Distribution of antimicrobial lipopeptides in *Bacillus* and
Pseudomonas spp., two genera with antagonistic effects against
plant pathogens

by

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A thesis submitted to the Faculty of Graduate and Postdoctoral
Affairs in partial fulfillment of the requirements for the degree of

Master of Science

in

Chemistry

(Concentration in Food Science and Nutrition)

Carleton University
Ottawa, Ontario

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Abstract

Suppressive soils, composts, and compost teas have previously shown inhibitory effects against plant disease. A major reason for this suppressiveness has been their beneficial microbial populations. The present study was carried out to investigate the inhibitory activity of forty bacteria isolated from these three sources against the mycelial growth of six plant pathogens. Thirty-eight isolates inhibited at least one of the pathogens whereas sixteen of the isolates inhibited all the pathogens (36% average inhibition). Crude lipopeptides extracts were precipitated from bacterial liquid cultures. All tested lipopeptide samples inhibited the mycelial growth or conidial germination of at least one of the pathogens. Known antimicrobial lipopeptides in selected samples were identified by LC-MS. Results showed that all *Bacillus* and *Bacillus*-related spp. produced one or more lipopeptides from the fengycin, iturin, and surfactin families. In addition, certain *Pseudomonas* spp. produced lipopeptides from the amphisin and putisolvin families.

Acknowledgments

It is with immense gratitude that I acknowledge the support and help of my supervisor, Dr. Tyler Avis. He has been the pivotal point of my academic growth. Dr. Avis' passion and positive attitude towards learning and teaching science has encouraged my educational aspirations. I sincerely thank him for allowing me to pursue research in his lab for five years.

I would also like to express my deep and sincere gratitude to my co-supervisor, Dr. Apollinaire Tsopmo. A great amount of his valuable time was used to give me constructive suggestions to complete this thesis. More specifically, I appreciate his warm and gentle approach to any issues that occurred with the data.

As well, I wish to acknowledge each of my past and present colleagues from Dr. Avis' lab for their friendly and open attitudes that made the lab a pleasant working environment.

And, I wish to thank the Chemistry Department and each of my undergraduate professors at Carleton University. A special thanks to Professor J. David Miller, and his lab members, for allowing the use of his specialized equipment.

Additionally, I would like to thank the Natural Sciences and Engineering Research Council (NSERC) of Canada, Les Fraises de l'Île d'Orléans, and Berger Peat Moss for funding this research project.

Finally, I want to appreciate my family and friends for their continuous interest in my research-oriented life. More specifically, I owe my most loving thanks to my mother. Without her encouragement, support, and assistance, this work would not have been completed work. Lastly, my thesis is dedicated to my nephew, Yasseen.

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Chapter 1: Introduction

1.1. Emerging fungal diseases

In the last few decades, an increase in crop diseases, caused by emerging species of fungi in plants, was observed around the globe from 1995 to 2010 leading to major economic losses and starvation (Fisher et al., 2013; Figure 1). Fungal infections presently destroy at least 125 million tonnes of the top five food crops (rice, wheat, maize, potatoes, and soybeans) (Fisher et al., 2013). Because 59 percent of calories consumed by humans are derived from just four essential plant species (rice, wheat, maize, and potatoes), fungal diseases in these staple crops can catastrophically threaten local and global food security (Strange and Scott, 2005; Vurro et al., 2010). The ability of many plant pathogens, including fungi, to persist in the environment for long periods of time makes it difficult to eradicate fungal diseases, resulting in an economic burden to agricultural communities (Fisher et al., 2013, Figure 1b).

Figure 1. Worldwide reporting trends in emerging fungal diseases, (a) an increase in alerts concerning fungal infections in plants and animals in the past few decades and (b) the global distribution of alerts in the emergence of fungal pathogens (plants and animals) (Fisher et al., 2013).

1.2. Infections in plants

Consumption of fruits and vegetables products has dramatically increased in the United States by more than 30% during the past few decades. It is also estimated that about 20% of all fruits and vegetables produced is lost each year to spoilage, representing 18.9 billion pounds of lost produce (Barth et al., 2009). Fungi are the predominant pathogens in plants (Avis, 2007) including fruits and vegetables. Some common fruit and vegetable pathogens of economic importance are *Alternaria solani* Sorauer, *Botrytis cinerea* Pers., *Fusarium sambucinum* Fuckel, *Rhizopus stolonifer* (Ehrenb.: Fr.) Vuill. and *Verticillium dahliae* Kleb. These pathogens are all fungi. In addition to these fungal pathogens, *Pythium sulcatum* is another important vegetable pathogen that was classified previously as a fungus, but has been reclassified and currently belongs to the oomycete class in the Chromalveolata kingdom. Taxonomic classification of the plant pathogens used in this thesis are listed in Table 1. These pathogens infect the host plants and cause symptoms such as: rotting, spotting, drying, wilting, and discoloration (Figure 2) as described in the following sections.

Table 1. Taxonomic classification of the plant pathogens used in this study

Kingdom	Fungi					Chromalveolata
Phylum	Ascomycota	Ascomycota	Ascomycota	Zygomycota	Ascomycota	Heterokontophyta
Class	Dothideomycetes	Leotiomycetes	Sordariomycetes	Zygomycetes	Sordariomycetes	Oomycetes
Subclass	Pleosporomycetidae	N/A	Hypocreomycetidae	N/A	Hypocreomycetidae	N/A
Order	Pleosporales	Helotiales	Hypocreales	Mucorales	Incertae	Pythiales
Family	Pleosporaceae	Sclerotiniaceae	Nectriaceae	Mucoraceae	Plectosphaerell	Pythiaceae
Genus	<i>Alternaria</i>	<i>Botrytis</i>	<i>Fusarium</i>	<i>Rhizopus</i>	<i>Verticilium</i>	<i>Pythium</i>
Species	<i>Solani</i>	<i>cinerea</i>	<i>sambucinum</i>	<i>stolonifer</i>	<i>dahliae</i>	<i>sulcatum</i>

All information was obtained from the National Center for Biotechnology Information (NCBI, 2015)

Figure 2. Symptoms and signs of plant diseases in different food crops (a) *Alternaria solani* causal agent of early blight of tomato fruit (Stevenson, 1993); (b) *Botrytis cinerea* causal agent of gray mold of strawberries (Caltex Mold Services, 2007); (c) *Fusarium sambucinum* causal agent of potato dry rot (Loria, 1993); (d) *Pythium sulcatum* causal agent of cavity spot in carrot roots (Davison and McKay, 2003); (e) *Rhizopus stolonifer* causal agent of *Rhizopus* rot of tomato fruit (Boyer, 2015); (f) *Verticillium dahliae* causal agent of *Verticillium* wilt in strawberry plants (Horst, 2001).

1.2.1. Physiopathology of plant diseases

Most plant diseases are caused by fungi and fungus like pathogens, compare to losses to other classes of pathogens such bacteria and viruses. In this study, we focused on six plants pathogens that cause loss to important crops (Fisher et al., 2013) as described in the following sections.

1.2.1.1. *Alternaria solani*

Alternaria solani causes blight disease in tomatoes and potatoes, and produces various phytotoxins such as solanapyrones (Hideaki et al., 1998). In tomatoes, infected plants develop dark spots on older lower leaves and blackish-brown lesions with a yellow halo appear on older leaves and stems. Although these lesions are initially circular, they can become angular when they grow between leaf veins. Infection of the tomato fruit normally occurs through the calyx and lesions of a considerable size can be found on the fruit (Kemmitt, 2002) (Figure 2a). It is also a major concern for potato growers because the pathogen can persist and survive in the soil between crop harvests and can therefore be present to infect the next crop (Stevenson et al., 2001).

1.2.1.2. *Botrytis cinerea*

Botrytis cinerea is a phytopathogenic fungus which causes gray mold (Figure 2b) or *Botrytis* blight and is found in more than 200 host plant species. The fungus mostly affects vegetable and fruit crops such as grapes, strawberries and tomatoes (Nakajima and Akutsu, 2013).

1.2.1.3. *Fusarium sambucinum*

Fusarium sambucinum is one of the most common fungi that causes dry rot of potatoes (Figure 2c) in Europe and North America (Ripperger et al., 1975). This pathogen produces many mycotoxins (Bojanowski et al., 2013) such as trichothecene and the mycotoxin 4,15-diacetoxyscirpenol (DAS), which has teratogenic effects in animals (Sokolova and Voznesenskiĭ, 2011).

1.2.1.4. *Pythium sulcatum*

Pythium sulcatum is the most common cause of cavity spot in carrot roots (Davidson and McKay, 2003). *Pythium sulcatum* is not a fungus. It is a fungus-like organism from the oomycete class (kingdom Chromalveolata). The hyphae of *Pythium* have the ability to penetrate the carrot roots through cracks produced by the outgrowth of the lateral roots or other wounds (Zamski and Peretz, 1995). Characteristic symptoms of the disease are a cooked-tuber texture, while a discolored water-soaked area appears around the wound (Figure 2d). The rotted tissues are spongy and extremely watery (Du Toit and Derie, 2014). Diseased tissues are separated from healthy tissue by a dark line and affected tissues change their color from gray, to brown, and finally, to black when exposed to air (Christ, 1998).

1.2.1.5. *Rhizopus stolonifer*

Rhizopus stolonifer is a heterotrophic, thread-like mold. *Rhizopus stolonifer* produces a mass of mycelium, the vegetative filaments of the fungus, and a characteristic fruiting structure that contains spores. *Rhizopus stolonifer* breaks down soft fruits such as papaya and tomatoes (Figure 2e) (Fan and Tian, 2000).

1.2.1.6. *Verticillium dahliae*

Verticillium dahliae is one of the main wilt causing fungi in numerous plants including strawberries (Figure 2f), potatoes, tomatoes, mint, and other plants (Horst, 2001). *Verticillium dahliae* is a conidia-forming fungus, producing one-celled, hyaline, fibrose to ellipsoid conidia at tips of whorled branches and separating readily forming tips. *Verticillium dahliae* is the only species of the genus forming microsclerotia, which help differentiate this species from other *Verticillium* spp. such as *V. albo-atrum* (Isaac, 1967).

1.3. Control measures

1.3.1. Chemical measures

The most common strategies available to manage plant diseases are chemical based methods (compounds with fungicidal or oomycetal activity). For example, although costly, fumigation with chemical biocides (which control fungi, nematodes, and weeds) are practically the only economically viable measure available to growers to control plant pathogens in some crops such as strawberries. However, these chemical methods may have major negative impacts on the environment and human health (Bae et al., 2008). Indeed, pesticides have been linked to a wide range of human health hazards, ranging from short-term impacts such as headaches and nausea to chronic impacts like cancer, reproductive harm, and endocrine disruption. Impacts of pesticides on the environment are also well known and some pesticides were shown to be toxic to living organisms. Some pesticides can accumulate in water systems, pollute the air, and in some cases have other dramatic environmental effects (Toxic Action Center, 2012).

Furthermore, pesticide use can lead to the development of resistant plant pathogen strains (Trivedi, 2007).

1.3.2. Alternatives to chemical treatments

Many alternatives to chemical control measures seem promising. These alternative methods include: host resistance, crop rotation, and biological control (or biocontrol). Host resistance is the use of plants bred with improved genetic ability to stave off disease. A drawback of this method is that selection of resistance traits is delayed until later generations.

Rotation of crops with plants that are not susceptible to the particular disease (Martin and Bull, 2002) is another alternative. However, some plant pathogens persist for many years as they can survive extreme environmental conditions (Gomez-Alipzar, 2001). Thus, some pathogens may still be present when the main crop is returned to the field if the rotation was not sufficiently long (Njoroge et al., 2009).

In addition to the previous methods, biological control of pathogens with antagonists has been intensively investigated (Van Loon and Glick, 2004). According to Baker and Cook (1974), biocontrol is defined as “the reduction of inoculum density or disease producing activities of a pathogen or parasite in its active or dormant state, by one or more organisms accomplished naturally or through manipulation of the environment, host, or antagonist or by mass introduction of one or more antagonists”. Biocontrol has different modes of action including (i) parasitism i.e., the ability to penetrate through the fungal tissue and produce metabolic substance (such as digestive enzymes) that damage the tissues of the pathogen; (ii) competition for nutrients and space; (iii) antibiosis i.e., the production of volatile and or non-volatile antimicrobial compounds; and (iv) induced resistance i.e., the ability of a beneficial

microorganism or other elicitor to induce and/or enhance the host plant's ability to defend itself through plant defense mechanisms (Trivedi, 2007).

1.3.3. Market value of biocontrol

The global consumer market demands high quality food with reduced pesticide exposure. A recent study was conducted to report the state of the agricultural inoculants market (Markets and Markets, 2013). Agriculture inoculants are formulations containing one or more beneficial microorganism strains (or species), which help plants develop, directly or indirectly. They include plant growth promoting microorganisms, biocontrol agents, and plant resistance stimulants. The global market for agricultural inoculants was valued at \$232 million in 2013 (Markets and Markets, 2013). This market is projected to grow at 9.5% from 2014 to reach \$398 million by 2019. North America dominated in sales of these products in 2013. The Latin American demand for these inoculants are projected to emerge as the fastest growing market segment at rate of ~10% from 2014 to 2019 (Markets and Markets, 2013). This indicates that biological control products are growing rapidly, and there is still a need to develop and optimize naturally-derived products.

1.4. Sources of biological control agents

Microorganisms that naturally antagonise plant pathogens and suppress plant diseases are found in many environmental materials such as compost, compost tea, and suppressive soils.

1.4.1. Composts

According to Hoitink (1980) “composting is the breakdown of organic waste materials by mixed populations of microorganisms in a thermophilic aerobic environment”. Compost has agricultural value because it improves the structure and moisture-retention properties of soil and provides plants with nutrients. Composts produced from various agricultural wastes, such as plant residues and animal manure (Chen et al., 1988; Inbar et al., 1991) have also been shown to suppress soil-borne plant pathogens (Hoitink et al., 1997; Cotxarrera et al., 2002). Disease suppression in these composts are in part attributed to their microbial populations (Avilés et al., 2011). Two main uses of composts for disease control are (i) the direct use of suppressive composts and (ii) the use of compost tea.

