). To investigate the effects of PA23 on photosynthetic physiology, we extracted total pigment from PA23-treated and mock-treated tissue. The major photosynthetic pigments, chlorophyll α and chlorophyll β, were extracted and we identified a significant increase of 65% and 139% at 24hpt respectively. At 7 dpt, pigment levels accumulated to levels of 32% and 34% chlorophyll α and chlorophyll β, respectively in PA23-treated *B. napus* (Figure 2.5.A). Lastly, we measured the relative photosynthetic rates of PA23-treated and mock-treated *B. napus* seedlings at 7dpt to determine the relationship between transcript abundance, pigment concentration, and carbon fixation rates. Using IRGA and measuring the rate of CO_2 depletion, we determined that PA23-treated *B. napus* plants exhibited a 60% increase in photosynthetic rates at 7dpt (Figure 2.5.B). Taken together, *Pseudomonas chlororaphis* PA23 increased the pigment concentration and photosynthetic rates of *B. napus* seedlings which contribute to the observed growth phenotype.

2.4.4 *P. Chlororaphis* PA23 Promotes Root Hair Development and Nutrient Transporter Gene Expression

At the root interface between the plant and bacteria, we identified an enrichment of GO terms associated with nutrient uptake. For example, we observed an increase in the abundance of *AMT* (ammonium methyl transporter), *NRT* (nitrate transporter), and *PHT* (phosphorus transporter) transcripts in PA23-treated roots at 7 dpt. Lastly, we observed an enrichment of GO terms associated with cellular growth including cell wall biogenesis, cellulose synthase, and root hair elongation. Specifically, we identified an upregulation of the essential root hair development gene – *ACTIN2*. Here we observed an increase of 12% and 21% in *ACTIN2* transcript abundance at 24 hpt and 7 dpt respectively. To relate transcript abundance with phenotypic changes we used

the model species - *Arabidopsis thaliana*, a close relative of *B. napus* in the Brassicaceae family. Microscopic visualization of PA23-treated and mock-treated roots at 7dpt revealed differences in root hair abundance. Specifically, the mock treatment (0.9% saline) exhibited a lower abundance of root hairs (Figure 2.4.C). Whereas, PA23-treated plants had a higher abundance of root hairs along the primary root axis (Figure 2.4.B).

2.4.5 *P. Chlororaphis* PA23 Enhances Plant Growth Through the Modulation Of Phytohormone Signaling

Next, we investigated genes associated with CK, GA, and IAA signaling. Roots exposed to PA23 were enriched for GA, CK, and IA response genes at 24 hpt and 7 dpt. Notably, all transcripts related to GA response at 24 hpt and 7 dpt were homologs of the sucrose catabolism gene, *GLYCOSYL HYDROLASE FAMILY 32*, and their respective FPKM values increased by 43% and 86%. Genes associated with IAA signalling in the *AUXIN-INDUCED IN ROOT CULTURES* family were highly upregulated, with a 465% increase in PA23-treated roots at 24 hpt (Supplemental File 1). Furthermore, multiple homologs of the CK/IAA response integrator, *SHY2*, were upregulated in the roots of PA23-treated *B. napus* with a 54% and 9% increase at 24 hpt and 7 dpt respectively. Additionally, we measured the production of IAA by *P. chlororaphis* PA23 using colorimetric spectrophotometry. Bacterial cultures were grown in the presence of L-tryptophan and we determined that *P. chlororaphis* PA23 can produce IAA at a rate of 44.2 µg/hour (Figure 2.7).

2.5 DISCUSSION

Crop production is of growing global concern and recent interest in the integration of environmentally friendly agricultural adjuncts has highlighted the potential use of PGPBs (Spiertz and Ewert 2009; Babalola 2010). Currently, the mechanisms involved in growth

promotion are not well understood and building on our understanding of the genetic mechanisms that underlie PGPB-induced plant growth is a critical step for the acceptance of PGPBs by regulatory agencies and the agricultural sector (Backer et al. 2018). Our study aims to contribute to the growing body of information on the genetic mechanisms driving PGPB induced plant growth. Specifically, we investigated the mechanisms that drive enhanced growth during the *B. napus* – *P. chlororaphis* PA23 interaction. Our study identifies both molecular and phenotypic changes in the shoot and root systems of *B. napus* treated with *P. chlororaphis* PA23.

2.5.1 *P. Chlororaphis* PA23 Enhances Photosynthetic Capabilities of *B. Napus*

In the shoots, we identified increased concentrations of the major photosynthetic pigments – chlorophyll α, chlorophyll β, and carotenoids in the leaf tissue of *B. napus* treated with *P. chlororaphis* PA23. At the transcript level, our dataset identified increased abundance of photosynthetic transcripts such as *LHCB1.3*. This gene encodes for a pigment binding protein of light harvesting complex II, which is responsible for light capture in the antenna complex of plants (Jansson 1994). Other studies have identified changes in the expression of photosynthetic genes following PGPR treatment. For example, treatment of *Triticum aestivum* with *Dietzia natronolimnae* STR increased the concentrations of chlorophyll α, chlorophyll β, and carotenoids in photosynthetic tissues. Furthermore, PGPR treated *T. aestivum* were larger in size and was attributed to the increased pigment concentrations and photosynthetic rates (Bharti et al. 2016). The expression of photosynthetic genes, such as *LHCB1.3*, is influenced by abiotic and biotic factors (Xu et al. 2012). For example, plants acclimatize to changing light conditions through phytohormone signaling and differential expression of genes associated with photosynthesis. In *Hordeum vulgare*, the presence of exogenous cytokinin increased the expression rates of genes linked to chloroplast development and differentiation (Zubo et al. 2008). Tahir et al identified an

increase in the expression rates of phytohormone signaling genes and enhanced photosynthetic rates in *Solanum lycopersicum* following treatment of *Bacillus subtilis* (Tahir et al. 2017). These changes were due to the volatile organic compounds produced by *B. subtilis* which altered gene expression in *S. lycopersicum*. We identified a similar increase in photosynthetic rates of *B. napus* following treatment with *P. chlororaphis* PA23. The production of cytokinin has been previously reported in *P. fluorescens* G20-18, which bolstered plant defense through the activation of cytokinin mediated immune responses (Großkinsky et al. 2016). Together, these studies suggest that bacterial metabolites can alter phytohormone signaling, photosynthetic transcript abundance, and photosynthetic rates. In the current study, we identified an enrichment of genes linked to cytokinin response in PA23-treated *B. napus*; however, we did not detect differential expression of genes associated with cytokinin biosynthesis. Thus, the expression patterns identified in our data and previous reports of volatile organic compound and cytokinin production in other *Pseudomonas spp* suggest that *P. chlororaphis* PA23 may alter *B. napus* gene expression through a secondary or volatile compound (Santoro et al. 2011; Park et al. 2015). Despite these findings, the identities of the entire suite of plant growth promoting metabolites produced by PA23 are unknown and further studies are required to elucidate the active metabolites produced by PA23.

2.5.2 Heighted Levels of Photosynthesis Require Additional Reactive Oxygen Species Scavenging

Increased photosynthetic activity can have detrimental effects on cellular components and biological molecules. Under stress conditions such as drought, UV, and heat; ROS generation from the photosystems can increase (Das and Roychoudhury 2014). The prevention of ROS mediated damage is facilitated by both non-enzymatic methods which absorb excessive light

radiation, preventing the production of ROS, and enzymatic methods such as superoxide dismutases (SOD) that degrade cellular radicals(Das and Roychoudhury 2014). These SODs are the first line of defense against ROS-mediated oxidation and SOD1 is one of the most potent cellular antioxidants (Xing et al. 2013). In the current study, we identified enriched GO terms associated with ROS scavenging. For example, essential ROS scavenging genes such as *FSD 1*, *FSD III*, and *SOD* were upregulated in PA23-treated *B. napus* shoots 24 hpt suggesting an increased demand for ROS scavenging. Under copper limited conditions, the expression of SODs are downregulated in *A. thaliana* and *Brassica juncea*, as copper is an essential component of the catalytic core of these enzymes (Cohu and Pilon 2007). Following PA23-treatement, we observed increased expression of genes associated with copper binding, copper chaperones, and copper uptake in the root dataset. Together, this may suggest that in response to increased SOD activity, *B. napus* requires elevated rates of copper sequestration to replenish depleted copper pools to counteract heightened levels of ROS production from the photosystems. *FSD I* is a cytoplasmic antioxidant and is preferentially expressed in *A. thaliana* under copper deficient conditions and *FSD III* is essential for chloroplast development. Mutants deficient in *FSD III* are smaller in size, lack photosynthetic pigments, and are sensitive to oxidative stress (Myouga et al. 2008). Both *FSD I* and *FSD III* transcripts were more abundant in PA23-treated *B. napus*, further suggesting an increase in antioxidant demand. Together, the expression patterns of *SOD*, *FSD I*, and *FSD III* suggest that enhanced photosynthetic capabilities of PA23-treated *B. napus* is managed through increased expression rates of genes associated with antioxidants.

2.5.3 *P. Chlororaphis* Alters Phytohormone Signaling Through Auxin Secretion and Increases Abundance of Nutrient Transporter Transcripts in Roots

Growth regulation and patterning of the root is governed by concentration gradients of auxin and cytokinin. Crosstalk between these phytohormones is mediated by the signal integrator – SHY2, a transcriptional inhibitor of auxin response elements which regulate cell division (Ioio et al. 2008). The *ARR,* Arabidopsis response regulator, gene family is a group of cytokinin-induced transcription factors which increase expression rates of *SHY2* (Knox 2003; Li et al. 2016). In contrast, auxin promotes the degradation of SHY2 and the activation of auxin response factors which increase transcriptional rates of auxin response elements resulting in elevated rates of cellular division. Thus, phytohormone concentration gradients and SHY2 work to balance cellular differentiation and division in the developing root. The production of auxin has been previously reported in other *Pseudomonas spp* and PA23 carries the essential genes, *TRYPTOPHAN 2-MONOOXYGENASE* and *INDOLE ACETMIDE HYDROLASE*, for auxin biosynthesis via the indole acetamide pathway (Malik and Sindhu 2011; Dimkpa et al. 2012; Loewen et al. 2014). Further, we quantified the production of auxin by *P. chlororaphis* PA23 in liquid culture and observed moderate production of auxin or auxin homologs. Thus, the observed expression patterns of auxin response genes and *SHY2* in the root would suggest that growth promotion in the *B. napus – P. chlororaphis* PA23 interaction could be partially attributed to the presence of exogenous auxin. However, further studies characterizing the bacterial exudates, such as auxin, in the presence of developing plants are required.

The process of root hair development is separated into three phases: bulge formation, slow tip growth, and rapid tip growth (Dolan et al. 1993). Root hair extension occurs through asymmetric growth of the cytoskeletal element - actin. Application of chemicals that interfere

with actin formation results in abnormal root hair initiation and early termination (Miller et al. 1999; Braun et al. 1999; Baluška et al. 2000). Specifically, the interruption of *DER1* in *A. thaliana* results in the absence of root hair on the primary and lateral roots (Ringli et al. 2002). The *der1*$^{-}$ phenotype was rescued with an *ACT2* insertion under its native promoter. Together, these studies suggest that *ACT2* is vital for root hair initiation and elongation. As previously described, nutrient availability and uptake are some of the most significant factors governing plant growth (Prinzenberg et al. 2010). PGPRs can increase soil nutrient content and enhance nutrient uptake rates. For example, wheat treated with *P. fluorescens* under nutrient limited conditions produced similar yields when compared to optimally fertilized conditions; suggesting that *Pseudomonas spp* can mobilize organic and inorganic nutrients. Currently, the nutrient mobilization effects of *P. chlororaphis* PA23 and its effects on plant nutrient uptake is unknown. Nitrogen is an essential macronutrient and is primarily assimilated as nitrate. The uptake of nitrates occurs at the root hairs through two systems: high affinity transporters (HATs) and low affinity transporters (LATs) (Krapp et al. 2014). HAT expression is rapidly increased following the detection of increased soil nitrate levels; this response is accented in *A. thaliana* that are exposed to nitrate after long term nitrogen deficiency. Furthermore, *nrt2.1*$^{-}$ assimilated significantly lower levels of nitrogen, suggesting that HATs are essential for rapid and efficient nitrogen uptake (Cerezo et al. 2001). Ammonium is another source of nitrogen and uptake is facilitated by ammonium transporters (AMTs) at the root hairs. *A. thaliana amt1.1*$^{-}$ *amt1.3*$^{-}$ double mutants absorbed 70% less ammonium, while *AMT1.3* overexpression increased ammonium influx by 30% (Yuan et al. 2006). Together, these findings suggest that AMTs are essential for the uptake of ammonium and overexpression of *AMTs* enhance uptake rates. In the current study, we identified higher levels of *ACT2* transcript abundance in PA23-treated *B. napus*

and higher occurrence of root hairs on the primary and lateral roots of PA23-treated *A. thaliana.* Further, we detected increased expression levels of *NRTs* and *AMTs* in our dataset. Together, these findings suggest that PA23 increases the abundance of nutrient transporters present on the root hairs which may increase nutrient uptake rates. Application to other field crops including *Glycine max*, the crop species from which PA23 was originally isolated, may result in similar increases of nutrient uptake and decrease the dependence or application rates of chemical fertilizers.

