Characterization of bioactivity and antimicrobial metabolite production in bacteria antagonistic to plant and foodborne molds

by

Serine Ramlawi

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 with a concentration in Food Science

Carleton University
Ottawa, Ontario

Abstract

Biocontrol agents show promise in mitigating the drawbacks of synthetic chemicals. This study investigates potential biocontrol bacteria isolated from disease suppressive composts to reduce Fusarium dry rot on potato tubers, and to gain insight into the mode of action of *Arthrobacter* spp. The objectives were to (i) evaluate the antagonistic activity against *Fusarium sambucinum in vitro* and suppression of potato dry rot, (ii) assess the antimicrobial activity of *Arthrobacter* spp. *in vitro* and *in vivo*, and (iii) identify possible modes of action of these antagonistic bacteria. Results indicated that bacterial isolates from *Bacillus*, *Pseudomonas*, and *Arthrobacter* genera suppressed Fusarium dry rot. Additionally, *Arthrobacter humicola* M9-1A, M9-2, and M9-8, *A. psychrophenolicus* M9-17 and their cell-free filtrates possess antimicrobial properties suppressing various plant pathogens. Two antimicrobial compounds were isolated and structurally characterized from *A. psychrophenolicus* M9-17. Overall, this study reported that antibiosis may be a major mode of action of these bacteria.

Acknowledgements

To start, I would like to thank Dr. Tyler J. Avis for his guidance, support and mentorship over the course of my undergraduate and graduate experience. Not only have I learned laboratory techniques and grown my scientific knowledge from you, but you have helped me become a better presenter and a critical thinker through this experience as well as in class. I cannot thank you enough for the opportunities you have given me during this time and for the future. Your vow of confidence in me was much appreciated in times of high stress. Even through a pandemic, you were able to help me accomplish this amazing experience.

I would also like to thank Dr. David McMullin for his valued expertise in numerous chemical analysis and technical support. Your help in the identification of two compounds is tremendously appreciated. Thanks for always reminding me that "there's a light at the end of the tunnel". And a special thanks to Dr. J. David Miller for use of specialized infrastructure.

Thank you, Kim MacDonald for helping me through these two past years. Your knowledge and advice will always impress me. Having you by my side throughout the course of my graduate degree has been a blast. So happy to have made another close friend in this journey!

A special thanks to all my family and friends that have helped me get to this point. I will always cherish the loving support and encouragement you have given me during my graduate studies. Steph, I'm sorry to say that you will no longer hear me talk about my potatoes and tomatoes.

Last but not least, a big thank you to all the other graduate students that I have met along the way! Bakers breakfast and Mike's beers and nachos will be missed!

Table of Contents

List of Tables

List of Figures

List of Appendices

Chapter 1: Literature Review

1.1 Plant pathogens

Plant disease may be caused by bacteria, viruses, fungi, oomycetes, nematodes, parasitic plants or abiotic factors. Among causal agents, pathogenic fungi as well as pathogenic oomycetes have been shown to cause significant economic losses in various agricultural environments, whether it be temperate or tropical regions (Dean et al. 2012). Their ability to grow on a large scale, adapt to environmental changes and overcome previously effective control methods makes their control very challenging. Phytopathogens possess a wide range of methods to colonize plants and cause diseases, whether it be pre-harvest or post-harvest. They may affect different parts of the plants such as the roots, leaves, flowers and/or fruit (Scholz et al. 2018).

Most plant pathogens interact with host plants in a symbiotic relationship. Some plant pathogens establish a relationship with a plant host and obtain nutrients from living cells and tissues without killing the host (Spanu and Panstruga 2017). These types of phytopathogens are known as biotrophs and may cause milder symptoms while still causing economical deficits. On the other hand, necrotrophic phytopathogens destroy cells and utilize the dead plant material to support their own growth (Spanu and Panstruga 2017). Necrotrophic pathogens have been shown to secrete toxins and lytic enzymes to cause death of host plants (Koeck et al. 2011). Therefore, necrotrophs invade and destroy plant tissue rapidly, causing devastating economical losses of affected plants. Some necrotrophs may also live as saprophytes, meaning they survive on dead or decaying matter. Hemibiotrophic phytopathogens use a combination of both methods. These phytopathogens initially begin as biotrophs causing no severe symptoms to the plant (Scholz et al. 2018). As the fungi continues to spread throughout the infected plant, they shift to a necrotrophic

relationship where they begin to release toxins and lytic enzymes killing the host plant (Koeck et al. 2011).

The relationship between phytopathogens and host plants can help categorize the various plant pathogens. However, plant pathogens may also be categorized by taxonomy outlining their similarity in structure and function. Ascomycota, Basidiomycota and Mucorales are all orders or divisions found within the Fungi kingdom. Additionally, Oomycota, once within the Fungi kingdom, has now been categorized in the Chromista kingdom as a phylum that is fungus-like.

1.1.1 Ascomycota

Fungi within this division are named ascomycetes. These fungi can reproduce sexually, forming ascospores that are encapsulated in a sac known as an ascus. Typically, each ascus contains four to eight ascospores in the sexual stage. The ascospores are refractile spores and are resistant to heat, pressure and certain chemicals. Ascomycetes can also reproduce asexually by forming conidia, which are much less resistant to pressure and heat, but may still be as resistant to chemicals (Naranjo-Ortiz and Gabaldon 2019). In this research, ascomycetes such as *Alternaria alternata*, *Alternaria solani*, *Fusarium sambucinum*, *Fusarium oxysporum* f.sp. *radicis-lycopersici* (*FORL*), *Botrytis cinerea*, *Sclerotinia sclerotiorum*, and *Verticillium dahliae* will be studied.

1.1.1.1 *Alternaria alternata*

Alternaria alternata is a necrotrophic pathogen that has been shown to cause diseases in a variety of fruits and vegetables. Pre-harvest symptoms of *A. alternata* include leaf spots, rots, stem cankers, and blights on host plants (Jia et al. 2013). Additionally, *A. alternata* causes dark sunken lesions on fruit with grey-green mycelia and dark green conidia present (Wall and Biles 1993). The fungus infects fruits and vegetables such as tomatoes and bell peppers (Luo et al. 2019; Wang

et al. 2008). A main concern with this fungus is that it may produce toxins and cause allergies and respiratory problems due to its production of spores (da Cruz Cabral et al. 2016; Green et al. 2003).

1.1.1.2 *Alternaria solani*

Alternaria solani is a necrotrophic pathogen responsible for early blight of tomatoes and potatoes (Brouwer et al. 2020). Studies indicate that the loss of product can be as high as 50%, if the disease is not controlled (Leiminger and Hausladen 2012). *Alternaria solani* symptoms include dark spots on leaves, stems, and fruit. Similar to other *Alternata* spp., *A. solani* may produce toxins and may be responsible for allergies associated with its spores (da Cruz Cabral et al. 2016; Green et al. 2003; Stinson et al. 1980).

1.1.1.3 *Botrytis cinerea*

Gray mold disease is caused by *Botrytis cinerea*, a necrotrophic pathogen that impacts a large variety of host plants. It has been shown to impact crops located in temperate and subtropical regions such as grapes, strawberries, and tomatoes. Its ability to produce toxins and cell-wall degrading enzymes contribute to its post-harvest host destruction (Williamson et al. 2007). *Botrytis cinerea* is considered one of the ten most economically important plant pathogens on the planet (Dean et al. 2012).

1.1.1.4 *Fusarium sambucinum*

Fusarium sambucinum is another necrotrophic phytopathogen that is commonly known to cause dry rot in fruits and vegetables, such as potatoes. Dry rot symptoms consist of large brown cavities with wrinkled skin and mycelial growth beneath the cavity (Bojanowski et al. 2013; Stefanczyk et al. 2016). Studies have also indicated the production of mycotoxins from *F. sambucinum*

negatively affecting the health of animals and humans. Therefore, the control of this pathogen is essential (Stefanczyk et al. 2016; Xue et al. 2019).

1.1.1.5 *Fusarium oxysporum* f.sp. *radicis-lycopersici*

Fusarium oxysporum f.sp. *radicis-lycopersici* (FORL) is a necrotrophic pathogen affecting a broad range of host plants. FORL causes Fusarium wilt in many host plants, such as tomatoes. Symptoms include wilting, withering and death of leaves and flowers, in addition to the presence of a pink mycelial growth, which leads to a low product yield of the host plant (de Lamo and Takken 2020).

1.1.1.6 *Sclerotinia sclerotiorum*

Sclerotinia sclerotiorum is a necrotrophic phytopathogen that has been shown to infect sunflowers, soybeans, and carrots among other plants. Symptoms associated with this phytopathogen are water-soaked stem lesions with white fluffy mycelial growth giving this pathogen its common names, white mould, cottony rot or watery soft rot (Bolton et al. 2006). *Sclerotinia* spp. are able to produce sclerotia, which play an important role in their life cycle. Sclerotia consist of thick black rind cells containing melanin. This allows the fungus to survive for long periods under various environmental conditions and can protect the fungus against microbial degradation (Bolton et al. 2006). Its ability to produce sclerotia makes its control particularly difficult.

1.1.1.7 *Verticillium dahliae*

Verticillium dahliae is a hemibiotrophic phytopathogen that causes Verticillium wilt in host plant. Artichoke, cotton, pepper, strawberry and tomato crops are a few examples of hosts impacted by Verticillium wilt (Bhat and Subbarao 1999). Symptoms produced by this disease are curling and discolouration of the plant resulting in plant death and low fruit production yields (Bhat and Subbarao 1999).

1.1.2 Basidiomycota

Fungi belonging to this division are known as basidiomycetes. They reproduce sexually by forming basidiospores that are on a structure called a basidium. This group of fungi includes two well-known groups of plant pathogens that cause rust and smut diseases in plants. It has been shown that most edible fungi belong to this division, however, some basidiomycetes may produce highly toxic compounds (Naranjo-Ortiz and Gabaldon 2019). In this study, the basidiomycete *Rhizoctonia solani* will be investigated.

1.1.2.1 *Rhizoctonia solani*

Rhizoctonia solani is a soil-borne necrotrophic pathogen responsible for infecting cereals, sugar beets, rice, peas, beans, potatoes, and tomatoes (Tu et al. 1996). *Rhizoctonia solani* grows best in warmer and wet environments causing brown patches, root rot and most commonly, sheath blight (Gonzalez et al. 2011).

1.1.3 Mucorales

Members of this order are known as Mucoralean fungi. They possess distinct properties. Like other fungi, they reproduce asexually. They form asexual reproductive spores known as sporangiospores. The sporangiospores are produced in sacs known as sporangium. Most Mucoralean fungi grow very rapidly, taking about two to four days to fully grow. They typically grow rapidly on high water activity foods. Unlike other fungi, the mycelium of Mucoralean fungi grow without any septa allowing quick movement of cell contents. They have low resistance to heat, pressure and chemical treatments and rarely produce mycotoxins (Naranjo-Ortiz and Gabaldon 2019). In this research, the Mucoralean species studied was *Rhizopus stolonifer.*

1.1.3.1 *Rhizopus stolonifer*

Rhizopus stolonifer is another necrotrophic plant pathogen. It has a wide range of target host plants such as eggplants, tomatoes, capsicums and berries, specifically strawberries. Symptoms produced by *Rhizopus* rot have been shown as discolorations and the presence of dense, fluffy mycelia with dark sporangia (Avis et al. 2006). This phytopathogen may decay a whole container of fruit at once, giving it its name of "transit rot".

1.1.4 Oomycota

Previously, this group had been characterized as fungi based on traditional morphology-based grouping. However, it has been discovered that these oomycetes are not phylogenetically related to fungi and are now considered fungus-like organisms from the Chromista Kingdom. Similar to fungi, oomycetes retrieve their nutrients through absorption and produce mycelia. Like Mucoralean fungi, their mycelia do not have septa, which allows their cell contents to move quickly. Unlike true fungi, oomycetes produce zoospores asexually and when they mature, they produce sexual oospores. Zoospores are motile with the use of one or two flagella. They also structurally differ from true fungi such that their cell wall is composed of beta-glucans and cellulose, whereas fungi are predominantly composed of chitin.

1.1.4.1 *Pythium sulcatum*

Pythium sulcatum is a fungus-like necrotrophic pathogen. It is most commonly known for its devastating impact on carrot roots. Among other species, *P. sulcatum* infect host plants like carrots and cause cavity spot symptoms (El-Tarabily et al. 2004). This oomycete uses cell wall degrading enzymes in order to penetrate and colonize the host plant (El-Tarabily et al. 2004). This leads to cell wall damage and maceration of tissues, ultimately leading to a product loss due to plant death.

1.1.5 Cell structure of fungi and oomycetes

Fungi and oomycetes both possess cell membranes. Fungal membranes are typically composed of various lipids, specifically glycerophospholipids, sphingolipids and sterols (Sant et al. 2016). It has been shown that phosphatidylcholine and phosphatidylethanolamine are the most predominant phospholipids in fungal membranes (Avis 2007). The main sterol found in fungal membranes is ergosterol (Avis 2007). Conversely, oomycete membranes do not contain sterols. Within a cell membrane, these lipids produce a phospholipid bilayer contained enzymes and trans-membrane proteins (Avis 2007). The function of a cell membrane is to maintain cell order, integrity and essential functions. These properties are important for the growth and survival of fungi and oomycetes. Without its proper function, the fungus or oomycete cell may experience cell lysis or cell death (Wise et al. 2014). Therefore, understanding how the membrane may be disrupted by antimicrobials is of importance for the control of plant pathogens.

The cell wall is an essential structure that surrounds the plasma membrane of fungal and oomycete cells. This structure gives protection to the cells to external stressors and permits interaction with the environment to allow for nutrient uptake, host recognition, adhesion and colonization (Dehghanifar et al. 2019). The cell wall provides various biological functions to the cells such that it can control cellular permeability, protect the cell from osmotic stress and other environmental stresses as well as maintain the cell structure (Garcia-Rubio et al. 2020). The synthesis of the cell wall is very complex and involves numerous biosynthetic and signaling pathways (Garcia-Rubio et al. 2020). Even though the function of the cell wall of fungi and oomycetes is the same, their composition differ. Fungal cell walls are composed of various glucans, chitin, chitosan and glycoproteins. Oomycete cell walls differ such that they are predominantly composed of cellulose and varying glucans. In both fungi and oomycetes, the

disruption of the cell wall may lead to cell lysis as well as cell death due to the change in morphology and growth of the cell. Therefore, the study of cell wall disruption has been an important field in terms of protection of phytopathogens.

1.2 Food losses by fungal pathogens

As the population is rapidly increasing, reaching approximately 9.5 billion people by 2050, the world faces various challenges that need to be overcome. The steep population growth has been shown to influence the agricultural supply, such that the agricultural demand increases by 1.1% annually (Taheri et al. 2017). The demand comes from the stress to feed the increasing population. Other challenges arise due to the growing population, such as the urbanization of agricultural areas (Wan et al. 2018). Without arable land, agriculture cannot supply the increasing demand. Therefore, it is essential to make use of all currently available land in order to produce enough food. To maximize crop yield from the limited land, it is also important to reduce freshwater, energy consumption, and use of fertilizers, as well as reduce food loss by controlling pests (Kummu et al. 2012).

In order to have sufficient food available, food loss or waste is therefore a pressing challenge that must be overcome. In 2010, the FAO released a study indicating that approximately 1.3 billion tonnes of food annually do not reach the population, playing a large part in the food security issue (FAO, 2011). From all food loss, it is seen that fruits and vegetables have the highest estimated waste rates, where approximately 35-55% is lost at pre- and post-harvest stages (FAO, 2011). The causes of this loss may be due to climate change, human infiltration, and pests (Bourguet et al. 2016).

Food loss and food waste caused by plant pests (i.e., viruses, bacteria, insects and fungi) have been a world-wide issue that impacts crop yields and quality. Pests may affect crop yields pre-

harvest (e.g., in the fields or greenhouses) or post-harvest (e.g., during storage and throughout the supply chain) resulting in approximately 27-42% of loss world-wide, increasing to 48-83% without any control measure (Glare et al. 2012; Oerke 2006). Specifically, plant pathogens have been associated with 10-16% of food loss globally (Bourguet et al. 2016). Fortunately, by the use of appropriate control methods, losses may be reduced or suppressed.

1.3 Conventional control methods

1.3.1 Pre-harvest methods

Pre-harvest control measures are crucial to prevent and inhibit the development of phytopathogens. With the use of pre-harvest measures, crop quality and yields can increase in order to overcome food and/or nutrient deficit issues at hand.

1.3.1.1 Cultural control

Cultural control measures are sought out as preventative measures in order to reduce or eradicate the presence of phytopathogens, thus promoting a successful harvest. Cultural measures consist of a wide range of labor-intensive measures such as crop rotation, tillage, removal of diseased plants, trapping and/or border crops and burning or deep burial of crop residues (Dordas 2009). The benefit of these techniques is that they are deemed safer in terms of contaminating and polluting the environment compared to other methods (e.g., fungicides, insecticides and fumigants) (Katan 2000). They have also been shown to prevent certain plant diseases. These techniques may be used on their own or in conjunction with other methods (Katan 2000). Unfortunately, cultural control measures are highly labor-intensive, steering farmers away from these measures and using more efficient methods such as the use of synthetic chemicals.

1.3.1.2 Genetic control

Phytopathogens attack crop plants causing diseases, which can significantly impact crop plants, threatening food security. Therefore, exploring new ways to grow crops that can combat pathogens are desired. Two genetic control techniques have been used for pre-harvest control measures against phytopathogens. The first technique, which is considered the traditional mechanism is breeding. Breeding programs are often used prior to understanding the mode of action of resistance genes. Breeders identify and select plants with optimal traits and cross breed these plants. The produced plants are then evaluated in order to determine their resistance against pathogens (Staskawicz 2001). This method benefits farmers by limiting the use of pesticides when producing crops with resistant cultivars. However, the efficiency of this technique is inferior to others and is time-consuming to produce resistant crops (Staskawicz 2001).

Another genetic control measure, which stems from the traditional breeding method is genetic engineering of crops. This technique encompasses the incorporation of resistant genes into the plant's genome, thereby producing a genetically modified organism (GMO). Unlike traditional genetic control, genetic engineering allows for a single gene to be manipulated (Piquerez et al. 2014). The addition and/or alteration of a single gene can benefit cultivar of crops by becoming resistant against pathogens, as well as increase production yields (Piquerez et al. 2014). Even though this method has shown some promise, its use has been controversial as research is still required to determine whether the food produced is safe for long-term consumption and whether genetically-modified crops negatively impact the environment (Bawa and Anilakumar 2013). Therefore, the production and/or development of other new efficient control methods are still being researched.

1.3.1.3 Synthetic chemicals

Various strategies are being used in order to control and manage loss of agricultural products caused by plant pathogens. Currently, synthetic chemicals, such as fungicides, are heavily used pre- and post-harvest to prevent fungal diseases as they are effective, low in cost, and easy to use (Xia et al. 2006). However, other concerns arise with the excessive use of synthetic chemicals. Synthetic chemicals have been attributed to environmental contamination, risks associated with human health, as well as the development of pathogen resistance (Sui et al. 2016).

When sprayed in crop fields to reduce presence of fungal pathogens, some residues of pesticides may enter surrounding groundwater, soil and air affecting the environment and non-target organisms, such as humans. Some synthetic chemical residues have been classified as possible carcinogens. For example, mancozeb, a commercially used fungicide, showed adverse health implications associated with its carcinogenicity after long-exposure in rats (Belpoggi et al. 2002). Similarly, fumigants are biocides that are released as gas or vapors into the air or in the soil to eliminate or destroy pests. Without their use, crop yields may be greatly reduced. However, some fumigants such as methyl bromide have been banned due to its high human toxicity and its correlation to ozone depletion (Budnik et al. 2012). Additionally, the excessive use of synthetic chemicals has also been attributed to the development of resistant strains of plant pathogens (Avis 2007). Due to the pathogen resistance, lower efficiency and efficacy of some fungicides has gradually reduced their usefulness.

Without the use of pesticides, crops could be destroyed resulting in low crop yields. The lack of alternative control measures has increased their use due to the need of agricultural products for the growing population. However, strict regulations and the pressure from consumers for less risky and more effective options have driven research for novel control measures to combat pathogens.

1.3.2 Post-harvest control methods

Fruits and vegetables are susceptible to numerous post-harvest fungal pathogens that affect food waste issues. Pathogens may arise at any stage post-harvest: handling, storage, processing, and distribution. It is important to find and develop methods post-harvest in order to prevent and inhibit pathogen growth.

1.3.2.1 Physical control

Physical control has successfully been used as a means to control diseases and protect foods, such as fruits and vegetables after harvesting. Methods for inhibition or delayed development of pathogens include cold storage, the use of heat, ultraviolet radiation, hypobaric and hyperbaric pressures, and modified atmospheres (Usall et al. 2016). Placing fruits and vegetables in cold storage, also known as refrigeration, immediately after harvest can act directly and indirectly. Cold storage directly reduces the growth of pathogens and indirectly slows down ripening of the produce, which reduces fungal infection (Usall et al. 2016). The use of heat can be applied via hot water treatment, hot air treatment or microwave treatment. These techniques reduce post-harvest loss by inhibiting the mycelial growth and spore germination of pathogens present on the surface of the fruit or vegetable (Karabulut et al. 2010). Similarly to cold storage, the use of ultraviolet radiation, specifically ultraviolet-C light, acts directly and indirectly such that it inhibits pathogen growth, as well as activates induced resistance in the fruit or vegetable (Romanazzi et al. 2006).

1.3.2.2 Chemical control

In some cases, physical control methods are not adequate to suppress microbial invasions of fresh fruits and vegetables during long storage periods or extended movement from crop to market. In addition, some fruits or vegetables cannot withstand near-freezing temperatures that are required to reduce pathogenic fungi (Eckert and Ogawa 1985). In these cases, antifungal preservatives must

be used prior to storage to maintain the quality of these commodities. There are numerous organic compounds that have been used as post-harvest fungicides to control decay-causing pathogens, for example thiabendazole, imazalil, and sodium *o*-phenylphenate (Schirra et al. 2011). They may be applied to fruits and vegetables in a gaseous or water-based manner. Their application depends on their physical and chemical properties, as well as the type of crop to which they will be applied. The residues of these synthetic fungicides have increased the public's concern questioning their safety. Additionally, the efficacy of fungicides has diminished due to the presence of fungicide resistant pathogens (Avis 2007). Due to negative perception of synthetic chemicals from the public as well as potential health risks, governments have restricted their use for post-harvest control of phytopathogens (Janisiewicz and Korsten 2002) and thus, effective alternatives to control post-harvest diseases are still being investigated.

1.4 Biological control methods

Biological control or biocontrol is a term used to define the use of living organisms to suppress the population density of another harmful organism. Biocontrol includes the control of pests, weeds and plant pathogens by the use of predators, parasitoids, pathogens, as well as antagonistic microorganisms either pre-harvest and/or post-harvest (Eilenberg et al. 2001). Therefore, the biocontrol of plant diseases is the use of living organisms such as antagonistic microorganisms to reduce the populations of plant pathogens. Biological control can be categorized into three main types: natural, classical and augmentative. Natural biocontrol has been occurring for over 500 million years such that pests are prevented by natural enemies that are present in that time and place (Kohl et al. 2019; van Lenteren 2012). Classical biocontrol, also known as inoculative biological control, refers to the collection of natural enemies and using them against their known pests that have been introduced in a new region (Bale et al. 2008). In augmentative biological

control, the antagonistic organism is produced at a large scale to control the target harmful organism within the same niche from which it had been isolated. In terms of plant disease control, antagonistic microorganisms known to suppress specific plant pathogens are grown in mass quantities in biofactories on artificial media and are applied to crops in order to reduce diseases associated with phytopathogens (Kohl et al. 2019; van Lenteren 2012).

Over the last 120 years, approximately 230 biocontrol agents have been applied worldwide to fruit and vegetable crops, maize, cereals, cotton, sugarcane and soybean crops instead of synthetic chemical control methods for a more environmental and safer method (van Lenteren 2012; van Lenteren et al. 2018). In 2015, biological control agents comprised 2% of the total global pesticide market, estimated at about US$ 1.7 billion (van Lenteren et al. 2018). Since 2005, the market for biocontrol agents has increased by 15% per year.

Before becoming commercially available, biocontrol agents must be studied ensuring their efficacy against phytopathogens before product development. Therefore, identifying their mode of action is essential for understanding how they function, how they are efficient in combatting plant disease, and for regulatory processes to obtain a product registration.

1.4.1 Biological control mechanisms

Biological control agents manage plant disease and suppress phytopathogens through various direct and indirect modes of action. Typically, most successful biocontrol agents use one or more mechanisms to control plant pathogens. Two indirect mechanisms are induced resistance and competition for nutrients and/or space. Induced resistance refers to the presence of the microorganism (or other elicitor/inducer), which may stimulate the plant cells and tissues to more effectively combat a pathogen without the beneficial microorganism directly affecting the pathogen. Competition is another indirect mechanism referring to the microorganism's ability to

outcompete the pathogen for nutrients (e.g., oxygen, nitrogen and carbohydrates) and space thereby inhibiting pathogen proliferation. Biocontrol agents may also act directly against the pathogens via parasitism and antibiosis. Parasitism is a direct interaction where the antagonistic microorganism gains from the pathogen (e.g., nutrients) causing degradation or lysis of fungal structures (Spadaro and Droby 2016). Lastly, antibiosis refers to the production of secondary metabolites (Raaijmakers et al. 2010). These secondary metabolites may possess antimicrobial activity that directly inhibit pathogen growth (Raaijmakers and Mazzola 2012).

1.4.2 Induced resistance

Plants may co-exist with numerous mutualistic microbes. These mutualistic microbes benefit the plant by providing advantages such as promoting growth, as well as fighting off different pathogens (Pieterse et al. 2014). Biocontrol agents have been shown to benefit plants in this way by enhancing their resistance against pathogens. Certain biological control agents (BCA) colonize the plant and enhance the level of resistance of the plant against phytopathogens that may be present in the environment. In this case, the BCA has no direct impact on the pathogen and only enhances, or in other words prepares the plant's defense system to combat the pathogen (Kohl et al. 2019). Resistance from the host plant may be restricted to the location of induction or may also spread throughout the plant's tissues via plant signaling pathways (Kohl et al. 2019).

1.4.3 Competition

Most biocontrol agents overcome plant pathogens at the wound site by outcompeting for limiting resources and reducing the pathogens growth. Microorganisms such as plant pathogens may not survive without the appropriate nutrients. An essential element needed is iron, which is found in soil in its ferric form at low concentrations. A mode of action of some biocontrol agents is their ability to produce low-molecular-weight siderophores that have a high affinity to ferric iron. Thus,

the beneficial bacteria outcompetes pathogenic fungi by depleting iron concentrations (Kohl et al. 2019). Another mechanism by which biocontrol agents outcompete for resources is by their ability to colonize plants and therefore limiting the space available for phytopathogens to grow. In order to effectively compete for resources, the antagonistic bacteria must be in sufficient amounts at the appropriate time and location (Spadaro and Droby 2016).

1.4.4 Parasitism

Parasitism is a type of symbiosis where two organisms co-exist together for a period of time. In terms of biocontrol, hyperparasitism occurs when the pathogen is directly impacted by a beneficial microorganism (Spadaro and Droby 2016). The beneficial microorganism can either damage the pathogen's structure or even kill the pathogen thereby suppressing its associated disease. Some biocontrol agents directly attack the pathogens by degrading fungal cell walls via lytic enzymes such as chitinase, β-1,3-glucanase and proteases (Bonaterra et al. 2012; Choudhary et al. 2014).

Biocontrol agents may break down fungal cells in numerous ways. Antifungal enzymes have the ability to degrade fungal structures leading to suppression of the pathogen. The fungal cell wall is comprised of chitin and β-1,3-glucan as structural cell wall components. Within the cell wall, proteins such as glycoproteins are also synthesized and help maintain cell shape. Without the cell wall, fungal cells may not survive, and their growth is reduced. Therefore, antifungal enzymes such as β-1,3-glucanase, chitinase and proteases can impact the survival of phytopathogens (Spadaro and Droby 2016).

1.4.5 Antibiosis

Antimicrobial compounds are produced by numerous microorganisms, such as bacteria and fungi (Kohl et al. 2019). Bacterial biocontrol agents from some genera have been extensively studied to determine the amount of antibiotics produced and the diversity in the types of antimicrobial

compounds (Raaijmakers et al. 2002). These antimicrobial compounds have varied activities, because they encompass a large group of diverse chemicals that include non-peptide and peptide containing antimicrobial compounds.

1.4.5.1 Non-peptide-containing antimicrobials

Microorganisms, such as bacteria, produce numerous secondary metabolites with different bioactive chemical structures. These microorganisms may produce non-peptide containing antimicrobials. A large class of these secondary metabolites are polyketides, which consist of parasiticides, antibiotics, and antifungal compounds (Gomes et al. 2013). Polyenes, which are polyunsaturated organic compounds, are found within this class and over 200 polyenes have been shown to be produced by Actinomycetes (Hamdache et al. 2011; Milisavljevic et al. 2015). Polyenes have been shown to possess antifungal properties and inhibit the proliferation of pathogenic fungi in plants (Gomes et al. 2013; Harrison et al. 1986; Milisavljevic et al. 2015). Volatile organic compounds (VOCs) is another class of secondary metabolites produced by microorganisms. VOCs possess advantageous properties such as low molecular weight, high vapour pressure and low boiling point that facilitate their diffusion through the environment (Schulz-Bohm et al. 2017). VOCs produced by soil bacteria have been shown to suppress the growth of plant pathogenic fungi and reduce plant disease, contributing to biocontrol abilities of the bacteria (Ossowicki et al. 2017). In addition to non-peptide containing antimicrobials, biocontrol agents may also produce peptide-containing antimicrobials.

1.4.5.2 Peptide-containing antimicrobials

A wide range of peptide containing antimicrobials are produced by microorganisms such as bacteria. These antimicrobials consist of short sequence peptides that typically contain less than 50 amino acid residues. Bacteria may produce antimicrobial peptides via ribosomal synthesis or

as secondary metabolites, which are synthesized through non-ribosomal synthesis (Montesinos 2007).

Bacteriocins are a group of ribosomally synthesized antimicrobial peptides produced by all major groups of bacteria such as *Enterobacteriaceae*, Gram-positive bacteria, and lactic acid bacteria (Parret et al. 2005). Bacteriocin characteristics such as low molecular weight, their cationic and hydrophobic nature contribute to their main mode of action to inhibit plant pathogens, which is membrane permeability and inhibition of peptidoglycan synthesis (Dehghanifar et al. 2019; Parret et al. 2005).

Bacteria also produce various non-ribosomally synthesized secondary metabolites such as cyclopeptides. These peptides are formed by a cyclization of amino acid residues to produce a structure that is more rigid than its linear form (Lee and Kim 2015). An example of these are tyrocidines, which are a group of cyclic decapeptides that are produced by *Bacillus aneurinolyticus* and suppress growth of plant pathogens such as *Fusarium solani* and *B. cinerea* (Lee and Kim 2015). Cyclopeptides may also contain carbohydrate or lipid moieties, producing glycopeptides or lipopeptides, respectively. Cyclic glycopeptides such as occidiofungin produced by *Burkholderia contaminans* have been shown to inhibit the growth of plant pathogens such as *A. alternata* and *R. solani* (Lu et al. 2009).

The interest in cyclic lipopeptides (CLP) has increased over the years for their potent antifungal activity against plant pathogenic fungi. Cyclic lipopeptides are secondary metabolites that contain a cyclic oligopeptide complex covalently linked to differing chain lengths of fatty acid (Lee and Kim 2015). Major producers of these compounds are *Pseudomonas* and *Bacillus* spp. *Bacillus* cyclic lipopeptides can be categorized based on structure within three families known as fengycins, surfactins, and iturins (Ongena and Jacques 2008). *Pseudomonas* spp. also produce cyclic

lipopeptides, such as viscosins and amphisins (Raaijmakers et al. 2010). Because of their structures, CLPs have the ability to interact with fungal membranes leading to membrane permeability that causes an imbalance of the ionic potential across the fungal membrane, finally causing cell lysis. Their antifungal potential has been evaluated against various phytopathogens, for example, *F. oxysporum*, *B. cinerea*, *R. solani*, and *Phytophthora capsici* (Lee and Kim 2015; Montesinos 2007).

1.5 Biocontrol agents

1.5.1 *Bacillus* spp.

Bacillus spp. is a large and diverse genus belonging to the *Firmicutes* phylum. They are comprised of rod-shaped, Gram-positive endospore-forming, aerobic or facultative anaerobic bacteria naturally occurring in agricultural soils (Celandroni et al. 2019; Fira et al. 2018). The desire to use *Bacillus* spp. as a biocontrol agent is attributed to their ability to replicate quickly and their ability to live in various ecological niches, and because of they form spores that are resistant to extreme environmental conditions such as dry environments and high temperatures (Shafi et al. 2017). *Bacillus* spp. have demonstrated control against a wide range of plant pathogens through indirect and direct means. They can indirectly antagonize pathogens by inducing systemic resistance, by promoting plant growth, or by outcompeting for nutrients and/or space (Shafi et al. 2017). Whereas, they can directly antagonize pathogens by producing an array of secondary metabolites (Ntushelo et al. 2019). It has been shown that within the *Bacillus* genus, 5 to 8% of their genomes have been associated with the production of antagonistic secondary metabolites (Fira et al. 2018). *Bacillus* spp. have been shown to produce numerous peptide and non-peptide containing antifungal compounds, as well as antifungal enzymes to reduce or suppress the growth of pathogens (Falardeau et al. 2013; Hamdache et al. 2011; Ongena and Jacques 2008; Wise et al. 2012). Due

to these characteristics, many commercially available biological control products contain various *Bacillus* strains in order to suppress pathogenic fungi (Ntushelo et al. 2019).

1.5.2 *Pseudomonas* spp.

Pseudomonas spp. are Gram-negative, aerobic rhizobacteria that have been found naturally in agricultural soils (Weller 2007). Unlike *Bacillus* spp., *Pseudomonas* spp. do not produce endospores causing some issues with their use as biocontrol agents (Weller 2007). However, pseudomonads have been studied as plant pathogen antagonists as they are able to promote plant growth and have shown potential use as biocontrol agents by suppressing plant disease (Ganeshan and Kumar 2005; Nelkner et al. 2019). These beneficial pseudomonads often belong to the *Pseudomonas fluorescens* group (Ganeshan and Kumar 2005). Members of this group include *P. gessardii*, *P. moraviensis*, *P. koreensis*, *P. brenneri*, and *P. chlororaphis* (Mulet et al. 2010). Studies have indicated that *P. gessardii* suppressed symptoms of grey mold on strawberries, as well as *Alternaria* rot in bell peppers (Cloutier et al. 2020; Luo et al. 2019). *Pseudomonas moraviensis* and *P. koreensis* have been shown to reduce severity of symptoms of grey mold and *Rhizopus* rot in strawberries, as well as grey mold in bell peppers and blueberries (Cloutier et al. 2020; Kurniawan et al. 2018; Luo et al. 2019). *Pseudomonas brenneri* has also demonstrated antagonistic properties against grey mold in strawberries and blueberries, *Rhizopus* rot in strawberries, as well as *Alternaria* rot in bell peppers and blueberries (Cloutier et al. 2020; Kurniawan et al. 2018; Luo et al. 2019). *Pseudomonas chlororaphis* has shown biocontrol activity against *Fusarium oxysporum* f.sp. *radicis-lycopersici* foot and root rot in tomato (Chin-A-Woeng et al. 2001)

Pseudomonads have been studied in order to determine their mode of action as biocontrol agents. They have been shown to be plant growth-promoting rhizobacteria, and additionally, some

members of the *P. fluorescens* group have also been demonstrated to produce a variety of antifungal secondary metabolites (Nelkner et al. 2019). These include non-peptide-containing compounds such as pyrrolnitrin, pyoluteorin and phenazines, as well as peptide-containing compounds such as arthrofactin (Kurniawan et al. 2018; Ligon et al. 2000; Raaijmakers et al. 2010). *Pseudomonas* spp. have also been shown to produce antifungal enzymes in order to combat pathogenic fungi (Ligon et al. 2000). Because of their ability to antagonize fungal pathogens, their use as biocontrol agents are increasing.