1.4.1.1. Suppressive compost

A suppressive compost can be defined as a compost providing reduced disease development in the presence of a pathogen and a susceptible plant. Plant disease suppression can be the direct result of the activity of antagonistic microorganisms, which naturally colonize compost (Mandelbaum et al., 1988). For example, compost from marine waste has been shown to increase crop yields and suppress plant diseases caused by several plant pathogens (Niisawa et al., 2008). This compost was also found to contain beneficial *Bacillus* spp., which had the ability to inhibit pathogenic *Fusarium* spp. (Niisawa et al., 2008). Other work on olive and grape marc compost showed that this compost consistently suppressed *Fusarium* wilt of tomatoes and *Rhizoctonia* damping-off (Avilés et al., 2011).

1.4.1.2. Compost tea

Compost teas are “fermented watery extracts of composted materials that are used for their ability to decrease plant diseases” (Litterick et al., 2004). The resulting liquid can then be applied as a soil or foliar application (Scheuerell and Mahaffee, 2002). Compost tea is highly heterogeneous in nature and is a source of many microorganisms that compete with the pathogens, thereby reducing disease. Compost teas act on pathogens by inhibiting their spore germination, by increasing the amount of benign microorganisms that produces antifungal compounds, and/or by inducing systemic and local acquired resistance (Deepthi and Reddy, 2013). The disease suppressive ability of compost teas is mainly because of the presence of beneficial microorganisms (Dionne et al., 2012; On et al., 2015). For example, compost teas showed successful suppression of tomato plant pathogens because of the beneficial microorganisms they contained such as fluorescent *Pseudomonas* spp. and *Actinomycetes* (Koné et al., 2010). Bacteria of the genera *Bacillus*, *Pseudomonas* and *Serratia*, and filamentous fungi of the genus *Trichoderma* are commonly isolated antagonists from compost teas (Hoitink et al., 1991; Hoitink et al., 1997) and their antagonistic activity will be discussed in section 1.5.

1.4.2. Suppressive soils

A suppressive soil is one in which pathogens, although present, do not cause the expected damage due to the inhibitory activities of the soil (Termorshuizen and Jeger, 2008). Among the most reported causes for the disease suppressiveness in these soils is the presence of beneficial microorganisms (Martinez et al., 2002). Suppressive soils therefore provided a wealth of microbial resources with potential to be utilized as biological control agents of plant pathogens.

Recently, *B. subtilis* CU12 was isolated from an agricultural field with a history of low plant disease and the bacterium showed antagonistic activities against plant pathogens such as *A. solani*, *B. cinerea*, *F. sambucinum* and *P. sulcatum* (Wise et al., 2012). Furthermore, other researchers isolated *Bacillus cereus*, *Cellulomonas fimi*, *Kocuria varians*, *Pseudomonas putida*, *Rhodococcus erythropolis* and *Rhodococcus globerulus* from different suppressive soils, which exhibited *in vitro* reduction of mycelial growth of *Helminthosporium solani* (Martinez et al., 2002). Among the antagonistic bacteria isolated from suppressive soils *Bacillus* spp. and *Pseudomonas* spp. are among the most common and well-studied (Trotel-Aziz et al., 2007) and will be the focus of the following sections.

1.5. Antimicrobial metabolites from microorganisms

As previously mentioned, several reports showed that microbial antagonists isolated from compost, compost teas and suppressive soils can play a role in the suppression of plant diseases (Avilés et al., 2011). Many studies showed that this suppression is linked, in part, to the production of antimicrobial compounds. For example, antimicrobial metabolites are linked to suppression of plant disease such *Armillaria* root rot by *Trichoderma viride* and *Pythium* and *Rhizoctonia* damping-off, stem and root rot by *Pseudomonas fluorescens* (Zintani, 2005). There are numerous types of these antimicrobial compounds that are generally classified into two groups: peptide and non-peptide antimicrobials.

1.5.1. Non-peptide antimicrobials

Pseudomonas spp. are among the most active and dominant bacteria producing non-peptide antimicrobials such as pyrrolnitrin (Figure 3), pyoluteorin and phenazine-1-carboxylate.

Previous work showed that these antimicrobial compounds are linked to the suppression of plant diseases (Duffy and Defago, 1999). Similarly, bacteria producing siderophores, compounds that mediate competition for iron, have emerged as a sustainable approaches for integrated plant disease management (Sayyed and Patel, 2011). A study showed that *Pseudomonas* spp. inhibited the growth of black root rot of tobacco caused by *Chalara elegans* and that suppression was linked to the production of siderophore (Voisard et al., 1994). *Bacillus subtilis* has also been shown to produce the siderophore 2,3-dihydroxybenzoyl-glycine (Figure 3), which is implicated in the suppressive activity toward a wide spectrum of plant pathogens (Leong, 1986). Furthermore, *B. subtilis* strain CU12 showed the production of 3-hydroxypropionaldehyde compounds with inhibitory properties against mycelial growth of *A. solani*, *B. cinerea*, and *P. sulcatum* (Wise et al., 2012). Also reported by Hamdache et al. (2011) showed that *Bacillus* spp. produce diverse groups of none-peptide metabolites with inhibitory activities such as polyene antimicrobials.

Figure 3. Structure of two non-peptide biocontrol (a) 2,3-dihydroxybenzoyl-glycine and (b) pyrrolnitrin (Adapted from the National Library of Medicine Database, 2015).

1.5.2. Peptide antimicrobials

Antimicrobial peptides, also known as peptide antibiotics, have been further defined in the past half-century. There are two classes of peptides: non-ribosomally synthesized peptides

and ribosomally synthesized peptides. Antimicrobial peptides are largely produced by bacteria and other species as major components of the natural host defense system (Hancock and Chapple, 1999). Antimicrobial peptides are exceptional compounds due to their significant role in the health of plants. Living organisms secrete a wide range of antimicrobial peptides produced through ribosomal (defensins and small bacteriocins) or non-ribosomal synthesis (peptaibols, cyclopeptides and pseudopeptides). Many antimicrobial peptides have shown high levels of protection against plant diseases (Montesinos, 2007). For examples, *B. subtilis* isolates was shown to control *Pythium* damping-off on tomatoes and its inhibitory effects were attributed to the production of the peptide antimicrobial mycosubtilin (Leclère et al., 2005).

1.5.2.1. Cyclic lipopeptides (CLP)

Among peptide antimicrobials are a subcategory named cyclic lipopeptides (CLPs), which are biosynthesized by non-ribosomal peptide synthetases (NRPSs), which, unlike the ribosome, often utilize both regular and non-proteinogenic amino acids as substrates (Bockus et al., 2013). The general structure of CLP comprises a lactone or lactam ring (Bruner et al., 2002) with a fatty acyl tail. CLPs are characterized by structural diversity arising from differences in the carbon length (C₆-C₁₈), composition of the fatty acid moiety (β -OH groups, iso-, anteiso-methyl branched forms) and from variations in the number of (2-25 AA), type of (basic, acidic, aromatic, aliphatic, cyclic, OH/SO₃H-containing, α - or β -type), and configuration of (D, L) the amino acids in the peptide portion (Liu et al., 2008). The two major genera of bacteria that produce a wide range CLPs are *Pseudomonas* spp. and *Bacillus* spp. (Raaijmakers et al., 2010). In nature, antimicrobial biosynthesis by microorganisms can provide many different molecules (Schneider et al., 2009). Discovering and identifying which antagonists produce which

antimicrobial compounds as well as the effect of these compounds *in vitro* and *in vivo* will provide a better understanding on how the antimicrobial and, ultimately, the antagonist will behave in the environment in terms of its biological control ability.

1.5.2.1.1. *Bacillus* spp. and their CLP

Bacillus spp. are typically Gram positive, rod-shaped, heterotrophic, and usually motile. *Bacillus* spp. produce three major families of CLPs: fengycin, iturin, and surfactin (Malfanova et al., 2012). Each lipopeptide family is further sub-divided based on amino acid composition. The CLPs produced by *Bacillus* spp. are some of the most powerful CLP with regard to their antifungal and biosurfactant activity (Ongena and Jacques, 2008; Jacques, 2011).

1.5.2.1.1.1. Fengycin family

Fengycins are a family of biologically active CLPs produced by various species of *Bacilli*, such as *B. subtilis*, *B. cereus*, *B. amyloliquefaciens* and *B. globigii* (Hu et al., 2007; Romero et al., 2007; Bie et al., 2009; Pyoung et al., 2010). The peptide portion of fengycins consist of 10 amino acids forming a peptide ring attached to a C₁₄-C₁₈ fatty acid chain (Figure 4b and Table 2) (Lang, 2002). Fengycin is synthesized non-ribosomally by peptide synthetases. The biosynthesis of fengycin requires five fengycin synthetases encoded by *fenC*, *fenD*, *fenE*, *fenA* and *fenB* (Lin et al., 1998; Shu et al., 2002; Wu et al., 2007). Studies demonstrated that fengycins are implicated in the suppression of plant disease including preventing damping-off disease of bean seedlings caused by *Pythium ultimum* (Ongena et al., 2005).

1.5.2.1.1.2. Iturin family

Iturins consist of 7 amino acids, which are linked to a C₁₄-C₁₇ fatty acid chain (Figure 6c and Table 3) (Lang, 2002). The iturin operon is composed of four open reading frames (ORFs): *ituD*, *ituA*, *ituB*, and *ituC*. The iturin family consists of many subgroups including iturin A, mycosubtilin, and bacillomycin, which showed effective antifungal activity against numerous plant pathogens (Thimon et al., 1995; Duitman et al., 1999; Tsuge et al., 2001; Moyne et al., 2004).

1.5.2.1.1.3. Surfactin family

Surfactins are the most commonly studied *Bacillus* lipopeptide family and their structures consist of a 7 amino acid cyclic sequence connected to a C₁₃-C₁₆ fatty acid chain (Figure 4a and Table 2) (Kakinuma et al., 1969). Surfactins contain β -hydroxyl fatty acid groups, usually β -hydroxytetradecanoic acid (Figure 4a and Table 2), synthesized by the *urfA* operon, and involve non-ribosomal peptide synthetases with ORFs that catalyse multifunctional enzymes for surfactin synthesis (Cosmina et al., 1993). Surfactins seem to also be key factors in the establishment of stable biofilms as they can inhibit the biofilm formation of other bacteria, thus protecting the host plant, as shown in *Arabidopsis* against *Pseudomonas syringae* (Bais et al., 2004).

Figure 4. Structural representation of three families of lipopeptides produced by *Bacillus* spp. (a) surfactin (b) iturin and (c) fengycin (Ongena et al., 2005).

Table 2. Examples of amino acid composition and fatty acyl group length of the *Bacillus* spp. lipopeptide families' surfactin, iturin, and fengycin. Structures were adapted from (Mondol et al., 2013).

Lipopeptide family	Amino acid and fatty composition of variants and homologs*
Surfactin	$ \begin{array}{c} \text{C}_{10-16}\text{CHCH}_2\text{CO-L-Glu-L-Leu-D-Leu-L-Val-L-Asp-D-Leu-L-Leu} \\ \text{O} \quad \text{L-Ile C} \\ \quad \quad \text{L-Val B} \\ \quad \quad \text{A} \end{array} $
Iturin	$ \begin{array}{c} \text{C}_{14-17}\text{CHCH}_2\text{CO-L-Asn-D-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Ser} \\ \text{HN} \quad \text{L-Asp} \\ \quad \quad \text{B} \\ \quad \quad \text{A} \end{array} $
Fengycin	$ \begin{array}{c} \text{C}_{14-18}\text{CHCH}_2\text{CO-L-Glu-D-Orn-D-Tyr-D-Thr-L-Glu-D-Ala} \\ \text{OH} \quad \text{D-Val B} \\ \quad \quad \text{O} \quad \text{A} \\ \quad \quad \text{L-Ile-L-Tyr-L-Gln-L-Pro} \end{array} $

*A, B, and C indicate different homologs of lipopeptides within each family

Surfactin: (A) surfactin, (B) [Val7] surfactin, (C) [Ile7] surfactin

Iturin: (A) iturin A-1, (B) iturin C-1

Fengycin: (A) fengycin A, (B) fengycin B

1.5.2.1.2. *Pseudomonas* spp. and their CLP

Pseudomonas strains are one of the most intensively investigated sources of potential biocontrol agents. Among *Pseudomonas* spp., the most prevalent species that suppress plant diseases include *P. aureofaciens*, *P. cepacia*, *P. chlororaphis*, *P. fluorescens* and *P. putida* (Janisiewicz et al., 1997; 1992; Meena and Kanwar, 2015). Additionally, non-plant pathogenic isolates of *P. syringae* have been identified as useful biocontrol agents (Hildebrand et al., 1998). Strains of *Pseudomonas* spp. are typically Gram negative, chemoheterotrophic motile rods with polar flagella. They are non-spore forming and can produce pigments as defined by Palleroni et al. (1993). The extensive interest in *Pseudomonas* spp. is due in part to the production of a wide variety of metabolites, including enzymes, bacteriocins, toxins, antibiotics, and cyclic lipopeptides, which are a result of their interactions with multiple plant hosts (Haas and Défago 2005; Raaijmakers et al., 2006). The following sections include some of the most commonly reported families of antimicrobial CLP in *Pseudomonas* spp.

1.5.2.1.2.1. Amphisin family

Amphisins produced by *Pseudomonas* spp. have both biosurfactant and antifungal properties and inhibit plant pathogenic fungi (Sorensen et al., 2001). The primary structure of amphisin consists of 11 amino acids attached to 3-hydroxydecanoic acid (Figure 5b and Table 3). The structure of amphisin is distinguished by a cyclic peptide wrapping around a hydrogen-bonded water molecule, which is important for its function (Raaijmakers et al., 2010). Amphisin is synthesized from non-ribosomal proteins encoded by large multifunctional peptide synthetases (Marahiel, 1997). Previous work showed that amphisin has the ability to suppress plant diseases caused by *Pythium ultimum* and *Rhizoctonia solani* (Nielsen et al., 2002). The

amphisin family includes variants such as arthrofactin, tensin and lokisin (Table 3) (Sorensen et al., 2001).

