# *Pseudomonas chlororaphis* PA23 Biocontrol of *Sclerotinia sclerotiorum* on Canola: Understanding Populations and Enhancing Inoculation

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A Thesis

Submitted to the Faculty of Graduate Studies

In Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

Department of Plant Science

University of Manitoba

Winnipeg, Manitoba

## GENERAL ABSTRACT

**Reimer, Lori M. M.Sc., The University of Manitoba, June 2016. *Pseudomonas chlroraphis* PA23 Biocontrol of *Sclerotinia sclerotiorum* on Canola: Understanding Populations and Enhancing Inoculation. Supervisor, Dr. Dilantha Fernando.**

*Pseudomonas chlororaphis* strain PA23 has demonstrated biocontrol of *Sclerotinia sclerotiorum* (Lib.) de Bary, a fungal pathogen of canola (*Brassica napus* L.). This biocontrol is mediated through the production of secondary metabolites, of which the antibiotics pyrrolnitrin and phenazine are major contributers. The objectives of this research were two-fold: to optimize PA23 phyllosphere biocontrol and to investigate PA23's influence in the rhizosphere. PA23 demonstrated longevity, both in terms of *S. sclerotiorum* biocontrol and by having viable cells after 7 days, when inoculated on *B. napus* under greenhouse conditions. Carbon source differentially effected growth rate and antifungal metabolite production of PA23 in culture. PA23 grew fasted in glucose and glycerol, while mannose lead to the greatest inhibition of *S. sclerotiorum* mycelia and fructose lead to the highest levels of antibiotic production relative to cell density. Carbon source did not have a significant effect on *in vivo* biocontrol. PA23 demonstrated biocontrol ability of the fungal root pathogens *Rhizoctonia solani* J.G. Kühn and *Pythium ultimum* Trow in radial diffusion assays. PA23's ability to promote seedling root growth was demonstrated in sterile growth pouches, but in a soil system these results were reversed. Initial studies into the mechanisms of plant growth promotion included an assay for the enzyme ACC deaminase and production of the auxin IAA, for which PA23 tested negative and positive, respectively. This research is essential for developing PA23 into an effective biocontrol agent in the phyllosphere and it opens the door for use of PA23 as a rhizosphere seed treatment.

## ACKNOWLEDGEMENTS

First and foremost, I would like to thank my thesis supervisor Dr. Dilantha Fernando for giving me the opportunity to work in his lab and help me grow as a young scientist. I value the independence you allowed me to have and your encouragement to pursue topics that captured my interest. A big thank you goes to Dr. Teri de Kievit, a committee member who went above and beyond. Thank you for all of your guidance and support. I would also like to thank Drs. Mark Belmonte and Rob Duncan for their critical assessment of my project and helping me to think of things from a different point of view.

I've been lucky to be able to work in many different labs and learn from a diverse group of people. Thank you to everyone in the Fernando, de Kievit, and Belmonte labs. A big thanks to Paula, for your advice and expertise, and to Munmun, Kelly, and Nidhi, who adopted me as one of their own. I will look back on this period of study with fondness.

To my parents, thank you for encouraging me to pursue my goals, for lifting my spirits and reassuring me. To Spence, who kept me grounded, accountable, and excited- thanks for supporting 'my other boyfriend'. Finally, to my other family and friends- I am appreciative of your constant support and encouragement.

## FORWARD

This thesis was written manuscript style following the Applied and Environmental Microbiology style. There are two manuscripts presented, each with an abstract, introduction, methods and materials, results, discussion and conclusion. In addition, the thesis contains a general abstract and a literature review prior to the manuscripts and literature cited following the manuscripts.

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## ABBRIVIATIONS

AAFC- Agriculture and Agri-Food Canada
ABA- abscisic acid
ACC- 1-aminocyclopropane-1-carboxylate
AF- antifungal
AHL- acylated homoserine lactone
a.i.- active ingredient
ANOVA- analysis of variance
BCA- biological control agent
BS6- *Bacillus amyloliquefaciens* strain BS6
CFU- colony forming units
CHA0- *Pseudomonas florescens* strain CHA0
CK- cytokinin
$CO_2$- carbon dioxide
CRD- completely randomized esign
CV- coefficient variance
2,4-DAPG- 2,4-diacetylphloroglucinol
DF- degrees of freedom
DF41- *Pseudomonas brassicacearum* strain DF41
$dH_2O$- deionized water
EDP- Entner-Doudoroff pathway
$Fe^{3+}$- ferric ion
GA- gibberellins
GDP- gross domestic product
Gm- gentamicin
HCN- hydrogen cyanide
HPLC- high performance liquid chromatography
IAA- indole-3-acetic acid
IAM- indole-3-acetamide
IAN- indole-3-acetonitrile
IPyA- indole-3-pyruvic acid
ISR- induced systemic resistance
LB- Luria Bertani
M9- M9 minimal salts media
MAFRD- Manitoba Agriculture, Food and Rural Development
mL- millilitre
$N_2$- atmospheric nitrogen
NADH- nicotinamide adenine dinucleotide (reduced form)
NE- nutritional enhancement
$NH_4^+$- ammonium
OD- optical density
2-OH-PHZ- 2-hydroxyphenazine
PA23- *Pseudomonas chlororaphis* stain PA23
PCA- phenazine-1-carboxylic acid
PCR- polymerase chain reaction

PDA- potato dextrose agar
PGPB- plant growth-promoting bacteria
PGPR- plant growth-promoting rhizobacteria
PHL- 2,4-diacetylphloroglucinol
PHZ- phenazine
PLT- pylouteorin
PMRA- Pest Management Regulatory Agency
ppGpp- guanosine 5'-diphosphate 3'-diphosphate; guanosine tetraphosphate
pppGpp- guanosine pentaphosphate
(p)ppGpp- referring to ppGpp and/or pppGpp
PRN- pyrrolnitrin
PsrA- Pseudomonas sigma regulator A
PTS- phosphotransferase system
QS- quorum sensing
qRT-PCR- semi-quantitative reverse transcription polymerase chain reaction
QTL- quantitative trait loci
RDA- radial diffusion assay
Rif- rifampicin
RNA- ribonucleic acid
r.p.m.- revolutions per minute
SE- standard error
SR- stringent response
TAM- tryptamine
Tc- tetracycline
TSO- tryptophan side chain oxidase
UV- ultraviolet

# CHAPTER 1

LITERATURE REVIEW

## 1.1. PLANT DISEASE IN CANOLA

### 1.1.1. Canola

Canola was developed in western Canada in the 1970s. Traditional plant breeding techniques were used to obtain a high-quality edible oilseed that thrived in the Canadian prairies. Canola is differentiated from rapeseed, from which it was derived, by its oil having less than 2 % erucic acid and less than thirty micromoles per gram of glucosinolates (Phillips and Khachatourians, 2001; Rempel *et al*., 2014). It has quickly grown to be a staple in Canadian agriculture, with production exceeding 15 million tonnes in 2014 (Canola Council of Canada, 2014).

### 1.1.2. Plant disease in Canadian agriculture

Agriculture is an important part of the Canadian economy. Based on the 2012 consensus, the agriculture and agri-food sector provided jobs to 2.1 million Canadians, generated 103.5 billion, and accounted for 6.7 % of Canada's gross domestic product (GDP) [Agriculture and Agri-Food Canada (AAFC), 2015]. Because of agriculture's high value, it is important to minimize agricultural losses from pests; this includes weeds, insects, and plant diseases. When there are tighter crop rotations to maximize short-term profits, there is increased potential for plant pathogens to become more virulent, spread quickly, and cause increased economic losses. Currently, many strategies are implemented to control plant diseases in Canada. Major methods include petroleum-derived pesticides, crop rotation, and breeding crops for specific pathogen-resistance.

### 1.1.3. Canola diseases in western Canada

Plant resistance to pests is a key part of canola breeding programs (Phillips and Khachatourians, 2001). Microbial-mediated plant diseases are one of the greatest causes of reduced canola yield. Several diseases are of particular importance in Western Canada canola production, including sclerotinia, blackleg, and clubroot.

Blackleg is an oilseed rape disease that causes seedling death, lodging and early senescence of canola. Caused by *Leptosphaeria maculans* (Desm.) Ces (anamorph *Phoma lingam* (Tode: Fr./Desm.)), blackleg is responsible for yield losses in Australia, Canada, and Europe (West *et al*., 2001). Because canola is being grown more intensively in western Canada, in rotations less than the optimal one every four years, it results in an increase in both incidence and severity of blackleg (Kutcher *et al*., 2013). Breeding for resistance, stubble management, crop rotation and fungicide seed treatments are important techniques used to manage blackleg in western Canada (West *et al*., 2001).

Clubroot is caused by the obligate parasite *Plasmodiophora brassicae* Woronin. It is an important disease of plants in the Brassicaceae family; it is known to cause yield reductions worldwide (Dixon, 2009). In Canada, it was first detected on canola near Edmonton, Alberta in 2003 (Tewari *et al*. 2005). Since this initial report the disease has spread to southern Alberta, Saskatchewan, and Manitoba (Cao *et al*., 2009; Dokken-Bouchard *et al*., 2010; Hwang *et al*., 2009; Hwang *et al*., 2014; Strelkov *et al*., 2011). Clubroot has been shown to cause significant yield loss, up to 90%, and diminished seed quality (Hwang *et al*., 2010; Hwang *et al*., 2012; Strelkov *et al*., 2007). Management practices appropriate for canola production systems include genetic resistance to *P. brassicae*, crop rotation and appropriate cultural practices, and

minimization of the spread of the pathogen between canola fields by sanitation of field equipment and seed (Hwang *et al*., 2014).

*Sclerotinia sclerotiorum* (Lib.) de Bary is an economically important soil-borne fungus that causes stem rot of canola. This pathogen is known to cause infection in more than 400 plant species world-wide (Purdy, 1979). Infection of canola by *S. sclerotiorum* will be discussed in more detail in the follow section.

There are additional diseases that cause yield loss and disease in canola, though to less economically significant levels. These include damping off or seedling disease complex caused by fungi, including *Rhizoctonia solani* J.G. Kühn, *Fusarium* spp., and *Pythium* spp. (Canola Council of Canada, 2015). Downy mildew [*Hyaloperonospora parasitica* (Pers.) Constant.], white leaf spot [*Pseudocercosporella capsellae* (Ellis & Everh.) Deighton)], white rust [*Albugo candida* (Pers.) Kuntze], and recently verticillium stripe [*Verticillum longisporum* (C.Stark) Karapapa, Bainbr. & Heale] can all contribute to yield losses (Canola Council of Canada, 2015).

### 1.1.4. *Sclerotinia sclerotiorum*

*Sclerotinia sclerotiorum* (Lib.) de Bary is a necrotrophic fungal pathogen that causes disease on more than 400 plant species (Bolton *et al*., 2006). In western Canada, this includes many important agricultural crops, such as canola, sunflowers, soybeans, field peas, and potatoes [Manitoba Agriculture, Food and Rural Development (MAFRD), 2015]. In canola, yield losses of 5-100% have been attributed to sclerotinia infection (MAFRD, 2015).

Sclerotia bodies are the over-wintering inoculum of sclerotinia stem rot. Remaining viable for up to 8 years in soil, these hyphal aggregates ensure long-term survival of the pathogen (Adams and Ayers, 1979; Bolton *et al*., 2006). In summer, the sclerotia germinate to

produce apothecia, mushroom-shaped fruiting bodies, which release wind-borne ascospores (Yang, Srivastava, *et al.*, 2007). In order to have a food source and infect canola plants, the spores must initially land on flowers, fallen petals or pollen (MAFRD, 2015). Sclerotinia infection is favored by moist soil conditions, moderate temperatures ($15^oC – 25^oC$), and high humidity (Bolton *et al*., 2006; MAFRD, 2015).

*Sclerotinia sclerotiorum* has a large host range; as such, symptoms often vary on the host plant infected (Bolton *et al*., 2006). On canola, common symptoms include pale-grey or white lesions, premature ripening, and sclerotia formation within stems. Severe infection can lead to lodging of the canola crop and shattering of pods during swathing (McQuilken, *et al.,* 1995).

Sclerotinia stem rot is managed primarily by rotation with non-host crops and foliar fungicides. Because of the persistence of sclerotia bodies and the popularity of sclerotinia host crops in western Canada, rotations are often only partially effective (Bradley *et al*., 2006). Fungicides can be economically valuable if disease pressure and yield potential are both high (Bradley *et al*., 2006). Within western Canada, fungicides are generally applied at the full bloom stage to prevent infection of the senscing petals (Sharma, *et al*., 2015). Common synthetic fungicides include Astound, Lance, Proline, Quadris, Quash, Rovral Flo, and Vertisan (Canola Countil of Canada, 2016). As a complement or alternative to chemical fungicides, bio-fungicides are also used (Canola Council of Canada, 2016). Most notably is Contans WG, active ingredient *Coniothyrium minitans* Campbell, which is used as a soil treatment (Jing *et al.,* 2015). *C. minitans* acts as a mycoparasite, parasitizing the sclerotia bodies in the soil (Jing *et al.,* 2015).

Developing canola cultivars with resistance to Sclerotinia is difficult, with no commercial canola cultivars currently having complete resistance to *S. sclerotiorum* (Bradley *et al.*, 2006; Sharma *et al*., 2015). Although biochemical and genetic bases of resistance to *S. sclerotiorum* are

currently not well understood (Zhao *et al*., 2009), oxalic acid is thought to have an important role in facilitating pathogen invasion (Yang, Srivastava, *et al.*, 2007). Resistance to sclerotinia appears to be governed by multiple genes as found using quantitative trait loci (QTLs) associated with partial resistance, adding challenge to the breeding process (Zhao *et al.*, 2009).

## 1.2. PLANT GROWTH PROMOTING BACTERIA

Plants and microorganisms are members of complex communities characterized by frequent interactions with one another (Bloemberg and Lugtenberg, 2001; Glick, 1995). Plant-associated bacteria can be classified into three groups: beneficial, deleterious, and neutral (Dobbelaere *et al*., 2003). Deleterious or phytopathogenic bacteria induce deleterious symptoms on a plant while neutral bacteria have no detectable effect on plant growth and development (Glick, 1995). Beneficial, or mutualistic, bacteria are often known as plant growth-promoting bacteria (PGPB). Research on PGPB has been increasing since the term was first used by Kloepper and colleagues in the late 1970s (Kloepper and Scroth, 1978; Vessey, 2003).

Bacteria are attracted to plants, especially the roots, because of the exudation and accumulation of plant photosynthates, a rich source of energy and nutrients in an often limiting environment (Glick, *et al.*, 2007; Gray and Smith, 2005). These bacteria can provide nutrients or inhibit pathogenic bacteria, enhancing plant growth by either direct or indirect methods (Glick *et al*., 2007).

Bacteria that benefit plants are diverse, though *Bacillus* and *Pseudomonas* spp. are predominant (Podile and Kishore, 2006). These bacteria can either form a symbiotic relationship with the plant, for example rhizobia, or be free-living in the soil, though still found near, on, or even within the roots of the plant (Glick, 1995). PGPR (plant growth-promoting rhizobacteria)

and their interactions with plants can be exploited commercially and hold great promise for sustainable agriculture (Podile and Kishore, 2006; Lucy, Reed and Glick, 2004).

### 1.2.1. Direct plant growth promotion

Direct promotion of plant growth is facilitated by two main mechanisms, either through providing the plant with a compound that is synthesized by the bacterium or by facilitating the uptake of certain nutrients from the environment (Glick, 1995). These beneficial bacteria are often referred to as 'biofertilizers' (Vessey, 2003). A PGPR does not solely depend upon one mechanism for promoting plant growth, rather one or more mechanisms may be employed, particularly under varying environmental conditions (Glick, 1995).

A well-known mechanism of PGPR is through fixation of atmospheric nitrogen ($N_2$) that is supplied to plant cells in the form of ammonium ($NH_4^+$) (Richardson *et al*., 2009). Rhizobia are the most studied and longest exploited PGPR for their ability to fix $N_2$ in their legume hosts (Vessey, 2003). In symbiotic relationships, $N_2$ fixation is done in exchange for fixed carbon, a photosynthate (Glick, 1995). Free-living bacteria can also fix nitrogen to a biologically relevant form, but often this is only a portion of the bacteria's plant growth promoting ability (Chanway and Holl, 1991; Vessey, 2003).

Iron in the soil is largely unavailable for direct assimilation by living organisms. The ferric ion, $Fe^{3+}$, is the predominant form of iron in nature and it is sparingly soluble (Neilands *et al*., 1987). To combat this issue, microorganisms secrete iron-binding molecules (siderophores) that bind $Fe^{3+}$ and make it available for microbial growth (Neilands and Leong, 1986). While siderophores contribute to bacterial nutrition, in most instances their effect on plant growth is probably small because most plants can grow at much lower iron concentrations than

microorganisms (Glick, 1995; O'Sullivan and O'Gara, 1992). The beneficial effect of siderophores is likely related to biocontrol activities.

Phosphorus is an essential macronutrient for biological growth that can be a limiting factor in plant nutrition (Goldstein, 1986; Rodríguez and Fraga, 1999). The solubilization of phosphate by bacteria can promote plant growth. Biofertilizing strains from genera *Pseudomonas, Bacillus* and *Rhizobium* are among the most powerful phosphate solubilizers (Rodríguez and Fraga, 1999). Solubilization of both mineral phosphate and organic phosphate can be mediated by various bacterial species. Previously, confusion about the role of phosphate solubilization in plant growth promotion was primarily due to the ability of these strains to have additional mechanisms of growth promotion (Glick, 1995; Rodríguez and Fraga, 1999).

Bacteria are capable of modulating plant growth through the production or degradation of plant hormones that regulate growth and development (Dodd *et al*., 2010; Glick, 1995). These mechanisms don't directly enhance water or nutrient activity in the plant, but they can alter root elongation and architecture, thus increasing the capture of plant resources already available in the soil (Dodd *et al*., 2010). Many hormones have been implicated as playing a role in promoting plant growth. Cytokinins (CKs) are found to inhibit root growth, possibly due to stimulation of ethylene production (Cary *et al.*, 1995). Plant CK status is thought to be controlled by endophytic bacteria that produce adenine and adenine derivatives, which cause an increase in total CK concentrations (Dodd *et al*., 2010). The detrimental effect of CKs is generally found to be balanced by PGPR, which are able to decrease root ethylene production (Dodd *et al*., 2010). Abscisic acid (ABA) helps control the elongation of roots growing with low water potential (Dodd *et al*., 2010). Several rhizobacteria produce ABA in culture media or mediate plant ABA production and are found to increase both the plant tissue ABA levels and overall root mass

(Dodd *et al*., 2010). Gibberellins (GAs) are known to promote lateral root number and length. A number of PGPR produce physiologically active GAs or can hydroxlyze inactive 3-deoxy GAs present in roots to active forms, leading to improved seedling vigor and enhanced root and shoot elongation (Bottini *et al*., 2004). A more detailed discussion of GAs is found in a review by Bottini and colleagues (2004). The auxin indole-3-acetic acid (IAA) and the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminse, which modulates ethylene levels, have been extensively studied for their impact on plant growth and are discussed in more detail below.

#### 1.2.1.1. IAA

Auxin signaling is involved in virtually all aspects of plant growth and development (Taiz and Zeiger, 2010). Indole-3-acetic acid (IAA) is the principle auxin found in plants, being the most abundant and physiologically important (Spaepen *et al.*, 2007). Low levels of auxins promote root growth, while high concentrations can inhibit primary root growth (Lavenus *et al.*, 2013). In contrast, initiation of lateral and adventitious roots is stimulated by higher auxin concentrations (Taiz and Zeiger, 2010; Vacheron *et al.*, 2013). Other roles of auxin include stem elongation, apical dominance, root initiation, and fruit development (Taiz and Zeiger, 2010).

IAA production is common among plant rhizosphere-associated bacteria; production has been found in many genera, including *Azospirillum, Pseudomonas, Xanthomonas, Rhizobium, Alcaligenes, Enterobacter, Acetobacter,* and *Bradyrhizobium* species (Patten and Glick, 1996). Several different pathways can be used for IAA biosynthesis. The majority of pathways start with the amino acid tryptophan and use one to two intermediates, with corresponding enzymes, to create IAA (Patten and Glick, 1996; Spaepen *et al*., 2007). For example, the first pathway discovered in microbes was the indole-3-acetamide (IAM) pathway, in which tryptophan is

converted to indole-3-acetamide by tryptophan-2-monooxygenase, and then indole-3-acetamide is hydrolased to IAA by indoleacetamide hydrolase (Figure 1.1; Van Onckelen *et al.,* 1986, Patten and Glick, 1996). Other pathways include the indole-3-pyruvic acid (IPyA) pathway, the tryptophan side chain oxidase (TSO) pathway, the tryptamine (TAM) pathway, and the indole-3-acetonitrile (IAN) pathway (Patten and Glick, 1996). Most beneficial bacteria are linked to the IPyA pathway (Spaepen *et al.*, 2007). There are a few pathways that utilize other starting materials, namely indole or indole-3-glycerol phosphate, but they are not commonly found in nature (Normanly *et al*., 1993; Idris *et al*., 2007).

IAA has a diverse role in plant-microorganism interactions (Spaepen *et al*., 2007). In the past, bacterial auxins were associated with pathogenesis, namely gall formation (Jamison, 2000; Smidt and Kosuge, 1978). Later, it was found that IAA production extended to bacteria that were phytopathogenic or plant growth-promoting. Bacterial IAA producers can interfere with several aspects of plant physiology including cell enlargement and division, tissue differentiation, and responses to light and gravity (Spaepen *et al*., 2007).

Beneficial bacteria exert their main effect while colonizing the external surface of the plant root (Patten and Glick, 1996). Auxin production has been linked to increased bacterial root colonization and fitness on the host (Spaepen *et al*., 2007). Bacterial IAA production helps the bacteria circumvent the host defense system by derepressing auxin signalling (Manulis *et al*., 1998; Spaepen *et al*., 2007). Evidence of auxins as a signalling molecule in microorganisms has been growing. IAA may have a role in adaptation to nutrient-poor environments and biofilm formation in *Escherichia coli* (Wang *et al.*, 2001; Domka *et al*., 2006). IAA has also been connected to the Gac-Rsm posttransciptional regulatory pathway (Yang, Zhang, *et al*., 2007) and

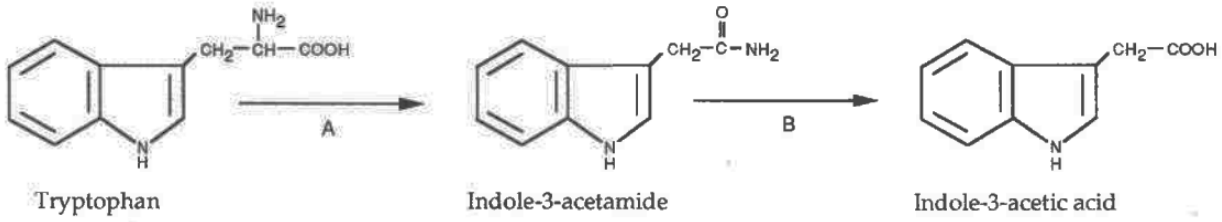



**Figure 1.1** Bacterial formulation of IAA from tryptophan through the intermediate indole-3-acetamide using the enzymes (A) tryptophan 2-monooxygenase and (B) indoleacetamide hydrolase (Patten and Glick, 1996).

the bacterial alarmone guanosine 5'-diphosphate 3'-diphosphate (ppGpp) that mediates stringent control upon stress conditions (reviewed by Braeken *et al*., 2006).

Many factors can influence IAA biosynthesis in bacteria including acidic pH, osmotic and matrix stress, and carbon limitation (Spaepen *et al.,* 2007). These factors can cause early entry into stationary phase; congruently, it was found that IAA production was enhanced by the overproduction of the stationary-phase sigma factor RpoS (Saleh and Glick, 2001; Patten and Glick, 2002a; Spaepen *et al*., 2007). Plant extracts or compounds such as flavonoids can also impact IAA production. In this category, tryptophan often has major effects. As the main precursor for IAA biosynthesis, tryptophan availability can dramatically increase IAA production levels in many bacteria (Spaepen *et al*., 2007).

#### 1.2.1.2. ACC deaminase

Ethylene is a simple organic molecule that plays a major biological role as a plant hormone. It acts as a growth regulator of plant development and is an important part of a plant's response to stress (Deikman, 1997). Ethylene biosynthesis is triggered by environmental and biological stresses and traumas (Morgan and Drew, 1997). Such triggers include: chemicals, temperature extremes, water stress, ultraviolet light, insect damage, disease, and mechanical wounding (Glick, 2005).

Once past germination, high levels of ethylene inhibit root elongation (Jackson, 1991). A number of plant growth-promoting bacteria contain the enzyme ACC deaminase. This enzyme can cleave the precursor of ethylene, ACC, which lowers the level of ethylene in the associated plant (Figure 1.2; Arshad *et al*., 2007; Glick, 1995; Glick *et al*., 1998; Glick, 2005). Keeping the ethylene level in roots lows can enhance the survival of some seedlings and protect stressed

plants from the deleterious effects of ethylene (Glick, 1995). ACC deaminase has been found in a wide range of beneficial microbes. This includes Gram negative bacteria, Gram positive bacteria, rhizobia, and endophytes (Glick, 1995).

### 1.2.2. Indirect plant growth promotion

An indirect method of plant growth promotion is through biocontrol of phytopathogens. More specifically, lessening or preventing the deleterious effects of one or more phytopathogenic organisms (Glick, 1995). This indirect stimulation of plant growth may be mediated through a variety of mechanisms, described in more detail in the following section.

## 1.3. BIOLOGICAL CONTROL

Biocontrol is a method of controlling agricultural pests that relies on biological organisms (Campbell, 1989; Höfte and Altier, 2010). Biofungicides, a category of biocontrol used to control fungal diseases, are important as they offer different modes of action from chemical pesticides, reducing the potential for pathogen resistance when used in rotation. They are also valuable in situations where there is currently no control available, where conventional pesticides cause re-entry or residue concerns, or where the product is certified organic (Fravel, 2005). These considerations, in addition to increased regulation stringency, environmental issues, and safety considerations, contribute to the attractiveness of biological control agents (BCAs) as part of a sustainable pest management strategy (Thakore, 2006).

Bacteria have been artificially introduced to improve plant growth and health for decades in both the plant growth promotion and biocontrol capacities (Weller, 1988; Kim, *et al*., 2011;

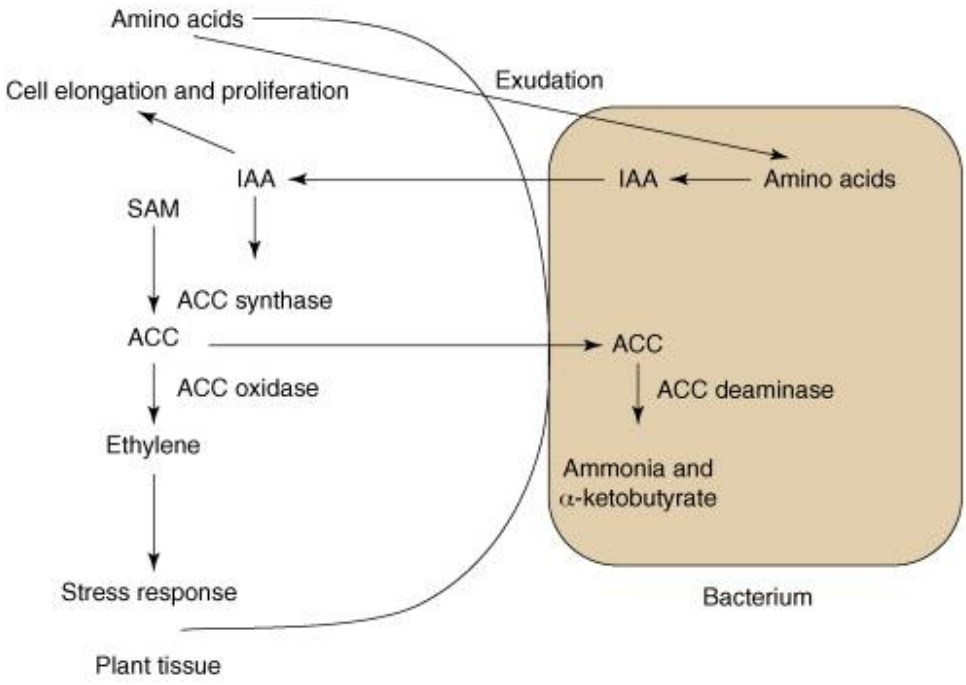



**Figure 1.2** Schematic representation of how bacteria containing ACC deaminase activity lower the ethylene concentration. Abbreviations: IAA, indole acetic acid; SAM, S-adenosyl-methionine (Arshad *et al*., 2007).

Andrews, 1992). Many bacterial genera have been studied and released in this capacity; these include *Acinetobacter, Agrobacterium, Alcaligenes, Arthrobacter, Azospirillum, Azoctobacter, Bacillus, Bradyrhizobium, Frankia, Pantoea, Pseudomonas, Rhizobium, Serratia, Stenotrophomonas, Streptomyces,* and *Thiobacillus* species (Raaijmakers, *et al*., 2002; Weller, 1988; Van Elsas and Heijnen, 1990; Whipps, 2001).

### 1.3.1. Biocontrol in Canada

In Canada, microbial biopesticides have been registered for 35 years but the number of registrations has significantly increased in the 21$^{st}$ century. The first biofungicide, with the active ingredient *Steptomyces griseoviridis* and trade name Mycostop, was registered in Canada in 1999 through the Pest Management Regulatory Agency (PMRA) (Bailey *et al*., 2010). Despite the increasing number of BCAs available to commercial agricultural producers, less than 2% of Canadian growers use biopesticides (Bailey *et al*., 2010; Cuddeford, 2007). This may be due in part to the unpredictable efficacy of BCAs under field conditions. It is therefore essential to investigate molecular mechanisms underlying biocontrol activity in order to optimize survival, stability, and antifungal (AF) action of these microbial biofungicides.

### 1.3.2. Mechanisms of biocontrol

Bacteria are a diverse group of organisms and as such, they have evolved varying mechanisms for survival. In the rhizosphere, a nutrient limiting environment, persistence depends on competing with other organisms for limited resources. Many different modes of action exist for control of the surrounding organisms including competition for iron, colonization sites, and nutrients supplied by plants; parasitism and predation; the production of AF

metabolites; and, the induction of plant resistance mechanisms. Many BCAs can use several modes of action at once to control plant pathogens (Compant *et al*., 2005; Glick, 1995; Haas and Défago, 2005; Raaijmakers *et al*., 2002; Whipps, 2001).

#### 1.3.2.1. Competition

The rhizosphere provides a source of carbon and nutrients in a sparse soil environment. As such, competition for these nutrients and niches represents a mechanism by which bacteria can indirectly protect plants from phytopathogens (Compant *et al*., 2005). The ability of bacteria to take advantage of a specific environment, to adapt to changing conditions, to produce bacterial lipopolysaccharides and vitamin $B_1$, to exude NADH dehydrogenases, and their high rate of growth all contribute to root colonization, which confers a competitive advantage (Compant *et al*., 2005).

Iron is an essential growth element that is of extremely limited availability in the rhizosphere. Microorganisms secrete low molecular mass iron-binding molecules, called siderophores, to bind $Fe^{3+}$ and make it available for microbial growth (Glick, 1995). Bacteria can produce siderophores with a very high affinity for iron, preventing the proliferation of phytopathogens or the germination of fungal spores by the resulting lack of this critical element (O'Sullivan and O'Gara, 1992).

#### 1.3.2.2. Parasitism/predation

Bacteria are known to parasitize and degrade spores of fungal plant pathogens (El-Tarabily *et al*., 1997; El-Tarabily and Sivasithamparam, 2006; Whipps, 2001), making nutrients derived from the pathogen available for bacterial use (Whipps, 2001). If fungal cells are lysed by

cell wall breakdown, cell wall-degrading or lytic enzymes produced by bacteria are held responsible. Such enzymes include cellulases (El-Tarabily *et al*., 1996), glucanases (Valois *et al*., 1996), chitinases (Frankowski *et al*., 2001; Pleban *et al.*, 1997), and proteases (Dunne *et al*., 1997).

#### 1.3.2.3. Antibiosis

Antibiotics are a chemically heterogeneous group of organic, low-molecular weight compounds that inhibit growth or metabolic activities of other microorganisms at low concentrations (Fravel, 1988; Raaijmakers *et al*., 2002). When produced by bacterial BCAs, antibiotics typically have broad-spectrum activity, inhibiting a range of phytopathogens (Raaijmakers et *al*., 2002).

There are numerous reports of the production of AF metabolites by BCAs (reviewed in: Morrissey et al. 2004; Raaijmakers et al., 2002). The main categories of antibiotic compounds include phenazines (PHZ), phloroglucinols, pyoluteorin (PLT), pyrrolnitrin (PRN), cyclic lipopeptides (all of which are diffusible) and hydrogen cyanide (HCN; which is volatile) (Figure 1.3; Raaijmakers *et al*., 2002). These antibiotics can be purified and chemically identified both *in vitro* and in the rhizosphere, and the regulatory genes controlling expression can be characterized and manipulated to test the effect of over-expression or non-production (Raaijmakers *et al*., 2002). Inactivation of antibiotic production resulting in reduced ability of a BCA to control a pathogen as well as over-production of an antibiotic leading to increased biocontrol ability both provide convincing evidence of antibiosis (Raaijmakers *et al*., 2002).

#### 1.3.2.4. Systemic resistance

Long-lasting and broad-spectrum control of plant pathogens can be achieved by treating the plant or seed with a BCA. Through induced systemic resistance (ISR), the bacteria can induce the plant immune system (Glick, 1995), resulting in enhanced protection without the need to activate specific and costly defenses (Pieterse *et al*., 2014). Induced resistance can lead to the direct activation of defences or to the priming of cells for a swift and strong response following pathogen attack (Walters *et al*., 2013). Several studies demonstrated that signals provided by root-inoculated bacteria cause a reduction in disease symptoms after pathogen challenge on the leaves (Alström, 1991; Pieterse *et al*., 2014; Van Peer *et al*., 1991; Wei *et al*., 1991). The PGPR and pathogen remained spatially separated and thus, the enhanced disease resistance was caused by a plant-mediated immune response: ISR (Alström, 1991; Pieterse *et al*., 2014; Van Peer *et al*., 1991; Wei *et al*., 1991). Since then, hundreds of studies have confirmed this phenomenon, featuring both bacteria and fungi, with a general disease reduction between 20 and 85 % (for reviews see: De Vleesschauwer and Höfte, 2009; Jung *et al*., 2012; Pozo and Azcon-Aguilar, 2007; Shoresh *et al*., 2005; Van Loon and Bakker, 2006; Walters *et al*., 2013).