1.5.3 *Arthrobacter* spp.

Members of the *Arthrobacter* genus are Gram-positive aerobes that belong to the *Actinobacteria* phylum. They are in the Actinomycetales order and are often referred to as actinomycetes. They are commonly found within agricultural soils and in extreme environmental conditions, which is associated with their ability to survive in stressful conditions (Mongodin et al. 2006). At their early stage in the morphological cycle, *Arthrobacter* spp. are rod-shaped, however, within older cultures, they become coccus-shaped making this genus pleomorphic (Yan et al. 2019). It has been demonstrated that their small coccus-shape benefits their survival under stressful conditions, as it is its most stable form (Mongodin et al. 2006). *Arthrobacter* spp. have sparked interest as they have been identified to be able to biodegrade various environmental pollutants, as well as herbicides found in soils (Mongodin et al. 2006).

Numerous actinomycetes produce various antifungal secondary metabolites and as a member of this group, *Arthrobacter* spp. have also shown antifungal activity (Keikha et al. 2015). Previous work has demonstrated antagonistic properties against plant pathogens such as *F. sambucinum*, *V. dahliae*, *A. alternata* and *B. cinerea* (Lu et al. 2009; Mohamed et al. 2017). Other work has indicated the production of the first non-ribosomally synthesized peptide-containing compound

produced by *Arthrobacter* sp. PGVB1, arthroamide, as well as its congener, turnagainolide (Igarashi et al. 2015). Additionally, an antimicrobial volatile organic compound, namely dimethylhexadecylamine, was isolated from *Arthrobacter agilis* UMCV2 and demonstrated inhibitory activity against *B. cinerea* and *P. cinnamomi* (Velazquez-Becerra et al. 2013). Although secondary metabolites have been identified from *Arthrobacter* spp., limited information is available regarding their antagonistic properties against fungal pathogens, their primary mode of action, as well as their potential use as a biocontrol agent.

1.6 Hypotheses and objectives

Our lab has previously isolated *Bacillus* spp. and other *Firmicutes* related to *Bacillus*, *Pseudomonas* spp., and *Arthrobacter* spp. from disease suppressive soils, composts and compost teas, which have shown antifungal activity against various phytopathogens *in vitro* and *in vivo*. This study investigates the antagonistic effect of 32 bacterial isolates against *Fusarium sambucinum in vitro* and potato dry rot *in vivo*. Additionally, *Arthrobacter* spp., namely *Arthrobacter humicola* strains M9-1A, M9-2, and M9-8, as well as *Arthrobacter psychrophenolicus* strain M9-17 were further investigated to determine their antimicrobial activity and determine their potential mode of action. Our hypotheses were:

1. *Verticillium dahliae* bacterial antagonists will suppress mycelial growth of *Fusarium sambucinum* and symptoms of Fusarium dry rot on potato tubers.
2. Extracts from the most inhibitory bacteria will suppress mycelial growth and conidial germination of *F. sambucinum* indicating antibiosis as a mode of action.
3. *Arthrobacter psychrophenolicus* is more efficient than *A. humicola* in inhibiting *A. alternata* and other phytopathogens.

4. Cell-free filtrates of *Arthrobacter* spp. will suppress black mold (*A. alternata*) on tomato fruit.
5. *Arthrobacter* spp. possess extracellular compounds and enzymes that contribute to their antifungal mode of action.

To test these hypotheses, our objectives were:

i. to investigate the antimicrobial activity of 32 bacterial isolates against *Fusarium sambucinum*
ii. to determine their suppressive effect against *Fusarium* dry rot in potato tubers
iii. to evaluate the extracellular antimicrobial compounds produced by the most inhibitory bacteria
iv. to investigate the antimicrobial activity of *A. humicola* strains M9-1A, M9-2, and M9-8 and *A. psychrophenolicus* strain M9-17 against fungal and oomycete pathogens
v. to determine the antimicrobial activity of *Arthrobacter* extracellular compounds
vi. to investigate the suppressive effect of *Arthrobacter* extracellular compounds on tomato black mold
vii. to identify the presence of extracellular lytic enzymes in *Arthrobacter* spp.
viii. to isolate and identify antifungal compounds in *A. psychrophenolicus* M9-17.

1.7 References

Avis TJ (2007) Antifungal compounds that target fungal membranes: applications in plant disease control. Canadian Journal of Plant Pathology 29: 323-329. doi: 10.1080/07060660709507478.

Avis TJ, Martinez C, Tweddell RJ (2006) Effect of chlorine atmospheres on the development of rhizopus rot *Rhizopus stolonifer* and gray mold *Botrytis cinerea* on stored strawberry fruits. Canadian Journal of Plant Pathology 28: 526-532. doi: 10.1080/07060660609507330.

Bale JS, van Lenteren JC, Bigler F (2008) Biological control and sustainable food production. Philosophical Transactions of the Royal Society B-Biological Sciences 363: 761-776. doi: 10.1098/rstb.2007.2182.

Bawa AS, Anilakumar KR (2013) Genetically modified foods: safety, risks and public concerns-a review. Journal of Food Science and Technology-Mysore 50: 1035-1046. doi: 10.1007/s13197-012-0899-1.

Belpoggi F, Soffritti M, Guarino M, Lambertini L, Cevolani D, Maltoni C (2002) Results of long-term experimental studies on the carcinogenicity of ethylene-bis-dithiocarbamate (Mancozeb) in rats. Carcinogenesis Bioassays and Protecting Public Health: Commemorating the Lifework of Cesare Maltoni and Colleagues 982: 123-136.

Bhat RG, Subbarao KV (1999) Host range specificity in *Verticillium dahliae*. Phytopathology 89: 1218-1225. doi: 10.1094/phyto.1999.89.12.1218.

Bojanowski A, Avis TJ, Pelletier S, Tweddell RJ (2013) Management of potato dry rot. Postharvest Biology and Technology 84: 99-109. doi: 10.1016/j.postharvbio.2013.04.008.

Bolton MD, Thomma B, Nelson BD (2006) *Sclerotinia sclerotiorum* (Lib.) de Bary: biology and molecular traits of a cosmopolitan pathogen. Molecular Plant Pathology 7: 1-16. doi: 10.1111/j.1364-3703.2005.00316.x.

Bonaterra A, Badosa E, Cabrefiga J, Frances J, Montesinos E (2012) Prospects and limitations of microbial pesticides for control of bacterial and fungal pomefruit tree diseases. Trees-Structure and Function 26: 215-226. doi: 10.1007/s00468-011-0626-y.

Bourguet D, Delmotte F, Franck P, Guillemaud T, Reboud X, Vacher C, Walker A-S, Consortium REX (2016) Combining Selective Pressures to Enhance the Durability of Disease Resistance Genes. Frontiers in Plant Science 7. doi: 10.3389/fpls.2016.01916.

Brouwer SM, Odilbekov F, Burra DD, Lenman M, Hedley PE, Grenville-Briggs L, Alexandersson E, Liljeroth E, Andreasson E (2020) Intact salicylic acid signalling is required for potato defence against the necrotrophic fungus *Alternaria solani*. Plant Molecular Biology 104: 1-19. doi: 10.1007/s11103-020-01019-6.

Budnik LT, Kloth S, Velasco-Garrido M, Baur X (2012) Prostate cancer and toxicity from critical use exemptions of methyl bromide: Environmental protection helps protect against human health risks. Environmental Health 11. doi: 10.1186/1476-069x-11-5.

Celandroni F, Vecchione A, Cara A, Mazzantini D, Lupetti A, Ghelardi E (2019) Identification of *Bacillus* species: Implication on the quality of probiotic formulations. PLOS ONE 14. doi: 10.1371/journal.pone.0217021.

Chin-A-Woeng TFC, Thomas-Oates JE, Lugtenberg BJJ, Bloemberg GV (2001) Introduction of the phzH gene of *Pseudomonas chlororaphis* PCL1391 extends the range of biocontrol ability of phenazine-1-carboxylic acid-producing *Pseudomonas* spp. strains. Molecular Plant-Microbe Interactions 14: 1006-1015. doi: 10.1094/mpmi.2001.14.8.1006.

Choudhary B, Nagpure A, Gupta RK (2014) Fungal cell-wall lytic enzymes, antifungal metabolite(s) production, and characterization from *Streptomyces exfoliatus* MT9 for controlling fruit-rotting fungi. Journal of Basic Microbiology 54: 1295-1309. doi: 10.1002/jobm.201400380.

Cloutier A, Tran S, Avis TJ (2020) Suppressive effect of compost bacteria against grey mould and *Rhizopus* rot on strawberry fruit. Biocontrol Science and Technology 30: 143-159. doi: 10.1080/09583157.2019.1695745.

da Cruz Cabral L, Terminiello L, Fernandez Pinto V, Fog Nielsen K, Patriarca A (2016) Natural occurrence of mycotoxins and toxigenic capacity of *Alternaria* strains from mouldy peppers. International Journal of Food Microbiology 236: 155-160. doi: 10.1016/j.ijfoodmicro.2016.08.005.

de Lamo FJ, Takken FLW (2020) Biocontrol by *Fusarium oxysporum* using endophyte-mediated resistance. Frontiers in Plant Science 11. doi: 10.3389/fpls.2020.00037.

Dean R, Van Kan JAL, Pretorius ZA, Hammond-Kosack KE, Di Pietro A, Spanu PD, Rudd JJ, Dickman M, Kahmann R, Ellis J, Foster GD (2012) The top 10 fungal pathogens in molecular plant pathology. Molecular Plant Pathology 13: 804-804. doi: 10.1111/j.1364-3703.2012.00822.x.

Dehghanifar S, Keyhanfar M, Emtiazi G (2019) Production and partial purification of thermostable bacteriocins from *Bacillus pumilus* ZED17 and DFAR8 strains with antifungal activity. Molecular Biology Research Communications 8: 41-49. doi: 10.22099/mbrc.2019.31563.1367.

Dordas C (2009) Role of Nutrients in Controlling Plant Diseases in Sustainable Agriculture: A Review. Sustainable Agriculture: 443-460. doi: 10.1007/978-90-481-2666-8_28.

Eckert JW, Ogawa JM (1985) The chemical control of postharvest diseases - sub-tropical and tropical fruits. Annual Review of Phytopathology 23: 421-454.

Eilenberg J, Hajek A, Lomer C (2001) Suggestions for unifying the terminology in biological control. Biocontrol 46: 387-400. doi: 10.1023/a:1014193329979.

El-Tarabily KA, Abouzeid MA, Hardy G, Sivasithamparam K (2004) *Pythium sulcatum* and *P. ultimum* as causal agents of cavity spot disease of carrots in Egypt. Canadian Journal of Plant Science 84: 607-614. doi: 10.4141/p03-014.

Falardeau J, Wise C, Novitsky L, Avis TJ (2013) Ecological and mechanistic insights into the direct and indirect antimicrobial properties of *Bacillus subtilis* lipopeptides on plant pathogens. Journal of Chemical Ecology 39: 869-878. doi: 10.1007/s10886-013-0319-7.

Fira D, Dimkic I, Beric T, Lozo J, Stankovic S (2018) Biological control of plant pathogens by *Bacillus* species. Journal of Biotechnology 285: 44-55. doi: 10.1016/j.jbiotec.2018.07.044.

Ganeshan G, Kumar AM (2005) *Pseudomonas fluorescens*, a potential bacterial antagonist to control plant diseases. Journal of Plant Interactions 1: 123-134. doi: 10.1080/17429140600907043.

Garcia-Rubio R, de Oliveira HC, Rivera J, Trevijano-Contador N (2020) The fungal cell wall: *Candida*, *Cryptococcus*, and *Aspergillus* species. Frontiers in Microbiology 10. doi: 10.3389/fmicb.2019.02993.

Glare T, Caradus J, Gelernter W, Jackson T, Keyhani N, Kohl J, Marrone P, Morin L, Stewart A (2012) Have biopesticides come of age? Trends in Biotechnology 30: 250-258. doi: 10.1016/j.tibtech.2012.01.003.

Gomes ES, Schuch V, de Macedo Lemos EG (2013) Biotechnology of polyketides: new breath of life for the novel antibiotic genetic pathways discovery through metagenomics. Brazilian Journal of Microbiology : 44: 1007-1034.

Gonzalez M, Pujol M, Metraux J-P, Gonzalez-Garcia V, Bolton MD, Borras-Hidalgo O (2011) Tobacco leaf spot and root rot caused by *Rhizoctonia solani* Kuhn. Molecular Plant Pathology 12: 209-216. doi: 10.1111/j.1364-3703.2010.00664.x.

Green BJ, Mitakakis TZ, Tovey ER (2003) Allergen detection from 11 fungal species before and after germination. Journal of Allergy and Clinical Immunology 111: 285-289. doi: 10.1067/mai.2003.57.

Hamdache A, Lamarti A, Aleu J, Collado IG (2011) Non-peptide metabolites from the genus *Bacillus*. Journal of Natural Products 74: 893-899. doi: 10.1021/np100853e.

Harrison PH, Noguchi H, Vederas JC (1986) Biosynthesis of polyene antibiotics - intact of C-13 labeled octanoate into fungichromin by stretomyces-cellulosae. Journal of the American Chemical Society 108: 3833-3834. doi: 10.1021/ja00273a050.

Igarashi Y, Yamamoto K, Fukuda T, Shojima A, Nakayama J, Carro L, Trujillo ME (2015) Arthroamide, a cyclic depsipeptide with quorum sensing inhibitory activity from *Arthrobacter* sp. Journal of Natural Products 78: 2827-2831. doi: 10.1021/acs.jnatprod.5b00540.

Janisiewicz WJ, Korsten L (2002) Biological control of postharvest diseases of fruits. Annual Review of Phytopathology 40: 411-441. doi: 10.1146/annurev.phyto.40.120401.130158.

Jia C, Zhang L, Liu L, Wang J, Li C, Wang Q (2013) Multiple phytohormone signalling pathways modulate susceptibility of tomato plants to *Alternaria alternata* f. sp *lycopersici*. Journal of Experimental Botany 64: 637-650. doi: 10.1093/jxb/ers360.

Karabulut OA, Smilanick JL, Crisosto CH, Palou L (2010) Control of brown rot of stone fruits by brief heated water immersion treatments. Crop Protection 29: 903-906. doi: 10.1016/j.cropro.2010.03.010.

Katan J (2000) Physical and cultural methods for the management of soil-borne pathogens. Crop Protection 19: 725-731. doi: 10.1016/s0261-2194(00)00096-x.

Keikha N, Ayatollahi Mousavi SA, Shahidi Bonjar GH, Fouladi B, Izadi AR (2015) *In vitro* antifungal activities of *Actinomyces* species isolated from soil samples against *Trichophyton mentagrophytes*. Current Medical Mycology 1: 33-38. doi: 10.18869/acadpub.cmm.1.3.33.

Koeck M, Hardham AR, Dodds PN (2011) The role of effectors of biotrophic and hemibiotrophic fungi in infection. Cellular Microbiology 13: 1849-1857. doi: 10.1111/j.1462-5822.2011.01665.x.

Kohl J, Kolnaar R, Ravensberg WJ (2019) Mode of action of microbial biological control agents against plant diseases: relevance beyond efficacy. Frontiers in Plant Science 10. doi: 10.3389/fpls.2019.00845.

Kummu M, de Moel H, Porkka M, Siebert S, Varis O, Ward PJ (2012) Lost food, wasted resources: Global food supply chain losses and their impacts on freshwater, cropland, and fertiliser use. Science of the Total Environment 438: 477-489. doi: 10.1016/j.scitotenv.2012.08.092.

Kurniawan O, Wilson K, Mohamed R, Avis TJ (2018) *Bacillus* and *Pseudomonas* spp. provide antifungal activity against gray mold and *Alternaria* rot on blueberry fruit. Biological Control 126: 136-141. doi: 10.1016/j.biocontrol.2018.08.001.

Lee DW, Kim BS (2015) Antimicrobial Cyclic Peptides for Plant Disease Control. Plant Pathology Journal 31: 1-11. doi: 10.5423/ppj.rw.08.2014.0074.

Leiminger JH, Hausladen H (2012) Early Blight Control in Potato Using Disease-Orientated Threshold Values. Plant Disease 96: 124-130. doi: 10.1094/pdis-05-11-0431.

Ligon JM, Hill DS, Hammer PE, Torkewitz NR, Hofmann D, Kempf HJ, van Pee KH (2000) Natural products with antifungal activity from *Pseudomonas* biocontrol bacteria. Pest Management Science 56: 688-695. doi: 10.1002/1526-4998(200008)56:8<688::aid-ps186>3.3.co;2-m.

Lu S-E, Novak J, Austin FW, Gu G, Ellis D, Kirk M, Wilson-Stanford S, Tonelli M, Smith L (2009) Occidiofungin, a unique antifungal glycopeptide produced by a strain of Burkholderia contaminans. Biochemistry 48: 8312-8321. doi: 10.1021/bi900814c.

Luo M, Purdy H, Avis TJ (2019) Compost bacteria provide antifungal activity against grey mold and *Alternaria* rot on bell pepper fruit. Botany 97: 221-230. doi: 10.1139/cjb-2018-0180.

Milisavljevic M, Zivkovic S, Pekmezovic M, Stankovic N, Vojnovic S, Vasiljevic B, Senerovic L (2015) Control of human and plant fungal pathogens using pentaene macrolide 32, 33-didehydroroflamycoin. Journal of Applied Microbiology 118: 1426-1434. doi: 10.1111/jam.12811.

Mohamed R, Groulx E, Defilippi S, Erak T, Tambong JT, Tweddell RJ, Tsopmo A, Avis TJ (2017) Physiological and molecular characterization of compost bacteria antagonistic to soil-borne plant pathogens. Canadian Journal of Microbiology 63: 411-426. doi: 10.1139/cjm-2016-0599.

Mongodin EF, Shapir N, Daugherty SC, DeBoy RT, Emerson JB, Shvartzbeyn A, Radune D, Vamathevan J, Riggs F, Grinberg V, Khouri H, Wackett LP, Nelson KE, Sadowsky MJ (2006) Secrets of soil survival revealed by the genome sequence of *Arthrobacter aurescens* TC1. PLOS Genetics 2: 2094-2106. doi: 10.1371/journal.pgen.0020214.

Montesinos E (2007) Antimicrobial peptides and plant disease control. FEMS Microbiology Letters 270: 1-11. doi: 10.1111/j.1574-6968.2007.00683.x.

Mulet M, Lalucat J, Garcia-Valdes E (2010) DNA sequence-based analysis of the *Pseudomonas* species. Environmental Microbiology 12: 1513-1530. doi: 10.1111/j.1462-2920.2010.02181.x.

Naranjo-Ortiz MA, Gabaldon T (2019) Fungal evolution: diversity, taxonomy and phylogeny of the Fungi. Biological Reviews 94: 2101-2137. doi: 10.1111/brv.12550.

Nelkner J, Torres Tejerizo G, Hassa J, Lin TW, Witte J, Verwaaijen B, Winkler A, Bunk B, Sproeer C, Overmann J, Grosch R, Puehler A, Schlueter A (2019) Genetic potential of the biocontrol agent *Pseudomonas brassicacearum* (Formerly *P. trivialis*) 3Re2-7 unraveled by genome sequencing and mining, comparative genomics and transcriptomics. Genes 10. doi: 10.3390/genes10080601.

Ntushelo K, Ledwaba LK, Rauwane ME, Adebo OA, Njobeh PB (2019) The mode of action of *Bacillus* species against *Fusarium graminearum*, tools for investigation, and future prospects. Toxins 11. doi: 10.3390/toxins11100606.

Oerke EC (2006) Crop losses to pests. Journal of Agricultural Science 144: 31-43. doi: 10.1017/s0021859605005708.

Ongena M, Jacques P (2008) *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. Trends in Microbiology 16: 115-125. doi: 10.1016/j.tim.2007.12.009.

Ossowicki A, Jafra S, Garbeva P (2017) The antimicrobial volatile power of the rhizospheric isolate *Pseudomonas donghuensis* P482. PLOS ONE 12. doi: 10.1371/journal.pone.0174362.

Parret AHA, Temmerman K, De Mot R (2005) Novel lectin-like bacteriocins of biocontrol strain *Pseudomonas fluorescens* Pf-5. Applied and Environmental Microbiology 71: 5197-5207. doi: 10.1128/aem.71.9.5197-5207.2005.

Pieterse CMJ, Zamioudis C, Berendsen RL, Weller DM, Van Wees SCM, Bakker P (2014) Induced systemic resistance by beneficial microbes. Annual Review of Phytopathology, Vol 52 52: 347-375. doi: 10.1146/annurev-phyto-082712-102340.

Piquerez SJM, Harvey SE, Beynon JL, Ntoukakis V (2014) Improving crop disease resistance: lessons from research on *Arabidopsis* and tomato. Frontiers in Plant Science 5. doi: 10.3389/fpls.2014.00671.

Raaijmakers JM, de Bruijn I, Nybroe O, Ongena M (2010) Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: more than surfactants and antibiotics. FEMS Microbiology Reviews 34: 1037-1062. doi: 10.1111/j.1574-6976.2010.00221.x.

Raaijmakers JM, Mazzola M (2012) Diversity and natural functions of antibiotics produced by beneficial and plant pathogenic bacteria. Annual Review of Phytopathology, 50: 403-424. doi: 10.1146/annurev-phyto-081211-172908.

Raaijmakers JM, Vlami M, de Souza JT (2002) Antibiotic production by bacterial biocontrol agents. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology 81: 537-547. doi: 10.1023/a:1020501420831.

Romanazzi G, Gabler FM, Smilanick JL (2006) Preharvest chitosan and postharvest UV irradiation treatments suppress gray mold of table grapes. Plant Disease 90: 445-450. doi: 10.1094/pd-90-0445.

Sant DG, Tupe SG, Ramana CV, Deshpande MV (2016) Fungal cell membrane-promising drug target for antifungal therapy. Journal of Applied Microbiology 121: 1498-1510. doi: 10.1111/jam.13301.

Schirra M, D'Aquino S, Cabras P, Angioni A (2011) Control of postharvest diseases of fruit by heat and fungicides: efficacy, residue levels, and residue persistence. A Review. Journal of Agricultural and Food Chemistry 59: 8531-8542. doi: 10.1021/jf201899t.

Scholz SS, Schmidt-Heck W, Guthke R, Furch ACU, Reichelt M, Gershenzon J, Oelmueller R (2018) *Verticillium dahliae-Arabidopsis* interaction causes changes in gene expression profiles and jasmonate levels on different time scales. Frontiers in Microbiology 9. doi: 10.3389/fmicb.2018.00217.

Schulz-Bohm K, Martin-Sanchez L, Garbeva P (2017) Microbial volatiles: small molecules with an important role in infra- and inter-kingdom interactions. Frontiers in Microbiology 8. doi: 10.3389/fmicb.2017.02484.

Shafi J, Tian H, Ji M (2017) *Bacillus* species as versatile weapons for plant pathogens: a review. Biotechnology & Biotechnological Equipment 31: 446-459. doi: 10.1080/13102818.2017.1286950.

Spadaro D, Droby S (2016) Development of biocontrol products for postharvest diseases of fruit: The importance of elucidating the mechanisms of action of yeast antagonists. Trends in Food Science & Technology 47: 39-49. doi: 10.1016/j.tifs.2015.11.003.

Spanu PD, Panstruga R (2017) Editorial: Biotrophic Plant-Microbe Interactions. Frontiers in Plant Science 8. doi: 10.3389/fpls.2017.00192.

Staskawicz BJ (2001) Genetics of plant-pathogen interactions specifying plant disease resistance. Plant Physiology 125: 73-76. doi: 10.1104/pp.125.1.73.

Stefanczyk E, Sobkowiak S, Brylinska M, Sliwka J (2016) Diversity of *Fusarium* spp. associated with dry rot of potato tubers in Poland. European Journal of Plant Pathology 145: 871-884. doi: 10.1007/s10658-016-0875-0.

Stinson EE, Bills DD, Osman SF, Siciliano J, Ceponis MJ, Heisler EG (1980) Mycotoxinproduction by *Alternaria* species grown on apples, tomatoes, and blueberries. Journal of Agricultural and Food Chemistry 28: 960-963. doi: 10.1021/jf60231a040.

Sui Y, Wisniewski M, Droby S, Norelli J, Liu J (2016) Recent advances and current status of the use of heat treatments in postharvest disease management systems: Is it time to turn up the heat? Trends in Food Science & Technology 51: 34-40. doi: 10.1016/j.tifs.2016.03.004.

Taheri F, Azadi H, D'Haese M (2017) A World without Hunger: Organic or GM Crops? Sustainability 9. doi: 10.3390/su9040580.

Tu CC, Hsieh TF, Chang YC (1996) Vegetable diseases incited by *Rhizoctonia* spp. *Rhizoctonia* Species: Taxonomy, Molecular Biology, Ecology, Pathology and Disease Control: 369-377.

Usall J, Ippolito A, Sisquella M, Neri F (2016) Physical treatments to control postharvest diseases of fresh fruits and vegetables. Postharvest Biology and Technology 122: 30-40. doi: 10.1016/j.postharvbio.2016.05.002.

van Lenteren JC (2012) The state of commercial augmentative biological control: plenty of natural enemies, but a frustrating lack of uptake. Biocontrol 57: 1-20. doi: 10.1007/s10526-011-9395-1.

van Lenteren JC, Bolckmans K, Kohl J, Ravensberg WJ, Urbaneja A (2018) Biological control using invertebrates and microorganisms: plenty of new opportunities. Biocontrol 63: 39-59. doi: 10.1007/s10526-017-9801-4.

Velazquez-Becerra C, Macias-Rodriguez LI, Lopez-Bucio J, Flores-Cortez I, Santoyo G, Hernandez-Soberano C, Valencia-Cantero E (2013) The rhizobacterium *Arthrobacter agilis* produces dimethylhexadecylamine, a compound that inhibits growth of phytopathogenic fungi in vitro. Protoplasma 250: 1251-1262. doi: 10.1007/s00709-013-0506-y.

Wall MM, Biles CL (1993) *Alternaria* fruit rot of ripening chile peppers. Phytopathology 83: 324-328. doi: 10.1094/Phyto-83-324.

Wan N-F, Cai Y-M, Shen Y-J, Ji X-Y, Wu X-W, Zheng X-R, Cheng W, Li J, Jiang Y-P, Chen X, Weiner J, Jiang J-X, Nie M, Ju R-T, Yuan T, Tang J-J, Tian W-D, Zhang H, Li B (2018) Increasing plant diversity with border crops reduces insecticide use and increases crop yield in urban agriculture. eLife 7. doi: 10.7554/eLife.35103.

Wang Y, Bao Y, Shen D, Feng W, Yu T, Zhang J, Zheng XD (2008) Biocontrol of *Alternaria alternata* on cherry tomato fruit by use of marine yeast *Rhodosporidium paludigenum* Fell & Tallman. International Journal of Food Microbiology 123: 234-239. doi: 10.1016/j.ijfoodmicro.2008.02.002.

Weller DM (2007) *Pseudomonas* biocontrol agents of soilborne pathogens: Looking back over 30 years. Phytopathology 97: 250-256. doi: 10.1094/phyto-97-2-0250.

Williamson B, Tudzynsk B, Tudzynski P, van Kan JAL (2007) *Botrytis cinerea*: the cause of grey mould disease. Molecular Plant Pathology 8: 561-580. doi: 10.1111/j.1364-3703.2007.00417.x.

Wise C, Falardeau J, Hagberg I, Avis TJ (2014) Cellular lipid composition affects sensitivity of plant pathogens to fengycin, an antifungal compound produced by *Bacillus subtilis* strain CU12. Phytopathology 104: 1036-1041. doi: 10.1094/phyto-12-13-0336-r.

Wise C, Novitsky L, Tsopmo A, Avis TJ (2012) Production and antimicrobial activity of 3-hydroxypropionaldehyde from *Bacillus subtilis* strain CU12. Journal of Chemical Ecology 38: 1521-1527. doi: 10.1007/s10886-012-0219-2.

Xia XH, Huang YY, Wang L, Huang LF, Yu YL, Zhou YH, Yu JQ (2006) Pesticides-induced depression of photosynthesis was alleviated by 24-epibrassinolide pretreatment in *Cucumis sativus* L. Pesticide Biochemistry and Physiology 86: 42-48. doi: 10.1016/j.pestbp.2006.01.005.

Xue HL, Bi Y, Prusky D, Raza H, Zhang R, Zhang S, Nan MN, Zong YY, Cheng XY (2019) The mechanism of induced resistance against *Fusarium* dry rot in potato tubers by the T-2 toxin. Postharvest Biology and Technology 153: 69-78. doi: 10.1016/j.postharvbio.2019.03.021.

Yan R, Liu D, Fu Y, Zhang Y, Ju H, Zhao J, Wang X, Zhang J, Xiang W (2019) *Arthrobacter celericrescens* sp. nov., isolated from forest soil. International Journal of Systematic and Evolutionary Microbiology 69: 3109-3115. doi: 10.1099/ijsem.0.003597.

Chapter 2: Suppression of Fusarium dry rot of potato using beneficial bacterial treatments

2.1 Abstract

Fusarium dry rot affects up to 60% of potato tubers during storage. Pathogen resistance development to synthetic chemical fungicides is a main contributing factor. Previous work has demonstrated the use of bacterial antagonistic against plant pathogens. In this study, bacteria isolated from disease suppressive composts were tested to evaluate their antagonistic activity against *F. sambucinum in vitro*, as well as to assess their ability to reduce Fusarium dry rot of potato tubers. To identify the potential implication of antifungal compounds in their inhibitory activity, bacterial extracts were produced and assayed against *F. sambucinum* mycelial growth and conidial germination. *In vitro* results showed that all tested bacteria suppressed *F. sambucinum* mycelial growth over six days of incubation. When testing the bacteria against potato dry rot, 30 of 32 bacteria reduced the symptoms of the disease at the higher bacterial concentration tested. The combined *in vitro* and *in vivo* studies indicated *Bacillus subtilis* B9-8*, Pseudomonas moraviensis* F9-6, *Pseudomonas koreensis* F9-9, and *Pseudomonas gessardii* M9-16 were the most effective strains. Extracts from these four bacteria were able to inhibit mycelial growth and/or conidial germination of the fungal mold. Results from this study suggests that multiple antagonistic bacteria may be useful in controlling Fusarium dry rot on potato tubers. Extracts from the four most effective bacteria indicated that antibiosis was a main mode of action against *F. sambucinum*.

Keywords antibiosis; antifungal; *Arthrobacter*; *Bacillus*; Fusarium dry rot; *Pseudomonas*

2.2 Introduction

Food safety, quantity, and quality is highly affected by numerous plant pathogens causing economical losses. Fusarium dry rot is a pre- and post-harvest fungal infection caused by *Fusarium* spp. The disease effects various types of potatoes resulting up to 60% of tubers affected during post-harvest storage and up to 25% loss in tubers due to spoilage (Delgado et al. 2010; Theron and Holz 1991). *Fusarium sambucinum* Fuckel has been identified as one of the two most common and most pathogenic causes of potato dry rot (along with *Fusarium solani* var. *coeruleum* (Lib. ex Sacc.) C. Booth) (Bojanowski et al. 2013; Delgado et al. 2010; Estrada et al. 2010). Skin breakage, such as wounds and lesions, can occur during harvest, transport, and storage, increasing the risk of Fusarium dry rot. Fusarium dry rot symptoms include large brown cavities with wrinkled skin that can have coloured mycelial growth below the cavity (Bojanowski et al. 2013; Stefanczyk et al. 2016). More specifically, dry rot appears on potato tubers as dark brown to black lesion from the exterior and a sponge-like rot from the interior. In addition to affecting potato tuber quantity and quality, mycotoxin production that may affect the health of animals and humans negatively has been reported in *F. sambucinum* infected potato tubers (Stefanczyk et al. 2016; Xue et al. 2019).

By minimizing bruising to the tubers during harvest and storage, exposure to dry rot can be reduced (Gachango et al. 2012). During the curing process, skin wound and bruise healing conditions such as 95-99% relative humidity, pulp temperature of 13-16 °C, and good ventilation can allow for rapid healing of infection sites, thereby preventing the disease from progressing (Hay et al. 2019; Ocamb et al. 2007). However, these temperatures and humidity do not prevent and, in some cases, may improve the growth of *F. sambucinum*. Therefore, healing conditions alone are insufficient to manage the post-harvest disease (Hay et al. 2019). Other reported pre- and post-

harvest control methods shown to prevent Fusarium dry rot include organic and inorganic salts (e.g., aluminium, sodium, potassium, and sulfite-containing salts) (Kolaei et al. 2013; Kolaei et al. 2012; Mecteau et al. 2002; Mecteau et al. 2008), antimicrobial films (amylose-hexadecyl ammonium chloride with poly vinyl alcohol) (Hay et al. 2019), crop rotation (Bojanowski et al. 2013), and synthetic fungicides, such as benzimidazoles (e.g., thiabendazole) (Hide et al. 1992; Ocamb et al. 2007).

Current control methods have some drawbacks. The use of salts may impact the flavour, as well as the processing capabilities of the tuber. In addition, some species of *Fusarium*, specifically *F. sambucinum*, have developed resistance to fungicides, thereby reducing or eliminating their usefulness to control dry rot disease (Gachango et al. 2012; Hide et al. 1992). Because of the development of fungicide resistance and potential impact of fungicides on the environment and health, new alternative control methods to synthetic fungicides are of considerable interest for the development of sustainable agricultural systems. While some countries do not allow any type of post-harvest treatment, others have shown an interest in biological methods to mitigate postharvest spoilage of produce. However, the use of beneficial bacteria as antagonists of potato dry rot pathogens has been investigated sparingly. Among the few examples, inoculation of potato tuber seeds with *Pseudomonas fluorescens* Migula and *Enterobacter cloacae* (Jordan) Hormaeche and Edwards reduced the severity of lesions caused by Fusarium dry rot in a pre-harvest study (Al-Mughrabi 2010). Similarly, post-harvest studies using *P. fluorescens* (Schisler et al. 2016) and *Bacillus subtilis* (Ehrenberg) Cohn (Mnif et al. 2015) showed antagonistic properties of these bacteria against dry rot caused by *F. sambucinum* and *F. solani*, respectively.