1.5.2.1.2.2. Putisolvin family

Pseudomonas putida PCL1445 was found to produce two surface-active cyclic lipopeptides, putisolvins I and II. The ORF encoding the synthesis of the putisolvins bears amino acid homology to various lipopeptide synthetases (Kuiper et al., 2003). Putisolvins are produced by putisolvin synthetases designated as *psoA*, *psoB* and *psoV* and were shown to encode NRPs (Dubern et al., 2008). Putisolvins I and II have a similar structure that consists of 12 amino acid polar peptide heads linked to hexanoic acid moiety (Figure 5c and Table 3). Putisolvins can inhibit the growth of microorganisms by inhibiting the formation of biofilms or breaking down previously formed biofilms (Kuiper et al., 2003).

1.5.2.1.2.3. Syringomycin family

Many strains of the bacterial pathogen *Pseudomonas syringae*, produce syringomycin lipopeptides (Segre et al., 1989). Syringomycin consists of 10 amino acid bonded to dodecanoic acid (Figure 5d and Table 3) (Guenzi et al., 1998). Syringomycin is synthesized by two non-ribosomal peptide (NRP) (*SyrB1* and *SyrE*) and three modifying protein systems (*SyrB2*, *SyrC* and *SyrP*) (Roongsawang et al., 2011). Syringomycin displays inhibitory activities against a broad spectrum of fungi, particularly yeasts (Sorensen et al. 1996; Lavermicocca et al. 1997). Syringomycin acts on membranes of the target fungus by forming voltage-dependent and nonselective ion channels (Takemoto et al., 2003).

1.5.2.1.2.4. Viscosin family

Viscosin was first isolated from a *Pseudomonas* spp. in 1951. The CLP consists of 9 amino acid cyclic oligopeptides linked to a 3-hydroxydecanoic acid (Figure 5a and Table 3). Viscosin is synthesized by NRP systems that are encoded by three large ORFs, termed *viscA*, *viscB* and *viscC*. Viscosin's mode of action is to induce surfactant activity by reducing the surface tension of water (Roongsawang et al., 2011). There are many variants of the viscosin family such as massetolide (C, G, A, H, L, B, F and D), white line inducing principle, viscosin, pseudophomin (A and B), and viscosinamine (Table 3) (Roongsawang et al., 2011).

Table 3. Primary structure of lipopeptide families and representative variants produced by *Pseudomonas* spp. Adapted from Roongsawang et al. (2011).

Group/Name	Structure
Viscosin^A	
Viscosin	FA-β-OH-L-Leu-D-Glu-D- <i>a</i> Thr-D-Val-L-Leu-D-Ser-L-Leu-D-Ser-L-Ile
Viscosinamide	FA-β-OH-L-Leu-D-Gln-D- <i>a</i> Thr-D-Val-L-Leu-D-Ser-L-Leu-D-Ser-L-Ile
Massetolide A	FA-β-OH-L-Leu-D-Glu-D- <i>a</i> Thr-D- <i>alle</i> -L-Leu-D-Ser-L-Leu-D-Ser-L-Ile
Pseudophomin A	FA-β-OH-L-Leu-D-Glu-D- <i>a</i> Thr-D- Ile-D-Leu-D-Ser-L-Leu-D-Ser-L-Ile
Pseudodesmin A	FA-β-OH-L-Leu-D-Gln-D- <i>a</i> Thr-D-Val-D-Leu-D-Ser-L-Leu-D-Ser-L-Ile
Syringomycin^B	
Syringomycin A	FA-β-OH-L-Ser-D-Ser-D-Dab-L-Dab-L-Arg-L-Phe-Z-Dhb-L-Asp _(3-OH) -L-Thr _(4-Cl)
Syringostatin A	FA-β-OH-L-Ser-D-Dab-L-Dab-D-Hse-L-Orn-L- <i>a</i> Thr-Z-Dhb-L-Asp _(3-OH) -L-Thr _(4-Cl)
Syringotoxin B	FA-β-OH-L-Ser-D-Dab-L-Gly-D-Hse-L-Orn-L- <i>a</i> Thr-Z-Dhb-L-Asp _(3-OH) -L-Thr _(4-Cl)
Pseudomycin A	FA-β-OH-L-Ser-D-Dab-L-Asp-D-Lys-L-Dab-L- <i>a</i> Thr-Z-Dhb-L-Asp _(3-OH) -L-Thr _(4-Cl)
Cormycin A	FA-β-OH-L-Ser-D-Orn-L-Asn-D-Hse-L-His-L- <i>a</i> Thr-Z-Dhb-L-Asp _(3-OH) -L-Thr _(4-Cl)
Amphisin^A	
Arthrofactin	FA-β-OH-D-Leu-D-Asp-D- <i>a</i> Thr-D-Leu-D-Leu-D-Ser-L-Leu-D-Ser-L-Ile-L-Ile-L-Asp
Amphisin	FA-β-OH-D-Leu-D-Asp-D- <i>a</i> Thr-D-Leu-D-Leu-D-Ser-L-Leu-D-Gln-L-Leu-L-Ile-L-Asp
Lokisin	FA-β-OH-D-Leu-D-Asp-D- <i>a</i> Thr-D-Leu-D-Leu-D-Ser-L-Leu-D-Ser-L-Leu-L-Ile-L-Asp
Pholipeptin	FA-β-OH-D-Leu-L-Asp-L- Thr-D-Leu-D-Leu-D-Ser-D-Leu-D-Ser-D-Leu-L-Ile-D-Asp
Tensin	FA-β-OH-D-Leu-D-Asp-D- <i>a</i> Thr-D-Leu-D-Leu-D-Ser-L-Leu-D-Gln-L-Leu-L-Ile-L-Glu
Putisolvin^C	
Putisolvin I	CH ₃ (CH) ₄ CO-D-Leu-D-Glu-D-Leu-D-Ile-D-Gln-D-Ser-D-Val-D-Ile-D-Ser-L-Leu-L-Val-X-Ser
Putisolvin II	CH ₃ (CH) ₄ CO-D-Leu-D-Glu-D-Leu-D-Ile-D-Gln-D-Ser-D-Val-D-Ile-D-Ser-L-Leu-L-Xle-X-Ser

^A the hydroxyl group of *a*Thr or Thr form an ester bond with the carboxyl group of the C-terminal amino acid

^B the hydroxyl group of L-Ser1 form an ester bond with the carboxyl group of the C-terminal amino acid

^C the hydroxyl group of D-Ser9 form an ester bond with the carboxyl group of the C-terminal amino acid

Figure 5: Representative structures of cyclic lipopeptides families produced by *Pseudomonas* spp. (a) viscosin (Gross and Loper, 2009), (b) amphisin (Sorensen et al., 2001), (c) putisolvin (Kuiper et al., 2003), and (d) syringomycin (Galonic et al., 2006).

1.6. Hypotheses and objectives

Most of the studies on biological control indicated the promise of strains of antagonists to decrease disease incidence or severity. However, the lack of information on secondary metabolites, such as antimicrobials, produced by microbial antagonists has hampered the development of biocontrol agents with consistent disease control.

Bacterial genomes produce many different of bioactive compounds. In *Bacillus* spp., 4-5% of their genomes are devoted towards producing more than two dozen antimicrobial compounds (Stein, 2005). In *Pseudomonas* spp., approximately 6% (Gross and Loper, 2009) of their genomes are devoted to produce antimicrobial compounds such as phenazine, siderophores, and HCN (Haas and Defago, 2005). Although are large proportion of these bacterial genomes are devoted to antimicrobial production, little is known of the different antimicrobials produced by different antagonistic species, as well as by different isolates or strains with the same species.

Currently, the major challenge in plant pathology is to successfully introduce biological control agents as disease strategies. Additional knowledge of the antimicrobial compounds involved in the inhibition of plant pathogens will undoubtable help in the selection of the most promising antagonists for successful biological control.

1.6.1. Hypotheses

- (I) Bacteria isolated from suppressive soils, composts, and compost teas will inhibit the growth of plant pathogens.
- (II) Compounds isolated from these bacteria will inhibit mycelial growth and spore germination of plant pathogens.

- (III) Conidia will be more susceptible than mycelia to bioactive compounds from *Bacillus*, *Bacillus*-related, and *Pseudomonas* spp.
- (IV) Compounds from bacterial extracts contain different families of known antimicrobial lipopeptides.

1.6.2. Objectives

The objectives of this study were (I) to investigate the potential antimicrobial activity in various bacteria isolated from suppressive soils, disease suppressive composts, and compost teas, (II) to assay lipopeptide fractions from selected bacteria against mycelial growth and spore germination of plant pathogens, and (III) to detect the presence of various known antimicrobial lipopeptides in the extracts from *Bacillus*, *Bacillus*-related, and *Pseudomonas* spp.

Chapter 2: Materials and methods

2.1. Microbial material

2.1.1. Bacteria

The bacteria *Bacillus subtilis* isolates B9-1, B9-5, B9-7, B9-8, B9-9A, and B9-14, *Paenibacillus favisporus* isolate B9-4, *Pseudomonas gessardii* isolate B9-3, and *Bacillus megaterium* isolate B9-9B were isolated from a disease suppressive compost prepared with bovine manure and peat moss. *Pseudomonas moraviensis* isolates F9-2 and F9-11, *Pseudomonas arsenicoxydans* isolate F9-7, *Pseudomonas koreensis* isolate F9-9, *Pseudomonas proteolytica* isolate F9-10, *Pseudomonas libanensis* isolate F9-13, and *B. subtilis* isolates F9-2, F9-8, and F9-12 were isolated from a disease suppressive compost composed of forestry residue and peat moss. *Arthrobacter humicola* isolates M9-1A, M9-2 and M9-8, *B. megaterium* isolate M9-1B, *B. subtilis* isolates M9-3, M9-4, M9-7, M9-9 and M9-14, *P. gessardii* isolate M9-16, *Arthrobacter psychrophenicus* isolate M9-17, *Brevibacillus borstelensis* isolate M9-18, *Rummeliibacillus pycnus* isolate M9-19 and *Bacillus badius* isolate M9-20 were isolated from a disease suppressive compost prepared from marine residue and peat moss. All composts were produced by Berger Peat Moss (Saint-Modeste, QC) (Figure 6).

Bacillus subtilis isolate CU12 (Wise et al. 2012) and *Bacillus licheniformis* isolate PSA1 were isolated from disease suppressive soils in Ottawa, ON (Figure 6).

Aminobacter aminovorans isolate IB-2, *B. subtilis* isolates IB-16 and IC-10, *Brevibacterium iodinum* isolates IB-48 and IC-23, and *Advenella kashmirensis* isolate IC-6 were isolated from a compost tea prepared from sheep manure compost (Koné et al., 2010; Dionne et al., 2012) (Figure 6).

All bacteria were stored in glycerol stocks at -80°C and maintained on tryptic soy agar (TSA, Becton Dickinson, Sparks, MD).

2.1.2. Fungi and oomycetes

The plant pathogenic fungi *A. solani*, *B. cinerea*, *F. sambucinum*, *R. stolonifer*, and *V. dahliae* were obtained from the *Laboratoire de diagnostic en phytoprotection* (MAPAQ, Québec, QC). The plant pathogenic oomycete *P. sulcatum* was isolated from an infected carrot root and is available at the Canadian Collection of Fungal Cultures (Agriculture and Agri-Food Canada, Ottawa, ON). The pathogens were stored as freeze-dried stocks and maintained on potato dextrose agar (PDA, Becton Dickinson).

Figure 6. Flow chart showing the experimental samples and methodology for determining antagonistic effects of bacterial isolates and the presence of lipopeptide compounds.

2.2. Antagonistic activity of bacterial isolates against pathogens

The six pathogens were confronted with the isolated bacteria to determine their antagonistic activity. The 40 bacterial isolates were individually inoculated at the four cardinal points on PDA by streaking 1-cm line of each bacterium at a distance of 2 cm (for *A. solani*), 3.5 cm (for *B. cinerea*, *F. sambucinum*, *P. sulcatum* and *R. stolonifer*), and 1 cm (for *V. dahliae*) from the center of the dish. A 5-mm diameter agar plug containing actively growing mycelium was placed in the center of each Petri dish. Control dishes were inoculated with the pathogens without bacterial streaks. Petri dishes were incubated for 5, 4, 8, 6, 2, and 10 days for *A. solani*, *B. cinerea*, *F. sambucinum*, *P. sulcatum*, *R. stolonifer* and *V. dahliae*, respectively, at 23°C in the dark. Following the incubation period, growth was measured and expressed as the average of two perpendicular diameters of the thallus. The experiments were conducted according to a completely randomized design with three replicates (Figure 6).

2.3. Extraction of lipopeptides

2.3.1. Preparation of medium optimized for lipopeptide production (MOLP)

Individual bacterial isolates were grown in medium optimized for lipopeptide production (MOLP) as described by Akpa et al. (2001). Briefly, MOLP contains 20 g sucrose, 30 g peptone, 7 g yeast extract, 1.9 g KH_2PO_4 , 0.45 g MgSO_4 , 9 ml Trace Element Solution (TES), and 9 ml of a citric acid solution (10 g/L ddH₂O). TES was prepared by adding CuSO_4 (0.001 g), FeCl_3 (0.005 g), Na_2MoO_4 (0.004 g), KI (0.002 g), ZnSO_4 (0.014 g), H_3BO_3 (0.01 g), MnSO_4 (0.0036 g) to 1 L of sterile ultra-pure water. MOLP components were added to 982 ml of ddH₂O and sterilized.