2.5.4 CONCLUSIONS

In conclusion, our data suggests that *P. chlororaphis* PA23 is a potent PGPB that alters both the root and shoot systems of *B. napus*. Furthermore, our data identified that secretion of auxin by *P. chlororaphis* PA23 may be the primary contributor for plant growth promotion and potentially alter gene expression associated with photosynthesis in the shoot. However, our current understanding of the suite of metabolites produced by *P. chlororaphis* PA23 remains unknown and requires further investigations to identify potential active metabolites or small molecules. These metabolites could be the determining factor that governs the dual action of plant growth promotion and biocontrol exhibited by *P. chlororaphis* PA23. Several factors may contribute to this phenomenon including population densities, quorum sensing, and differing host plant species. Further studies using growth, quorum sensing, and signaling mutants of *P. chlororaphis* PA23 could shed light on the underlying mechanism that determines its activity as a PGPB or as a biocontrol agent. Together, our study provides preliminary evidence for the mechanisms that drive enhanced plant growth by *P. chlororaphis* PA23, but further studies are required to determine the mechanisms that control metabolite production and biocontrol or plant growth promotion activity

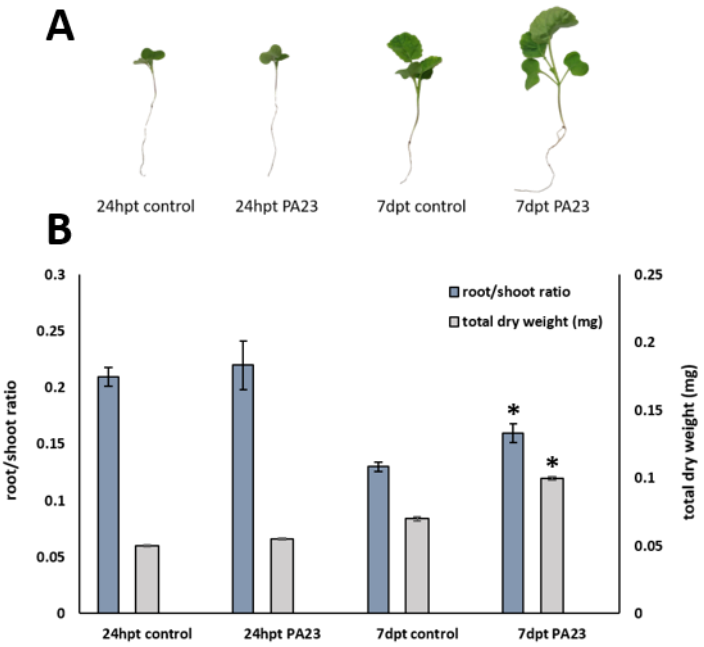


Figure 2.1. Agronomic traits of *B. napus* seedlings at 24hpt and 7dpt. *B. napus* treated with lysogeny broth (mock-treatment) or a PA23 suspension ($1x10^9$ cfu/mL). Plant material (n=15) was dried for 72 hours at 65ºC to determine dry weight. **A)** Representative *B. napus* seedlings showing phenotypic differences. **B)** Total dry weight of *B. napus* and root/shoot ratios at 24hpt and 7dpt * indicates significance in a Student's t-test ($p < 0.05$) and error bars represent standard error.

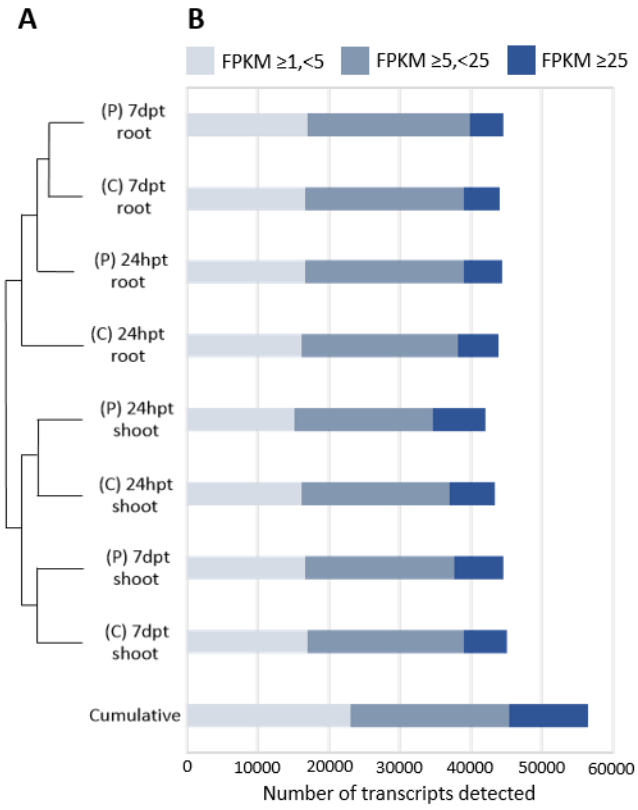


Figure 2.2. Hierarchical clustering and global gene activity in the *P.chlororaphis* PA23-*B. napus* interaction. **A)** Hierarchical clustering of all differentially expressed genes detected in the dataset. (P) indicates PA23 treatment and (C) indicates the mock-treatment group. **B)** Number of transcripts detected in both tissue systems across all treatments. Detected transcripts are subdivided into lowly (FPKM ≥1, <5), moderately (FPKM ≥5, <25), or highly (FPKM ≥ 25) expressed.

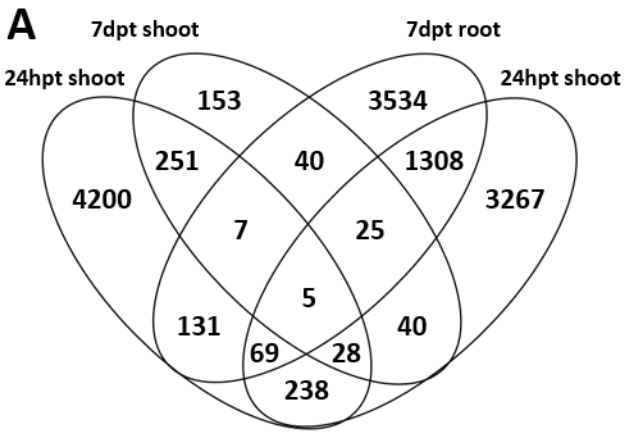


Figure 2.3. Differentially expressed genes identified in the PA23-treated *B. napus* seedlings in the root and shoot systems at 24hpt and 7dpt. Upregulated differentially expressed genes with overlapping regions showing the number of shared genes between tissue types and time points.

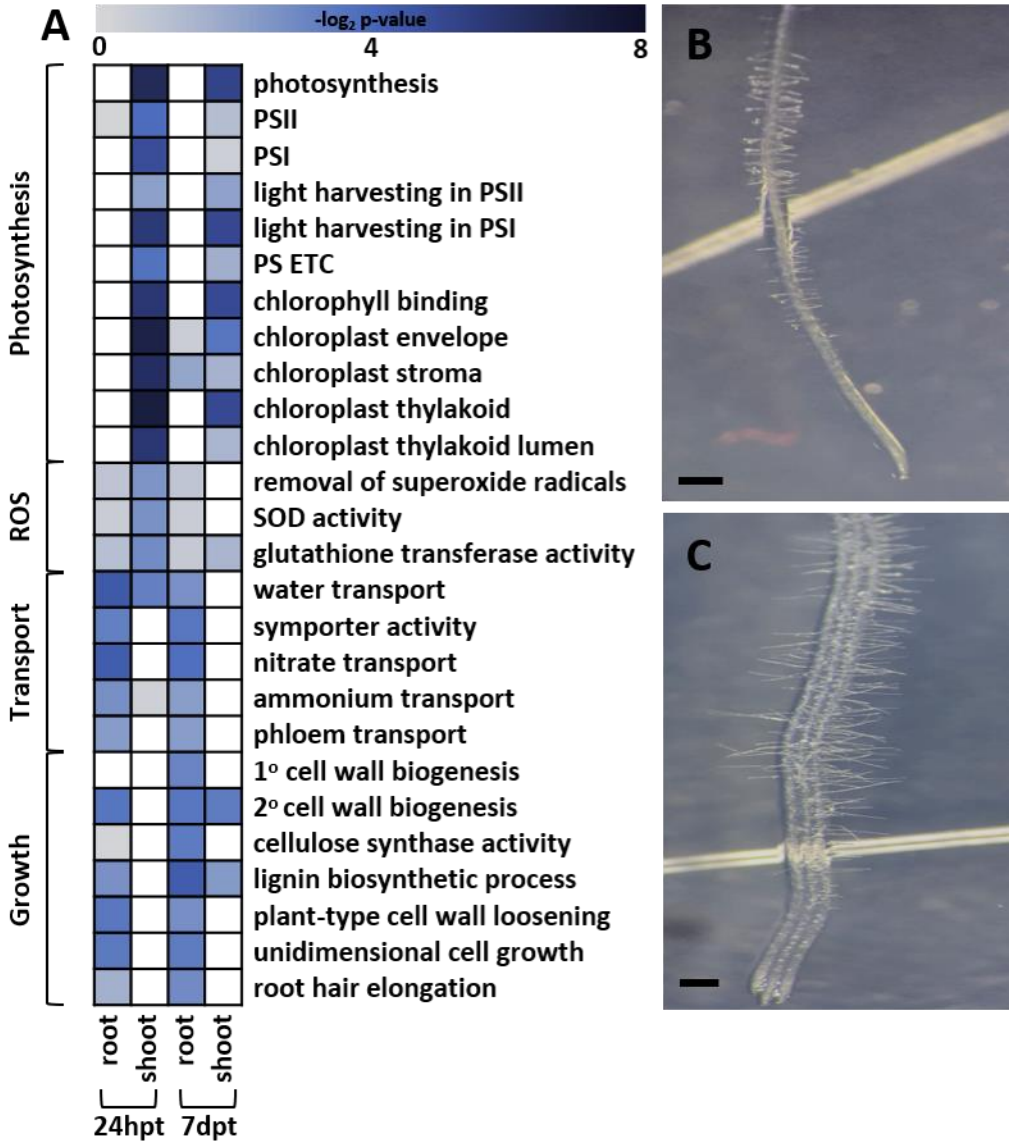


Figure 2.4. **A)** Heatmap of enriched Gene Ontology terms identified from upregulated genes with SeqEnrich. Darker colour represents a greater statistical enrichment **B)** *Arabidopsis thaliana* root imaged with a Leica M80 seven days after treatment with 2000 cfu of *P. chlororaphis* PA23. **C)** *Arabidopsis thaliana* root imaged with a Leica M80 seven days after treatment with 0.9% saline. Scale bar = 1000 µm. A complete list of enriched GO terms can be found in supplemental file 1.