Recently, various plant pathogen antagonistic bacteria were isolated from disease suppressive composts prepared from bovine manure, as well as marine or forestry residues (Mohamed et al. 2017). Whereas the use of suppressive composts may be implemented under field conditions, biocontrol of post-harvest disease of tubers in storage would require isolation and use of products based on inhibitory microbial strains from the composts. These bacterial isolates (mainly *Bacillus*, *Pseudomonas*, and *Arthrobacter* spp.) demonstrated reduced fungal mycelial growth and spore germination, with antibiosis i.e., the excretion of antimicrobial compounds, mentioned as a possible mode of action responsible for the observed suppressive activity (Cloutier et al. 2020; DeFilippi et al. 2018; Mohamed et al. 2017). *Bacillus* spp. and *Pseudomonas* spp. have been shown to previously produce peptide and non-peptide antimicrobial compounds (DeFilippi et al. 2018; Falardeau et al. 2013; Gross and Loper 2009). *Arthrobacter* spp. may also produce antimicrobial compounds (Igarashi et al. 2015; Velazquez-Becerra et al. 2013), however minimal information is available on this genus.

In this study, *Bacillus* spp. and related species, *Pseudomonas* spp., and *Arthrobacter* spp. isolated from disease suppressive composts were tested (i) to assess their ability to reduce mycelial growth of *F. sambucinum*, (ii) to evaluate their suppressive effects on post-harvest Fusarium dry rot on potato tubers, and (iii) to assess production of extracellular antimicrobial compounds from the most efficient bacteria.

2.3 Materials and methods

2.3.1 Microbial material

Fusarium sambucinum strain 2351 was provided by the Laboratoire de diagnostic en phytoprotection (MAPAQ, Quebec, QC). The fungus was isolated from an infected potato tuber (Saint-Paul, Quebec, Canada) and pathogenicity on potatoes was confirmed prior to starting the experiments. The fungus was maintained on potato dextrose agar (PDA, Becton Dickinson, Sparks, MD) at 20 °C in the dark.

Thirty-two bacterial antagonists were used throughout this study and are listed in Table 2-1. These bacteria were previously isolated from disease suppressive composts (Bernier-English et al. 2010; Martin-Lapierre et al. 2011) because of their antagonistic activity against different plant pathogens (Mohamed et al. 2017). More specifically, the bacteria were isolated using a bioassay-guided technique for their ability to antagonize *Verticillium dahliae* Kleb., another soil- and tuberborne pathogen causing disease in potato. The isolated bacteria include members of well-known plant pathogen antagonistic genera (e.g., *Bacillus*, *Pseudomonas*), as well as more sparsely reported genera (e.g., *Arthrobacter*, *Brevibacillus*, *Paenibacillus*, *Rummeliibacillus*). Bacterial isolates were maintained on tryptic soy agar (TSA, Becton Dickinson) at 20 °C in the dark.

Table 2-1. Antagonistic compost bacteria used in this study

Isolate	Compost source	GenBank Accession
Bacillus spp. and related Firmicutes		
Bacillus subtilis (Ehrenberg) Cohn B9-1	Bovine manure	KT382225
Bacillus subtilis B9-5	Bovine manure	KT382228
Bacillus subtilis B9-7	Bovine manure	KT382229
Bacillus subtilis B9-8	Bovine manure	KT382230
Bacillus subtilis B9-9A	Bovine manure	KT382231
Bacillus subtilis B9-14	Bovine manure	KT382233
Bacillus subtilis F9-2	Forest residues	KT382234
Bacillus subtilis F9-8	Forest residues	KT382237
Bacillus subtilis F9-12	Forest residues	KT382241
Bacillus subtilis M9-3	Marine residues	KT382246
Bacillus subtilis M9-4	Marine residues	KT382247
Bacillus subtilis M9-7	Marine residues	KT382248
Bacillus subtilis M9-9	Marine residues	KT382250
Bacillus subtilis M9-14	Marine residues	KT382251
Bacillus megaterium de Bary B9-9B	Bovine manure	KT382232
Bacillus megaterium M9-1B	Marine residues	KT382244
Bacillus badius Batchelor M9-20	Marine residues	KT382256
Paenibacillus favisporus Velázquez et al. B9-4	Bovine manure	KT382227
Brevibacillus borstelensis Shida et al. M9-18	Marine residues	KT382254
Rummeliibacillus pycnus (Nakamura et al.) Vaishampayan et al. M9-19	Marine residues	KT382255
Pseudomonas spp.		
Pseudomonas gessardii Verhille et al. B9-3	Bovine manure	KT382226
Pseudomonas gessardii M9-16	Marine residues	KT382252
Pseudomonas moraviensis Tvrzová et al. F9-6	Forest residues	KT382235
Pseudomonas moraviensis F9-11	Forest residues	KT382240
Pseudomonas arsenicoxydans Campos et al. F9-7	Forest residues	KT382236
Pseudomonas koreensis Kwon et al. F9-9	Forest residues	KT382238
Pseudomonas brenneri Baïda et al. F9-10	Forest residues	KT382239
Pseudomonas brenneri F9-13	Forest residues	KT382242
Arthrobacter spp.		
Arthrobacter humicola Kageyama et al. M9-1A	Marine residues	KT382243
Arthrobacter humicola M9-2	Marine residues	KT382245
Arthrobacter humicola M9-8	Marine residues	KT382249
Arthrobacter psychrophenolicus Margesin et al. M9-17	Marine residues	KT382253

Adapted from Mohamed et al. 2017

2.3.2 Effect of compost bacteria on mycelial growth of *F. sambucinum in vitro*

The bioassays were performed by inoculating 100 × 15 mm PDA plates with a 5-mm diameter plugs of actively growing *F. sambucinum* in the center of the plate. Two-day old bacterial cultures were used to individually inoculate the PDA plates with 1-cm streaks at the four cardinal points. The bacterial streaks were placed 3.5 cm away from the fungal plug. The control consisted of the fungal plug without the bacterial streaks. The plates were incubated at 20 °C for 5 and 6 days. After incubation elapsed, the diameter of fungal thallus was obtained as the average of two perpendicular measurements. The *in vitro* trial was performed as a randomized complete block design with three repetitions. The experiment was performed twice.

2.3.3 Effect of compost bacteria on Fusarium dry rot of potato

White potato (*Solanum tuberosum* L.) tubers cv. 'Superior' were surface sanitized by submerging in a 0.5% sodium hypochlorite solution for 10 minutes, followed by 70% ethanol for 10 minutes, and finally rinsed with sterile distilled water. The tubers were air dried for 30 minutes.

Bacterial suspensions were prepared by transferring two-day old bacteria from TSA plates to 1 mL sterile distilled water into a 1.5-mL centrifuge tubes. Using a hemocytometer, the bacterial suspensions were adjusted to a concentration of 10^8 or 10^{10} cells/mL using sterile distilled water. These concentrations were chosen as they were previously reported to provide suppressive effect by some of these bacteria strains or species in potato or other plant pathosystems (Cloutier et al. 2020; Kurniawan et al. 2018; Luo et al. 2019; Recep et al. 2009). A bacterial suspension was obtained for each compost bacterium individually. Sterile water served as the control treatment.

Prior to inoculation, a 0.5-cm diameter cork borer was used to wound the tubers creating a small hole in the surface 0.2-cm deep. Each wound was inoculated with 50 µL of the appropriate bacterial suspension and allowed to absorb for 5 minutes. A 0.5-cm diameter cork borer was then

used to obtain *F. sambucinum* plugs, taken from the margins of the fungal thallus. The fungal plugs were placed inverted into the wound, so the mycelium was in contact with the peripheral skin and the internal flesh of the tuber. Wounds containing bacterial treatments without fungal inoculation were used to determine potential adverse effect of the bacterium on the tuber.

Each tuber was individually transferred to a sanitized plastic container. A high relative humidity was maintained by using a paper towel saturated with sterile distilled water. The plastic containers were closed and allowed to incubate at 20 °C for 7 days in the dark. Following incubation, two perpendicular measurements of the surface lesion were taken. The depth of rot was measured by cutting the tuber through the middle of the surface lesion. The depth of rot was measured from each half of the tuber. The average for surface lesion and depth of rot were calculated prior to statistical analysis. The experiment was completed in triplicates as a randomized complete block design. The experiment was performed twice.

2.3.4 Antimicrobial extracts from antagonistic bacteria

2.3.4.1 Preparation of antimicrobial extracts

The most effective bacteria from the *in vitro* and *in vivo* trials were further analyzed to assess their ability to produce antimicrobial compounds. Individual bacterial strains (*B. subtilis* B9-8, *P. gessardii* M9-16, *P. koreensis* F9-9, and *P. moraviensis* F9-6) were inoculated into MOLP medium as described previously (Akpa et al. 2001; Mohamed et al. 2017). Each strain was inoculated, in triplicate, into 50 mL of sterilized MOLP broth in 250 mL Erlenmeyer flask. Inoculated samples were incubated at 30 °C and 130 rpm for 72 hours.

Incubated samples were transferred into sterile 50-mL capped conical-bottom plastic centrifuge tubes and centrifuged at 4,400 ×*g* for 30 minutes. The supernatant obtained was filtered through a disposable, sterile, 250-mL flask filter (0.45 µm). The filtered supernatants were

precipitated by acidifying the medium to pH 1.9-2.0 using a 5M solution of HCl at 4 °C overnight. The precipitated samples were centrifuged for 30 minutes at 4,400 ×*g* and 4 °C. The supernatants were discarded and the precipitated pellet was suspended in 5 mL of methanol and centrifuged for an additional 30 minutes at 4,400 ×*g* and 4 °C. The supernatants were recovered and stored at 4 °C until use. These 10× concentrated extracts were used in bioassays to determine their inhibitory activity on mycelial growth and conidial germination of *F. sambucinum*.

2.3.4.2 Antifungal activity of extracts on mycelial growth of *F. sambucinum*

The antifungal activity was assessed using a disk diffusion bioassay. Briefly, 25 µL of each extract was individually placed on sterilized filter paper disks (0.5 cm diameter) and left to dry for 1 hour. Methanol was used as the control. Petri dishes containing PDA were inoculated with a 5-mm diameter agar plug containing actively growing *F. sambucinum*. The dried paper disks containing the extracts or control were placed at a distance of 3.5 cm from the fungal plug. Petri dishes were incubated at 20 °C in the dark for 5 days.

Following incubation, mycelial growth was measured as the radius of the fungal thallus facing the paper disk containing treatments. The experiment was conducted according to a completely randomized design with three replicates.

2.3.4.3 Antifungal activity of extracts on conidial germination of *F. sambucinum*

The antifungal activity was determined using a conidial germination bioassay. Each extract (25 µL) was placed in a sterile 1.5 mL conical snap-cap tube, the solvent was evaporated under a stream of nitrogen, and resuspended in 90 µL of sterile water. 90 µL of sterile water without extracts served as the control. Conidia were obtained by aspiration following flooding of *F. sambucinum* mycelium with sterile water. Using a hemocytometer, concentration was adjusted to 5×10^5 conidia/mL with sterile distilled water. Ten µL of suspension was added to each treatment.

The conidia were incubated at 20 °C for 30 minutes and 100 µL of the suspension was spread on water agar (containing 15 g/L agar). Petri dishes were incubated for 48 hours at 20 °C in the dark.

Following the incubation period, dishes were observed under visible light using an inverted microscope. Conidia were considered germinated when the germ tube length was at least equal to the length of the conidium. The percentage of germinated cells was calculated as follows: percent germination = germinated conidia/total conidia × 100. At least 25 conidia were counted for each treatment. The experiment was performed according to a completely randomized design with three replicates.

2.3.5 Statistical analysis

Analysis of variance (ANOVA) was completed separately for *in vitro*, *in vivo*, and antimicrobial extract experiments. In repeated experiments, when analysis showed no significant difference between two experiments, the data were combined and analysed as a single experiment. When the ANOVA was significant ($P \leq 0.05$), means were separated using Fisher's Least Significant Difference (LSD) test (α-level=0.05). All statistical analysis was performed using SAS software version 9.4 (SAS Institute, Cary, NC).

2.4 Results

2.4.1 Effect of compost bacteria on mycelial growth of *F. sambucinum in vitro*

Compost bacteria were generally able to inhibit *F. sambucinum* growth. Following 5 days of incubation, 31 of the 32 bacteria significantly inhibited the growth of the fungus (Fig. 2-1). Only *B. megaterium* B9-9B was unable to inhibit mycelial growth. Among the inhibitory bacteria, a group composed of *B. subtilis* B9-1, B9-5, B9-7, B9-8, B9-9A, F9-2, F9-8, M9-3, M9-9, and M9-14, *P. moraviensis* F9-6 and F9-11, *P. koreensis* F9-9, *A. humicola* M9-1A, *P. gessardii* M9-16, *B. borstelensis* M9-18, and *B. badius* M9-20 was the most inhibitory, reducing mycelial growth by an average of 32% (Fig. 2-1). After 6 days of incubation, all bacteria significantly inhibited *F. sambucinum* growth (Fig. 2-2). A group composed of *B. subtilis* B9-1, B9-5, and B9-8, *P. moraviensis* F9-6, *P. koreensis* F9-9, and *P. gessardii* M9-16 revealed the highest activity, inhibiting the growth of *F. sambucinum* by 39% (Fig. 2-2).

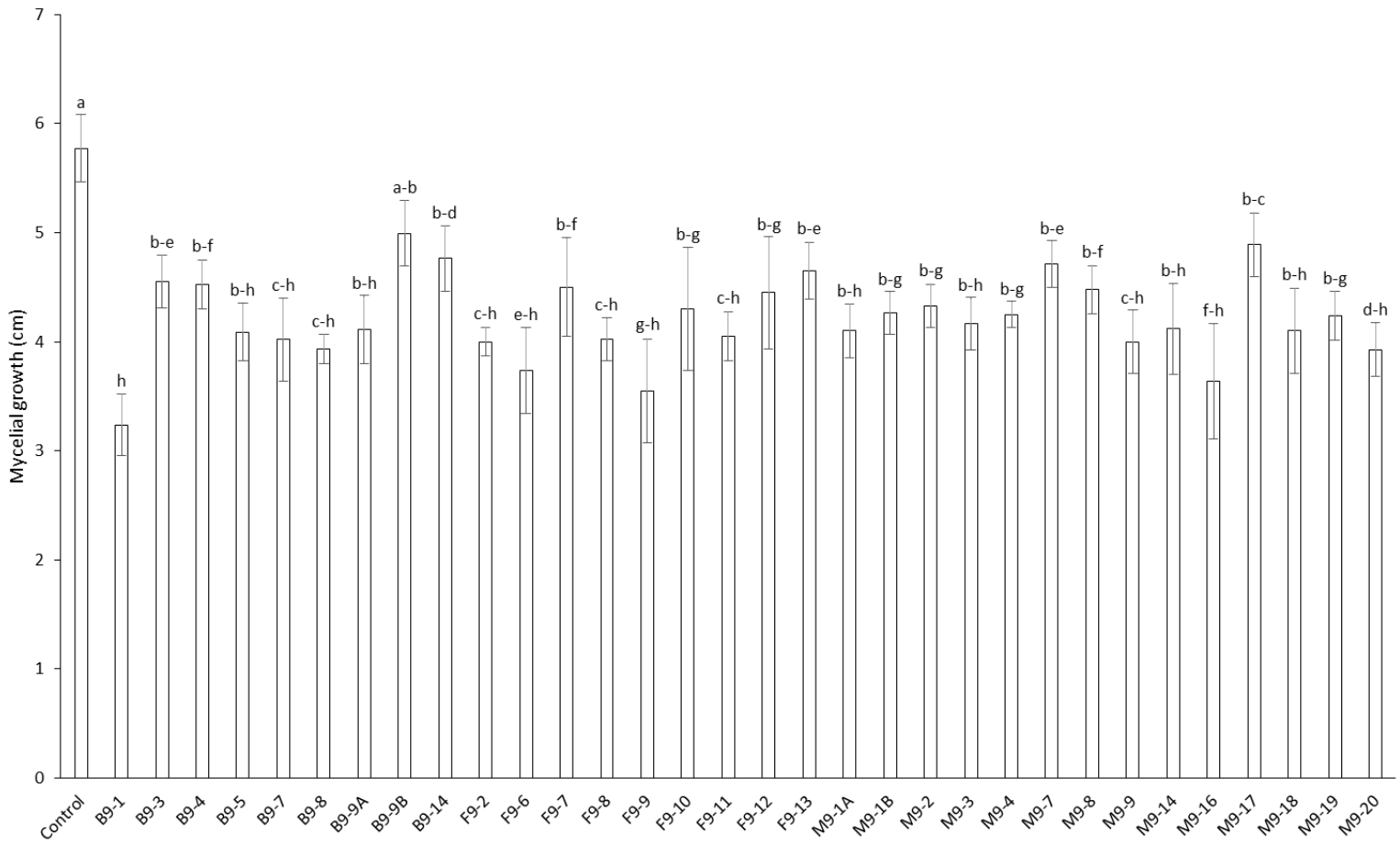


Figure 2-1. Effect of bacterial antagonists on mycelial growth of *Fusarium sambucinum* following a 5 days incubation. B9-1, B9-5, B9-7, B9-8, B9-9A, B9-14, F9-2, F9-8, F9-12, M9-3, M9-4, M9-7, M9-9 and M9-14 = *Bacillus subtilis*; B9-9B and M9-1B = *Bacillus megaterium*; M9-20 = *Bacillus badius*; B9-4 = *Paenibacillus favisporus*; M9-18 = *Brevibacillus borstelensis* M9-19 = *Rummeliibacillus pycnus*; B9-3 and M9-16 = *Pseudomonas gessardii*; F9-6 and F9-11 = *Pseudomonas moraviensis*; F9-7 = *Pseudomonas arsenicoxydans*, F9-9 = *Pseudomonas koreensis*; F9-10 and F9-13 = *Pseudomonas brenneri*; M9-1A, M9-2 and M9-8 = *Arthrobacter humicola*; M9-17 = *Arthrobacter psychrophenolicus*. Bars sharing a same letter are not significantly different according to Fisher's protected LSD test (α level = 0.05). Error bars are standard errors.

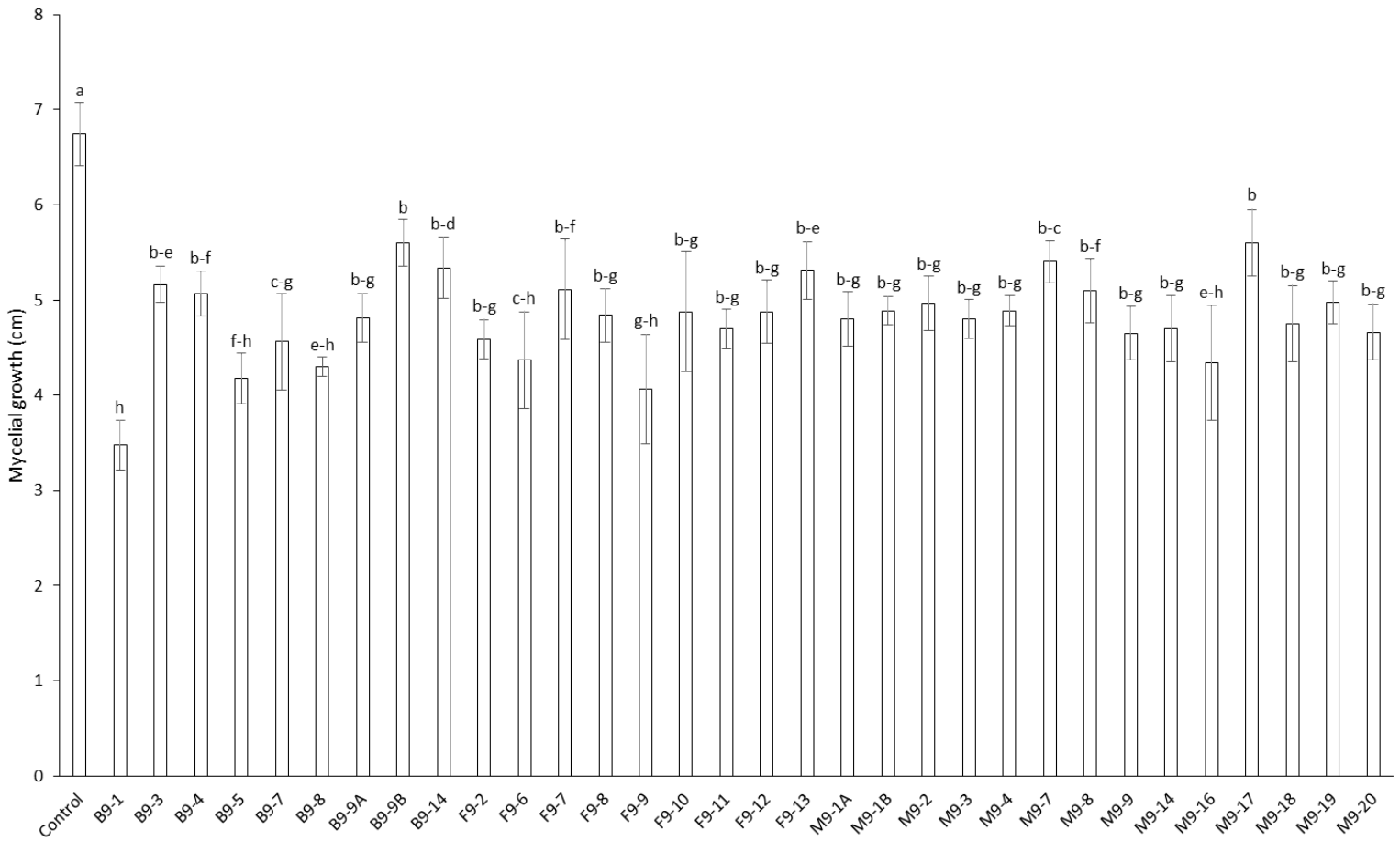


Figure 2-2. Effect of bacterial antagonists on mycelial growth of *Fusarium sambucinum* following a 6 days incubation. B9-1, B9-5, B9-7, B9-8, B9-9A, B9-14, F9-2, F9-8, F9-12, M9-3, M9-4, M9-7, M9-9 and M9-14 = *Bacillus subtilis*; B9-9B and M9-1B = *Bacillus megaterium*; M9-20 = *Bacillus badius*; B9-4 = *Paenibacillus favisporus*; M9-18 = *Brevibacillus borstelensis* M9-19 = *Rummeliibacillus pycnus*; B9-3 and M9-16 = *Pseudomonas gessardii*; F9-6 and F9-11 = *Pseudomonas moraviensis*; F9-7 = *Pseudomonas arsenicoxydans*, F9-9 = *Pseudomonas koreensis*; F9-10 and F9-13 = *Pseudomonas brenneri*; M9-1A, M9-2 and M9-8 = *Arthrobacter humicola*; M9-17 = *Arthrobacter psychrophenolicus*. Bars sharing a same letter are not significantly different according to Fisher's protected LSD test (α level = 0.05). Error bars are standard errors.

When comparing results from both time periods (Table 2-2), it was found that *B. subtilis* B9-7, B9-14, F9-2, F9-8, M9-3, M9-9, and M9-14, *B. megaterium* B9-9A, *P. moraviensis* F9-11, *A. humicola* M9-1A, *B. borstelensis* M9-18, and *B. badius* M9-20, while still inhibitory, were no longer among the most inhibitory of the tested strains following the longer incubation period. By contrast, *B. subtilis* B9-1, B9-5, B9-8, *P. moraviensis* F9-6, *P. koreensis* F9-9, and *P. gessardii* M9-16 where the most inhibitory of the bacterial treatments at both tested time periods (Table 2-2).

Table 2-2. Summary of antagonistic effects of bacteria on mycelial growth of *Fusarium sambucinum* and lesion suppression of Fusarium dry rot of potato

	Strain	GI-5	HGI-5	GI-6	HGI-6	SSL-8	HSSL-8	SSL-10	HSSL-10	SIL-10	HSIL-10
Bacillus subtilis	B9-1	×	∗	×	∗			×		×	
P. gessardii	B9-3	×		×		×		×		×	∗
P. favisporus	B9-4	×		×		×	∗	×	∗	×	∗
B. subtilis	B9-5	×	∗	×	∗	×		×		×	
B. subtilis	B9-7	×	∗	×		×	∗	×	∗	×	∗
B. subtilis	B9-8	×	∗	×	∗	×	∗	×	∗	×	∗
B. subtilis	B9-9A	×	∗	×		×	∗	×		×	
B. megaterium	B9-9B			×		×		×	∗	×	∗
B. subtilis	B9-14	×		×		×	∗	×		×	∗
B. subtilis	F9-2	×	∗	×		×	∗			×	∗
P. moraviensis	F9-6	×	∗	×	∗	×	∗	×	∗	×	∗
P. arsenicoxydans	F9-7	×		×		×	∗	×	∗	×	∗
B. subtilis	F9-8	×	∗	×				×	∗	×	
P. koreensis	F9-9	×	∗	×	∗	×	∗	×	∗	×	∗
P. brenneri	F9-10	×		×		×	∗	×		×	∗
P. moraviensis	F9-11	×	∗	×		×		×	∗	×	∗
B. subtilis	F9-12	×		×		×	∗	×	∗	×	∗
P. brenneri	F9-13	×		×		×	∗	×	∗	×	
A. humicola	M9-1A	×	∗	×		×	∗	×	∗	×	∗
B. megaterium	M9-1B	×		×		×		×		×	
A. humicola	M9-2	×		×		×		×	∗	×	∗
B. subtilis	M9-3	×	∗	×		×	∗	×		×	∗
B. subtilis	M9-4	×		×		×	∗	×	∗	×	∗
B. subtilis	M9-7	×		×						×	∗
A. humicola	M9-8	×		×		×	∗	×		×	∗
B. subtilis	M9-9	×	∗	×		×		×	∗	×	∗
B. subtilis	M9-14	×	∗	×		×	∗	×		×	
P. gessardii	M9-16	×	∗	×	∗	×	∗	×	∗	×	∗
A. psychrophenolicus	M9-17	×		×		×	∗	×		×	
B. borstelensis	M9-18	×	∗	×		×		×		×	∗
R. pycnus	M9-19	×		×		×		×		×	
B. badius	M9-20	×	∗	×		×	∗	×	∗	×	∗

Legend: × = statistically significant when compared to the control; ∗ = highest inhibitory or suppressive effect among treatments

GI-5 = Growth inhibition - Day 5; HGI-5 = Highest growth inhibition - Day 5; GI-6 = Growth inhibition - Day 6; HGI-6 = Highest growth inhibition - Day 6; SSL-8 = Suppression of surface lesions (10^8 cell/ml); HSSL-8 = Highest suppression of surface lesions (10^8 cells/ml); SSL-10 = Suppression of surface lesions (10^{10} cells/ml); HSSL-10 = Highest suppression of surface lesions (10^{10} cells/ml); SIL-10 = Suppression of internal lesions (10^{10} cells/ml); HSIL-10 = Highest suppression of internal lesions (10^{10} cells/ml)

2.4.2 Effect of compost bacteria on Fusarium dry rot on potato

Bacteria treatments alone did not affect the potato tuber. More specifically, there were no phytotoxic or other adverse effects, such as visible bacterial growth, offensive odours or other spoilage symptoms. The suppressive activity against dry rot was dependent on bacterial concentration. At the lower of the tested concentrations (10^8 cell/mL), there was no significant effect on suppression of the depth of dry rot lesions by the bacteria (Fig. 2-3). However, 29 of the 32 bacteria reduced dry rot lesions at the surface of the tuber (Fig. 2-4). Only, *B. subtilis* B9-1, F9-8, and M9-7 did not significantly reduce surface dry rot. Among the inhibitory bacteria, *P. favisporus* B9-4, *B. subtilis* B9-7, B9-8, B9-9A, B9-14, F9-2, F9-12, M9-3, M9-4, and M9-14, *P. arsenicoxydans* F9-7, *P. koreensis* F9-9, *P. brenneri* F9-10 and F9-13, *A. humicola* M9-1A and M9-8, *P. gessardii* M9-16, *A. psychrophenolicus* M9-17, and *B. badius* M9-20 were the most efficient, reducing surface dry rot by 26% (Fig. 2-4).

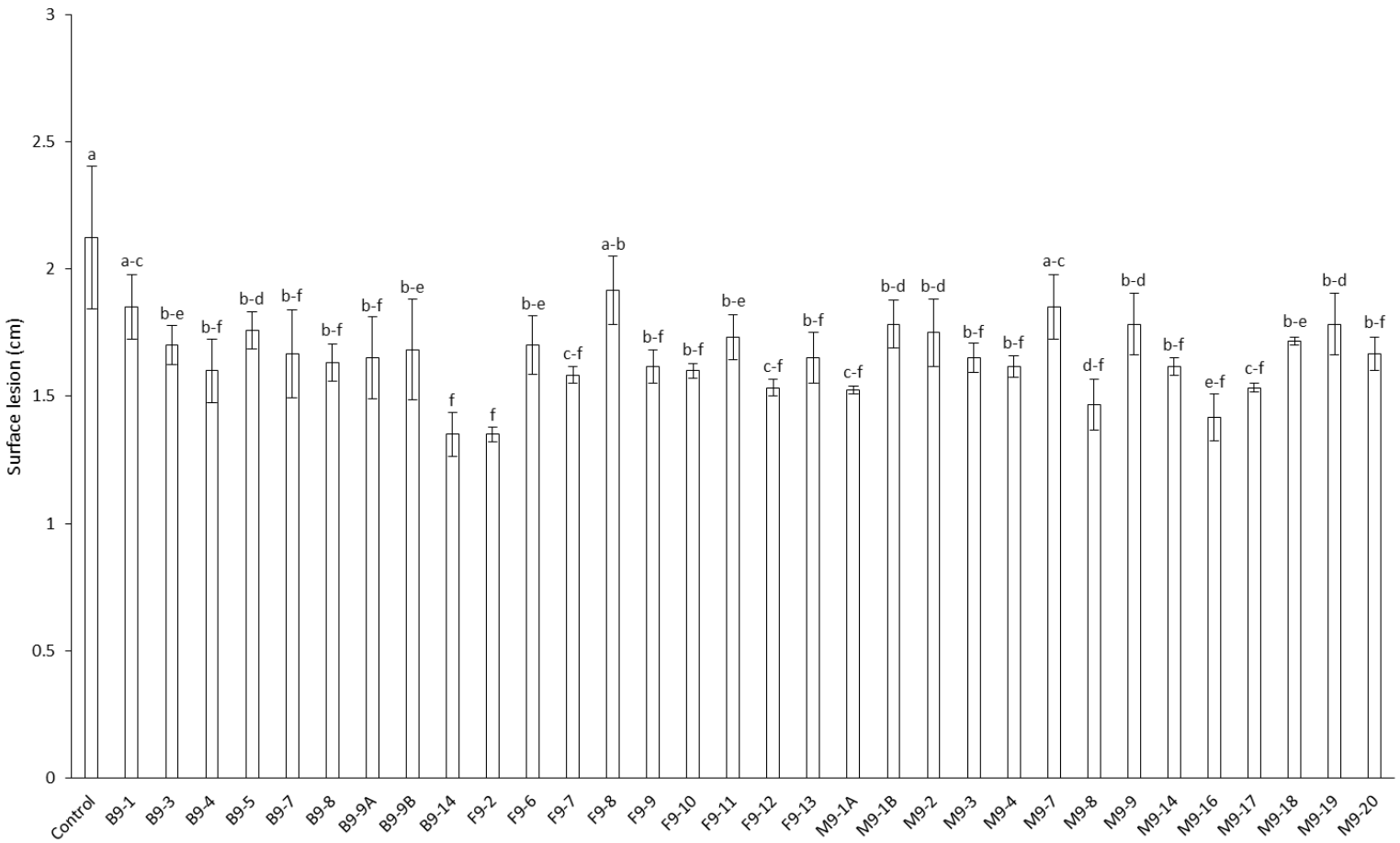


Figure 2-3. Effect of a lower concentration (10^8 cells/mL) of bacterial antagonists on surface lesions of potato dry rot. B9-1, B9-5, B9-7, B9-8, B9-9A, B9-14, F9-2, F9-8, F9-12, M9-3, M9-4, M9-7, M9-9 and M9-14 = *Bacillus subtilis*; B9-9B and M9-1B = *Bacillus megaterium*; M9-20 = *Bacillus badius*; B9-4 = *Paenibacillus favisporus*; M9-18 = *Brevibacillus borstelensis* M9-19 = *Rummeliibacillus pycnus*; B9-3 and M9-16 = *Pseudomonas gessardii*; F9-6 and F9-11 = *Pseudomonas moraviensis*; F9-7 = *Pseudomonas arsenicoxydans*, F9-9 = *Pseudomonas koreensis*; F9-10 and F9-13 = *Pseudomonas brenneri*; M9-1A, M9-2 and M9-8 = *Arthrobacter humicola*; M9-17 = *Arthrobacter psychrophenolicus*. Bars sharing a same letter are not significantly different according to Fisher's protected LSD test (α level = 0.05). Error bars are standard errors.

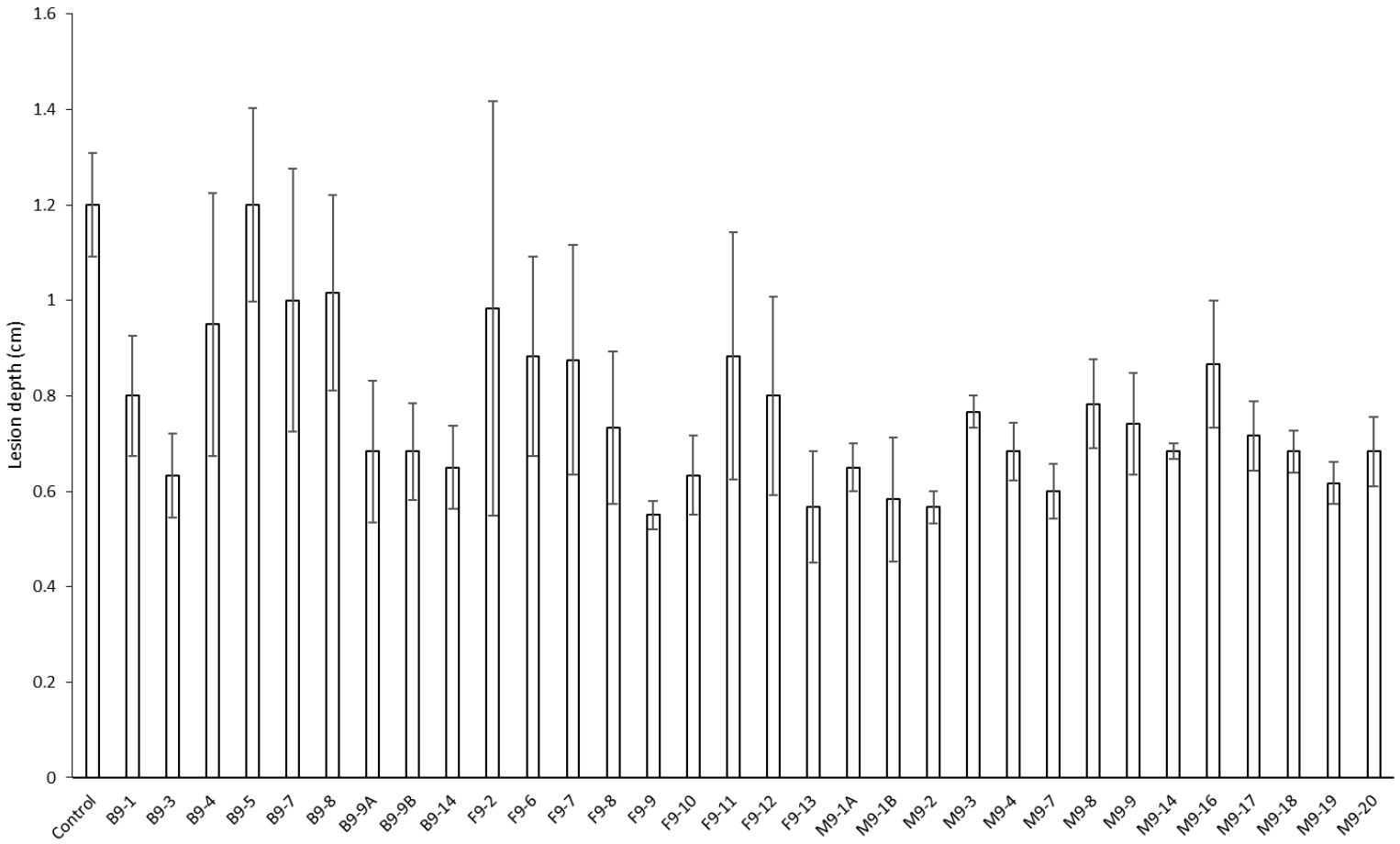


Figure 2-4. Effect of a lower concentration (10^8 cells/mL) of bacterial antagonists on internal lesions of potato dry rot. B9-1, B9-5, B9-7, B9-8, B9-9A, B9-14, F9-2, F9-8, F9-12, M9-3, M9-4, M9-7, M9-9 and M9-14 = *Bacillus subtilis*; B9-9B and M9-1B = *Bacillus megaterium*; M9-20 = *Bacillus badius*; B9-4 = *Paenibacillus favisporus*; M9-18 = *Brevibacillus borstelensis* M9-19 = *Rummeliibacillus pycnus*; B9-3 and M9-16 = *Pseudomonas gessardii*; F9-6 and F9-11 = *Pseudomonas moraviensis*; F9-7 = *Pseudomonas arsenicoxydans*, F9-9 = *Pseudomonas koreensis*; F9-10 and F9-13 = *Pseudomonas brenneri*; M9-1A, M9-2 and M9-8 = *Arthrobacter humicola*; M9-17 = *Arthrobacter psychrophenolicus*. Result were not significantly different according to the ANOVA ($P > 0.05$). Error bars are standard errors.