### 1.3.3. Environmental variability

Because BCAs show differential control depending on their environmental parameters, it makes disease control difficult, unpredictable, and liable to fail. BCAs are initially tested for *in vitro* inhibition of pathogens, but this doesn't necessarily reflect disease suppression in field settings. The organisms may be ecologically unsuited to the environments in which the pathogens impart the greatest impact. In order to be successful, a BCA needs to both survive in a given environment and execute the required mechanism of biocontrol. The ideal conditions for

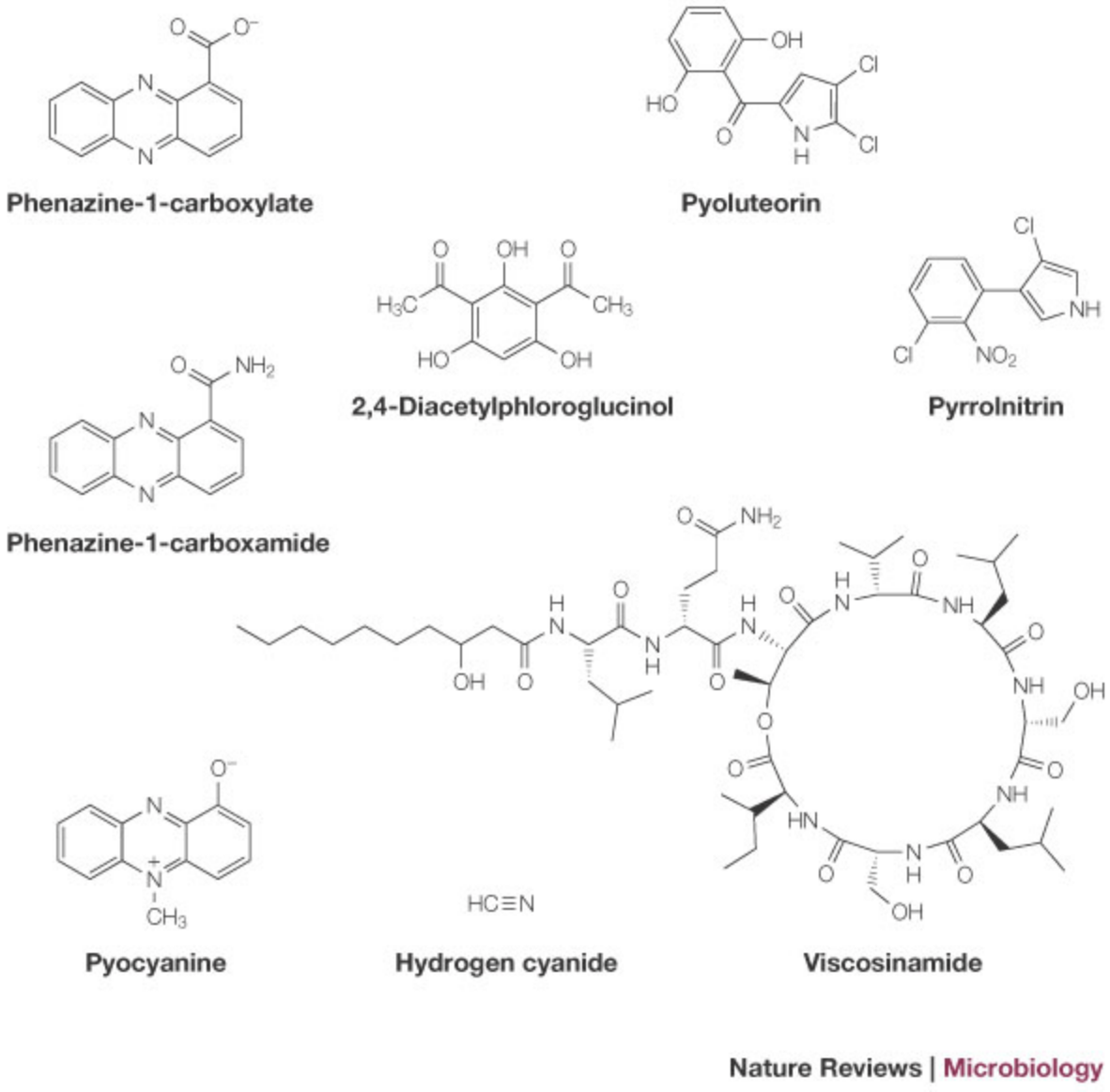



**Figure 1.3** Common antibiotic compounds produced by BCAs that are relevant for biocontrol (Haas and Défago, 2005)

pathogen development and damage to the host plant must therefore correspond with those associated with BCA-mediated pathogen suppression for this to be successful.

Variables in the rhizosphere that have been implicated to play a role in an agent's success include temperature (Landa *et al*., 2004; Mao *et al*., 1997; Weller, 1988), moisture (Dupler and Baker, 1984; Narisawa *et al*., 2005), pH (Park *et al*., 1988; Tjeerd van Rij *et al*., 2004), oxygen levels (Kim *et al*., 1996; Rosenzweig and Stotzky, 1979; Strigul and Kravchenko, 2006), and the soil nutrient status (Duffy and Défago, 1992; Tjeerd van Rij *et al*., 2004) and structure (Bashan and Vazquez, 2000; Meyer *et al*., 2010). This is contrasted to the more inconstant and cyclic phyllosphere, where once again temperature (Guetsky *et al*., 2001; Yuen *et al*., 1994), moisture (Cabrefiga *et al.*, 2011), and nutrient status (Cabrefiga *et al*. 2011; Ji and Wilson, 2002) are involved. Moreover, wind (Lindemann and Upper, 1985), radiation (Larkin and Fravel, 2002; Sundin *et al*., 1996), and the spacial distribution of the bacterium of interest (Collins *et al*., 2003; Pujol *et al.*, 2007) can all affect pathogen suppression. Biotic parameters that may impact the BCA include the host plant (Madloo, *et al*., 2013; Notz *et al.*, 2001), the pathogen (Mazzola and Cook, 1991; McSpadden *et al*., 2001), the native microbial community (Strigul and Karvchenko, 2006), as well as other BCAs (Guetsky *et al*., 2002; Kim *et al*., 2011; Yang *et al*., 2013).

### 1.3.4. Biocontrol formulation

One of the challenges of BCAs is that of application and survival. Ideally, the formulation provides a high efficacy and a long shelf life, is easy to handle and apply, is safe, and has low production costs (de Vrije *et al*., 2001). Formulation is dependent on the type of fermentation used to grow the BCA and is chosen based on the intended use (Spadaro and Gullion, 2005). A liquid formulation is preferred when the inoculant is to be applied through a

soilless culture or irrigation. A granular material is appropriate for potting mix or soil application. A wettable powder is often chosen for root dips or sprays (Spadaro and Gullion, 2005).. Another option is applying the BCA as a seed treatment (Spadaro and Gullino, 2005). The biofungicide recipe is complex and includes many components such as: carriers, diluents, bulking additives, membrane stabilizers, growth and contaminant suppressants, buffering systems, binders, dispersants, lubricants, activators, food sources and coating compounds (Paau, 1998)

## 1.4. *PSEUDOMONAS CHLORORAPHIS* PA23

### 1.4.1. *Pseudomonas* spp.

*Pseudomonas* is a diverse and abundant bacterial genus, which includes 128 species (Weller, 2007; O'Sullivan and O'Gara, 1992; Hofte and Altier, 2010). Plant-associated *Pseudomonas* spp. include both beneficial (PGPR) and pathogenic isolates. Pathogenic, saprophytic, and plant-growth-promoting strains are often found on or within the same plant species (Hofte and Altier, 2010). *Pseudomonas* spp. are known to be effective BCAs against a wide range of phytopathogens (Someya, *et al*., 2013). They are particularly suited to biocontrol because they are abundantly present in natural soils, they can use many exudates as nutrient sources, they possess high growth rates, and they are capable of controlling diseases by a variety of mechanisms (Hofte and Altier, 2010). Secondary metabolites are the primary agents by which fluorescent pseudomonads control phytopathogens (Someya, *et al*., 2013).

### 1.4.2. Origin of PA23

*Pseudomonas chlororaphis* PA23 was first isolated from the root tips of soybeans plants in 1995 (Savchuk and Fernando, 2004). It is a fluorescent pseudomonad that has been shown to protect *B. napus* from disease caused by the fungal pathogen *S. sclerotiorum* in both greenhouse and field studies (Savchuk and Fernando, 2004; Fernando *et al*., 2007). *P. chlororaphis* PA23 has similarly proven to be an antagonistic bacterial strain against *L. maculans,* the causative agent of canola blackleg (Ramarathnam *et al*., 2011).

### 1.4.3. Diffusible antibiotics: pyrrolnitrin and phenazines

Antibiosis is a common mechanism underlying the AF activity of fluorescent pseudomonads. Diffusable antibiotics include: 2,4-diacetylphloroglucinol (2,4-DAPG), PHZ, PRN, and PLT, among others (Dowling and O'Gara, 1994; Haas and Défago, 2005). These antibiotics inhibit fungal pathogens, but can also be active against bacteria and higher organisms (Haas and Défago, 2005). PA23 produces the diffusible compounds PRN and PHZ (Poritsanos *et al.*, 2006; Selin *et al.*, 2010).

PRN is a common AF antibiotic that is derived from tryptophan with a broad-spectrum of activity (Figure 1.4; Kirner *et al.*, 1998). PRN has shown activity against a wide range of Basidiomycetes, Deuteromycetes, and Ascomycetes (Raaijmakers *et al.*, 2002) and has been described as an inhibitor of fungal respiratory electron transport chains (Tripathi and Gottlieb, 1969). At the inhibitory concentration of 10 µg/mL, PRN inhibited endogenous and exogenous respiration in *Saccharomyces cerevisiae*; in mitochondrial preparations, it was found to inhibit succinate oxidase, NADH oxidase, succinate-cytochrome c reductase, NADH-cytochrome c reductase, and succinate-coenzyme $Q_6$ reductase (Tripathi and Gottlieb, 1969). This antibiotic

has been developed for therapeutic purposes against human pathogenic bacteria and fungi while synthetic PRN analogues have been used as agricultural fungicides (Raaijmakers *et al*., 2002; Ligon *et al*., 2000; Haas and Défago, 2005).

Many *Pseduomonas* strains with AF activity produce PHZ, including *P. synxantha* 2-79 (Thomashow and Weller, 1988), *P. chlororaphis* strains 30-84 (Pierson *et al*., 1995) and PCL1391 (Chin-A-Woeng *et al.*, 1998), among others (Cartwright et al, 1995; Tambong and Höfte, 2001; Shanmugaiah *et al.*, 2010). Many BCAs produce more than one PHZ derivative (Figure 1.5; Chin-A-Woeng *et al.*, 2003).

PA23 produces two PHZs, namely phenazine-1-carboxylic acid (PCA) and 2-hydroxyphenazine (2-OH-PHZ). A phenotypic outcome of this PHZ production is an orange coloring (Zhang *et al*., 2006). PHZs exhibit their AF activity through the inhibition of electron transport (Haas and Défago, 2005). It is assumed that they either diffuse across or insert into membranes and act as a reducing agent (Chin-A-Woeng *et al*., 2003). When ferripyochelin, a siderophore, is present, PHZs can catalyse the formation of hydroxyl radicals that cause damage to lipids and other macromolecules (Britigan *et al*., 1992, Haas and Défago, 2005). In addition to AF activity, PHZs play a role in survival and ability to compete with resident microflora, imparting ecological competence to producing strains (Mazzola *et al*., 1992).

In a study by Selin and colleagues (2010), mutant PA23-63 with a *phzE*-Tn insertion that prevented production of PHZ ($PHZ^-$), exhibited increased AF activity compared to the wild type. It was discovered that in PA23, PRN production is increased over twofold in the $PHZ^-$ background. In contrast, a PRN-deficient mutant, PA23-8, had wild-type PHZ levels. Monitoring the transcriptional fusions *prnA-lacZ* and *phzA-lacZ* revealed that PRN and PHZ are neither subject to autoregulation nor cross-regulation. When the PA23 antibiotic mutants, PA23-63

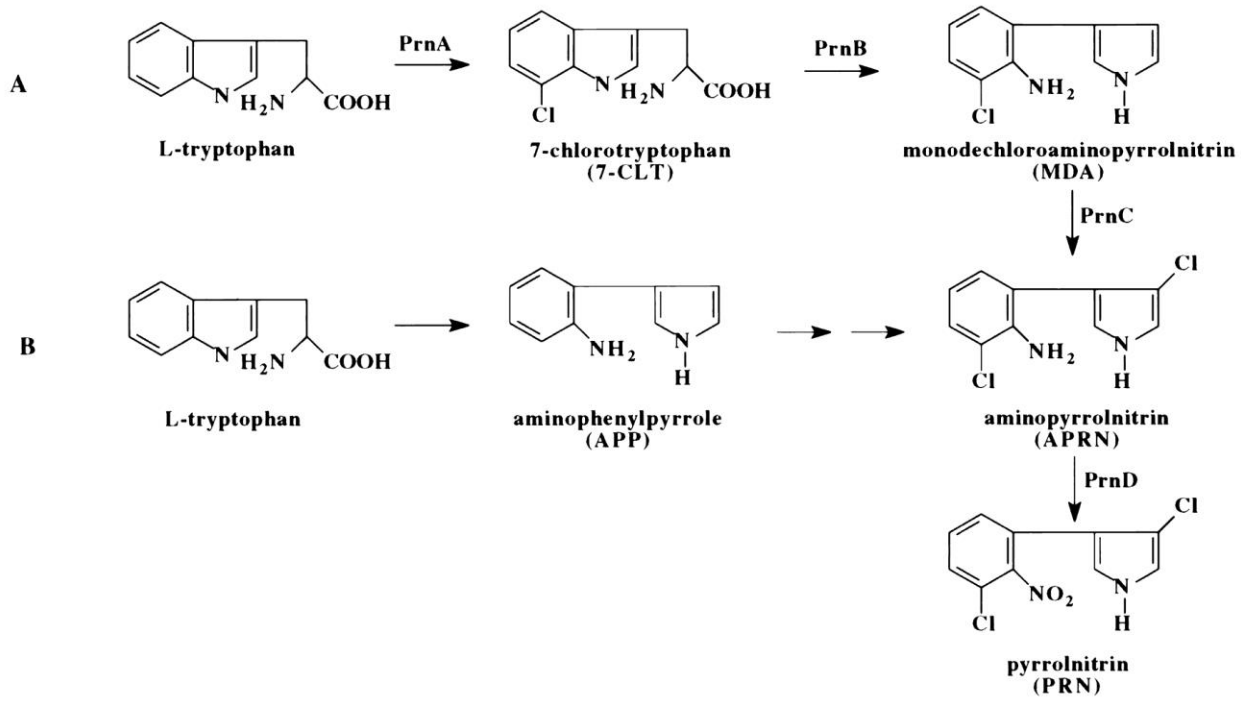



**Figure 1.4** Proposed pyrrolnitrin biosynthesis pathway (Kirner *et al*., 1998)

Reprinted with permission from American Society for Microbiology (Sabine Kirner, Philip E. Hammer, D. Steven Hill et al., 2001. Functions Endcoded by Pyrrolnitrin Biosynthetic Genes from *Pseudomonas fluorescens*. J. Bacteriology. 180(7): 1939-1943.)

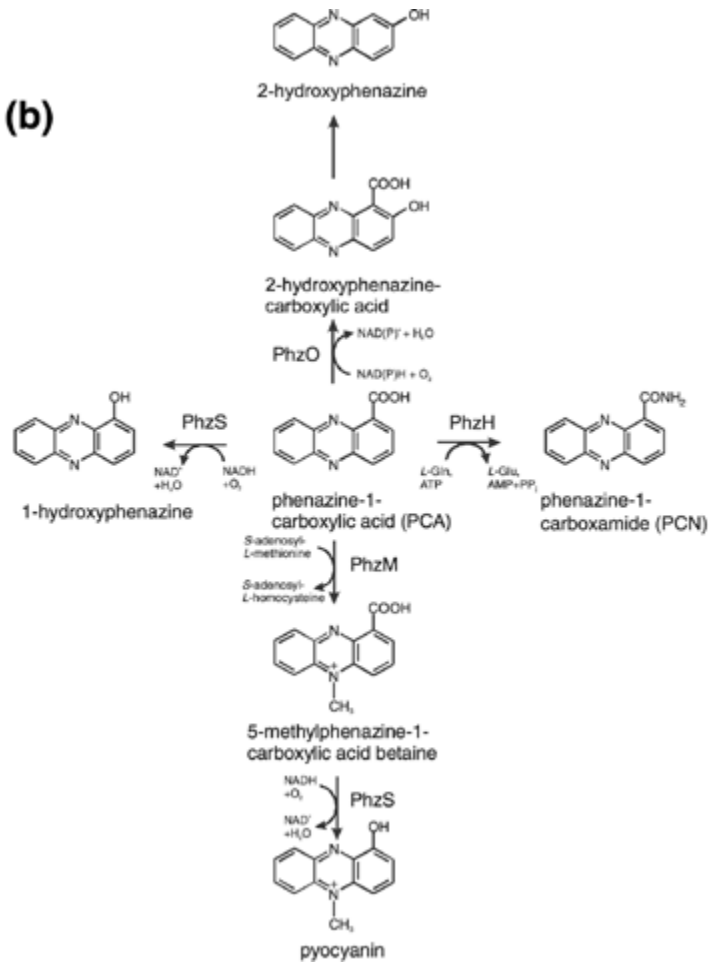



**Figure 1.5** Biosynthesis of derivatives of phenazine-1-carboxylic acid (PCA) (Chin-A-Woeng *et al*., 2003)

(PHZ$^-$) and PA23-8 (PRN$^-$) and the double mutant PA23-63-1 (PHZ$^-$/PRN$^-$), were evaluated for their ability to protect canola from fungal stem rot caused by *S. sclerotiorum*, it was discovered that PRN is the primary AF antibiotic responsible for PA23 biocontrol. PHZ inhibited the fungus to some degree, but played a more minor role in disease suppression (Selin *et al*., 2010).

### 1.4.4. Volatile antibiotics

Hydrogen Cyanide (HCN) is a volatile antibiotic that has been implicated as an important metabolite in biocontrol (Voisard *et al*., 1989; Dowling and O'Gara, 1994; Flaishman *et al*., 1996). The cyanide ion that is derived from HCN is an inhibitor of many metaloenzymes, especially cytochrome *c* oxidases (Blumer and Haas, 2000; Haas and Défago, 2005). The enzyme complex that converts glycine to HCN and $CO_2$, HCN synthase, was found to be membrane-associated (Figure 1.6; Blumer and Haas, 2000). Interestingly, the HCN synthase of *Pseudomonas* spp. is inhibited in whole cells by PRN (Wissing, 1974; Blumer and Haas, 2000). Moreover, production of this compound in *P. aeruginosa* and *P. fluorescens* cultures is induced under oxygen limiting conditions (Blumer and Hass, 2000; Castric, 1983; Laville *et al*., 1998). PA23 has been shown to produce HCN, but its role in fungal suppression has yet to be established (Manuel *et al*., 2012; Poritsanos *et al*., 2006).

PA23 also produces nonanal, benzothiazole, and 2-ethyl-1-hexanol (Figure 1.7; Athukorala *et al*., 2010; Fernando *et al*., 2005). These volatile antibiotics showed 100% inhibition of mycelial and sclerotial germination of *S. sclerotiorum* (Fernando *et al*., 2005). Mutations in regulatory genes and diffusible antibiotics did not appear to affect production of these three metabolites, although quantitative analysis was not performed (Athukorala *et al*., 2010).

### 1.4.5. Siderophores

Siderophores are secondary metabolites involved in iron transport that can efficiently complex with environmental iron (Kloepper *et al*., 1980). Siderophores can be produced with catecholate (carboxylate) iron (III)-binding ligands or with hydroxamate iron(III)-binding groups (Leong, 1986). In addition to their role in iron transport, siderophores can act as growth factors and antibiotics (Leong, 1986).

Siderophore production is known to be an important mechanism for promoting plant growth (Leong, 1986; Schippers *et al*., 1988; El-Tarabily and Sivasithamparam, 2006; Bhattacharyya and Jha, 2012). Siderophore production plays a role in limiting the available trace elements, especially iron, thus suppressing the growth of various phytopathogens by competition (Kloepper *et al*., 1980; Bhattacharyya and Jha, 2012). These molecules may also indirectly stimulate the biosynthesis of other secondary metabolites, including those that are AF or antimicrobial, by increasing the availability of limiting minerals to the bacteria (Duffy and Défago, 1999). Additionally, siderophores may act as signals that induce local and systemic host resistance in associated plants (Leeman *et al*., 1996; Duffy and Défago, 1999). In regards to direct plant growth promotion, siderophores can presumably deliver iron to the plants, as seen by the lack of iron chlorosis in associated plants (Leong, 1986).

When PA23 was tested for the presence of siderophores *in vitro*, it was found to produce the hydroxamate type of siderophores to a higher intensity than the carboxylate ligand type (Mathiyazhagan *et al*., 2004). In a study by Poritsanos and colleagues (2006), a *gacS* mutant exhibiting increased siderophores showed a loss AF activity, leading to the conclusion that enhanced siderophore production alone is not sufficient for *S. sclerotiorum* biocontrol (Poritsanos *et al*., 2006).

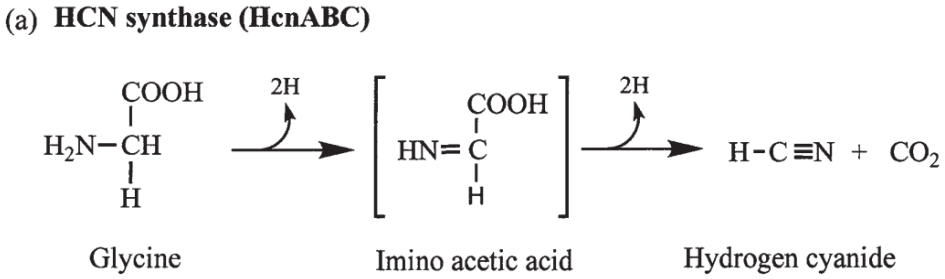



**Figure 1.6** HCN synthesis as proposed by Laville and colleagues in 1998 (Blumer and Haas, 2000)

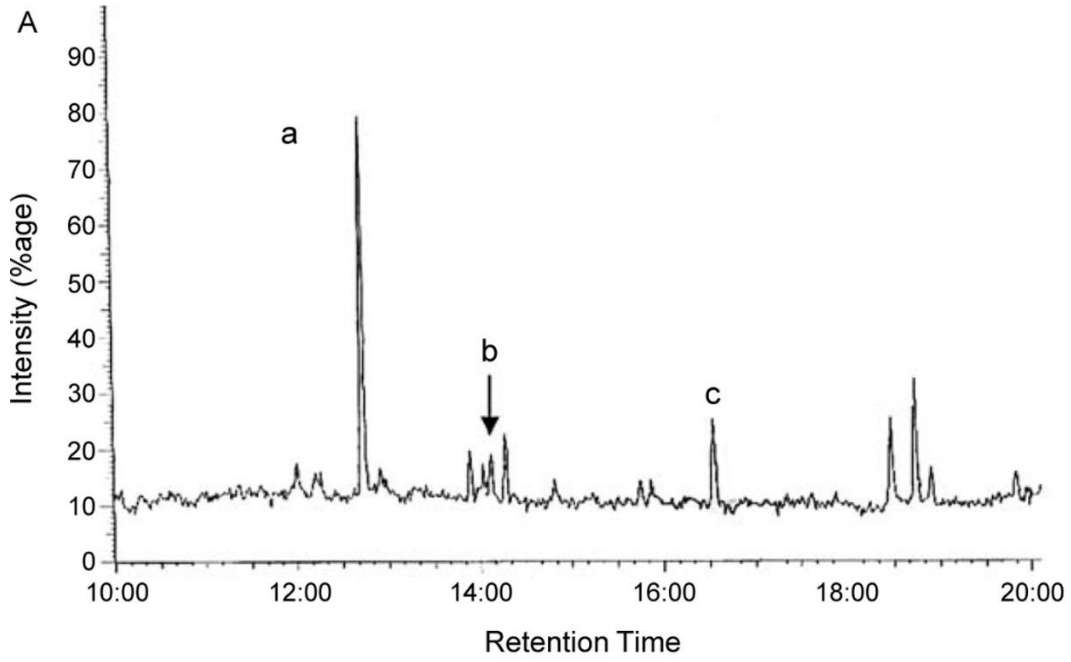



**Figure 1.7** Chromatogram of volatiles collected from PA23 showing (a) 2-ethyl-1-hexanol, (b) nonanal, and (c) benzothiozole. (Athukorala *et al*., 2010)

### 1.4.6. Antifungal enzymes: proteases and lipases

Extracellular enzymes produced by biocontrol bacteria can play a role in control of fungal pathogens through mycoparatism (Spadaro and Gullino, 2005). These enzymes lyse fungal cells and degrade cells walls (Whipps, 2001) *P. chlororaphis* PA23 has been shown to produce lipases and proteases, both of which may contribute to AF activity (Poritsanos *et al*., 2006).

### 1.4.7. Gac-Rsm regulatory control

The GacS-GacA two-component signal transduction system is a common regulatory system in a wide variety of Gram-negative bacteria (Heeb and Haas, 2001; Poritsanos *et al*., 2006). GacS encodes a sensor kinase that senses an environmental signal and activates, through a phosphorelay mechanism, the GacA transcription regulator (Heeb and Haas, 2001). In turn, GacA activates the transcription of small RNA molecules, RsmX/Y/Z, which function to antagonize the post-transcriptional repression proteins RsmA and RsmE (Kay *et al*., 2006; Lapouge *et al*., 2007). RsmA and RsmE are RNA-binding proteins that bind to the ribosome binding sites of target mRNA, thereby blocking translation. Because RsmX/Y/Z are able to titrate out RsmA and E, translational repression is relieved through the GacS-GacA system (Reimmann *et al*., 2005; Lapouge *et al*., 2008).

Many secondary metabolites are regulated by the Gac system. For example, in the well-studied biocontrol bacteria *P. fluorescens* CHA0, the Gac/Rsm system is shown to govern production of HCN, PRN, PLT, motility and overall biocontrol (Lapouge *et al*., 2008). In *P. aeruginosa*, HCN, pyocyanin, elastase, chitinase and the quorum-sensing signal *N*-butanoyl-homoserine lactone are all governed by the Gac-Rsm system (Kay *et al*., 2006). PHZs, HCN,

surfactants, proteases and other biocontrol processes were found to be under control of this system in *P. chlororaphis* (Lapouge *et al.*, 2008). Additional examples can be found in a review by Lapouge and colleagues (2008).

In PA23, the Gac-Rsm system oversees the production of AF metabolites and degradative enzymes, including PCA, 2-OH-PHZ, PRN, proteases, lipases, HCN, and siderophores (Poritsanos *et al*., 2006). PA23 has the repressor proteins RsmA and RsmE and the regulatory RNA RsmZ (Selin *et al*., 2014). Characterization of the Rsm circulatory revealed that RsmZ and RsmE were positively controlled by GacA together with RpoS, PsrA, the stringent response (SR) and quorum sensing (QS). In contrast, RsmA was activated by GacA but was negatively controlled by the SR (Selin *et al*., 2014).

### 1.4.8. RpoS and PsrA regulatory control

The RNA polymerase stationary-phase sigma factor RpoS has been known to regulate transcription of genes in response to starvation and stress conditions (Heeb et al., 2005; Lazazzera, 2000). Within *Pseudomonas* spp., RpoS accumulates during the late logarithmic-phase and is widely known to play a role in the production of secondary metabolites, including those involved in biocontrol (Kang *et al*., 2004; Oh *et al*., 2013; Venturi, 2003). In addition, RpoS interacts with global regulation systems such as the Gac/Rsm system, quorum sensing, and the TetR family regulator, PsrA (Girard et al., 2006; Heeb et al., 2005; Venturi, 2003).

PsrA (Pseudomonas sigma regulator A) is a positive regulator of RpoS transcription that binds to and activates the *rpoS* promoter during stationary phase (Venturi, 2003). A *psrA* knock-out mutant displayed a 90% decrease of *rpoS* promoter activity in stationary phase in both *Pseudomonas putida* and *P. aeruginosa* (Kojic and Venturi, 2001).

In PA23, RpoS is positively regulated by QS and the SR (Manuel *et al*., 2012; Selin *et al*., 2012). RpoS has a complex relationship with the Gac-Rsm system, showing positive regulation by GacA and also playing a role in RsmZ and RsmE activation (Selin *et al*., 2014). Characterization of an *rpoS* knock-out mutant revealed enhanced AF activity *in vitro* and increased expression of PRN, protease and lipase, highlighting that this sigma factor has an overall negative influence on AF activity. Although the mutant showed decreased PHZ production, this did not seem to be involved in fungal antagonism (Manuel *et al*., 2012). RpoS has also been shown to repress biofilm production, as the mutant showed enhanced biofilm production compared with wild type (Selin *et al*., 2012).

### 1.4.9. Phz quorum sensing

Quorum sensing (QS) is the regulation of gene expression in response to changes in population density (Miller and Bassler, 2001). Bacteria produce and release autoinducers and chemical signal molecules that increase in concentration as a function of population density. Once a minimal threshold concentration is reached, changes in gene expression result (Miller and Bassler, 2001). Gram-negative bacteria generally use acylated homoserine lactones (AHLs) as autoinducers. These AHLs are generated by an autoinducer synthase, the product of a *luxI*-type gene (Bassler, 2002). AHLs bind to LuxR proteins once threshold levels have accumulated, enabling the R-proteins to dimerize and regulate target genes (Bassler, 2002).

In PA23, as with other *Pseudomonas* spp., a PhzI/PhzR QS system controls the PHZ biosynthetic operon (Selin *et al*., 2012). It was discovered that in addition to PHZ, PRN, protease and biofilm production are upregulated by the PhzI/PhzR QS system. QS and RpoS exhibit cross-regulation, adding to the complexity of the global regulation systems present in PA23.

Specifically, RpoS activates *phzI* while repressing *phzR* and QS positively controls *rpoS* transcription (Selin *et al*, 2012).

### 1.4.10 Stringent response

The SR is a mechanism used by bacteria to adapt to nutrient stress, allowing them to alter their metabolism from a reproductive mode to one that promotes survival (Manuel *et al*., 2012; Potrykus and Cashel, 2008). The accumulation of guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp), collectively known as (p)ppGpp, signals nutritional stress and leads to adjustments in gene expression (Potrykus and Cashel, 2008). Two proteins are responsible for intracellular (p)ppGpp levels, namely RelA and SpoT. RelA synthetase is responsible for the synthesis of (p)ppGpp, while hydrolysis of this metabolite is mediated by the enzyme SpoT (Manuel *et al*., 2012; Potrykus and Cashel, 2008).

In a study by Manuel and colleagues (2012), it was discovered that the AF activity of PA23 is affected by the SR. Two SR mutants were created, PA23*relA* and PA23*relAspoT*, that no longer produced (p)ppGpp. These mutants exhibited increased AF activity and elevated production of AF metabolites, including PRN, lipase, and protease. Thus, the SR showed an overall negative influence on fungal antagonism (Manuel *et al*., 2012). As previously mentioned, the SR showed positive control over *rpoS* expression (Figure 1.8; Manuel *et al*., 2012).

## 1.5. THESIS OBJECTIVES

Biocontrol strains provide an attractive alternative to chemical pesticides for control of plant diseases. *P. chlororaphis* strain PA23 has demonstrated excellent biocontrol activity against *S. sclerotiorum* (Savchuk and Fernando, 2004; Fernando *et al*., 2007) through the

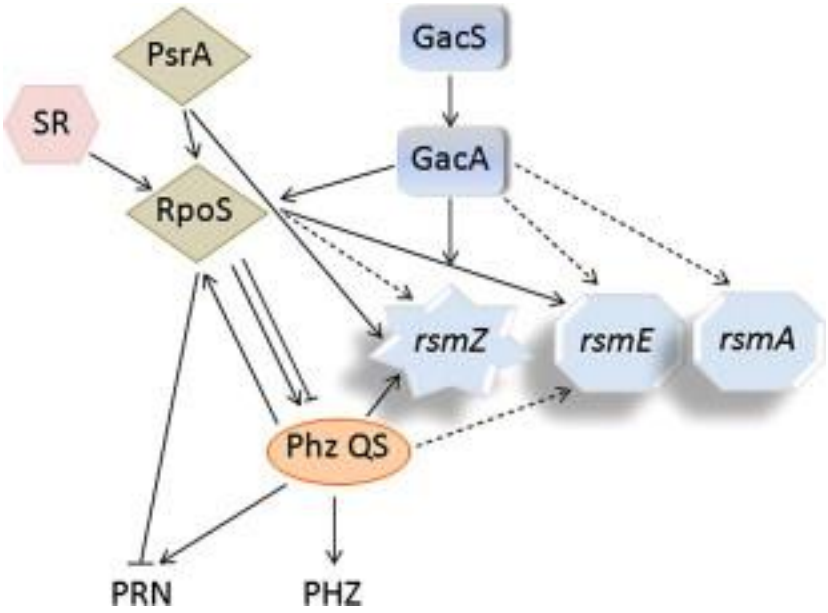



**Figure 1.8** Current model of the genetic regulatory cascade governing antifungal metabolite production in *Pseudomonas chlororaphis* PA23. Abbreviations are those used in the text. (Selin et al., 2014)

production of secondary metabolites that include the antibiotics PHZ and PRN (Zhang *et al*., 2006) as well as HCN, proteases and siderophores (Poritsanos *et al*., 2006). The regulatory cascade overseeing production of PA23 AF metabolites is complex (Figure 1.8). To date, most of this research has been based on studies performed in the lab. Little is known about what promotes AF metabolite gene expression and production *in planta*. Also, PA23 has never been studied as a PGPB beyond its fungal suppression. Thus, the main objectives of this thesis are as follows:

1) To investigate the influence of carbon source on PA23-mediated AF metabolite production and biocontrol
2) To explore the influence of inoculation time on PA23 populations on *B. napus* and the influence of growth stage on secondary metabolite production
3) To determine the influence of PA23 on growth promotion of *B. napus*, both in gnotobiotic conditions and *in planta*.
4) To determine the mechanisms that PA23 uses to directly promote plant growth in the absence of pathogens.