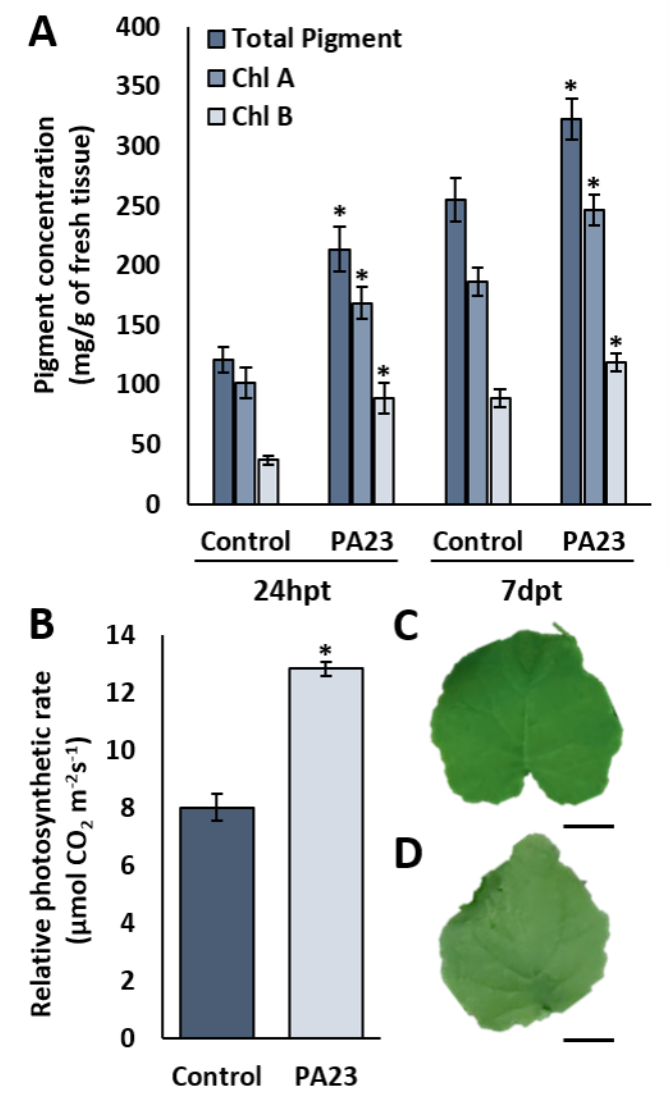


Figure 2.5. A) Pigment concentration of PA23-treated and mock-treated leaves at 24hpt and 7dpt. Absorbance values were measured at 663 nm and 645 nm and used to determine concentrations which were normalized to leaf input weight. * indicate statistical significance in a Student's t-test ($p < 0.05$) and error bars represent standard error (n=15). **B)** Relative photosynthetic rates determined with IRGA equipment and CO_2 depletion. * indicate statistical significance in a Student's t-test ($p<0.05$) and error bars represent standard error (n=10). **C)** PA23-treated leaf at 7dpt, Scale bar = 1 cm. D) Control treated leaf at 7dpt, Scale bar = 1 cm

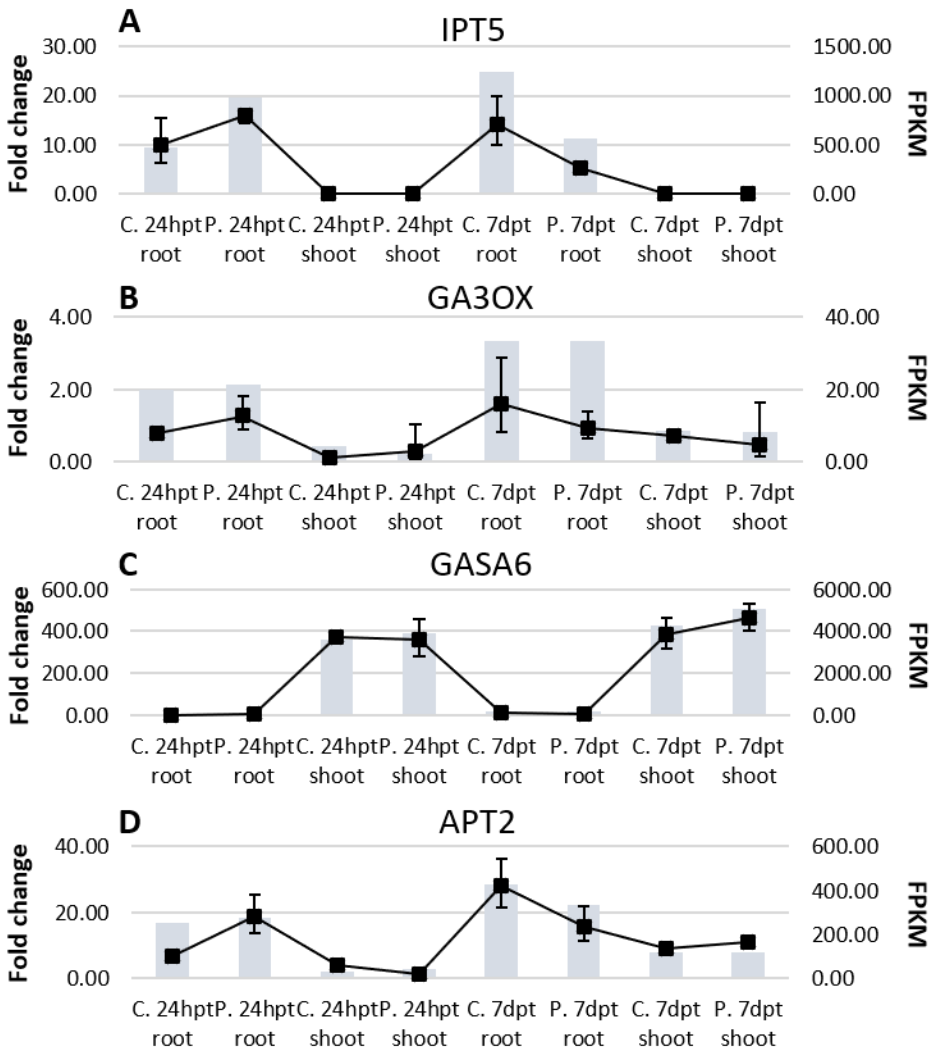
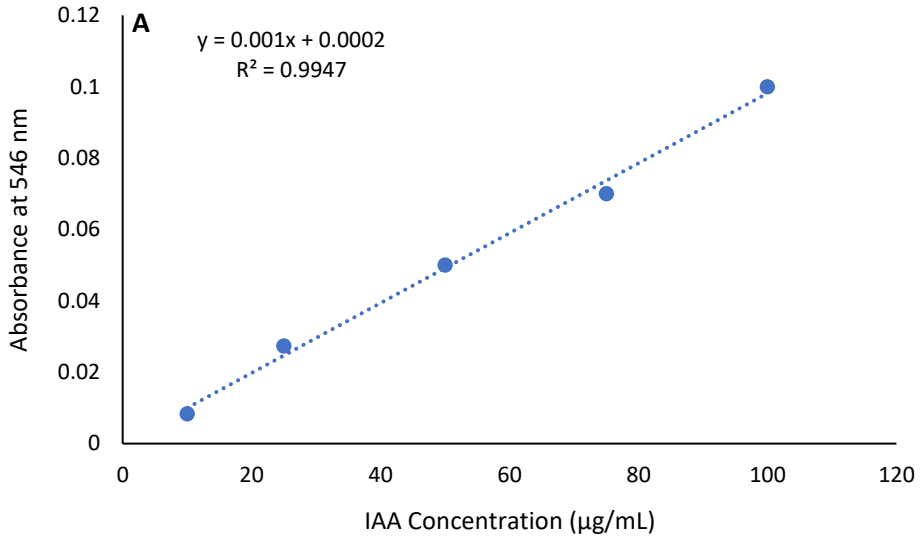


Figure 2.6. Relative fold change against the endogenous control gene β-actin and FPKM values of select genes from root and shoot tissue systems of *B. napus* treated with lysogeny broth (C) or a PA23 suspension (P) at two timepoints. Relative fold changes are represented as points and lines. FPKM values are represented as bars. **A)** *Isopentenyltransferase* 5. **B**) *Gibberelin-3-oxidase.* **C)** *GA-stimulated Arabidopsis 6*. **D)** *Adenine Phosphoribosyl transferase 2*



B

Sample	**Absorbance at 546 nm**	**Auxin Concentration (µg/mL)**	**Auxin Synthesis Rate (µg/hour)**
A	0.008	10	N/A
B	0.027	25	N/A
C	0.050	50	N/A
D	0.070	75	N/A
E	0.100	100	N/A
PA23	1.900	1900	44.2

Figure 2.7. Linear modelling of auxin concentration and absorbance values at 546 nm as measured by a spectrophotometer. Linear regression was used to estimate auxin production rate of *P. chlororaphis* PA23. **A)** Linear modelling of auxin concentration and absorbance values. **B)** Absorbance values for varying auxin concentrations and *P. chlororaphis* PA2

Table 2.1. *B. napus* gene targets and primer sequences used for directed qPCR validation of RNA sequencing data.

Target	**Primer sequence (5'-3')**	**Primer efficiency**
IPT5-F	CGCTGTAATTGAGGAACCGG	87.3
IPT5-R	CGAGGAGAAGAAGCTGACGA	
GA3OX-F	ACGCCAAGTGAAGTTAGCGA	83.5
GA3OX-R	TCACCTTAACAACTACTGCGACA	
GASA6-F	CAGAAAGTTATGGAGCTGGAAGT	101.35
GASA6-R	ATGGTACTTGGTGTTGCTGC	
APT2-F	AGCTTACAATGCCCCTTGAA	115
APT2-R	GGAACACTCTCCGCTTCCAT	
βActin-F	ATCTCTTGGTTCTGGCATCG	80.52
βActin-R	GCAATGTGCGTTCAAAGATT	

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Chapter 3: Effects of *Pseudomonas chlororaphis* PA23 seed treatment on the soybean rhizosphere microbiome

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3.1 ABSTRACT

Plant growth promoting bacteria (PGPB) are a heterogeneous group of soil microbes that confer beneficial effects to plants. Recent interest in sustainable and organic farming strategies has highlighted the potential use of PGPB in agricultural settings. Novel PGPBs are difficult to isolate from environmental samples and previous techniques are time-consuming and tedious. The advent of next-generation sequencing has allowed for the rapid identification of novel microbes and the investigation of the effects of PGPB on endogenous microbial communities. The present study was carried out to identify potential changes in bacterial and fungal communities found in the rhizosphere and bulk soil in a *G. max* cropping rotation in response to a *Pseudomonas chlororaphis* PA23 seed treatment. Using a combination of marker-based Illumina sequencing and bioinformatic analyses, we profiled biodiversity metrics in the bulk and rhizosphere soil in response to *P. chlororaphis* PA23 treatment and across the cropping season. We did not detect significant differences in microbial community composition or species diversity in response to PA23 seed treatmnent. Moreover, we identified subtle but gradual changes in the bacterial and fungal composition over time regardless of the presence or absence of PA23 treatment. Together, the results of our study suggest that *P. chlororaphis* PA23 does not alter the endogenous microbial communities and contributes additional information for the utilization of this PGPB as an agricultural adjunct.

3.2 INTRODUCTION

Agricultural soils are a complex ecosystem that hosts a range of organisms such as single-celled eukaryotes, fungi, prokaryotes, and archaea (Beckers et al. 2017). The population composition of the soil is dependent on abiotic and biotic factors that include soil type, nutrient content, soil moisture content, local flora and fauna. The root system provides a unique ecological niche known as the rhizosphere for microorganisms (Philippot et al. 2013). Root exudates, compounds secreted by the plant root, act as signalling molecules and substrates for microbial metabolism. The bacterial and fungal members of the rhizosphere are well defined. Fungal communities are defined by all six fungal phyla; however, a majority of detected species belong to Basidiomycota, Ascomycota, and Zygomycota (Wang et al. 2017) while bacterial members are dominated by the Firmicutes, Acidobacteria, Verrucomicrobia, Bacteroidetes, and Proteobacteria (Liu et al. 2019).

Plant growth promoting bacteria (PGPB) are a heterogeneous group of beneficial microbes that are found in the bulk soil, rhizosphere, and on the root surface (Backer et al. 2018). These microbes enhance plant growth through the secretion of metabolites, nutrient mobilization, and alleviation of biotic and abiotic stressors (Garcia et al. 2001; Rijavec and Lapanje 2016; Tahir et al. 2017). The recent interest in sustainable and organic farming has highlighted the potential use of PGPB in larger agricultural settings. Currently, crop production heavily relies on the application of chemical fertilizers and pesticides. These agricultural inputs can have deleterious effects on the environment that may have unintended off-target effects, nutrient leeching, and ecological disturbances (Carpenter et al. 1998; Wennergren and Stark 2000). Several PGPB products have been developed and are primarily used in European countries

(Radhakrishnan et al. 2017). These products include liquid formulations of *Bacillus* spp that are dispersed as aerosals and granular formulations of *Rhizobium* spp that are applied in furrow. Granular formulations of *Rhizobium* and *Bradyrhizobium* are commonly used in soybean crop rotations and reduce fertilizer dependency while increasing crop yield (Caldwell and Vest 1970; Ulzen et al. 2016; Santos et al. 2019). However, their effects on endogenous fungal and bacterial populations is not well studied. Given the link between soil microbes and plant health, a holistic study investigating the effects of PGPB products on soil microbes is required to determine potential ecological risks prior to widespread implementation of PGPBs for agricultural purposes. The identification of novel PGPB relied on *in-vitro* cultivation of environmental samples on selective media which is time-consuming and difficult as a small minority of soil bacteria (< 1%) are cultivatable (Kalam et al. 2017). Furthermore, the study of microbial communitites and interactions under lab conditions do not provide a complete understanding of the complex microbial dynamics in the field which are influenced by environmental conditions and microbial mobility. The advent and implementation of marker-based next-generation sequencing technologies, has allowed for more robust study of field samples and it is now possible to capture the biological diversity, potential PGPB taxa, and microbial dynamics more easily (Caporaso et al. 2012).