With the higher bacterial concentration (10^{10} cells/mL), all but *B. subtilis* F9-2 and M9-7 significantly reduced surface dry rot (Fig. 2-5). The most inhibitory of the bacteria were a group comprised of *P. favisporus* B9-4, *B. subtilis* B9-7, B9-8, F9-8, F9-12, M9-4, and M9-9, *B. megaterium* B9-9B, *P. moraviensis* F9-6 and F9-11, *P. arsenicoxydans* F9-7, *P. koreensis* F9-9, *P. brenneri* F9-13, *A. humicola* M9-1A, and M9-2, *P. gessardii* M9-16, and *B. badius* M9-20, which reduced surface dry rot by an average of 27% (Fig. 2-5). Contrary to the lower dose tested, all bacterial antagonists reduced depth of dry rot into the interior of the tuber (Fig. 6). More precisely, *P. gessardii* B9-3 and M9-16, *P. favisporus* B9-4, *B. subtilis* B9-7, B9-8, B9-14, F9-2, F9-12, M9-3, M9-4, M9-7, and M9-9, *B. megaterium* B9-9B, *P. moraviensis* F9-6 and F9-11, *P. arsenicoxydans* F9-7, *P. koreensis* F9-9, *P. brenneri* F9-10, *A. humicola* M9-1A, M9-2, and M9-8, *A. psychrophenolicus* M9-17, and *B. badius* M9-20 were the most suppressive, reducing dry rot depth by an average of 52% (Fig. 2-6).

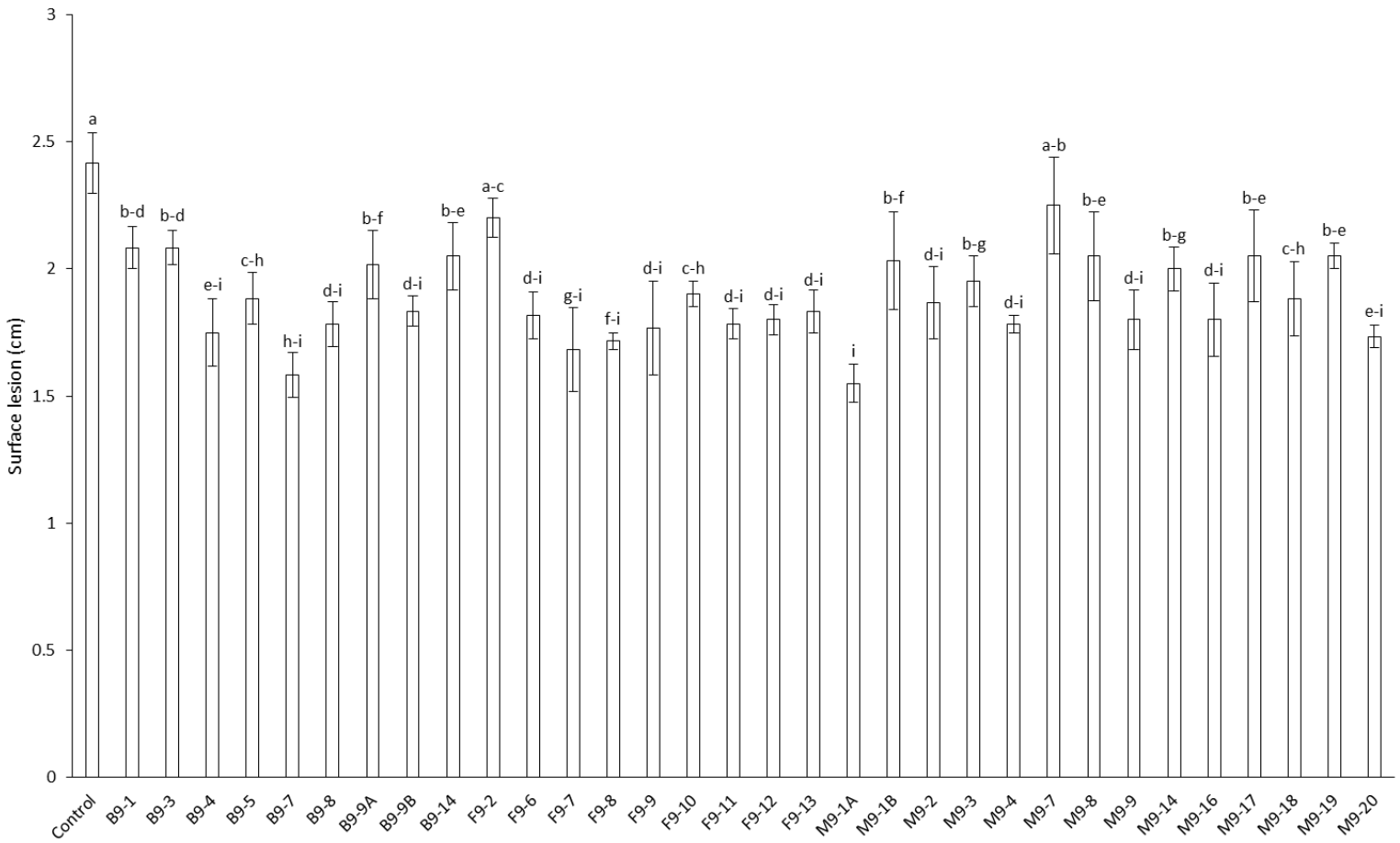


Figure 2-5. Effect of a higher concentration (10^{10} cells/mL) of bacterial antagonists on surface lesions of potato dry rot. B9-1, B9-5, B9-7, B9-8, B9-9A, B9-14, F9-2, F9-8, F9-12, M9-3, M9-4, M9-7, M9-9 and M9-14 = *Bacillus subtilis*; B9-9B and M9-1B = *Bacillus megaterium*; M9-20 = *Bacillus badius*; B9-4 = *Paenibacillus favisporus*; M9-18 = *Brevibacillus borstelensis* M9-19 = *Rummeliibacillus pycnus*; B9-3 and M9-16 = *Pseudomonas gessardii*; F9-6 and F9-11 = *Pseudomonas moraviensis*; F9-7 = *Pseudomonas arsenicoxydans*, F9-9 = *Pseudomonas koreensis*; F9-10 and F9-13 = *Pseudomonas brenneri*; M9-1A, M9-2 and M9-8 = *Arthrobacter humicola*; M9-17 = *Arthrobacter psychrophenolicus*. Bars sharing a same letter are not significantly different according to Fisher's protected LSD test (α level = 0.05). Error bars are standard errors.

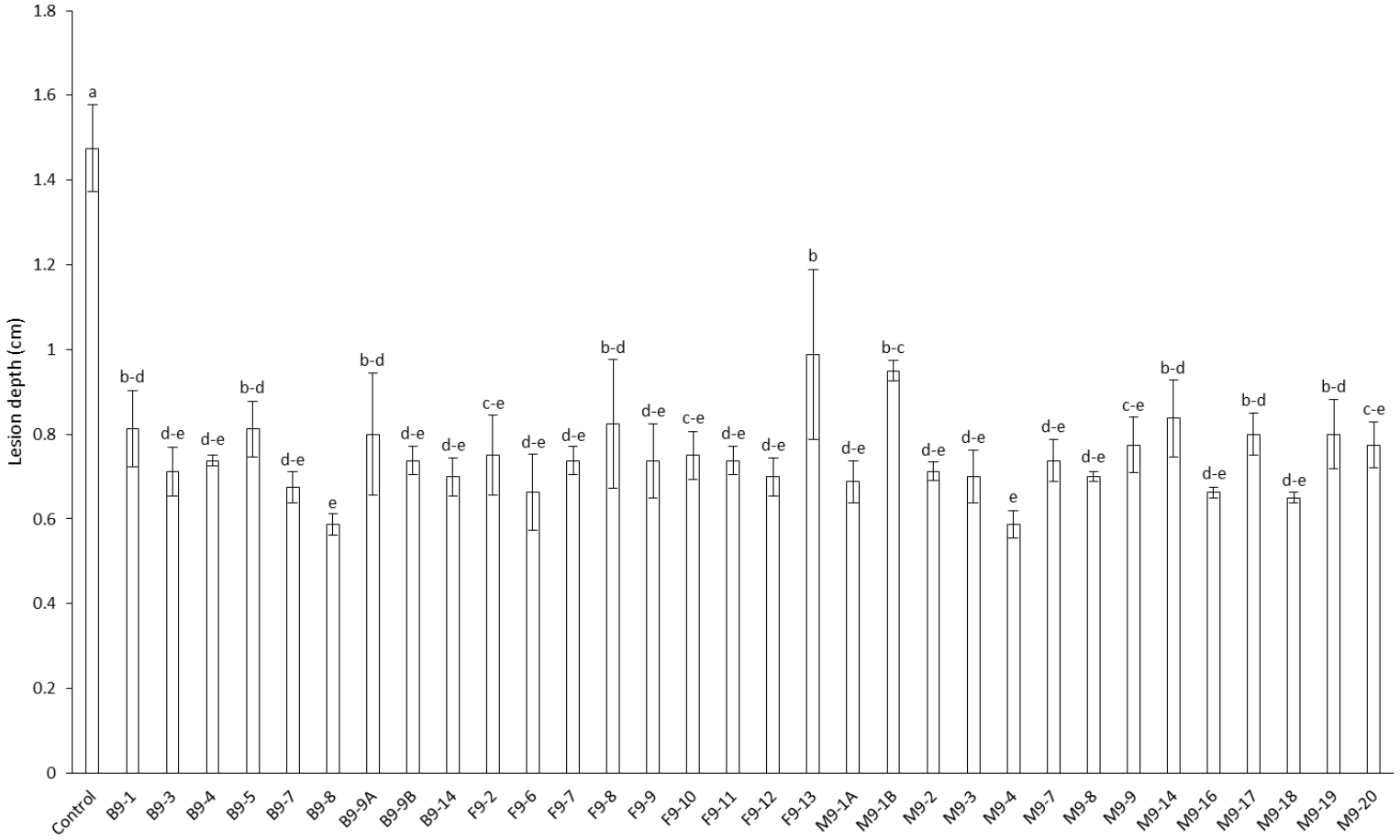


Figure 2-6. Effect of a higher concentration (10^{10} cells/mL) of bacterial antagonists on internal lesions of potato dry rot. B9-1, B9-5, B9-7, B9-8, B9-9A, B9-14, F9-2, F9-8, F9-12, M9-3, M9-4, M9-7, M9-9 and M9-14 = *Bacillus subtilis*; B9-9B and M9-1B = *Bacillus megaterium*; M9-20 = *Bacillus badius*; B9-4 = *Paenibacillus favisporus*; M9-18 = *Brevibacillus borstelensis* M9-19 = *Rummeliibacillus pycnus*; B9-3 and M9-16 = *Pseudomonas gessardii*; F9-6 and F9-11 = *Pseudomonas moraviensis*; F9-7 = *Pseudomonas arsenicoxydans*, F9-9 = *Pseudomonas koreensis*; F9-10 and F9-13 = *Pseudomonas brenneri*; M9-1A, M9-2 and M9-8 = *Arthrobacter humicola*; M9-17 = *Arthrobacter psychrophenolicus*. Bars sharing a same letter are not significantly different according to Fisher's protected LSD test (α level = 0.05). Error bars are standard errors.

When comparing efficacy of the bacteria at both concentrations (Table 2-2), *B. subtilis* B9-1 and F9-8 were found to suppression surface dry rot only at the higher concentration. The most effective bacterial treatments also varied depending on the concentration tested. Generally, bacteria that were the most effective in inhibiting surface dry rot at the lowest concentration were also the most effective against surface and internal dry rot at the highest concentration. This was the case for *P. favisporus* B9-4, *B. subtilis* B9-7, B9-8, F9-12, and M9-4, *P. arsenicoxydans* F9-7, *P. koreensis* F9-9, *A. humicola* M9-1A, *P. gessardii* M9-16, and *B. badius* M9-20 (Table 2-2). Conversely, three strains (*B. subtilis* B9-9A and M9-14, and *A. psychrophenolicus* M9-17) were the most suppressive at low concentration, but were not among the most surface and internal dry rot suppressive bacteria at the higher concentration. Finally, *B. megaterium* B9-9A, *P. moraviensis* F9-6 and F9-11, *A. humicola* M9-2, and *B. subtilis* M9-9 were shown to be among the most suppressive bacteria against surface and internal dry rot when their concentration was increased from 10^8 to 10^{10} cells/mL (Table 2-2).

2.4.3 Antifungal activity of extracts on mycelium and conidia of *F. sambucinum*

The four bacteria that were the most effective in inhibiting *F. sambucinum in vitro* and in suppressing dry rot *in vivo* (*P. moraviensis* F9-6, *P. koreensis* F9-9, *P. gessardii* M9-16, and *B. subtilis* B9-8; Table 2-2) were tested for their ability to produce antimicrobial compounds. In disk diffusion assays, extracts from *P. gessardii* M9-16, *P. moraviensis* F9-6, and *P. koreensis* F9-9 inhibited mycelial growth of *F. sambucinum* relative to the control (Table 3). *Bacillus subtilis* B9-8 extract did not exhibit inhibition. More precisely, extracts from *P. moraviensis* F9-6 and *P. koreensis* F9-9 were the most inhibitory, reducing mycelial growth by 18% (Table 2-3).

In conidial germination assays, extracts from *P. moraviensis* F9-6, *P. koreensis* F9-9, and *B. subtilis* B9-8 significantly reduced *F. sambucinum* germination (Table 2-3). *Pseudomonas gessardii* M9-16 did not inhibit fungal germination. Extracts from *P. koreensis* F9-9 and *B. subtilis* B9-8 were the most inhibitory, reducing conidial germination by 60% (Table 2-3).

Table 2-3. Effect of bacterial extracts on the mycelial growth and conidial germination of *Fusarium sambucinum*

Treatment	Mycelium (cm)	Germination (%)
Control	3.5 a	86 a
Bacillus subtilis B9-8	3.2 ab	34 c
Pseudomonas gessardii M9-16	3.1 bc	76 ab
Pseudomonas koreensis F9-9	2.7 d	35 c
Pseudomonas moraviensis F9-6	2.9 cd	65 b

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

2.5 Discussion

Bacteria from *Bacillus* spp. and related genera, as well as *Pseudomonas* spp. and *Arthrobacter* spp. have previously shown antimicrobial properties against phytopathogens (Mohamed et al. 2017). This study investigated the antagonistic properties of bacterial isolates from disease suppressive composts against *F. sambucinum*, their suppressive effects against Fusarium dry rot on potato tubers, and the inhibitory effects of extracellular antimicrobial compounds produced by the most efficient bacterial isolates.

This work demonstrated that all 32 of the tested bacterial isolates were able to inhibit the mycelial growth of *F. sambucinum* (Table 2-2). Specifically, all four *Arthrobacter* spp. showed antagonistic effects toward the fungus at both incubation periods. Previously, *A. humicola* and *A. psychrophenolicus* had demonstrated antagonistic effects against *F. sambucinum in vitro* (Mohamed et al. 2017). A previous study also showed that *Arthrobacter methylotrophus* inhibited the growth of *Fusarium graminearum* (Verma et al. 2015).

Similar to the *Arthrobacter* spp. results, all *Pseudomonas* spp. inhibited *F. sambucinum in vitro* at days 5 and 6. This agrees with other results, where various *Pseudomonas* spp. have shown antagonistic properties against *Fusarium* spp. Specifically, *P. fluorescens* showed strong inhibition of *Fusarium solani* f.sp. *phaseoli* (Kalantari et al. 2018). Additionally, *Pseudomonas putida* has been reported to reduce the growth of *Fusarium* species such as *Fusarium culmorum*, *Fusarium oxysporum*, and *F. sambucinum* (Kotan et al. 2011). *Pseudomonas brenneri*, *P. gessardii* and *P. moraviensis* had also been shown to reduce growth of *F. sambucinum* (Mohamed et al. 2017).

The *in vitro* study determined that *Bacillus* spp. as well as related bacteria (*P. favisporus*, *B. borstelensis*, *R. pycnus*) inhibited the mycelial growth of *F. sambucinum* at both incubation periods, with the exception of *B. megaterium* B9-9B, which only inhibited growth after 6 days of

incubation. To our knowledge, this is the first report indicating the antagonistic effects of *P. favisporus* and *B. badius* against *F. sambucinum*. Previous work had shown that *B. subtilis, B. megaterium*, *B. borstelensis*, and *R. pycnus* were able to reduce the mycelial growth of *F. sambucinum* (Kotan et al. 2011; Mohamed et al. 2017; Wise et al. 2012). Additionally, multiple studies indicated antagonistic effects of *B. subtilis* by reducing mycelial growth of various *Fusarium* spp. including *F. oxysporum*, *F. culmorum* and *F. solani* (Kalantari et al. 2018; Mohamed et al. 2017; Wise et al. 2012). Another *Bacillus* sp. (tentatively identified as *Bacillus amyloliquefaciens*) showed inhibition of mycelial growth of *F. solani* (Baez-Vallejo et al. 2020).

To determine the suppressive effects of the bacteria against the disease caused by *F. sambucinum*, each bacterial isolate was tested for its reduction of potato dry rot at two concentrations. The results indicated that at the lower bacterial concentration (10^8 cells/mL), the depth of the dry rot lesions were not significantly different than the control. However, all bacterial isolates except for three *B. subtilis* (B9-1, F9-8, and M9-7) were able to reduce the surface lesion. This suggests that at this concentration the bacteria could not sufficiently prevent the advance of the fungus in the potato flesh to reduce the lesion depth, but could reduce the surface lesions near the skin surface of the tuber. This result may be due to the combination of bacterial activity with the known suppressive effect of the potato tuber periderm (Bojanowski et al. 2013; Secor and Gudmestad 1999). Previous work has indicated that potato tubers are able to slow infections caused by *F. sambucinum* by defenses such as pre-existing suberin structures in the periderm (Chaves et al. 2009; Lulai and Corsini 1998). Therefore, a lower concentration of bacteria may aid in preventing expansion of surface lesions, but may not be sufficient to stop penetration into the tuber, if the skin wounds are sufficiently deep to give the fungus access to the underlying flesh such as in our trial.

At the higher bacterial concentration (10^{10} cells/mL), it was shown that all bacterial isolates reduced depth of dry rot penetration into the potato flesh. Moreover, all bacteria reduced surface lesions, except for *B. subtilis* F9-2 and M9-7. For *B. subtilis* B9-1, this indicated that a higher concentration was needed to suppress surface lesions caused by Fusarium dry rot, as the lower concentration failed to provide control (Table 2-2). In addition, this suggest that *B. subtilis* M9-7 would require a concentration higher than 10^{10} cells/mL to reduce symptoms of Fusarium dry rot or that the bacterium is simply not effective in controlling the disease (Table 2-2).

Similar to the strains tested in our study, other *B. subtilis* strains were shown to have comparable, yet variable suppressive effects against Fusarium dry rot (23-82% suppression) on refrigerated potato seeds or tubers (Recep et al. 2009; Wharton and Kirk 2014), as well as other *Fusarium* diseases (Lecomte et al. 2016; Raza et al. 2017). Similar to our results with *B. badius*, previous work also showed that other non-*subtilis Bacillus* spp., such as *B. amyloliquefaciens*, *Bacillus cereus*, *Bacillus pumilus*, and *Bacillus thuringiensis*, could reduce potato dry rot caused by *Fusarium* spp. (Cherif et al. 2002; Recep et al. 2009) by 18-50%. *Pseudomonas gessardii* M9-16, *P. koreensis* F9-9, *P. moraviensis* F9-6, *P. arsenicoxydans* F9-7, and *A. humicola* M9-1A were also among the most inhibitory bacteria to potato dry rot. Within the *Pseudomonas* genus, previous studies have indicated suppressive abilities of *P. fluorescens* (Al-Mughrabi 2010; Vatankhah et al. 2019) and *P. putida* (Recep et al. 2009) against Fusarium dry rot on potato tubers and potato seeds by 23-47%. To our knowledge, this is the first report of *Arthrobacter* spp. showing suppressive effects toward Fusarium dry rot.

By testing the bacteria in the presence of actively growing *F. sambucinum*, it is possible to visualize putative diffusible antimicrobial compounds produced by the bacteria that suppress fungal growth. Our *in vitro* results indicated that by day 6, each bacterium was able to reduce the

growth of *F. sambucinum*, indicating antibiosis as a potential mode of action. Competition for nutrients might also explain our observed results (Dukare et al. 2019). In contrast, some bacteria (e.g., *P. favisporus* B9-4, *B. subtilis* strains B9-14, F9-12, and M9-4, as well as *P. arsenicoxydans* F9-7) were grouped as some of the most inhibitory against potato dry rot, but reduced mycelial growth to a lesser extent (Table 2-2). This may indicate that other mechanisms of suppression are at work, such as induction of potato defense mechanisms (Kumar et al. 2020; Zhang et al. 2008) or that these bacteria outcompete fungi for space and/or nutrients on the potato tuber (Dukare et al. 2019).

Overall, our results showed that disease suppressive bacteria mainly provided a moderate and generalistic antagonistic effect against Fusarium potato dry. However, some strains provided higher efficacy than others. Indeed, our results also demonstrated that four bacterial isolates, *B. subtilis* B9-8, *P. gessardii* M9-16, *P. koreensis* F9-9, and *P. moraviensis* F9-6 were the most effective in reducing *F. sambucinum* growth, as well as the most suppressive toward potato dry rot (Table 2-2). In order to determine if the observed antagonistic effects were a result of antimicrobial compound production, extracts from these four isolates were tested in disk diffusion and conidial germination assays. Our results showed that bacterial extracts reduced mycelial growth (*P. gessardii* M9-16), inhibited conidial germination (*B. subtilis* B9-8) or both (*P. moraviensis* F9-6 and *P. koreensis* F9-9), thereby indicating the involvement of extracellular antimicrobial compounds in the antagonistic activity of these bacteria. Although all strains produced antimicrobial extracts, *B. subtilis* B9-8 and *P. gessardii* M9-16 were unable to inhibit mycelial growth and conidial germination, respectively. This may be due to the insufficient or variable production of antimicrobials under certain conditions. For example, previous work indicated that the production of antimicrobial compounds from *B. subtilis* increased slowly over time when

grown in the presence of germinating conidia of *F. sambucinum* and the quantity and types of antimicrobials produced was dictated by the presence or absence of specific fungal aggressors (DeFilippi et al. 2018).

Nevertheless, the reduction of mycelial growth and/or conidial germination supports antibiosis as a mode of action involved in the observed effects. Previous work has indicated that bacteria from the genera *Bacillus* and *Pseudomonas* produce various secondary metabolites such as peptide and/or non-peptide containing antimicrobial compounds (Falardeau et al. 2013; Gross and Loper 2009; Hamdache et al. 2011; Raaijmakers et al. 2010; Wise et al. 2012). Our results revealed that *P. koreensis* F9-9 provided the overall highest inhibition of *F. sambucinum* conidia and mycelium, which may be partly provided by arthrofactin homologs, which have been previously identified in this bacterium (Kurniawan et al. 2018).

Taken together, our results showed that bacteria isolated from disease suppressive composts inhibited growth of *F. sambucinum* and reduced dry rot disease on potato tubers. The bacterial extracts of the most inhibitory bacteria indicated that antibiosis i.e., the production of antimicrobial compounds, may be partly responsible for the observed activity. Additionally, competition for space and/or nutrients, as well as induced resistance of the tubers might play a role in the suppression of Fusarium dry rot. Future work will attempt to clarify the relationship between the bacteria and defense mechanisms of the tubers, as well as to identify the antimicrobial compounds produced by these bacteria. Finally, determination of optimal bacterial concentrations, as well as testing of bacterial treatments alone or in combination with conventional control strategies (e.g., cold storage, preventative control treatments) will be performed to obtain additional data toward use of these bacteria as potential biological control agents against potato dry rot in a sustainable production system.

2.6 References

Akpa E, Jacques P, Wathelet B, Paquot M, Fuchs R, Budzikiewicz H, Thonart P (2001) Influence of culture conditions on lipopeptide production by *Bacillus subtilis*. Appl Biochem Biotechnol 91-93:551-561. https://doi.org/10.1385/abab:91-93:1-9:551

Al-Mughrabi KI (2010) Biological control of *Fusarium* dry rot and other potato tuber diseases using *Pseudomonas fluorescens* and *Enterobacter cloacae*. Biol Control 53:280-284. https://doi.org/10.1016/j.biocontrol.2010.01.010

Baez-Vallejo N, Camarena-Pozos DA, Monribot-Villanueva JL, Ramirez-Vazquez M, Carrion-Villarnovo GL, Guerrero-Analco JA, Partida-Martinez LP, Reverchon F (2020) Forest tree associated bacteria for potential biological control of *Fusarium solani* and of *Fusarium kuroshium*, causal agent of *Fusarium dieback*. Microbiol Res 235:126440-126440. https://doi.org/10.1016/j.micres.2020.126440

Bernier-English V, Avis TJ, Mimee B, Antoun H, Tweddell RJ (2010) Compost amendment, a potential alternative to soil fumigation for the control of strawberry verticillium wilt. Phytopathology 100:S193-S193. https://doi.org/10.1094/PHYTO.2010.100.6.S193

Bojanowski A, Avis TJ, Pelletier S, Tweddell RJ (2013) Management of potato dry rot. Postharvest Biol Technol 84:99-109. https://doi.org/10.1016/j.postharvbio.2013.04.008

Chaves I, Pinheiro C, Paiva JAP, Planchon S, Sergeant K, Renaut J, Graca JA, Costa G, Coelho AV, Ricardo CPP (2009) Proteomic evaluation of wound-healing processes in potato (*Solanum tuberosum* L.) tuber tissue. Proteomics 9:4154-4175. https://doi.org/10.1002/pmic.200700649

Cherif M, Sadfi N, Benhamou N, Boudabbous A, Boubaker A, Hajlaoui MR, Tirilly Y (2002) Ultrastructure and cytochemistry of *in vitro* interactions of the antagonistic bacteria

Bacillus cereus X16 and *B. thuringiensis* 55T with *Fusarium roseum* var. *sambucinum*. J Plant Pathol 84:83-93. https://doi.org/10.4454/jpp.v84i2.1091

Cloutier A, Tran S, Avis TJ (2020) Suppressive effect of compost bacteria against grey mould and *Rhizopus* rot on strawberry fruit. Biocontrol Sci Technol 30:143-159. https://doi.org/10.1080/09583157.2019.1695745

DeFilippi S, Groulx E, Megalla M, Mohamed R, Avis TJ (2018) Fungal competitors affect production of antimicrobial lipopeptides in *Bacillus subtilis* strain B9-5. J Chem Ecol 44:374-383. https://doi.org/10.1007/s10886-018-0938-0

Delgado JA, Schwarz PB, Gillespie J, Rivera-Varas VV, Secor GA (2010) Trichothecene mycotoxins associated with potato dry rot caused by *Fusarium graminearum*. Phytopathology 100:290-296. https://doi.org/10.1094/phyto-100-3-0290

Dukare AS, Paul S, Nambi VE, Gupta RK, Singh R, Sharma K, Vishwakarma RK (2019) Exploitation of microbial antagonists for the control of postharvest diseases of fruits: a review. Crit Rev Food Sci Nutr 59:1498-1513. https://doi.org/ 10.1080/10408398.2017.1417235

Estrada R, Gudmestad NC, Rivera VV, Secor GA (2010) *Fusarium graminearum* as a dry rot pathogen of potato in the USA: prevalence, comparison of host isolate aggressiveness and factors affecting aetiology. Plant Pathol 59:1114-1120. https://doi.org/10.1111/j.1365-3059.2010.02343.x

Falardeau J, Wise C, Novitsky L, Avis TJ (2013) Ecological and mechanistic insights into the direct and indirect antimicrobial properties of *Bacillus subtilis* lipopeptides on plant pathogens. J Chem Ecol 39:869-878. https://doi.org/10.1007/s10886-013-0319-7

Gachango E, Hanson LE, Rojas A, Hao JJ, Kirk WW (2012) *Fusarium* spp. causing dry rot of seed potato tubers in Michigan and their sensitivity to fungicides. Plant Dis 96:1767-1774. https://doi.org/10.1094/pdis-11-11-0932-re

Gross H, Loper JE (2009) Genomics of secondary metabolite production by *Pseudomonas* spp. Nat Prod Rep 26:1408-1446. https://doi.org/10.1039/b817075b

Hamdache A, Lamarti A, Aleu J, Collado IG (2011) Non-peptide metabolites from the genus *Bacillus*. J Nat Prod 74:893-899. https://doi.org/10.1021/np100853e

Hay WT, Fanta GF, Rich JO, Schisler DA, Selling GW (2019) Antifungal activity of a fatty ammonium chloride amylose inclusion complex against *Fusarium sambucinum*; control of dry rot on multiple potato varieties. Am J Potato Res 96:79-85. https://doi.org/10.1007/s12230-018-9683-8

Hide GA, Read PJ, Hall SM (1992) Resistance to thiabendazole in *Fusarium* species isolated from potato-tubers affected by dry rot. Plant Pathol 41:745-748. https://doi.org/10.1111/j.1365-3059.1992.tb02558.x

Igarashi Y, Yamamoto K, Fukuda T, Shojima A, Nakayama J, Carro L, Trujillo ME (2015) Arthroamide, a cyclic depsipeptide with quorum sensing inhibitory activity from *Arthrobacter* sp. J Nat Prod 78:2827-2831. https://doi.org/10.1021/acs.jnatprod.5b00540

Kalantari S, Marefat A, Naseri B, Hemmati R (2018) Improvement of bean yield and *Fusarium* root rot biocontrol using mixtures of *Bacillus*, *Pseudomonas* and *Rhizobium*. Trop Plant Pathol 43:499-505. https://doi.org/10.1007/s40858-018-0252-y

Kolaei EA, Cenatus C, Tweddell RJ, Avis TJ (2013) Antifungal activity of aluminium-containing salts against the development of carrot cavity spot and potato dry rot. Ann Appl Biol 163:311-317. https://doi.org/10.1111/aab.12056

Kolaei EA, Tweddell RJ, Avis TJ (2012) Antifungal activity of sulfur-containing salts against the development of carrot cavity spot and potato dry rot. Postharvest Biol Technol 63:55-59. https://doi.org/10.1016/j.postharvbio.2011.09.006

Kotan R, Sahin F, Demirci E, Eken C (2011) Biological control of the potato tubers dry rot caused by *Fusarium* species using PGPR strains. Biol Control 59:390-390. https://doi.org/10.1016/j.biocontrol.2011.09.006

Kumar P, Sharma N, Sharma S, Gupta R (2020) Rhizosphere stochiometry, fruit yield, quality attributes and growth response to PGPR transplant amendments in strawberry (*Fragaria* x *ananassa* Duch.) growing on solarized soils. Sci Hortic 265:109215. https://doi.org/10.1016/j.scienta.2020.109215

Kurniawan O, Wilson K, Mohamed R, Avis TJ (2018) *Bacillus* and *Pseudomonas* spp. provide antifungal activity against gray mold and *Alternaria* rot on blueberry fruit. Biol Control 126:136-141. https://doi.org/10.1016/j.biocontrol.2018.08.001

Lecomte C, Alabouvette C, Edel-Hermann V, Robert F, Steinberg C (2016) Biological control of ornamental plant diseases caused by *Fusarium oxysporum*: a review. Biol Control 101:17-30. https://doi.org/10.1016/j.biocontrol.2016.06.004

Lulai EC, Corsini DL (1998) Differential deposition of suberin phenolic and aliphatic domains and their roles in resistance to infection during potato tuber (*Solanum tuberosum* L.) wound-healing. Physiol Mol Plant Pathol 53:209-222. https://doi.org/10.1006/pmpp.1998.0179

Luo M, Purdy H, Avis TJ (2019) Compost bacteria provide antifungal activity against grey mold and *Alternaria* rot on bell pepper fruit. Botany 97:221-230. https://doi.org/10.1139/cjb-2018-0180

Martin-Lapierre A, Mimee B, Tweddell RJ (2011) Evaluation of combined effect of compost amendments and fumigation on strawberry verticillium wilt. Phytopathology 101:S261-S261. https://doi.org/10.1094/PHYTO.2011.101.6.S257

Mecteau MR, Arul J, Tweddell RJ (2002) Effect of organic and inorganic salts on the growth and development of *Fusarium sambucinum*, a causal agent of potato dry rot. Mycol Res 106:688-696. https://doi.org/10.1017/s0953756202005944

Mecteau MR, Arul J, Tweddell RJ (2008) Effect of different salts on the development of *Fusarium solani* var. *coeruleum*, a causal agent of potato dry rot. Phytoprotection 89:1-6. https://doi.org/10.7202/000377ar

Mnif I, Hammami I, Triki MA, Azabou MC, Ellouze-Chaabouni S, Ghribi D (2015) Antifungal efficiency of a lipopeptide biosurfactant derived from *Bacillus subtilis* SPB1 versus the phytopathogenic fungus, *Fusarium solani*. Environ Sci Pollut Res 22:18137-18147. https://doi.org/10.1007/s11356-015-5005-6

Mohamed R, Groulx E, Defilippi S, Erak T, Tambong JT, Tweddell RJ, Tsopmo A, Avis TJ (2017) Physiological and molecular characterization of compost bacteria antagonistic to soil-borne plant pathogens. Can J Microbiol 63:411-426. https://doi.org/10.1139/cjm-2016-0599

Ocamb CM, Hamm PB, Johnson DA (2007) Benzimidazole resistance of *Fusarium* species recovered from potatoes with dry rot from storages located in the Columbia basin of Oregon and Washington. Am J Potato Res 84:169-177. https://doi.org/10.1007/bf02987140

Raaijmakers JM, de Bruijn I, Nybroe O, Ongena M (2010) Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: more than surfactants and antibiotics. FEMS Microbiol Rev 34:1037-1062. https://doi.org/10.1111/j.1574-6976.2010.00221.x

Raza W, Ling N, Zhang R, Huang Q, Xu Y, Shen Q (2017) Success evaluation of the biological control of *Fusarium* wilts of cucumber, banana, and tomato since 2000 and future research strategies. Crit Rev Biotechnol 37:202-212. https://doi.org/10.3109/07388551.2015.1130683

Recep K, Fikrettin S, Erkol D, Cafer E (2009) Biological control of the potato dry rot caused by *Fusarium* species using PGPR strains. Biol Control 50:194-198. https://doi.org/10.1016/j.biocontrol.2009.04.004

Schisler DA, Slininger PJ, Olsen NL (2016) Appraisal of selected osmoprotectants and carriers for formulating Gram-negative biocontrol agents active against *Fusarium* dry rot on potatoes in storage. Biol Control 98:1-10. https://doi.org/10.1016/j.biocontrol.2016.03.009

Secor GA, Gudmestad NC (1999) Managing fungal diseases of potato. Can J Plant Pathol 21:213-221. https://doi.org/10.1080/07060669909501184

Stefanczyk E, Sobkowiak S, Brylinska M, Sliwka J (2016) Diversity of *Fusarium* spp. associated with dry rot of potato tubers in Poland. Eur J Plant Pathol 145:871-884. https://doi.org/10.1007/s10658-016-0875-0

Theron DJ, Holz G (1991) Prediction of potato dry rot based on the presence of *Fusarium* in soil adhering to tubers at harvest. Plant Dis 75:126-130. https://doi.org/10.1094/pd-75-0126

Vatankhah M, Riseh RS, Eskandari MM, Sedaghati E, Alaei H, Afzali H (2019) Biological control of *Fusarium* dry rot of potato using some probiotic bacteria. J Agric Sci Technol 21:1301-1312

Velazquez-Becerra C, Macias-Rodriguez LI, Lopez-Bucio J, Flores-Cortez I, Santoyo G, Hernandez-Soberano C, Valencia-Cantero E (2013) The rhizobacterium *Arthrobacter agilis* produces dimethylhexadecylamine, a compound that inhibits growth of phytopathogenic fungi in vitro. Protoplasma 250:1251-1262. https://doi.org/10.1007/s00709-013-0506-y

Verma P, Yadav AN, Khannam KS, Panjiar N, Kumar S, Saxena AK, Suman A (2015) Assessment of genetic diversity and plant growth promoting attributes of psychrotolerant bacteria allied with wheat (*Triticum aestivum*) from the northern hills zone of India. Ann Microbiol 65:1885-1899. https://doi.org/10.1007/s13213-014-1027-4

Wharton PS, Kirk WW (2014) Evaluation of biological seed treatments in combination with management practices for the control of *Fusarium* dry rot of potato. Biol Control 73:23-30. https://doi.org/10.1016/j.biocontrol.2014.03.003

Wise C, Novitsky L, Tsopmo A, Avis TJ (2012) Production and antimicrobial activity of 3-hydroxypropionaldehyde from *Bacillus subtilis* strain CU12. J Chem Ecol 38:1521-1527. https://doi.org/10.1007/s10886-012-0219-2

Xue HL, Bi Y, Prusky D, Raza H, Zhang R, Zhang S, Nan MN, Zong YY, Cheng XY (2019) The mechanism of induced resistance against *Fusarium* dry rot in potato tubers by the T-2 toxin. Postharvest Biol Technol 153:69-78. https://doi.org/10.1016/j.postharvbio.2019.03.021

Zhang HM, Xie XT, Kim MS, Kornyeyev DA, Holaday S, Pare PW (2008) Soil bacteria augment *Arabidopsis* photosynthesis by decreasing glucose sensing and abscisic acid levels in planta. Plant J 56:264-273. https://doi.org/10.1111/j.1365-313X.2008.03593.x

Chapter 3: Chemical and biological characterization of antimicrobial compounds from *Arthrobacter* spp.