# CHAPTER 2

UNDERSTANDING *PSEUDOMONAS CHLORORAPHIS* STRAIN PA23 POPULATIONS AND ENHANCING INOCULATION

## 2.1. ABSTRACT

*Pseudomonas chlororaphis* strain PA23 has been shown to effectively control *Sclerotinia sclerotiorum* (Lib.) de Bary on canola (*Brassica napus* L.). Biocontrol is mediated through the production of secondary metabolites, notably the antibiotics pyrrolnitrin (PRN) and phenazine (PHZ). Little is known about what promotes expression of secondary metabolites *in planta*. When PA23 was applied to *B. napus* prior to *S. sclerotiorum* ascospores, highest disease suppression was observed when PA23 was able to establish itself for 7 days. It was observed that genes involved with PA23 biocontrol are differentially regulated with respect to time. When carbon sources were investigated for their influence on PA23 growth, glucose and glycerol promoted the fastest growth, while mannose and fructose supported intermediate and slow growth, respectively. Propagation on mannose lead to the greatest inhibition of *S. sclerotiorum* mycelia, while fructose yielded the lowest inhibition; the latter finding is surprising since PRN and PHZ production were highest when grown in fructose. While carbon source effected antifungal metabolite production and regulation in culture, it didn't have an effect on biocontrol of *S. sclerotiorum* when PA23 was applied to *B. napus*. This research provides important insights into PA23 survival on *B. napus* plants, together with an understanding of factors regulating expression of essential antibiotic genes. Such findings are essential for designing an effective biocontrol agent against *S. sclerotiorum*.

## 2.2. INTRODUCTION

*Sclerotinia sclerotiorum* (Lib.) de Bary is a soil-borne fungal pathogen known to cause disease on more than 400 plant species (Bolton *et al*., 2006; Purdy, 1979). In western Canada, canola (*Brassica napus* L.) yield losses of 5-100% have been attributed to sclerotinia stem rot [Manitoba Agriculture, Food and Rural Development (MAFRD), 2015]. With canola, *S. sclerotiorum* infection usually starts on the blossoms, where spores land on flowers, fallen petals, or pollen, all of which act as food sources (Turkington and Morrall, 1993). These infected organs fall onto leaves or stems, where infection progresses, and *S. sclerotiorum*'s necrotrophic activity disrupts the plant's transport systems. Currently, sclerotinia stem rot is mainly managed by rotation with non-host crops and by foliar fungicides. Rotations are often only partially effective due to the persistence of sclerotia bodies, the overwintering structures of *S. sclerotiorum*, and the popularity of *S. sclerotiorum* host crops in western Canada (Bradley *et al*., 2006). Foliar fungicides, sprayed during flowering to prevent spore germination, can be economically valuable only if disease pressure and yield potential are both high (Bradley *et al*., 2006). Due to an increasing demand for sustainable tools with minimal adverse environmental impact, other methods, including biological control, are being explored for sclerotinia stem rot management.

*Pseudomonas chlororaphis* PA23, a bacterium isolated from the root tips of soybeans, has been shown to protect canola from disease caused by *S. sclerotiorum* in both greenhouse and field studies (Savchuk and Fernando, 2004; Fernando *et al*., 2007). PA23 produces a number of secondary metabolites that contribute to fungal antagonism including the antibiotics pyrrolnitrin (PRN), phenazine (PHZ), and HCN, as well as proteases, lipases and siderophores (Poritsanos *et al*., 2006; Zhang *et al*., 2006; Selin *et al*., 2010). Specifically, PRN has been implicated as the primary anti-fungal (AF) metabolite, while PHZ takes a secondary role being involved in biofilm

development (Selin *et al*., 2010). The regulatory cascade overseeing production of PA23 AF metabolites is complex. Systems found to be involved are the Gac-Rsm two-component signal transduction system (Poritsanos *et al*., 2006), the stationary-phase RNA polymerase sigma factor RpoS (Manuel *et al*., 2012), the PhzI/PhzR quorum sensing (QS) system (Selin *et al*., 2012), and the stringent response (SR) (Manuel *et al*., 2012).

A major challenge of biological control is the ability of the biocontrol agent (BCA) to both survive and execute the required mechanism of biocontrol in the environment of the pathogen of interest. The level of epiphytic growth of the BCA on the host plant is subject to both the physical environment and the microbiological environment (Pujol *et al*., 2007). Evaluation of the capacity of an introduced bacterial antagonist to become established, remain viable, and control the pathogen of interest is required prior to wide-spread use.

Secondary metabolites have been shown to be differentially produced in the presence of several minerals and carbon sources. In *Pseudomonas fluorescens* CHA0, the carbon sources glucose, fructose, mannitol, and glycerol caused differential expression of both antibiotics and siderophores (Duffy and Défago, 1999). When this study was extended to different biocontrol strains in the same study, some factors were strain-dependent while others had a general effect. Additional examples of BCAs with differential production of antibiotics due to carbon source include *P. chlororaphis* PCL1391 (Tjeerd van Rij *et al*., 2004), *P. fluorescens* strain 2-79 (Slininger and Shea-Wilbur, 1995), and *Pseudomonas* sp. strain F113 (Shanahan *et al*, 1992).

To date, the majority of research on PA23 has been performed in the lab. Little is known about what promotes AF metabolite gene expression and production *in planta*. In the present work, we explore the influence of time on PA23 populations on *B. napus* and the influence of

growth stage on secondary metabolite production. We also investigate the influence of carbon source on PA23-mediated AF metabolite production and biocontrol.

## 2.3. MATERIALS AND METHODS

### 2.3.1. Bacterial strains and growth conditions

*Pseudomonas chlororaphis* PA23 wild type, which is a naturally rifampicin resistant strain originally isolated from soybeans (Savchuk and Fernando, 2004) was used in this study. Additional PA23 isolates harbouring plasmids were also used, as outlined in Table 2.1. Strains were cultured and maintained on Lennox Luria Bertani (LB) media (Difco Laboratories, Detroit, MI) at 28 $^{o}$C. *Sclerotinia sclerotiorum* (Lib.) de Bary was cultured and maintained on Potato Dextrose Agar (PDA; IBI Scientific, Peosta, IA) at room temperature. Rifampicin (rif; Research Products International Corp, Prospect, IL) was used as needed at the concentration of 100 µg/mL. For all phenotypic assays, strains were grown in M9 minimal salts media (M9; Difco Laboratories, Detroit, MI) supplemented with 1 mM $MgSO_4$ and 0.2% glucose.

### 2.3.2. Growth rate analysis

Cultures of wild-type PA23 were adjusted to a concentration of 1 x $10^7$ CFU/mL from overnight cultures. Calculations were based off of 2 x $10^9$ CFU/mL equaling an $OD_{600}$ of 1. Growth curve analysis of cultures grown in 30 ml M9 minimal media supplemented with 1 mM $MgSO_4$ and 0.2% glucose, glycerol, mannose, or fructose were performed. $OD_{600}$ readings were taken every 12 hours for cell population density, up until 60 hours. The number of live cells was analyzed by plating diluted cultures on agar plates using an Autoplate 4000 spiral plater (Spiral Biotech Inc, Norwood, MA) and counting the number of colonies that formed using the ACOLyte colony counter 7500 (Synbiosis, Frederick, MD). Triplicate samples were analyzed and the experiment was repeated three times.

**Table 2.1** Bacterial strains and plasmids used in Chapter 2 methods and experiments.

| **Strain** | **Relevant characteristics** | **Source or reference** |
|---|---|---|
| *Pseudomonas chlororaphis* | | |
| PA23 | $Phz^{+}Rif^{R}$ wild type (soybean plant isolate) | Savchuk and Fernando, 2004 |
| **Plasmids** | | |
| pRPOS-*lacZ* | pLP170 containing the *rpoS* promoter region from PA23 | Poritsanos *et al*., 2006 |
| pPRNA-*lacZ* | pLP170 containing the *prnA* promoter region from PA23 | Selin *et al*., 2010 |
| pPHZA-l*acZ* | pLP170 containing the *phzA* promoter region from PA23 | Selin *et al*., 2010 |
| pPHZI-*lacZ* | 674 bp fragment containing the *phzI* promoter in pLP170 | Selin *et al*., 2012 |
| pLP170 | Promoterless *lacZ* transcriptional fusion vector | Preston *et al*., 1997 |

### 2.3.3. Antifungal assays

To study the AF activity of PA23 against *S. sclerotinia*, radial diffusion assays (RDAs) were performed (Poritsanos *et al*., 2006). PA23 was grown in M9 salts media supplemented with 1 mM $MgSO_4$ and 0.2% of either glucose, glycerol, fructose, or mannose. A 125 mL flask containing 30 mL of media was inoculated with an overnight culture of PA23 to 1 x $10^7$ cfu/mL and grown for 24 hrs. Cultures were then adjusted to an $OD_{600}$ of 0.1 in their respective media, after which 5 µL was spotted onto 1/5 strength PDA plates and incubated at 28ºC. Twenty-four hours later, a *S. sclerotinia* mycelial plug was placed in the centre of the agar plate. Plates were then incubated at 22 ºC to allow for growth of the fungus. Fungal inhibition was assessed by measuring the zone of clearing between the edge of the bacterial colony and the fungal growth front 3 days after the placement of the fungal plug. Twelve replicates of each strain were analyzed and the experiment was repeated three times.

### 2.3.4. Analysis of carbon source on fungal growth

In order to analyze the influence of carbon source on *S. sclerotiorum,* mycelial growth was measured. M9 agar media was made (11.3 g M9 and 12 g agar-agar per litre); after autoclaving, glucose, glycerol, fructose, or mannose was added to a 0.2% concentration and $MgSO_4$ to 1 mM after which plates were poured. A *S. sclerotiorum* plug was placed at the centre of each plate and the diameter of mycelial growth was measured after 6 days. Six plates were used for each carbon source and the experiment was repeated 2 times.

### 2.3.5. Quantitative analysis of phenazine

Production of PCA and 2-OH-PHZ was quantified according to the methods outlined by Chancey *et al.* (1999). PA23 was grown in M9 salts media supplemented with 1 mM $MgSO_4$ and 0.2% of either glucose, glycerol, fructose, or mannose. A 125 mL flask containing 30 mL of media was inoculated with an overnight culture of PA23 to 1 x $10^7$ cfu/mL. After 24 or 48 hours of growth, cells were spun down and 10 mL of cell supernatant was combined with 10 mL of benzene and 30 µL of concentrated HCl. Following one hour of shaking, the top organic layer was removed and dried under air. The dried extracts were resuspended in 1 mL 0.1 M NaOH and filtered to remove particulate matter. Spectrophotometric quantification was performed at 367 nm and 490 nm for PCA and 2-OH-PHZ, respectively, according to Maddula *et al*. (2008). To determine the relative amounts of PHZ present, absorption maxima were divided by their standard extinction coefficients (PCA: 3019 $M^{-1}cm^{-1}$; 2-OH-PHZ: 7943 $M^{-1}cm^{-1}$). Each treatment was tested in biological triplicate and the experiment was repeated three times.

### 2.3.6. High performance liquid chromatography (HPLC) pyrrolnitrin quantification

Production of the antibiotic PRN was quantified according to the methods outlined by Selin and colleagues (2010). A 125 mL flask containing 30 mL of M9 minimal salts, 1 mM of $MgSO_4$, and 0.2% of either glucose, glycerol, fructose, or mannose was diluted to 1 x $10^7$ CFU/mL with an overnight culture. The cultures were grown for 5 days on a 28 $^oC$ shaker after which PRN was extracted with an equal volume of ethyl acetate. PRN UV absorption maxima were recorded at 225 nm with a Varian 335 diode array detector. PRN peaks were detected at 4.1 min. Samples were analyzed in duplicate.

### 2.3.7. Semi-quantitative reverse transcription-PCR

Semi-quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to monitor expression of several genes involved in biocontrol of PA23. PA23 was grown in M9 salts media supplemented with 1 mM $MgSO_4$ and 0.2% of glucose. RNA was extracted from 12, 24, and 48 hour glucose cultures using a RNeasy Mini Kit (Qiagen, Valencia, USA). Residual DNA was removed by treatment with the Ambion TURBo RNAse-free DNAse kit (Life Technologies, Carlsbad, USA). Only RNA samples with an $A_{260}/A_{280}$ between 1.8-2.0 were used in subsequent steps. cDNA was generated with the Maxima First Strand Synthesis Kit (ThermoScientific, Rockford, USA) in a 20 uL reaction volume. Sequences for the PA23 genes of interest were obtained from Genbank. Primers are listed in Table 2.2. PCR was performed using the CFX Connect Real-Time system (Bio-Rad, Hercules, USA). The final 10 µL volume mixture contained 0.4 µL of both forward and reverse primers (12 mM), 1 µL of 1:20 diluted cDNA, 5 µL SSOFast$^{TM}$ EvaGreen$^{®}$ Supermix (Bio-Rad, Hercules, USA) and 3.2 µL nuclease-free water. qRT-PCR reaction conditions included an initial denaturation at 98 $^{o}$C for 2 min, followed by 40 cycles of 98 $^{o}$C for 5 sec and 60 $^{o}$C for 30 sec. Melt-curve analysis was performed each run to assess the formation of primer dimers and other artefacts. Each reaction was performed in triplicate and experiments were repeated three times with three biological replicates. Relative gene expression was calculated using the ΔΔCt method as described by Livak and Schmittgen (2001).

### 2.3.8. β-Galactosidase assay to measure gene expression

To monitor the gene expression of *rpoS*, *prnA*, *phzA*, and *phzI*, PA23 isolates containing expression vectors that included the promoter regions for the aforementioned genes upstream

of *lacZ* were utilized (Table 2.1). Cultures harboring these plasmids were grown in M9 minimal media with 1 mM $MgSO_4$ and 0.2% of either glucose, glycerol, mannose, or fructose. A promoterless vector, pLP170, was used to control for background absorbance. β-Galactosidase assays were performed after 24 and 48 hours of growth, with the exception of glucose that was also analyzed after 12 hours, from starting cultures of 1 x $10^7$ CFU/mL (Miller, 1972). Samples were analyzed in triplicate and the experiment was repeated for a total of two experiments.

### 2.3.9. *B. napus* growth parameters

For all experiments the *Brassica napus* variety Westar was used (Klassen *et al*., 1987). Plants were seeded in Sunshine Advanced Growing Mix #4 (Sun Gro Horticulture, Agawam, MA) and transplanted at the 3-leaf stage into a 6" pot of Sunshine mix #4. For all experiments, inoculations were carried out at the 30-50 % bloom stage and plants were incubated in a humidity chamber. After lesions began to develop, plants were placed in a growth chamber and grown at 22 $^oC$ with a 16-h photoperiod, with the temperature decreasing to 16 $^oC$ during the night.

### 2.3.10. Greenhouse analysis of PA23 biocontrol

PA23 was grown overnight in a M9 solution with 0.2 % carbon source and 1 mM $MgSO_4$. The cell suspension was centrifuged for 10 min at 6000 r.p.m., then the pellet was resuspended in $dH_2O$ to a concentration of 1 x $10^8$ CFU/mL, after which 0.02 % Tween 80 (Difco Laboratories, Detroit, IL) was added. Ascospores were suspended in water to a concentration of 8 x $10^4$ spores/mL with 0.02 % Tween 80. Both PA23 and ascospores were applied with a spray bottle until plants were saturated. The number of infected leaves was

**Table 2.2** Primers used in semi-quantitative qRT-PCR to study *P. chlororpahis* PA23 promoter activity

| **Name** | **Sequence** | **Source or Reference** |
|---|---|---|
| rpoS FWD | 5′- TGGCTTTCCGAATTGACC-3′ | Klaponski, 2014 |
| rpoS REV | 5′- CAGACGCTTGAGACCTTC-3′ | Klaponski, 2014 |
| prnA FWD | 5′- CCGGCAAAGATGCAGTAGTAG -3′ | This study |
| prnA REV | 5′- CATCAAAGAGAAGGTTCAGCG -3′ | This study |
| phzA FWD | 5’- GCGAAAACCATTACATCCATTC-3’ | This study |
| phzA REV | 5’ –GGATACCTTCACGCTTGATTC-3’ | This study |
| phzI FWD | 5′- GCGATGCCGTTGTTCTGG-3′ | Klaponski, 2014 |
| phzI REV | 5′- AGCCGTTCGTAGTGGACTC-3′ | Klaponski, 2014 |
| rpsL FWD | 5’-TGATAACGAACACCTGGCAA-3’ | This study |
| rpsL REV | 5’-AACTCGGCACTGCGTAAAGT-3’ | This study |
| proC FWD | 5’-GAACGACTTGATCGCAGCTT-3’ | This study |
| proC REV | 5’-GGCGTATTTCTTCCTGCTGA-3’ | This study |
| recF FWD | 5’-ACACCTGGCAGCGTAAGTCT-3’ | This study |
| recF REV | 5’-GGCGCGGTCAGTGTATTTAT-3’ | This study |
| rpoD FWD | 5′- GCCAGTGACGACGAAGAAG-3′ | Klaponski, 2014 |
| rpoD REV | 5′- GCCTTGCGGGTGATTTCC-3′ | Klaponski, 2014 |

measured at 3-5 days post ascospore inoculation, once lesions began to develop, and the plants were moved from the humidity chamber to the growth chamber. Fourteen days post ascospore inoculation, plants were analyzed for disease severity based on the following scale: 0: no visible stem or leaf infection, 1: leaf infection with no visible stem infection, 2-7: leaf infection present and disease severity based on size of stem lesion on main stem (mm), 2: 1-20, 3: 21-40, 4: 41-60, 5: 61-80, 6: 81-100, 7: >100 or plant death (Savchuck *et al*., 2004; Selin *et al*., 2010).

#### 2.3.10.1. Carbon supplements and PA23 biocontrol

When analyzing the effect of carbon source on PA23 biocontrol, the dependent variable was the carbon source in which PA23 was grown prior to inoculation on *B. napus*. PA23 was grown in M9 and 1 mM $MgSO_4$ supplemented with 0.2 % glucose, fructose, or mannose. A check treatment was included in which plants were inoculated with 0.02 % Tween 80 in water. Ascospores were applied to plants 1 day post PA23 application. Ten plants were used for each treatment and the experiment was repeated 2 times.

#### 2.3.10.2. The effect of PA23 application time on biocontrol

PA23 grown in M9 with 0.2 % glucose and 1 mM $MgSO_4$ was applied 1, 4, and 7 days prior to ascospores (Treatments A-C; Table 2.3). A positive disease control, in which no PA23 was applied, was included (Treatment D; Table 2.3). Ten plants were used for each treatment and the experiment was repeated three times.

**Table 2.3** The timing of inoculation of *P. chlororaphis* PA23 and *S. sclerotiorum* ascospores onto *B. napus* plants when analysing PA23 biocontrol with respect to time.

| | **Days** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Treatment** | **7** | **6** | **5** | **4** | **3** | **2** | **1** | **0** |
| **A** | PA23 | | | | | | | Ascospores |
| **B** | | | | PA23 | | | | Ascospores |
| **C** | | | | | | | PA23 | Ascospores |
| **D** | | | | | | | | Ascospores |

### 2.3.11. Population quantification on *B. napus*

A 1 x $10^8$ CFU/mL solution of rifampicin resistant PA23 in $dH_2O$ and 0.02 % Tween 80 (Difco Laboratories, Detroit, IL), prepared as described in earlier (2.3.10), was sprayed on *B. napus* at 20 % flowering to establish populations. Flowers that were near senescence were placed in a 50 mL conical tube 1, 4, and 7 days post-inoculation and 20 mL of $dH_2O$ was added. The mixture was vortexed for 1 min to wash the bacterial cells off of the plant material. The solution was plated onto LB plates that included rifampicin (50 µg/mL) and the fungicide Azoxystrobin (1 µg active ingredient [a.i.]/mL; Quadris, 250 g/L a.i.; Syngenta Canada Inc. Guelph, ON) by an Autoplate 4000 spiral plater (Spiral Biotech Inc, Norwood, MA). Colonies were counted with ACOLyte colony counter 7500 (Synbiosis, Frederick, MD). The experiment was completed 3 times and each time 10 samples of 10 flowers were analyzed.

## 2.4. RESULTS

### 2.4.1. Expression of antifungal metabolites over time

Growth curves were created to see how PA23 cell numbers changed over time in culture. Absorbance was used to measure the total number of cells that were produced while colony forming units (CFUs) were used to visualize the number of live cells in culture (Figure 2.1). A minimal media was used with low levels of carbohydrate in an effort to mimic for the nutrient-limiting conditions of the phyllosphere.

β-galactosidase assays were used to see promoter activity of genes involved with AF activity, either with production of metabolites (*prnA* and *phzA*) or regulation (*rpoS* and *phzI*) (Figure 2.2). The relative expression of the *rpoS*, *prnA*, *phzA*, and *phzI* promoters was analyzed over three time points: 12, 24, and 48 hours, corresponding to log, early stationary and late stationary phase, respectively (Figure 2.3). All the genes showed a similar pattern of β-galactosidase production increasing over time. Gene activity at 48 hours was significantly higher than the earlier times for all of the promoters.

Fold change of the mRNA expression level was calculated by semi-quantitative RT-PCR at 12, 24 and 48 hours. The $C_t$ of the target gene transcripts were normalized to those of housekeeping genes. The housekeeping genes, *proC* and *rpsL*, were chosen due to their low M-values and low coefficient variance (CV) values (Table 2.4). For heterogeneous samples such as ours, M-values of less than 1 and CV values of less than 0.5 are desired.

All of the genes were found to have a change of gene expression over time (*rpoS* and *prnA*, Pr>F = 0.0001; *phzA*, Pr>F = 0.0107; *phzI*, Pr>F = 0.0006). *rpoS* expression increased over time. The expression of *prnA* was lower during log phase than stationary phase. Contrastingly, *phzA* and *phzI* had higher expression at 12 hours versus 24 and 48 hours. In

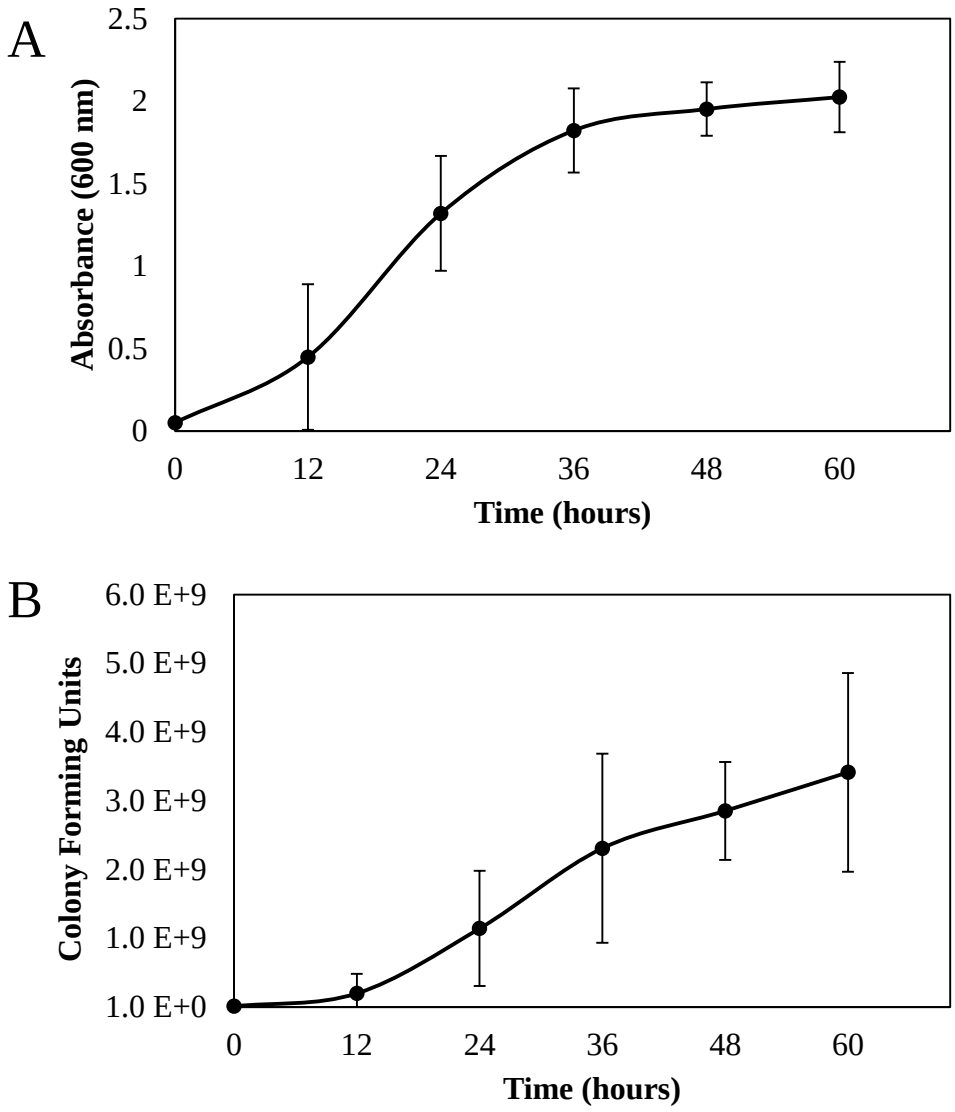



**Figure 2.1** Growth curve of *P. chlororaphis* PA23 grown in M9 minimal media supplemented with 0.2% glucose. The (A) absorbance at 600 nm and (B) the number of live cells were taken every 12 hours. Error bars represent standard deviation.

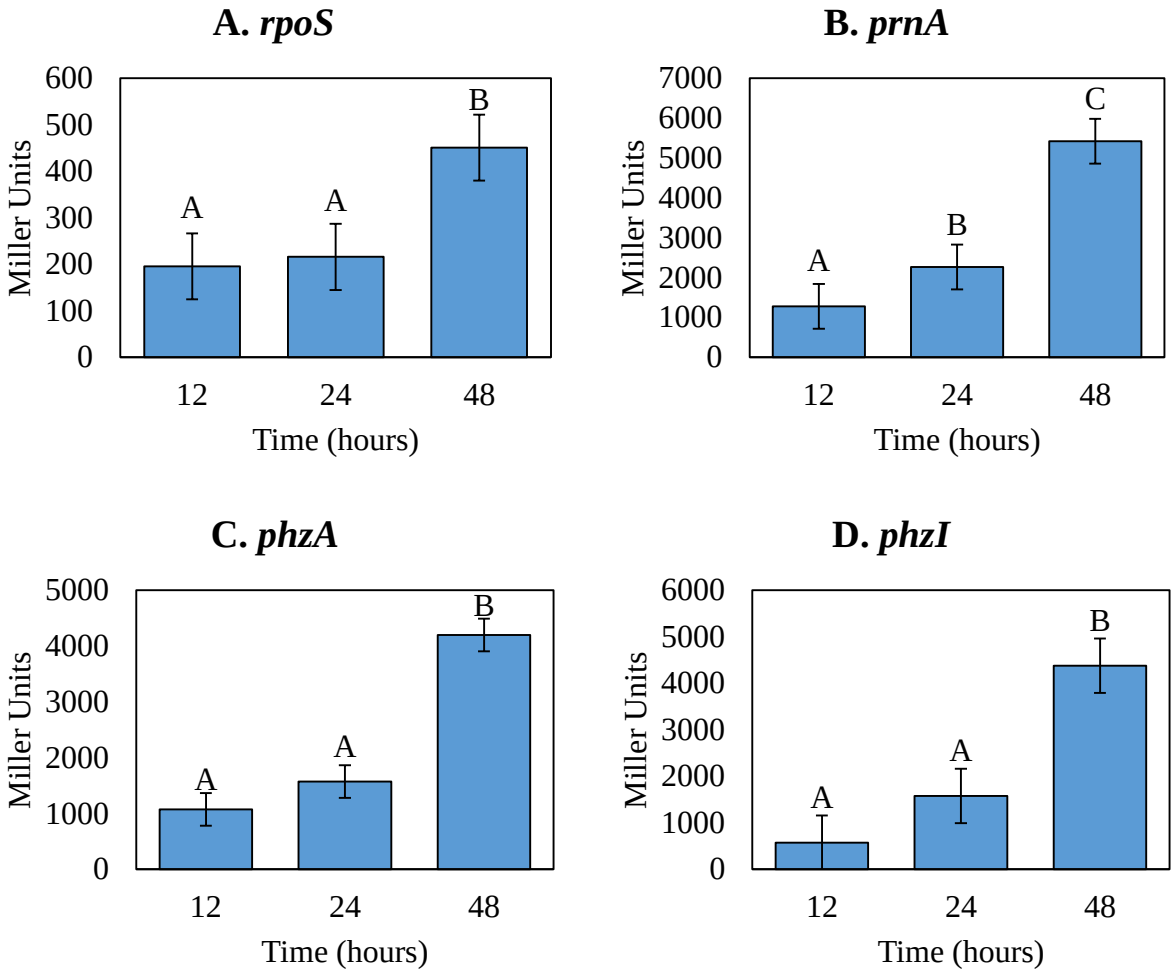



**Figure 2.2** B-galactosidase assays of the promoter regions of (A) *rpoS,* (B) *prnA,* (C) *phzA,* and (D) *phzI* in *P. chlororaphis* PA23. Miller units of the bacterial promoter were determined over the course of time. Values are means of two experiments, each with three biological replicates. Values with different letters are significantly different ($P < 0.05$) using Tukey's post-hoc test for multiple variance. Error bars represent the standard error of the mean.

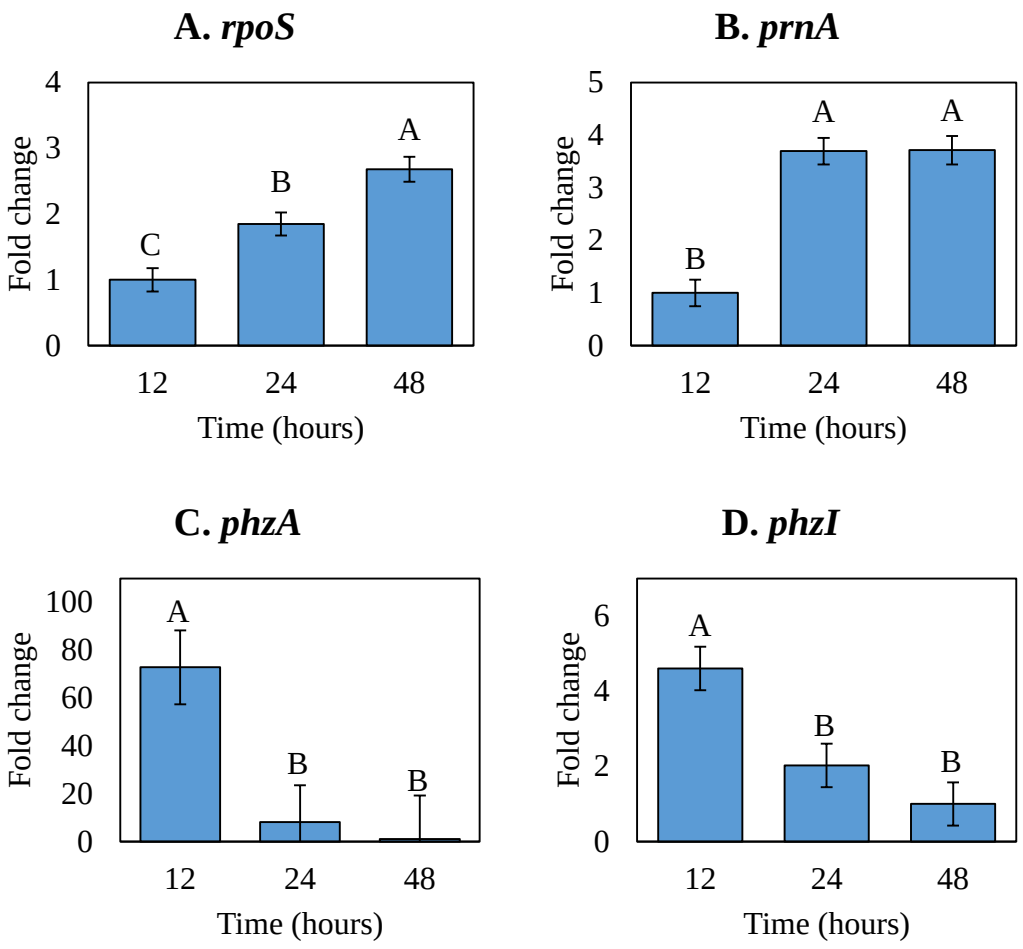



**Figure 2.3** Relative expression levels of (A) *rpoS*, (B) *prnA*, (C) *phzA*, and (D) *phzI* in *P. chlororaphis* PA23 normalized to *proC* and *rpsL* gene expression. Fold change of the bacterial target gene was determined over the course of time. Values are means of three experiments, each with 3 biological replicates. Values with different letters are significantly different ($P < 0.05$) using Tukey's post-hoc test for multiple variance. Error bars represent the standard error of the mean.

**Table 2.4.** Analysis of the stability of housekeeping genes in *P. chlororaphis* PA23 after 12, 24, and 48 hours of growth in M9 minimal media supplemented by 1 mM $MgSO_4$ and 0.2 % glucose.

| Standard | M Value | Coefficient Variance |
|---|---|---|
| *rpoD* | 0.7399 | 0.3415 |
| *recF* | 0.7403 | 0.2989 |
| *proC* | 0.6470 | 0.2474 |
| *rpsL* | 0.6470 | 0.2012 |

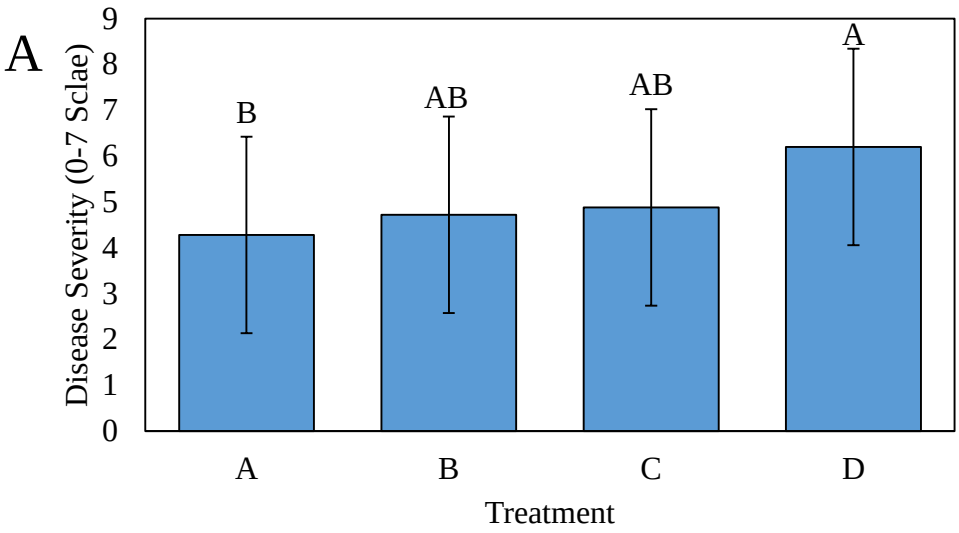
general, the expression fold change was moderate with the exception of *phzA*, which had a fold change of over 70 at 12 hours compared with 48 hours.