The Pseudomonads are a group of bacteria that are universally found in agricultural fields. Members of this genus have potent biocontrol and plant growth-promoting activity (Choi et al. 2008; Simonetti et al. 2012). *Pseudomonas chlororaphis* PA23 was originally isolated from the soybean rhizosphere. This bacterium has been studied extensively as a biocontrol agent against fungal pathogens. Biocontrol activity is associated with the synthesis and secretion of antibiosis compounds such as phenazine, hydrogen cyanide, and pyrrolnitrin (Ramarathnam et al.

2011, Nandi et al. 2015). Furthermore, Pseudomonads have been shown to induce systemtic acquired resistance and may provide broader protection against plant pathogens (De Meyer et al. 1999; Duke et al. 2017b). Interestingly, *P. chlororaphis* PA23 also exhibits plant growth promoting activity. Application of *P. chlororaphis* PA23 to *Brassica napus* and *Glycine max* seedlings under greenhouse conditions resulted in accelerated growth and more abundant and greener foliage. Plant growth promoting activity was linked to the synthesis and secretion of auxin which altered gene expression within the host plant (Chapter 2). Although the mechanisms of biocontrol and plant growth promotion of *P. chlororaphis* PA23 are well described; we have yet to fully investigate its beneficial effects on crop production under field conditions and potential effects on the endogenous microbial communities present in the field.

Given the unknown impact of a bacterial seed treatment on the endogenous microbial community, a holistic understanding of community dynamics throughout the growing season and in response to *P. chlororaphis* PA23 is required. In this chapter, we used a combination of marker-based, 16s and ITS, sequencing to identify potential shifts in the bacterial and fungal communities of the rhizosphere and soil over time and to *P. chlororaphis* PA23 seed treatment. Specifically, we identified no significant differences in the species diversity between the bulk soil and rhizosphere; moreover, we did not detect any shifts in diversity due to *P. chlororaphis* PA23 treatment. Furthermore, we did not detect differences in the proportional community composition of the rhizosphere due to *P. chlororaphis* PA23 treatment. However, we report significant differences in the proportional community composition between the bulk soil and rhizosphere suggesting an active selection of microbial members in the rhizosphere by *G. max*. Together, the results of this study provide a deeper understanding of the microbial communities present in the bulk soil and rhizosphere, how these communities shift over time, and changes in

these dynamics in response to *P. chlororaphis* PA23 treatment that have applications for global crop management strategies.

3.3 MATERIALS AND METHODS

3.3.1 Bacterial and Plant Materials

Bacterial cultures were prepared from frozen stocks of *P. chlororaphis* PA23 stored in 10% skim milk (ThermoFisher, Waltham, MA) at -80°C. Cultures were streaked onto lysogeny broth (LB) agar plates (Difco Laboratories, Detroit, MI) and incubated at 28°C for 24 hours. Isolated colonies were used to inoculate large volumes of liquid LB and incubated for 24 hours in a rotary shaker at 28°C at 250 rotations per minute. Cell density was determined by measuring the optical density at 600 nm (OD_{600}) with a spectrophotometer. The final treatment solution was adjusted to an $OD_{600} = 1$ or approximately 2×10^9 cfu/mL with sterile distilled water.

Naked, untreated with fungicide or pesticide, Soybean (*Glycine max*) cv. Sperling RR24 seeds were treated with *P. chlororaphis* PA23 suspension, at a concentration of 1×10^9 cfu/g of dry seed, for 30 minutes. Mock-treated seeds were submerged in sterile LB for 30 minutes. After the treatment period seeds were dried in a laminar flow hood for 24 hours. Any remaining moisture due to the treatment was removed by further drying the seeds in a 37°C oven for 48 hours. Prior to seeding, the presence of *P. chlororaphis* PA23 was confirmed through seed homogenization in sterile 0.9% saline, serial dilution, plating onto Pseudomonas Isolation Agar (Difco Laboratories, Detroit, MI), and colony counting.

3.3.2 Planting and In-Field Measurements

The field study was conducted at the Ian N. Morrison Research Farm located in Carmen, Manitoba, Canada. A complete randomized plot was designed for the study area containing four crop rotations: corn-soybean (CS), canola-soybean (CanS), wheat-soybean (WS), and soybean-

soybean (SS). The main plots measured 8 meters by 8 meters and were divided into sub-plots measuring 2 meters x 8 meters with 30" spacing between planting rows (Figure 3.1). Seeds were planted at a target plant population of 170, 000 plants per acre with the Haldrup SP35 Precision Vacuum Planter. A granular additive of *Bradyrhizobium* spp, Nodulator CP SCG (BASF, Ludwigshafen, Germany), was added in-furrow according to the manufacturer's recommendation. General plant health was determined at the R1 and R6 growth stages with a SPAD (soil plant analysis development) meter – a measure of chlorophyll content. SPAD values were measured for a total of 50 plants per rotation and treatment; values between *P. chlororaphis* PA23 and mock-treated plants were compared within their respective rotations with a Student's t-test. Seed pods were collected from a total of 50 R6 plants per rotation and treatment; the total number of pods, one, two, three, and four seed pods were counted and compared to the mock-treated plants with a Student's t-test.

3.3.3 Bulk Soil and Rhizosphere Sample Collection

Soil samples for microbial community analyses were collected at four different stages: Pre-seeding, V6, R1, and R6. Bulk soil samples were collected at a depth of eight inches from areas between plant rows. Five samples were collected along the length of each plot area and pooled together to generate one biological replicate; with a total of 15 sample points and three biological replicates per plot. Soil samples were flash-frozen and stored at -80^{o}C until processing. Rhizosphere samples were collected from the soil adhered to the roots of *P. chlororaphis* PA23 treated and mock-treated plants. Five root systems were collected to generate one biological replicate; with a total of 15 plants and three biological replicates per plot. Root samples were flash-frozen and stored at -80^{o}C until needed; at which point the samples were

slowly thawed on ice and rhizosphere soil was brushed off with sterile paintbrushes and collected for downstream experiments.

3.3.4 DNA Extraction, Library Synthesis and Cleanup, and Illumina Sequencing

Total genomic DNA was extracted from 0.5 g of soil with the Qiagen DNeasy PowerSoil Kit (Qiagen, Hilden, Germany). Extracted template DNA was quantified with a NanoDrop 2100 Spectrophotometer and when higher sensitivity was required the Quant-iT dsDNA PicoGreen Assay (Invitrogen, Carlsbad, CA) and the NanoDrop 3300 Flurospectrophotometer was used. Samples that returned low quantity were re-extracted with 2-4x the amount of starting material. Both bacterial (v4) and fungal (ITS) libraries were performed following the Earth Microbiome Project protocol (http://www.earthmicrobiome.org/). Briefly, the v4 region of bacterial and archaeal 16S rRNA gene was amplified using bar-coded 515F/806R primers (Caporaso et al. 2011, 2012). Shotgun amplicon synthesis was performed with Platinum Hot Start PCR II Mix and the following thermocycling conditions: 94^{o}C for three minutes, 35 cycles of 94^{o}C for 45 seconds, 50^{o}C for 60 seconds, 72^{o}C for 90 seconds, and 72^{o}C for ten minutes. Sample PCR amplification was carried out in triplicate to ensure sufficient product for downstream experiments. These triplicates were pooled together, and gel electrophoresis was performed to confirm proper amplification and absence of unintended PCR products. The ITS1 and ITS2 sequences were amplified with barcoded ITS1/ITS2 primers (Bokulich and Mills 2013). PCR amplification was performed as previously with the following modifications: 94^{o}C for 1 minute, 35 cycles of 94^{o}C for 30 seconds, 52^{o}C for 30 seconds, 68^{o}C for 30 seconds, and 68^{o}C for 10 minutes. Both v4 and ITS libraries were cleaned with the Qiagen UltraClean 96 PCR Cleanup Kit (Qiagen, Hilden, Germany) according to the manufacturers recommended protocol. Following cleanup, library concentration was measured with the Quant-iT PicoGreen dsDNA

Assay Kit and the NanoDrop 3300 Flurospectrophotometer. Samples were pooled based off concentration and size distribution of sequencing pools were determined on an Agilent DNA 1000 Chip and Agilent 2100 Bioanalyzer. Library pools, multiplex index of 60, were sent to Genome Quebec and 100 bp paired end sequencing was conducted on the Illumina HiSeq 4000.

3.3.5 Microbiome Bioinformatics

Microbiome sequencing data were analyzed for the canola-soybean rotation using the Quantitative Insights Into Microbial Ecology (QIIME) v2.1 suite (Bolyen et al. 2019). First, raw sequencing reads were subsampled with SeqTK (https://github.com/lh3/seqtk) to a depth of 150,000 reads per sample. Briefly, demultiplexed sequences and associated metadata were imported into QIIME via q2-import. Imported sequences were quality filtered using the q2-demux plugin followed by sequence background denoising with DADA2 via q2-dada2. Demultiplexed sequences and associated metadata was imported into QIIME2 via q2-import. Imported sequences were quality filtered, trimming the first five nucleotide reads and reads that fell below the quality threshold, using the q2-demux plugin followed by sequence denoising with DADA2 via q2-dada2 (Callahan et al. 2016).

Identified amplicon sequence variants (ASVs) were aligned with mafft (via q2-alignment) and fasttree (via q2-phylogeny) was used to construct phylogenetic trees (Katoh 2002; Price et al. 2010). Biodiversity metrics were calculated on rarefied samples that were subsampled to 14000 sequences without replacement generating alpha diversity values – observed OTUs and Faith's Phylogenetic Diversity (measures of microbiome richness) and beta diversity values – weighted and unweighted UniFrac, Bray-Curtis Dissimilarity (for fungal communities, and principal coordinates analysis via the q2-diversity plugin (Faith 1992; Lozupone and Knight 2005; Lozupone et al. 2007). Alpha rarefication, a measure of observed

OTUs against sequence count, was performed to ensure that sufficient sequencing depth was achieved to capture the complete biodiversity of the sample (Willis 2019) and determined that the sampling depth of 14,000 was sufficient to capture the biological diversity present in the samples. Analysis of composition of microbiome (ANCOM) was used to identify differentially abundant species or taxa between samples (Mandal et al. 2015). Following, taxonomy was assigned to ASVs via q2-feature-classifer (Bokulich et al. 2018) sklearn naïve Bayes taxonomy classifier against the GreenGenes 13_5 99% OTU refence sequences and UNITE 8.2 Dynamic reference sequences for bacterial and fungal ASVs respectively (DeSantis et al. 2006; Nilsson et al. 2019). Lastly, proportional taxa bar plots were generated via q2-barplots; detected taxa were averaged across three biological replicates.

3.4 EXPERIMENTAL RESULTS

3.4.1 MICROBIOME METRICS

3.4.1.1 Effect of *P. chlororaphis* PA23 seed treatment on bacterial community dynamics

First, we investigated the effects of *P. chlororaphis* PA23 seed treatment on bacterial community dynamics. Species richness represented by the alpha diversity metric – Faith's Phylogenetic Diversity (FPD) was calculated for both rhizosphere and bulk soil samples over developmental time (pre seed, V6, R1, and R6). Statistical significance of FPD values was determined with a pairwise Kruskal-Wallis One-Way Analysis of Variance. Our dataset did not detect significant differences in FPD values between rhizosphere and bulk soil samples ($P = 0.476$) (Figure 3.4). Further, the dataset did not detect significant differences in FPD values between *P. chlororaphis* PA23 treated and control rhizosphere samples ($P = 0.0933$) (Figure 3.4).