3.1 Abstract

Antagonistic bacteria can act as biocontrol agents against various phytopathogens. Recently, *Arthrobacter* spp. demonstrated antifungal activity, but were not further characterized. In this work, the antimicrobial activity of *Arthrobacter humicola* strains M9-1A, M9-2, and M9-8, and *Arthrobacter psychrophenolicus* strain M9-17 were evaluated against nine plant pathogens *in vitro*, and their cell-free filtrates were additionally assessed against disease caused by *Alternaria alternata* on tomato fruit. Results indicated that *A. humicola* M9-1A and *A. psychrophenolicus* M9-17 were the most inhibitory, suppressing seven pathogens. Additionally, the cell-free filtrates of *A. psychrophenolicus* M9-17 reduced the growth of the most pathogens. All bacterial filtrates except *A. humicola* M9-2 suppressed black mold in tomato fruit equally. Extracellular proteases were produced by each bacterial isolate, with *A. humicola* M9-1A forming the largest digestion zone. Disk diffusion assays with crude ethyl acetate extracts of each bacteria reduced the mycelial growth of *A. alternata*. Large inhibition zones were observed for *A. humicola* M9-1A and *A. psychrophenolicus* M9-17 extracts on drop bioassays. Two antifungal compounds were isolated and purified from *A. psychrophenolicus* M9-17. Compound 1 was characterized as *N*-acetyltryptamine, whereas compound 2 was partially characterized as a tetrapeptide compound (Pro-Leu-Hyp-Tyr). Both compounds showed antifungal effects on disk assays using *A. alternata.* This study suggests that antibiosis may be a key role in the antimicrobial activity of *Arthrobacter* spp.

Keywords: *Arthrobacter* spp., antifungal, antimicrobial, protease, *N*-acetyltryptamine, *cyclo*-(Pro-Leu-Hyp-Tyr), tetrapeptide, biocontrol

3.2 Introduction

As the global population increases, food loss and waste are of great concern. Plant pathogens have been a primary cause resulting in 10-16% of food loss yearly, world-wide (Bourguet et al. 2016). Among plant pathogens, fungi and oomycetes generally cause the highest losses overall (Derevnina et al. 2016; Thornton and Wills 2015). The presence of these pathogens, whether it be pre-harvest and/or post-harvest, results in plant disease causing low yields and food losses. Fortunately, with proper pre- and post-harvest control methods such as synthetic chemicals, crop rotation, physical and genetic control methods, an increase in yields and decrease in food loss can be observed. Currently, the most effective and efficient manner to mitigate fungal pathogens is with the use of synthetic fungicides. However, the increase in fungicide costs, the accumulation of their residues and the increase in fungicide resistant pathogens are issues that have risen due to the overuse of synthetic chemicals (Avis 2007). Additionally, the use of fungicides has been associated with potential negative environmental effects, as well as risks to human health (Belpoggi et al. 2002; Sui et al. 2016). Therefore, alternative control methods are needed to reduce the use of synthetic chemicals.

A promising alternative control method currently is biological control. This technique mitigates fungal pathogens by, for example, employing antagonistic beneficial bacteria. Antagonistic bacteria have previously demonstrated effective control against post-harvest plant diseases caused by pathogenic fungi (Cloutier et al. 2020; Kurniawan et al. 2018; Luo et al. 2019). Biological control agents (BCA) typically combat phytopathogens through induced resistance, competition for nutrients and space, parasitism and/or antibiosis (Kohl et al. 2019; Raaijmakers and Mazzola 2012; Spadaro and Droby 2016).

Antibiosis is the antagonistic association between two living organisms where one is negatively impacted by the other because of the production of antimicrobial compounds. Numerous antimicrobial metabolites have been found in *Bacillus* and *Pseudomonas* spp. and are generally categorized in peptide or non-peptide antimicrobial compounds groups (Falardeau et al. 2013; Hamdache et al. 2011; Ongena and Jacques 2008). In addition to antibiosis, the production of lytic enzymes may indicate parasitism as a mechanism by which antagonistic bacteria reduce phytopathogen growth. Fungal cell walls are predominantly composed of chitin and beta-glucans and to a lesser amount glycoproteins and chitosan, whereas oomycetes cell walls are predominantly composed of cellulose and glucans. Therefore, the production of lytic enzymes such as chitinase, glucanases, proteases, and cellulases may target and disrupt fungal and oomycetal cell walls, leading to cell lysis (Dunne et al. 1997; El-Bendary et al. 2016).

Previously, *Arthrobacter* spp. have been shown to inhibit phytopathogens, such as *Botrytis cinerea*, *Alternaria alternata*, *Fusarium sambucinum*, *Verticillium dahliae* and *Pythium sulcatum* (Luo et al. 2019; Mohamed et al. 2017). Additionally, *Arthrobacter* spp. have demonstrated suppression of grey mold and *Alternaria* rot on bell peppers (Luo et al. 2019). Similarly to other actinomycetes (e.g., *Streptomyces* spp.), *Arthrobacter* spp. have revealed antimicrobial activity through the production of antimicrobial metabolites. Indeed, arthroamide, a peptide-containing compound, and its congener turnagainolide have previously been isolated from *Arthrobacter* sp. PGVB1 and shown to participate in the antimicrobial properties of this bacterium (Igarashi et al. 2015). Additionally, *Arthrobacter agilis* UMCV2 has been found to produce a volatile organic compound, dimethylhexadecylamine, with strong antifungal activity against *B. cinerea* and *P. cinnamomi* (Velazquez-Becerra et al. 2013). Even though these antimicrobial metabolites have previously been identified from *Arthrobacter* spp., it is likely that additional compounds would be

isolated from other species of this bacterium. Additionally, limited information is available on the production of extracellular lytic enzymes that may be associated with the antifungal activity of *Arthrobacter* spp. (Adamitsch et al. 2003; Latzko and Hampel 1993).

Previously in our laboratory, four *Arthrobacter* spp. strain have been isolated from a disease suppressive marine compost. Although some studies have indicated the antimicrobial activity of these bacteria, there have not been previous studies identifying active antimicrobial compounds they may produce. The objectives of the present study were (i) to investigate the antimicrobial activity of *Arthrobacter humicola* strains M9-1A, M9-2 and M9-8 as well as *Arthrobacter psychrophenolicus* strain M9-17, (ii) to evaluate the efficacy of their extracellular compounds against *Alternaria* rot of tomatoes, (iii) to determine the presence of extracellular lytic enzymes, and (iv) to isolate and identify bioactive compounds responsible for their antimicrobial activity.

This study was conducted with *Alternaria alternata*, a fungal phytopathogen that targets a vast number of host plants such as tomatoes. The fungal pathogen causes *Alternaria* rot disease (sometimes referred to as black mold) in tomato fruit with symptoms including dark sunken lesions on tomato fruit, as well as leaf spots, rots, and stem cankers on tomato plants (Jia et al. 2013; Wall and Biles 1993).

3.3 Materials and methods

3.3.1 Microbial material

Arthrobacter humicola Kageyama et al. strains M9-1A, M9-2, M9-8 and *A. psychrophenolicus* Margesin et al. strain M9-17 were isolated from a disease suppressive compost composed of marine residues (from the fishing industry) and peat moss. The bacteria were maintained on tryptic soy agar (TSA, Becton Dickson, Sparks, MD, USA) at 23°C prior to use. The bacteria were identified using nucleotide sequence data from the small subunit (16*S*) of ribosomal RNA (GenBank Accession nos. KT382243, KT382245, KT382249, KT382253).

The plant pathogenic fungal species *Alternaria alternata* (Fr.) Keissl., *Alternaria solani* Sorauer, *Botrytis cinerea* Pers., *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL) Jarvis & Shoemaker, *Fusarium sambucinum* Fuckel, *Rhizoctonia solani* Khün, *Rhizopus stolonifer* (Ehrenb.) Vuill., *Sclerotinia sclerotiorum* (Lib.) de Bary, and *Verticillium dahliae* Kleb. were provided by the Laboratoire de diagnostic en phytoprotection (MAPAQ, Québec, Canada). The pathogenic oomycete, *Pythium sulcatum* R.G. Pratt & J.E. Mitch was isolated from an infected carrot root and is available at the Canadian Collection of Fungal Cultures (Agriculture and Agri-Food Canada, Ottawa, Ontario, Canada). All fungi and the oomycete were maintained on potato dextrose agar at 23°C (PDA, Becton Dickson, Sparks, Maryland, USA).

3.3.2 Effect of *Arthrobacter* spp. on mycelial growth of phytopathogens *in vitro*

To assess the inhibitory spectrum of *A. humicola* strains M9-1A, M9-2 and M9-8 as well as *A. psychrophenolicus* strain M9-17, the bacteria were tested against an assemblage of phytopathogens. A 0.5-cm diameter plug of actively growing phytopathogen was placed in the center of a 100 × 15 mm PDA plate. Two-day old bacterial cultures were used to inoculate the PDA with 1 cm streaks at the four cardinal points. The bacterial streaks were placed at a distance

of 1 cm from the plug for *V. dahliae*, 2 cm for *A. solani*, 2.5 cm for *A. alternata* and *R. solani*, 3 cm for FORL, *S. sclerotiorum* and *P. sulcatum*, and 3.5 cm from *B. cinerea* and *R. stolonifer*. The plates were incubated at 23°C for 2 days for *R. stolonifer*, *S. sclerotiorum*, 5 days for *B. cinerea*, 6 days for *A. alternata*, FORL, and *P. sulcatum*, and 7 days for *A. solani* and *V. dahliae.* The control consisted of a fungal plug without the presence of bacterial streaks. The diameter of mycelial growth was measured as the average of two perpendicular measurements. *In vitro* trial was performed as a completely randomized design with three repetitions.

3.3.3 Effect of cell-free filtrates against phytopathogens *in vitro*

To test for antimicrobial activity of extracellular compounds produced by *A. humicola* M9-1A, M9-2, M9-8 and *A. psychrophenolicus* M9-17, the cell-free filtrates were tested against phytopathogens *in vitro*. The bacteria were cultured in medium optimal for lipopeptide production (MOLP) containing 20 g/L sucrose, 30 g/L peptone, 7 g/L of yeast extract, 1.9 g/L of KH_2PO_4, 0.45 g/L $MgSO_4$, 9 mL/L of trace element solution (TES), and 9 mL/L of citric acid solution (10 g/L ddH_2O), as previously described (Akpa et al. 2001; Mohamed et al. 2017). TES was prepared by adding 0.001 g of $CuSO_4$, 0.005 g of $FeCl_3$, 0.004 g Na_2MoO_4, 0.002 g KI, 0.014 g of $ZnSO_4$, 0.01 g of H_3BO_3 and 0.0036 g of $MnSO_4$ in 1 L of sterile ultra-pure water. Five 500-mL Erlenmeyer flasks containing 100 mL of sterile MOLP were inoculated with each bacterium to a concentration of approximately 1×10^4 cells/mL. Inoculated samples were incubated for 72 hours at 30°C with shaking conditions of 120 rpm. To obtain cell-free filtrates, inoculated samples were first centrifuged at 10,000 rpm for 30 minutes. The supernatants were subsequently filtered through a sterile 0.45-µm PES filter.

Treatments were prepared by incorporating 5, 10 and 15% v/v of cell-free filtrate into 55°C sterilized PDA. The control consisted of only PDA without filtrate. To confirm MOLP did not

have an inhibitory effect, 15% of sterile MOLP was incorporated to PDA and was tested against each phytopathogen. A 0.5-cm plug of mycelium for each phytopathogen was placed in the center of each dish and incubated at 23°C in the dark for the appropriate time for each pathogen as indicated above (Section 3.3.2).

Following incubation, the mycelial growth was measured as the average of two perpendicular diameters. *In vitro* trial was performed as a completely randomized design with three repetitions.

3.3.4 Effect of cell-free filtrates against *Alternaria* rot (black mold) of tomato fruit

The cell-free filtrate of each bacterium, produced above, was tested *in vivo* against *Alternaria* rot on tomatoes.

Prior to inoculation, tomato fruit (*Solanum lycopersicum* L. cultivar 'Roma') were surface-sanitized by submerging in 70% ethanol for 15 minutes and rinsed with sterile distilled water. The tomatoes were air dried for 30 minutes. Once tomatoes were dry, three wounds were formed using a 0.3-cm diameter cork borer to a depth of 0.3 cm, resulting in a 0.3 × 0.3 cm hole. The three wounds on a single tomato were inoculated with 100 µL of cell-free supernatant of a single bacterium. The control tomato was inoculated with 100 µL of sterile distilled water. A 0.3-cm plug of actively growing *A. alternata* was placed mycelial side down into each wound. Each tomato was transferred to a sterile plastic container maintaining high relative humidity (>95%) with a paper towel saturated with sterile distilled water. The tomatoes were allowed to incubate for 4 days at 23°C in the dark.

After 4 days, horizontal measurements of the surface lesion of each wound were taken. The average of three wounds on each tomato were used as a repetition. The experiment was conducted as a randomized complete block design with five repetitions.

3.3.5 Identification of extracellular lytic enzyme production

To identify the presence of extracellular lytic enzymes, each bacterium was grown on skim milk powder medium, chitosan medium, cellulase medium, and β-glucanase medium. The skim milk powder, cellulase and β-glucanase media were prepared by incorporating 2% skim milk powder, cellulose and laminarin separately into TSA, respectively. The chitosan medium was prepared by incorporating 0.2% of chitosan into TSA. Approximately 1×10^6 cells of two-day old bacteria were placed in the center of the plate. The plates were incubated at 23°C for 6 days in the dark. Following incubation, the zone of digestion was measured as the average of two perpendicular diameters. *In vitro* trial was performed as a completely randomized design with three repetitions.

3.3.6 Antimicrobial extracts from *Arthrobacter* spp.

To determine the antimicrobial effects of the extracellular compounds of each bacterium, 1 L of MOLP was inoculated with *A. humicola* M9-1A, M9-2 and M9-8 as well as *A. psychrophenolicus* strain M9-17 separately and incubated at 30°C with shaking (120 rpm) for 72 hours. Following incubation, the media was centrifuged for 30 minutes at 10,000 rpm. The supernatant was filtered through a sterile 0.45-µm PES filter. Each liter of supernatant was extracted with 600 mL of ethyl acetate and re-extracted with 400 mL of ethyl acetate. The ethyl acetate extracts were combined and dried using a Heidolph 4000 rotary evaporator (Schwabach, Germany). The extracts were resuspended in methanol and collected in amber vials. Samples were further dried under a stream of nitrogen gas.

3.3.6.1 Disk diffusion bioassay

To quantify the antimicrobial activity of each fraction, a disk diffusion bioassay was performed. Each bacterial extract was resuspended in ethyl acetate to a concentration of 50 mg/mL. A 0.5-cm

sterile filter paper disk was inoculated with 20 µL of each extract separately. The control consisted of a disk with 20 µL of ethyl acetate. The disks were allowed to air dry in an aseptic environment. Once dried, the disks were placed at a distance of 2.5 cm from a 0.3-cm plug of actively growing *A. alternata*. The plates were left to incubate in the dark at room temperature for 7 days. The radius of the mycelium facing the disk was measured daily. *In vitro* trial was performed as a completely randomized design with three biological repetitions.

3.3.6.2 Drop bioassay

Each bacterial extract was directly bioassayed on a thin-layer chromatography (TLC) plate to determine the antifungal potential. The bacterial extracts were resuspended in ethyl acetate to a concentration of 50 mg/mL. Drops of approximately 4 µL were spotted on a TLC plate and left to air dry. Ethyl acetate was used as the control. A spore suspension was prepared by adding 10 mL of ultrapure water to an actively growing plate of *A. alternata*. The mycelium was gently scraped dislodging the spores. Molten PDA was made by adding 0.4 g of PDA to 10 mL of ultrapure water and heated to the boiling point. Once cooled, the spore suspension was incorporated into the PDA thoroughly. The mixture was thinly spread over TLC plate. Once the media solidified, the TLC plate was transferred to a moist chamber (>95% RH) and incubated in the dark at room temperature. Observations were taken over a 7-day period observing inhibition zones around the active bacterial extracts. A rating system was used to distinguish between the different sizes of inhibition zones from no inhibition (-) to largest inhibition zone (+++).

3.3.7 Separation of compounds and antimicrobial determination of *A. humicola* strain M9-1A via normal-phase flash chromatography

Initial separation and purification of antimicrobial compounds was accomplished via normal-phase flash chromatography of *A. humicola* M9-1A bacterial extract. The column was packed with silica

submerged in 80:20 hexane:ethyl acetate solvent. The crude sample was dissolved in minimal 3:1 hexane:ethyl acetate solvent and transferred to the column. The mobile phase consisted of 80:20, 60:40, 50:50, 40:60, 20:80, 0:100 hexane:ethyl acetate, followed by 95:5, 90:10, 85:15, 70:30, 60:40 and 50:50 of ethyl acetate:methanol. Any fractions with similar banding patterns were combined producing fractions 1&2, 3&4, 5&6, 7, 8, 9 and 10.

All fractions were bioassayed via drop bioassays and disk assays, as previously described, to determine presence of active antimicrobial fractions.

3.3.8 Large scale extraction of antimicrobial compounds from *A. psychrophenolicus* strain M9-17

A total of 12 L of MOLP media was inoculated with *A. psychrophenolicus* strain M9-17 and incubated at 30°C with shaking of 120 rpm for 72 hours. After 72 hours of growth, the media was centrifuged for 30 minutes at 10,000 rpm. The supernatant was filtered through a sterile PES 0.45 µm filter. Each liter of supernatant was extracted with 600 mL of ethyl acetate and reextracted with 400 mL of ethyl acetate. The ethyl acetate extracts were combined and dried using a Heidolph 4000 rotary evaporator (Schwabach, Germany). The extracts were resuspended with methanol and collected in amber vials. Samples were further dried under a stream of nitrogen gas.

3.3.8.1 Isolation and purification of antimicrobial compounds from *A. psychrophenolicus* strain M9-17 extract

3.3.8.1.1 Separation of compounds via normal-phase flash chromatography

Initial separation and purification of antimicrobial compounds was accomplished via normal-phase flash chromatography. The column was packed with silica gel (Silicycle; 40-63 µm) submerged in 80:20 hexane:ethyl acetate solvent. The crude sample was dissolved in minimal 3:1 hexane:ethyl

acetate solvent and transferred to the column. The step elution system consisted of 80:20, 60:40, 50:50, 40:60, 20:80 and 0:100 hexane:ethyl acetate solvent, followed by 5, 10, 15, 30 and 50% of MeOH-EtOAc (v/v).

All fractions were bioassayed via drop bioassays, as previously described, to determine presence of active antimicrobial fractions.

3.3.8.1.2 Separation of bioactive fractions via preparative thin-layer chromatography

The active fractions (fractions 1, 4 and 7) were each subject to preparative thin-layer chromatography to isolate compounds. Fractions were dissolved in 1 mL of ethyl acetate and transferred to in a horizontal line 1 inch up from the bottom of the 20 × 20 cm glass back TLC plate. The TLC plate was allowed to air dry for 1 hour. The glass chamber was filled with 200 mL of 9:1 chloroform:methanol. The TLC plate was placed in the glass chamber and let run for approximately an hour until the solvent front reached an inch from the top. Following drying, each band visualized under a short wavelength (254 nm) was removed using a utility knife and the silica containing the separated compounds was placed in an Erlenmeyer flask.

Compounds were extracted from the silica by suspending in 150 mL of 2:1 ethyl acetate:methanol for 3 hours with shaking conditions of 150 rpm at room temperature. The suspensions were then filtered by gravity using Whatman Grade 1 filter paper. All fractions were dried using a Heidolph 400 rotary evaporator (Schwabach, Germany) and further dried under a stream of nitrogen.

3.3.8.1.3 Purification of compounds via high-performance liquid chromatography (HPLC)

The most abundant fractions from flash chromatography were further purified by semi-preparative HPLC using an Agilent 1100 HPLC instrument with a photodiode array detector (200-600 nm). Compounds were separated using a Phenomenex Kinetex C_{18} (250 × 10 mm, 5 μm, 100 Å) column (Torrance, California, USA) using acetonitrile-water (ACN-H_2O) as the mobile phase with a flow rate of 4 mL/min. Fractions were resuspended in HPLC grade methanol to a concentration of 50 mg/mL and 50 μL volumes were repeatedly injected. Linear HPLC elution profiles to obtain purified compounds were metabolite specific. The solvent gradient began at 30% ACN and held for 2 minutes, increased to 70% ACN over 10 minutes and held for 1 minute and finally, decreased back to 30% ACN. The run was 15 minutes.

3.3.9 Characterization of compounds

3.3.9.1 Nuclear magnetic resonance (NMR) analysis

^{1}H, ^{13}C-NMR, HSQC, HMBC, COSY and NOESY experiments were recorded using a JEOL ECZS 400 MHz NMR spectrometer (Akishima, Tokyo, Japan) using an auto-tuning broadband probe. Samples were dissolved in methanol-*d_4* or dimethyl sulfoxide-*d_6* (CDN Isotopes, Pointe-Claire, Quebec). The spectra were referenced to the solvent peak: δ_H 3.31 and δ_C 49.1 for methanol-d_4 or δ_H 2.50 and δ_C 39.5 for DMSO-d_6.

3.3.9.2 Optical rotation and UV-Visible spectra

Optical rotations were acquired with an Autopol IV polarimeter (Rudolph Analytical, Hackettown, NJ) and UV spectra were recorded with a Varian Cary 3 UV-VIS spectrophotometer (190-800nm).

3.3.10 Determination of antimicrobial activity of isolated compounds through disk diffusion bioassay

To quantify the antimicrobial activity of compound 1, a disk bioassay was performed. Compound 1 and an *N*-acetyltryptamine standard were resuspended in methanol to a concentration of 5 mg/mL and 50 mg/mL. A 0.5-cm sterile filter paper disk was inoculated with 20 µL of each extract separately. Methanol was used as the control. The disks were allowed to air dry in an aseptic environment. Once dried, the disks were place at a distance of 2.5 cm from a 0.3-cm plug of actively growing *A. alternata*. Three biological repetitions were performed for each concentration of the isolated compound and standard. The plates were left to incubate in the dark at room temperature for 8 days. The radius of the mycelium facing the disk was measured daily. *In vitro* trial was performed as a completely randomized design with three biological repetitions.

The *N*-acetyltryptamine standard was used in a dose-dependent study. The standard was resuspended in methanol to concentrations of 0.005, 0.05, 0.5, 5, 50 mg/mL. Methanol was used as the control. The disk diffusion assay was performed as described above. *In vitro* trial was performed as a completely randomized design with three biological repetitions.

Compound 2 was subjected to a disk diffusion assay to determine its antimicrobial activity. Compound 2 was resuspended in methanol to concentrations of 0.0025, 0.025, 0.25, 2.5, 25 mg/mL. Methanol was used as the control. The disk diffusion assay was performed as described above. *In vitro* trial was performed as a completely randomized design with three biological repetitions.

3.3.11 Statistical analysis

For all *in vitro* and *in vivo* studies, analysis of variance (ANOVA) was performed. When significant ($P \leq 0.05$), treatment means were separated using Fisher's protected least significant

difference test (α level = 0.05). Statistical analysis was performed using SAS 9.4 (SAS Institute, Cary, North Carolina, USA). Interactions analyses were performed in the *in vitro* experiments to investigate the relationship between mold and bacteria. If the interactions revealed significant differences, data was presented for each mold.

3.4 Results

3.4.1 Effect of *Arthrobacter* spp. on mycelial growth of phytopathogens *in vitro*

To determine the antagonistic properties of the bacteria, each bacterium was tested against different plant pathogens *in vitro*. The tested *Arthrobacter* strains showed inhibition to various plant pathogens. *Arthrobacter humicola* M9-1A and *A. psychrophenolicus* M9-17 significantly inhibited seven of nine of the tested pathogens. *Arthrobacter humicola* M9-2 and M9-8 reduced four of nine of the tested pathogens (Fig. 3-1).

More specifically, *A. humicola* M9-1A and M9-2, and *A. psychrophenolicus* M9-17 significantly inhibited growth of *A. alternata. Arthrobacter humicola* M9-1A was the most inhibitory against *A. alternata*, reducing growth by 23%. None of the bacterial strains reduced the growth of *A. solani. Arthrobacter humicola* M9-1A and M9-8, and *A. psychrophenolicus* M9-17 significantly inhibited the growth of *B. cinerea. Arthrobacter humicola* M9-1A and *A. psychrophenolicus* M9-17 were the most inhibitory, suppressing *B. cinerea* growth by an average of 14%. *Arthrobacter humicola* M9-1A and *A. psychrophenolicus* M9-17 were the only bacteria inhibiting *FORL*, reducing growth by an average of 11%. All three *A. humicola* (M9-1A, M9-2 and M9-8) significantly reduced the growth of *P. sulcatum.* They were equally inhibitory suppressing *P. sulcatum* growth by an average of 21%. *Arthrobacter humicola* M9-1A and *A. psychrophenolicus* M9-17 were the only inhibitory bacteria against *R. solani*, suppressing its growth by an average of 20%. Only *A. psychrophenolicus* M9-17 reduced the growth of *R. stolonifer* (11% inhibition). All bacterial strains were equally inhibitory for *S. sclerotiorum*, suppressing its growth by an average of 16%. All bacterial strains significantly reduced *V. dahliae. Arthrobacter humicola* M9-1A and *A. psychrophenolicus* M9-17 were the most inhibitory, suppressing *V. dahliae* growth by an average of 50%.

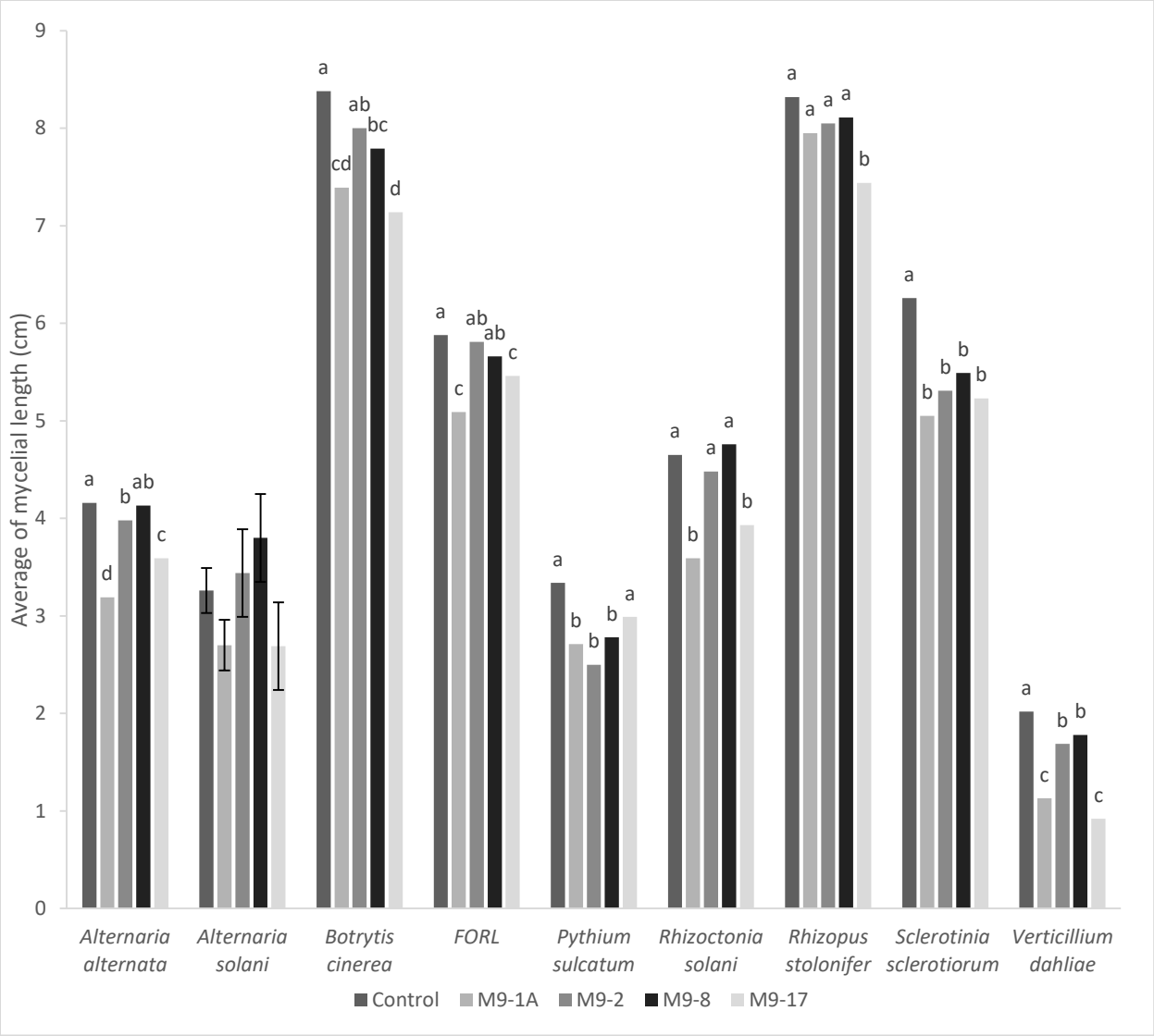


Figure 3-1. Effect of bacterial strains on the mycelial growth of fungal plant pathogens. M9-1A, M9-2 and M9-8 = *Arthrobacter humicola*; M9-17 = *Arthrobacter psychrophenolicus*. Means with a letter in common within the same plant pathogen are not significantly different according to Fisher's protected LSD test (α level = 0.05). When ANOVA was not significant (P > 0.05), standard error bars were provided.

3.4.2 Effect of cell-free filtrates against phytopathogens *in vitro*

To evaluate the production of antimicrobials from *Arthrobacter* spp., each plant pathogen was tested *in vitro* on PDA plates incorporated with cell-free filtrates from each bacterium. The uninoculated control medium showed no significant inhibition of any phytopathogen. It was

determined that 15% cell-free filtrates showed the highest inhibition. *Arthrobacter humicola* M9-1A filtrates significantly reduced the growth of five phytopathogens and *A. psychrophenolicus* M9-17 filtrates significantly reduced the growth of six, whereas *A. humicola* M9-2 and M9-8 filtrates each inhibited three of the plant pathogens tested (Fig. 3-2).

All bacterial filtrates significantly suppressed the growth of *A. alternata* equally by an average of 29%. *Arthrobacter humicola* M9-8 and *A. psychrophenolicus* M9-17 were the only filtrates that significantly suppressed *A. solani. Arthrobacter psychrophenolicus* M9-17 was the most inhibitory, reducing growth by 19%. *Arthrobacter humicola* M9-1A and *A. psychrophenolicus* M9-17 provided the only filtrates to significantly reduce the mycelial growth of *B. cinerea*, where *A. psychrophenolicus* M9-17 was the most inhibitory (10% inhibition). *Arthrobacter humicola* M9-1A and *A. psychrophenolicus* M9-17 were the only filtrates to significantly suppress *P. sulcatum* with *A. humicola* M9-1A being the most inhibitory (20% inhibition). All bacterial filtrates except *A. humicola* M9-2 inhibited the growth of *R. stolonifer. Arthrobacter humicola* M9-8 increased the growth of *R. stolonifer* compared to the control whereas, *A. humicola* M9-1A and *A. psychrophenolicus* M9-17 were the most inhibitory by an average of 31%. All bacterial filtrates significantly reduced the growth of *S. sclerotiorum* equivalently by an average of 32%. None of the bacterial filtrates significantly reduced the mycelial growth of *FORL*, *R. solani* or *V. dahliae*.