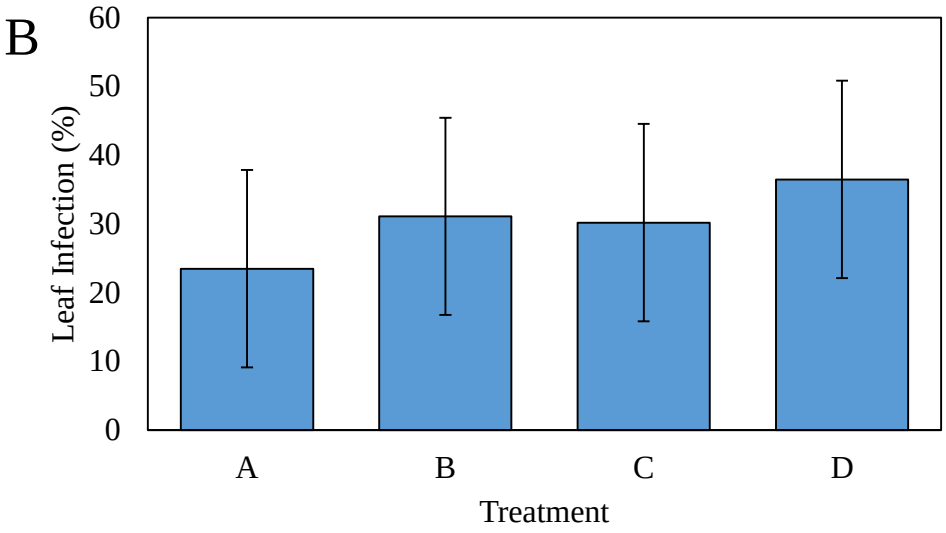
### 2.4.2. Effect of inoculation time on PA23 biocontrol of *S. sclerotiorum* in the greenhouse

PA23 was evaluated for its ability to protect *B. napus* from stem rot disease caused by *S. sclerotiorum* when applied varying amounts of time prior to ascospore challenge (Figure 2.4). Stem rot disease severity showed statistical significance between treatments ($F= 4.17$; $Pr>F= 0.0081$; $DF= 3, 96$), while incidence of leaf infection did not ($F= 2.24$; $Pr>F= 0.0886$; $DF= 3, 94$) (Figure 2.4). Both measurements did show the same trend, with both disease severity and leaf infection incidence decreasing as PA23 inoculation time prior to ascospores inoculation time increased. Only when PA23 was applied 7 days prior to ascospores were significantly lower levels of disease severity measured.

### 2.4.3. *P. chlororaphis* PA23 populations on *B. napus* flowers

The population dynamics of *P. chlororaphis* strain PA23 after its inoculation onto *B. napus* plants are shown in Figure 2.5. After 1 day, the BCA agent was present at high population levels, averaging $4.0 \times 10^5$ viable cells per blossom. These levels dropped off, with an average of $1.2 \times 10^5$ and $1.4 \times 10^4$ cells being found after 4 and 7 days, respectively. Importantly, it was found that PA23 can survive and remain viable on *B. napus* flowers for at least 7 days under greenhouse conditions.




**Treatments:**
A: PA23 inoculated 7 days prior to ascospores
B: PA23 inoculated 4 days prior to ascospores
C: PA23 inoculated 1 days prior to ascospores
D: No PA23 inoculated prior to ascospores

**Figure 2.4.** (A) Disease severity and (B) leaf infection as influenced by time of *P. chlororaphis* PA23 inoculation prior to presence of *S. sclerotiorum* ascospores onto *B. napus* plants. Values are means of three experiments, each with 10 biological replicates per treatment. Values with different letters are significantly different ($P < 0.05$) using Tukey's post-hoc test for multiple variance. Error bars represent the standard error of the mean.

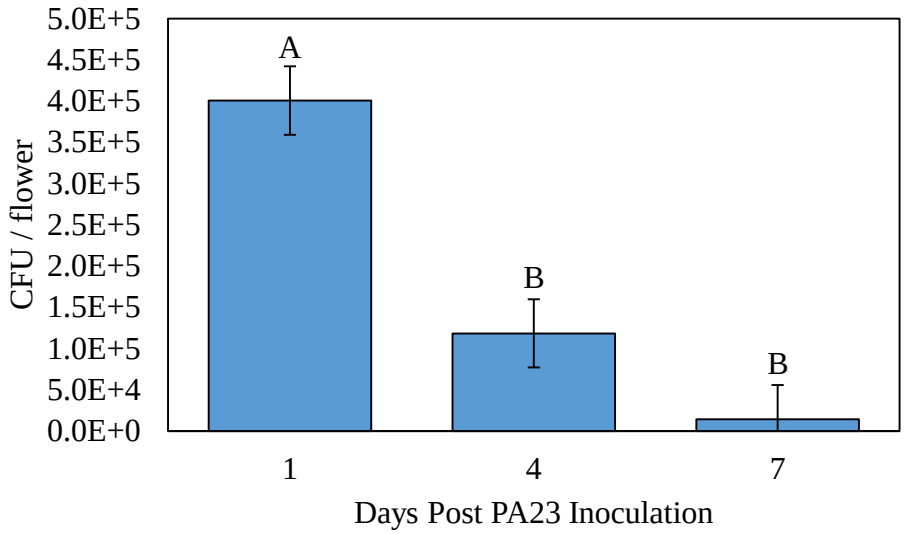



**Figure 2.5** *P. chlororaphis* PA23 populations on *B. napus* flowers over time. Values are means of three experiments, each with 10 samples consisting of 10 flowers for each day. Values with different letters are significantly different ($P < 0.05$) using Tukey's post-hoc test for multiple variance. Error bars represent the standard error of the mean.

supplemented in the minimal media (Figure 2.6). Total cells were visualized using absorbance while CFUs were used to measure the total amount of viable cells in culture. Generation times were calculated based on absorbance during the exponential growth phase (Table 2.5). Glucose caused PA23 to have the fastest multiplication rate, producing a generation time of approximately 2 hours. Glycerol and mannose were intermediate in ability to promote PA23 growth, having generation times of 2.5 and 4.2 hours, respectively. Fructose, as seen in both the growth curves and generation time (6.7 hours), didn't promote efficient PA23 growth. Interestingly, glycerol reached high cell numbers, as seen when measuring cell density using absorbance, but didn't promote long-term cell viability, as reflected by the lower number of CFUs in plate counts.

### 2.4.5. Influence of carbon source on antifungal activity and metabolites

PA23 grown in minimal media containing either glucose, glycerol, mannose, or fructose was tested for AF activity using radial diffusion assays. It was found that carbon source had a significant effect on the ability of PA23 to inhibit *S. sclerotiorum* mycelial growth (Figure 2.7; $F = 38.16$, $Pr>F = <0.0001$, $DF = 3, 138$). Mannose, glycerol and glucose caused PA23 to produce the highest amount of AF metabolites, with mannose enabling the highest level of inhibition. Similar to generation time, fructose caused PA23 to lag behind, having a significant reduction in mycelial inhibition.

### 2.4.6. Antifungal metabolite production

With differences in *in vitro* AF activity, it was of interest to determine whether the PA23 AF metabolite production was also influenced by carbon source. When PRN production was

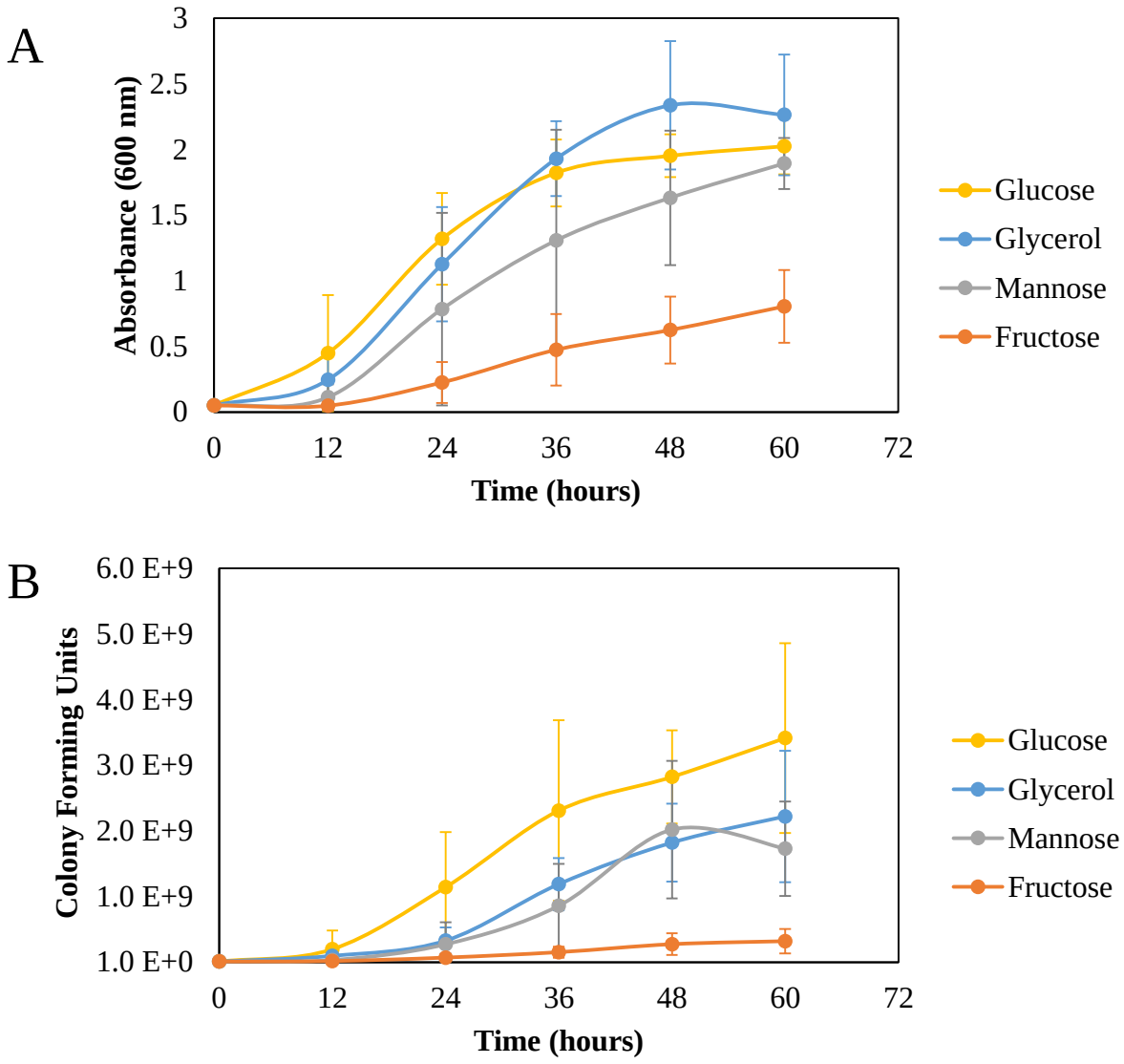



**Figure 2.6** Growth curves of *P. chlororaphis* PA23 grown in M9 minimal media supplemented with 1 mM $MgSO_4$ and 0.2 % of glucose, glycerol, mannose, or fructose. (A) Measured by absorbance at 600 nm and (B) measured by CFU per mL. Samples were analyzed in triplicate and the experiment was completed three times. Error bars represent the standard deviation of the measurements.

**Table 2.5** Generation time of *P. chlororaphis* PA23 as influenced by carbon source added at 0.2 % to M9 minimal media with 1 mM $MgSO_4$. Values with different letters are significantly different ($P < 0.05$) using Tukey's post-hoc test for multiple variance.

| Carbon source | Generation time (SD) in min | Statistical group |
|---|---|---|
| Glucose | 117 (31) | A |
| Glycerol | 152 (29) | A |
| Mannose | 254 (20) | B |
| Fructose | 397 (87) | C |

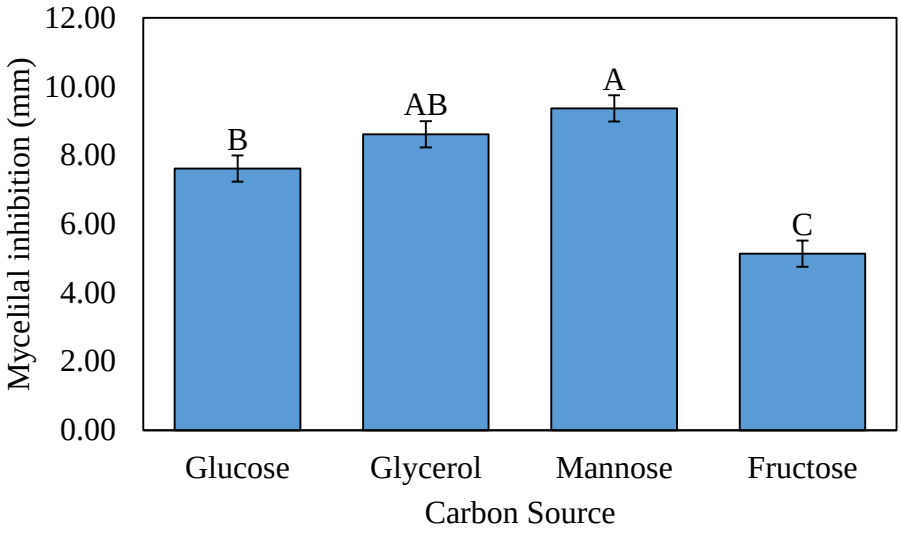



**Figure 2.7** *P. chlororaphis* PA23 inhibition of *S. sclerotiorum* mycelial growth as influenced by carbon source. PA23 was grown on M9 minimal media supplemented with 1 mM $MgSO_4$ and 0.2 % of glucose, glycerol, mannose or fructose before being plated along the edge of the agar plate for the radial diffusion assay. Values are means of three experiments each with 12 replicates per treatment. Values with different letters are significantly different ($P < 0.05$) using Tukey's post-hoc test for multiple variance. Error bars represent the standard error of the mean.

analyzed after 5 days of culture growth, there was a significant difference due to carbon source (Figure 2.8; F= 45.29, Pr>F= 0.0015, DF = 3, 4). Fructose and glucose induced the highest levels of PRN production. Interestingly, mannose, which had the highest levels of inhibition of *S. sclerotiorum* mycelium, had the lowest levels of production. Relative to cell number, fructose had the greatest PRN production.

PHZ production was measured after 24 and 48 hours of growth in minimal media supplemented with either glucose, glycerol, mannose, or fructose. As seen in Figure 2.9 and Table 2.6, total PHZ, as well as PCA and 2-OH-PCA, production were generally increased from 24 to 48 hours. Carbon source also has a significant effect on PHZ production. Glucose and glycerol produce higher levels of PHZ. For both total PHZ production and 2-OH-PCA production, there is a significant interaction between time and carbon source. This is because time doesn't affect PA23's PHZ production in the same way for each carbon source. There is a much smaller increase in PHZ levels in glucose and glycerol than in mannose and fructose from 24 to 48 hours. Relative to cell number, fructose had the greatest levels of PHZ production (Figure 2.9).

### 2.4.7. Effect of carbon source on antifungal metabolite expression

Once measurements of AF metabolites were made, there was interest if these results would correspond to expression of the corresponding gene promoters and if regulatory genes would be similarly expressed. β-galactosidase assays were performed that measured the promoter activity of regulatory genes *rpoS* and *phzI*, and production genes *prnA* and *phzA* (Figure 2.10). These assays measured activity when PA23 was grown for 24 or 48 hours in minimal media supplemented with the different carbon sources. *rpoS* had the greatest promoter activity when

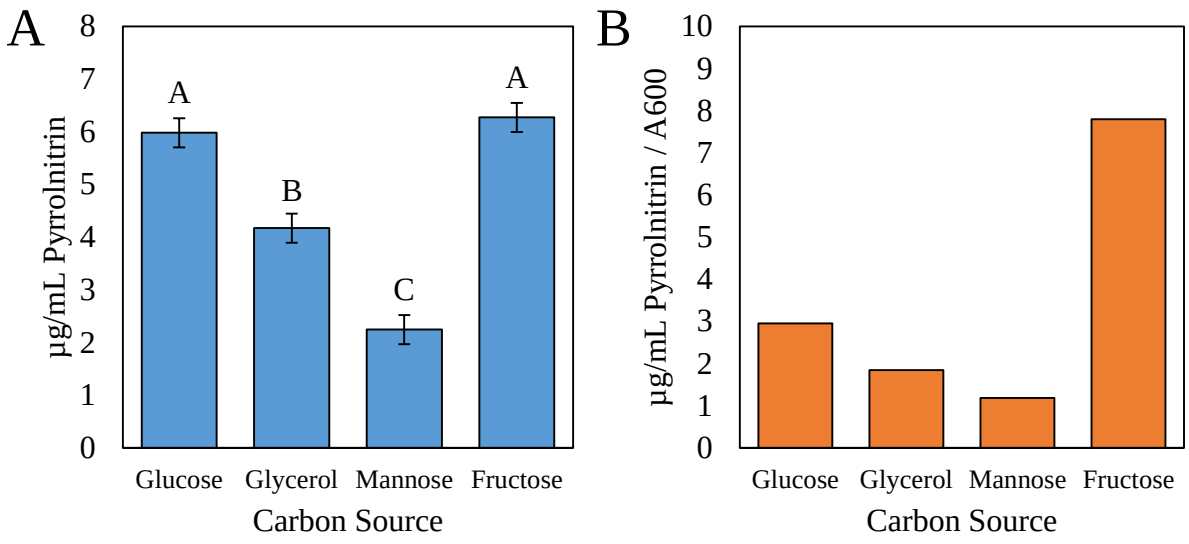



**Figure 2.8** Pyrrolnitrin production by *P. chlororaphis* PA23 after 5 days of growth in M9 minimal media supplemented with 1 mM $MgSO_4$ and 0.2 % of glucose, glycerol, mannose, or fructose. (A) Total pyrrolnitrin produced, (B) pyrrolnitrin divided by cell density at 48 hours. Values with different letters are significantly different ($P < 0.05$) using Tukey's post-hoc test for multiple variance. Error bars represent the standard error of the mean.

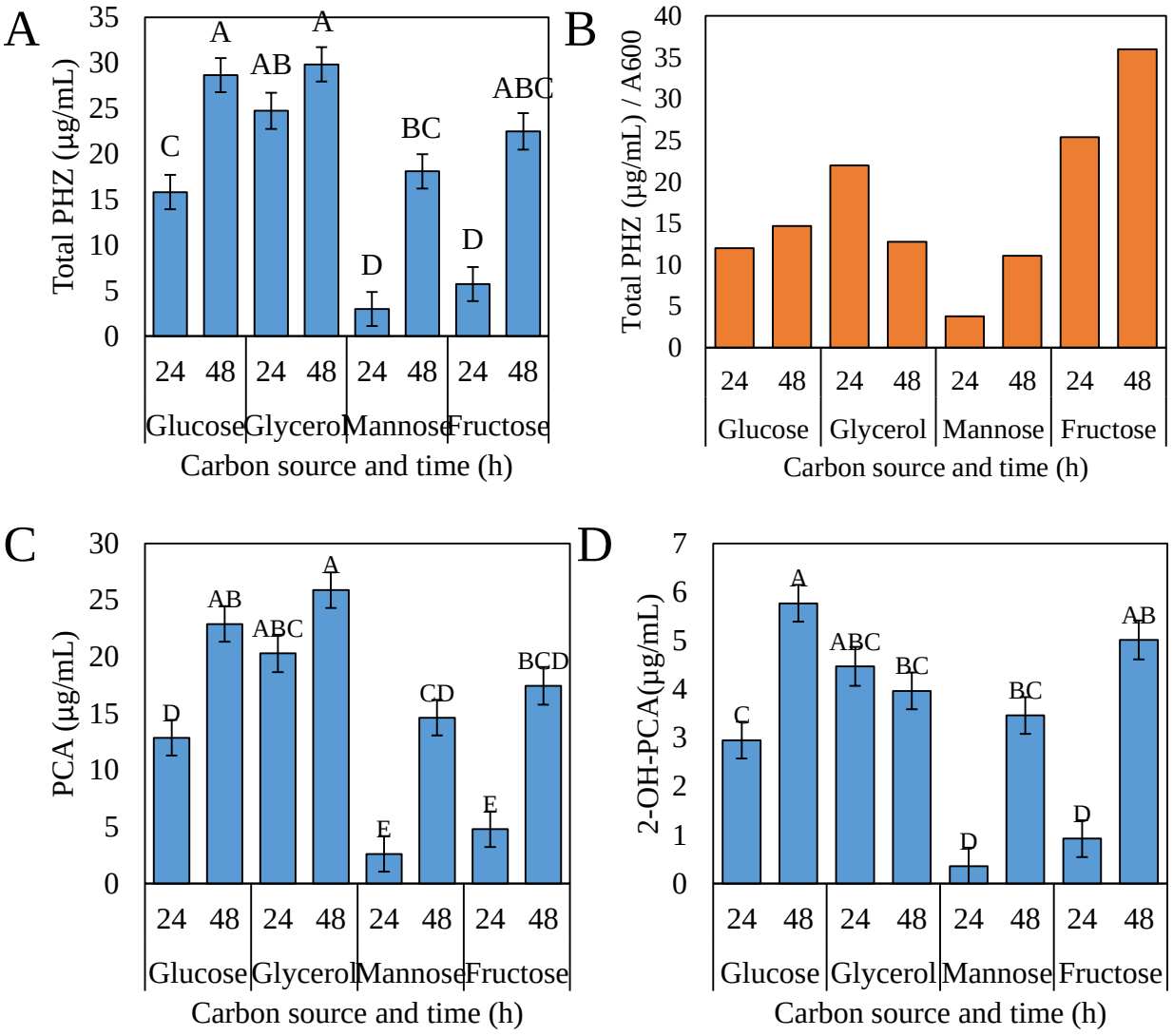



**Figure 2.9** Phenazine production by *P. chlororaphis* PA23 after growth in M9 minimal media supplemented with 1 mM $MgSO_4$ and 0.2 % of glucose, glycerol, mannose, or fructose. Samples were taken after 24 or 48 hours. Measurements were taken of (A) total PHZ, (B) PHZ divided by population density at 24 and 48 hours for each carbon source (C) PCA and (D) 2-OH-PCA production. Values are means of three experiments each with 3 replicates per treatment. Values with different letters are significantly different ($P < 0.05$) using Tukey's post-hoc test for multiple variance. Error bars represent the standard error of the mean.

**Table 2.6** Phenazine (PHZ) production by *P. chlororaphis* PA23 was measured after growth in M9 minimal media supplemented with 1 mM $MgSO_4$ and 0.2 % of glucose, glycerol, mannose, or fructuse. Samples were taken after 24 or 48 hours. Results of the type 3 tests of fixed effects for a 2-way ANOVA on the measurements, where variables included were carbon source and time of sample. Significant results are italicized.

| Effect | Numerator DF | Denumerator DF | F Value | Pr > F |
|---|---|---|---|---|
| **Total PHZ** | | | | |
| Carbon source | 3 | 60 | 31.9 | *<.0001* |
| Time | 1 | 60 | 85.55 | *<.0001* |
| Carbon*time | 3 | 60 | 3.58 | *0.0189* |
| **PCA** | | | | |
| Carbon source | 3 | 60 | 34.87 | *<.0001* |
| Time | 1 | 60 | 82.56 | *<.0001* |
| Carbon*time | 3 | 60 | 2.03 | 0.1191 |
| **2-OH-PCA** | | | | |
| Carbon source | 3 | 60 | 19.12 | *<.0001* |
| Time | 1 | 60 | 79.7 | *<.0001* |
| Carbon*time | 3 | 60 | 13.69 | *<.0001* |

DF, degrees of freedom

PA23 was grown with fructose. The other genes, *prnA, phzA,* and *phzI,* appeared to have the greatest promoter activity when grown in glucose. In glycerol, *prnA* promoter activity was greatest at 24 hours, whereas the other carbon sources had greater activity at 48 hours.

### 2.4.8. Influence of sugars on *Sclerotinia* growth

It was of interest to see if carbon source had an influence on *S. sclerotiorum* mycelial growth. When mycelial plugs were placed on minimal agar media containing different carbon sources, there were significant differences in the growth (Figure 2.11; F= 38.49, Pr>F= <0.0001, DF= 3,66). The *S. sclerotiorum* preferred growing on media supplemented with glucose, followed closely by fructose and mannose. *Sclerotinia sclerotiorum* did not appear to grow well when glycerol was used as a food source.

### 2.4.9. *In-vivo* influence of carbon source

The final study included observing if growing PA23 in different carbon sources had an effect on *S. sclerotiorum* biocontrol once inoculated under greenhouse conditions. Measurements of disease severity and the percentage of leaves with *S. sclerotiorum* lesions showed the same trends (Figure 2.12). In both cases, PA23 had a significant reduction of disease compared to the negative control, regardless of the carbon source it was grown in prior to inoculation. There was no significant distinction between the pre-inoculation conditions.

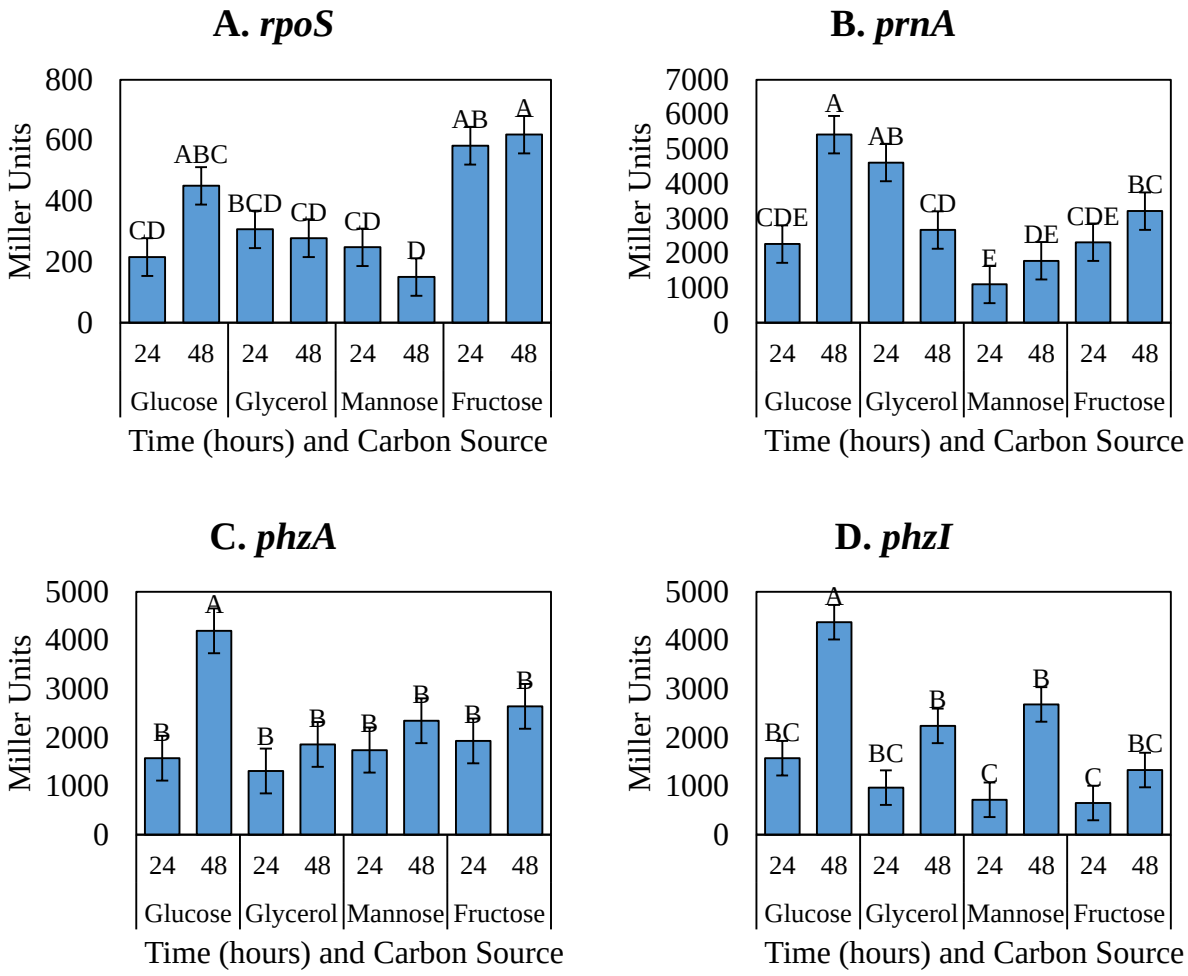



**Figure 2.10** B-galactosidase assays of the promoter regions of (A) *rpoS*, (B) *prnA*, (C) *phzA*, and (D) *phzI* in *P. chlororaphis* PA23 when grown in M9 minimal media supplemented with 1 mM $MgSO_4$ and 0.2 % of glucose, glycerol, mannose, or fructose. Activity of the bacterial promoters was determined after 24 and 48 hours of growth. Values are means of two experiments, each with 3 biological replicates. Values with different letters are significantly different ($P < 0.05$) using Tukey's post-hoc test for multiple variance. Error bars represent the standard error of the mean.

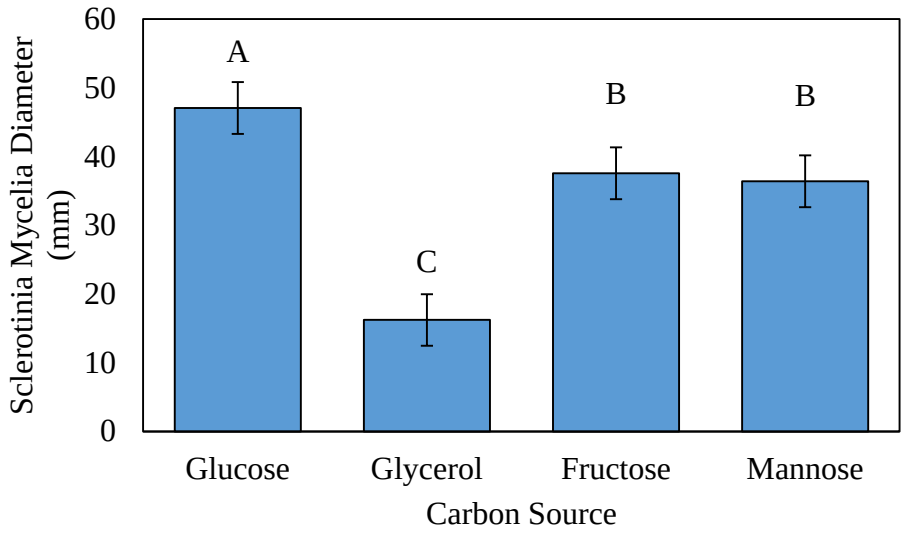



**Figure 2.11** Assay measuring *S. sclerotiorum* mycelial growth on M9 minimal media agar containing 1 mM $MgSO_4$ and 0.2 % of glucose, glycerol, mannose, or fructose. Values are means of three experiments each with 6 replicates per treatment. Values with different letters are significantly different ($P < 0.05$) using Tukey's post-hoc test for multiple variance. Error bars represent the standard error of the mean.

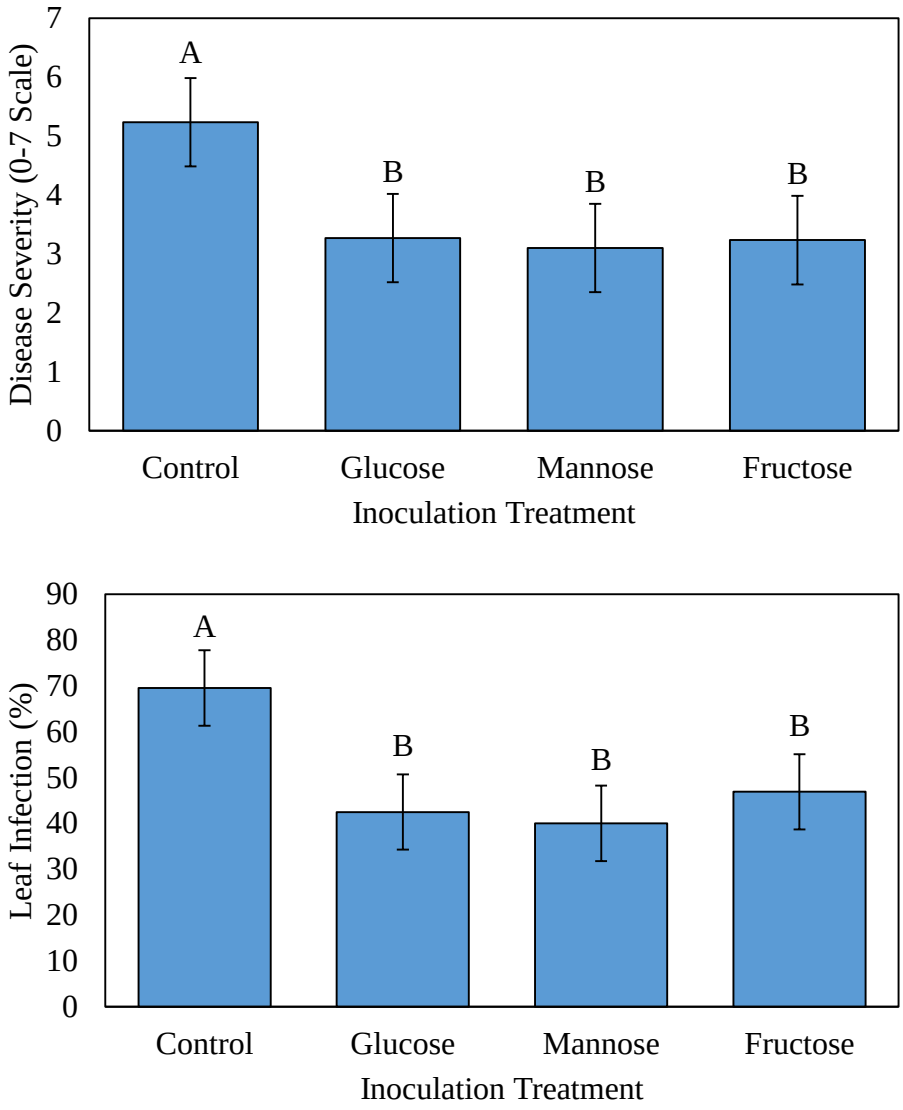



**Figure 2.12** (A) Disease severity and (B) percent leaf infection as influenced by the carbon source *P. chlororaphis* PA23 was grown in prior to inoculation onto *B. napus* plants. *S. sclerotiorum* ascospores were applied to the *B. napus* plants one day after PA23 was inoculated. Values are means of three experiments, each with 10 biological replicates per treatment. Values with different letters are significantly different ($P < 0.05$) using Tukey's post-hoc test for multiple variance. Error bars represent the standard error of the mean.