Next, we calculated beta diversity metrics to examine differences in the community composition of the rhizosphere and bulksoil samples over developmental time. Quantitative comparisons of weighted UniFrac and unweighted UniFrac metrics revealed differences in bacterial community compositions. First, weighted UniFrac values, which consider phylogenetic relationship, presence, absence, and species abundance, revealed a significant difference in the bacterial population structure between rhizosphere and bulk soil (P = 0.033). Unweighted UniFrac values, which considers phylogenetic relationship, presence or absence, confirmed differences in the bacterial composition between the rhizosphere and bulk soil (P = 0.008). After establishing differences in population composition between the rhizosphere and bulk soil; the effects of *P. chlororaphis* PA23 on rhizosphere composition were investigated. Pairwise comparisons between *P. chlororaphis* PA23 treated and control rhizosphere samples revealed no significant differences in community structure (P = 0.287, P = 0.252, for weighted and unweighted UniFrac values respectively). We then visualized differences in weighted and unweighted UniFrac values with principle coordinate analyses which revealed bacterial community compositions clustered primarily by developmental time point and sample type (bulksoil versus rhizosphere) (Figure 3.5). Analysis of the composition of microbes (ANCOM) between treated and untreated soybean rhizosphere revealed no differentially abundant taxa or species. Specifically, the individual levels of detected bacterial phyla did not vary significantly based on *P. chlororaphis* PA23 treatment, developmental time, or sample type (Figure 3.6).

3.4.1.2 Effect of *P. chlororaphis* PA23 seed treatment on fungal community dynamics

We then studied fungal species diversity and community composition in response to PA23 seed treatment. The fungal alpha diversity, FPD, metrics were not significantly different between the rhizosphere and bulk soil samples (P = 0.7243); further, *P. chlororaphis* PA23

treatment did not alter the FPD detected in the rhizosphere (P = 0.5797). Three beta diversity metrics were used to determine significant differences in taxa proportions and community composition. Weighted (P = 0.002) and unweighted UniFrac (P = 0.001) reported significant differences in the fungal community structure between the rhizosphere and bulk soil. Bray-Curtis Dissimilarity (P = 0.001), a more commonly used metric for fungal beta diversity, further supports the difference in fungal community structure between the rhizosphere and bulk soil. Population structure of treated and control rhizosphere were compared with weighted uniFrac (P = 0.175), Bray-Curtis Dissimilarity (P = 0.075), and unweighted UniFrac (P = 0.004). Bray-Curtis dissimilarity distance matrices were used for downstream principal coordinate analyses which showed that samples cluster based on time point then sample point. Lastly, differentially abundant fungal taxa between treated and control rhizosphere were identified with ANCOM. One differently abundant ASV was identified and BLAST results predictively aligns to *Mortierella beljakovae* (98% percent identity). Proportional taxa bar plots were generated and show small variations of the major fungal phyla based on sample type and time point (Figure 3.9).

3.4.2 *P. chlororaphis* PA23 increases chlorophyll content and pod formation

To determine if the increase in photosynthetic pigments observed in the *B. napus – P. chlororaphis* PA23 interaction would translate to a different crop species under field conditions. We observed an 8.4% increase in SPAD values in the treated plants of the CanS rotation. Raw SPAD values were used to calculate statistical significance with a Student's t-test between *P. chlororaphis* PA23 treated and mock-treatment; where we observed a significant increase in the CanS ($P < 0.05$) and WS ($P < 0.05$) rotations.

Next, we investigated the extent of plant growth-promoting action by *P. chlororaphis* PA23 by counting the number of pods formed by the R6 flowering stage of soybean. Here we report an average increase of 30% for the total number of pods in the treated plants of the CanS rotation. Statistical significance was determined by comparing the pod group (total pod, one seed pod, two seed pod, three seed pod, four seed pod) against their respective mock-treatment controls. We observed significant increases in all five groups for the canola-soybean rotation ($P < 0.05$).

3.5 DISCUSSION

Plant growth promoting bacteria (PGPB) are a group of soil microbes that confer beneficial effects onto plants. These PGPB are used as environmentally friendly and organic agricultural adjuncts aiding in crop production and plant health (Backer et al. 2018). However, the effects of PGPBs on endogenous organisms are unknown; specifically, their effects on microbes that reside within the soil. Given the link between soil microbes, soil health, and crop production (Chaparro et al. 2012a), it is important to gain an understanding of how PGPBs affect soil microbes before their wide-spread adoption into crop management strategies. Our study aims to investigate the effects of a novel PGPB seed treatment, *P. chlororaphis* PA23, on fungal and bacterial communities found in agricultural fields. Specifically, we treated *G. max* with *P. chlororaphis* PA23 which did not alter the fungal and bacterial species richness or community composition within the rhizosphere. However, we did detect differences in the in both the bacterial and fungal compositions between the rhizosphere and bulk soil suggesting that *G. max* plays a role in the selection of rhizosphere members.

3.5.1 Rhizosphere diversity is inherited from the surrounding bulk soil

Previous studies have shown that root structure, morphology, and exudates can directly affect the community structure and species diversity of the rhizosphere microbiome (Korenblum et al. 2020). Bacterial and fungal taxa found within the rhizosphere are inherited from the surrounding soil suggesting that environmental conditions are one of the largest drivers in rhizosphere species diversity (Xue et al. 2018). Previous studies suggest that sediment type and moisture are considered some of the most significant determinants in microbial species diversity (Zhang et al. 2020). In the current study, FPD values suggest similar levels of species diversity between bulk soil and rhizosphere samples. Given that rhizosphere members are horizontally transferred from the bulk soil it is likely that the environmental conditions and soil type of the field study could be a driver of species diversity (Lloyd et al. 2016). However, the current study did not investigate soil type, moisture content, or nutrient profile. Thus, a multi-year study that implements an soil analysis and metagenomics could provide more concrete insights into the factors that drive species diversity within the bulk soil and rhizosphere.

3.5.2 *P. chlororaphis* PA23 does not alter rhizosphere microbial diversity

The presence of an exogenous microbe can impact soil and rhizosphere biodiversity (Whipps 2001; Schreiter et al. 2018). The extent of this effect varies and is dependent on the metabolite profile of the microbe. PGPBs can synthesize and secrete metabolites and phytohormones which alter plant signalling systems (Garcia et al. 2001; Glick 2014; Chauhan et al. 2015). The induction or modulation of plant signalling systems can lead to increased growth rates of root tissue resulting in higher root volume in the soil (Bulgarelli et al. 2013). This increase in root tissue volume coincides with increases in the habitable surface area and results in higher microbe cell density found within the rhizosphere. For example, *Zea mays* treated with

Azospirillum brasilense, a nitrogen-fixing PGPB, exhibited denser root systems and increased bacterial density. However, increases in root volume and cell density did not alter species diversity within the rhizosphere (Herschkovitz et al. 2005). Together, these findings suggest that induction of root growth by PGPBs do not result in changes in species diversity. In the previous chapter, we describe *P. chlororaphis* PA23 plant growth promotion of *B. napus* which resulted in increased root volume and root hair abundance and was driven by the synthesis and secretion of auxin into the rhizosphere. Given these findings, it is possible that plant growth promotion in *G. max* is achieved in a similar fashion; however, the current study did not investigate the phenotypic differences in the plants grown under field conditions. Thus, an additional greenhouse study is required to confirm a potential root growth phenotype in *P. chlororaphis* PA23 treated *G. max*

Other methods of plant growth promotion can be linked to antibiotic compounds that alter soil microbial compositions. Previous studies investigating the effects of antibiotic-producing pseudomonads have discovered that changes in rhizosphere species diversity are transient and are limited both spatially and temporally (De Leij et al. 1995; Natsch et al. 2006). Furthermore, the effects of the antibiotic compound – phenazine was shown to have minimal effects on the population dynamics and species diversity of the rhizosphere during *in-vivo* experiments (Moënne-Loccoz et al. 2001; Bakker et al. 2002). Together, these findings suggest that the presence of an antibiotic-producing PGPB may only have temporary and non-significant effects on rhizosphere biodiversity. Previous studies on *P. chlororaphis* PA23 have identified the production of phenazine, pyrrolnitrin, and hydrogen cyanide which have antibiotic effects on fungi and bacteria under *in-vitro* and greenhouse conditions (Selin et al. 2010; Nandi et al. 2015b; Duke et al. 2017b). In the current study, no differences in FPD values between *P.*

chlororaphis PA23 treated and control rhizospheres were detected, suggesting that the production of antibiotic compounds does not alter rhizosphere diversity. It is possible that the antibiotic compounds produced by *P. chlororaphis* PA23 had minimal and transient effects on the fungal and bacterial members within the rhizosphere. However, the resolution of our study was not capable of capturing these changes. Thus, additional studies profiling the rhizosphere microbiome with shorter sampling intervals may capture the potential effects of *P. chlororaphis* PA23 antibiotic compounds on rhizosphere diversity and provide a more robust understanding of shifts in rhizosphere diversity.

3.5.3 Trends in microbial community composition

Microbial members found within the bulk soil and rhizosphere can change over time in response to environmental conditions and active selection by the plant root. These differences in community composition or relative taxa abundance are represented by beta diversity metrics and can play an important role in soil and plant health (Lozupone et al. 2007; Lebeis 2014). Plants select for microbes that can preferentially use root exudates leading to defined composition differences between the rhizosphere and bulk soil (York et al. 2016). These differences are established early in the plant life cycle and alterations may continue through developmental time leading to defined differences in the microbial communities throughout a growing season. In the current study, the microbial compositions of the rhizosphere and bulk soil were compared. We observed significant differences in the weighted UniFrac (bacterial composition) and Bray-Curtis Dissimilarity (fungal composition) metrics suggesting that the rhizosphere composition differs significantly from the bulk soil. Principle coordinate analyses revealed distinct clustering patterns based on developmental time and sample type. Weighted UniFrac and Bray-Curtis

Dissimilarity values clustered tightly based on time suggesting that microbial communities in the soil change over a growing season regardless of *P. chlororaphis* PA23 treatment. In the rhizosphere, changes over developmental time are likely linked to changes in the root exudate profile and root morphology. For example, studies using the model plant system *A. thaliana* show changes in microbial populations in response to shifts in root exudate profile and structure across the plant lifecycle (Chaparro et al. 2013; Hu et al. 2018). While changes in the bulk soil are likely associated with changes in soil properties such a moisture, pH, and nutrient content (Sessitsch et al. 2001; Ashworth et al. 2017; Xue et al. 2018). Future studies focusing on the root exudate profiles of *G. max* over developmental time and its effects on microbial taxa under gnotobiotic conditions will provide the necessary information required to fully understand microbial composition changes in the rhizosphere.

Similar to FPD, the addition of *P. chlororaphis* PA23 did not affect the composition of bacterial and fungal communities. Weighted UniFrac and Bray-Curtis Dissimilarity metrics suggest that the addition of *P. chlororaphis* PA23 did not significantly alter community dynamics over one growing season. However, other studies suggest that the addition of an exogenous microbe can reduce bacterial biodiversity and composition of the rhizosphere. For example, the addition of *P. fluorescens* F113Rif reduced the number of unique bacterial species and altered the community composition within the rhizosphere (Walsh et al. 2003). Further, the addition of *Bacillus amyloliquefaciens* BNM122 for the biocontrol of *Rhizoctohnia* spp resulted in minor changes in the soybean rhizosphere (Correa et al. 2009). Lastly, the addition of a commercially available and undefined microbial soil amendment – VESTA resulted in signiciantly altered species richness and community composition of the strawberry rhizosphere (Deng et al. 2019). Together, these studies suggest that the addition of some 'active' bacterial

agricultural adjuncts may affect the species diversity and composition of the plant rhizosphere. Given the nature of our study, it is difficult to extrapolate the potential effects of multi-year *P. chlororaphis* PA23 application on soil microbial populations. Further studies spanning multiple growing seasons are required to fully determine potential changes in soil microbes and health.

3.5.4 Potential mechanisms for increased photosynthesis and pod formation following *P. chlororaphis* PA23 treatment

In chapter two, we show that *P. chlororaphis* PA23 increased the concentration of photosynthetic pigments and photosynthetic rates in canola. The previous study utilized a higher cell count treatment solution when compared to the seed treatement protocol used in the current study for *G. max*. Given that some metabolites produced by Pseudomonads (Venturi 2006; Lee and Zhang 2014), in particular *P. chlororaphis* PA23 (Shah et al. 2020), are driven through quorum sensing, it is possible that the lower cell density present in the treated *G. max* seed and under field condition may reduce or eliminate the plant growth promoting phenotype. Interestingly, we observe the contrary, with a robust increase in the SPAD values at the R1 stage suggesting that a lower number of *P. chlororaphis* PA23 cells could elevate the photosynthetic pigment concentrations in *G. max* foliage. However, we did not detect differentially abundant ASV counts for *P. chlororaphis* PA23 between treated and control rhizosphere samples at the R1 suggesting that indirect actions of *P. chlororaphis* PA23 may be driving this phenotype. Due to the predictive nature of the study, we can only postulate that the initial influx of *P. chlororaphis* PA23 may have impacted *G. max* seedling phytohormone signllaing and indirect effects on other microbial members in the soil may be driving plant growth promotion. Additional studies are required to determine the persistence of *P. chlororaphis* PA23 under field conditions, its prolonged effect on *G. max* growth, and potential effects on other beneficial microbial taxa.