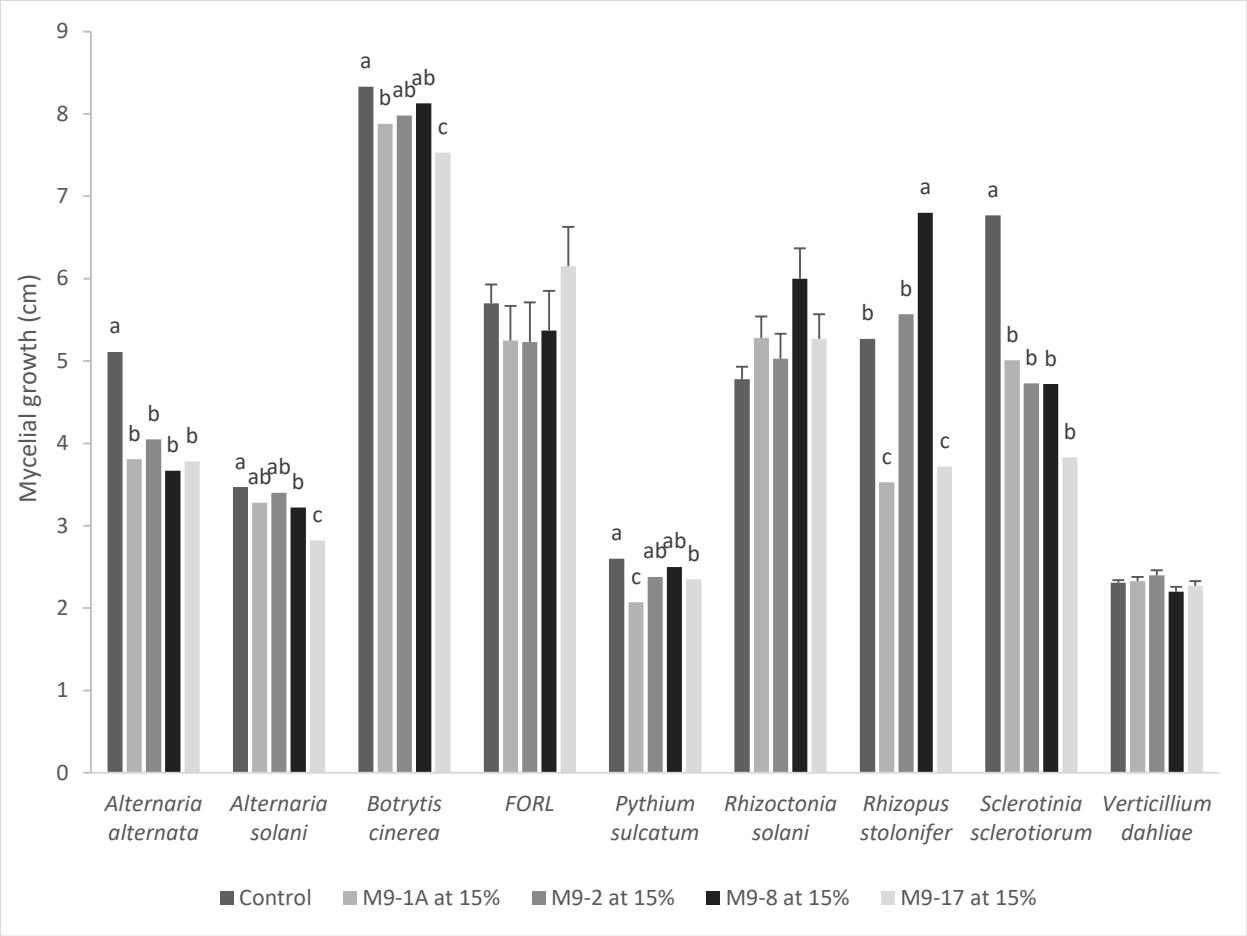


Figure 3-2. Effect of 15% cell-free filtrates from *Arthrobacter humicola* M9-1A, M9-2, M9-8 and *Arthrobacter psychrophenolicus* M9-17 on the mycelial growth of fungal plant pathogens. Means with a letter in common within the same plant pathogen are not significantly different according to Fisher's protected LSD test (α level = 0.05). When ANOVA was not significant (P > 0.05), standard error bars were provided.

Cell-free filtrate bioassays demonstrated a range of inhibitory effects depending on concentration (Fig. 3-3). Each bacterial strain displayed inhibition against *A. alternata. Arthrobacter humicola* M9-1A showed higher inhibition when increasing the concentration, where 5% cell-free filtrates showed no inhibition and 15% showed the most inhibition. The 10 and 15% filtrates of *A. humicola* M9-1A significantly reduced *A. alternata* by 16% and 25%, respectively. At 10 and 15% cell-free filtrates of *A. humicola* M9-2 significantly reduced *A. alternata* equally by an average of 20%. *Arthrobacter humicola* M9-8 showed a dose response, where 5% showed

the lowest inhibition and 15% showed the highest. The 15% cell-free filtrate of *A. humicola* M9-8 significantly reduced the growth of *A. alternata* by 32%. The 5% cell-free filtrate of *A. psychrophenolicus* M9-17 showed no inhibition, whereas 10 and 15% equally suppressed the growth of *A. alternata* by an average of 23% (Fig. 3-3).

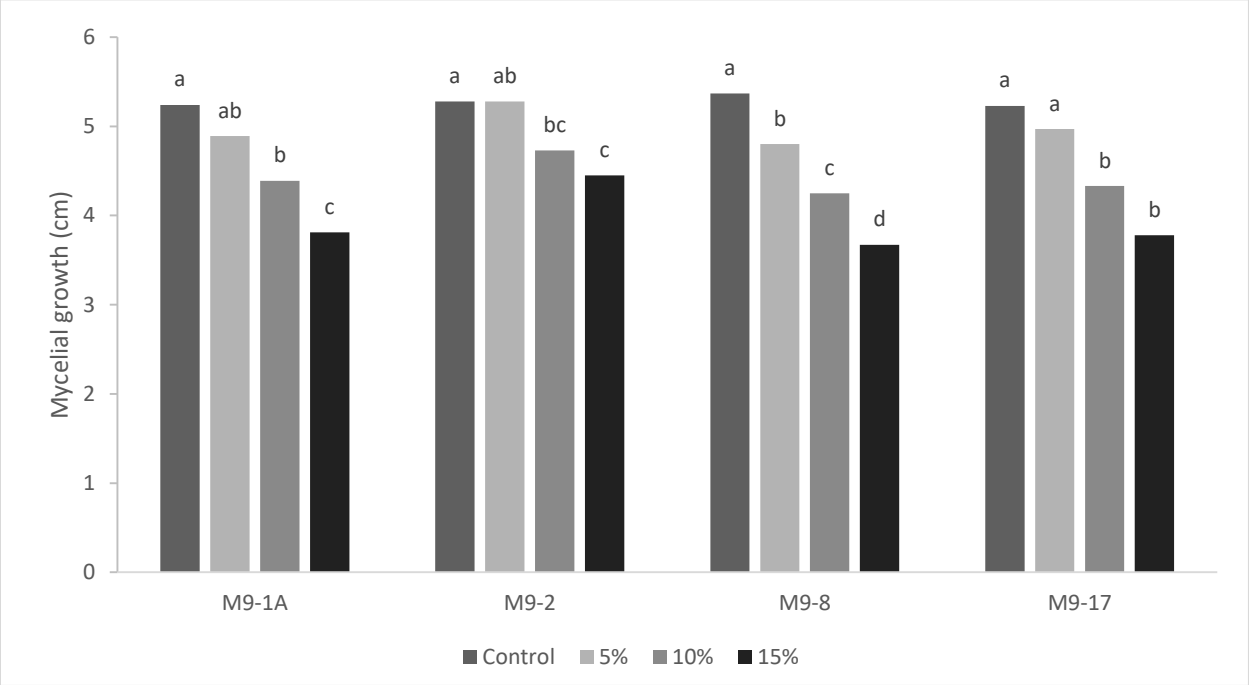


Figure 3-3. Cell-free filtrate concentrations effect on the mycelial growth of *Alternaria alternata*. M9-1A, M9-2 and M9-8 = *Arthrobacter humicola*; M9-17 = *Arthrobacter psychrophenolicus*. Means with a letter in common within the same bacterial filtrate are not significantly different according to Fisher's protected LSD test (α level = 0.05).

Arthrobacter humicola M9-2 and M9-8 did not reduce the growth of *A. solani* at any filtrate concentration (P = 0.0862 and 0.1422). *Arthrobacter humicola* M9-1A filtrates had variable effects on *A. solani*. Lower concentrations (5 and 10%) increased growth whereas 15% filtrates did not affect the growth of the fungus (Fig. 3-4). *Arthrobacter psychrophenolicus* M9-17 did not inhibit the growth of *A. solani* at 5 and 10%, however significantly reduced the growth by 19% at 15% cell-free filtrate (Fig. 3-4).

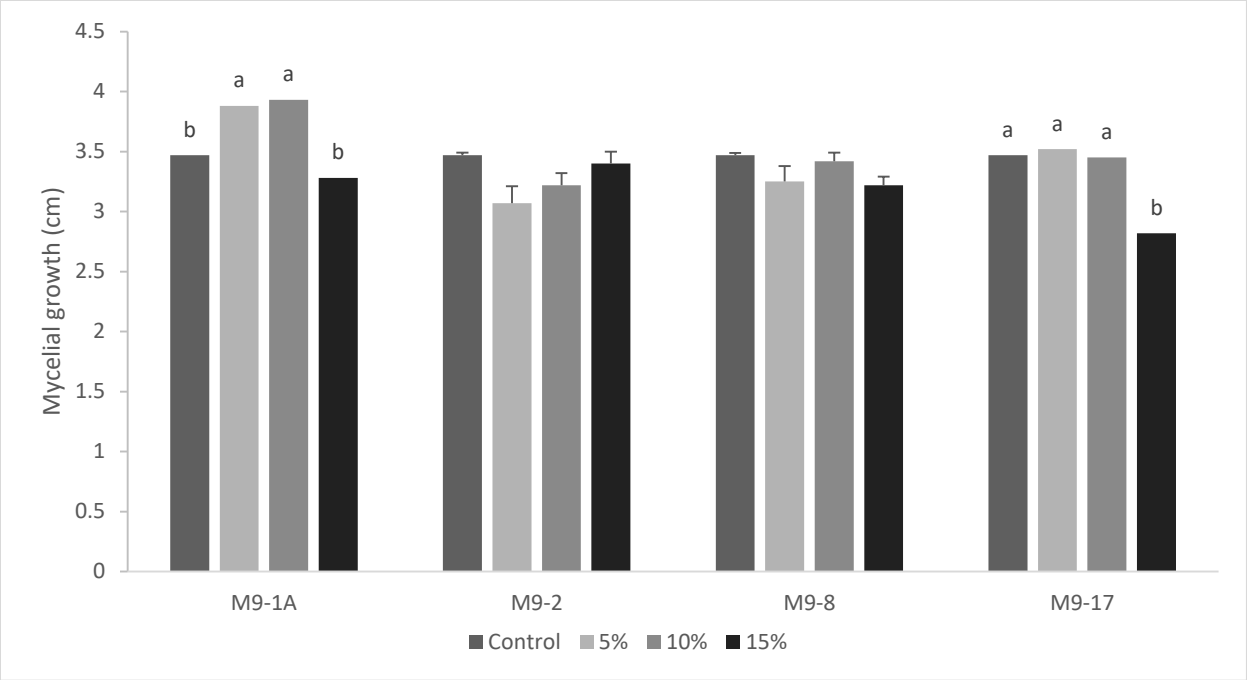


Figure 3-4. Cell-free filtrate concentrations effect on the mycelial growth of *Alternaria solani*. M9-1A, M9-2 and M9-8 = *Arthrobacter humicola*; M9-17 = *Arthrobacter psychrophenolicus*. Means with a letter in common within the same bacterial filtrate are not significantly different according to Fisher's protected LSD test (α level = 0.05). When ANOVA was not significant (P > 0.05), standard error bars were provided.

Arthrobacter humicola M9-2, and M9-8 as well as *A. psychrophenolicus* M9-17 did not suppress the growth of *B. cinerea* at any filtrate concentration (P = 0.1433, 0.3199, and 0.4422, respectively). *Arthrobacter humicola* M9-2 did not reduce *B. cinerea* at filtrate concentrations of 5 and 10%, however it did reduce the growth at 15% by 6% (Fig. 3-5).

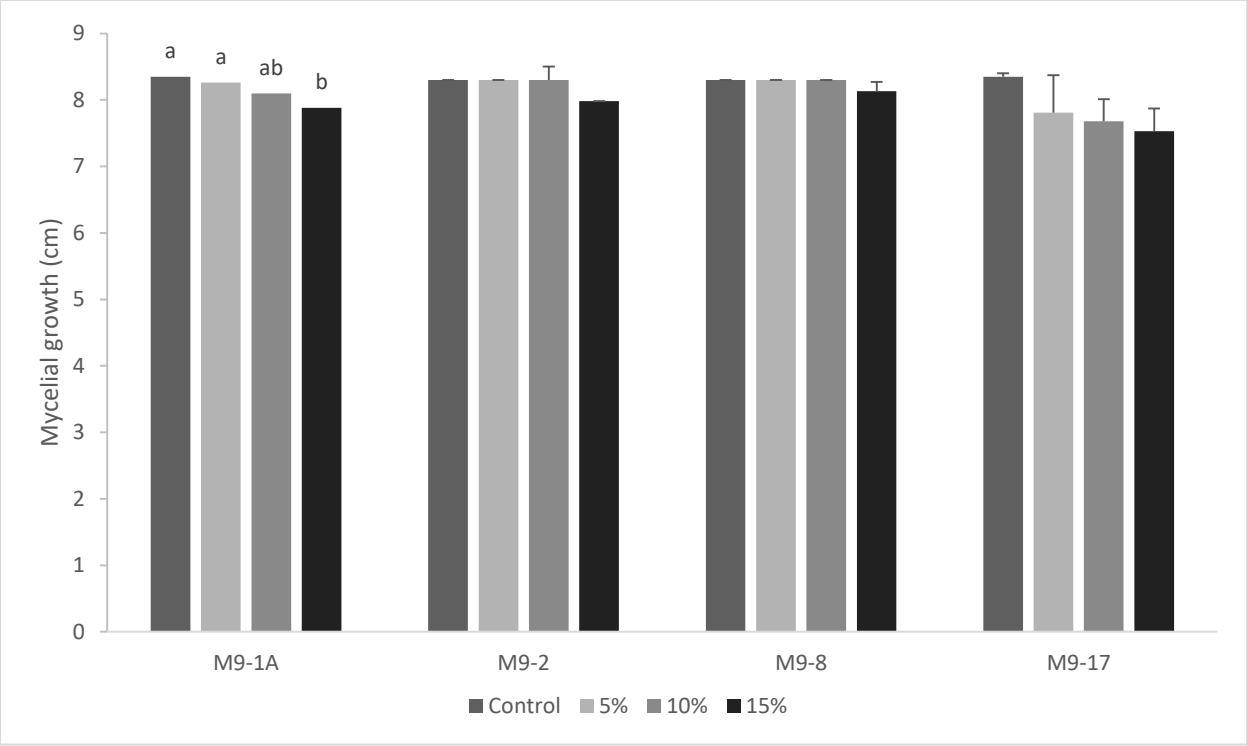


Figure 3-5. Cell-free filtrate concentrations effect on the mycelial growth of *Botrytis cinerea*. M9-1A, M9-2 and M9-8 = *Arthrobacter humicola*; M9-17 = *Arthrobacter psychrophenolicus*. Means with a letter in common within the same bacterial filtrate are not significantly different according to Fisher's protected LSD test (α level = 0.05). When ANOVA was not significant (P > 0.05), standard error bars were provided.

At all filtrate concentrations, *A. humicola* M9-8 did not inhibit *P. sulcatum* (P = 0.0747). *Arthrobacter humicola* M9-1A inhibited the growth of *P. sulcatum* at 15% filtrates by 20%, whereas at 5% filtrate, the growth of *P. sulcatum* increased and there was no suppression seen at 10% filtrate. *Arthrobacter psychrophenolicus* M9-17 inhibited the growth of *P. sulcatum* at 15% cell-free filtrates equally by an average of 10% (Fig. 3-6).

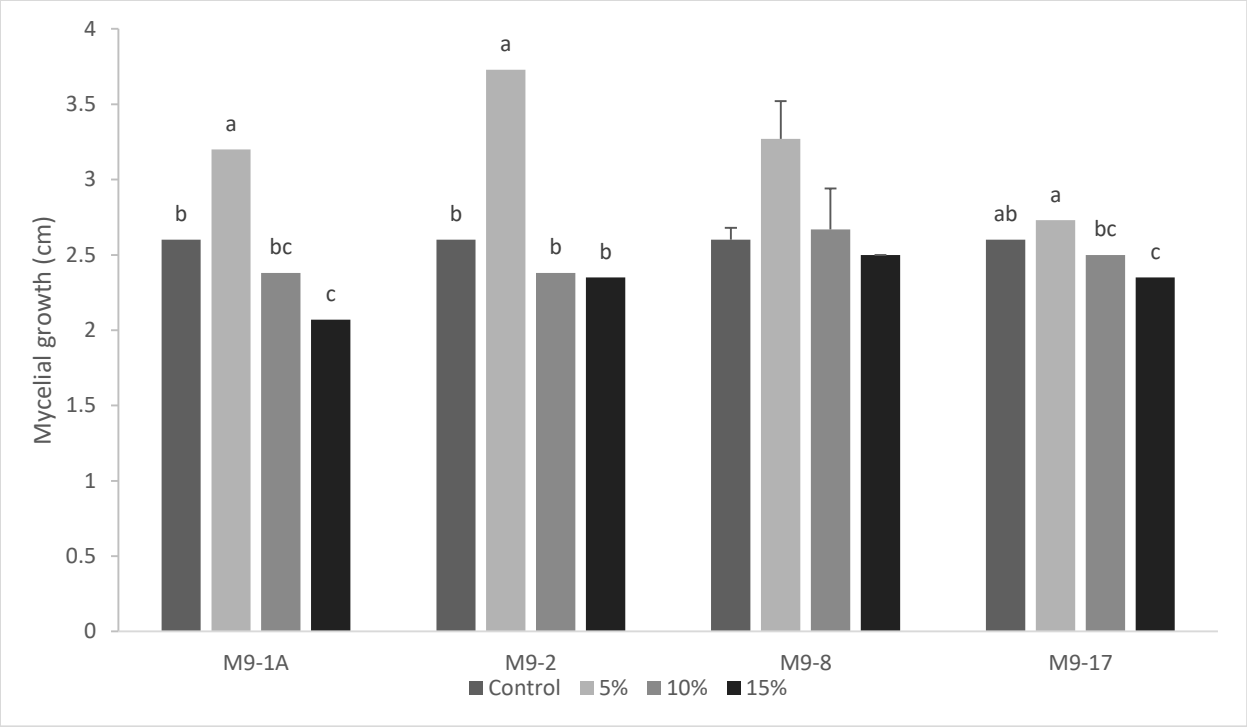


Figure 3-6. Cell-free filtrate concentrations effect on the mycelial growth of *Pythium sulcatum*. M9-1A, M9-2 and M9-8 = *Arthrobacter humicola*; M9-17 = *Arthrobacter psychrophenolicus*. Means with a letter in common within the same bacterial filtrate are not significantly different according to Fisher's protected LSD test (α level = 0.05). When ANOVA was not significant (P > 0.05), standard error bars were provided.

Arthrobacter humicola M9-2 and M9-8 did not suppress the growth of *R. stolonifer* (P = 0.2796 and 0.4847). All filtrates of *A. humicola* M9-1A reduced the growth of *R. stolonifer*, whereas the 15% cell-free filtrate revealed the highest inhibition (33%). The 10 and 15% cell-free filtrates of *A. psychrophenolicus* M9-17 were the only filtrates to suppress the growth of *R. stolonifer* by an average of 27% (Fig. 3-7).

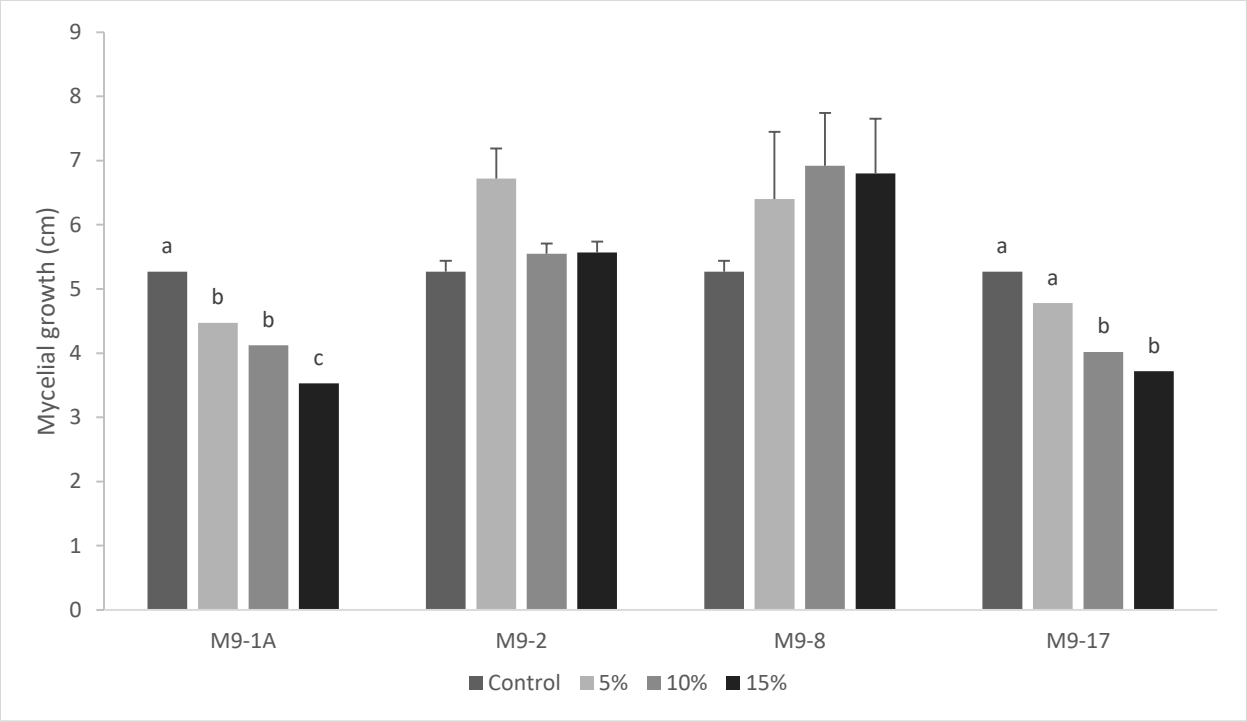


Figure 3-7. Cell-free filtrate concentrations effect on the mycelial growth of *Rhizopus stolonifer.* M9-1A, M9-2 and M9-8 = *Arthrobacter humicola*; M9-17 = *Arthrobacter psychrophenolicus*. Means with a letter in common within the same bacterial filtrate are not significantly different according to Fisher's protected LSD test (α level = 0.05). When ANOVA was not significant (P > 0.05), standard error bars were provided.

Arthrobacter humicola M9-1A, M9-2 and M9-8 did not reduce the growth of *S. sclerotiorum* at any filtrate concentration (P = 0.0547, 0.1922, and 0.1487, respectively). All filtrate concentrations of *A. psychrophenolicus* M9-17 equally reduced the growth of *S. Sclerotinia* by an average of 36% (Fig. 3-8).

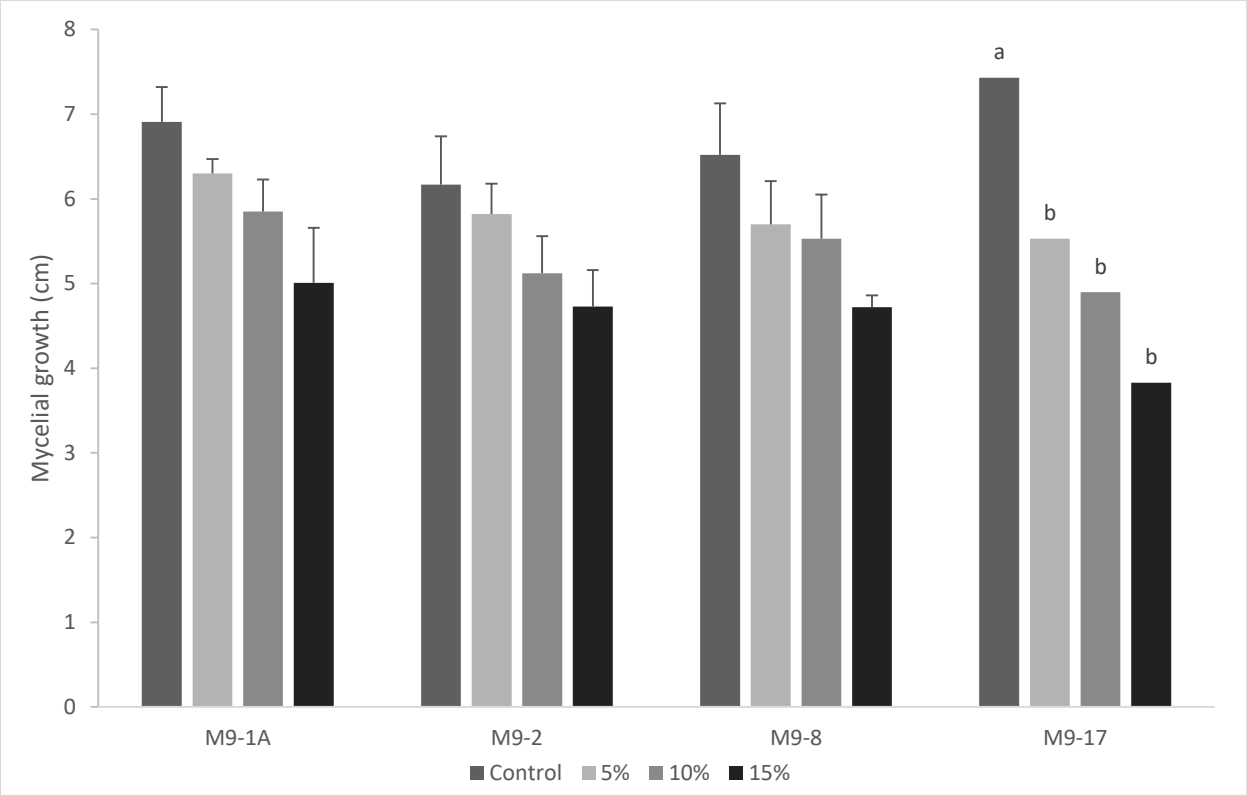


Figure 3-8. Cell-free filtrate concentrations effect on the mycelial growth of *Sclerotinia sclerotiorum*. M9-1A, M9-2 and M9-8 = *Arthrobacter humicola*; M9-17 = *Arthrobacter psychrophenolicus*. Means with a letter in common within the same bacterial filtrate are not significantly different according to Fisher's protected LSD test (α level = 0.05). When ANOVA was not significant (P > 0.05), standard error bars were provided.

Statistical analysis revealed no significant inhibition against *FORL* (M9-1A P = 0.8507, M9-2 P = 0.9909, M9-8 P = 0.8241, M9-17 P = 0.9973), *R. solani* (M9-1A P = 0.1820, M9-2 P = 0.9097, M9-8 P = 0.4971, M9-17 P = 0.8964) and *V. dahliae* (M9-1A P = 0.4846, M9-2 P = 0.8722, M9-8 P = 0.1367, M9-17 P = 0.2110) for any bacterial filtrate, at any concentration.

3.4.3 Effect of cell-free filtrates against *Alternaria* rot (black mold) of tomato fruit

To evaluate the antimicrobial properties of *Arthrobacter* spp., cell-free filtrates were used to suppress symptoms of *Alternaria* rot on tomato fruit (Fig. 3-9). In absence of pathogen inoculation, the cell-free filtrates alone did not produce spoilage symptoms on the tomato fruit over a 4-day

incubation period. *Arthrobacter humicola* M9-1A, M9-2 and *A. psychrophenolicus* M9-17 significantly suppressed lesions of *Alternaria* rot on tomato fruit. *Arthrobacter humicola* M9-1A and M9-8, as well as *A. psychrophenolicus* M9-17 were equally the most inhibitory, reducing lesions by an average of 31%. *Arthrobacter humicola* M9-2 did not significantly reduce growth of *Alternaria* fruit rot on tomato fruit.

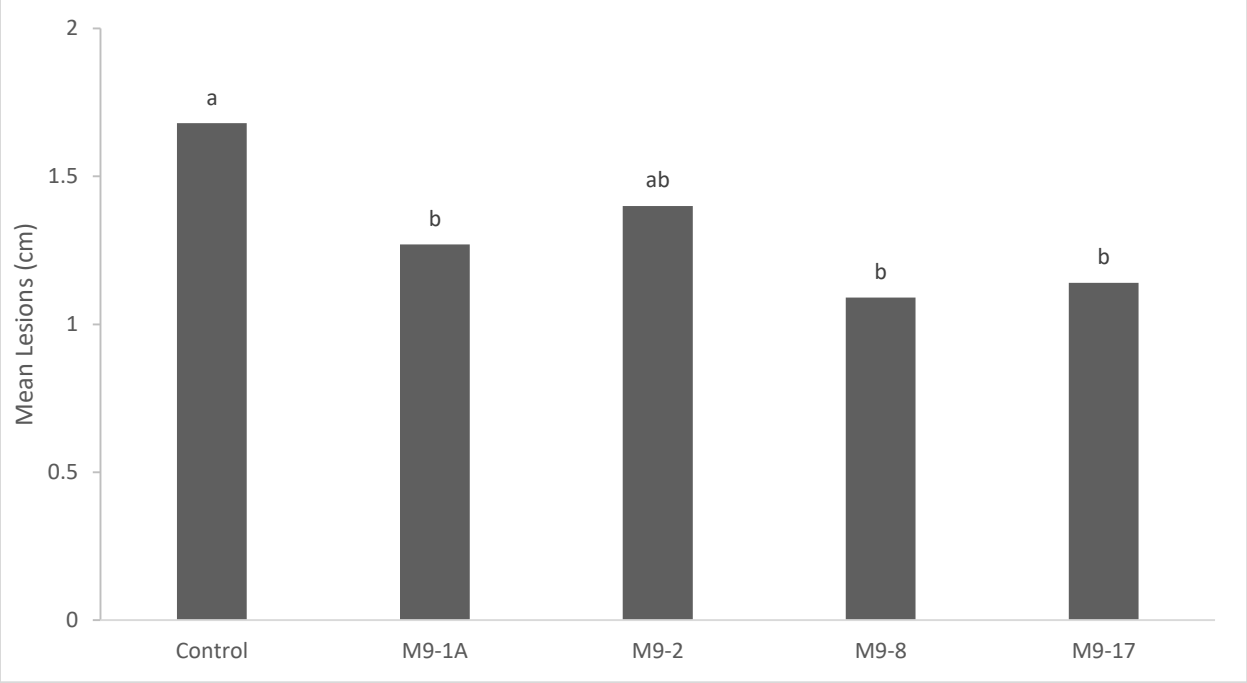


Figure 3-9. Effect of cell-free filtrates from *Arthrobacter humicola* M9-1A, M9-2, M9-8 and *Arthrobacter psychrophenolicus* M9-17 on the diameter of black mold lesions from *Alternaria alternata* on tomato fruit. Means with a letter in common are not significantly different according to Fisher's protected LSD test (α level = 0.05).

3.4.4 Identification of extracellular lytic enzyme production

To determine the presence of extracellular enzymes potentially involved in their antimicrobial activity, each bacterium was plated on TSA incorporated with 2% of skim milk powder, laminarin or cellulose, or 0.2% chitosan individually. *Arthrobacter humicola* M9-1A, M9-2, M9-8 and *A. psychrophenolicus* M9-17 showed a zone of digestion on TSA plates with 2% skim milk powder after a 5-day incubation period (Fig. 3-10). *Arthrobacter humicola* M9-1A showed the largest zone

of digestion compared to the other bacteria. *Arthrobacter humicola* M9-8 and *A. psychrophenolicus* M9-17 presented the smallest clearing zones. None of the bacteria produced a zone of clearance on TSA with 2% laminarin, cellulose or with 0.2% chitosan.

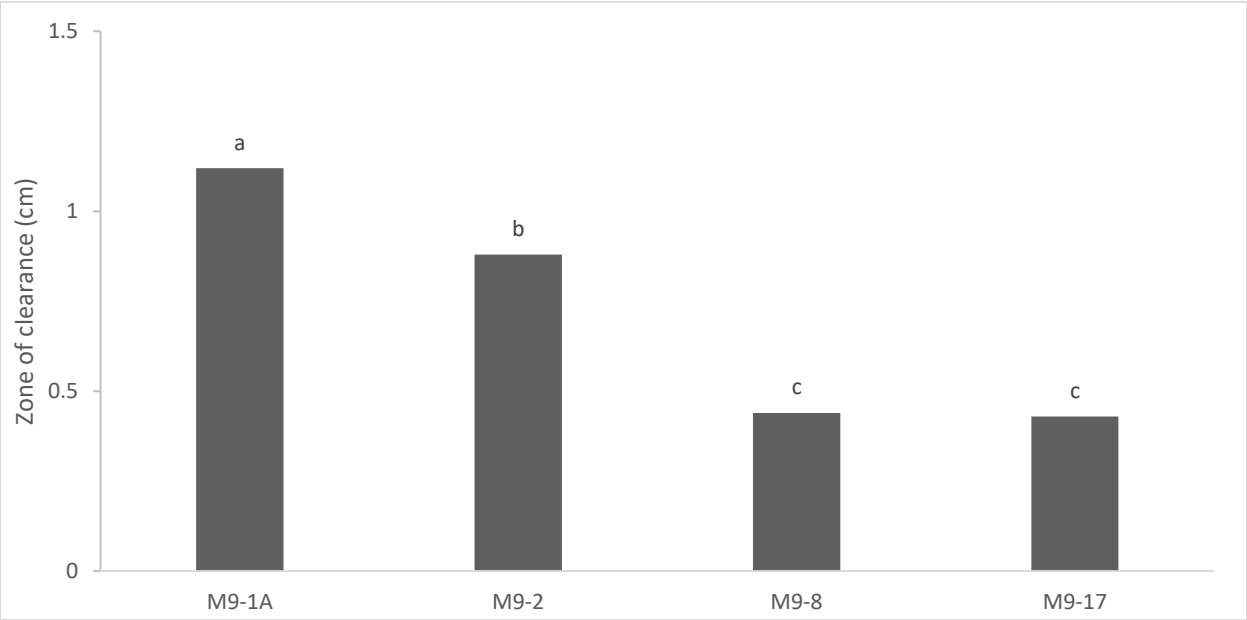


Figure 3-10. Detection of extracellular protease production from *Arthrobacter humicola* strains M9-1A, M9-2, M9-8 and *Arthrobacter psychrophenolicus* M9-17. Means with a letter in common are not significantly different according to Fisher's protected LSD test (α level = 0.05).