2.3.2. Bacterial growth and recovery of cell-less culture medium

Selected isolates representing *Pseudomonas*, *Bacillus* and *Bacillus*-related spp. were individually inoculated into three 250-ml Erlenmeyer flasks containing 50 ml of sterilized MOLP. Inoculated samples were incubated at 30°C and 130 rpm for 72 h (for isolates B9-1, B9-3, B9-4, B9-5, B9-7, B9-8, B9-9A, B9-9B, B9-14, CU12, IB16, IC10, M9-3, M9-4, M9-7, M9-9, M9-14, M9-16, M9-18, M9-19, and M9-20) or 96 h (for isolates F9-2, F9-6, F9-7, F9-8, F9-9, F9-10, F9-11, F9-12, F9-13 and PSA1). Incubated isolates were transferred to 50-ml sterile plastic tubes and centrifuged at 4,300 rpm for 30 min at 4°C. The supernatant was then filtered through sterile, 500-ml 0.45 µm polyether sulfone (PES) filter units (Celltreat, Mandel Scientific, Guelph, ON). The cell-free filtrates were stored at 4°C until use (Figure 6).

2.3.3. Lipopeptide precipitation

The lipopeptides in the filtrates were precipitated using 30% w/v ammonium sulfate [(NH₄)₂SO₄] by adding 15 g of (NH₄)₂SO₄ to each tube. The tubes were then shaken and incubated at 4°C overnight. Samples were centrifuged for 60 min at 4,300 rpm and 4°C to recover lipopeptides. Supernatants were discarded and the precipitated lipopeptides were suspended in 5 ml of 10 mM phosphate buffer (pH = 6.1) (Figure 6).

2.4. Antimicrobial activity of the lipopeptide extracts

Fourteen bacterial isolates were chosen to test the antimicrobial activity of lipopeptide extracts in the inhibition of mycelial growth and conidial germination. Three categories representing isolates from different inhibition class (from section 2.2) were chosen: class 1

(containing *B. subtilis* B9-5, B9-14, IC10, CU12, M9-9, *P. proteolytica* F9-10 and *P. koreensis* F9-11) reduced the growth of all pathogens; class 2 (containing *B. subtilis* M9-7, F9-8, F9-2 and F9-12, *P. koreensis* F9-9 and *P. arsenicoxydans* F9-7) reduced the growth of some, but not all of the pathogens; and class 3 (containing *B. badius* M9-20) did not reduce the growth of any of the tested pathogens. Tests were performed to assess the extracts against different morphological structures (mycelium and/or conidia) of the pathogens.

2.4.1. Effect of crude lipopeptide extracts on mycelia growth

The antifungal activities of lipopeptide extracts were tested using a disc diffusion assay with *A. solani*, *B. cinerea*, *F. sambucinum*, *P. sulcatum*, *R. stolonifer* and *V. dahliae*. Briefly, 25 μ L of each crude extract (10 $\times$ concentrated) was placed on sterile filter paper disks (0.5 cm diameter) and left to dry for 1 h. Sterile ultra-pure water was used as a control. Petri dishes containing PDA were inoculated with a 5-mm diameter agar plug containing actively growing mycelium. The dried paper disks containing lipopeptide extracts or controls were placed at a distance of 2 cm (for *A. solani*), 3.5 cm (for *B. cinerea*, *F. sambucinum*, *P. sulcatum* and *R. stolonifer*), and 1 cm (for *V. dahliae*) from the agar plugs. All Petri dishes were incubated at 23°C in the dark for 6, 3, 5, 6, 2 and 12 days for *A. solani*, *B. cinerea*, *F. sambucinum*, *P. sulcatum*, *R. stolonifer* and *V. dahliae*, respectively. Mycelial growth was measured as the radius of the thallus facing the paper disc containing samples or controls. The experiments were conducted according to a completely randomized design with three replicates (Figure 6).

2.4.2. Effect of crude lipopeptide extracts on spore germination

Antifungal activities were tested using a spore germination assay with *A. solani*, *B. cinerea*, *F. sambucinum*, *R. stolonifer*, and *V. dahliae*. Each sample was concentrated 10 times and 25 µL was placed in a sterile 1.5 mL conical snap-cap tube (Eppendorf tube) evaporated and re-suspended in 90 µL of sterile water. 90 µL of sterile water without sample served as the control. 10-µL spore suspensions (5×10^5 spores/mL in sterile water) of *A. solani*, *B. cinerea*, *F. sambucinum*, *R. stolonifer* and *V. dahliae* were individually added to each treatment. The treated spores were incubated at 23°C for 30 min and 100 µL of the suspension was spread on water agar (containing 15 g/L agar). The Petri dishes were incubated for 24 h (*R. stolonifer*), 28 h (*B. cinerea* and *F. sambucinum*) or 48 h (*V. dahliae* and *A. solani*) at 23°C in the dark. Following the incubation period, Petri dishes were observed under visible light using an inverted microscope. Spores were considered germinated when the length of the germ tube equaled at least the length of the spore. The percentage of germinated cells was calculated as follows: percent germination = germinated spores/total spores $\times$ 100. The experiment was performed according to a completely randomized design with three replicates (Figure 6).

2.5. Fractionation of lipopeptide extracts

Reverse-phase sterile 5 mL C₁₈ cartridges (Bond Elut, Varian, Mississauga, ON) were used for fractionation. The extracts in phosphate buffer were concentrated 5 times by bringing samples to dryness using a rotary evaporator at 150 rpm and 35-40°C and resuspended in 1 mL of phosphate buffer (pH 6.4). The column was conditioned twice with 5 mL of ultra-pure water. Samples were placed on the column and the flow through was collected. The column was sequentially eluted with 5 mL of various ratios of methanol:water as follows: 0:100, 20:80,

50:50, 80:20, and 100:0. Five fractions (F1-F5) obtained were brought to dryness using a rotary evaporator at 150 rpm and 35-40°C. Samples were suspended in 200 µl of 100% methanol, passed through syringe filters (0.45 µm) and transferred to auto-sampler vials containing 250-µl inserts (Figure 6).

To characterize the presence of lipopeptides produced by the antagonistic bacterial isolates, preliminary study was done to screen all the fractions from the various ratios of methanol:water as follows: 0:100, 20:80, 50:50, 80:20, and 100:0. Previous work on these lipopeptide extracts with this methodology showed that the 80% and 100% methanol fractions (F4 and F5) contained the lipopeptides. Therefore, these two fractions were the samples analyzed by LCMS.

2.6. Characterization of lipopeptides in bacterial extracts

Liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) was performed using a Waters 2795 separations module and MicroMass Quattro Ultima triple quadrupole mass spectrometer. Samples were separated by a Phenomenex Kinetix C₁₈ (100 × 4.6 mm, 2.6 µm, 100 Å) column (Torrance, California) using a mobile phase consisting of acetonitrile-water (ACN-H₂O) with formic acid (FA) [0.1%, (v/v)]. The solvent gradient was linear and programmed from 5 to 100% ACN over 13 min with a flow rate of 1 ml min⁻¹.

Positive ESI conditions included: capillary voltage 3.5 kV, cone voltage 20 V, source temperature 80°C, desolvation temperature 180°C, cone gas flow (N₂) 90 l/h, desolvation gas flow (N₂) 540 l/h, and multiplier voltage of 650 V. The mass range examined was *m/z* 900-1600. Lipopeptides were identified by comparison with authentic standards and MS data available in the literature.

Comparative analysis of mass spectra of the two methanolic fractions (80% and 100%), revealed quantitative variability of m/z ions masses and series of homologs corresponding to lipopeptides families belonging *Bacillus* and *Pseudomonas* spp. Identification of lipopeptides was further confirmed by comparing to retention time to specific region of spectra that belongs to each family of the lipopeptides and by comparing these to authentic standards (Figure 6).

2.7. Statistical analysis

For the antagonistic confrontation trials, the disc diffusion assay, and spore germination assay, analysis of variance (ANOVA) was performed and, when significant ($P < 0.05$), means were compared using Fisher's protected least significant difference (LSD) test (α level = 0.05) (Figure 6).

Chapter 3: Results

3.1. Antagonistic activity of bacterial isolates against fungi

Antifungal activities of forty bacterial isolates were investigated by testing their potential to inhibit the mycelial growth of *A. solani*, *B. cinerea*, *F. sambucinum*, *P. sulcatum*, *R. stolonifer* and *V. dahliae* *in vitro* (Table 4). The following sixteen isolates were able to reduce the growth of all six tested pathogens (Table 4): *P. proteolytica* F9-10 (61% inhibition), *P. libanensis* F9-13 (56%), *B. subtilis* IC10 and IB2 (39 %), CU12 and B9-5 (37%), B9-14 and M9-14 (36%), B9-7 and B9-9A (35%), M9-4 and M9-9 (33%), and M9-3 (29%), and *P. moraviensis* F9-6 (37%), *A. kashmirensis* IC6 (34%) and F9-11 (36 %).

In addition, nine of the bacterial isolates inhibited five of the six pathogens. Specifically, *B. subtilis* B9-1 (39 % average inhibition), F9-12 (37%), F9-8 and IB16 (36%), F9-2 and B9-8 (34%) reduced the growth of all pathogens with the exception of *R. stolonifer*. Also, *R. pycnus* M9-19 (29 %) reduced the growth of all pathogens with the exception of *P. sulcatum*. *P. koreensis* F9-9 (31% inhibition) reduced the growth of all pathogens with the exception of *F. sambucinum*, and *B. subtilis* M9-7 (36%) reduced the growth of all pathogens with the exception of *B. cinerea* (Table 4).

Furthermore, five bacterial isolates inhibited four of the six pathogens. *B. subtilis* M9-2 (42% inhibition) reduced the growth of all pathogens with the exception of *F. sambucinum* and *R. stolonifer*. Additionally, *P. arsenicoxydans* F9-7 (28 %) reduced the growth of all pathogens with the exception of *B. cinerea* and *R. stolonifer*. *P. gessardii* B9-3 (27%) reduced the growth of all pathogens with the exception of *P. sulcatum* and *V. dahliae*. *B. borstelensis* M9-18 (22%) reduced the growth of all pathogens with the exception of *P. sulcatum* and *R. stolonifer*. Finally,

A. psychrophenicus M9-17 (16%) reduced the growth of all pathogens with the exception of *A. solani* and *P. sulcatum* (Table 4).

Moreover, two bacterial isolates inhibited three of the six pathogens. *B. megaterium* B9-9B (30% inhibition) reduced the growth of *A. solani*, *B. cinerea* and *F. sambucinum*. *P. gessardii* M9-16 (30 %) reduced the growth of *A. solani*, *F. sambucinum* and *V. dahliae* (Table 4).

Three other bacterial isolates inhibited two of the six pathogens. *Arthrobacter humicola* M9-1A (26% inhibition) reduced the growth of *F. sambucinum* and *V. dahliae*. *B. iodinum* IB48 (19%) reduced the growth *A. solani* and *F. sambucinum*. Finally, *B. subtilis* M9-8 (24%) reduced the growth of *B. cinerea* and *F. sambucinum* (Table 4).

Three additional bacterial isolates inhibited only one of the six pathogens. *B. megaterium* M9-1B only inhibited the growth of *P. sulcatum* (25%), *B. iodinum* IC 23 only reduced the mycelial growth of *R. stolonifer* (13%), and *B. licheniformis* PSA1 only inhibited the growth of *A. solani* (25%) (Table 4).

Finally, two bacterial isolates (*P. favisporus* B9-4 and *B. badius* M9-20) showed no significant reduction on the mycelial growth of any of the six pathogens (Table 4).

With regard to the inhibition of *A. solani*, *P. proteolytica* F9-10, *B. subtilis* IB-2 and *P. libanensis* F9-13 showed the highest inhibitory effect (68% inhibition) whereas *P. favisporus* B9-4, *B. badius* M9-20, *A. humicola* M9-1A, *B. megaterium* M9-1B, *B. iodinum* IC-23, *B. subtilis* M9-8 and *A. psychrophenicus* M9-17, showed no significant inhibitory effect on its mycelial growth (Table 4).

In *B. cinerea* trials, *P. libanensis* F9-13, *A. humicola* M9-2 and *P. koreensis* F9-9 showed the highest inhibition (49%) of mycelial growth whereas *P. favisporus* B9-4, *B. badius* M9-20, *A. humicola* M9-1A, *B. megaterium* M9-1B, *B. iodinum* IC-23, *P. gessardii* M9-17 and B9-3,

B. subtilis M9-7, *B. iodinum* IB48 and *B. licheniformis* PSA1 had no significant inhibitory effect on its mycelial growth (Table 4).

Pseudomonas proteolytica F9-10 showed the highest inhibitory effect on the mycelial growth of *F. sambucinum* (58%) whereas *P. favisporus* B9-4, *B. badius* M9-20, *B. megaterium* M9-1B, *B. iodinum* IC-23, *P. koreensis* F9-9, *A. humicola* M9-2, and *B. licheniformis* PSA1 had no significant inhibitory effect on its mycelial growth (Table 4).

Two isolates, *P. proteolytica* F9-10 and *P. libanensis* F9-13, decreased the mycelial growth of *P. sulcatum* by 68%. Conversely, *P. favisporus* B9-4, *B. megaterium* B9-9B, *P. arsenicoxydans* F9-7, *B. iodinum* IB48 and IC23, *B. badius* M9-20, *A. humicola* M9-1A, M9-8, *P. gessardii* M9-16, *A. psychrophilicus* M9-17, *B. borstelensis* M9-18, *R. pycnus* M9-19 and *B. licheniformis* PSA1 had no significant inhibitory effect on its mycelial growth (Table 4).

For *R. stolonifer*, *P. proteolytica* F9-10, *P. libanensis* F9-13, *P. moraviensis* F9-11 and *R. pycnus* M9-19 showed the highest reduced the mycelial growth (50%) whereas *P. favisporus* B9-4, *B. badius* M9-20, *A. humicola* M9-2, M9-1A and M9-8, *B. megaterium* M9-1B, *P. gessardii* M9-16 and B9-3, *B. subtilis* B9-1, IB16, F9-2, B9-8, F9-8 and F9-12 *B. megaterium* B9-9B, *B. iodinum* IB48, *B. borstelensis* M9-18 and *B. licheniformis* PSA1 had no significant inhibitory effect on its mycelial growth (Table 4).