## 2.5. DISCUSSION

RpoS, a RNA polymerase stationary-phase sigma factor, had promoter activity that was low at 12 and 24 hours, significantly increasing at 48 hours. *prnA, phzA,* and *phzI* showed similar trends when promoter activity was measured with β-galactosidase assays, with expression increasing over time. It is expected that the genes would follow the same trends, as the regulation genes *phzI* and *rpoS* are cross-regulated and *prnA* is upregulated by *phzI* and downregulated by *rpoS* and *phzA* is upregulated by *phzI* (Selin *et al*., 2014). *P. chlororaphis* PA23 follows a typical bacterial life cycle in culture as it has a lag phase with slow growth and adjustment to the conditions followed by rapid growth during log phase. Stationary phase, characterized by limited resources, slower growth, and secondary metabolite production, follows. When PA23 was grown under similar conditions under a previous experiment, all promoter activity appeared to peak at 24 hours then drop off slightly by 36 hours (Selin *et al*., 2012). Selin and colleagues did not state their starting inoculation concentration of bacteria, thus we hypothesize that a higher initial concentration could account for the earlier peak in promoter activity. When *rpoS* expression was measured in *P. putida* and *P. aeruginosa* strains, expression increased as population density increased in both strains (Bertani *et al.,* 2003). In *Pseudomonas* sp. M18, antibiotic production peaked between 36 and 48 hours in minimal media (Zhang *et al*., 2005).

These results were partially reflected by qRT-PCR data measuring mRNA concentrations. *RpoS* expression increased over time. This was expected; as an RNA polymerase stationary-phase sigma factor, we would expect the *rpoS* to be both produced and active to greater levels in stationary phase than prior growth phases. *prnA*, a gene encoding an enzyme involved in the conversion of L-tryptophan to PRN, was also upregulated during the stationary phase. In contrast, both *phzA*, a biosynthetic gene in the PHZ pathway, and *phzI*, which is

involved in the PhzI/PhzR QS system, were downregulated with time. As previously mentioned, we would have expected all of the genes to have been expressed similarly (Selin *et al*., 2014). Possible explanations for the decrease in expression over time of *phzI* and *phzA* include primers binding to the incorrect mRNA, the chosen housekeeping genes not being equally expressed in all the samples, and the mRNA longevity not matching the activity of the promoters. Due to the fact that *rpoS* and *prnA* have the expected relative expression over time, the housekeeping genes likely do not account for the unexpected expression of *phzI* and *phzA.* While mRNA levels do not always match promoter activity due to transcript stability and degradation, it would be unexpected to have them be completely opposite, as is our case. Thus, we suspect promoter specificity accounts for the discrepancies of *phzA* and *phzI*, and thus would disregard the data.

It has previously been concluded that best disease management was provided when bacteria were inoculated prior to ascospores infecting the petals (Savchuk and Fernando, 2004), but there was interest in studying the optimal time of inoculation. When applied onto *B. napus* plants prior to *S. sclerotiorum* ascospore application, PA23 exhibited the greatest control when it had 7 days to establish populations, as compared to 4 or 1 days prior to application. The BCA being applied considerably in advance of the pathogen resulting in enhanced disease control has been observed before (Lindow and Suslow, 2003). This may be, in part, due to AF metabolite production occurring later in the growth cycle of PA23, as this typically occurs during late exponential or early stationary phase within the growth cycle of Gram-negative bacteria (Chater and Bibb, 1997; Martin and Loper, 1999). It can be concluded that PA23 has good longevity in terms of biocontrol and the best biocontrol is provided when PA23 has time to establish itself. We hypothesize that this period may be shorter under field conditions, where survival is more difficult due to variable environmental conditions.

When PA23 population levels were measured on *B. napus* flowers, by 7 days the populations had dropped significantly from 1-day post inoculation. In instances of BCA population levels being measured on flowers under field or low humidity conditions, the populations generally seem to stay relatively steady or slowly decrease over time (Cabrefiga *et al*., 2011; Lindow and Suslow, 2003; Pujol *et al*., 2007). In the study by Lindow and Suslow (2003), the colonization of secondary flowers, ones that had not yet blossomed at the time of inoculation, by *Pseudomonas fluorescens* strain A506 on pear trees was impressive, reaching colonization levels of $10^5$ to $10^6$. They believed that bacteria were transferred between flowers by insects, as others have also previously concluded (Johnson *et al*., 1993; Thomson *et al*., 1992). It is important that PA23 can survive and provide biocontrol up to 7 days post inoculation, as long-term biocontrol is an essential quality of a BCA.

Optimizing a BCA for field inoculation can involve different processes. We focused on culture growth in an attempt to optimize both viability and efficacy of the biocontrol agent. Osmoadaptation, by cultivation under suboptimal conditions, has previously been reported to increase the growth rate, carrying capacity, and stress tolerance of BCAs on host plants (Bonaterra *et al*., 2005; Bonaterra *et al*., 2007; Cabrefiga *et al*., 2011). As such, a minimal media was used for growth of PA23 in preparation for inoculation. Also involved with inoculation preparation, AF metabolites are known to be differentially produced with carbon sources (Shanahan *et al*, 1992; Slininger and Shea-Wilbur, 1995; Duffy and Défago, 1999; Tjeerd van Rij *et al*., 2004). Using minimal media supplemented with one of four different carbon sources, PA23 growth and AF metabolite regulation and production were monitored.

In the *Pseudomonas* genera, different carbon sources are transported and metabolized in peripheral pathways which feed into the central Entner-Doudoroff pathway (EDP) (Temple *et*

*al*., 1998; Browne *et al*., 2010). Notably, glucose, fructose and glycerol all have different transport systems (Figure 2.13; Temple *et al*., 1998; Browne *et al.* 2010). Mannose is less well known. As a 6-carbon sugar, it is similar in structure to both fructose and glucose. It had previously been predicted in *P. aeruginosa* that mannose is converted into fructose 6-P, a catabolic product of fructose (Eagon and Williams, 1960). Due to the predicted presence of the PTS (phosphotransferase system) mannose transporter subunit IIC in the PA23 genome [complement (5538282..5540819) in Locus CP008696], we hypothesize that mannose is transported into the bacterial cell independently. This enzyme catalyzes the phosphorylation of mannose and simultaneously facilitates it's translocation across the cell membrane (Huber and Erni, 1996).

Growth curves were created, measuring both the total number of bacterial cells and the number of viable cells, using minimal media containing a low concentration of glucose, mannose, glycerol, or fructose. When growth rates were calculated for the absorbance of the cell culture, glucose provided the fastest rate of growth, followed by glycerol, mannose, and then fructose. This corresponded with literature on *P. chlororaphis* PCL1391, where glucose, glycerol and fructose produced generation times of 60, 93, and 186 minutes, respectively (Tjeerd van Rij *et al*., 2004). In Tjeerd van Rij and collegues study (2004), the generation times are different due to different media and carbon concentrations, but the trends are parallel.

As observed with the growth curves, glycerol had a high growth rate as seen with absorbance measurements, but the cell longevity was low, as seen in the lower curve when measuring CFUs. In our cultures, we used very low concentrations of carbohydrate sources. Glucose, mannose, and fructose are all six carbon molecules, while glycerol is a three carbon sugar. Based on the molar weights (180.16 g $mol^{-1}$ for the 6 carbon sugars; 92.09 g $mol^{-1}$ for

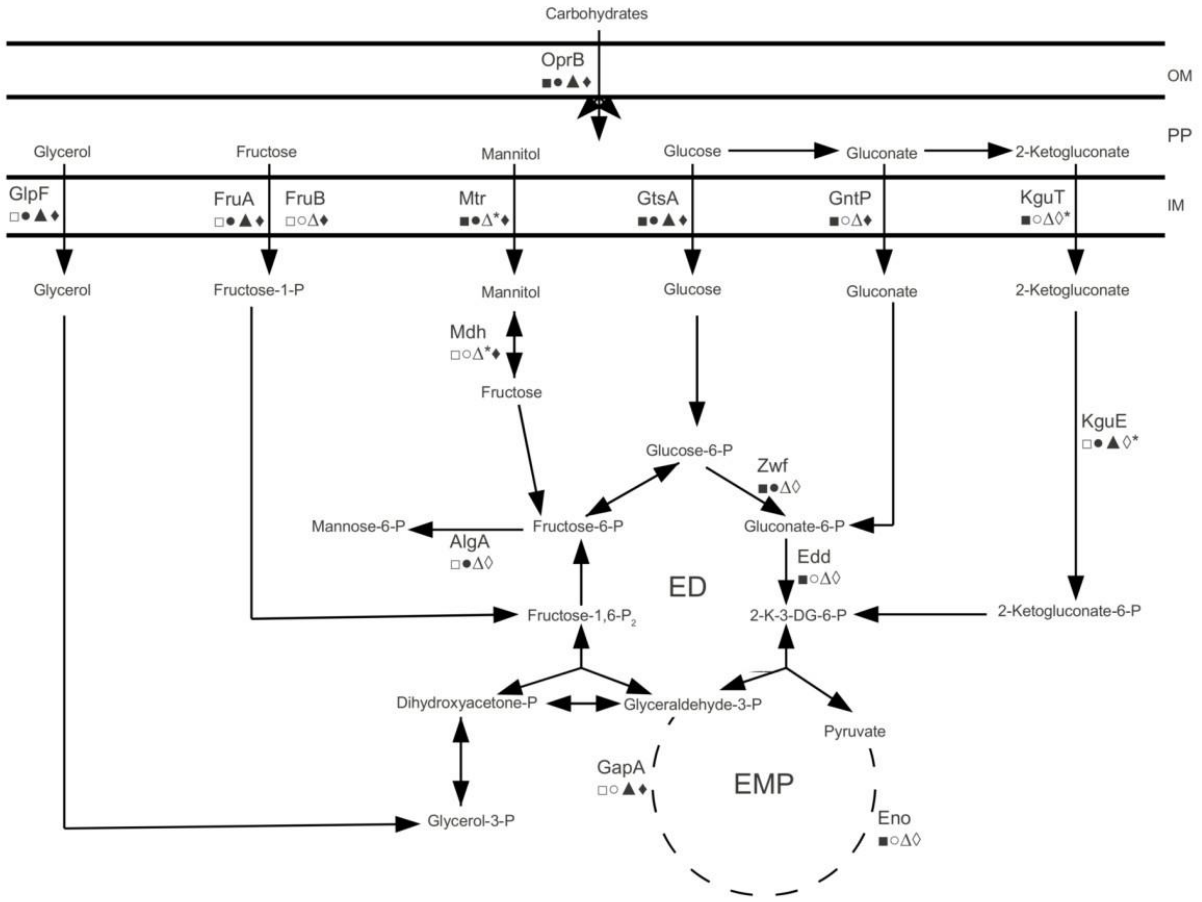



**Figure 2.13** Predicted carbohydrate metabolism in *Pseudomonas* spp. Selected genes involved in carbohydrate transport and metabolism are shown. Genes from *P. aeruginosa* (squares), *P. fluorescens* (circles), *P. putida* (triangles), and *P. syringae* (diamonds) are shown. OM- outer membrane; PP- periplasm; IM- inner membrane; ED- Entner-Doudoroff pathway; EMP- Embden-Meyerhoff pathway; 2-K-3-DG-6-P-2 – 2-keto-3-deoxygluconate-6-phosphate. OprB- carbohydrate porin B; GlpF- glycerol transporter; FruAB- fructose phosphotransferase system; Mtr- mannitol transporter subunit; GtsA- glucose transporter subunit; GntP- gluconate transporter; KguT- 2-ketogluconate transporter; Mdh- mannitol dehydrogenase; AlgA- mannose-6-P isomerase; Zwf- glucose-6-P dehydrogenase; Edd- gluconate-6-P dehydratase; KguE- xylose isomerase; GapA- glyceraldehyde-3-P dehydrogenase; Eno- phosphorpyruvate hydratase.

Reprinted from BMC Microbiology (Computational prediction of the Crc regulon identifies genus-wide and species-specific targets of catabolite repression control in *Pseudomonas* bacteria. Patrick Browne, Matthieu Barret, Fergal O'Gara, and John P Morrissey, 2010, 10:300) with permission from BioMed Central.

glycerol), this lack of longevity wouldn't be due to less carbon, as there would have been twice as many glycerol molecules added to the media. We are unsure as to why glycerol promotes faster cell death.

Due to the fact that PA23 can grow on fructose, the enzymes responsible for fructose catabolism must be present. Reasons for the very slow growth on fructose include: (A) a fructose bioproduct or enzyme involved in its catabolism enhances secondary metabolite regulation and production, taking energy away from cell growth, or (B) the cells are stressed, and as such put energy towards secondary metabolite production.

PRN is the primary antibiotic involved in biocontrol in PA23, while PHZ plays a minor role in antagonism but a major role in biofilm formation (Selin *et al*., 2010). Having fructose as a carbon source led to the highest levels of PRN in the culture, especially relative to its much lower cell number. This result was mirrored with PHZ. It was expected that the two antibiotics would show similar trends, as they do not cross-regulate one another (Selin *et al*., 2010) and are similarly regulated (Selin *et al*., 2014).

In *P. chlororaphis* PCL1391, relative to the optical density of the culture, the primary antibiotic phenazine-1-carboxamide (PCN) is produced at low levels with fructose as the carbon source (0.35 μM/$OD_{620}$), while glucose and glycerol stimulate much higher levels of production (7.90 and 3.00 μM/$OD_{620}$, respectively) (Tjeerd van Rij *et al*., 2004). *P. protegens* CHA0, after 5 days of growth, showed approximately double the production of PRN with fructose compared to glycerol and glucose (Duffy and Défago, 1999). CHA0's other two antibiotics, 2,4-diacetylphloroglucinol (PHL) and pyoluteorin (PLT) showed different tendencies: PHL had high production with fructose and glycerol, while PLT had the greatest production with glycerol and intermediate production with fructose (Duffy and Défago, 1999). Contrastingly, when *P.*

*fluorescens* Pf-5 was tested with glucose and glycerol, it had the greatest production of PHL with glucose, while PLT and PRN were upregulated with glycerol (Nowak-Thompson and Gould, 1994). Production of PCA in *P. fluorescens* 2-79 was greatest in glucose (0.26 g/g), intermediate in mannose and glycerol (0.19 and 0.17 g/g) and lowest in fructose (0.09 g/g) (Nowak-Thompson and Gould, 1994). Thus, the effect of carbon source is varied, depending on the bacterial strain and the specific antibiotic in question.

Initially found on the roots of soybeans, PA23 is naturally a rhizosphere bacterium. The root composition of two soybean varieties can be found in Table 2.7. At 10 days, glucose has high levels but they drop off by 21 days (Yaryura *et al*., 2008). Contrastingly, fructose levels increase over the 11-day period (Yaryura *et al*., 2008). We can hypothesize that in its initial environment, PA23 could use glucose to promote rapid population growth in order to become established, then use fructose as a carbon source to enhance AF metabolite production.

There was interested in whether the promoter activity of genes controlling regulation and production of AF metabolites would be affected by carbon source. Not surprisingly, the production of PHZ and PRN, the genes responsible for regulating and creating these products were variable based on both time and carbon source. Even though cell density is taken into consideration with β-galactosidase assays for promoter activity, fructose did not stimulate high levels of activity from either the *prnA* or the *phzA* promoter. Fructose did have the highest levels of activity for *rpoS*. This was unexpected because *rpoS* is a stationary-phase sigma factor- it isn't generally expressed until higher absorbances are achieved.

Carbon source had a significant impact on PA23 inhibition of *S. sclerotiorum* mycelial growth on agar plates. Fructose produced the lowest level of inhibition; due to the fact it produced high levels of both PRN and PHZ, this low level of inhibition would primarily be due

**Table 2.7** Carbohydrate composition of the root exudates from two soybean varieties, FN 4.10 and 4.85, as determined by high-performance liquid chromatography. Analyses performed at the Department of Organic Chemistry, School of Sciences, University of Buenos Aires, Argentina (Yaryura et al., 2008)

| | **FN 4.10 (nmol plant$^{-1}$)** | | **FN 4.85 (nmol plant$^{-1}$)** | |
|---|---|---|---|---|
| | **10 days** | **21 days** | **10 days** | **21 days** |
| Arabinose | 41.41 | 130.58 | 9.11 | 177.84 |
| Galactose | 27.35 | 3.69 | 30.34 | 19.94 |
| Glucose | 138.32 | 71.10 | 130.48 | 59.83 |
| Mannose | 19.89 | 41.77 | 39.45 | 31.42 |
| Fructose | 41.03 | 539.66 | 160.82 | 287.78 |

to slow cell growth. Interestingly, mannose, which produced the lowest levels of PRN and PHZ,had the greatest inhibition of *S. sclerotiorum*. We hypothesize that this is due to the production of AF metabolites other than diffusible antibiotics. PA23 is known to produce siderophores, degradative enzymes, and HCN, which may contribute to fungal inhibition (Poritsanos *et al*., 2006; Zhang *et al*., 2006; Selin *et al*., 2010). It has previously been reported that siderophores are expressed opposite of other AF metabolites (Poritsanos *et al*., 2006). This may partially explain the high levels of biocontrol with mannose as the carbon source.

To increase the fitness of the BCA upon delivery to the field, nutritional enhancement (NE) can be used (Cabrefiga *et al*., 2011; Janisiewicz *et al*., 1992). NE is the use of an inoculation formulation that includes nutrients that are more efficiently used by the BCA than the pathogen (Cabrefiga *et al*., 2011). It was of interest to determine what carbon sources the pathogen could efficiently utilize. *S. sclerotiorum* mycelial growth was greatest on minimal media agar supplemented with glucose followed closely by fructose and mannose. Glycerol was not a good food source for the pathogen; after 6 days of growth, the mycelia had only slightly extended off the agar plug. If, in the future, the inoculation formula contained a carbon source, glycerol would be of interest.

While *in vitro* work is important for understanding the building blocks of a BCA's mode of action and its regulation, the *in vivo* or *in planta* work is imperative before the BCA is used in a commercial setting. If the biocontrol bacterium can't control the desired pathogen in a field or greenhouse setting, it is of limited use. Glucose, mannose, and fructose were tested to see if they affected biocontrol when used as carbon sources for PA23 pre-inoculation. Glucose was chosen due to its fast growth rate, mannose because of its inhibition of *S. sclerotiorum* mycelia, and fructose since it had the highest levels of PRN and PHZ production. Due to the fact that the

bacteria were resuspended in $dH_2O$ prior to inoculation, the effect of glycerol inhibiting the pathogen would be nominal and space in the humidity chamber was limited. As such, glycerol was not tested in the greenhouse.

The carbon source that PA23 was grown in didn't have a significant effect on disease severity or the percent leaf infection. All of the carbon sources allowed PA23 to significantly reduce disease compared to the control in which no BCA was applied. While many studies have looked at the effect of carbon source on AF activity (Duffy and Défago, 1999; Nowak-Thompson and Gould, 1994; Tjeerd van Rij *et al*., 2004), it is novel, as far as we could find, to test if carbon sources that have an effect on AF activity *in vivo* have an effect under greenhouse conditions. We can conclude that while carbon sources have an effect on growth and production of AF metabolites *in vitro,* there is no significant effect of carbon source once PA23 is applied as a biocontrol agent under greenhouse conditions. While no significant effect under this experiment, in an optimized formulation carbon source may play a larger role in PA23 biocontrol performance. When combined with the many components of a commercial biocontrol formulation, such as carriers, diluents, bulking additives, membrane stabilizers, binders, dispersants, etc., carbon source may have a significant effect (Paau, 1998).

## 2.6. CONCLUSIONS AND FUTURE DIRECTIONS

Overall, this study continues to expand our knowledge on the biocontrol agent *P. chlororaphis* PA23, connecting *in vitro* and *in vivo* research. It had previously been concluded that best control of *S. sclerotiorum* was provided when PA23 was inoculated prior to pressure from *S. sclerotiorum* ascospores (Savchuk and Fernando, 2004). This study demonstrates that PA23 can survive on *B. napus* for 7 or more days under greenhouse conditions and optimal biocontrol is provided when PA23 has a chance to establish itself for 7 days prior to ascospore inoculation. Steps were taken towards optimizing PA23 for inoculation onto *B. napus* plants. Focusing on the culture conditions, we found that while carbon source had a significant effect on the production and regulation of AF metabolites in culture and of control of *S. sclerotiorum* mycelia on agar plates, these effects didn't affect greenhouse biocontrol.

Further research on PA23 biocontrol would be beneficial. We found that PA23 populations last for 7 days under greenhouse conditions. Studies on exactly how long populations last and how these are effected by environmental conditions such as temperature and humidity would be useful in understanding how PA23 would react under field conditions. These could be connected to outdoor field studies also mapping PA23 populations. PA23 should be applied considerable before ascospore inoculation in order to to provide the greatest level of disease protection. It would be interesting to study if PA23 could be inoculated on seeds or pre-flowering plants and still provide biocontrol; how long could PA23 be inoculated prior to disease pressure and still be effective? With regard to PA23 inoculation formulation, we removed all substrates and sprayed PA23 in sterilized water with a small amount of Tween80. Studies where the direct culture is inoculated, thus putting the carbon sources and other culture media into the environment, would be of value. In addition, creating and monitoring formulas where PA23

could be stored and sprayed at a later date, mimicking what would happen in an industrial process, would be beneficial.

# CHAPTER 3

# ELICITING PLANT GROWTH PROMOTION BY *PSEUDOMONAS CHLORORAPHIS* PA23

## 3.1. ABSTRACT

*Pseudomonas chlororaphis* stain PA23, *Pseudomonas brassicacearum* strain DF41, and *Bacillus amyloliquifaciens* strain BS6 have been studied as phyllosphere biocontrol agents, most notably against *Sclerotinia sclerotiorum* (Lib.) de Bary in the canola (*Brassica napus* L.) phyllosphere. *In vitro* analysis showed the potential for their use in suppressing rhizosphere pathogens. PA23 demonstrated significantly higher control of *Pythium ultimum* Trow mycelia, creating a zone of inhibition of 3.3 mm, compared to DF41 and BS6, which inhibited *P. ultimum* 0.8 mm and 0.0 mm, respectively. DF41 significntly suppressed growth of *Rhizoctonia solani* J.G. Kühn to 9.3 mm of inhibition, while PA23 showed inhibition of 1.5 mm and BS6 0.0 mm. All PA23 and BS6 promoted *B. napus* root growth to significant levels in growth pouches, with PA23 stimulating seedling growth to 127 % of the control. The three bacteria inhibited root growth compared to the bacteria-free control when root growth was measured in soil. DF41 had the highest level of *B. napus* growth reduction as well as the greatest root populations, at 1.2 x $10^8$ CFU/g of root material. Analysis into possible mechanisms of plant growth modification involved an assay for ACC deaminase and quantification of IAA production. Only DF41 was found to have the enzyme ACC deaminase, as seen by its growth on media with ACC as the sole nitrogen source. All of the bacteria produced IAA, but PA23 produced significantly more, producing 41.7 ug IAA/mL M9 culture, compared to 29.8 and 26.2 ug/mL for BS6 and DF41, respectively. Interestingly, while in DF salts minimal media, tryptophan caused IAA production and antifungal activity to significantly drop in PA23, 300 μg tryptophan/mL of culture causing IAA production to drop by 89%. Through mutant analysis, PA23's PhzI/PhzR quorum-sensing system was found to likely be involved in this novel observation. This research begins the

exploration of using PA23, DF41, and BS6 as rhizosphere biocontrol agents and their potential effects on plant growth when no pathogens are present.

## 3.2. INTRODUCTION

Plants and microorganisms are members of complex communities characterized by frequent interactions with one another (Kinkel *et al*., 1989). Plant-associated bacteria can be classified into three groups: beneficial, deleterious, and neutral (Dobbelaere *et al*., 2003). Beneficial, or mutualistic, bacteria are often known as plant growth-promoting bacteria (PGPB). Research on PGPB has been increasing since the term was first used by Kloepper and colleagues in the late 1970s (Kloepper and Scroth, 1978; Vessey, 2003). Bacteria are attracted to plants, especially the roots, because of the exudation and accumulation of plant photosynthates, a rich source of energy and nutrients in an often limiting environment (Glick, *et al.*, 2007; Gray and Smith, 2005). These PGPB can provide nutrients or inhibit pathogenic bacteria, enhancing plant growth by either direct or indirect methods (Glick *et al*., 2007)

Direct promotion of plant growth is facilitated by two main mechanisms, either through providing the plant with a compound that is synthesized by the bacterium or by facilitating the uptake of certain nutrients from the environment (Glick, 1995). More specifically, direct mechanisms include fixation of atmospheric nitrogen (Richardson *et* al., 2009), production of siderophores for recruitment of iron (Neilands and Leong, 1986), solubilisation of phosphate (Rodríguez and Fraga, 1999), and the modulation of plant growth through the production or degradation of plant hormones (Dodd *et al.*, 2010). Plant hormones that have been shown to be influenced by PGP include auxins (Patten and Glick, 1996; Spaepen *et al*., 2007), ethylene (Arshad *et al*., 2007; Glick, 2005), cytokinins (Dodd *et al*., 2010), abscisic acid (Dodd *et al*., 2010), and gibberellins (Bottini *et al*., 2004). A PGPB does not solely depend upon one mechanism for promoting plant growth, rather one or more mechanisms may be employed, particularly under varying environmental conditions (Glick, 1995).

An indirect method of PGP is that of biocontrol: lessening or preventing the deleterious effects of one or more phytopathogenic organisms through use of a biological control agent (BCA) (Glick, 1995). Bacteria that provide biocontrol are diverse, though *Bacillus* and *Pseudomonas* spp. are predominant (Podile and Kishore, 2006). In the control of *S. sclerotiorum* (Lib.) de Bary, a fungal pathogen of the canola phyllosphere, three bacteria that have been shown to provide biocontrol are *P. chlororaphis* strain PA23, *P. brassicacearum* strain DF41, and *B. amyloliquefaciens* strain BS6 (Savchuk and Fernando, 2004; Fernando *et al*., 2007). PA23's biocontrol is mediated through the production of secondary metabolites, notably the antibiotics pyrrolnitrin (PRN) and phenazine (PHZ) (Selin *et al*., 2010). DF41 provides control against *S. sclerotiorum* mainly through the production of a lipopeptide molecule (Berry *et al*., 2010). BS6's control mechanism is thought to be antibiotics, namely fengycins and surfactins (Zhang et al., 2006; Athukorala *et al*., 2009).

While PA23 was initially isolated from the soybean root tip, DF41 from the canola root tip, and BS6 from a canola leaf, all have only been tested as biocontrol agents in the phyllosphere (Savchuk, 2002; Savchuk and Fernando, 2004; Zhang, 2004). This leads to questions of biocontrol in the rhizosphere. Therefore, the objectives of this study were (i) to investigate biocontrol of fungal root pathogens of *Brassica napus*, (ii) to study the effect of BCAs on *B. napus* in the absence of pathogens, and, if BCAs show promotion of seedling growth (iii) to identify direct mechanisms of plant growth promotion.

## 3.3. MATERIALS AND METHODS

### 3.3.1. Bacterial strains and growth conditions

All bacterial strains used in this study are outlined in Table 3.1. Bacteria were maintained on Lennox Luria Bertani (LB) media (Difco Laboratories, Detroit, MI) at 28ºC. Dworkin-Foster (DF) salts minimal media was used to check for ACC deaminase and for IAA analysis according to Penrose and Glick (2003). *Sclerotinia sclerotiorum* (Lib.) de Bary, *Pythium ultimum* Trow, and *Rhizoctonia solani* J.G. Kühn were cultured and maintained on Potato Dextrose Agar (PDA; IBI Scientific, Peosta, IA). All antibiotics used were from Research Products International Corp. (Prospect, IL) and supplemented at the following concentrations: gentamicin (Gm; 20 µg/mL), tetracycline (Tc; 15 µg/mL), and rifampicin (Rif; 100 µg/mL). For all other phenotypic assays, strains were grown in M9 Minimal Salts Media (M9; Difco Laboratories, Detroit, MI) supplemented with 1 mM $MgSO_4$ and 0.2% glucose.

### 3.3.2. Antifungal assays to assess the influence of tryptophan on PA23 AF activity

Radial diffusion assays were performed to investigate the effect of tryptophan on antifungal (AF) activity of PA23. PA23 was grown in DF salts media supplemented with 2 g/L $(NH_4)_2SO_4$ and either 0, 100, or 300 µg/mL L-tryptophan (Sigma-Aldrich, St. Louis, USA). A test tube containing 5 mL of media was inoculated with 20 µL of overnight culture and grown for 48 hours. A 5 µL volume of culture was plated onto 1/5 strength PDA plates and then incubated at 28 ºC for 24 hours prior to the placement of a *S. sclerotiorum* mycelial plug in the centre of the plates. Plates were incubated at 22 ºC to allow for growth of the fungus. Three days after the *S. sclerotiorum* plug was added, AF activity was assessed by measuring the zone of

**Table 3.1** Bacterial strains used in Chapter 3 methods and experiments.

| Strain | Relevant characteristics | Source or reference |
|---|---|---|
| *Pseudomonas chlororaphis* | | |
| PA23 | $Phz^{+}Rif^{R}$ wild type (soybean, root) | Savchuk and Fernando, 2004 |
| PA23-314 | $Phz^{-}$ $Rif^{R}$ *gacS*::Tn5-OT182 genomic fusion | Poritsanos *et al*., 2006 |
| PA23*rpoS* | PA23 with pKNOCK-Tc vector inserted into *rpoS*, $Tet^{R}$ | Selin *et al*., 2012 |
| PA23-6863 | PA23 carrying pME6863, $Tet^{R}$ | Selin *et al*., 2012 |
| PA23*phzR* | PA23 with $Gm^{R}$ marker inserted into *phzR*, $Gm^{R}$ | Selin *et al*., 2012 |
| *Pseudomonas brassicacearum* | | |
| DF41 | $Rif^{R}$ wild type (canola, root tip) | Savchuk and Fernando, 2004 |
| *Bacillus amyloliquefaciens* | | |
| BS6 | $Rif^{R}$ wild type (canola, leaf) | Zhang, 2004; Fernando *et al*., 2007 |

clearing between the edge of the bacterial colony and the advancing tips of fungal mycelia growth. Twelve replicates of each tryptophan concentration were analyzed per experiment. The experiment was completed three times.

### 3.3.3. Antifungal assays to assess biocontrol of *S. sclerotiorum*, *P. ultimum*, and *R. solani.*

Radial diffusion assays were performed to assess the AF activity of bacterial strains against different fungal pathogens, similar to section 3.3.2. PA23, DF41 and BS6 were grown overnight in LB media. Overnight cultures (5 µL) were spotted onto 1/5 PDA for *P. ultimum* and *S. sclerotiorum*, and on PDA for *R. solani*. The zones of clearance were measured after 2 days, 3 days or 10 days, respectively. Six replicates of each bacterial strain were analyzed for each fungal species and the experiment was completed three times in total.

### 3.3.4. Gnotobiotic analysis of plant growth promoting ability

*B. napus* seeds (Westar) were sterilized in 70% ethanol by soaking seeds for 15 minutes, after which they were rinsed 2 times in 95% ethanol and allowed to dry in a laminar flow hood. After sterilization, seeds were placed on an agar plate and incubated at 4 $^{o}$C for 4 days for vernalisation, to ensure even germination. PA23, DF41, and BS6 were grown overnight in LB media, after which they were centrifuged at 6000 rpm for 10 minutes, and the cell pellets were resuspended in $dH_2O$. Cultures were adjusted to $10^5$, $10^7$, and $10^9$ cfu/mL for each bacterial strain; water was included as a negative control. Sterilized, vernalized seeds were added to the bacterial preparations and allowed to shake at 28 $^{o}$C for 1 hour. Seeds were then placed in CYG seed germination pouches (Mega International, West St. Paul, USA). The pouches were arranged in a random order and stored in a sterile, dark environment at room temperature. After 3 days,

un-germinated seeds were removed. Five days post seeding, root length and shoot length were measured. Each treatment had 3 pouches and each pouch had 8 seeds. The experiment was completed 3 times in total.

### 3.3.5. Plant growth promoting ability in soil

Bacterial cultures of PA23, DF41, and BS6 were grown overnight in 30 mL of M9 minimal media supplemented with 1 mM $MgSO_4$ and 0.2% glucose. Cells were pelleted and resuspended in $dH_2O$ to a concentration of 1 x $10^9$ cfu/mL. A consortium culture was also created, that contained 1 x $10^9$ cfu/mL total bacteria, consisting of equal parts PA23:DF41:BS6. Ethanol sterilized seeds (see 3.1.4) were placed in the cultures and the $dH_2O$ negative control and allowed to shake at 28 $^oC$ for 1 hour. Cell flats of sterilized soil (Sunshine Advanced Growing Mix #4, Sun Gro Horticulture, Agawam, MA) were prepared. The cell flats were organized in a completely randomized design (CRD) and placed in a growth chamber (16h/8h, 22$^oC$/16$^oC$). After 2 weeks, the seedling roots were washed to remove soil and stained with 1% w/v toluidine blue. Roots were placed in glycerol on a clear glass plate and manipulated with tweezers to ensure all roots were clearly separated. Subsequently, they were scanned against a white background using a flatbed scanner (HP Scanjet 8300, Hewlett-Packard, Mississauga, ON). Assess 2.0 imaging software (American Phytopathological Society, St. Paul, USA) was used to determine total root length and area. The primary root length was also measured. The experiment was repeated once with 3 cell flats for each treatment containing 6 seeds in each experiment.

### 3.3.6. Population analysis of PGP bacteria

Bacteria were prepared and planted as above (section 3.1.5). After two weeks, loose soil was shaken from the seedling roots and each cell-flat was divided into two sections. Roots and tightly adherent soil were weighed, after which they were placed in a 50 mL conical tube. Twently mL of $dH_2O$ was added and the samples were vortexed vigorously for 1 minute. The resulting solution was plated onto LB plates that included rifampicin (50 µg/mL) and the fungicide Azoxystrobin (1 µg active ingredient [a.i.]/mL; Quadris, 250 g/L a.i.; Syngenta Canada Inc. Guelph, ON) by an Autoplate 4000 spiral plater (Spiral Biotech Inc, Norwood, MA). Colonies were counted with an ACOLyte colony counter 7500 (Synbiosis, Frederick, MD) to find the CFU / g of roots and closely adherent soil. The experiment was completed 3 times, with 3 cell flats of each treatment, each producing two samples.