Varying levels of bacterial taxa may contribute to plant growth promotion under field conditions. When comparing the treated and control rhizosphere samples at their respective time points, we report non-significant differences in the number of ASVs assigned to Actinobacteria and Proteobacteria. Members of the Actinobacteria have been reported to contribute to plant growth promotion. For example, *Rhizobiaceae* and *Bradirhizobiaceae* are common symbionts for legumunious plants and act as nitrogen fixators; contributing additional nutrients that can drive plant growth, fruit formation, and seed set (Sathya et al. 2017). Furthermore, phytohormone production has been reported in other Actinobacteria (El-Tarabily and Sivasithamparam 2006; El-Tarabily 2008) and may work in conjuction with endogenous plant signaling mechanisms and *P. chlororaphis* PA23 metabollites to modulate plant growth. Members of the Proteobacteria have similar mechanisms and contribute to plant growth through nutrient mobilization and phyhormone production (Bruto et al. 2014). Thus, the observed differences in Actinobacteria and Proteobacteria abundance present in the *P. chlororaphis* PA23 treated rhizosphere may partially contribute to the observed differences in SPAD values and pod set. Further studies under gnotobiotic conditions are required to isolate potential interactions between *P. chlororaphis* PA23 and beneficial Actinobacteria and Proteobacteria. Furthermore, isolation of Proteobacteria and Actinobacteria species from field samples may provide additional insights into the complex network of plant growth promoting microbes present within the *P. chlororaphis* PA23 – *G. max* interaction.

3.6 CONCLUSIONS

In conclusion, our study reveals the addition of *P. chlororaphis* PA23 as a seed treatment on *G. max* did not result in significant changes in species richness or rhizosphere composition

under field conditions. Specifically, we did not identify differences in bacterial or fungal richness and observed no differences in the compositions of *P. chlororaphis* PA23 treated and control rhizospheres. However, we identified distinct clustering of the beta diversity metrics over developmental time which suggests that *G.* max plays a active role in modulating the microbial members present in the rhizosphere. Although no significant changes in response to *P. chlororaphis* PA23 were detected in this study, we report small shifts in the relative abundance of the benefibial microbial taxas – Actinobacteria and Proteobacteria which may contribute to plant growth promotion. Thus, further studies are required to isolate potential interactions with beneficial taxa and to investigate the potential long-term effects of *P. chlororaphis* PA23 on soil microbial populations. Our dataset provides the preliminary information needed to establish long-term field studies that integrate *P. chlororaphis* PA23 and gnotiobiotic studies to investigate potential interactions with Actinobacteria and Proteobacteria.

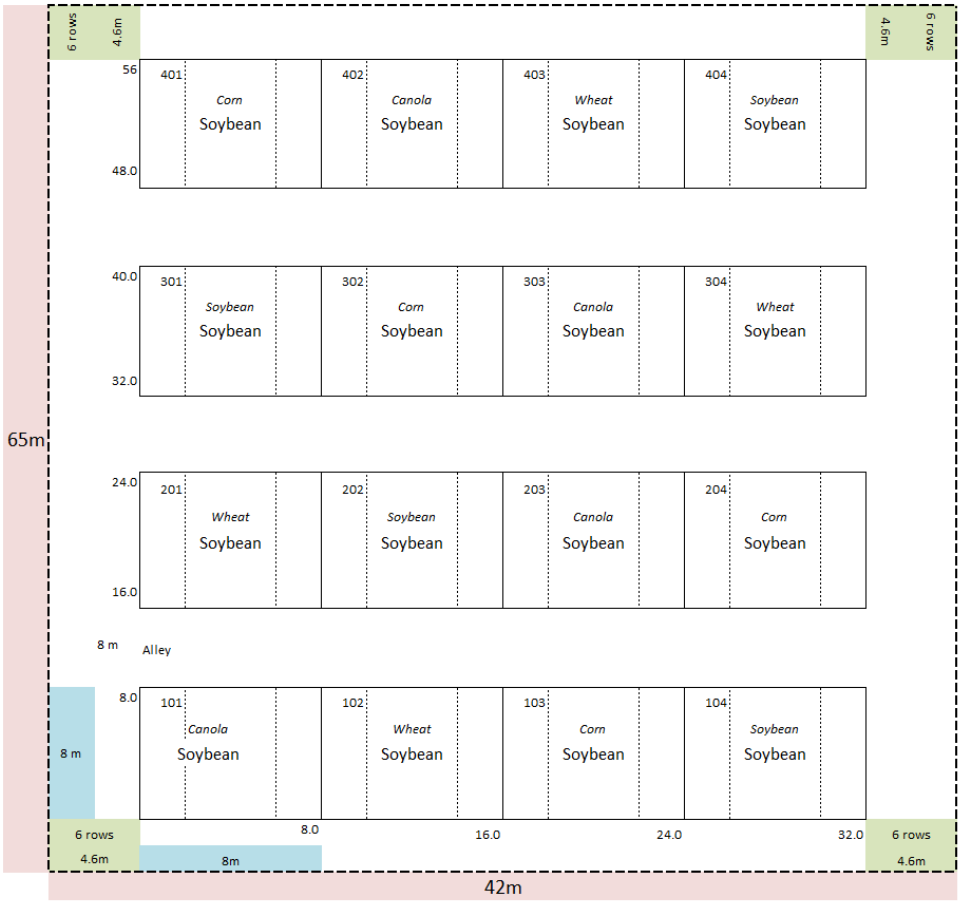

Figure 3.1. Schematic layout of the complete randomized field study located at the Ian N. Morrison Research Farm located in Carmen, Manitoba, Canada.

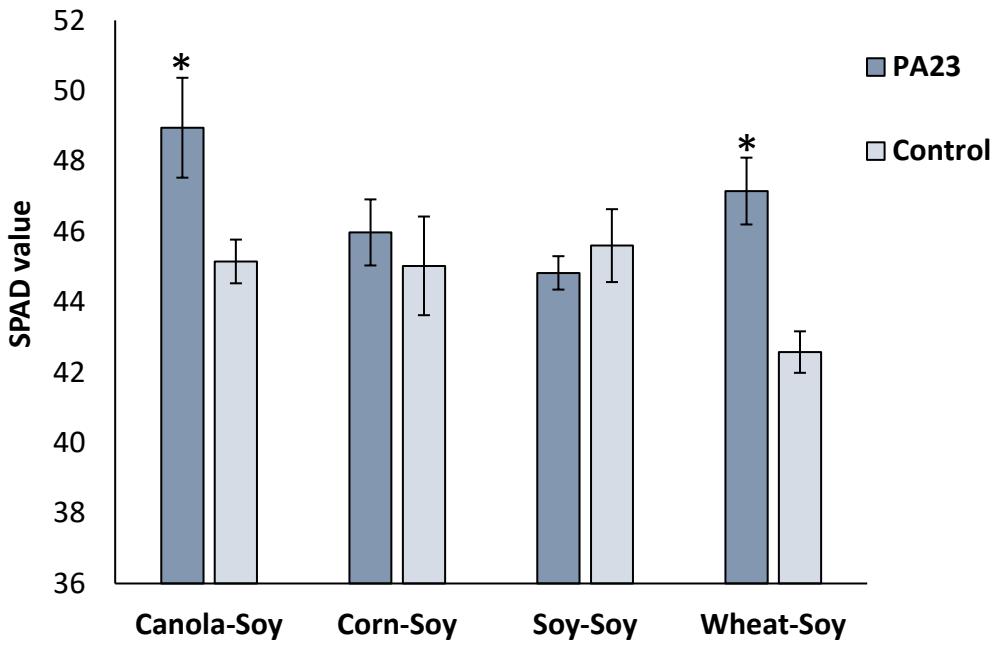

Figure 3.2 Average SPAD (soil plant analysis development) values at the R6 stage of *G. max* showing elevated photosynthetic pigment concentrations in *P. chlororaphis* PA23 treated soybean in two crop rotations. Higher value represents increased levels of chlorophyll content. * indicates statistical significance when compared against the respective mock-treated control ($P < 0.05$) n = 50.

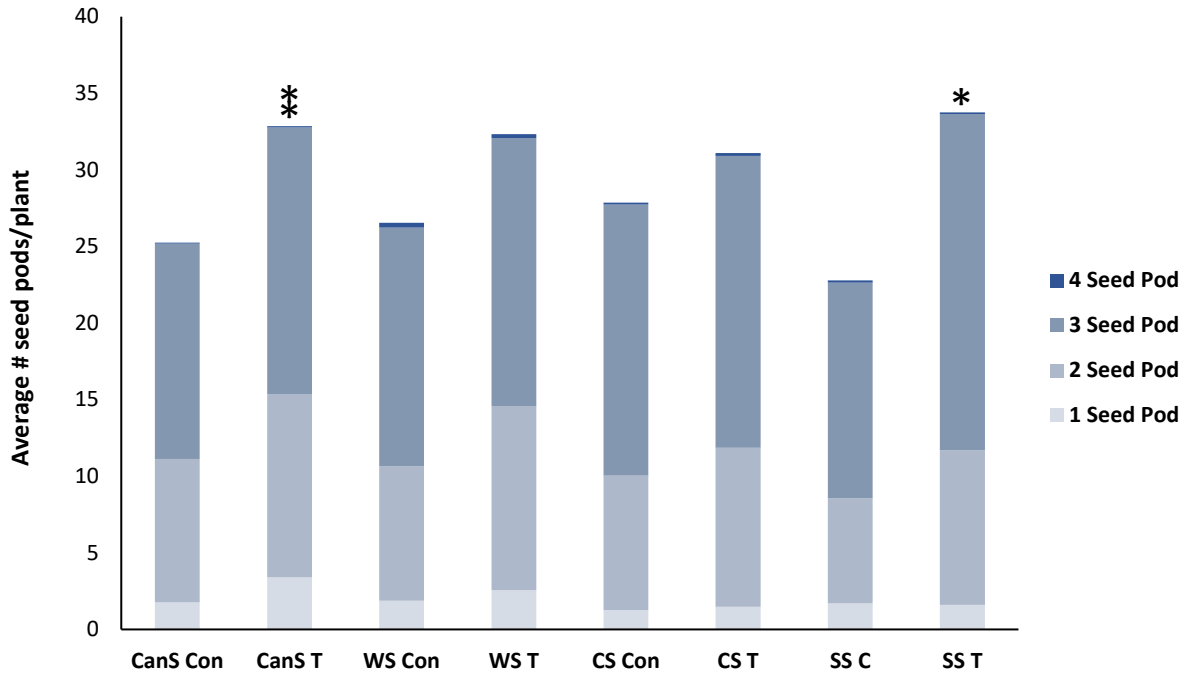

Figure 3.3 Average pod counts harvested at the R6 stage of growth. Con = mock treatment. T = *P. chlororaphis* PA23 seed treatment. ** indicates statistical significance at all levels (total pods, one seed, two seed, three seed, four seed) using a Student's t-test against mock-treated control ($P < 0.05$) ($n = 50$). * indicate statistical significance in one group (two-seed pods) using a Student's t-test against mock-treated control ($P < 0.05$).