Pseudomonas proteolytica F9-10, *P. libanensis* F9-13 and *P. moraviensis* F9-6 showed the highest inhibition of mycelial growth of *V. dahliae* (57%) whereas *P. favisporus* B9-4, *B. badius* M9-20, *A. humicola* M9-8, *B. megaterium* M9-1B, *B. megaterium* B9-9B, *B. iodinum* IB48, *B. iodinum* IC23, *P. arsenicoxydans* F9-7 and *B. licheniformis* PSA1 revealed no significant inhibitory effect on its mycelial growth (Table 4).

In addition to measurement of mycelial growth shown in Table 4, relative inhibitory activity was calculated for the isolates in three groups (*Bacillus* spp. and *Bacillus*-related spp., *Pseudomonas* spp., and other tested species) (Figure 7). The *Pseudomonas* spp. group showed the greatest relative efficiency in inhibiting combined pathogens. *Bacillus* and *Bacillus*-related spp. showed less total relative inhibition in mycelial growth. Finally, isolates from species other than *Bacillus* and *Bacillus*-related or *Pseudomonas* spp. showed the least total relative inhibition of the pathogens (Figure 7).

Table 4. *In vitro* inhibitory activities of forty bacterial isolates from composts, compost tea, and soil against six pathogens: *Alternaria solani*, *Botrytis cinerea*, *Fusarium sambucinum*, *Pythium sulcatum*, *Rhizopus stolonifer* and *Verticillium dahliae*.

Mycelial growth inhibition (cm)						
Isolates	<i>A. solani</i>	<i>B. cinerea</i>	<i>F. sambucinum</i>	<i>P. sulcatum</i>	<i>R. stolonifer</i>	<i>V. dahliae</i>
Control	4.0±0.1a	7.1±0.1a	7.1±0.3a	6.9±0.1a	7.1±0.1abc	2.1±0.1a
B9-1	1.8±0.1nop	5.3±0.1cdefg	5.0±0.04fghijlm	4.0±0.1fgijk	6.5±0.1bcdefgh	1.2±0.1ghijkl
B9-3	3.0±0.3efgh	7.0±0.0a	5.5±0.6defghi	6.1±0.3b	7.0±0.1abcd	1.1±0.1jklm
B9-4	3.6±0.2abcd	7.0±0.0a	6.7±0.2a	7.0±0.0a	7.3±0.0a	1.9±0.0abc
B9-5	1.7±0.0op	4.9±0.1fghi	4.7±0.2klmn	3.8±0.2hijk	5.8±0.1ijklmn	1.5±0.0efg
B9-7	2.0±0.1lmnop	5.3±0.0cdef	4.6±0.2klmn	4.0±0.1fghijk	5.6±0.2lmno	1.4±0.2fghij
B9-8	2.2±0.2ijklmno	5.2±0.1defgh	4.9±0.3hijklm	4.6±0.1ef	6.7±0.0abcdef	1.4±0.1fghi
B9-9A	2.2±0.3ijklmno	5.0±0.0fghi	5.1±0.3efghijkl	4.1±0.3fghijk	5.1±0.4no	1.4±0.1fghi
B9-9B	2.7±0.2ghijk	5.3±0.4cdefg	4.7±0.1klmn	6.7±0.2ab	7.0±0.1abcd	1.9±0.1abc
B9-14	1.9±0.1mnop	5.3±0.0.1cdefg	5.0±0.3ghijklm	3.5±0.1jk	5.6±0.2klmn	1.3±0.1ghijkl
CU12	2.0±0.0mnop	5.0±0.04fghi	4.3±0.4mn	4.1±0.1efghijk	6.2±0.6fghijkl	1.2±0.2ijklm
F9-2	2.2±0.0ijklmno	5.2±0.1efghi	5.0±0.3fghijklm	4.3±0.1efghi	7.1±0.1abc	1.4±0.0fghi

Mycelial growth inhibition (cm)						
Isolates	<i>A. solani</i>	<i>B. cinerea</i>	<i>F. sambucinum</i>	<i>P. sulcatum</i>	<i>R. stolonifer</i>	<i>V. dahliae</i>
F9-6	3.0±0.0defgh	4.30±0.2k	4.8±0.2ijklm	4.6±0.2ef	4.9±0.1o	0.92±0.0mn
F9-7	3.3±0.0bcdef	4.3±0.1k	4.7±0.1efghijkl	7.0±0.0a	5.2±0.0mno	2.0±0.0ab
F9-8	2.7±0.7ghij	4.9±0.0ghij	4.7±0.3jklm	3.8±0.4ghijk	6.5±0.4bcdefg	1.3±0.0ghijkl
F9-9	2.8±0.1fghi	3.6±0.7m	6.3±0.5abcd	5.3±0.1cd	4.9±0.1o	1.3±0.2ghijkl
F9-10	0.9±0.2q	4.1±0.0kl	2.9±0.0o	2.0±0.1l	3.7±0.2p	0.70±0.0n
F9-11	2.6±0.0ghijkl	4.5±0.1jk	5.5±0.1efghij	4.5±0.4efgh	3.9±0.3p	1.2±0.0hijkl
F9-12	1.8±0.1nop	5.2±0.2defgh	4.6±0.1klmn	4.5±0.0efg	7.0±0.1abc	1.3±0.0ghijkl
F9-13	1.5±0.1pq	3.6±0.3m	3.9±0.3n	2.4±0.3l	3.5±0.3p	0.78±0.0n
IB-2	1.4±0.2pq	5.0±0.0fghi	5.0±0.1hijklm	3.8±0.1ghijk	5.5±0.1lmno	1.3±0.1ghijkl
IB-16	2.2±0.1ijklmno	5.2±0.0defgh	4.7±0.2jklmn	3.9±0.1fghijk	6.5±0.2bcd fgh	1.5±0.1efgh
IB-48	3.1±0.1cdefg	6.9±0.1a	5.9±0.1bcde	7.0±0.0a	6.8±0.1abcdef	2.0±0.0ab
IC-6	2.5±0.2hijklm	4.8±0.1ij	4.7±0.2jklm	4.8±0.4de	5.9±0.6ghijkl	1.1±0.0lm
IC-10	1.9±0.1mnop	4.8±0.1hij	4.7±0.2jklm	3.6±0.2ijk	5.9±0.04ijklmn	1.1±0.1jklm

Mycelial growth inhibition (cm)						
Isolates	<i>A. solani</i>	<i>B. cinerea</i>	<i>F. sambucinum</i>	<i>P. sulcatum</i>	<i>R. stolonifer</i>	<i>V. dahliae</i>
IC-23	3.5±0.1abcde	6.9±0.0ab	6.7±0.2a	7.0±0.0a	6.3±0.3efghijk	1.9±0.1abc
M9-1A	3.4±0.1abcde	7.0±0.0a	5.8±0.5bcdefg	7.0±0.1a	6.9±0.2abcde	1.4±0.2ghijk
M9-1B	3.4±0.1abcde	6.9±0.1a	6.4±0.4abc	6.0±0.8bc	7.0±0.0abcd	2.0±0.0ab
M9-2	2.4±0.1hijklm	3.4±0.1m	6.4±0.1ab	3.5±0.8jk	6.4±0.4cdefghi	1.4±0.0efgh
M9-3	2.3±0.0ijklmn	5.5±0.1cde	4.7±0.3klmn	4.2±0.3efghij	5.5±0.1lmno	1.7±0.2cde
M9-4	2.0±0.1mnop	5.3±0.1cdefg	4.8±0.3ijklm	3.7±0.1ijk	6.3±0.4defghij	1.5±0.1efg
M9-7	1.8±0.2nop	7.0±0.0a	4.7±0.1jklm	4.1±0.1efghijk	5.9±0.1ghijkl	1.4±0.1ghijk
M9-8	3.5±0.1abcde	5.2±0.0defghi	5.6±0.2cdefgh	6.9±0.1a	7.0±0.0abcd	1.8±0.2abcd
M9-9	2.1±0.1klmno	5.2±0.0defgh	5.5±0.0defghi	3.4±0.1k	5.8±0.3hijklm	1.4±0.0fghij
M9-14	2.0±0.3mnop	5.3±0.1cdefg	4.5±0.4lmn	3.6±0.1ijk	5.7±0.1jklmn	1.4±0.1fghi
M9-16	3.0±0.0efgh	7.0±0.0a	5.8±0.7bcdef	7.0±0.3ab	6.9±0.1abcde	1.2±0.1hijkl
M9-17	3.7±0.1abc	6.5±0.2b	5.1±0.2efghijkl	6.9±0.1a	6.1±0.4fghijkl	1.8±0.1bcd
M9-18	3.1±0.2cdefg	5.7±0.04c	5.3±0.2efghijk	7.0±0.0a	6.4±0.5bcdefgh	1.6±0.1def

Mycelial growth inhibition (cm)						
Isolates	<i>A. solani</i>	<i>B. cinerea</i>	<i>F. sambucinum</i>	<i>P. sulcatum</i>	<i>R. stolonifer</i>	<i>V. dahliae</i>
M9-19	3.0±0.6efgh	5.6±0.0cd	4.9±0.5hijklm	6.9±0.0a	3.7±0.0p	1.8±0.1bcd
M9-20	3.9±0.1ab	7.0±0.0a	7.0±0.0a	7.0±0.0a	7.1±0.1abc	2.0±0.0ab
PSA1	3.0±0.4efgh	7.0±0.1a	7.1±0.1a	7.0±0.1a	7.1±0.1ab	2.0±0.0ab

Values are growth diameter of the pathogen thallus (averages of three replicates ± SEM).

Within a column, values sharing the same letter are not significantly different according to Fisher's protected LSD test (α level = 0.05).

Figure 7. Relative inhibitory activity on pathogen mycelial growth by bacterial isolates from *Bacillus* and *Bacillus*-related spp., *Pseudomonas* spp. and other isolates. Values were calculated from Table 4. Relative inhibitory activity = [(growth of mycelial radius control)-(growth of mycelial from the treatment)]/(growth of mycelial radius control).

3.2. Effect of lipopeptide extracts on mycelial growth

In order to assess the involvement of lipopeptide compounds in the observed inhibition, extracts from fourteen isolates representing different inhibition groups were used to examine their inhibitory effect on mycelial growth of the pathogens.

Among the tested bacterial isolates, samples from *B. subtilis* isolates F9-8, M9-7, M9-9, CU12 and B9-5 showed the highest antagonist activity against *A. solani*, reducing mycelial growth by 29% (Table 5). A group consisting of *B. subtilis* IC10 and B9-1, *B. badius* M9-20, *P. arsenicoxydans* F9-7, *P. proteolytica* F9-10 and *P. moraviensis* F9-11, also reduced the mycelial growth of *A. solani*. Samples from *P. koreensis* F9-9 and *B. subtilis* F9-2 and F9-12, were not significantly inhibitory to *A. solani* mycelial growth (Table 5).

Furthermore, samples from *B. subtilis* M9-9, CU12, IC10, B9-5, and *P. koreensis* F9-9 showed a significant reduction (10%) of the mycelial growth of *B. cinerea* (Table 5). The remaining isolates exhibited no inhibitory effect on *B. cinerea* mycelial growth.

Four lipopeptide samples were equally efficient in reducing mycelial growth of *F. sambucinum*. Indeed, *B. subtilis* CU12, B9-14, and M9-7 and *P. koreensis* F9-9 caused a 17% reduction in mycelial growth (Table 5). No other samples showed inhibitory effect against *F. sambucinum* (Table 5).

Ten lipopeptide samples showed significant reduction in the mycelial growth of *P. sulcatum* when compared to the control group (Table 5). The highest inhibition found for samples from *P. koreensis* F9-9 and *B. subtilis* M9-9, CU12, and IC10 (16% inhibition). *P. proteolytica* F9-10, *B. subtilis* B9-5, B9-14 and M9-7, *B. badius* M9-20, *P. arsenicoxydans* F9-7 also demonstrated significant inhibition when compared to the control. Conversely, samples from *B. subtilis* F9-2, F9-8 and F9-12, and *P. moraviensis* F9-11, exhibited no inhibitory effect on the mycelial growth of *P. sulcatum*.

Furthermore, eleven samples showed significant reduction of mycelial growth of *R. stolonifer*. Among these samples *B. subtilis* F9-8 and F9-12 and *P. koreensis* F9-9 exhibited the highest reduction (8%) in mycelial growth. A group consisting of *B. subtilis* B9-5, B9-14, F9-2, M9-7, and M9-9, *P. proteolytica* F9-10, *B. badius* M9-20, and *P. moraviensis* F9-11 also reduced the mycelial growth. Three samples including *B. subtilis* CU12, IC10 and *P. arsenicoxydans* F9-7, showed no inhibitory effect on the mycelial growth of *R. stolonifer* (Table 5).

Moreover, ten of the fourteen treatments with lipopeptide extracts from antagonistic bacteria showed a marked reduction of the mycelial growth of *V. dahliae* (Table 5). Among the ten lipopeptide extracts, samples from *B. subtilis* F9-12 and M9-9 and *P. moraviensis* F9-11

showed the highest inhibitory activity against *V. dahliae* (82% inhibition). *B. subtilis* B9-5, B9-14, IC10, M9-7, and CU12, *P. koreensis* F9-9, *P. proteolytica* F9-10, also significantly inhibited its growth. However, *B. subtilis* F9-2, F9-8, *P. arsenicoxydans* F9-7, and *B. badius* M9-20 revealed no inhibitory effect on the mycelial growth of *V. dahliae* (Table 5).