### 3.3.7. Assay for ACC deaminase

The protocol for testing for ACC deaminase was followed according to Penrose and Glick (2003). Briefly, PA23, DF41, and BS6, were grown overnight in sterile DF salts minimal medium containing 2 g/L $(NH_4)_2SO_4$ as the nitrogen source. Ten µL of this culture was then transferred into DF salts minimal media containing 3 mM ACC and grown for 48 hours. As a negative control, the overnight culture was also transferred into DF salts media containing no nitrogen source. Cultures with an optical density below 0.05 at 600 nm were considered not to have grown.

### 3.3.8. IAA assay

The IAA assay was done according to Patten and Glick (2002). Briefly, 5µL of an overnight culture was transferred into 5 mL media containing filter sterilized L-tryptophan

(Sigma-Aldrich, St. Louis, USA). Following growth for 48 hours, the spectrophotometric density of each culture was measured at 600 nm. Bacterial cells were then removed from the culture media (12000 rpm for 5 minutes) and 1 mL of supernatant was mixed vigorously with 4 mL of Salkowski's reagent (150 mL of 1 M $H_2SO_4$, 250 mL $dH_2O$, 15 mL of 0.25 M $FeCl_3 \bullet 6H_2O$). After standing at room temperature for 20 minutes, the absorbance was read at 535 nm with a spectrophotometer. The concentration of IAA in each culture was determined by comparison with a standard curve generated using purified IAA (Sigma-Aldrich, St. Louis, USA). The IAA assay was performed on PA23, DF41, and BS6 cultures grown in either M9 minimal media or DF salts minimal media. Overnight cultures grown in the same media were sub-cultured into fresh media containing 0, 100, or 300 µg/mL of L-tryptophan. Standard curves were prepared in both M9 and DF salts minimal media. Three samples were used for each tryptophan concentration and the experiment was repeated three times. In addition to the PA23 wildtype, PA23*phzR*, PA23-6863, PA23*rpoS*, and PA23-314 were all tested in both M9 and DF salts media using L-tryptophan concentrations of 0 and 300 µg/mL. Three samples were used for each treatment in the experiment and the experiment was repeated two additional times.

## 3.4. RESULTS

### 3.4.1. Fungal inhibition by biocontrol bacteria

DF41 had the greatest ability to inhibit mycelial growth of *S. sclerotiorum* and *R. solani,* creating zones of inhibition with radiui of 7.8 mm and 9.3 mm, respectively (Figure 3.1). In contrast, PA23 produced the greatest inhibition of *P. ultimum,* with a zone of inhibition of 3.3 mm. BS6 had very limited control of any of the fungal pathogens, with its largest zone of inhibition being 0.1 mm for any of the fungi.

### 3.4.2. Gnotobiotic analysis of plant growth promoting ability

The effect of the PA23, DF41, and BS6 on *B. napus* seedling growth was tested under controlled conditions (Figure 3.2). PA23 had a significant effect on *B. napus* root growth, with all concentrations of bacteria significantly influencing the primary root length. No significant effect was observed on shoots. DF41 had a positive effect on root growth with bacterial concentrations of $1 \times 10^5$ CFU/mL, but high bacterial concentrations of $1 \times 10^9$ CFU/mL appeared to shorten root lengths. DF41 had an overall negative effect on shoot length; length was negatively associated with bacterial concentration. A positive effect was observed when BS6 was inoculated onto *B. napus* seeds. Root length increased, though only significantly with high culture concentrations. BS6 had no significant effects on shoot length.

### 3.4.3. Plant growth promoting ability in soil

After treating seeds in the same manner as used for gnotobiotic analysis, measurements were taken after 14 days under greenhouse conditions. Because the previously observed growth

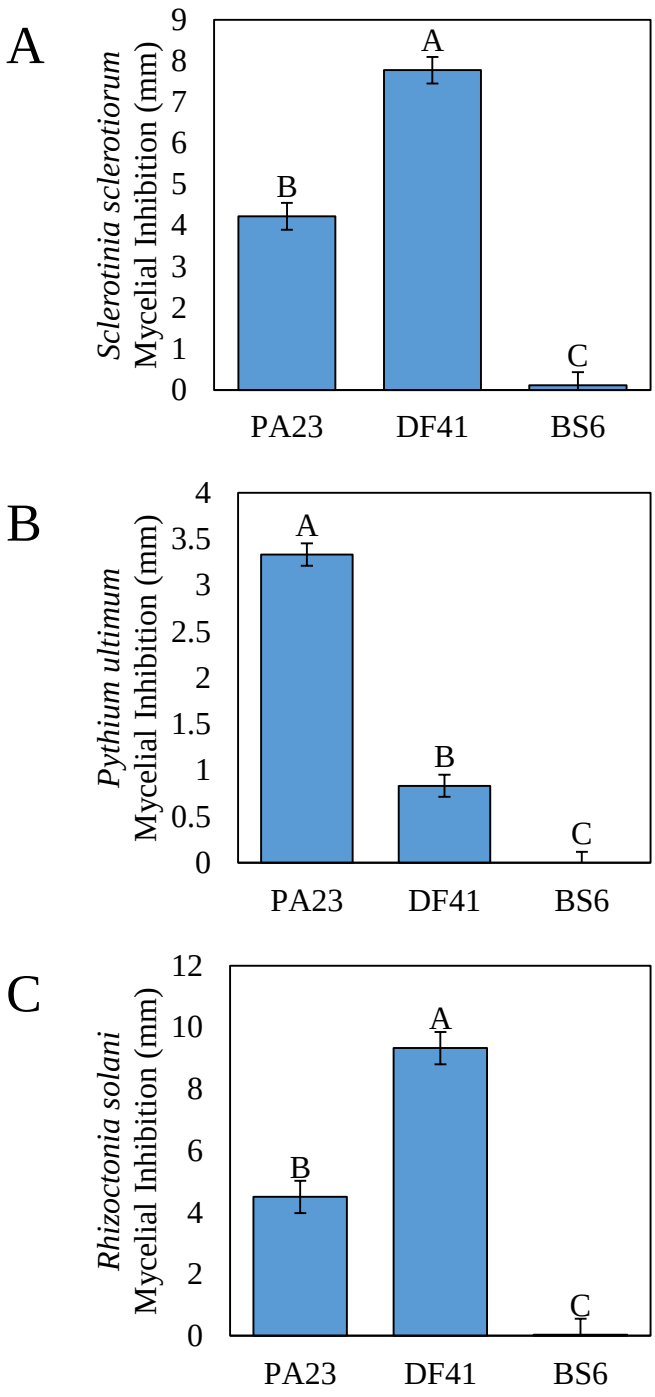



**Figure 3.1** Radial diffusion assays to assess to ability of *P. chlororaphis* PA23, *P. brassicacearum* DF41, and *B. amyloliquifaciens* BS6 to inhibit the mycelial growth of (A) *S. sclerotiorum*, (B) *P. ultimum*, and (C) *R. solani*. Values are means of three experiments, each with 6 biological replicates per treatment. Values with different letters are significantly different ($P < 0.05$) using Tukey's post-hoc test for multiple variance. Error bars represent the standard error of the mean.

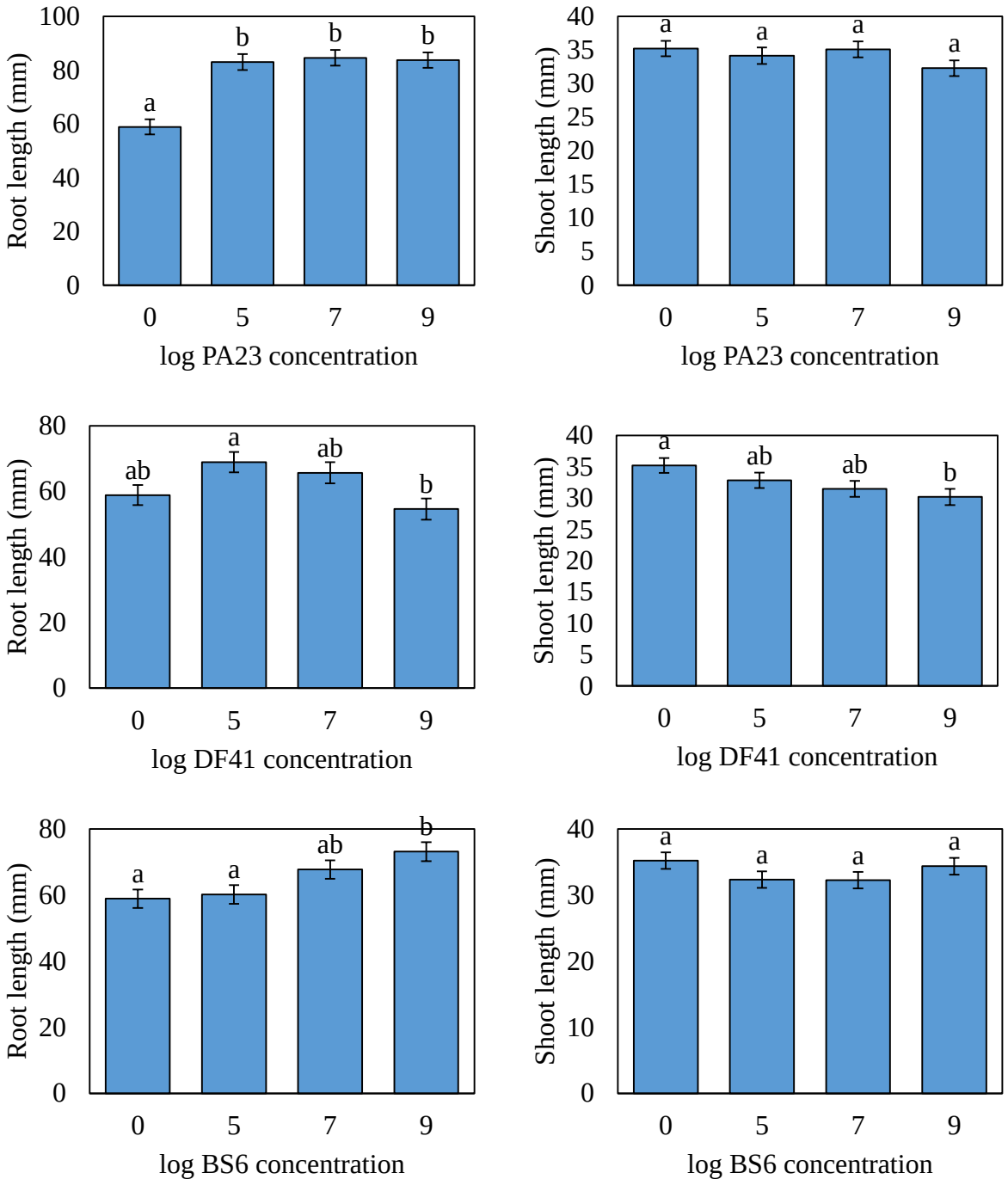



**Figure 3.2** The effect of plant growth promotion on *B. napus* seedling root and shoot length in gnotobiotic conditions. Log bacterial concentration refers to the bacterial concentration in which seeds were soaked in prior to placement in growth pouch. *P. chlororaphis* PA23 influence on (A) root length and (B) shoot length, *P. brassicacearum* DF41 influence on (C) root length and (D) shoot length, *B. amyloliquifaciens* BS6 influence on (E) root length and (F) shoot length. Values are means of three experiments, each with 3 pouches which contained 8 seeds for each treatment. Values with different letters are significantly different ($P < 0.05$) using Tukey's post-hoc test for multiple variance. Error bars represent the standard error of the mean.

promotion was seen mainly in the root system, total and primary root length were measured, as well as root area (Figure 3.3). There were no significant differences seen in primary root length between the control, which had no bacteria inoculated, compared with the bacterial treatments. In contrast, all bacterial treatments caused a decrease in total root length, though only DF41 reduced length to a significant level. This result was also seen when root area was measured, with PA23, DF41, and BS6 all reducing root area by significant measures, while a consortium of the bacteria did not reduce the area by a significant amount. The ratio of total root length divided by primary root length provided significant results of DF41 compared to the control. Based off the significance of the total root length, this would primarily be due to a reduction of lateral root growth.

### 3.4.4. Population analysis of PGP bacteria in soil

Bacteria were washed off *B. napus* roots and closely associated soil in order to find the number of bacteria surviving in the rhizosphere (Figure 3.4). DF41 grew at the highest density on *B. napus* roots, reaching $1.2 \times 10^8$ CFU/ g of root material. The consortium of bacteria reached intermediate populations levels of $8.9 \times 10^7$ CFU/ g of root material. PA23 and BS6 had lower population levels, growing to $6.1 \times 10^7$ and $6.7 \times 10^7$ CFU/ g of roots and closely associated soil, respectively. Visualizing the consortium plates, we could see a mix of orange and white cell colonies, indicating that a mixture of bacterial species was present, but this was not analyzed quantitatively.

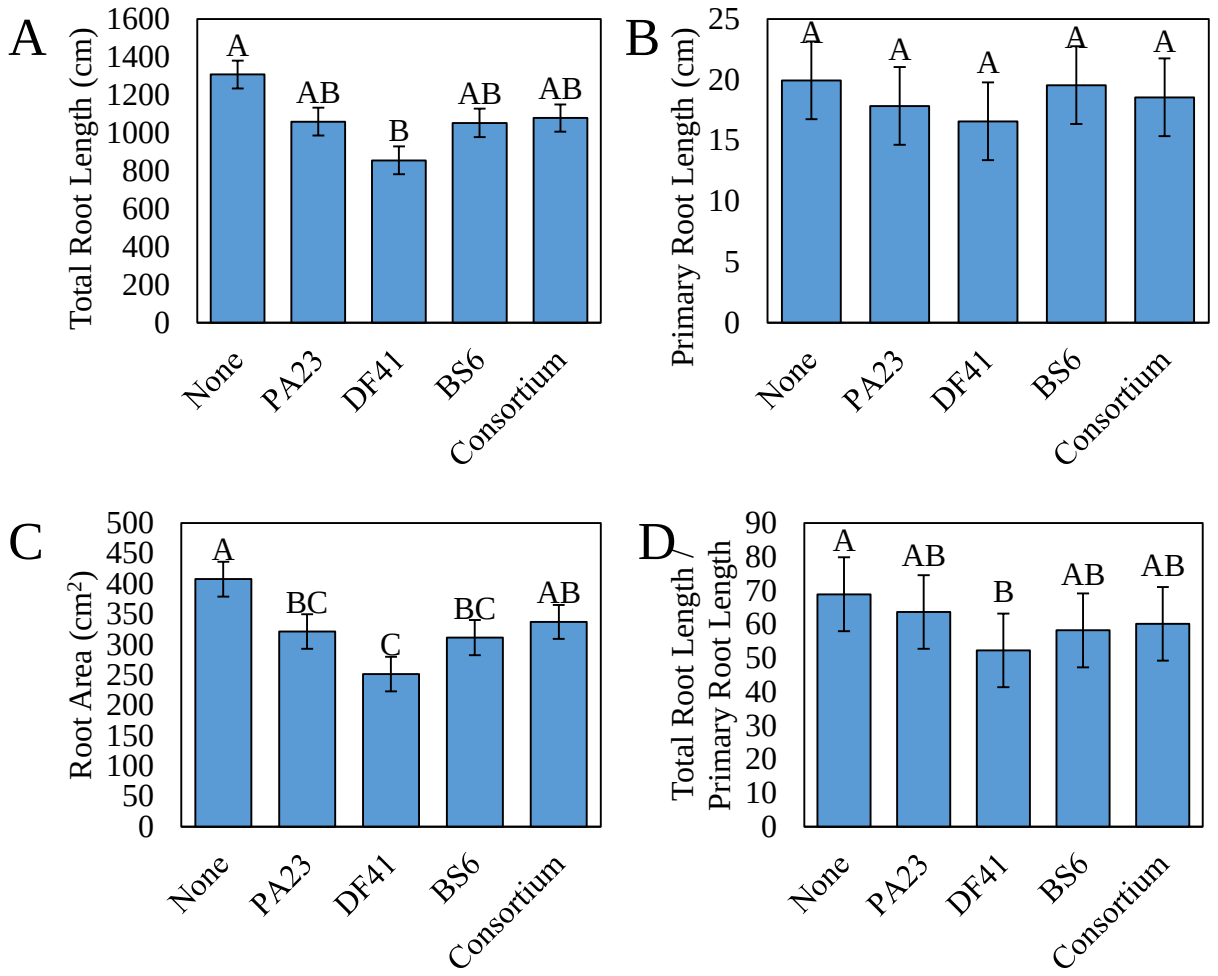



**Figure 3.3** The effect of PGP on root length and area by *P. chlororaphis* PA23, *P. brassicacearum* DF41, *B. amyloliquefaciens* BS6, and a consortium of PA23, DF41, and BS6. Areas measured were: (A) total root length, (B) primary root length, (C) root area, and (D) ratio of total root length to primary root length. Values are means of two experiments, each with 18 biological replicates per treatment. Values with different letters are significantly different ($P < 0.05$) using Tukey's post-hoc test for multiple variance. Error bars represent the standard error of the mean.

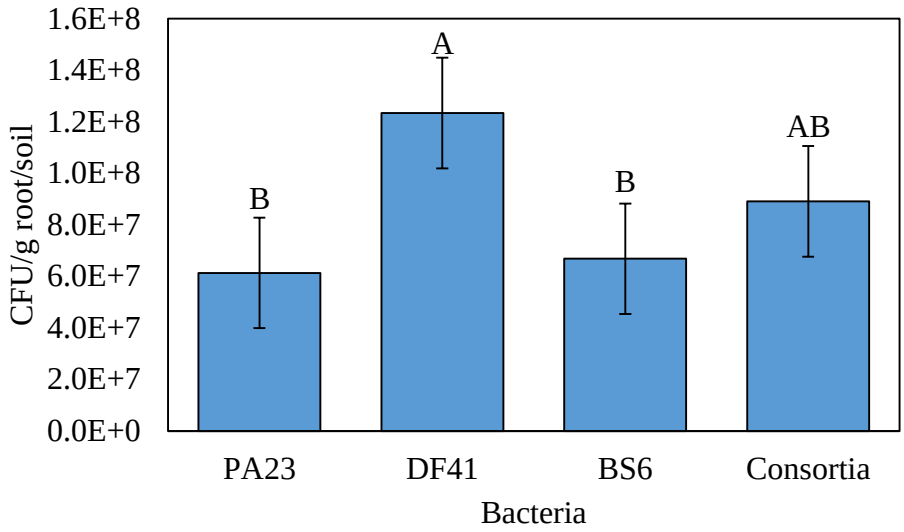



**Figure 3.4** Analysis of bacterial populations of *P. chlororaphis* PA23, *P. brassicacearum* DF41, *B. amyloliquefaciens* BS6, and a consortium of PA23, DF41, and BS6 on *B. napus* seedling roots 2 weeks post seed treatment and planting. Values are means of three experiments, each with 6 biological replicates per treatment. Values with different letters are significantly different ($P < 0.05$) using Tukey's post-hoc test for multiple variance. Error bars represent the standard error of the mean.

### 3.4.5. Assay for ACC deaminase

The assay for ACC deaminase is simply the observation of whether bacteria can grow in media that has ACC as the sole nitrogen source. ACC deaminase was detected in DF41 through its growth on DF salts media containing ACC. Both PA23 and BS6 were unable to grow on this media, demonstrating that they did not have functional ACC deaminase enzymes.

### 3.4.6. IAA assay

A spectrophotometric assay was performed for the presence of IAA in the culture media of PA23, DF41, and BS6 under varying levels of tryptophan (Figure 3.5). The strains had a significant effect (F value= 36.57, Pr>F= <0.0001, DF= 2, 140), as well as tryptophan concentrations (F value= 4.34, Pr>F= <0.0148, DF= 2, 140) and media (F value= 39.46, Pr>F= <0.0001, DF= 1, 140). In M9 minimal media, all bacteria show the trend of IAA concentration increasing as the tryptophan concentration increases. DF41 and BS6 also show this trend in DF salts minimal media, but PA23 shows the opposite trend- IAA production drastically drops with the addition of tryptophan to culture media. Overall, IAA production is higher in M9 minimal media than in DF salts.

### 3.4.7. Influence of tryptophan on PA23 AF activity

When PA23 was grown in DF salts media, the color production, related to the production of PHZ, varied. When no tryptophan was present the culture was orange, while when tryptophan was present the culture was white. It was of interest whether this change in secondary metabolites reflected a change in the AF activity of the PA23. These cultures were used in radial diffusion assays, to observe if they had different levels of inhibition of *S. sclerotiorum* mycelia.

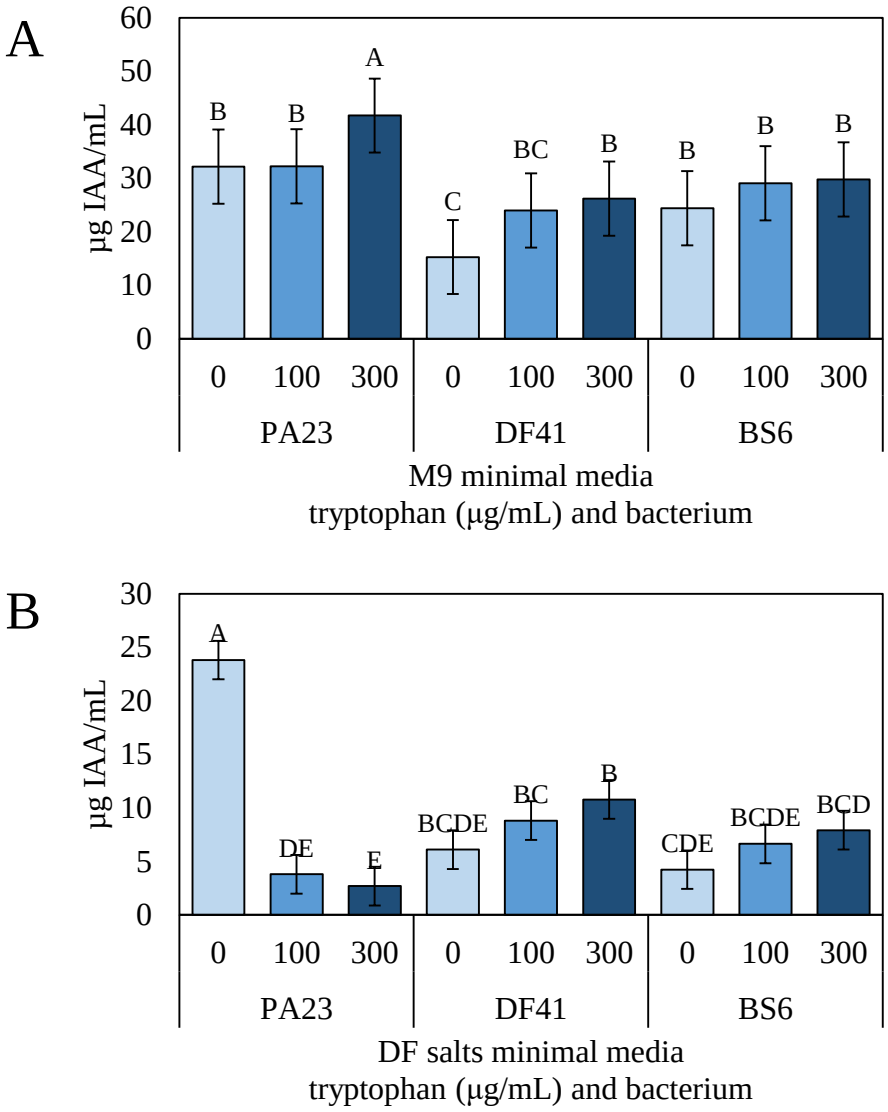



**Figure 3.5** IAA production by *P. chlororaphis* PA23, *P. brassicacearum* DF41, and *B. amyloliquefaciens* BS6 under varying levels of tryptophan in (A) M9 minimal media and (B) DF salts minimal media. Values are means of three experiments, each with 3 biological replicates per treatment. Values with different letters are significantly different ($P < 0.05$) using Tukey's post-hoc test for multiple variance. Error bars represent the standard error of the mean.

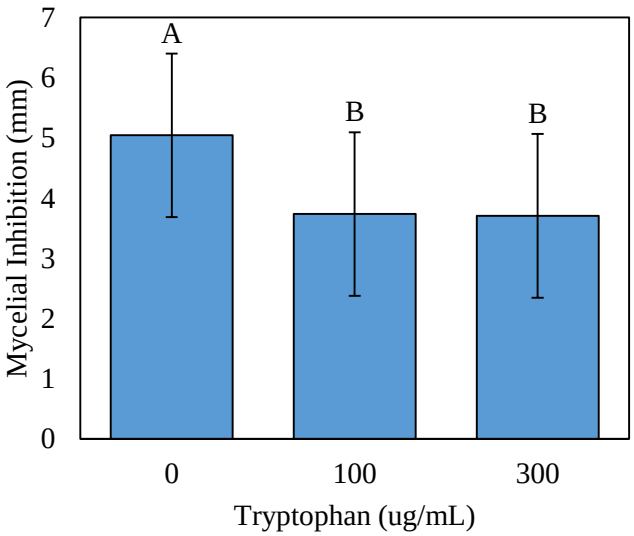



**Figure 3.6** Radial diffusion assays to assess the ability of *P. chlororaphis* PA23 to inhibit *S. sclerotiorum* mycelial growth in the presence of tryptophan. Values are means of three experiments, each with 10 biological replicates per treatment. Values with different letters are significantly different ($P < 0.05$) using Tukey's post-hoc test for multiple variance. Error bars represent the standard error of the mean.

As seen in Figure 3.6, the addition of tryptophan to DF salts media caused a decrease in inhibition of *S. sclerotiorum* mycelia.

### 3.4.8. PA23 mutants IAA assay

Mutants in *rpoS*, *gacS*, *phzR*, and *phzI*, were tested to see if their production of IAA was different than that of wild-type PA23. IAA production was influenced by the PA23 isolates (F= 31.1, Pr>F= <0.0001, DF= 4, 157). It was seen in Figure 3.7 that in both M9 and DF salts minimal medias, the production of IAA by the *rpoS* knockout mutant is not significantly different from that of wild type. In M9 minimal media, the remaining Δ*gacS*, *ΔphzR*, and PA23(pME6863) strains appeared to lower production of IAA. Interestingly, in DF salts media, when no tryptophan was present, *gacS*, *phzR*, and PA23(pME6863) exhibited lowered IAA production, but when tryptophan was present, the *phzR* and AI-deficient strains once again caused an increase in IAA production compared to when no tryptophan was present.

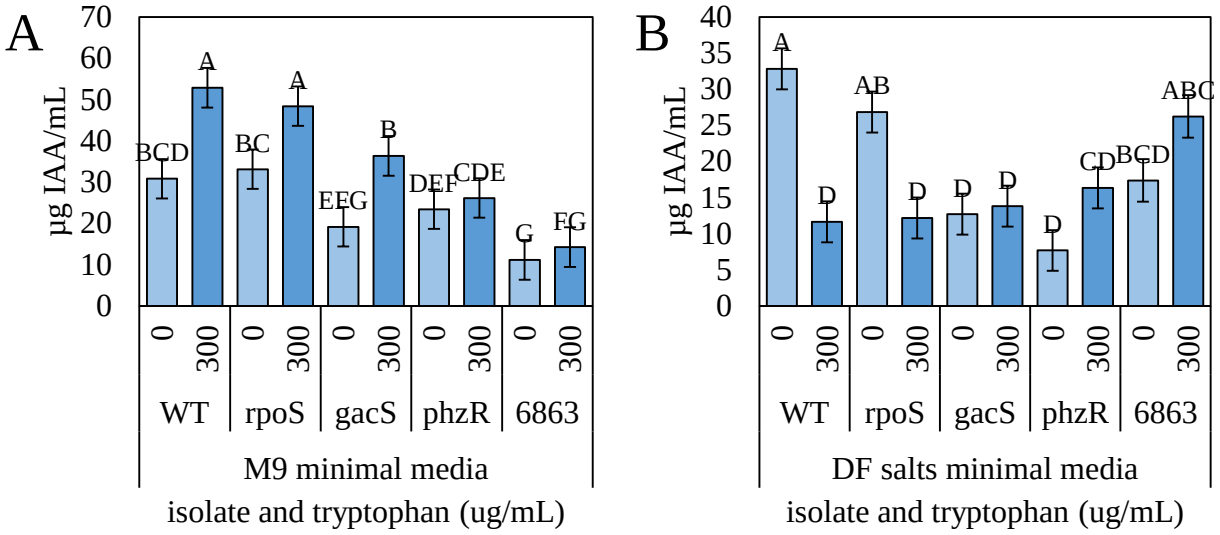



**Figure 3.7** Analysis of *P. chlororaphis* PA23 and derivative strains effect on IAA production in DF salts minimal media with either the absence or presence of tryptophan. Bacterial strains used were PA23 WT (WT), PA23-314 (gacS), PA23*rpoS* (rpoS), PA23*phzR* (phzR), and PA23-6863 (6863). Values are means of three experiments, each with 3 biological replicates per treatment. Values with different letters are significantly different ($P < 0.05$) using Tukey's post-hoc test for multiple variance. Error bars represent the standard error of the mean.

## 3.5. DISCUSSION

This study continues to explore the ability of bacterial biocontrol agents to control fungal pathogens in agriculture. Namely, PA23, DF41, and BS6 were tested for their ability to control *B. napus* pathogens. In addition, these bacterium were tested for their ability to modulate plant growth in the absence of pathogens. In gnotobiotic growth pouches, PA23 stimulated *B. napus* seedling root growth to the greatest level, but in a soil system PA23 had a negative effect on growth. Due to these differences in root growth in pouches versus soil systems, bacterial hormones were the suspected as modulating seedling growth. DF41 was found to have the enzyme ACC deaminase, while PA23 and BS6 did not. All the bacteria produced IAA, but PA23 produced the greatest levels.

PA23 demonstrated the greatest ability to inhibit *P. ultimum* mycelial growth. *Pythium* spp. is a diverse genus of oomycete fungi, of which many species are plant pathogens (Martin and Loper, 1999; Bardin *et al*., 2003). *P. ultimum* is known to infect a number of economically valuable crops, including canola (Martin and Loper, 1999). Biocontrol agents have been previously tested against *Pythium* spp., with successful BCAs including many *Pseudomonas* and *Bacillus* species (Nakkeeran *et al*., 2006; Loper, 1988; Martin and Loper, 1999). Siderophores have been implicated in playing a major role in *Pythium* biocontrol (Loper, 1988; Buysens *et al*., 1996). Antibiotics produced by BCAs, including pyoluteorin and 2,4-diacetylphloroglucinol, have also been shown to contribute to controlling *Pythium* spp. (Martin and Loper, 1999). As PA23 produces both siderophores and antibiotics, this high level of control is expected. (Poritsanos *et al*., 2006). DF41 and BS6 both currently have not been reported to produce siderophores.

*Rhizoctonia solani* is another common fungal pathogen of canola root systems (Kataria and Verma, 1992). When *P. cepacia* strain 5.5B was tested as a BCA against *R. solani*, it provided control mainly through the antibiotics PRN and PHZ (Cartwright *et al*., 1995). Research on *P. cepacia* R55 and R85 as well as *P. putida* R104 biocontrol of *R. solani* suggested antibiosis activity to contribute to plant protection (Renato de Freitas and Germida, 1991). *B. subtilis* RB14 showed antibiotic activity, specifically iturin A and surfactin, as a method of control of *R. solani* (Asaka and Shoda, 1996). DF41 showed the greatest control of *R. solani* mycelial growth, as well as *S. sclerotiorum*. This could be due to either greater production of antibiotics or the production antibiotics of greater strength.

While BCAs are initially tested for *in vitro* inhibition of pathogens, such as radial diffusion assays, this does not necessarily reflect the bacteria's ability to suppress disease *in planta*, in a greenhouse or field setting. This lab work hints at how BCAs may manage disease, especially mechanisms such as antibiosis or enzymes, but certain methods of control, such as competition, can't be mimicked in a test tube. Also, the BCAs may be ecologically unsuited to environmental conditions in which the pathogens impart the greatest damage. It would be recommended that in the future PA23 and DF41 be tested for the control of *P. ultimum* and *R. solani* in a soil system on plant roots.

We know that PA23, DF41, and BS6 play a role in indirect promotion of plant growth through their production of secondary metabolites to control plant pathogens, most notably through antibiotics (Selin *et al*., 2010; Berry *et al*., 2010; Athukorala *et al*., 2009). We were interested in studying whether they also affect plant growth directly. The first study we undertook was determining if the bacteria had an influence on *B. napus* seedling growth under gnotobiotic, controlled conditions as initially described by Lifshitz and colleagues in 1987. PA23

showed the greatest promotion of plant growth, with all concentrations of bacteria causing a significant increase in root length of approximately 30%. BS6 also promoted the length of *B. napus* roots, but only to significant levels at high bacterial concentrations. DF41 had no significant changes in root length compared to the wild type. PA23 and BS6 both had no effect on shoot length while DF41 reduced the length of shoots, but only to a significant measure at high bacterial concentrations. While these results, particularly with PA23, demonstrated increase in *B. napus* seedling length, it was slightly less than the 35-50% increase demonstrated by other PGP bacteria (Lifshitz *et al*., 1987; Glick *et al*., 1997, Patten and Glick, 2002). The experiment used in this study used room temperature, moderate light, and water for solution. Adding environmental plant stresses, such as salt, heavy metals minerals, or polycyclic aromatic hydrocarbons have been shown to allow PGPB to increase seedling length and health compared to un-inoculated controls (Gamalero *et al*., 20010; Gamalero *et al*., 2009; Reed and Glick, 2005)

Seeing that the bacteria had an effect on *B. napus* seedling length in gnotobiotic pouches, it was of interest whether these trends would be reflected in a soil system. While primary root length wasn't affected to significant measures, the total root length was. DF41 had a significantly smaller root system than water-treated seeds while PA23, BS6, and a consortium of the three bacteria appeared to reduce the total root length though not to statistically significant measures. These results were mirrored when root area was measured, with the exception that PA23 and BS6 now had significantly less root area. It was surprising that the trends in growth pouches were not reflected in a soil system. When *P. putida* GR12-2 was inoculated in a similar manner on *B. napus* seeds, it produced increased root growth in both pouches and in soil (Glick *et al*., 1997). A possible reason for a decrease in root length in a soil system compared to a growth pouch includes the difference in time at which measurements were taken. With the growth

pouches, measurements were taken prior to lateral root formation while the root system in the soil was considerably more developed. If the bacteria helped with germination, this would lead to significant increases in root length at 5 days post seeding. Having the seeds germinate on day 3 versus day 4 would show increased root length. This would be hidden the farther from the seeding date the measurements were taken. Increases in germination by PGP have previously been observed (Fages and Arsac, 1991; Polyanskaya *et al*., 2000; Gholami *et al*., 2009).