3.4.5 Effects of antimicrobial extracts from *Arthrobacter* spp. against *Alternaria alternata*

Prior to day 6 of incubation, no significant difference was observed for radial mycelial growth of *A. alternata* for any of the treatments. At day 6, all extracts from *A. humicola* as well as *A. psychrophenolicus* M9-17 significantly reduced the growth of *A. alternata* by an average of 20% (Fig. 3-11). By day 7, all bacterial extracts significantly reduced the growth of *A. alternata* equally by an average of 36% (Fig. 3-12).

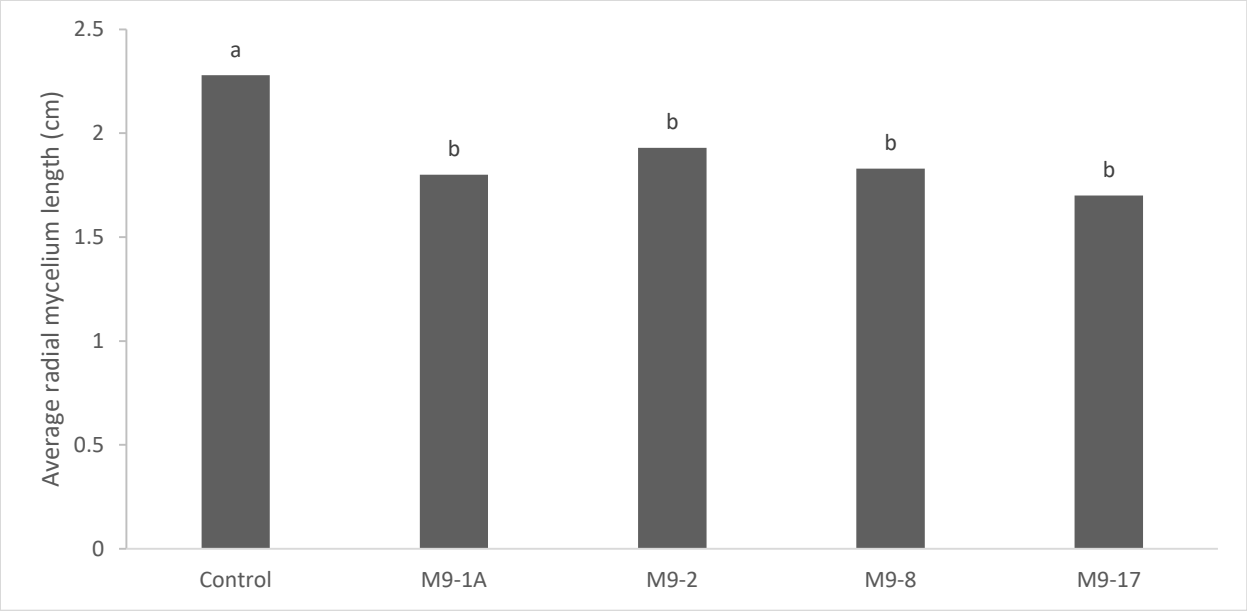


Figure 3-11. Effect of bacterial extracts from *Arthrobacter humicola* strains M9-1A, M9-2, M9-8 and *Arthrobacter psychrophenolicus* M9-17 on mycelial growth of *Alternaria alternata* at day six. Control is ethyl acetate. Means with a letter in common are not significantly different according to Fisher's protected LSD test (α level = 0.05).

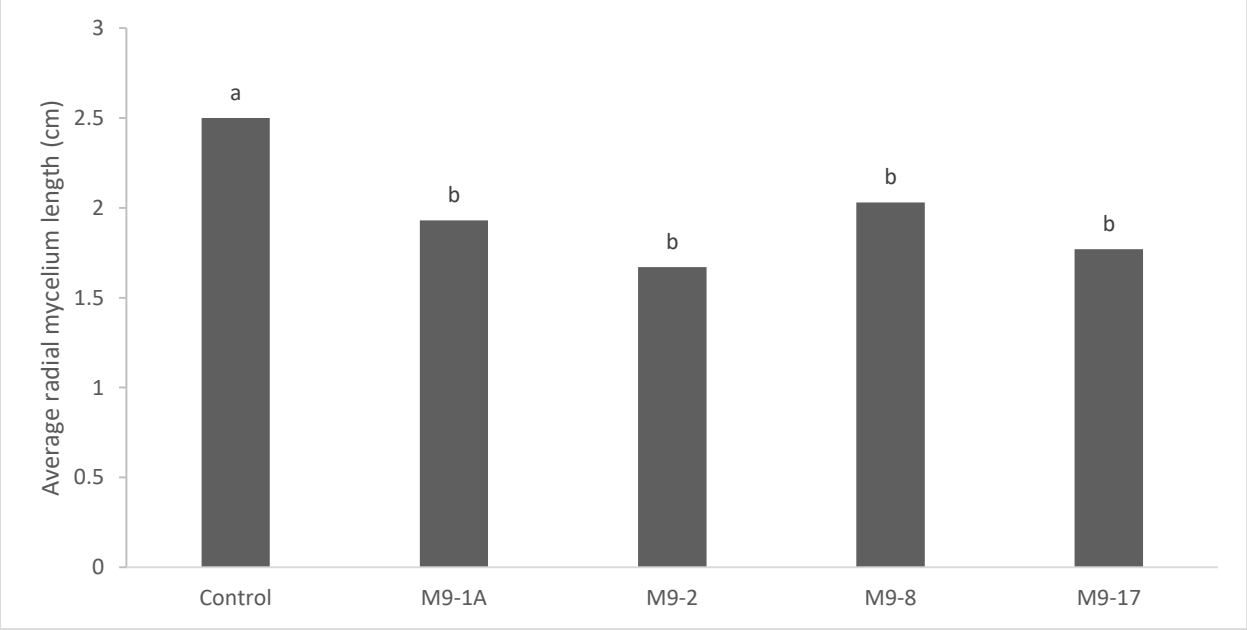


Figure 3-12. Effect of bacterial extracts from *Arthrobacter humicola* strains M9-1A, M9-2, M9-8 and *Arthrobacter psychrophenolicus* M9-17 on mycelial growth of *Alternaria alternata* at day seven. Control is ethyl acetate. Means with a letter in common are not significantly different according to Fisher's protected LSD test (α level = 0.05).

Results showed that the ethyl acetate control did not suppress the growth of *A. alternata*. However, bacterial extracts from *A. humicola* M9-1A, and *A. psychrophenolicus* M9-17 demonstrated zones of inhibition (Fig. 3-13). *Arthrobacter humicola* strain M9-2 and M9-8 showed no inhibition. *Arthrobacter humicola* M9-1A and *A. psychrophenolicus* M9-17 revealed the largest inhibition zones (Fig. 3-13 and Table 3-1).

Table 3-1. Drop bioassay observations of zones of inhibition from bacterial extracts of *Arthrobacter humicola* M9-1A, M9-2, M9-8 and *Arthrobacter psychrophenolicus* M9-17 assayed against *Alternaria alternata* at day four. Control is ethyl acetate.

Strain	Observations
Control	-
M9-1A	+++
M9-2	-
M9-8	-
M9-17	+++

Legend: - = no inhibition zone
+ = minimal inhibition zone
++ = clearly visible inhibition zone
+++ = largest inhibition zone

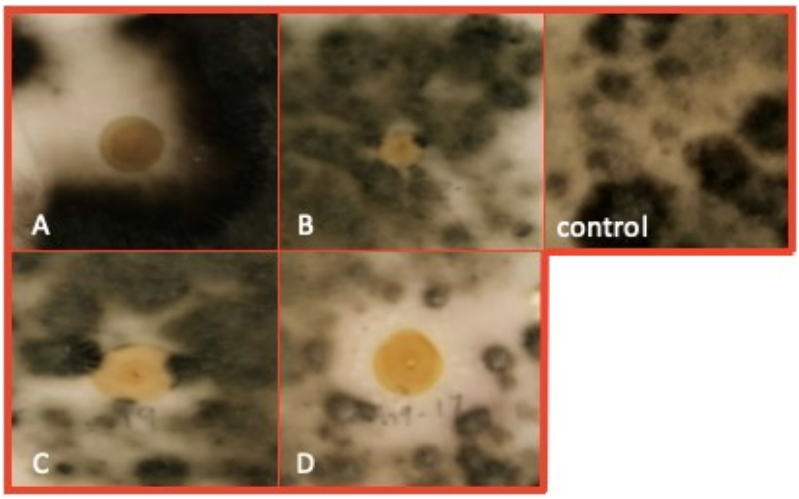


Figure 3-13. Drop bioassay of *Arthrobacter humicola* M9-1A (A), M9-2 (B), M9-8 (C) and *Arthrobacter psychrophenolicus* M9-17 (D) after four days of incubation. Control is ethyl acetate.

Fractions showing similar TLC profiles were combined. Results indicated the ethyl acetate control did not suppress the growth of *A. alternata.* However, fractions 1&2, 3&4 and 5&6 from *A. humicola* M9-1A exhibited the largest zones of inhibition (Figure 3-14 and Table 3-2). Fraction 7 also revealed clear inhibition, whereas fractions 8, 9 and 10 did not show any zones of inhibition (Figure 3-14 and Table 3-2).

Table 3-2. Drop bioassay observations of zones of inhibition from fractions of *Arthrobacter humicola* M9-1A assayed against *Alternaria alternata* at day four. Control is ethyl acetate.

Bacterial fraction	Observations
Control	-
1&2	+++
3&4	+++
5&6	+++
7	++
8	-
9	-
10	-

Legend: - = no inhibition zone
+ = minimal inhibition zone
++ = clearly visible inhibition zone
+++ = largest inhibition zone

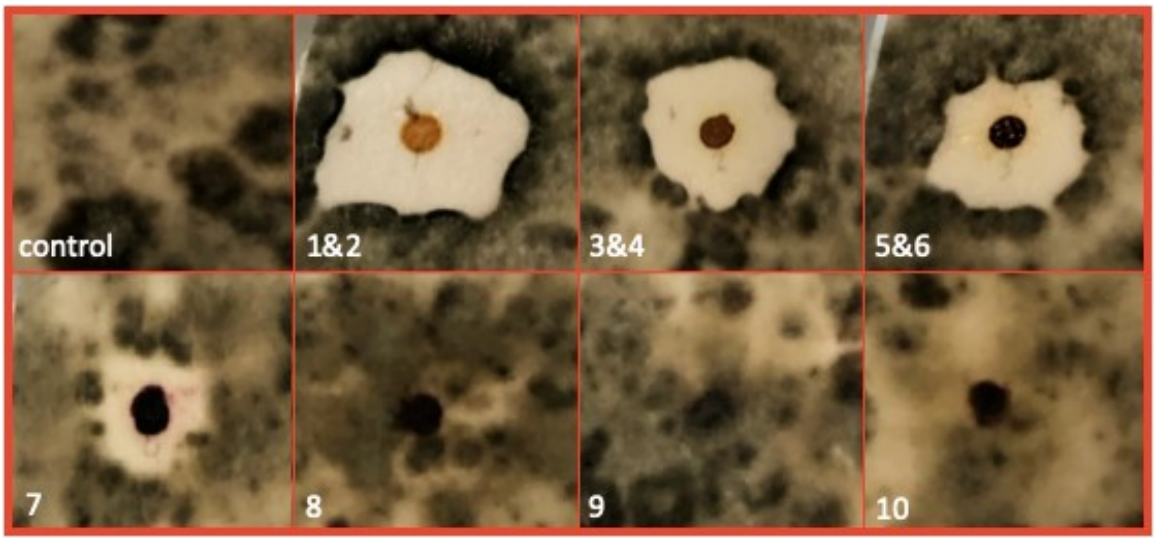


Figure 3-14. Drop bioassay of *Arthrobacter humicola* M9-1A fractions after four days of incubation. Control is ethyl acetate.

Prior to day 4 of incubation, no significant difference was observed between the radius length of *A. alternata* from the bacterial fractions of *A. humicola* M9-1A and the control. At day 4, fractions 1&2, 3&4 and 5&6 significantly reduced the growth of *A. alternata* by an average of 14% (Fig. 3-15). By day 5, fractions 1&2, 3&4 and 5&6 significantly reduced the growth of *A. alternata* equally. Their inhibitory activity was maintained, suppressing the growth by an average of 18% (Fig. 3-16). Fraction 7 did not significantly reduce the mycelial growth at either time period (Figure 3-15 and Fig. 3-16).

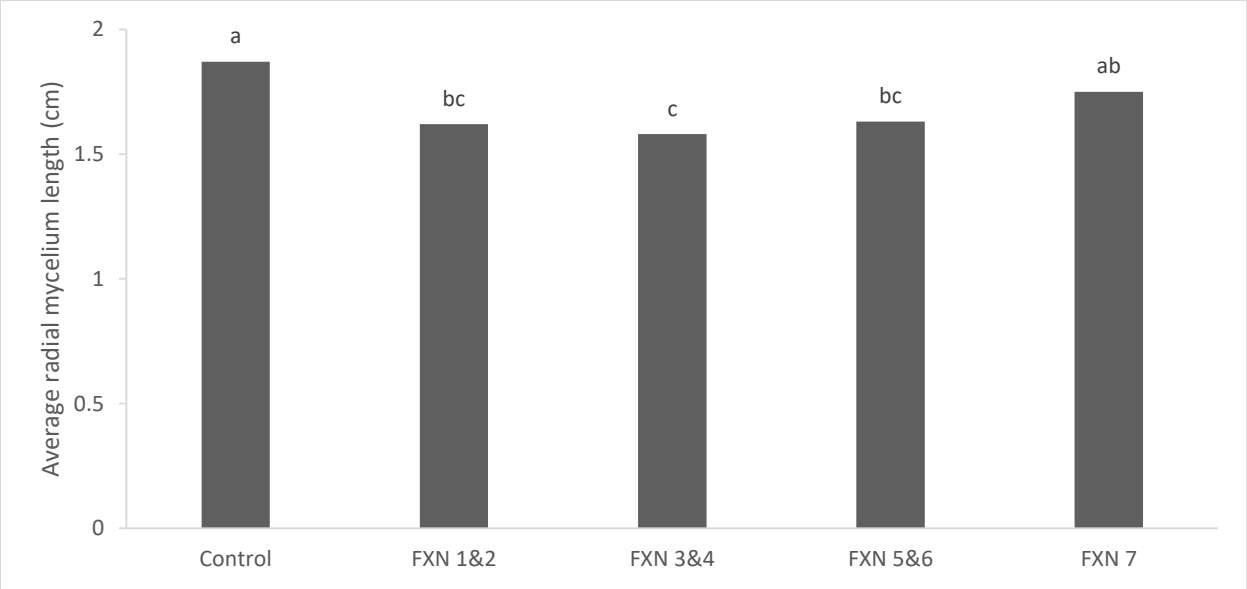


Figure 3-15. Effect of bacterial fractions from *A. humicola* M9-1A on mycelial growth of *A. alternata* at day four. Control is ethyl acetate. Means with a letter in common are not significantly different according to Fisher's protected LSD test (α level = 0.05).

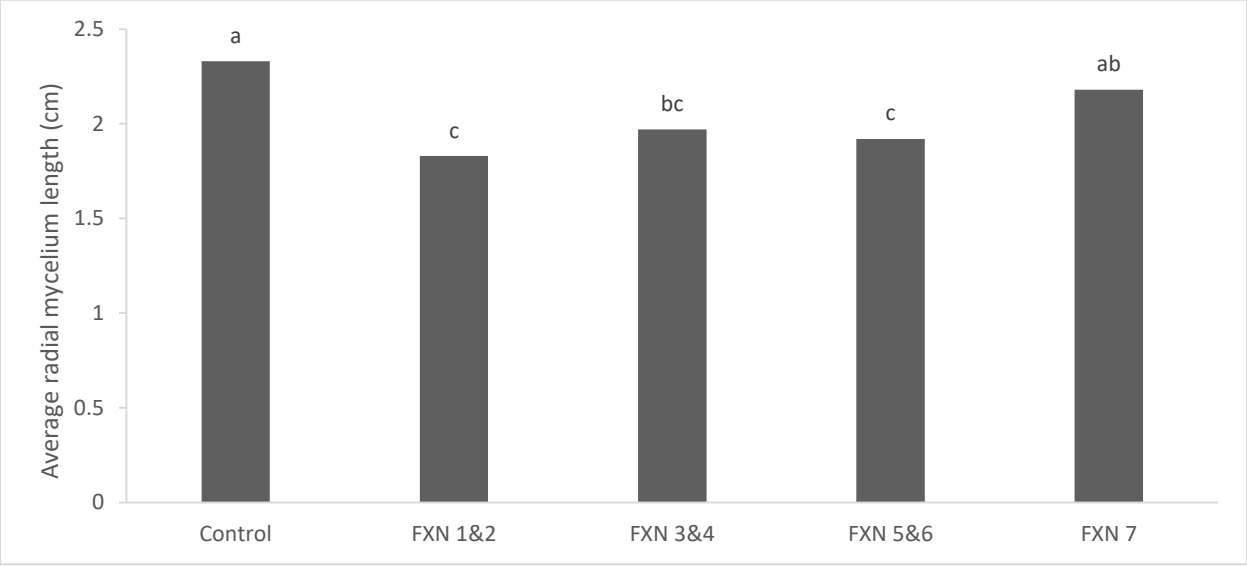


Figure 3-16. Effect of bacterial fractions from *Arthrobacter humicola* M9-1A on mycelial growth of *Alternaria alternata* at day five. Control is ethyl acetate. Means with a letter in common are not significantly different according to Fisher's protected LSD test (α level = 0.05).

The ethyl acetate crude extract (dark yellow oil, 2.021 g) was subject to normal-phase flash chromatography producing nine fractions. Results indicated the ethyl acetate control did not

suppress the growth of *A. alternata.* However, fractions 1, 2, 4, 5, and 7 from *A. psychrophenolicus* M9-17 exhibited zones of inhibition (Fig. 3-17 and Table 3-3). Fractions 1, 4, and 7 provided the largest inhibition zones. Fractions 3, 6, and 9 did not show any zones of inhibition.

Table 3-3. Drop bioassay observations of zones of inhibition from bacterial fractions of *A. psychrophenolicus* M9-17 assayed against *A. alternata* at day three. Control is ethyl acetate.

Bacterial fraction	Observations
Control	-
1	+++
2	++
3	-
4	+++
5	+
6	-
7	++
8	-
9	-

Legend: - = no inhibition zone
+ = minimal inhibition zone
++ = clearly visible inhibition zone
+++ = largest inhibition zone

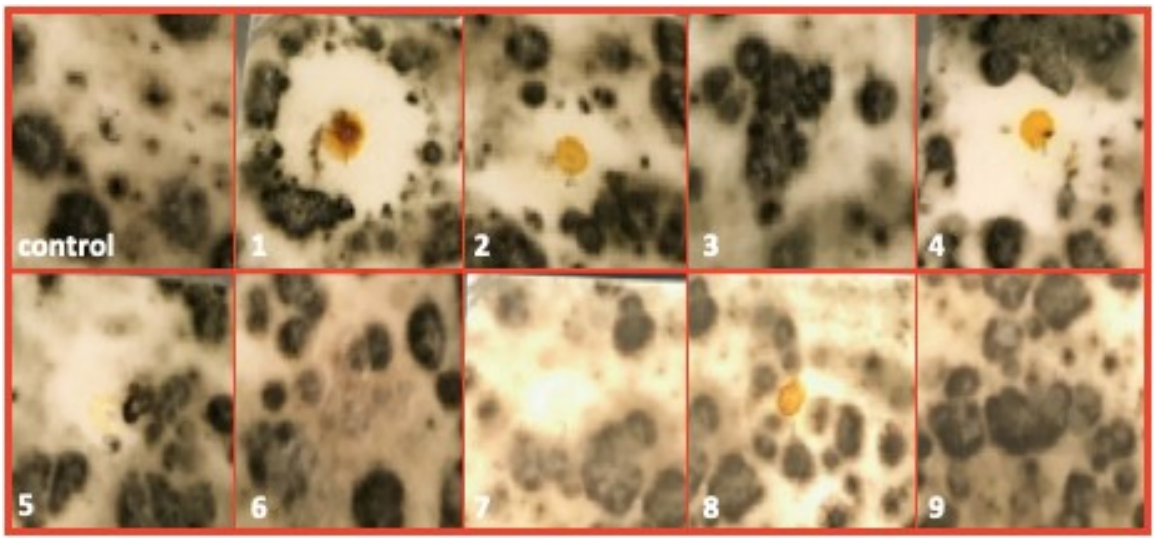


Figure 3-17. Drop bioassay of *Arthrobacter psychrophenolicus* M9-17 fractions after four days of incubation. Control is ethyl acetate.

3.4.6 Characterization of purified compounds from *Arthrobacter psychrophenolicus* M9-17

Fraction 4 (yellow oil, 0.1400 g) was the fourth fraction eluted from the normal-phase column and eluted with 90:10 ethyl acetate/methanol. Fraction 4 revealed 13 bands in preparative TLC analysis with band 8 being the major and most abundant compound. Band 8 from fraction 4 (compound 1) was the first compound isolated as a white powder (14.9 mg) and purified, and possessed a retention time of 6.2 minutes using HPLC. Compound 1 was determined to be *N*-acetyltryptamine following interpretation of homonuclear (^{1}H, ^{13}C, COSY) and heteronuclear (HSQC, HMBC) NMR spectra. The ^{1}H and ^{13}C NMR assignments for *N*-acetyltryptamine are reported in Table 3-4. The ^{1}H and ^{13}C spectra are provided in the annex (Fig. A-1 and Fig. A-2). The identity of the purified compound was confirmed via retention time and UV-profile against a reference standard (Sigma-Aldrich).

Table 3-4. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data for *N*-acetyltryptamine isolated from *Arthrobacter psychrophenolicus* M9-17 in CD_3OD.

Position	δ_C, type	δ_H (*J* in Hz)
2	123.4, CH	7.06, s
3	113.2, C	
4	128.8, C	
5	119.2, CH	7.54, d (8.0)
6	119.6, CH	6.99, ddd (8.0, 7.0, 1.0)
7	122.3, CH	7.08, m
8	112.2, CH	7.32, dd (8.2, 1.0)
9	138.2, C	
10	26.2, CH_2	2.93, t (7.3)
11	41.6, CH_2	3.46, t (7.3)
13	173.3, C	
14	22.6, CH_3	1.91, s

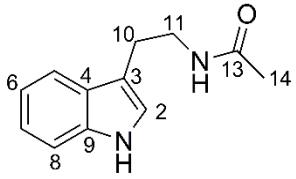


Figure 3-18. Structure of compound 1 isolated from the cell-free filtrate of *Arthrobacter psychrophenolicus* M9-17.

Fraction 7 (light yellow oil, 0.1780 g) was the seventh fraction eluted from the normal-phase column and eluted with 70:30 ethyl acetate/methanol. Fraction 7 produced 10 bands through preparative TLC analysis with band 8 being the major compound. Band 8 from fraction 7 (compound 2) was the second compound isolated and purified, and possessed a retention time of 5.6 minutes using HPLC. Compound **(2)** was isolated as a white solid (9.4 mg) with a molecular formula $C_{14}H_{18}O_4$, determined based on HRMS data; *m/z* 487.2555 $[M+H]^+$. Interpretation of homonuclear and heteronuclear NMR spectra identified the purified compound as cyclo(Pro-Leu-

Hyp-Tyr) (**2**), a new natural product (Fig. 3-19). The ^{1}H and ^{13}C spectra are provided in the annex (Fig. A-3 and Fig. A-4). The configuration of residues within the structure of **2** will be determined by Marfey's analysis.

Table 3-5. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data for cyclo(Pro-Leu-Hyp-Tyr) characterized from *Arthrobacter psychrophenolicus* M9-17 in DMSO-d_6

Residue	**Position**	**δ_C, type**	**δ_H (*J* in Hz)**
Pro	α	57.3, CH	2.83, dd (10.2, 6.4)
	β	28.6, CH_2	1.94, m
			1.57, m
	γ	21.4, CH_2	1.77, m
			1.54, m
	δ	44.7, CH_2	3.40, m
			3.20, m
	CO	168.5, C	
Leu	NH		8.38, d (4.4)
	α	55.3, CH	3.62, m
	β	42.3, CH_2	1.58, m
			1.43, ddd (13.7, 8.5, 5.4)
	γ	24.0, CH	1.69, m
	δ	21.6, CH_3	0.87, d (6.6)
	δ	22.9, CH_3	0.91, d (6.6)
	CO	166.4, C	
γHyp	α	56.0, CH	4.34, dd (11.3, 6.3)
	β	37.7, CH_2	2.06, dd (13.0, 6.3)
			1.86, m
	γ	66.6, CH	4.27, m
	γ-O<u>H</u>		5.15, bs
	δ	54.3, CH_2	3.57, dd (12.0, 4.6)
			3.18, d (12.0)
	CO	169.2, C	
Tyr	NH		8.12, d (3.9)
	α	58.2, CH	3.92, q (4.8)
	β	38.7, CH_2	2.92 dd (13.7, 5.4)
			2.75, dd (13.7, 4.9)
	1	126.0, C	
	2/6	130.9, CH	6.90, d (8.4)
	3/5	115.1, CH	6.66, d (8.4)
	4	156.4, C	
	4-O<u>H</u>		9.37, bs
	CO	165.0, C	

Cyclo(Pro-Leu-Hyp-Tyr) (**2**): 9.4 mg; white powder; $[\alpha]^{25}{}_{D}$ 2.721 (c 0.15, methanol), UV (methanol) λ_{max} (log ε) 230 (38.44), 276 (10.82), 322 (0.8027); for ^{1}H and ^{13}C NMR data see Table 3-5; HRMS *m/z* 487.2555 $[M+H]^+$ (calc. for $[C_{25}H_{35}N_4O_6]^+$, 487.2551), *m/z* 509.2368 $[M+Na]^+$ (calc. for $[C_{25}H_{34}N_4O_6Na]^+$, 509.2371).

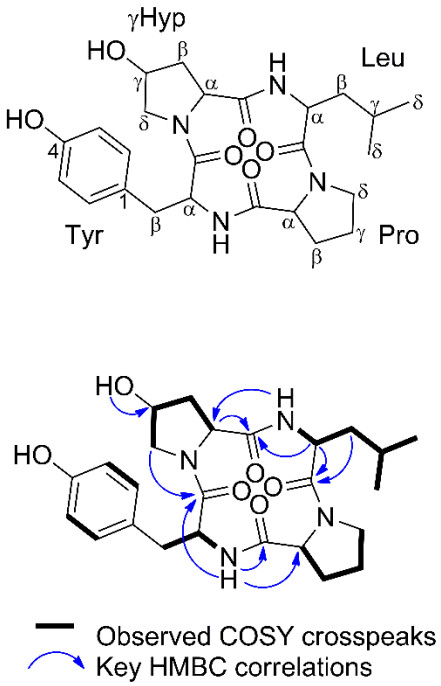


Figure 3-19. Structure of compound 2 isolated from the cell-free filtrate of *Arthrobacter psychrophenolicus* M9-17.

3.4.7 Determination of antimicrobial activity of isolated compounds through disk diffusion bioassay

The antimicrobial activity of compound 1 and the *N*- acetyltryptamine standard was tested against *A. alternata* using a disk diffusion assay. Prior to day 6 at a concentration of 5 mg/mL, no significance difference was found between the control and *N*-acetyltryptamine standard or compound 1. By day 6, at a concentration of 5 mg/mL, the *N*-acetyltryptamine standard and compound 1 significantly reduced the growth of *A. alternata* equally by an average of 7% (Fig. 3-20A). At day 8, the control reached the edge of the disk and the *N*-acetyltryptamine standard and compound 1 significantly reduced the growth of mycelium equally by an average of 13% (Fig. 3-

20B). At both days, the standard and compound 1 were observed to have the same antimicrobial activity.

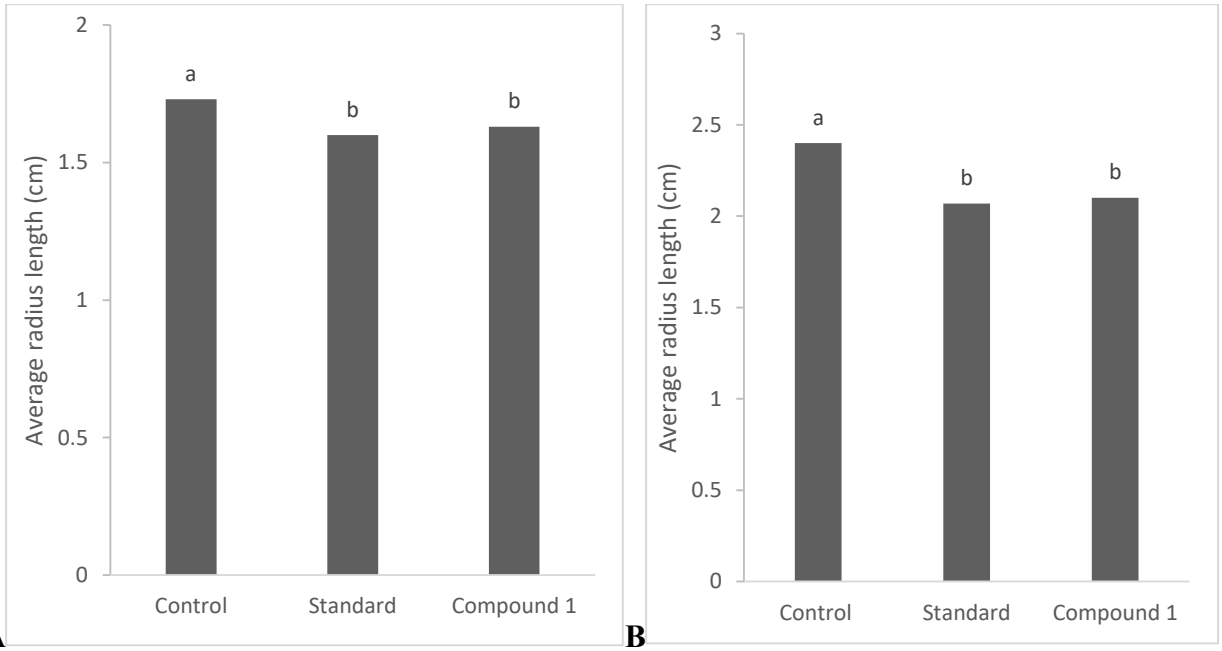


Figure 3-20. Effect of the *N*-acetyltryptamine standard and compound 1 from *A. psychrophenolicus* M9-17 at a concentration of 5 mg/mL on mycelial growth of *A. alternata* at day six (A) and at day eight (B). Control is methanol. Means with a letter in common are not significantly different according to Fisher's protected LSD test (α level = 0.05).

Prior to day 6 at a concentration of 50 mg/mL, no significant difference was observed between the control and *N*-acetyltryptamine standard or compound 1. By day 6, with a concentration of 50 mg/mL, the *N*-acetyltryptamine standard and compound 1 significantly reduced the growth of *A. alternata* equally by an average of 6% (Fig. 3-21A). By day 8, the control reached the distance of the disk and the *N*-acetyltryptamine standard and compound 1 significantly reduced the growth of mycelium equally. Their inhibitory activity increased to an average of 16% at day 8 (Fig. 3-21B). At both days, the standard and compound 1 demonstrated the same antimicrobial activity.

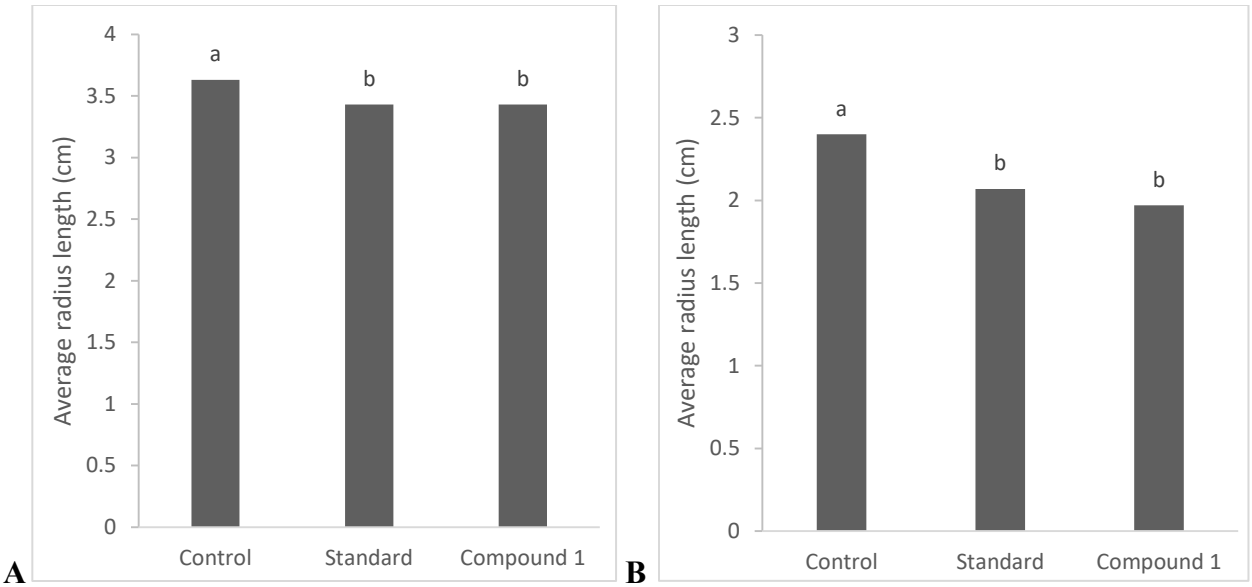


Figure 3-21. Effect of the *N*-acetyltryptamine standard and compound 1 from *Arthrobacter psychrophenolicus* M9-17 at a concentration of 50 mg/mL on mycelial growth of *Alternaria alternata* at day six (A) and at day eight (B). Control is methanol. Means with a letter in common are not significantly different according to Fisher's protected LSD test (α level = 0.05).

A dose-dependent study was performed to determine the antimicrobial potential of *N*-acetyltryptamine. Prior to day 6, no significant difference was observed. At day 6, concentrations of 0.5 mg/mL, 5 mg/mL and 50 mg/mL showed equal inhibition against *A. alternata* compared to the control (Fig. 3-22A). These concentrations significantly reduced the growth by an average of 9%. Lower concentrations, namely 0.005 and 0.05 mg/mL, did not show any inhibition against the fungus at day 6 (Fig. 3-22A). At day 8, all concentrations showed significant antimicrobial activity compared to the control (Fig. 3-22B). The concentrations that were the most inhibitory were 5 and 50 mg/mL. These concentrations equivalently reduced the mycelial growth by an average of 14%.