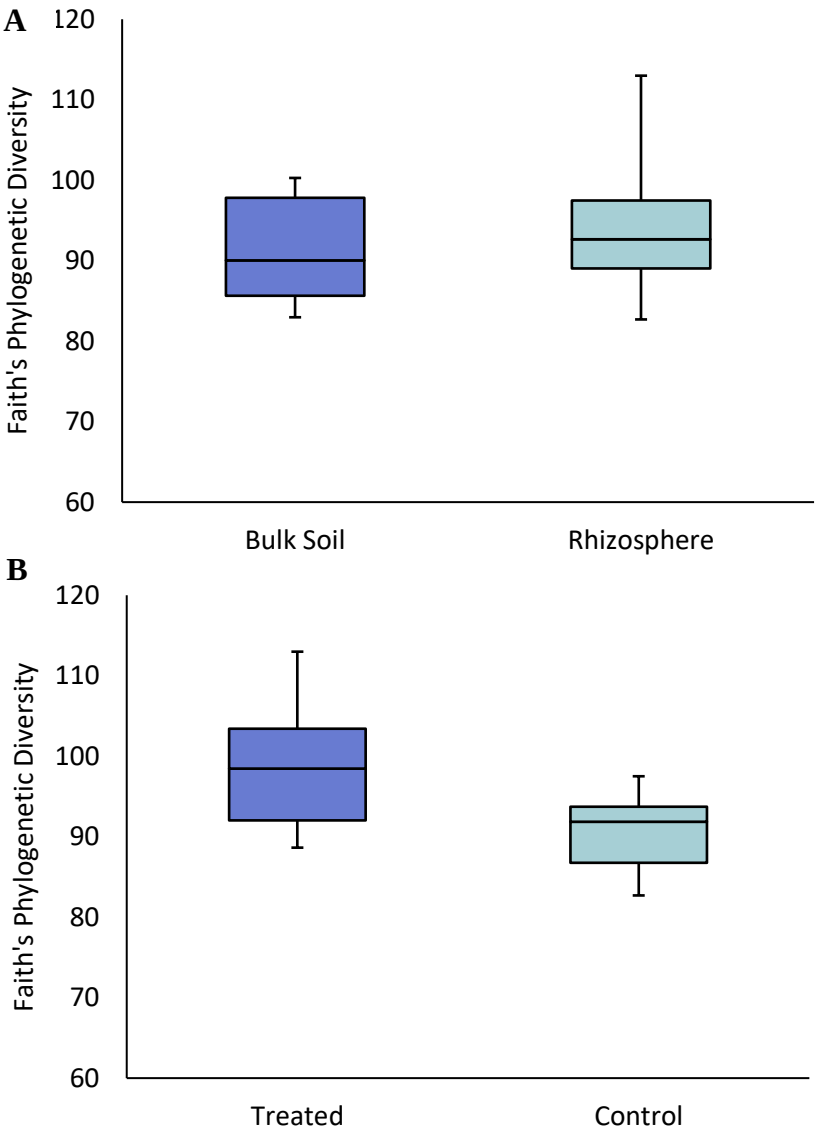

Figure 3.4 Quantitative comparisons of bacterial species richness represented by the alpha diversity metric – Faith's Phylogenetic Diversity – in canola-soybean samples. A) Comparison of cumulative bulk soil and rhizosphere samples. B) Comparison of cumulative *P. chlororaphis* PA23 (treated) and control rhizosphere samples. No statistical significance was identified in either comparison with a Kruskal-Wallis One-Way Analysis of Variance.

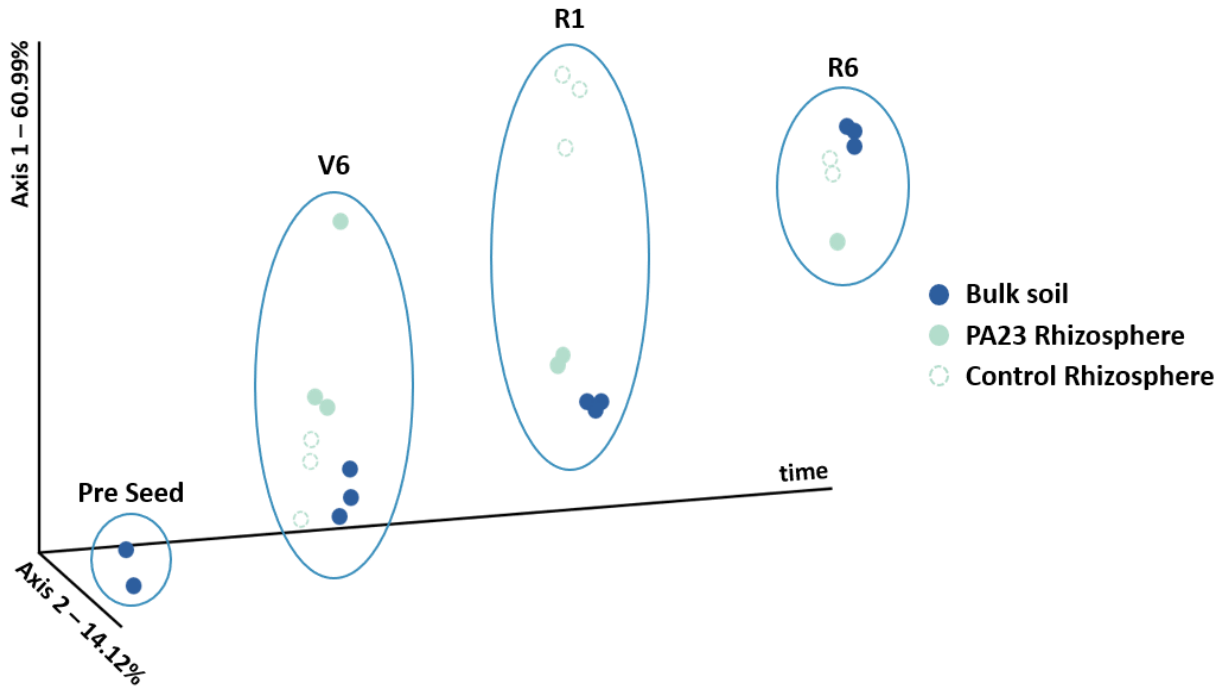

Figure 3.5 Principal coordinate analysis (PCoA) comparing the bacterial community composition, represented by the beta diversity metric – Weighted UniFrac, of canola-soybean rotation samples over developmental time. Solid blue line shows clustering of samples along the horizontal time (developmental time) axis, followed by clustering based off sample type (rhizosphere versus bulk soil: filed circles), and loose clustering based off *P. chlororaphis* PA23 treatment (unfilled circles)

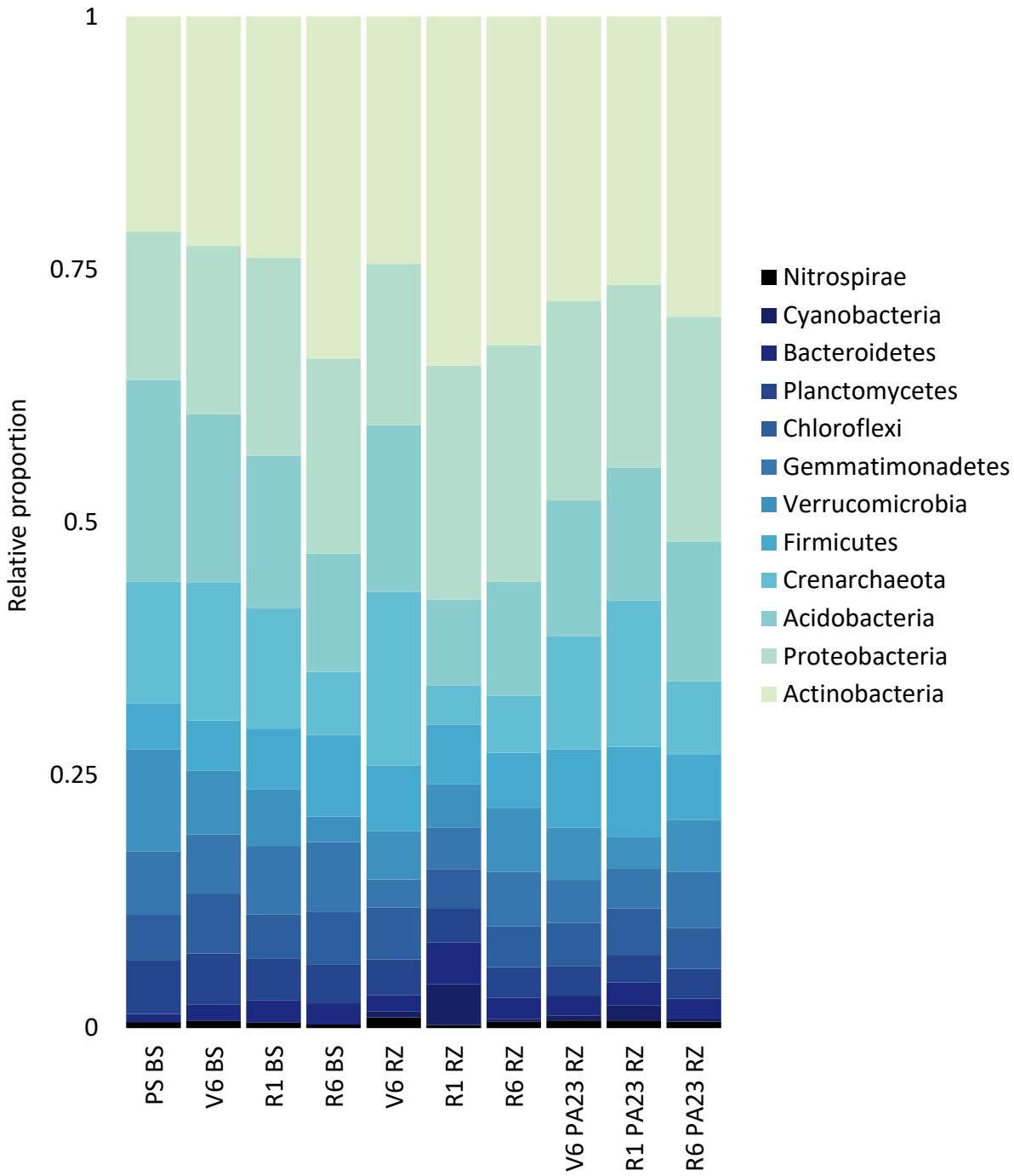

Figure 3.6 Bacterial proportional taxa bar plots showing shifts in the major bacterial phyla for canola-soybean samples in response to *P. chlororaphis* PA23 treatment and over developmental time. Bar plots were generated from 140,000 amplicon sequencing variants and baseline filter was set to exclude taxa with less than 2500 hits. Each bar represents an average of three

biological replicates. PS = Pre seeding, V6 = sixth trifoliate, R1 = Beginning of flowering, R6 = Full seed, BS = Bulk soil, RZ = Rhizosphere.

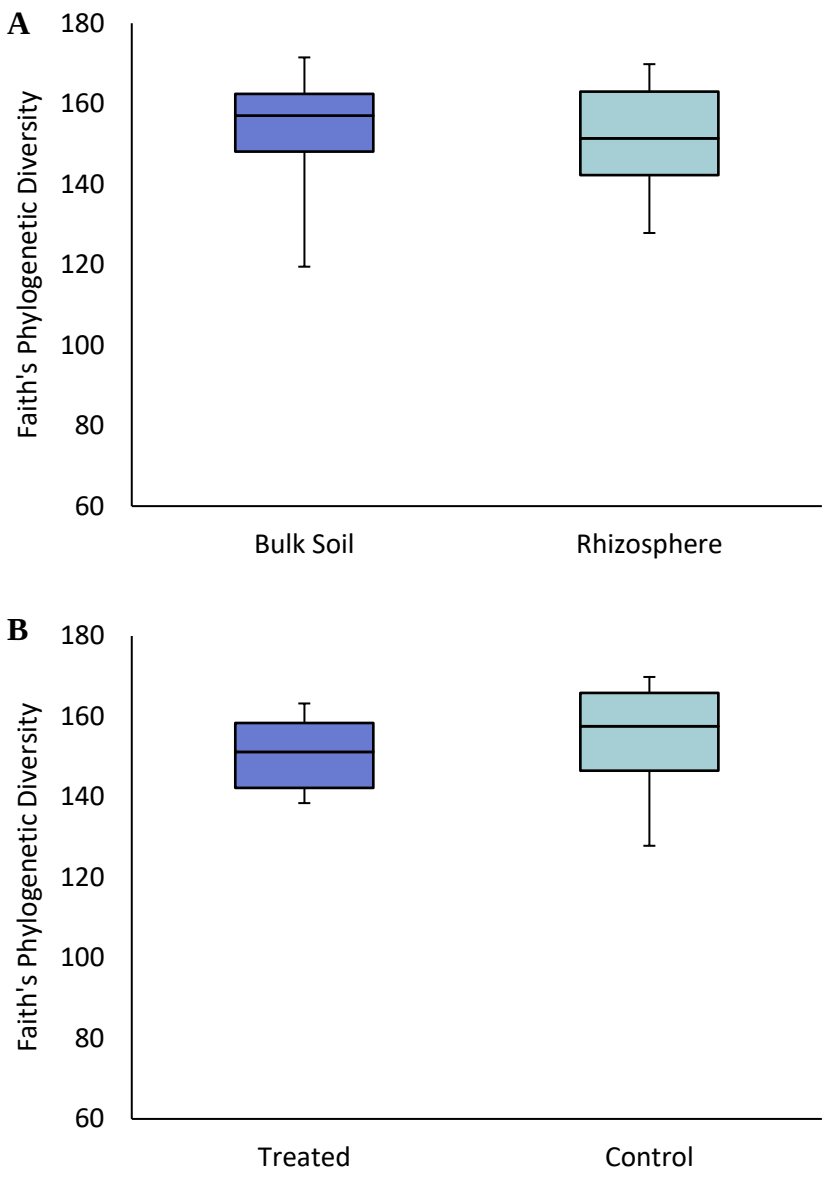

Figure 3.7 Quantitative comparisons of fungal species richness represented by the alpha diversity metric – Faith's Phylogenetic Diversity – in canola-soybean samples. A) Comparison of cumulative bulk soil and rhizosphere samples. B) Comparison of cumulative *P. chlororaphis* PA23 (treated) and control rhizosphere samples. No statistical significance was identified in either comparison with a Kruskal-Wallis One-Way Analysis of Variance.