Beneficial *Pseudomonas* strains have previously been shown to alter *Arabidopsis* root development; for example, *P. fluorescens* WCS417 enhanced the auxin response of the root, reducing primary root length but enhancing lateral roots and root hairs (Zamioudis *et al*., 2013). As such, it was of interest whether PA23, BS6, and DF41 would have an effect on the ratio of total root length to primary root length. All of the ratios for *B. napus* seedlings that had been inoculated with bacteria were lower than the control, demonstrating that these bacteria did not increase lateral root development compared to primary root length.

When in a soil system, we were interested in the population numbers of the bacteria on the root system. After 14 days of growth, seedlings and closely associated rhizosphere soil were measured for bacteria. All of the bacteria survived in the soil system for 14 days, but attained different population levels. DF41 had approximately double the number of viable cells compared to PA23 or BS6 per gram of root and rhizosphere soil. The consortium of all three bacterial species included an intermediate number of viable cells. With DF41's high level of growth and negative effect on root length and area, DF41 demonstrates the preliminary signs of being a plant pathogen in the absence of disease.

The consortium of bacteria appeared to hamper root growth to a lesser degree, as seen by the greater root area than singularly inoculated bacterial species. This hints at a synergistic

interaction between the different bacterial species. There is a current trend to mix BCAs of diverse microbial species to achieve increased efficacy, reliability and consistency under diverse soil and environmental conditions (Stockwell *et al*., 2011; Surma *et al*., 2015). This is due to ability of compatible microbes to utilize different methods of promoting plant growth as well as preferring different environments, thus ensuring that at least one microbe will be able to promote plant growth either directly or indirectly (Surma *et al*., 2015). We were unable to quantitatively differentiate the bacterial species and strains in the consortium, so cannot conclude if the bacteria had a positive or negative effect on one another's growth. That said, by visualizing the bacteria, PA23 colonies have a distinct orange color that could be seen and there were also white colonies present, demonstrating that multiple species of bacteria were surviving and were thus compatible.

With the bacteria modulating plant growth, both in growth pouches and in greenhouse conditions, there was interest in finding the mechanism(s) that cause the changes. Often, a PGPR does not simply depend upon one mechanism for promoting plant growth, but rather one or more mechanisms are employed (Glick, 1995). Due to the fact that root growth was modulated differently in gnotobiotic growth pouches compared to a soil system, plant hormones may play a role.

When assayed for the degradative enzyme ACC deaminase, DF41 had the enzyme while PA23 and BS6 did not. Following germination, ethylene inhibits root elongation (Jackson, 1991). Bacterial ACC deaminase can cleave the precursor of ethylene, ACC, which lowers the ethylene in the associated plant (Glick, 1995; Glick, 2005). Keeping ethylene levels in roots low can enhance the survival of seedlings and protect stressed plants from the deleterious effects of ethylene (Glick, 1995). This may partially explain why DF41 had a beneficial effect on the

seedlings in the root pouches and not in a soil system. In a growth pouch with just water, the seedlings would have been stressed with the lack of nutrients and sunlight, producing ethylene. DF41 could cleave the ethylene precursor, thus lowering ethylene levels from a deleterious level, and promoting the growth of the seedlings.

Indole-3-acetic acid (IAA) producing bacteria have previously been found to promote plant growth (Patten and Glick, 2002; Idris *et al*., 2007). As such, an assay for IAA was performed to see if our bacteria of interest were producing this plant hormone. PA23, DF41, and BS6 all produced IAA, with PA23 producing the highest levels. IAA production is common among rhizosphere bacteria and has previously been found in both *Pseudomonas* and *Bacillus* sp. (Patten and Glick, 1996; Idris *et al*, 2007). Ali and colleagues (2009) reported *Bacillus* spp. producing levels from 14.3 – 92.7 µg/mL of IAA and *Pseudomonas* spp. producing 32.4 – 106.0 µg/mL IAA. Bharucha and colleagues (2013) reported production levels of over 500 µg/mL IAA with a *P. putida* isolate, while other unidentified bacterial cultures also had triple digit levels. As the levels are moderate in our species, while IAA production likely plays a role in promoting plant growth, it may not be the whole story.

IAA is known to increase in levels as tryptophan concentrations increase (Patten and Glick, 2002; Spaepen *et al*., 2007; Goswami *et al*., 2013). This is because the majority of IAA production pathways start with the amino acid tryptophan and use one to two intermediates, with corresponding enzymes, to create IAA (Patten and Glick, 1996; Spaepen *et al*., 2007). In M9 minimal media, all bacteria demonstrated this correlation- an increase in precursor led to an increase in product. Surprisingly, in DF salts minimal media, while DF41 and BS6 demonstrated this trend, PA23 revealed the opposite. The presence of tryptophan dramatically decreased

production of IAA. This is the first observation of the addition of tryptophan decreasing IAA production, as far as we could find.

In addition to observing decreased IAA production in the presence of tryptophan when PA23 was in DF salts media, the color of the media was also changed. When no tryptophan was present, the media turned orange, indicative of PA23's production of PHZ, an antibiotic involved in both biofilm formation and biocontrol. When tryptophan was added, the culture media stayed white. Because the regulation of biocontrol metabolites in PA23 is complex, with an interconnected regulatory cascade governing AF metabolite production (Selin *et al*., 2014), we suspected that if tryptophan decreased PHZ production, that AF activity would also be decreased. Radial diffusion assays using these cultures confirmed this suspicion- the presence of tryptophan with DF salts media significantly decreased the mycelial inhibition of PA23. This observation indicated that PRN, the primary inhibiting antibiotic produced by PA23 (Selin *et al*., 2012), may also be downregulated when tryptophan was present. As tryptophan is also the precursor of PRN (Kirner *et al.*, 1998), it is surprising that the addition of tryptophan would cause a decrease of AF activity. Due to multiple AF metabolites likely being produced at lower levels, it may be that tryptophans addition to DF salts media decreases expression of suppressive compounds.

To explore the possibility of a regulator being affected by tryptophan, IAA production by PA23 mutants was assayed. Both M9 and DF salts minimal media were used, and IAA was measured in the presence and absence of tryptophan. In M9 minimal media, all the mutants showed the expected trend of IAA production increasing with the addition of tryptophan. The *rpoS* mutant, a knock-out mutant for the RpoS stationary-phase sigma factor, demonstrated the same production of IAA as wild-type PA23, indicating it wasn't involved in IAA regulation. The

other mutants, *gacS*, *phzR,* and the AI-deficient PA23(pME6863), all showed a lower level of IAA production, hinting that they may play a role in stimulating IAA production. GacS is a sensor kinase that senses an environmental signal and activates GacA, which regulates transcription (Heeb and Haas, 2001). PhzR is a transcriptional regulator involved in quorum sensing, while PME6863 encodes a lactonase enzyme that degrades acylated homoserine lactones (AHLs), which collect and cause a change in gene expression once a minimal threshold concentration is reached (Miller and Bassler, 2001)

In DF salts minimal media, IAA production was once again unaffected by the *rpoS* mutant when compared to wild type. Both show the trend of IAA levels dramatically decreasing with the addition of tryptophan. This indicates that the RpoS stationary phase sigma factor, which is known to control many secondary metabolites under stress or starvation conditions, isn't controlling the secondary metabolite IAA. The *gacS* mutant showed low levels of IAA production, both in the presence and absence of tryptophan. Of particular interest, when *phzR* or AHLs were knocked out, both of which are involved with quorum sensing, the opposite trend is seen- IAA once again increases with the presence of tryptophan. The PhzR/PhzI quorum sensing appears to be responsible for the decrease of IAA production of PA23 in DF salts minimal media with tryptophan. Interestingly, previous work has found that IAA production was enhanced by the overproduction of RpoS, the one mutant studied that didn't affect IAA production (Saleh and Glick, 2001; Patten and Glick, 2002a; Spaepen *et al*., 2007). As the PhzI/PhzR quorum-sensing system is also required for PRN and PHZ production (Selin *et al*., 2012), having this system affected by DF salts media and tryptophan explains the observed drop in AF activity as shown by the RDAs.

Because the trend of tryptophan decreasing IAA production is only seen when PA23 is grown in DF salts minimal media, there is a compound in the media that is not present in M9 minimal media that must induce this trend. When comparing the composition of the media (Table 3.2), we can see that there are differences. Four major components (sodium phosphate diabasic, monopotassium phosphate, magnesium sulphate, and glucose) are quite similar between the media. While both media have ammonium and sodium, both of these molecules are compounded with chloride in DF salts compared with sulphate and phosphate, respectively, in M9 minimal media. DF salts has additional compounds when compared with M9 minimal media, namely gluconic and citric acids, iron sulphate, and trace elements. Future analysis should be completed to see which unique compound in DF salts minimal media triggers this response in PA23 with the addition of tryptophan.

**Table 3.2** Composition of M9 minimal media and DF salts minimal media.

| | Concentration (mM) | |
|---|---|---|
| **Compound** | **M9 minimal media** | **DF salts minimal media** |
| Sodium phosphate dibasic | 47.75993 | 42.26543 |
| Monopotassium phosphate | 22.04488 | 29.39318 |
| Magnesium sulphate | 1 | 0.811359 |
| Glucose | 0.20% | 0.20% |
| Ammonium chloride | 18.69508 | N/A |
| Ammonium sulphate | N/A | 15.13546 |
| Sodium chloride | 8.555784 | N/A |
| Gluconic Acid | N/A | 10.19576 |
| Citric Acid | N/A | 10.41016 |
| Iron Sulphate | N/A | 0.003597 |
| Trace Elements | N/A | very low |

N/A: not applicable to the respective media

## 3.6. CONCLUSIONS AND FUTURE DIRECTIONS

Overall, this study continues to expand our knowledge of *Pseudomonas chlororaphis* stain PA23, *Pseudomonas brassicacearum* strain DF41, and *Bacillus amyloliquifaciens* strain BS6, both in their biocontrol abilities and their plant growth modulating capacity. While these strains had previously been used as biocontrol agents in the phyllosphere, this study begins to explore their aptitude for rhizosphere fungal suppression. PA23 showed *in vitro* ability to suppress *Pythium ultimum* while DF41 demonstrated the highest level of *Rhizoctonia solani* control. When no pathogens were present, these bacteria had variable effects on *B. napus* seedling growth. In gnotobiotic growth pouches, the bacteria stimulated root growth, with PA23 having the highest levels of growth promotion. This was reversed with an inhibition of root growth in a soil system. Interestingly, when the bacteria were applied as consortia in the greenhouse, root growth was returned to near negative control measurements. All the bacteria survived for 2 weeks on *B. napus* roots in a soil system. Due to the differences in root growth between growth pouches and soil, it was postulated that the bacteria may be modulating root hormones. DF41 was found to have the enzyme ACC deaminase, which can lower ethylene levels in roots, decreasing ethylene's negative effects on root growth. While all the bacteria produced the auxin IAA, PA23 produced the highest levels, though still quite moderate. While in DF salts media, when tryptophan was added to the media, PA23 had IAA production decrease. This combination of media and tryptophan also caused a decrease in PA23 AF activity. Through mutant analysis, PA23's PhzI/PhzR quorum-sensing has been implicated as being involved in this novel observation.

Further research would be beneficial for many sections of this study. While *R. solani* and *P. ultimum* mycelia were inhibited by our biocontrol agents on agar plates, it is important to see

if this is mimicked in a soil system. Greenhouse and then field studies would be beneficial to see if the BCAs can control these root pathogens of *B. napus* in their native environments. The BCAs may be ecologically unsuited to control the fungal pathogens in the environment where they impart the greatest damage. While the BCAs promoted growth in growth pouches, they decreased growth in a soil system. It would be interesting to see how these effects would translate to an adult plant. Would the biocontrol agents have an effect on flowering time, canola yield, etc.? The consortium appeared to have the least deleterious effects on *B. napus* root growth while in a soil system. As such, further research on the consortium would be valuable. Exploring the effect that bacteria have on one another, if they can work together to control plant pathogens, and the nature of their synergistic effects would all be useful. With respect to the mechanism of plant growth promotion, deeper exploration is needed. We tested for two hormonal mechanisms- additional plant hormone production as well as nutritional mechanisms could be studied in the BCAs of interest. IAA analysis led to interesting observations within PA23. Follow up analysis could include studying PA23's IAA synthesis pathway, quantifying DF salts minimal media and tryptophan's effect on PA23's AF metabolites and regulators, as well as finding the component in DF salts minimal media that leads to a drop in IAA production when tryptophan is present.

# CHAPTER 4

LITERATURE CITED

## 4. LITERATURE CITED


Agriculture and Agri-Food Canada. 2015. An Overview of the Canadian Agriculture and Agri-Food System 2014. Available from http://www.agr.gc.ca/eng/about-us/publications/economic-publications/alphabetical-listing/an-overview-of-the-canadian-agriculture-and-agri-food-system-2014/?id=1396889920372: [cited 27 April 2015].

Ali, B., A.N. Sabri, K. Ljung, and S. Hasnain. 2009. Auxin production by plant associated bacteria: impact on endogenous IAA content and growth of *Triticum aestivum* L. Let. Appl. Microbiol. 48: 542-547.

Alström, S. 1991. Induction of disease resistance in common bean susceptible to halo blight bacterial pathogen after seed bacterization with rhizosphere pseudomonads. J. Gen. Appl. Microbiol. 37: 495-501.

Arshad, M., M. Saleem, and S. Hussain. 2007. Perspectives of bacterial ACC deaminase in phytoremediation. TRENDS in Biotechnol. 25(8): 356-362.

Asaka, O., and M. Shoda. 1996. Biocontrol of *Rhizoctonia solani* damping-off of tomato with *Bacillus subtilis* RB14. Appl. Environ. Microbiol. 62(11): 4081-4085.

Athukorala, S.N.P., W.G.D. Fernando, K.Y. Rashid. 2009. Identification of antifungal antibiotics of *Bacillus* species isolated from different microhabitats using polymerase chain reaction and MALDI-TOF mass spectrometry. Can. J. Microbiol. 55:1021-2032.

Athukorala, S.N.P., W.G.D. Fernando, K.Y. Rashid, and T. de Kievit. 2010. The role of volatile and non-volatile antibiotics produced by *Pseudomonas chlororaphis* strain PA23 in its root colonization and control of *Sclerotinia sclerotiorum*. Biocontrol Sci. Technol. 20(8): 875-890.

Bailey, K.L., S.M. Boyetchko, and T. Längle. 2010. Social and economic drivers shaping the future of biological control: A Canadian perspective on the factors affecting the development and use of microbial biopesticides. Biological Control. 52:221-229.

Bardin, S.D., H.C. Huang, L. Liu, and L.J. Yanke. 2003. Control, by microbial seed treatment, of damping-off caused by *Pythium* sp. on canola, safflower, dry pea, and sugar beet. Can. J. Plant Pathol. 25(3): 268-275.

Bashan, Y. and P. Vazquez. 2000. Effect of calcium carbonate, sand and organic matter levels on mortality of five species of *Azospirillum* in natural and artificial bulk soils. Biol. Fertil. Soils. 30:450-459.

Beever, R.E., and D.J.W. Burns. 1980. Phosphorus uptake, storage and utilization by fungi. Adv Bot Res. 8:127-219.

Berry, C., W.G.D. Fernando, P.C. Loewen, and T.R. de Kievit. 2010. Lipopeptides are essential for *Pseudomonas* sp. DF41 biocontrol of *Sclerotinia sclerotiorum*. Biol. Control. 55:211-218.

Bertani, I., M. Sevo M. Kojic, and V. Venturi. 2003. Role of GacA, LasI, RhlI, Ppk, Vfr and ClpXP in the regulation of the stationary-phase sigma factor *rpoS*/RpoS in *Pseudomonas*. Arch. Microbiol. 180: 264-271.

Bharucha, U., K. Patel, and U.B. Trivedi. 2013. Optimization of indole acetic acid production by *Pseudomonas putida* UB1 and its effect as plant growth-promoting rhizobacteria on mustard (*Brassica nigra).* Agric Res. 2(3): 215-221.

Bhattacharyya, P.N., and D.K. Jha. 2012. Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. World J. Microbiol. Biotechnol. 28:1327-1350.

Bloemberg, G.V. and B.J.J. Lugtenberg. 2001. Molecular basis of plant growth promotion and biocontrol by rhizobacteria. Cur. Opin. Plant Biol. 4(1): 343-350.

Blumer, C. and D. Haas. 2000. Mechanism, regulation, and ecological role of bacterial cyanide biosynthesis. Arch. Microbiol. 173:170-177.

Bolton, M.D., B.P.H.J. Thomma, and B.D. Nelson. 2006. *Sclerotinia sclerotiorum* (Lib.) de Bary: biology and molecular trains of a cosmopolitan pathogen. Mol Plant Pathol. 7(1):1-16.

Bonaterra, A., J. Camps, and E. Montesinos. 2005. Osmotically induced trehalose and glycine betaine accumulation improves tolerance to desiccation, survival and efficacy of the postharvest biocontrol agent *Pantoea agglomerans* EPS125. FEMS Microbiol. Lett. 250: 1-8.

Bonaterra, A., J. Cabrefiga, J. Camps, and E. Montesinos. 2007. Increasing survival and efficacy of a biocontrol agent of fire blight of rosaceous plants by means of osmoadaptation. FEMS Microbiol. Ecol. 61: 185-195.

Bottani, R., F. Cassán, and P. Piccoli. 2004. Gibberellin production by bacteria and its involvement in plant growth promotion and yield increase. Appl. Microbiol. Biotechnol. 65: 497-503.

Bradley, C.A., R.A. Henson, P.M. Porter, D.G. LeGare, L.E. del Río, and S.D. Khot. 2006. Response of canola cultivars to *Sclerotinia sclerotiorum* in controlled and field environments. Plant Disease. 90(2):215-219.

Bradley, C.A., H.A. Lamey, G.J. Endres, R.A. Henson, B.K. Hanson, K.R. McKay, M. Halvorson, D.G. LeGare, and P.M. Porter. 2006. Efficacy of fungicides for control of sclerotinia stem rot of canola. Plant Disease. 90: 1129-1134.

Braeken, K., M.Moris, R. Daniels, J. Vanderleyden, and J. Michiels. 2006. New horizons for (p)ppGpp in bacterial and plant physiology. Trends Microbiol. 14:45-54.

Britigan, B.E., T.L. Roeder, G.T. Rasmussen, D.M. Shasby, M.L. McCormick, and C.D. Cox. 1992. Interaction of the *Pseudomonas aeruginosa* secretory products pyocyanin and pyochelin

generates hydroxyl radical and causes synergistic damage to endothelial cells. Implications for *Pseudomonas*- associated tissue injury. J. Clin. Invest. 90:2187-2196.

Brown, M.E.. 1974. Seed and root bacterization. Annu. Rev. Phytopathol. 12:181-197.

Browne, P., M. Berret, F. O-Gara, and J.P. Morrissey. 2010. Computational prediction of the Crc regulon identifies genus-wide and species-specific targets of catabolite repression control in *Pseudomonas* bacteria. BMC Microbiol. 10: 300.

Buysens, S., K. Heungens, J. Poppe, and M. Höfte. 1996. Involvement of pyochelin and pyoverdin in suppression of *Pythium*-induced damping-off of tomato by *Pseudomoans aeruginosa* 7NSK2. Appl. Environ. Microbiol. 62(3): 865-871.

Cabrefiga, J., J. Francés, E. Montesinos, and A. Bonaterra. 2011. Improvement of Fitness and Efficacy of a Fire Blight Biocontrol Agent via Nutritional Enhancement Combined with Osmoadaptation. Appl. Environ. Microbiol. 77(10):3174-3181.

Campbell, R. 1989. Biological Control of Microbial Plant Pathogens. Cambridge University Press.

Canola Council of Canada. 2014. Innovation in Action: 2014 Annual Report.

Canola Council of Canada. 2015. Canola Encyclopedia: Diseases. Available from: http://www.canolacouncil.org/canola-encyclopedia/diseases/ [cited 15 September 2015]

Cao, T., V.P. Manolii, S.F. Hwang, R.J. Howard, and S.E. Strelkov. 2009. Virulence and spread of *Plasmodiophora brassicae* [clubroot] in Alberta, Canada. Can J Plant Pathol. 31:321-329.

Cartwright, D.K., W.S. Chilton, and D.M. Benson. 1995. Pyrrolnitrin and phenazine production by *Pseudomonas cepacia*, strain 5.5B, a biocontrol agent of *Rhizoctonia solani.* Appl. Microbiol. Biotechnol. 43:211-216.

Cary, A.J., W. Liu, and S.H. Howell. 1995. Cytokinin action is coupled to ethylene in its effects on the inhibition of root and hypocotyl elongation in *Arabidopsis thaliana* seedlins. Plant Physiol. 107: 1075-1082.

Castric, P.A., 1983. Hydrogen cyanide production by *Pseudomonas aeruginosa* at reduced oxygen levels. Can. J. Microbiol. 29: 1344-1349.

Chancey, S.T., D.W. Wood, and L.S. Pierson 3rd. 1999. Two-component transcriptional regulation of N-acyl-monoserine lactone production in *Pseudomonas aureofaciens*. Appl. Environ. Microbiol. 65(6): 2294-2299.

Chanway, C.P. and F.B. Holl. 1991. Biomass increase and associative nitrogen fixation of mycorrhizal *Pinus contorta* seedlings inoculated with a plant growth promoting *Bacillus* strain. Can. J. Bot. 69: 507-511.

Chater, K.F., and M.J. Bibb. 1997. Regulation of bacterial antibiotic production. In: Biotechnology, vol. 7, Products of Secondary Metabolism. Pp. 57-105. H. Kleinkauf and H. von Dohren, Eds., VCH, Weinheim, Germany.

Chin-A-Woeng, T.F.C., G.V. Bloemberg and B.J.J. Lugtenberg. 2003. Phenazines and their role in biocontrol by *Pseudomonas* bacteria. New Phytologist. 157: 503-523.

Chin-A-Woeng, T.F.C., G.V. Bloemberg, A.J. van der Bij, K.M.G.M. van der Drift, J. Schripsema, B. Kroon, R.J. Scheffer, C. Keel, P.A.H.M. Bakker, H.V. Tichy, F.J. de Bruijn, J.E. Thomas-Oates, and B.J.J. Lugtenberg. 1998. Biocontrol by phenazine-1-carboxamide-producing *Pseudomonas chlororaphis* PCL1391 of tomato root rot caused by *Fusarium oxysporum* f.sp. *radices-lycopersici.* Molecular Plant-Microbe Interactions. 11: 1069-1077.

Collins, D.P., B.J. Jacobsen, and B. Maxwell. 2003. Spatial and temporal population dynamics of a phyllosphere colonizing *Bacillus subtilis* biological control agent of sugar beet cercospora leaf spot. Biological Cont. 26:224-232.

Compant, S., B. Duffy, J. Nowak, C. Clément, and E.A. Barka. 2005. Use of Plant Growth-Promoting Bacteria for Biocontrol of Plant Diseases: Principles, Mechanisms of Action, and Future Prospects. Appl. Environ. Microbiol. 71(9):4951-4959.

Costacura, A. and J. Vanderleyden. 1995. Synthesis of phytohormones by plant-associated bacteria. Crit. Rev. Microbiol. 21:1-18.

Cuddeford, V. 2007. Biocontrol Files, Issue 10, June 2007. Co-published by World Wildlife Fund, the Biocontrol Network, and Agriculture and Agri-Food Canada. Pp. 1-2.

Deikman, J. 1997. Molecular mechanisms of ethylene regulation of gene transcription. Physiol. Plant. 100: 561-566.

De Vleesschauwer, D., and M. Höfte. 2009. Rhizobacteria-induced systemic resistance. Adv. Bot. Res. 51:223-281.

De Vrije, T., N. Antoine, R.M. Buitelaar, S. Bruckner, M. Dissevelt, A. Durand, M. Gerlagh, E.E. Jones, P. Lüth, J. Oostra, W.J. Ravensberg, R. Renaud, A. Rinzema, F.J. Weber, and J.M. Whipps. 2001. The fungal biocontrol agent *Coniothyrium minitans*: production by solid-state fermentation, application and marketing. Appl. Microbiol. Biotechnol. 56: 58-68.

Dixon, G.R. 2009. The occurrence and economic impact of *Plasmodiophora brassicae* and clubroot disease. J Plant Growth Regul. 28:194-202.

Dobbelaere, S., J. Vanderleyden, and Y. Okon. 2003. Plant Growth-Promoting Effects of Diazotrophs in the Rhizosphere. Crit. Rev. Plant Sci. 22(2): 107-149.

Dodd, I.C., N.Y. Zinovkina, V.I. Safronova and A.A. Belimov. 2010. Rhizobacterial mediation of plant hormone status. Ann. Appl. Biol. 157: 361-379.

Dokken-Bouchard, F.L., A.J, Bouchard, J. Ippolito, G. Peng, S.E. Strelkov, C.. Kirkham, and H.R. Kutcher. 2010. Detection of *Plasmodiophora brassicae* in Saskatchewan, 2008. Can Plant Dis Surv. 90, 126.

Domka, J., J. Lee, T.K.Wood. 2006. YliH (BssR) and YceP (BssS) regulate *Escherichia coli* K-12 biofilm formation by influencing cell signaling. Appl. Environ. Microbiol. 72: 2449-2459.

Dowling, D.N. and F. O'Gara. 1994. Metabolites of *Pseudomonas* involved in the biocontrol of plant disease. Trends Biotechnol. 12: 133-144.

Duffy, B.K. and G. Défago. 1999. Environmental factors modulating antibiotic and siderophore biosynthesis by *Pseudomonas fluorescens* biocontrol strains. Appl. Enviorn. Microbiol. 65(6): 2429-2438.

Dunne, C., J.J. Crowley, Y. Moënne-Loccoz, D.N. Dowling, F.J. de Bruijn, and F. O'Gara. 1997. Biological control of *Pythium ultimum* by *Stenotrophomonas maltophilia* W81 is mediated by extracellular proteolytic activity. Microbiol. 143:3921-3931.

Dupler, M. and R. Baker. 1984. Survival of *Pseudomonas putida*, a biological control agent, in soil. Phytopathol. 74(2):195-200.

Eagon, R.G., and A.K. Williams. 1960. Enzymatic patterns of adaptation to fructose, glucose, and mannose exhibited by *Pseudomonas aeruginosa*. J. Bacteriol. 79(1): 90-94.

El-Tarabily, K.A., G.E.St.J. Hardy, K. Sivasithamparam, A.M. Hussein, and D.I Kurtböke. 1997. The potential for the biological control of cavity-spot disease of carrots, caused by *Pythium coloratum*, by streptomycete and non-streptomycete actinomycetes. New Phytol. 137: 495-507.

El-Tarabily, K.A. and K Sivasithamparam. 2006. Non-streptomycete actinomycetes as biocontrol agent of soil-borne fungal plant pathogens and as plant growth promoters. Soil Biol. Biochm. 38: 1505-1520.

El-Tarabily, K.A., M.L. Sykes, I.D. Kurtböke, G.E.St.J. Hardy, A.M. Barbosa, and R.F.H. Dekker. 1996. Synergistic effects of a cellulose-producing *Micromonospora carbonacea* and an antibiotic-producing *Streptomyces violascens* on the suppression of *Phytophthora cinnamomi* root rot of *Banksia grandis*. Can. J. Bot. 74:618-624.

Fages, J. and J.F. Arsac. 1991. Sunflower inoculation with *Azospirillum* and other plant growth promoting rhizobacteria. Plant. Scoil. 137: 87-90.

Fernando, W.G.D., S. Nakkeeran, Y. Zhang, and S.Savchuk. 2007. Biological control of *Sclerotinia sclerotiorum* (Lib.) de Bary by *Pseudomonas* and *Bacillus* species on canola petals. Crop Protection. 26:100-107.

Fernando, W.G.D., R. Ramarathnam, A.S. Krishnamoorthy, and S.C. Savchuk. Identification and use of potential bacterial organic antifungal volatiles in biocontrol. Soil Bio. Biochem. 37: 955-964.

Frankowski, J., M. Lorito, F. Scala, R. Schmidt, G. Berg, and H. Bahl. 2001. Purification and properties to two chitinolytic enzymes of *Serratia plymuthica* HRO-C48. Arch. Microbiol. 176:421-426.

Fravel, D. 1988. Role of antibiosis in the biocontrol of plant diseases. Annu. Rev. Phytopathol. 26:75-91.

Fravel, D. 2005. Commercialization and Implementation of Biocontrol. Annu Rev Phytopathol. 43: 337-359.

Gamalero, E., G. Berta, N. Massa, B.R. Glick, and G. Lingua. 2010. Interactions between *Pseudomonas putida* UW4 and *Gigaspora rosea* BEG9 and their consequences for the growth of cucumber under salt-stress conditions. J. Appl. Microbiol. 108: 236-245.

Gamalero, E., G. Lingua, G. Berta, and B.R. Glick. 2009. Beneficial role of plant growth promoting bacteria and arbuscular mycorrhizal fungi on plant responses to heavy metal stress. Can. J. Microbiol. 55: 501-514.

Gholami, A., S. Shahsavani, and S. Nezarat. 2009. The effect of plant growth promoting rhizobacteria (PGPR) on germination, seedling growth and yield of maize. WASET. 49: 19-24.

Girard, G., E. Tjeerd van Rij, B.J.J. Lugtenbery, and G.V. Bloemberg. 2006. Regulatory roles of *psrA* and *rpoS* in phenazine-1-carboxamide synthesis by *Pseudomonas chlororaphis* PCL 1391. Microbiol. 152: 43-58.

Glick, B.R. 1995. The enhancement of plant growth by free-living bacteria. Can J Microbiol. 41:109-117.

Glick, B.R. 2005. Modulation of plant ethylene levels by the bacterial enzyme ACC deaminase. FEMS Microbiol. Letters. 251:1-7.

Glick, B.R., C. Liu, S. Ghosh, and E.B. Dumbroff. 1997. Early development of canola seedlings in the presence of the plant growth-promoting rhizobacterium *Pseudomonas putida* GR12-2. Soil Biol. Biochem. 29(8): 1233-1239.

Glick, B.R., D.M Penrose, and J. Li. 1998. A model for the lowering of plant ethylene concentrations by plant growth promoting bacteria. J. Theor. Biol. 190: 63-68.

Glick, B.R., B. Todorovic, J. Czarny, A. Cheng, J. Duan, and B. McConkey. 2007. Promotion of Plant Growth by Bacterial ACC Deaminase. Crit Rev Plant Sci. 26: 227-242.

Goldstein, A.H. 1986. Bacterial solubilization of mineral phosphates: historical perspectives and future prospects. Am. J. Altern. Agri. 1:51-57.

Goswami, D., H. Vaghela, S. Parmar, P. Dhandhukia, and J.N. Thakker. 2013. Plant growth promoting potentials of *Pseudomonas* spp. strain OG isolated from marine water. J. Plant Interact. 8(4): 281-290.

Gray, E.J. and D.L. Smith. 2005. Intracellular and extracellular PGPR: commonalities and distinctions in the plant-bacterium signalling processes. Soil Biol. Biochem. 37: 395-412.

Guetsky, R., D. Shtienberg, Y. Elad, and A. Dinoor. 2001. Combining biocontrol agents to reduce the variability of biological control. Phytopathol. 91(7):621-627.

Guetsky, R. D. Shtienbery, Y. Elad, E. Fischer, and A. Dinoor. 2002. Improving biological control by combining biocontrol agents each with several mechanisms of disease suppression. Biological Control. 92(9):976-985.

Flaishman, M.A., Z. Eyal, A. Zilberstein, C. Voisard, and D. Haas. 1996. Suppression of *Septoria tritici* blotch and leaf rust of wheat by recombinant cyanide-producign strains of *Pseudomonas putida*. Mol. Plant-Microbe Interact. 9:642-645.

Haas, D. and G. Défago. 2005. Biological control of soil-borne pathogens by fluorescent pseudomonads. Nat. Rev. Microbiol. 3: 307-319.

Heeb, S. and D. Haas. 2001. Regulatory Roles of the GacS/GacA Two-Component System in Plant-Associated and Other Gram-Negative Bacteria. Molecular Plant-Micro. Interac. 14(12):1351-1363.

Heeb, S., C. Valverde, C. Gigot-Bonnefoy, and D. Haas. 2005. Role of the stress sigma factor RpoS in GacA/RsmA-controlled secondary metabolism and resistance to oxidative stress in *Pseudomonas fluorescens* CHA0. REMS Microbiol. Let. 243: 251-258.

Höfte, M. and Altier, N. 2010. Fluorescent pseudomonads as biocontrol agents for sustainable agricultural systems. Res Microbiol. 161: 464-471.

Hwang, S.-F., H.U. Ahmed, S.E. Strelkov, G.D. Gossen, G.D. Turnbull, G. Peng, and R.J. Howard. 2010. Seedling age and inoculum density affect clubroot severity and seed yield in canola. Can J Plant Sci. 91:183-190.

Hwang, S.-F., R.J. Howard, S.E. Strelkov, B.D. Gossen, and G. Peng. 2014. Management of clubroot (*Plasmodiophora brassicae*) on canola (*Brassica napus*) in western Canada. Can J Plant Pathol. 36:49-65.

Hwang, S.-F., S.E. Strelkov, J. Feng, B.D. Gossen, and R.J. Howard. 2012. *Plasmodiophora brassicae*: a review of an emerging pathogen of the Canadian canola (*Brassica napus*) crop. Mol Plant Pathol. 13(2):105-113.

Huber, F. and B. Erni. 1996. Membrane topology of the mannose transporter of *Escherichia coli* K12. Eur. J. Biochem. 239: 810-817.

Indris, E.E., D.J. Iglesias, M. Talon, and R. Borriss. 2007. Tryptophan-dependent production of indole-3-acetic acid (IAA) affects level of plant growth promotion by *Bacillus amyloliquefaciens* FZB42. Mol. Plant-Microbe Interac. 20(6):619-626.

Jackson, M.B. 1991. Ethylene and seed germination. In: The Plant Hormone Ethylene. Eds: A.K. Matoo and J.C. Suttle. Pp. 133-157. CRC Press. Boco Raton, FL.

Jamison, P. 2000. Cytokinins and auxins in plant-pathogen interactions- An overview. Plant Growth Reg. 32: 369-380.

Janisiewicz, W.J., J. Usall, and B.Bors. 1992. Nutritional enhancement of biocontrol of blue mold on apples. Phytopathol. 82 :1364-1370.

Ji, P., and M. Wilson. 2002. Assessment of the importance of similarity in carbon source utiliation profiles between the biological control agent and the pathogen in biological control of Bacterial Speck of tomato. Appl. Environ. Microbiol. 68: 4383-4389.

Jing, L., S.S. Navi, and X. Yang. 2015. Effects of colonization by different strains of *Coniothyrium minitans* on the viability of sclerotia of *Sclerotinia sclerotiorum*. Biocontrol Sci. Technol. 25(4):460-474.