Table 5. *In vitro* inhibitory activities of lipopeptide extracts from *Pseudomonas arsenicoxydans* F9-7, *Pseudomonas koreensis* F9-9, *Pseudomonas proteolytica* F9-10, *Pseudomonas moraviensis* F9-11, *Bacillus subtilis* B9-5, B9-14, CU12, F9-2, F9-8, F9-12, IC10, M9-7, and M9-9, and *Bacillus badius* M9-20 on the mycelial growth of *Alternaria solani*, *Botrytis cinerea*, *Fusarium sambucinum*, *Pythium sulcatum*, *Rhizopus stolonifer* and *Verticillium dahliae*

Mycelial growth inhibition zone (cm)						
Treatment	<i>A. solani</i>	<i>B. cinerea</i>	<i>F. sambucinum</i>	<i>P. sulcatum</i>	<i>R. stolonifer</i>	<i>V. dahliae</i>
Control	2.0±0.1a	3.5±0.0a	3.4±0.0ab	3.5±0.0a	3.5±0.0a	1.0±0.0a
B9-5	1.5±0.1cdef	3.2±0.0bcd	3.3±0.1abcd	3.0±0.0def	3.4±0.0b	0.6±0.1bcd
B9-14	1.6±0.1bcde	3.4±0.0ab	2.8±0.2de	3.2±0.2cde	3.4±0.0bc	0.5±0.1cde
CU12	1.4±0.1def	3.2±0.1bcd	2.6±0.3e	3.0±0.0efg	3.5±0.0a	0.5±0.2cde
IC10	1.7±0.1bc	3.2±0.0bcd	3.4±0.1abc	3.0±0.2efg	3.5±0.0a	0.6±0.2cd
M9-7	1.7±0.1bc	3.3±0.0abc	2.9±0.2cde	3.1±0.0def	3.4±0.0b	0.5±0.2cde
M9-9	1.3±0.1ef	3.0±0.3d	3.0±0.2abcde	2.9±0.2fg	3.4±0.0b	0.2±0.1ef
F9-2	2.0±0.0a	3.5±0.0a	3.2±0.1abcde	3.5±0.0abc	3.4±0.0bc	0.8±0.1abc
F9-7	1.6±0.0bcd	3.3±0.0abc	3.2±0.1abcde	3.3±0.0bcd	3.5±0.0a	0.7±0.0abc
F9-8	1.3±0.0f	3.4±0.0ab	3.2±0.1abcde	3.5±0.0ab	3.2±0.1d	0.9±0.1ab
F9-9	2.0±0.0a	3.1±0.0cd	2.7±0.4de	2.8±0.0g	3.3±0.6cd	0.4±0.1cde
F9-10	1.6±0.0bcd	3.5±0.0a	3.1±0.2abcde	3.1±0.0def	3.4±0.0b	0.50±0.1cde

F9-11	1.7±0.bc	3.5±0.0a	3.0±0.1bcde	3.5±0.0abc	3.4±0.0b	0.30±0.2def
F9-12	1.8±0.ab	3.5±0.0a	3.5±0.0a	3.4±0.0abc	3.1±0.03e	0.07±0.0f
M9-20	1.5±0.bcde	3.3±0.0abc	3.4±0.ab	3.3±0.9bcd	3.4±0.03bc	0.73±0.1abc

Values are expressed as radial growth of the mycelial in the direction of the disc containing the extracts (averages of three replicates ± SEM)

Within a column, values sharing the same letter are not significantly different according to Fisher's protected LSD test (α level = 0.05).

3.3. Effect of lipopeptide extracts on conidial germination

The fourteen selected lipopeptide extracts were also assayed against conidia to determine the relative activity of the extract against both morphological structures.

All treatments showed a significant inhibition of the conidial germination on *A. solani* (Table 6). Among the test samples, those from *P. arsenicoxydans* F9-7, *P. koreensis* F9-9, *P. moraviensis* F9-11, *B. subtilis* isolates B9-14, F9-2, F9-8, F9-12, IC10, and M9-7 (86% inhibition) exhibited the greatest reduction on conidial germination.

Similarly, all the extracts showed significant decrease on the conidial germination growth of *B. cinerea* (Table 6). Among the tested extracts, those from *P. arsenicoxydans* F9-7, *P. koreensis* F9-9, *P. proteolytica* F9-10, *P. moraviensis* F9-11, *B. subtilis* isolates B9-5, B9-14, CU12, F9-2, F9-8, IC10, M9-7, and *B. badius* M9-20 (89%) revealed the greatest reduction in conidial germination.

On the other hand, only six of the fourteen tested isolates showed significant reduction of conidial germination in *F. sambucinum* (Table 6). *B. subtilis* B9-5 and F9-8, *P. arsenicoxydans* F9-7, and *P. koreensis* F9-9 (76%) exhibited the highest reduction of *F. sambucinum* conidial germination. *B. subtilis* F9-2 and M9-9 also exhibited inhibition of *F. sambucinum* conidial germination when compared to the control whereas *B. subtilis* B9-14, CU12, F9-12, IC10 and M9-7, *P. moraviensis* F9-11, *P. proteolytica* F9-10 and *B. badius* M9-20 revealed no inhibitory effect on the conidial germination (Table 6).

B. subtilis CU12 showed a significant reduction (74%) of conidial germination of *R. stolonifer* (Table 6). No other tested isolate was significantly inhibitory to *R. stolonifer* conidial germination (Table 6). Finally, thirteen of the tested samples showed marked reduction of *V. dahliae* conidial germination (Table 6). Among the inhibitory extracts, those of *P.*

arsenicoxydans F9-7, *P. koreensis* F9-9, *P. moraviensis* F9-11, *B. subtilis* isolates B9-14, F9-2, F9-8, F9-12, IC10, M9-7, and *B. badius* M9-20 revealed the highest inhibitory effect on *V. dahliae* conidial germination (81% inhibition). *B. subtilis* M9-9 was the only sample that did not provide inhibitory effects on *V. dahliae* conidial germination (Table 6).

This data was also used to establish any correlation between the effectiveness of the extract in inhibiting mycelial growth and conidial germination (Figure 8). The results indicated no linear correlation, indicating a differential effect of the extract on inhibition of the two morphological structures.

Table 6. *In vitro* inhibitory activities of lipopeptide extracts from *Pseudomonas arsenicoxydans* F9-7, *Pseudomonas koreensis* F9-9, *Pseudomonas proteolytica* F9-10, *Pseudomonas moraviensis* F9-11, *Pseudomonas libanensis* F9-13, *Bacillus subtilis* isolates B9-5, B9-14, CU12, F9-2, F9-8, F9-12, IC10, M9-7, M9-9, and *Bacillus badius* M9-20 on conidial germination of *Alternaria solani*, *Botrytis cinerea*, *Fusarium sambucinum*, *Rhizopus stolonifer* and *Verticillium dahliae*

Conidial germination (%)					
Treatment	<i>A. solani</i>	<i>B. cinerea</i>	<i>F. sambucinum</i>	<i>R. stolonifer</i>	<i>V. dahliae</i>
Control	87±6.1a	91±1.5a	83±8.9a	97±1.2a	90±3.5a
B9-5	27±2.0cde	10±0.7bc	34±16cde	84±7.5ab	42±21bcd
B9-14	21±7.3cdef	15±6.4bc	91±1.7a	83±14ab	9.0±1.0e
CU12	37±15bc	9.3±4.4bc	90±3.8a	72±14b	42±23bc
IC10	5±0.9f	9.0±4.0bc	80±13a	92±3.2ab	20±1.8cde
M9-7	17±6.5def	18±2.7bc	87±1.9a	99±0.7a	1.7±0.3e
M9-9	46±11b	28±12b	64±20bcd	77±21ab	71±13ab
F9-2	4.7±0.9f	6.7±0.9bc	43±23bcd	97±1.5ab	14±3.8cde
F9-7	11±5.8ef	9.7±0.7bc	20±5.2de	80±2.9ab	13±4.0cde
F9-8	21±7.9cdef	3.7±0.9c	6.0±1e	98±0.6a	10±4.4e
F9-9	11±3.2ef	6.7±1.5bc	35±10cde	93±3.3ab	10±5.6e
F9-10	16±4.7def	25±21bc	76±4.9ab	96±2.5a	56±18b

Conidial germination (%)					
Treatment	<i>A. solani</i>	<i>B. cinerea</i>	<i>F. sambucinum</i>	<i>R. stolonifer</i>	<i>V. dahliae</i>
F9-11	18±2.9def	5.7±1.9bc	65±16abc	95±2.0b	12±4.3de
F9-12	15±3.9def	28±18b	70±23abc	88±3.8ab	8.0±3.3e
M9-20	34±3.0bcd	9.7±5.7bc	94±0.7a	97±0.3ab	14±1.3cde

Values are percent conidial germination (average of three replicates ± SEM)

Within a column, values sharing the same letter are not significantly different according to Fisher's protected LSD test (α level = 0.05)

Figure 8. Correlation between reduction of mycelial growth and conidial germination (% inhibition) of the pathogens. Data were obtained from Table 6 and 7. Presented data are means from three biological repeats

3.4. Identification of lipopeptides in the extracts

LC-MS was performed on lipopeptide fractions from C₁₈ cartridges. Results showed that lipopeptides were concentrated in fractions F4 and F5 and eluted with 80 and 100% methanol, respectively. Fengycin homologs were produced by *B. subtilis* B9-1, CU12, IC10, M9-7, M9-9, B9-5, M9-4, M9-14, B9-14, B9-7, B9-9A, *B. megaterium* M9-1B and *B. badius* M9-20, as well as by *Bacillus*-related species *P. favisporus* B9-4, *B. borstelensis* M9-18, and *R. pycnus* M9-19. In *Bacillus* and *Bacillus*-related spp., LC-MS data showed multiple fengycin homologs with side chain lengths from C₁₃-C₁₉. The most common side chains observed were C₁₆ and C₁₈ with fengycin molecular weight of 1463 and 1491 Da. Both protonated ion peaks ([M+H]⁺ ions 1463, 1477, and 1491) and sodiated ion peaks ([M+Na]⁺ ions 1485, 1499 and 1514), observed in fractions from *B. subtilis* CU12, M9-4, and M9-14, varied by 14 Da (CH₂-) indicating fengycin homologs with identical peptide sequence, but variable beta hydroxyl fatty acid chain lengths (Table 7) (Malfanova et al., 2012).

Additionally, LC-MS data showed the presence of iturin in *B. subtilis* F9-8, M9-9, and CU12 (Table 8). The homologs of iturin identified had side chain length of C₁₄, C₁₅, C₁₆, C₁₇ and C₁₈. The most common homologs were C₁₄ and C₁₇ with molecular weight 1043 and 1085 Da and is consistent with data from literature (Velho et al., 2011; Zhang et al., 2012)

Moreover, the LC-MS analysis showed the presence of surfactin. Surfactin peaks were observed in the following bacteria: *B. subtilis* B9-1, F9-2, F9-8, M9-7, M9-9, F9-12, M9-3, M9-4, M9-14, B9-8, B9-7, B9-9A, IC10 and IB16, *B. badius* M9-20, *B. borstelensis* M9-18, *R. pycnus* M9-19, *B. licheniformis* PSA1 and *B. megaterium* B9-4 and M9-1B (Table 9). The most common protonated ions ([M+H]⁺) were 994, 1008, 1022, 1036, 1050 and 1064 Da indicating homologs with side chains of C₁₃-C₁₈. The most common sodiated adducts ([M+Na]⁺) were 1016, 1030, 1044, and 1058 corresponding to surfactin homologs with side chain of C₁₃-C₁₆ (Table 9). Overall, the most commonly occurring family cyclic lipopeptide in the *Bacillus* and *Bacillus*-related spp. was surfactin followed by fengycin and lastly iturin.

B. subtilis CU12 and M9-9 revealed the presence of all three families (surfactin, iturin, and fengycin). *B. subtilis* F9-8 produced iturin and surfactin, but not fengycin. Moreover, *B. subtilis* B9-1, M9-7, IC10, M9-3, M9-4, M9-14, B9-14, B9-7 and B9-9A, *B. badius* M9-20, *B. borstelensis* M9-18, *R. pycnus* M9-19 and *B. megaterium* B9-4 and M9-1B produced fengycin and surfactin, but not iturin. Other extracts from *B. subtilis* F9-2, F9-12, IB16, B9-8, and M9-3 and *B. licheniformis* PSA1 produced only surfactin, while *B. subtilis* B9-5, produced only fengycin (Table 7 and Table 9).

In *Pseudomonas* spp. isolates, amphisin was produced by *P. moraviensis* F9-11 and *P. koreensis* F9-9. LC-MS analysis showed amphisin homologs from C₉-C₁₃ ([M+H]⁺ ions 1326, 1340, 1354, 1368, and 1382 Da), with the most observed homolog being C₁₁ (1354 Da) (Table

10). The data also showed that *P. moraviensis* F9-11 also produced putisolvin (Table 10), with side chains of C₆ and C₇ with molecular weight of 1380 and 1393 Da, respectively (Table 10).

Finally, *P. gessardii* B9-3 and M9-16, *P. moraviensis* F9-6, *P. arsenicoxydans* F9-7, *P. moraviensis* F9-10, and *P. libanensis* F9-13 did not reveal any known *Pseudomonas* lipopeptides from the amphisin, putisolvin, viscosin or syringomycin families.

Table 7. Fengycin homologs detected by LC-MS in *Bacillus* and *Bacillus*-related species.

CLP	Molecular mass [M-H] ⁺ /[M+Na] ⁺	Homolog	Microorganism
Fengycin	1463, 1477, 1491, 1505	C16, C17, C18, C19	<i>Bacillus badius</i> M9-20
	1462, 1476, 1490	C16, C17, C18	<i>Bacillus subtilis</i> CU12
	1441, 1454, 1467, 1481, 1495, 1508	C14, C15, C16, C17, C18, C19	<i>Bacillus subtilis</i> M9-7
	1462, 1476, 1490	C16, C17, C18	<i>Bacillus subtilis</i> M9-9
	1449, 1463	C15, C16	<i>Bacillus subtilis</i> IC-10
	1463, 1477, 1491	C16, C17, C18	<i>Paenibacillus favisporus</i> B9-4
	1448, 1461, 1475, 1489, 1503, 1517	C15, C16, C17, C18, C19, C20	<i>Brevibacillus borstelensis</i> M9-18
	1461, 1475, 1489	C16, C17, C18	<i>Rummeliibacillus pycnus</i> M9- 19
	1479, 1493, 1507	C17, C18, C19	
	1479, 1493, 1506	C17, C18, C19	<i>Bacillus subtilis</i> M9-4
	1463, 1477, 1491	C16, C17, C18	
	1485, 1499, 1514	C18, C19, C20	
	1463, 1477, 1491, 1506	C16, C17, C18, C19	<i>Bacillus subtilis</i> M9-14
	1449, 1463, 1477, 1491, 1505	C15, C16, C17, C18, C19	<i>Bacillus subtilis</i> B9-14
	1448, 1462, 1476, 1489	C15, C16, C17, C18	<i>Bacillus subtilis</i> B9-7
	1464, 1478, 1492, 1506	C16, C17, C18, C19	<i>Bacillus subtilis</i> B9-9B
	1463, 1491	C16, C18	<i>Bacillus megaterium</i> M9-1B
	1448, 1464, 1478, 1492, 1506	C15, C16, C17, C18, C19	<i>Bacillus subtilis</i> B9-1
	1504, 1521	C17, C18	<i>Bacillus subtilis</i> B9-5

Table 8. Iturin homologs detected by LC-MS in *Bacillus* and *Bacillus*-related species.