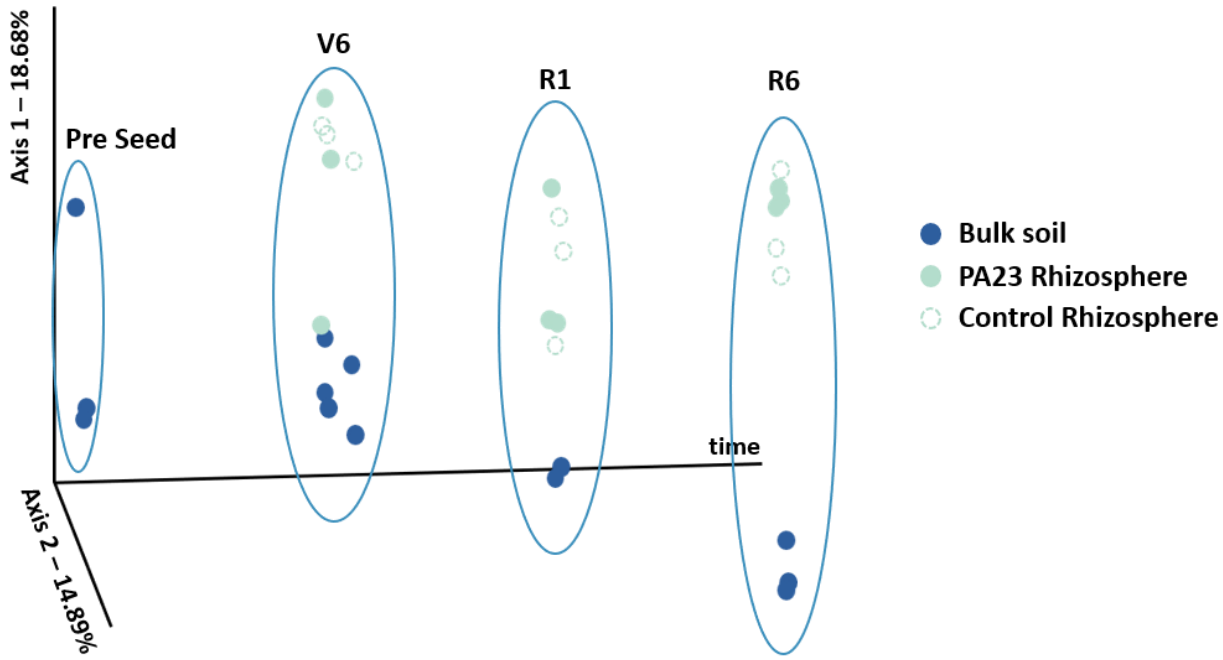

Figure 3.8 Principal coordinate analysis (PCoA) comparing the fungal community composition, represented by the beta diversity metric –Bray Curtis Dissimilarity, of canola-soybean rotation samples over developmental time. Solid blue line shows clustering of samples along the horizontal time (developmental time) axis, followed by clustering based off sample type (rhizosphere versus bulk soil: filed circles), and loose clustering based off *P. chlororaphis* PA23 treatment (unfilled circles).

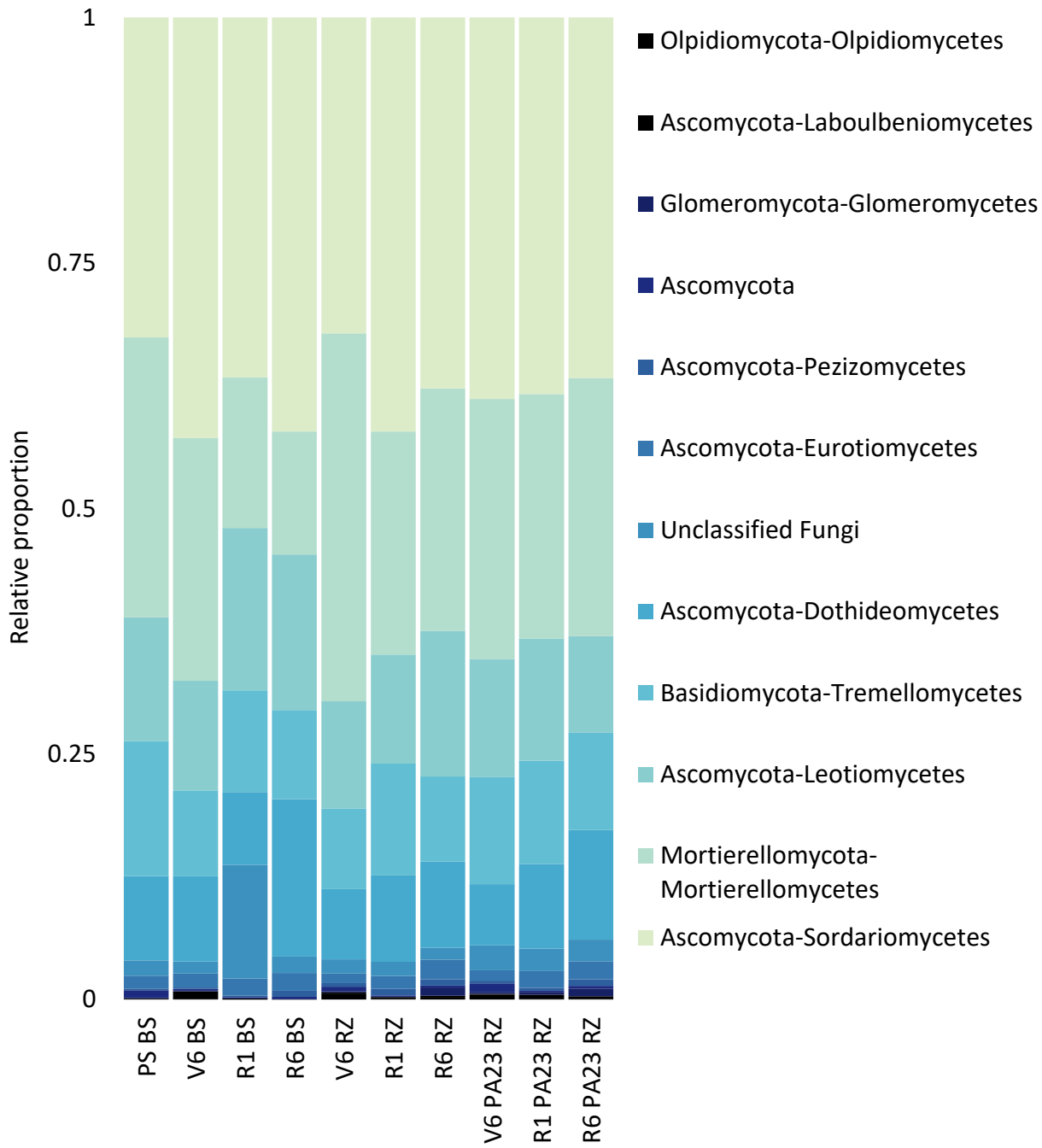

Figure 3.9 Fungal proportional taxa bar plots showing shifts in the major bacterial phyla for canola-soybean samples in response to *P. chlororaphis* PA23 treatment and over developmental time. Bar plots were generated from 140,000 amplicon sequencing variants and baseline filter was set to exclude taxa with less than 2500 hits. Each bar represents an average of three

biological replicates. PS = Pre seeding, V6 = sixth trifoliate, R1 = Beginning of flowering, R6 = Full seed, BS = Bulk soil, RZ = Rhizosphere.

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CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

Next-generation RNA sequencing and computational analyses have allowed for the extensive examination of PGPB mechanisms. In our study, we used these techniques to investigate the mechanisms that underlay the *P. chlororaphis* PA23 – *B. napus* interaction. The results of this study suggest that *P. chlororaphis* PA23 plant growth promotion is driven by the induction of phytohormone signalling, expression of photosynthetic genes, and development of root hairs. Further, we identified one of the primary metabolites, auxin, that likely contribute to plant growth promoting activity. However, more work is required to fully understand the genetic mechanisms that drive plant growth promotion and to identify other potential plant growth-promoting metabolites. For example, bioinformatic analyses of transcription factor networks and interacting gene partners can lead to exciting opportunities in the manipulation of the *P. chlororaphis* PA23 – plant interaction. Also, a complete screening of the metabolites produced by *P. chlororaphis* PA23 with Gas Chromatography – Mass Spectrophotometry may lead to the discovery of novel plant growth-promoting molecules including antibiotics for agricultural applications.

The field study performed in this thesis provides the first report of *P. chlororaphis* PA23 plant growth promotion under field settings. The addition of *P. chlororaphis* PA23 to *G. max* resulted in increased pod set but the underlying genetic mechanisms that drive this phenotype remain unknown. Future studies in the transcriptomic response of *G. max* to *P. chlororaphis* PA23 are needed to identify changes in gene expression that lead to increased pod set and other beneficial agronomic measures. Marker-based sequencing showed that the addition of an antibiotic-producing PGPB does not affect the bacterial and fungal populations in the soil. The results of this study show that the species diversity and community composition remain

unchanged in response to *P. chlororaphis* PA23; however, the data suggests community shifts over developmental time that are likely linked to changes in root exudates and root morphology. The study has its limitations, samples for microbiome analyses were collected within a growing season. Previous studies have shown that long-term utilization of biological inoculums may have lasting effects on microbial populations in the soil. Thus, future studies should focus on multi-year rotations that involve *P. chlororaphis* PA23 to provide a more robust understanding of long-term microbial community dynamics.

Currently many PGPB products belong to the Actinobacteria and Proteobacteria (with a focus on Bacillus and Pseudomonas species); with traditional methods for discovery and isolation of novel microbes being tedious and time consuming. The dataset generated in this study lay the framework for the rapid identification of potential beneficial organisms from field samples. Future datamining of this metagenomics database could lead to more rapid and targeted isolation methods of microbes for functional testing under greenhouse conditions. Although the experiments performed in chapter three provide insight into the members of the rhizosphere there remains the question of the function for each bacterial or fungal taxa present in the rhizosphere. An integrated approach that includes a metatranscriptomic study would allow for the discovery of active gene fragments found within the soil. For example, the data generated from metatranscriptomics may shed light on the degree of nutrient mobilization, nutrient fixation, or phytohormone production present in the soil. These gene fragments can then be aligned with metagenomics data to identify the most metabolically active members of the rhizosphere that contribute to plant growth promotion. Moreover, the combination of metagenomics and metatranscriptomics would allow for a more holistic understanding of interactions between a novel PGPB, such as *P. chlororaphis* PA23, with other microbial species found in the soil and

rhizosphere. The identification of active gene fragments through metatranscriptomics and metagenomics data mining may also lead to the discovery of beneficial gene inserts for the development of transgenic PGPB products. . For example, metatranscriptomics would facilitate the discovery of transcript fragments that could be altered or inserted into a more potent PGPB, generating a more robust product for a multitude of environmental conditions and field conditions. Together, future work in metagenomics data mining and metatranscriptomics could serve to be a critical tool in the safe development of agricultural adjuncts for global food production and safety.

In conclusion, the data generated in this study provide the initial information needed to understand the *P. chlororaphis* PA23 – plant interaction. Future work should focus on multi-plant species transcriptomic analyses to fully determine mode of action and to potentially identify key regulators in the *P. chlororaphis* PA23 – plant interaction. The metagenomics database presented here lays the foundation for the rapid identification of non-traditional plant growth promoting microbes that do not belong to the Actinobacteria or Proteobacteria. Future work implementing a metatranscriptomic study will increase our knowledge of the effects of *P. chlororaphis* PA23 on soil health and function with a focus on its effects on other microbial partners within the bulk soil and rhizosphere. The dataset generated from a metatranscriptomic study may also lead to exciting opportunities for the development of transgenic PGPBs that exhibit multiple modes of action or exhibit increased tolerance to environmental stress. Lastly, the combination of metagenomics and metatranscriptomics is necessary for a complete understanding of the potential effects of a novel PGPB, such as *P. chlororaphis* PA23, on soil microbes and ecological health which will lead to the development of safer and more effective PGPB products for agricultural purposes and crop production