Johnson, K.B., V.O. Stockwell, D.M. Burgett, D. Sugar, and J.E. Loper. 1993. Dispersal of *Erwinia amylovora* and *Pseudomonas fluorescens* by honeybees to apple and pear blossoms. Phytopathology 83: 478-484.

Jung, S.C., A. Martinez-Medina, J.A. Lopez-Raez, and M.J. Pozo. 2012. Mycorrhiza-induced resistance and priming of plant defenses. J. Chem. Ecol. 38- 651-664.

Kang, B.R., B.H. Cho, A.J. Anderson, and Y. C. Kim. 2004. The global regulator GacS of a biocontrol bacterium *Pseudomonas chlororaphis* O6 regulates transcription from the *rpoS* gene encoding a stationary-phase sigma factor and affects survival in oxidative stress. Gene. 325: 137-143.

Kataria, H.R. and P.R. Verma. 1992. *Rhizoctonia solani* damping-off and root rot in oilseed rape and canola. Crop Protection. 11: 8-13.

Kay, E., B. Humair, V. Dénervaud, K. Riedel, S. Spahr, L. Eberl, C. Valverde, and D. Haas. 2006. Two GacA-dependent small RNAs modulate the quorum-sensing response in *Pseudomonas aeruginosa*. J. Bacteriol. 188(16): 6026-6033.

Kim, Y.C., Leveau, J., McSpadden Gardener, B.B., Pierson, E.A., Pierson III, L.S., and Ryu, Choong-Min. 2011. The Multifactorial Basis for Plant Health Promotion by Plant-Associated Bacteria. App. Environ. Microbiol. 77(5): 1548-1555.

Kim, D.H., I.J. Misachi, and E.A. Pierson. 1996. Fluorescent pseudomonad populations in modified rhizosphere atmospheres. Soil Biol. Biochem. 28:497-501.

Kinkel, L.L., J.H. Andrews, and E.V. Nordheim. 1989. Fungal immigration dynamics and community development on apple leaves. Microbiol Ecol. 18:45-58.

Kirner, S., P.E. Hammer, D.S. Hill, A. Altmann, I. Fischer, L.J. Weislo, M. Lanahan, K.H. van Pée, and J.M. Ligon. 1998. Functions encoded by pyrrolnitrin biosynthetic genes from *Pseudomonas fluorescens.* J. Bacteriol. 180(7): 1939-1943.

Klaponski, N. 2014. Functional characterization of two divergently transcribed genes: *ptrA*, encoding a LysR-type transcitional regulator, and *scd*, encoding a short chain dehydrogenase in *Pseudomonas chlororaphis* PA23. Master of Science thesis, University of Manitoba, Winnipeg.

Klassen, A.J., R.K. Downey, and J.J. Capcara. 1987. Westar Summer Rape. Can. J. Plant Sci. 67: 491-493.

Kloepper, J.W., J. Leong, M. Teintze, and M.N. Schroth. 1980. Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. Nature. 286: 885-886.

Kloepper, J.W., and M.N. Schroth. 1978. Plant growth-promoting rhizobacteria on radishes. In Proceedings of the 4th International Conference on Plant Pathogenic Bacteria. Pp. 879-882. Gilbert-Clarey, Tours, France.

Kojic, M., and V. Venturi. 2001. Regulation of *rpoS* gene expression in *Pseudomonas*: Involvement of the TetR family regulator. J. Bacteriol. 183(12): 3712-3720.

Kutcher, H.R., S.A. Brandt, E.G. Smith, D. Ulrich, S.S. Malhi, and A.M. Johnston. 2013. Blackleg disease of canola mitigated by resistant cultivars and four-year crop rotations in western Canada. Can J Plant Pathol. 35(2):209-221.

Landa, B.B., J.A. Navas-Cortés, and R.M. Jiménez-Diaz. 2004. Influence of temperature on plant-rhizobacteria interactions related to biocontrol potential for suppression of fusarium wilt of chickpea. Plant Pathol. 53:341-352.

Lapouge, K., M. Schubert, F.H.-T. Allain, and D. Haas. 2008. Gac/Rsm signal transduction pathway of γ-proteobacteria: from RNA recognition to regulation of social behavior. Mol. Microbiol. 67(2): 241-253.

Lapouge, K., E. Sineva, M. Lindell, K. Starke, C.S. Baker, P. Babitzke, and D. Haas. 2007. Mechanism of *hcnA* mRNA recognition in the Gac/Rsm signal transduction pathway of *Pseudomonas fluorescens*. Mol. Microbiol. 66(2): 341-356.

Larkin, R.P., and D.R. Fravel. 2002. Effects of varying environmental conditions on biological control of Fusarium wilt of tomato by nonpathogenic *Fusarium* spp. Phytopathol. 92(11): 1160-1166.

Lavenus, J., T. Goh, I. Roberts, S. Guyomarc'h, M. Lucas, I. De Smet, H. Fukaki, T. Beeckman, M. Bennett, and L. Laplaze. Lateral root development in *Arabidopsis*: fifty shades of auxin. Trends in Plant Sci. 18(8): 450-458.

Laville, J., C. Blumer, C. Von Schroetter, V. Gaia, G. Défago, C. Keel, and D. Haas. 1998. Characterization of the *hcnABC* gene cluster encoding hydrogen cyanide synthase and anaerobic regulation by ANF in the strictly aerobic biocontrol agent *Pseudomonas fluorescens* CHA0. J. Bacteriol. 180: 3187-3196.

Lazazzera, B. 2000. Quorum sensing and starvation: signals for entry into stationary phase. Current Opinion Microbiol. 3(2): 177-182.

Leeman, M., F.M. Den Ouden, J.A. Van Pelt, F.P.M. Dirkx, H. Steijl, P.A.H.M. Bakker, and B. Schippers. 1996. Iron availability affects induction of systemic resistance to Fusarium wilt of radish in commericial greenhouse trials by seed treatment with *Pseudomonas fluorescens* WCS374. Phytopathology. 85:149-155.

Leong, J. 1986. Siderophores: Their biochemistry and possible role in the biocontrol of plant pathogens. Ann. Rev. Phytopathol. 24:187-209.

Lifshitz, R., J.W. Kloepper, M. Kozlowski, C. Simonson, J. Carlson, E.M. Tipping, and I. Zaleska. 1987. Growth promotion of canola (rapeseed) seedlings by a strain of *Pseudomonas putida* under gnotobiotic conditions. Can. J. Microbiol. 33: 390-395.

Ligon, J.M., D.S. Hill, P.E. Hammer, N.R. Torkewitz, D. Hofmann, H.J. Kempf, and K.H. van Pée. 2000. Natural products with antifungal activity from *Pseudomonas* biocontrol bacteria. Pest Manag. Sci. 56:688-695.

Lindemann, J., and C.D. Upper. 1985. Aerial dispersal of epiphytic bacteria over bean plants. Appl. Environ. Microbiol. 50(5):1229-1232.

Livak, K.J., and T.D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods. 25: 402-408.

Loper, J.E. 1988. Role of fluorescent siderophore production in biological control of *Pythium ultimum* by a *Pseudomonas fluorescens* strain. Disease Control and Pest Management. 78(2): 166-172.

Lucy, M., E. Reed, and B.R. Glick. 2004. Applications of free living plant growth-promoting rhizobacteria. Antonie van Leewenhoek. 86:1-25.

Maddula, V.S., E.A. Pierson, and L.S. Pierson, 3rd. 2008. Altering the ratio of phenazines in *Pseudomonas chlororaphis (aureofaciens)* strain 30-84: effects on biofilm formation and pathogen inhibition. J. Bacteriol. 190(8):2759-2766.

Maddula, V., Z. Zhang, E. Pierson, and L. Pierson III. 2006. Ouorum Sensing and Phenazines are Involved in Biofilm Formation by *Pseudomonas chlororaphis (aureofaciencs)* Strain 30-84. Microb Ecol. 52: 289-301.

Madloo, P.B., K. Behboudi, M. Tohidfar, G.S. Jouzani, and M. Ahmadzadeh. 2013. Response of some important Iranian wheat cultivars to *Fusarium culmorum* under genetic diversity of indigenous bio-control agent fluorescent *Pseudomonas* spp. AJCS. 7(7):1003-1009.

Manitoba Agriculture, Food and Rural Development. 2015. Crops- Plant Disease. Sclerotinia in Canola. Available from http://www.gov.mb.ca/agriculture/crops/plant-diseases/sclerotinia-canola.html: [cited 16 April 2015]

Manulis, S., A. Haviv-Chesner, M.T. Brandl, S.E. Lindow, and I. Barash. 1998. Differential involvement of indole-3-acetic acid biosynthetic pathways in pathogenicity and epiphytic fitness of *Erwinnia herbicola* pv. *gypsophilae*. Mol. Plant-Micro. Interact. 11(7): 634-642.

Mao, W., J.A. Lewis, P.K. Hebbar, and R.D. Lumsden. 1997. Seed treatment with a fungal or a bacterial antagonist for reducing corn damping-off caused by species of *Pythium* and *Fusarium*. Plant Disease. 81(5):450-454.

Martin, F.N., and J.E. Loper. 1999. Soilborne plant diseases caused by *Pythium* spp.: Ecology, epidemiology, and prospects for biological control. Crit. Rev. Plant Sci. 18(2): 111-181.

Mathiyazhagan, S., K. Kavitha, S. Nakkeeran, G. Chandrasekar, K. Manian, P. Renukadevi, A.S. Krishnamoorthy, and W.G.D. Fernando. 2004. PGPR mediated management of stem blight of *Phyllanthus amarus* (Schum and Thonn) caused by *Corynespora cassiicola* (Berk and Curt) Wei. Archives Phytopathol. Plant Protection. 37:183-199.

Mazzola, M., and R.J. Cook. 1991. Effects of fungal root pathogens on the populalation dynamics of biocontrol strains of fluorescent pseudomonads in the wheat rhizosphere. Appl. Environ. Microbiol. 57(8):2171-2178.

Mazzola, M., R.J. Cook, L.S. Thomashow, D.M. Weller, and L.S. Pierson 3rd. 1992. Contribution of phenazine antibiotic biosynthesis to the ecological competence of fluorescent pseudomonads in soil habitats. Appl. Environ. Microbiol. 58(8):2616-2624.

McQuilken, M.P., S.J. Mitchell, S.P. Budge, J.M. Whipps, J.S. Fenlon, and S.A. Archer. 1995. Effect of *Coniothyrium minitans* on sclerotial survival and apothecial production of *Sclerotinia sclerotiorum* in field grown oilseed rape. Plant Pathol. 44: 883-396.

McSpadden Gardener, B.B., and D.M. Weller. 2001. Changes in populations of phyizosphere bacteria associated with take-all disease of wheat. Appl. Environ. Microbiol. 67(10):4414-4425.

Meyer, J.B., M.P. Lutz, M. Frapolli, M. Péchy-Tarr, L. Rochat, C. Keel, G. Défago, and M. Maurhofer. 2010. Interplay between wheat cultivars, biocontrol Pseudomonads, and soil. Appl. Environ. Microbiol. 76(18):6196-6204.

Miller, M.B., and B.L. Bassler. 2001. Quorum sensing in bacteria. Ann. Rev. Microbiol. 55: 165-199.

Morgan, P.W. and C.D. Drew. 1997. Ethylene and plant responses to stress. Physiol. Plant. 100. 620-630.

Morrissey, J.P., M. Cullinana, A. Abbas, G.L. Mark, and F. O'Gara. 2004. In *Pseudomonas* Vol. 3. Ed. Ramos, J.L. Kluwer Academic/Plenum Publishers, New York. pp. 637-670.

Nakkeeran, S., K. Kavitha, G. Chandrasekar, P. Renukadevi, and W.G.D. Fernando. 2006. Induction of plant defence compounds by *Pseudomonas chlororaphis* PA23 and *Bacillus subtilis* BSCBE4 in controlling damping-off of hot pepper caused by *Pythium aphanidermatum*. Biocontrol Sci. Technol. 16(4): 403-416.

Narisawa, K., M. Shimura, F. Usuki, S. Fukuhara, and T. Hashiba. 2005. Effects of pathogen density, soil moisture, and soil pH on biological control of Clubroot in Chinese Cabbage by *Heteroconium chaetospira*. Plant Disease. 89(3):285-290.

Neilands, J.B. and S.A. Leong. 1986. Siderophores in relation to plant growth and disease. Annu. Rev. Plant Physiol. 37: 187-208.

Neilands, J.B., K. Konopka, B. Schwyn, M. Coy, R.T. Francis, B.H. Paw, and A. Bagg. 1987. Comparative biochemistry of microbial iron assimilation. In Iron transport in microbes, plants and animals. G. Winkelmann, D. van der Helm, and J.B. Heilands (eds). Verlagsgesellschaft mbH, Weinheim, Germany. Pp 3-33.

Normanly, J., J.D. Cohen, and G.R. Fink. 1993. *Arabidopsis thaliana* auxotrophs reveal a tryptophan-independent biosynthetic pathway for indole-3-acetic acid. PNAS. 90:10355-10359.

Notz, R., M. Maurhofer, U. Schnider-Keel, B. Duffy, D. Haas, and G. Défago. 2001. Biotic factors affection expression of the 2,3-diacetylphloroglucinol biosynthesis gene *phlA* in *Pseudomonas fluorescens* biocontrol strain CHA0 in the rhizosphere. Biological control: Phytopathol. 91(9):873-881.

Nowak-Thompson, B., and S.J. Gould. 1994. Production of 2,4-diacetylphloroglucinol by the biocontrol agent *Pseudomonas fluorescens* Pf-5. Can. J. Microbiol. 40: 1064-1066.

O'Sullivan, D.J. and F. O'Gara. 1992. Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. Microbiol. Rev. 56:662-676.

Oh, S.A., J.S. Kim, S.H. Han, J.Y. Park, C. Dimkpa, C. Edlund, A.J. Anderson, and Y.C. Kim. 2013. The GacS-regulated sigma factor RpoS governs production of several factors involved in biocontrol activity of the rhizobacterium *Pseudomoans chlororpahis* O6. Can. J. Microbiol. 59(8): 556-562.

Paau, A.S. 1998. Formulation of Beneficial Organisms Applied to Soil. In: Formulation of Microbial Biopesticides (Ed. H.D. Burges). Springer Netherlands. Pp. 235-254.

Pallai, R., R.K. Hynes, B. Verma, and L.M. Nelson. 2012. Phytohormone production and colonization of canola (*Brassica napus* L.) roots by *Pseudomonas fluorescens* 6-8 under gnotobiotic conditions. Can. J. Microbiol 58: 170-178.

Park, C.S., T.C. Paulitz, and R. Baker. 1988. Biocontrol of Fusarium Wilt of cucumber resulting from interactions between *Pseudomonas putida* and nonpathogenic isolates of *Fusarium oxysporum*. Phytopathol. 78(2):190-194.

Patten, C.L., and B.R. Glick. 1996. Bacterial biosynthesis of indole-3-acetic acid. Can. J. Microbiol. 42:207-220.

Patten, C.L., and B.R. Glick. 2002. Regulation of indoleacetic acid production in *Pseudomonas putida* GR12-2 by tryptophan and the stationary-phase sigma factor RpoS. Can J Microbiol. 48:625-642.

Penrose, D.M., and B.R. Glick. 2003. Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. Physiologia Plantarum. 118: 10-15.

Phillips, P.W.B, and G.G. Khachatourians. 2001. The Biotechnolgoy Revolution in Global Agriculture: Invention, Innovation and Investment in the Canola Sector. CABI Publishing.

Pierson L.S. III., T. Gaffney, S. Lam, and F. Cong. 1995. Molecular analysis of genes encoding phenazine biosynthesis in the biological control bacterium *Pseduomonas aureofaciens* 30-84. FEMS Microbiol. Letters. 134:299-307.

Pieterse, C.M.J., C. Zamioudis, R.L. Berendsen, D.M. Weller, S.C.M. Van Wees, and P.A.H.M. Bakker. 2014. Induced systemic resistance by beneficial microbes. Annu. Rev. Phytopathol. 52:347-375.

Pleban, S., L. Chernin, and I. Chet. 1997. Chitonolytic activity of an endophytic strain of *Bacillus cereus.* Let. Appl. Microbiol. 25: 284-288.

Podile, A.R., and G.K. Kishore. 2006. Plant growth-promoting rhizobacteria. In: Gnanamanickam, S.S. (ed). Plant-Associated Bacteria. Springer, Netherlands. Pp. 195-230.

Polyanskaya, L.M., O.T. Vedina, L.V. Lysak, and D.G. Zvyagintev. 2000. The growth-promoting effect of *Beijerinckia mobilis* and *Clostridium* sp. cultures on some agricultural crops. Microbiol. 71: 109-115.

Poritsanos, N., C. Selin, W.G.D. Fernando, S. Nakkeeran, and T.R. de Kievit. 2006. A GacS deficiency does not affect *Pseudomonas chlroraophis* PA23 fitness when growing on canola in aged batch culture or as a biofilm. Can. J. Microbiol. 52:1177-1188.

Potrykus, K., and M. Cashel. 2008. (p)ppGpp: still magical? Annu. Rev. Microbiol. 62: 35-51.

Pozo, M.J., and C. Azcon-Aguilar. 2007. Unraveling mycorrhiza-induced resistance. Curr. Opion. Plant Biol. 10:393-398.

Preston, M.J., P.C. Seed, D.S. Toder, B.H. Iglewski, D.E. Ohman, J.K. Gustin, J.B. Goldberg, and G.B. Pier. 1997. Contribution of proteases and LasR to the virulence of *Pseudomonas aeruginosa* during corneal infections. Infect. Immun. 65: 3086-3090.

Pujol, M., E. Badosa, and E. Montesinos. 2007. Epiphytic fitness of a biological control agent of fire blight in apple and pear orchards under Mediterranean weather conditions. FEMS Microbiol. Ecol. 59:186-193.

Purdy, L.H. 1979. *Sclerotinia sclerotiorum*: history, disease and symptomatology, host range, geographic distribution and impact. Phytophathol. 69: 875-880.

Raaigmakers, J.M., M. Vlami, and J.T. de Souza. 2002. Antibiotic production by bacterial biocontrol agents. A Van Leeuw J Microb. 81: 537-547.

Ramarathnam, R., W.G.D. Fernando, and T. de Kievit. 2011. The role of antibiosis and induced systemic resistance, mediated by strains of *Pseudomonas chlororaphis*, *Bacillus cereus* and *B. amyloliquefaciens*, in controlling blackleg disease of canola. BioControl. 56: 225-235.

Reed, M.L.E., and B.R. Glick. 2005. Growth of canola (*Brassica napus*) in the presence of plant growth-promoting bacteria and either copper or polycyclic aromatic hydrocarbons. Can. J. Microbiol. 51: 1061-1069.

Reimmann, C., N. Ginet, L. Michel, C. Keel, P. Michaux, V. Krishnapillai, M. Zala, K. Heurlier, K. Triandafillu, H. Harms, G. Défago, and D. Haas. 2002. Genetically programmed autoinducer destruction reduces virulence gene expression and swarming motility in *Pseudomonas aeruginosa* PAO1. Microbiology. 148:923-932.

Reimmann, C., C. Valverde, E. Kay, and D. Haas. 2005. Posttranscriptional repression of GacS/GacA-controlled genes by the RNA-binding protein RsmE acting together with RsmA in the biocontrol strain *Pseudomonas fluorescens* CHA0. J. Bacteriol. 187(1): 276-285.

Rempel, C.B., S.N. Hutton, and C.J. Jurke. reClubroot and the importance of canola in Canada. Can. J. Plant Pathol. 36:19-26.

Renato de Freitas, J., and J.J. Germida. 1991. *Pseudomonas cepacia* and *Pseudomonas putida* as winter wheat inoculants for biocontrol of *Rhizoctonia solani*. Can. J. Microbiol. 37(10): 780-784.

Richardson, A.E., J.-M. Barea, A.M. McNeill, and C. Prigent-Combaret. 2009. Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. Plant Soil. 321: 305-339.

Rodríguez, H. and R. Fraga. 1999. Phozphate solubilizing bacteria and their role in plant growth promotion. Biotech. Advances. 17: 319-339.

Rosenzweig, W.D., and G. Stotzky. 1979. Influence of environmental factors on antagonism of fungi by bacteria in soil: clay minerals and pH. Appl. Environ. Microbiol. 38(6):1120-1126.

Saleh, S.S., and B.R. Glick. 2001. Involvement of *gacS* and *rpoS* in enhancement of the plant growth-promoting capabilities of *Enterobacter cloacae* CAL2 and UW4. Can. J. Microbiol. 47:698-705.

Sarma, B.K., S.K. Yadav, S. singh, and H.B. Singh. 2015. Microbial consortium-mediated plant defense against phytopathogens: readdressing for enhancing efficacy. Soil Biol. Biochem. 87: 25-33.

Savchuk, S.C. 2002. Evaluation of biological control of *Sclerotinia sclerotiorum* on Canola (*Brassica napus*) in the lab, in the greenhouse, and in the field. Msc. thesis. University of Manitoba, pp. 49-83.

Savchuk, S., and Fernando, W.G.D. 2004. Effect of timing of application and population dynamics on the degree of biological control of *Sclerotinia sclerotiorum* by bacterial antagonists. FEMS Microbiol Ecol. 49: 379-88.

Schippers, B., J.M. Lynch, P. Neuenschwander, J.W. Deacon, B.C. Hemming, J.A. Lucas, and G. Defago. 1988. Biological Control of Pathogens and Rhizobacteria. Phil. Trans. R. Soc. London. 318:283-293.

Schnider, U., C. Keel, C. Voisard, G. Défago, and D. Haas. 1995. Tn5-directed cloning of *pqq* genes from *Pseudomonas fluorescens* CHA0: Mutational inactivation of the genes results in overproduction of the antibiotic pyoluteorin. Appl. Environ. Microbiol. 61(11): 3856-3864.

Selin, C., W.G.D. Fernando, and T. de Kievit. 2012. The PhzI/PhzR quorum-sensing system is required for pyrrolnitrin and phenazine production, and exhibits cross-regulation with RpoS in *Pseudomoans chlororaphis* PA23. Microbiol. 158:896-907.

Selin, C., R. Habibian, N. Poritsanos, S.N.P. Athukorala, D. Fernando, and T.R. de Kievit. 2010. Phenazines are not essential for Pseudomonas chlororaphis PA23 biocontrol of *Sclerotinia sclerotiorum*, but do play a role in biofilm formation. FEMS Microbiol Ecol. 71:73-83.

Selin, C., J. Manuel, W.G.D. Fernando, and T. de Kievit. 2014. Expression of the *Pseudomonas chlororaphis* strain PA23 Rsm expression is under control of GacA, RpoS, PsrA, quorum sensing and the stringent response. Biological Control. 69: 24-33.

Shanahan, P., D.J. O'Sullivan, P. Simpson, J.D. Glennon, and F. O'Gara. 1992. Isolation of 2,4-diacetylphloroglucinol from a fluorescent pseudomonad and investigation of physiological parameters influencing its production. Appl. Environ. Micrbiol. 58:353-358.

Shanmugaiah, V., N. mathivanan, and B. Varghese. 2010. Purification, crystal structure and antimicrobial activity of phenazine-1-carboxamide produced by a growth-promoting biocontrol bacterium, *Pseudomonas aeruginosa* MML2212. J. Appl. Microbiol. 108:703-711.

Sharma, P., P.D. Meena, P.R. Verma, G.S. Saharan, H. Mehta, D. Singh, and A. Kumar. 2015. *Sclerotinia sclerotiorum* (Lib.) de Bary causing Sclerotinia rot in oilseed *Brassicas*: A review. J. Oilseed Brassica. 6: 1-44.

Shoresh, M., G.E. Harman, and F .Mastouri. 2010. Induced systemic resistance and plant responses to fungal biocontrol agents. Annu Rev Phytopathol. 48: 21-43.

Slininger, P.J., and M.A. Shea-Wilbur. 1995. Liquid-culture pH, temperature, and carbon (not nitrogen) source regulated phenazine productivity of the take-all biocontrol agent *Pseudomonas fluorescens* 2.79. Appl. Microbiol. Biotechnol. 43:794-800.

Smitdt, M. and T. Kosuge. The role of indole-3-acetic acid accumulation by alpha methyl tryptophan-resistant mutants of *Pseudomonas savastanoi* in gall formation on oleanders. Physiol. Plant Pathol. 13(2): 203-210.

Someya, N., Ikeda, S. and Tsuchiya, K. 2013. Pseudomonas Inoculants as Agents for Plant Disease Management. In: *Bacteria in Agrobiology: Disease Management.* Berlin Heidelberg: Springer-Verlag.

Spadaro, D., and M.L. Gullino. 2005. Inproving the efficacy of biocontrol agents against soilborne pathogens. Crop Protection. 24:601-613.

Spaepen, S., J. Vanderleyden, and R. Remans. 2007. Indole-3-acetic acid in microbial and microorganism-plant signaling. FEMS Microbiol. Rev. 31: 425-448.

Stockwell, V.O., K.B. Johnson, D. Sugar, and J.E. Loper. 2011. Mechanistically compatible mixtures of bacterial antagonists improve biological control of fire blight of pear. Phytopathol. 101: 113-123.

Strelkov, S.E., V.P. Manolii, T. Cao, S. Zue, and S.F. Hwang. 2007. Pathotype classification of *Plasmodiophora brassicae* populations from Alberta, Canada. Can J Plant Pathol. 28: 467-474.

Strelkov, S.E., V.P. Manolii, D.C. Rennie, Q. Xiao, D. Cui, and S.F. Hwang. 2011. The occurrence of clubroot on canola in Alberta in 2010. Can Plant Dis Surv. 91.

Strigul, N.S., and L.V. Kravchenko. 2006. Mathematical modeling of PGPR inoculation into the rhizosphere. Environ. Modelling & Software. 21: 1158-1171.

Sundin, G.W., S.P. Kidambi M. Ullrich, and C.L. Bender. 1996. Resistance to ultraviolet light is *Pseudomonas syringae*: sequence and functional analysis of the plasmid-encoded *rulAB* genes. Gene. 177:77-81.

Taiz, L. and E. Zeiger. 2010. Plant Physiology, 5th Ed. Sinauer Associates Inc.. Sunderland, Massachusetts. Pp. 546-580.

Tambong, J.T., and M. Höfte. 2001. Phenazines are involved in biocontrol of *Pythium myriotylum* on cocoyam by *Pseudomonas aeruginosa* PNA1. Eur. J. Plant Pathol. 107:511-521.

Temple, L.M., A.E. Sage, H.P. Schweizer, and P.G. Phibbs, Jr. 1998. Carbohydrate Catabolism in *Pseudomonas aeruginosa*. In: Pseudomonas (Ed. Tomas C. Montie). Plenum Press, New York.

Tewari, J.P., S.E. Strelkov, D. Orchard, M. Hartman, R.M. Lange, and T.K. Turkington. 2005. Indentification of clubroot of crucifers on canola (*Brassica napus*) in Alberta. Can. J. Plant Pathol. 27: 143-144.

Thakore, Y. 2006. The biopesticide market for global agricultural use. Industrial Biotechnol. 2:194-208.

Thomashow, L.S., and D.M. Weller. 1988. Role of a phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaeumannomyces graminis* var. *tritici*. J. Bacteriol. 170:3499-3508.

Thomson, S.V., D.R. Hansen, K.M. Flint, and J.D. Vandenberg. 1992. Disseminiation of bacteria antagonistic to *Erwinia amylovora* by honey bees. Plant Dis. 776: 1052-1056.

Tjeerd van Rij, E., M. Wesselink, T.F.C. Chin-A-Woeng, G.V. Bloemberg, and B.J.J. Lugtenberg. 2004. Influence of environmental conditions on the production of phenazine-1-carboxamide by *Pseudomonas chlororaphis* PCL1391. Mol. Plant-Microbe Interact. 17(5): 557-566.

Tripathi, R.K. and D. Gottlieb. 1969. Mechanism of action of the antifungal antibiotic pyrrolnitrin. J. Bacteriol. 100:310-318.

Turkington, T.K. and R.A.A. Morrall. 1993. Use of petal infestation to forecast sclerotinia stem rot of canola: the influence of inoculum variation over the flowering period and canopy density. Phytopathol. 83(6): 682-689.

Vacheron, J., G. Desbrosses, M. Bouffaud, B. Touraine, Y. Moënne-Loccoz, D. Muller, L. Legendre, F. Wisniewski-Dyé, and C. Prigent-Combaret. 2013. Plant growth-promoting rhizobacteria and root system functioning. Frontiers in Plant Sci. 4 (356): 1-19.

Valois, D., K. Fayad, T. Barbasubiye, M. Garon, C. Déry, R. Brzezinski, and C. Beaulieu. 1996. Blucanolytic actinomycetes antagonistic to *Phytophthora fragariae* var. *rubi*, the causal agent of raspberry root rot. Appl. Environ. Microbiol. 62: 1630-1635.

van Elsas, J.D. and Heignen, C.E. 1990. Methods for the introduction of bacteria into soil- a review. Biol Fertil Soils. 10: 127-133.

Van Loon, L.C., and P.A.H.M. Bakker. 2006. Root-associated bacteria inducing system resistance. In Plant-Associated Bacteria. Ed. S.S. Gnanamanickam. Pp. 269-316. Dordrecht, Neth. Springer.

Van Onckelen, H., E. Prinsen, D. Inzé, P. Rüdelsheim, M. Van Lijsebettens, A. Follin, J. Schell, M. Van Montagu, and J. De Greef. 1986. *Agrobacterium* T-DNA gene *1* codes for tryptophan 2-monooxygenase activity in tobacco crown gall cells. FEBS Letters. 198(2): 357-360.

Van Peer, R. G.J. Niemann, and B. Schippers. 1991. Induced resistance and phytoalexin accumulation in biological control of *Fusarium* wilt of carnation by *Pseudomonas* sp. strain WCS417r. Phytopathol. 81:728-734.

Venturi, V. 2003. Control of *rpoS* transcription in *Escherichia coli* and *Pseudomonas*: why so different? Mol. Microbiol. 49(1): 1-9.

Vessey, J.K. 2003. Plant growth promoting rhizobacteria as biofertilizers. Plant and Soil. 255: 571-586.

Voisard, C., C. Keel, D. Haas, and G. Défago. 1989. Cyanide production by *Pseudomonas fluorescens* helps suppress black rot of tobacco under gnotobiotic conditions. EMBO Journal. 8(2): 351-358.

Walters, D.R., J. Ratsep, and N.D. Havis. 2013. Controlling crop diseases using induced resistance: challenges for the future. J. Exp. Bot. 64: 1263-1280.

Wang, D., X. Ding, and P.N. Rather. 2001. Indole can act as an extracellular signal in *Escherichia coli*. J. Bacteriol. 183:4210-4216.

Wei, G., J.W. Kloepper, and S. Tuzen. 1991. Induction of systemic resistance of cucumber to *Colletrotrichum orbiculare* by select strains of plant-growth promoting rhizobacteria. Phytopathol. 81: 1508-1512.

Weller, D.M. 1988. Biological Control of Soilborne Plant Pathogens in the Rhizosphere with Bacteria. Ann. Rev. Phytopathol. 26:379-407.

Weller, D.M. 2007. *Pseudomonas* biocontrol agents of soilborne pathogens: Looking back over 30 years. Phytopathol. 97(2): 250-256.

West, J.S., P.D. Kharbanda, M.J. Barbetti, and B.D.L. Fitt. 2001. Epidemiology and management of *Leptosphaeria maculans* (phoma stem canker) on oilseed rape in Australia, Canada and Europe. Plant Pathol. 50: 10-27.

Whipps, J.M. 2001. Microbial interactions and biocontrol in the rhizosphere. J. Experimental Bot. 52:487-511.

Wissing, F. 1974. Cyanide formation from oxidation of glycine by a *Pseudomonas* species. J. Bacteriol. 117:1289-1294.

Yang, B., S. Srivastava, M.K. Deyholos, and N.N.V. Kav. 2007. Transcriptional profiling of canola (*Brassica napus* L.) responses to the fungal pathogen *Sclerotinia sclerotiorum*. Plant Sci. 173:156-171.

Yang, P., Z. Sun, S. Liu, H. Lu, Y. Zhou, and M. Sun. 2013. Combining antagonistic endophytic bacteria in different growth stages of cotton for control of Verticillium wilt. Crop Protect. 47:17-23.

Yang, S., Q. Zhang, J. Guo, A.O. Charkowski, B.R. Glick, A.M. Ibekwe, D.A. Cooksey, C.H. Yang. 2007. Global effect of indole-3-acetic acid biosynthesis on multiple virulence factors of *Erwinina chrysanthemi* 3937. Appl. Environ. Microbiol. 73:1079-1088.

Yaryura, P.M., M. León, O.S. Correa, N.L. Kerber, N.L. Pucheu, and A.F. García. 2008. Assessment of the role of chemotaxis and biofilm formation as requirements for colonization of roots and seeds of soybean plants by *Bacillus amyloliquefaciens* BNM339. Curr. Microbiol. 56(6): 625-632.

Yuen, G.Y., M.L. Craig, E.D. Kerr, and J.R. Steadman. 1994. Influences of antagonist population levels, blossom development stage, and canopy temperature on the inhibition of *Sclerotinia sclerotiorum* on dry edible bean by *Erwinia herbicola*. Phytopathol. 84(5):495-501.

Zamioudis, C., P. Mastranesti, P. Dhonukshe, I. Blilou, and C.M.J. Pieterse. 2013. Unraveling root developmental programs initiated by beneficial *Pseudomonas* spp. bacteria. Plant Physiol. 162: 304-318.

Zhang, W., S. Wang, H. Geng, Y. Ge, X. Huang, H. Hu, and Y. Xu. 2005. Differential regulation of *rsmA* gene on biosynthesis of pyoluteorin and phenazine-1-carboxylic acid in *Pseudomoans* sp. M18. W. J. Microbiol. Biotechnol. 21: 883-889.

Zhang, Y. 2004. Biocontrol of Sclerotinia stem rot of canola by bacterial antagonists and study of biocontrol mechanisms involved. Msc. thesis, University of Manitoba, MB, p. 147.

Zhang, Y., W.G.D. Fernando, T. de Kievit, C. Berry, F. Daayf, and T.C. Paulitz. 2006. Detection of antibiotic-related genes from bacterial biocontrol agents using polymerase chain reaction. Can. J. Microbiol. 52:476-481.

Zhao, J., L. Buchwaldt, S.R. Rimmer, A. Sharpe, L. McGregor, D. Bekkaoui, and D. Hegedus. 2009. Patterns of differential gene expression in *Brassica napus* cultivars infected with *Sclerotinia sclerotiorum*. Mol Plant Pathol. 10(5): 635-649.