CLP	Molecular mass [M-H] ⁺	Homolog	Microorganism
Iturin	1043, 1057	C14, C16	<i>Bacillus subtilis</i> F9-8
	1079, 1093	C17, C18	
	1044, 1085	C14, C17	<i>Bacillus subtilis</i> M9-9
	1049, 1063	C14, C15	
	1084, 1099	C17, C18	<i>Bacillus subtilis</i> CU12
	1069, 1083	C16, C17	

Table 9. Surfactin homologs detected by LC-MS in *Bacillus* and *Bacillus*-related species.

CLP	Molecular mass [M-H] ⁺ / [M+Na] ⁺	Homolog	Microorganism
Surfactin	994, 1008, 1022, 1036	C13, C14, C15, C16	<i>Bacillus subtilis</i> F9-2
	994, 1008, 1022, 1036	C13, C14, C15, C16	<i>Bacillus subtilis</i> F9-8
	994, 1008, 1022, 1036, 1050	C13, C14, C15, C16, C17	<i>Bacillus badius</i> M9-20
	994, 1008, 1022, 1036, 1050	C13, C14, C15, C16, C17	<i>Bacillus subtilis</i> M9-7
	1008, 1022, 1036	C14, C15, C16	<i>Bacillus subtilis</i> M9-9
	922, 950, 964, 992	C8, C10, C11, C13	<i>Bacillus subtilis</i> IC-10
	994, 1008, 1022, 1036, 1050	C13, C14, C15, C16, C17	<i>Bacillus subtilis</i> F9-12
	994, 1008, 1022, 1036, 1050	C13, C14, C15, C16, C17	<i>Paenibacillus favisporus</i> B9-4
	994, 1008, 1022, 1036, 1050, 1064	C13, C14, C15, C16, C17, C18	<i>Brevibacillus borstelensis</i> M9-18
	994, 1008, 1022, 1036	C13, C14, C15, C16	<i>Bacillus megaterium</i> B9-9B
	994, 1008, 1022, 1036, 1050	C13, C14, C15, C16, C17	<i>Rummeliibacillus pycnus</i> M9-19
	994, 1008, 1022, 1036	C13, C14, C15, C16	<i>Bacillus subtilis</i> M9-3
	994, 1008, 1022, 1036	C13, C14, C15, C16	<i>Bacillus subtilis</i> M9-4
	994, 1008, 1022, 1036, 1050	C13, C14, C15, C16, C17	<i>Bacillus subtilis</i> M9-14
	994, 1008, 1022, 1036	C13, C14, C15, C16	<i>Bacillus subtilis</i> B9-7
	994, 1008, 1022, 1036	C13, C14, C15, C16	<i>Bacillus subtilis</i> B9-8
	994, 1008, 1022, 1036	C13, C14, C15, C16	<i>Bacillus subtilis</i> B9-9A
	994, 1008, 1022, 1036	C13, C14, C15, C16	<i>Bacillus subtilis</i> IB-16
	994, 1008, 1022, 1036	C13, C14, C15, C16	<i>Bacillus licheniformis</i> PSA1
	994, 1008, 1022, 1036	C13, C14, C15, C16	<i>Bacillus megaterium</i> M9-1B
	994, 1008, 1022, 1036	C13, C14, C15, C16	<i>Bacillus subtilis</i> CU12
	1008, 1022, 1036	C14, C15, C16	<i>Bacillus subtilis</i> B9-1

Table 10. Amphisin and putisolvin homologs detected by LC-MS in *Pseudomonas* species.

CLP	Molecular mass [M-H] ⁺	Homolog	Microorganism
Amphisin	1326, 1340, 1354, 1368, 1382	C9, C10, C11, C12, C13	<i>Pseudomonas koreensis</i> F9-9
	1376, 1390, 1404	C12, C13, C14	
	1326, 1340, 1354, 1368	C9, C10, C11, C12	<i>Pseudomonas moraviensis</i> F9-11
Putisolvin	1354, 1382, 1397	C4, C6, C7	<i>Pseudomonas moraviensis</i> F9-11
	1380, 1393	C6, C7	

Chapter 4: Discussion

Currently, with increasing awareness of possible deleterious effect of fungicides on the ecosystem, there is a growing interest in pesticide-free agricultural products (David, 2008). A major challenge in plant pathology is to introduce or develop new strategies to control plant diseases as more traditional controls and methods become obsolete (Pal and Gardener, 2006), and to create solutions that reduce or exclude the use of synthetic chemical products. As an alternative to the use of synthetic chemicals, biological control offers many advantages to growers and to society. Among the benefits, biocontrol is generally deemed a more sustainable and safer method to produce and preserve food. An important aspect for commercial purposes that should be kept in mind is that in order for biocontrol products to be accepted in agricultural communities, they must be safe and economical (Wilkinson and Fitzgerald, 1997). These issues can be addressed by understanding how the biological controls agents act, their inhibitory properties, and through the understanding and analysis of the production of antimicrobial secondary metabolites. Such antimicrobial compounds are widespread in nature and are produced by a wide spectrum of microorganisms. Compounds that are produced by biocontrol agents as treatments for plant pathogens may have advantages over their synthetic counterparts because they are often more biodegradable, effective at extreme temperatures and pH values (Cameotra and Makkar, 1998), and may be more active and selective as membrane-permeabilizers (Nielsen et al., 2009). In the past few years, biocontrol agents from *Bacillus* and *Pseudomonas* isolates have become a highly effective and environmentally friendly alternative to traditional pesticides. The antimicrobial activities are linked, in part, to the production of molecules that have the ability to suppress plant diseases.

This study was carried out to investigate the antifungal ability of various bacterial isolates that inhibit the *in vitro* growth of plant pathogens and to gain further knowledge on the means by which these bacterial antagonists exert their antifungal activity.

4.1. Evaluation of various bacterial antagonists through general plate inhibition assays

Forty bacterial isolates were tested *in vitro* for their ability to reduce the mycelial growth of six plant pathogens: *A. solani*, *B. cinerea*, *F. sambucinum*, *P. sulcatum*, *R. stolonifer* and *V. dahliae*. Among the eighteen different species of bacteria that were tested for inhibitory properties, sixteen showed significant reduction of the mycelial growth of at least one of the six pathogens. The presence of an inhibition zone in solid culture media indicated that growth reduction was most likely due to the production of diffusible extracellular metabolites.

All six tested *Pseudomonas* spp. were members of the *P. fluorescens* group (Mulet et al., 2010) and showed marked reduction of the pathogens. *P. gessardii*, *P. moraviensis*, *P. arsenicoxydans*, *P. koreensis*, *P. proteolytica* and *P. libanensis* caused significant reduction in growth of at least three of the plant pathogens. Of particular note, *P. proteolytica* F9-10 and *P. libanensis* F9-13 were generally the best inhibitors of pathogen growth of all the bacterial antagonists tested. Previous studies have shown that different *Pseudomonas* spp. have the ability to reduce the mycelial growth of many plant pathogens (Haas and Defago, 2005) including those from genera tested in this study. For example, *P. fluorescens* has been reported to inhibit *Pythium ultimum* (Mezaache-Aichour et al., 2012), and *A. solani* (El Sayed et al., 2008) mycelial growth *in vitro*. Other *Pseudomonas* spp. were reported to inhibit the mycelial growth of *B. cinerea* (Bryk et al., 2004), *V. dahliae* (Martin-Lapierre, 2011; Trivedi, 2007) and *Fusarium* spp. (Duijff

et al., 1993). To our knowledge, this is the first time that mycelial inhibition of *R. stolonifer* by *Pseudomonas* spp. has been reported.

In addition to *Pseudomonas* spp., three of the four *Bacillus* spp. tested (thirteen isolates of *B. subtilis*, two isolates of *B. megaterium* and one isolate of *B. licheniformis*), showed inhibition of at least one of the tested plant pathogens. *Bacillus* spp. are well-known for their ability to reduce the growth of plant pathogens (Ongena and Jacques, 2008; Falardeau et al. 2013). For example, *B. subtilis* has been previously shown to inhibit the growth of *A. solani* and *B. cinerea* (Wise et al., 2012; On et al., 2015) as well as *F. sambucinum* and *P. sulcatum* (Wise et al., 2012). Previous studies have also shown that *B. licheniformis* was antagonistic to *Alternaria alternata* and *Fusarium solani* (Lira-de León et al., 2014). *B. megaterium* has previously shown inhibitory effects on the growth of *Aspergillus flavus* (Kong et al., 2014). Although *B. badius* had previously shown inhibitory effects on *V. dahliae* (Martin-Lapierre, 2011), it did not inhibit mycelial growth of the plant pathogen in our test conditions.

Among the non-*Bacillus* bacteria isolated from compost teas, *A. aminovorans* IB2 and *A. kashmirensis* IC6 both decreased the mycelial growth of the six tested pathogens and *B. iodinum* isolates inhibited 1 or 2 of these pathogens. Previous work reported that these bacterial isolates inhibited the mycelial growth of *A. solani* and *B. cinerea* (On et al., 2015). To our knowledge, this is the first report of *A. aminovorans*, *A. kashmirensis* and *B. iodinum* inhibiting the growth of *F. sambucinum*, *P. sulcatum*, *R. stolonifer* and *V. dahliae* (Table 4).

In *Arthrobacter* spp. tested in this study, *A. humicola* M9-2 decreased the mycelial growth of all the fungal pathogens tested except *F. sambucinum*. Furthermore, *A. humicola* M9-1A showed inhibition to *F. sambucinum* and *V. dahliae* and *A. humicola* M9-8 reduced the growth of *F. sambucinum* and *B. cinerea*. Finally, *A. psychrophilicus* revealed inhibition of

all the tested pathogens with exception of *A. solani* and *P. sulcatum* (Table 4). Previous work showed that *Arthrobacter citreus*, *Arthrobacter nicotianae*, *Arthrobacter oxydans*, *Arthrobacter viscosus* and an unidentified *Arthrobacter* sp. have been shown to possess activity against plant pathogens such as *F. oxysporum*, *Cylindrocarpon destructans* and *Pythium* spp. (Axelrood et al., 1996).

The remaining isolates *B. borstelensis* (Wang et al., 2015), *P. favisporus* (Velázquez et al., 2004) and *R. pycnus* (Vaishampayan et al., 2009) are aerobic spore-forming bacteria that are related to *Bacillus*. *B. borstelensis* M9-18 and *R. pycnus* M9-19 both reduced the mycelial growth of five pathogens (but not *P. sulcatum*) (Table 4). To the best of our knowledge this is the first report that these bacteria have antagonistic activities against plant pathogens, however it has been reported previously that a strain of *B. borstelensis* was inhibitory to foodborne microorganisms that include *Listeria monocytogenes*, *B. subtilis*, *Clostridium perfringens*, *Staphylococcus aureus*, and others (Sharma et al., 2014). *P. favisporus* did not inhibit any of the tested plant pathogens in our test conditions. Other studies showed that the *Paenibacillus* genus displayed efficient biocontrol activity against oomycete pathogens (Li et al., 2007).

Finally, we also compared the relative inhibitory activity of the different bacterial groups which revealed that the *Pseudomonas* spp. group exhibited higher inhibitory activity than the *Bacillus* spp. and *Bacillus*-related spp. group. These two groups were also more inhibitory to the pathogens than the group composed of the tested bacterial species (Figure 7). The higher inhibitory effect of *Pseudomonas* spp. is likely because the group produce various potent antimicrobial compounds such as those from the two families of lipopeptides (amphisin and putisolvin) identified in our work (Table 10). Some *Pseudomonas* spp. also showed inhibitory effects against the pathogens, but did not reveal lipopeptides from the families of amphisin,

putisolvin, syringomycin and viscosin. Their activity may be due to the presence of other reported inhibitory compound such as HCN, siderophores, phenazine (Haas and Defago, 2005) and proteases (Bruijin and Raaijmakers, 2009). Further investigation is needed to identify the presence of these antimicrobial compounds from our four isolates. As mentioned previously, all lipopeptide extracts from *Bacillus* and *Bacillus*-related spp. revealed the presence at least of one of the three families of lipopeptide fengycin, iturin or surfactin. We hypothesized that inhibitory activities from *Bacillus* and *Bacillus*-related spp., are due, at least in part, to the presence of the lipopeptides. However, the presence of other bioactive compounds may also be implicated in the observed activity (Wise et al., 2012).

4.2. Effect of lipopeptide extracts on mycelial growth

Lipopeptide samples from the four *Pseudomonas* isolates (*P. arsenicoxydans* F9-7, *P. koreensis* F9-9, *P. proteolytica* F9-10, and *P. moraviensis* F9-11) significantly inhibited the mycelial growth of two out of six of the tested pathogens *in vitro*. This suggested that extracellular antimicrobial products were released when the bacteria were confronted with a competing microorganism. Previous work showed that extracts from *Pseudomonas chlororaphis* resulted in the inhibition of the growth of *P. ultimum* and *F. oxysporum* (Mezaache-Aichour et al., 2012). Moreover, *P. fluorescens* isolates were previously shown to produce antibiotics as well as volatile compounds such as hydrogen cyanide that decreased the mycelial growth of *V. dahliae* (Tehrani et al., 2001). Another study showed that a *Pseudomonas chlororaphis* strain producing pyrrolnitrin was able to control the spread of *B. cinerea* in tomatoes (Ajouz et al., 2011). Furthermore, *Pseudomonas* isolates producing phenazine-1-carboxylic acid were shown

to be inhibitory to *A. solani* (El Sayed et al., 2008). This is, to the best of our knowledge, the first report of compounds from *Pseudomonas* inhibiting the growth of *R. stolonifer*.

The tested *Bacillus* spp. isolates demonstrated that the lipopeptide extracts were also inhibitory to at least one of the pathogen assayed. Our laboratory previously indicated similar results showing extracts from *B. subtilis* were able to reduce the mycelial growth of *A. solani*, *B. cinerea*, and *P. sulcatum* (Wise et al., 2012). In addition, On et al. (2015) showed that extracts from *B. subtilis* inhibited mycelial growth of *A. solani* and *B. cinerea*. Multiple isolates of *B. subtilis* (B9-5, B9-14, M9-7, M9-9, F9-2, F9-8, and F9-12) as well as *B. badius* M9-20 inhibited the growth of *R. stolonifer* in this work. A previous study showed the *in vitro* antagonistic effect of another *Bacillus* species (*B. amyloliquefaciens*) on the mycelial growth of *B. cinerea*, *P. expansum* and *R. stolonifer* due to the presence of antifungal secondary metabolites in its culture filtrates (Arrebola et al., 2009).

Two bacterial isolates *B. badius* M9-20 and *P. favisporus* B9-4 showed no inhibitory effect against of the six pathogens (Table 4), but the samples from *B. badius* M9-20 showed inhibitory properties against mycelial growth of the pathogens. It is hypothesized that these bacterial isolates were not effective in inhibiting the growth of the mycelium because the antimicrobial compounds were produced at a low concentration by the bacterium when the bacteria and pathogens were confronted on Petri dishes whereas extracts were enriched in lipopeptide that are known to possess antifungal activity. Moreover, the lower concentration of antimicrobial compounds produced by these bacterial isolates may be completely degraded by the pathogens (no inhibition) (Sonnenbichler et al., 1997; Martinez et al., 2006), whereas a higher concentration is more difficult to degrade completely, thus providing inhibition.

4.3. Antifungal activity of lipopeptide extracts on fungal pathogen conidial germination

Extracts from *P. arsenicoxydans* F9-7, *P. koreensis* F9-9, *P. proteolytica* F9-10, and *P. moraviensis* F9-11 reduced the amount of conidial germination in *A. solani*, *B. cinerea*, and *V. dahliae*. *P. arsenicoxydans* F9-7 and *P. koreensis* F9-9 extracts also reduced the conidial germination of *F. sambucinum*. Previous work showed that culture filtrate of *P. fluorescens* inhibited spore germination in *A. alternata*, *Curvularia andropogonis*, *Fusarium moniliforme* and *Colletotrichum acutatum* (Mishra et al., 2011). Previous work also indicated that *P. chlororaphis* producing pyrrolnitrin was able to inhibit spore germination of *B. cinerea* (Ajouz et al., 2011). Furthermore, this is, to the best of our knowledge, the first report of compounds from *Pseudomonas* showing inhibitory effect on the conidial germination of *V. dahliae*.

Extracts from various isolates of *B. subtilis* as well as *B. badius* M9-20 reduced the spore germination in *A. solani*, *B. cinerea* and *V. dahliae*. Previous work showed that extracts from a strain of *B. subtilis* also inhibited the spore germination of *A. solani* and *B. cinerea* (On et al., 2015). Moreover, samples from isolates *B. subtilis* B9-5, M9-9, F9-2 and F9-8, decreased the conidial germination of *F. sambucinum*. Previous work showed that volatile compounds from *B. amyloliquefaciens* also inhibited the spores of *F. oxysporum* (Yuan et al., 2012). Lastly, samples from the strain *B. subtilis* CU12 and M9-9, showed decreased growth rate on the conidial germination of *R. stolonifer*. A previous study showed that *B. subtilis* compounds reduced the conidial germination of *R. stolonifer* (Zhou et al., 2011). To the best of our knowledge, this is the first report of *Bacillus* compounds inhibiting *V. dahliae* conidial germination.

4.4. Correlation of lipopeptides extracts and inhibitory effects

The effect of fourteen lipopeptide extracts from the bacterial isolates of *Bacillus*, *Bacillus*-related and *Pseudomonas* spp. showed inhibitory activities against mycelial growth and conidial germination with conidial germination showing higher sensitivity to the extracts. We hypothesized that lipopeptide extracts have more significant effects on the early stages of the plant pathogen development (i.e., the fragile germinating conidium) compared to the more tough structure that is the mycelium. There was no correlation between the inhibition of mycelium growth and conidial germination, indicating that extracts did not affect these two morphological structures in the same way (Figure 8). Therefore, it would seem necessary to perform antimicrobial testing on both structures in order to more completely understand the inhibitory activity of the extracts.

4.5. Identification of lipopeptides in extracts from bacterial antagonists

The success of biocontrol depends on the screening process for the biocontrol agent, such as its potential ability to produce antimicrobial compounds, which can affect its ability to survive in different environments containing multiple biotic and abiotic stressors (Pal et al., 2006; Delfosse, 2005). Lipopeptides extracts from thirty two bacterial isolates were submitted to reverse-phase column chromatography and were screened for the presence of known lipopeptides using LC-MS. In this study, we have described the use of *Bacillus*, *Bacillus*-related and *Pseudomonas* spp. isolates that showed inhibitory activity against plant pathogens. It is well known that several species of *Bacillus* and *Pseudomonas* are capable of producing biologically active molecules (Chen et al., 2009; Niu et al., 2011; Rückert et al., 2011), including lipopeptides (Gardener, 2004). LC-MS analysis showed the presence the three lipopeptides families

(fengycin, iturin, and surfactin) in *Bacillus* or *Bacillus*-related spp. Every isolate tested produced lipopeptides belonging to one, two or all three main *Bacillus* lipopeptide families.

Some isolates showed inhibitory activities against the tested pathogens and showed the presence of lipopeptides. Specifically, *B. subtilis* B9-1, B9-5, and B9-14 and *B. megaterium* M9-1B produced fengycin, *B. subtilis* B9-9A, F9-12, F9-2, B9-8 and IB16 and *B. licheniformis* PSA1 produced surfactin, *B. subtilis* B9-7, M9-4 and M9-7, *R. pycnus* M9-19 and *B. borstelensis* M9-18 and *B. megaterium* B9-9B produce fengycin and surfactin, *B. subtilis* F9-8 produce surfactin and iturin, and *B. subtilis* CU12 and M9-9 produce fengycin, iturin and surfactin as possible compounds leading to their inhibitory effects on the pathogens. Previous studies showed that *B. subtilis* and *B. amyloliquefaciens* revealed the presence of lipopeptides fengycin, iturin, and surfactin (Cawoy et al., 2014). A previous study also demonstrated that *Paenibacillus* spp. IIRAC30 produced surfactins, which proved to be effective in controlling *R. solani* *in vitro*, through its ability to form pores in membranes (Canova et al., 2010; Mandal et al., 2013). Other work done on characterization of lipopeptides from *B. licheniformis* revealed the production of the fengycin and surfactin families (Pecci et al., 2010; Thaniyavarn et al., 2003). Additionally, *Brevibacillus brevis*, a species of *Brevibacillus* different from the one we tested in our study, was shown to produce the surfactin family of lipopeptides (Wang et al., 2010). *R. pycnus* M9-19, *B. badius* M9-20 and *B. borstelensis* M9-18, revealed the presence of these lipopeptides (fengycins, iturins or surfactins) and are reported, to the best of our knowledge, for the first time.

The *Pseudomonas* isolates exhibited the presence of two families of lipopeptides. *P. koreensis* F9-9 revealed the presence of the amphisin family and *P. moraviensis* F9-11 showed both the amphisin family and the putisolvin family. Because we observed the antagonistic effect of these two bacterial isolates, we attributed the inhibitory activity to the production of these

antimicrobial lipopeptides that are present in the samples. However, other types of antimicrobial compounds may also contribute to the observed inhibitory activity. Many studies have linked *Pseudomonas* lipopeptides and their major role in inhibiting plant pathogens. Cyclic lipopeptides produced by *P. fluorescens* strains showed the production of amphisin (Gross and Loper, 2009), while isolates such as *P. putida* produced lipopeptides that belong to the putisolvin family (Kuiper et al., 2003).

P. moraviensis F9-6, *P. arsenicoxydans* F9-7, *P. gessardii* B9-3 and M9-16, *P. proteolytica* F9-10, and *P. libanensis* F9-13 showed inhibition through confrontation assays, but no production of lipopeptides was detected. This result may be because of the production of other types of secondary metabolites that were not screened using our identification methodology. Some of the more common antimicrobial compounds such as 2,4-diacetylphloroglucinol and phenazine (Trivedi, 2007) may be responsible for this observed inhibition. We also hypothesize that the media used for inducing lipopeptides production (MOLP) was developed for the isolation of *Bacillus* lipopeptides. Its use may therefore not be optimal for isolation of lipopeptides from *Pseudomonas*.

Two bacterial isolates *B.adius* M9-20 and *P. favisporus* B9-4 showed no inhibitory effect against any of the six pathogens (Table 4), but when lipopeptide extracts were screened using LC-MS, they were shown to produce fengycins and surfactins (Table 7 and 9). We hypothesized that the concentration of lipopeptides produced was too low to show inhibition in the confrontation assay and/or that the presence of lipopeptides alone is not sufficient to provide inhibition.

It was found that lipopeptide extracts from some of isolates were not inhibitory against mycelial growth or conidial germinations despite the bacterial antagonists showing inhibitory

effect against the pathogens (Table 2). It was found that these samples did not reveal any of the specifically tested lipopeptides. As previously mentioned, it is hypothesized that other antimicrobial compounds were being produced by these isolates to provide this inhibitory effect.

In summary, our results strongly suggest that lipopeptides are partly responsible for the inhibitory activity showed *in vitro* by *Bacillus*, *Bacillus*-related and *Pseudomonas* spp. toward multiple plant pathogens. Our data suggests that the inhibitory effects were not limited to the presence of the identified lipopeptides as we also noticed inhibitory effects in the absence of antimicrobial lipopeptides in the samples. We concluded that other types of bioactive compounds are present in our samples such as non-peptide antimicrobials.

Finally, this study also showed that three families of lipopeptides (fengycin, iturin, and surfactin) were produced by *Bacillus* spp. and *Bacillus*-related species. Also, two other families of lipopeptides (amphisin and putisolvin) were produced by certain *Pseudomonas* spp.

Chapter 5: Conclusions and future directions

Overall, this study showed that biological control agents from *Bacillus* spp., *Pseudomonas* spp., *Brevibacillus* spp., *Rummeliibacillus* spp., *Arthrobacter* spp., *Aminobacter* spp., *Brevibacterium* spp., and *Advenella* spp. reveal antagonistic activities against plant pathogens. A total of sixteen bacterial isolates *B. subtilis* CU12, M9-9, B9-5, B9-7, M9-3, M9-4, M9-14, B9-14, IC-10, IB-2 and B9-9A and *Pseudomonas* isolates consisting *P. moraviensis* F9-6, *P. proteolytica* F9-10, *P. moraviensis* F9-11 and *P. libanensis* F9-13, and from other isolates *A. kashmirensis* IC6, exhibited inhibitory effects against the six pathogens. Furthermore, some lipopeptide extracts from these bacteria were able to inhibit mycelial growth and conidial germination of the plant pathogens. A possible explanation for these effects could be the presence of lipopeptides in all of these isolates. Previous literature indicates a strong correlation between lipopeptides and the reduction of various phytopathogens (Malfanova et al., 2012; Meena and Kanwar, 2015). We also concluded that some of the bacterial isolates exhibit an inhibitory effect because of the presence of other types of antimicrobial compounds.

Future investigation is needed to purify additional bioactive compounds from these extracts in order to identify unknown antimicrobials. These purified compounds may also be used to test their effectiveness against pathogens as well as to test their combined (potentially synergistic) effects with the lipopeptides. In addition, *Bacillus* and *Pseudomonas* spp. produce a heterogeneous mixture of homologous lipopeptides, which makes it difficult to identify sub-families, which is why additional work is needed to identify sub groups using techniques such as nuclear magnetic resonance (NMR). This may be complemented with analytical, chromatographic, and bioassay methods with known antimicrobial compounds from these species to determine if the metabolites produced by these isolates are known or novel

compounds. Finally, the discovery of the precise mode of action of any novel antimicrobial compounds from these bacterial isolates and *in vivo* testing in greenhouse or field trials would be necessary for the registration of these bacteria as biocontrol agents. Additionally, safety testing for practical application of these potential biocontrol agents will need to be performed.

Chapter 6: References

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Appendix I

Figure A1. Lipopeptides families produced by *Bacillus subtilis* CU12. A. Chromatogram showing the three families of lipopeptides; B. Mass spectrum iturin, $m/z = 1084$ and 1099 ; C. Mass spectrum of fengycins, $m/z = 1462$, 1476 , 1490 ; D. Mass spectrum of surfactin $m/z = 1008$, 1022 , 1036 .

Figure A2. Lipopeptides families produced by *Bacillus megaterium* B9-9B. A. Chromatogram showing the two families of lipopeptides; B. Mass spectrum surfactin, $m/z = 1008, 1022, 1036$; C. Mass spectrum of fengycin, $m/z = 1476, 1490, 1492, 1506$.

Figure A3. Lipopeptides families produced by *Pseudomonas moraviensis* F9-11. A. Chromatogram showing the three families of lipopeptides; B. Mass spectrum amphisin, $m/z = 1340, 1354, 1368, 1382$; C. Mass spectrum of putisolvin, $m/z = 1383$ and 1397 .