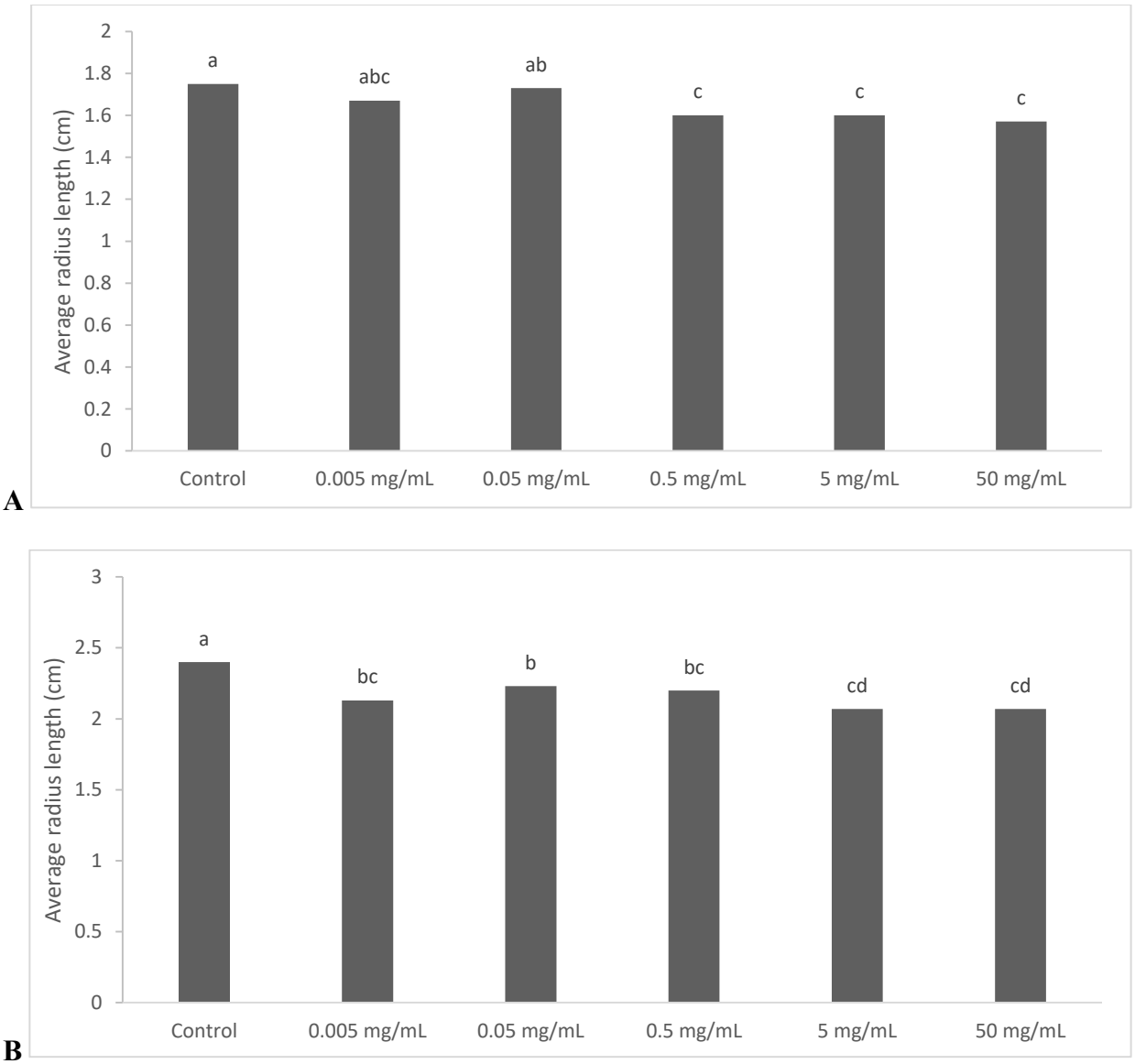
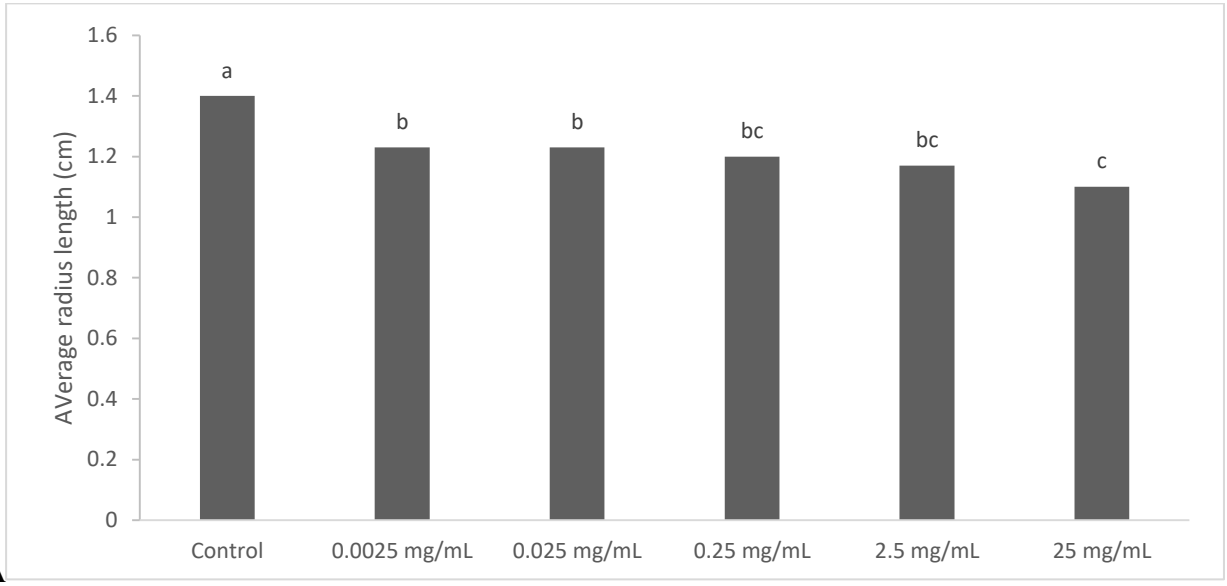


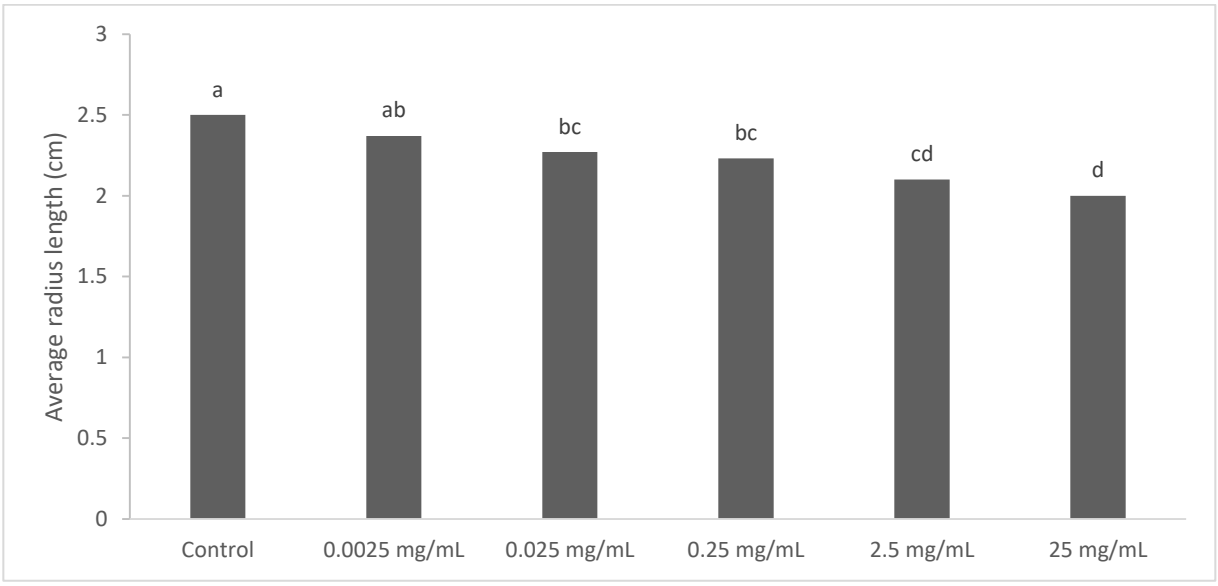
Figure 3-22. Dose-dependent effect of *N*-acetyltryptamine standard on mycelial growth of *Alternaria alternata* at day six (A) and at day eight (B). Control is methanol. Means with a letter in common are not significantly different according to Fisher's protected LSD test (α level = 0.05).

A dose-dependent study was performed to determine the antimicrobial potential of compound 2. At day 3, all concentrations showed a significant inhibition compared to the control. Concentrations at 0.25 mg/mL, 2.5 mg/mL and 25 mg/mL showed the most inhibition against *A. alternata* (Fig. 3-23A). These concentrations significantly reduced the growth by an average of 17%. Lower concentrations, namely 0.0025 and 0.025 mg/mL, showed inhibition albeit to a lesser

degree at day 3 (Fig. 3-23A). By day 8, the control grew to the disk. All concentrations except the lowest showed significant antimicrobial activity compared to the control (Figure 3-23B). The concentrations that were the most inhibitory were 2.5 and 25 mg/mL. These concentrations equivalently reduced the mycelial growth by an average of 18%.



A



B

Figure 3-23. Dose-dependent effect of compound 2 on mycelial growth of *Alternaria alternata* at day three (A) and at day eight (B). Control is methanol. Means with a letter in common are not significantly different according to Fisher's protected LSD test (α level = 0.05).

3.5 Discussion

In order to mitigate the health risks, environmental concerns, and pathogen resistance associated with synthetic chemical pesticides, microbial antagonists have been previously tested to determine their antagonistic properties against plant pathogens (Cloutier et al. 2020; Luo et al. 2019; Mohamed et al. 2017). In this study, the antifungal and anti-oomycete activity of *A. humicola* strains M9-1A, M9-2, and M9-8 as well as *A. psychrophenolicus* strain M9-17 were tested against various plant pathogens. In addition, the extracellular compounds produced by these bacteria were investigated *in vitro* and *in vivo* on tomato fruit to determine their antimicrobial activity, and the extracellular compounds were isolated and characterized in order to determine chemical compounds responsible for the antifungal activity.

Among the four tested bacterial strains, *A. humicola* M9-1A and *A. psychrophenolicus* M9-17 were the most inhibitory suppressing seven of nine tested phytopathogens whereas *A. humicola* M9-2 and M9-8 each only reduced four of nine plant pathogens (Table 3-6). Previous work has demonstrated antimicrobial activity from bacteria within the *Arthrobacter* genus. Specifically, *Arthrobacter agilis* reduced *B. cinerea* (Velazquez-Becerra et al. 2013), unspecified *Arthrobacter* spp. showed strong antimicrobial activity against *Pythium debaryanum* and *Fusarium oxysporum* (Gorlach-Lira and Stefaniak 2009), *Arthrobacter* sp. suppressed *R. solani* and *V. dahliae* (Bhattacharyya et al. 2020; Papasotiriou et al. 2013) and *Arthrobacter nicotianae* inhibited mycelial growth of *S. sclerotiorum* and *Pythium* sp. group G strain 87-202-3 (Axelrood et al. 1996; Kim et al. 2013). Previous studies from our lab showed that *A. humicola* inhibited the growth of *R. stolonifer*, *A. alternata*, *B. cinerea* and *V. dahliae* whereas *A. psychrophenolicus* inhibited *B. cinerea* and *V. dahliae* (Cloutier et al. 2020; Luo et al. 2019; Mohamed et al. 2017). To the best of

our knowledge, this is the first report of *A. humicola* suppressing *R. solani*, and *S. sclerotiorum* and *A. psychrophenolicus* inhibiting *R. solani*, *R. stolonifer,* and *S. sclerotiorum*.

Table 3-6. Effect of bacterial strains and bacterial filtrates on the mycelial growth of nine plant pathogens.

Pathogen	*M9-1A*		*M9-2*		*M9-8*		*M9-17*	
	Streak	Filtrate	Streak	Filtrate	Streak	Filtrate	Streak	Filtrate
A. alternata	×	×	×	×		×	×	×
A. solani						×		×
B. cinerea	×	×			×		×	×
FORL	×						×	
P. sulcatum	×	×	×		×			×
R. solani	×						×	
R. stolonifer		×		×			×	×
S. sclerotiorum	×	×	×	×	×	×	×	×
V. dahliae	×		×		×		×	

Legend: × = statistically significant when compared to the control;
Streak = confrontational bioassay; Filtrate = cell-free filtrate bioassay

The cell-free filtrate study determined the effects of extracellular antimicrobial compounds produced by *A. humicola* M9-1A, M9-2, M9-8, and *A. psychrophenolicus* M9-17 against a variety of phytopathogens (Table 3-6). The results confirmed that antibiosis may be a plausible mode of action used by *Arthrobacter* spp. The data indicated that when comparing the 15% cell-free filtrates to the control, *A. humicola* M9-1A suppressed five pathogens, *A. humicola* M9-2 and M9-8 reduced three pathogens and *A. psychrophenolicus* M9-17 inhibited the most with six pathogens. To our knowledge, this is the first report of the use of cell-free filtrates from *A. humicola* M9-1A, M9-2, M9-8 and *A. psychrophenolicus* M9-17 showing inhibition to various pathogens.

Each pathogen was also evaluated against lower concentrations to investigate the inhibitory effects of each bacterial filtrate. All bacterial filtrates showed no inhibition to *FORL*, *R. solani* and

V. dahliae indicating that antibiosis may not be a mechanism involved in their suppression. Conversely, *A. humicola* M9-8 inhibited the growth of *A. alternata* at the lowest concentration indicating that *A. humicola* M9-8 may produce a compound that is more inhibitory at lower concentrations or that the combination of compounds in the filtrate were acting in an additive or synergistic manner. *Pythium sulcatum* was the most sensitive towards the extracellular compounds produced by *A. psychrophenolicus* M9-17 at lower filtrate concentrations, whereas *R. stolonifer* was the most sensitive to the extracellular compounds produced by *A. humicola* M9-1A at lower concentrations. Inhibition was observed by all bacterial filtrates of *A. psychrophenolicus* M9-17 indicating that this bacterial species may produce extracellular compounds with higher efficacy than those of *A. humicola* against *S. sclerotiorum.*

An increase in growth was observed in *A. humicola* M9-1A at 5 and 10% filtrates against *A. solani*, as well as in *A. humicola* M9-1A and M9-2 at 5% filtrates against *P. sulcatum*. This might be attributed to the pathogen attempting to grow away from the antimicrobial compounds by quickly moving to a location more favourable to its growth. Alternatively, the bacteria may produce compounds that promote growth in the filtrate and the quantity of antifungal compounds that may be present may be insufficient to mitigate this effect.

Previous work reported the inhibition of two aquatic pathogenic fungi, *Saprolegnia australis* and *Mucor hiemalis*, by the cell-free supernatant of *Arthrobacter stackerbrandtii* and *Arthrobacter psychrolactophilus* (Lowrey et al. 2015). Another study indicated that the cell-free supernatant of *Arthrobacter polychromogenes* suppressed the growth of *F. oxysporum*, *Botrytis* sp., *R. solani*, and *Phytophthora infestans* (Santos et al. 2020). In the same study investigating Antarctic actinobacteria, *Arthrobacter globiformis*, *Arthrobacter psychrolactophilus*, *Arthrobacter scleromae*, and *Arthrobacter* sp. inhibited *Botrytis* sp. and *R. solani* (Santos et al. 2020). Minimal

data is available on the effect of cell-free filtrates from *Arthrobacter* spp. against other phytopathogens.

Antimicrobial activity was observed in both confrontation and 15% cell-free filtrate assays for a pathogen 11 times. However, differences in antimicrobial activity can be observed between the confrontation and 15% cell-free filtrates bioassays (Table 3-6). Some bacteria predominantly only inhibited pathogens in the confrontational bioassays, which was observed 11 times. The main difference between these bioassays are that the confrontational bioassay evaluates the antimicrobial activity of the bacterial isolates, which may partake in competition for nutrients and/or space, and/or antibiosis, whereas the cell-free filtrates evaluates the antimicrobial activity of the extracellular compounds produced (i.e., generally only antibiosis). For all bacteria tested, the bacterial cells inhibited more of the pathogens than the cell-free filtrates. This may be due to the additional mode of action of competition for nutrients and/or space. Additionally, the bacterial cells may need to be present in order to continuously produce antimicrobial compounds during pathogens growth in order to observe an antimicrobial effect. A previous study has also indicated that the presence of a pathogen can increase the production of antimicrobial metabolites by an antagonistic microorganism (DeFilippi et al. 2018). Finally, different antifungal compounds may be produced depending on the presence or absence of the pathogen (Martinez et al. 2006).

A possible reason for observing antimicrobial activity in bacterial filtrates in absence of bacterial activity in confrontation assays may be the type of antimicrobial compounds produced. To produce the bacterial filtrates, the bacteria are grown in a medium optimal for lipopeptide production. Therefore, the conditions and media may benefit the production of antimicrobial compounds. A study has indicated that the type of media may influence the production of antimicrobial compounds (Martinez et al. 2006).

When the cell-free filtrates of each bacteria were tested *in vivo*, all of the bacterial filtrates except *A. humicola* M9-2 suppressed the incidence of black mold on tomatoes. This study showed that filtrates from *A. humicola* M9-1A and M9-8 as well as *A. psychrophenolicus* M9-17 were more inhibitory to black mold than *A. humicola* M9-2 filtrates. Conversely, no significant difference was observed between the bacterial filtrates at 15% for *A. alternata*. Previous work on bell peppers indicated that *A. humicola* strains M9-1A and M9-2 reduced the incidence of *Alternaria* rot (Luo et al. 2019). To the best of our knowledge, this is the first study to use cell-free filtrates from *Arthrobacter* spp. showing inhibition to black rot on tomato fruit.

Antimicrobial compounds may not be the sole source of the antimicrobial activity of cell-free filtrates. Reports have indicated that some bacteria produce extracellular lytic enzymes such as glucanases, proteases, chitinases and cellulases that may target the cell wall of fungi and oomycetes. Previous work reported that *Arthrobacter* spp., namely *Arthrobacter aureus* and *Arthrobacter luteus*, produced extracellular proteases (Aizono and Funatsu 1978; Michotey and Blanco 1994). *Arthrobacter* sp. and *A. luteus* have been previously shown to produce β-1,3-glucanases, which have resulted in the lysis of yeast cells (Ferrer 2006; Kitamura et al. 1972; Latzko and Hampel 1993). Previous work has also indicated the production of chitinases in *Arthrobacter* sp. (Morrissey et al. 1976). Two genes coding for the production of two major chitinases, ChiA and ChiB (ArChiA and ArChiB) have been reported from *Arthrobacter* sp. strain TAD20 (Lonhienne et al. 2001). The production of cellulases was reported in *Arthrobacter* sp. HPG166 (Huang et al. 2015). Our results demonstrated that none of the tested bacteria indicated the production of glucanases, chitinases or cellulases. However, all bacterial isolates indicated the production of extracellular proteases by producing digestion zones around the colonies on skim milk agar. *Arthrobacter humicola* M9-1A produced the largest digestion zone, indicating it may

produce different proteolytic enzymes and/or produce these enzymes at a higher concentration. Therefore, a potential mode of action of *A. humicola* M9-1A, M9-2, and M9-8 and *A. psychrophenolicus* M9-17 against fungi and oomycetes could be the disruption of the cell wall through protein hydrolysis. The produced proteases may hydrolyze peptide bonds of proteins present in the fungal and oomycetal cell wall causing disruption and eventually, cell lysis (Mabood et al. 2014). To help support this possible mode of action, the bacterial isolates with proteolytic activity should be grown in the presence of fungal and oomycetal proteins. If the quantity of fungal and oomycetal proteins is reduced, this may indicate that the secreted proteases impact the fungal cell wall and cause cell lysis (Schonbichler et al. 2020).

Antibiosis was further confirmed as a potential mode of action when the extracts from all *Arthrobacter* spp. indicated equal inhibition against *A. alternata* at day 7. *Arthrobacter humicola* strains M9-2 and M9-8 did not show any inhibition zone in TLC bioassays. This could be due to the lower amount of extract used in comparison to the disk assays or the conidia used in this assay may be less sensitive to the antimicrobial compounds than the mycelia.

Studies have demonstrated that the extract of *A. humicola* M9-1A, M9-8 and *A. psychrophenolicus* M9-17 inhibited the mycelial growth of *V. dahliae* and *F. sambucinum*, and the extract of *A. humicola* M9-2, M9-8 and *A. psychrophenolicus* M9-17 suppressed *P. sulcatum* (Mohamed et al. 2017). To our knowledge, this is the first report of *Arthrobacter* spp. extracts inhibiting the mycelial growth of *A. alternata*.

Following purification, an extracellular compound produced by *A. psychrophenolicus* strain M9-17 was isolated and identified as *N*-acetyltryptamine. NMR (^{1}H and ^{13}C) spectra of the compound were similar to those published by Van Nam et al. (2019). In that report, the compound was isolated from a marine actinomycete, namely *Micromonospora* sp. A258. Notably, *A.*

psychrophenolicus strain M9-17 was isolated from a disease suppressive marine compost produced from residues of the fishing industry. *N*-acetyltryptamine is a known analog of melatonin, which has been previously reported to provide antibacterial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa, Acinetobacter baumannii* (Tekbas et al. 2008). It is derived from tryptophan via distributed aromatic amino acid decarboxylase and either arylalkylamine *N*-acetyltransferase, or arylamine *N*-acetyl transferase 1 or 2 (Backlund et al. 2017). It is also a reaction product from the biosynthesis of melatonin via the serotonin *N*-acetyltransferase (De Angelis et al. 1998). *N*-acetyltryptamine is a member of the indoles group. It therefore possess an indole structure, which has previously been associated with bioactivity in medicine and identified as a plant growth regulator (Dekker et al. 1975). Substituted indoles have previously indicated antifungal activity against *Botrytis allii*, *Cladosporium cucumerinum*, *Penicillium italicum* and *Aspergillus niger* (Dekker et al. 1975). In addition, a previous study also evaluated the antifungal activity of indoles derivatives and reported their antifungal activity against *Fusarium graminearum*, *A. alternata*, *Helminthosporium sorokinianum*, *Pyricularia oryzae*, *F. oxysporum* f. sp. *vasinfectum*, *F. oxysporum* f. sp. *cucumerinum*, and *Alternaria brassicae* (Xu et al. 2010). *N*-acetyltryptamine has previously been reported to possess antibacterial properties, showing suppression against *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas syringae* pv. *actinidiae* and methicillin-resistant *Staphylococcus aureus* (MRSA) (Zhang et al. 2013).

Our study showed the antifungal activity of *N*-acetyltryptamine from *A. psychrophenolicus* against *A. alternata* (Fig. 3-20, 3-21). This is the first report of *N*-acetyltryptamine suppressing mycelial growth of *A. alternata*. Previous work reported the inhibition of *Phoma lingam* by *N*-acetyltryptamine (Pedras and Okanga 2000). *N*-acetyltryptamine has previously been isolated from

fungi such as *Fusarium proliferatum* (Silva et al. 2020) and *Fusarium chlamydosporum* (Wang et al. 2020), which may result in more resistance to this compound from this species. However, more testing is required to determine the effects of the compound against *Fusarium* spp.

A second extracellular antimicrobial compound was isolated from *A. psychrophenolicus* M9-17 and characterized as a novel cyclic tetrapeptide, specifically *cyclo*-(Pro-Leu-Hyp-Tyr). Our data reports the antifungal activity of *cyclo*-(Pro-Leu-Hyp-Tyr) against *A. alternata* (Fig. 3-23). Cyclic peptides have been isolated from plants, fungi, bacteria, sponges, algae and mammals in the past (Abdalla and McGaw 2018). Reports have shown bioactivity of cyclic peptides, specifically anticancer, antiviral, antibiotic, and antifungal activity (Khedr et al. 2015). More specifically, previous studies have indicated the identification of different cyclic tetrapeptides from marine microorganisms (Gao et al. 2014; He et al. 2013; Pinzon-Espinosa et al. 2017; Rungprorn et al. 2008; Xie et al. 2018). Similarly to our compound, cyclic tetrapeptides namely saccharopolytide A, androsamide and *cyclo*-(Ile-Pro-Leu-Pro), have been isolated from actinobacteria from marine sources, *Saccharopolyspora cebuensis* MCC 1A09850, *Nocardiopsis* strain CNT-189 and *Nocardiopsis* sp., respectively (Lee et al. 2020; Shin et al. 2003; Xie et al. 2018). Antifungal activity against *Glomerella cingulate*, *Colletotrichum gloeosporoides* Penzig, and *C. lagenarium* was reported from glomecidin, a cyclic tetrapeptide isolated from *Streptomyces lavendulae* H698 SY2 (Kunihiro and Kaneda 2003). Additionally, cereusitin A also demonstrated antifungal activity against *C. gloeosporoides* C26 (Pinzon-Espinosa et al. 2017). Minimal information is available on the antimicrobial activity against other phytopathogens.

Overall, our results show that *Arthrobacter* spp. can inhibit fungal and oomycetal pathogens *in vitro*, and suppress black mold on tomato fruit *in vivo*. This study suggests that antibiosis may be the primary mode of action of the tested *Arthrobacter* spp. The identification and antifungal

activity of compound 1 (*N*-acetyltryptamine) and of compound 2 (a cyclic tetrapeptide; Pro-Leu-Hyp-Tyr) further support that these compounds are involved in the inhibition of *A. alternata* by *A. psychrophenolicus*. Additionally, other results in this study indicate that other modes of action such as competition for nutrients and/or space, and parasitism may also play a role in the inhibition of plant pathogens by *A. humicola* and *A. psychrophenolicus*. Future work will attempt (i) to characterize specific proteases from each *Arthrobacter* spp., and (ii) to investigate the mechanism by which *N*-acetyltryptamine and *cyclo*-(Pro-Leu-Hyp-Tyr) suppress fungal pathogens.

3.6 References

Abdalla MA, McGaw LJ (2018) Natural cyclic peptides as an attractive modality for therapeutics: A mini review. Molecules 23. doi: 10.3390/molecules23082080.

Adamitsch BF, Karner F, Hampel W (2003) Proteolytic activity of a yeast cell wall lytic *Arthrobacter* species. Letters in Applied Microbiology 36: 227-229. doi: 10.1046/j.1472-765X.2003.01300.x.

Aizono Y, Funatsu M (1978) Biochemical studies on rice bran lipase.6. Studies on active groups of rice bran lipase. Agricultural and Biological Chemistry 42: 757-762. doi: 10.1080/00021369.1978.10863057.

Akpa E, Jacques P, Wathelet B, Paquot M, Fuchs R, Budzikiewicz H, Thonart P (2001) Influence of culture conditions on lipopeptide production by *Bacillus subtilis*. Applied Biochemistry and Biotechnology 91-3: 551-561. doi: 10.1385/abab:91-93:1-9:551.

Avis TJ (2007) Antifungal compounds that target fungal membranes: applications in plant disease control. Canadian Journal of Plant Pathology 29: 323-329. doi: 10.1080/07060660709507478

Axelrood PE, Clarke AM, Radley R, Zemcov SJV (1996) Douglas-fir root-associated microorganisms with inhibitory activity towards fungal plant pathogens and human bacterial pathogens. Canadian Journal of Microbiology 42: 690-700. doi: 10.1139/m96-094.

Backlund PS, Urbanski HF, Doll MA, Hein DW, Bozinoski M, Mason CE, Coon SL, Klein DC (2017) Daily rhythm in plasma *N*-acetyltryptamine. Journal of Biological Rhythms 32: 195-211. doi: 10.1177/0748730417700458.

Belpoggi F, Soffritti M, Guarino M, Lambertini L, Cevolani D, Maltoni C (2002) Results of long-term experimental studies on the carcinogenicity of ethylene-bis-dithiocarbamate (Mancozeb) in rats. Carcinogenesis Bioassays and Protecting Public Health: Commemorating the Lifework of Cesare Maltoni and Colleagues 982: 123-136. doi: 10.1111/j.1749-6632.2002.tb04928.x.

Bhattacharyya C, Banerjee S, Acharya U, Mitra A, Mallick I, Haldar A, Haldar S, Ghosh A, Ghosh A (2020) Evaluation of plant growth promotion properties and induction of antioxidative defense mechanism by tea rhizobacteria of Darjeeling, India. Scientific Reports 10. doi: 10.1038/s41598-020-72439-z.

Bourguet D, Delmotte F, Franck P, Guillemaud T, Reboud X, Vacher C, Walker A-S, Consortium REX (2016) Combining selective pressures to enhance the durability of disease resistance genes. Frontiers in Plant Science 7. doi: 10.3389/fpls.2016.01916.

Cloutier A, Tran S, Avis TJ (2020) Suppressive effect of compost bacteria against grey mould and *Rhizopus* rot on strawberry fruit. Biocontrol Science and Technology 30: 143-159. doi: 10.1080/09583157.2019.1695745.

De Angelis J, Gastel J, Klein DC, Cole PA (1998) Kinetic analysis of the catalytic mechanism of serotonin *N*-acetyltransferase (EC 2.3.1.87). Journal of Biological Chemistry 273: 3045-3050. doi: 10.1074/jbc.273.5.3045.

DeFilippi S, Groulx E, Megalla M, Mohamed R, Avis TJ (2018) Fungal competitors affect production of antimicrobial lipopeptides in *Bacillus subtilis* strain B9-5. Journal of Chemical Ecology 44: 374-383. doi: 10.1007/s10886-018-0938-0.

Dekker WH, Selling HA, Overeem JC (1975) Structure-activity-relationships of some antifungal indoles Journal of Agricultural and Food Chemistry 23: 785-791. doi: 10.1021/jf60200a041.

Derevnina L, Petre B, Kellner R, Dagdas YF, Sarowar MN, Giannakopoulou A, De la Concepcion JC, Chaparro-Garcia A, Pennington HG, van West P, Kamoun S (2016) Emerging oomycete threats to plants and animals. Philosophical Transactions of the Royal Society B-Biological Sciences 371. doi: 10.1098/rstb.2015.0459.

Dunne C, Crowley JJ, Moenne-Loccoz Y, Dowling DN, de Bruijn FJ, O'Gara F (1997) Biological control of *Pythium ultimum* by *Stenotrophomonas maltophilia* W81 is mediated by an extracellular proteolytic activity. Microbiology-UK 143: 3921-3931. doi: 10.1099/00221287-143-12-3921.

El-Bendary MA, Hamed HA, Moharam ME (2016) Potential of *Bacillus* isolates as bio-control agents against some fungal phytopathogens. Biocatalysis and Agricultural Biotechnology 5: 173-178. doi: 10.1016/j.bcab.2016.02.001.

Falardeau J, Wise C, Novitsky L, Avis TJ (2013) Ecological and mechanistic insights into the direct and indirect antimicrobial properties of *Bacillus subtilis* lipopeptides on plant pathogens. Journal of Chemical Ecology 39: 869-878. doi: 10.1007/s10886-013-0319-7.

Ferrer P (2006) Revisiting the *Cellulosimicrobium cellulans* yeast-lytic beta-1,3-glucanases toolbox: A review. Microbial Cell Factories 5. doi: 10.1186/1475-2859-5-10.

Gao C-H, Chen Y-N, Pan L-X, Lei F, Long B, Hu L-Q, Zhang R-C, Ke K, Huang R-M (2014) Two new cyclic tetrapeptides from deep-sea bacterium *Bacillus amyloliquefaciens* GAS 00152. Journal of Antibiotics 67: 541-543. doi: 10.1038/ja.2014.27.

Gorlach-Lira K, Stefaniak O (2009) Antagonistic activity of bacteria isolated from crops cultivated in a rotation system and a monoculture against *Pythium debaryanum* and *Fusarium oxysporum*. Folia Microbiologica 54: 447-450. doi: 10.1007/s12223-009-0062-1.

Hamdache A, Lamarti A, Aleu J, Collado IG (2011) Non-peptide metabolites from the genus *Bacillus*. Journal of Natural Products 74: 893-899. doi: 10.1021/np100853e.

He F, Bao J, Zhang X-Y, Tu Z-C, Shi Y-M, Qi S-H (2013) Asperterrestide A, a cytotoxic cyclic tetrapeptide from the marine-derived fungus *Aspergillus terreus* SCSGAF0162. Journal of Natural Products 76: 1182-1186. doi: 10.1021/np300897v.

Huang S, Deng G, Yang Y, Wu Z, Wu L (2015) Optimization of endoglucanase production from a novel bacterial isolate, *Arthrobacter* sp HPG166 and characterization of its properties. Brazilian Archives of Biology and Technology 58: 692-701. doi: 10.1590/s1516-89132015050256.

Igarashi Y, Yamamoto K, Fukuda T, Shojima A, Nakayama J, Carro L, Trujillo ME (2015) Arthroamide, a cyclic depsipeptide with quorum sensing inhibitory activity from *Arthrobacter* sp. Journal of Natural Products 78: 2827-2831. doi: 10.1021/acs.jnatprod.5b00540.

Jia C, Zhang L, Liu L, Wang J, Li C, Wang Q (2013) Multiple phytohormone signalling pathways modulate susceptibility of tomato plants to *Alternaria alternata* f. sp *lycopersici*. Journal of Experimental Botany 64: 637-650. doi: 10.1093/jxb/ers360.

Khedr AIM, Mohamed GA, Orabi MAA, Ibrahim SRM, Yamada K (2015) Staphylopeptide A, a new cyclic tetrapeptide from culture broth of *Staphylococcus* sp. Phytochemistry Letters 13: 11-14. doi: 10.1016/j.phytol.2015.05.007.

Kim J-W (2013) Biological control of *Sclerotinia sclerotiorum* in lettuce using antagonistic bacteria. Research in Plant Disease 19: 12-20. doi: 10.5423/rpd.2013.19.1.012.

Kitamura K, Kaneko T, Yamamoto Y (1972) Lysisof viable yeast-cells by enzymes of *Arthrobacter*-luteus .1. Isolation of lytic strain and studies on its lytic activity. Journal of General and Applied Microbiology 18: 57. doi: 10.2323/jgam.18.57.

Kohl J, Kolnaar R, Ravensberg WJ (2019) Mode of action of microbial biological control agents against plant diseases: relevance beyond efficacy. Frontiers in Plant Science 10. doi: 10.3389/fpls.2019.00845.

Kunihiro S, Kaneda M (2003) Glomecidin, a novel antifungal cyclic tetrapeptide produced by *Streptomyces lavendulae* H698SY2. Journal of Antibiotics 56: 30-33. doi: 10.7164/antibiotics.56.30.

Kurniawan O, Wilson K, Mohamed R, Avis TJ (2018) *Bacillus* and *Pseudomonas* spp. provide antifungal activity against gray mold and *Alternaria* rot on blueberry fruit. Biological Control 126: 136-141. doi: 10.1016/j.biocontrol.2018.08.001.

Latzko F, Hampel W (1993) Enxyme formation by a yeast-cell wall lytic *Arthrobacter* sp.: Formation of beta(1,3)-glucanase. Applied Microbiology and Biotechnology 40: 12-16.

Lee J, Gamage CDB, Kim GJ, Hillman PF, Lee C, Lee EY, Choi H, Kim H, Nam S-J, Fenical W (2020) Androsamide, a cyclic tetrapeptide from a marine *Nocardiopsis* sp., suppresses motility of colorectal cancer cells. Journal of Natural Products 83: 3166-3172. doi: 10.1021/acs.jnatprod.0c00815.

Lonhienne T, Mavromatis K, Vorgias CE, Buchon L, Gerday C, Bouriotis V (2001) Cloning, sequences, and characterization of two chitinase genes from the antarctic *Arthrobacter* sp

strain TAD20: Isolation and partial characterization of the enzymes. Journal of Bacteriology 183: 1773-1779. doi: 10.1128/jb.183.5.1773-1779.2001.

Lowrey L, Woodhams DC, Tacchi L, Salinas I (2015) Topographical mapping of the rainbow trout (*Oncorhynchus mykiss*) microbiome reveals a diverse bacterial community with antifungal properties in the skin. Applied and Environmental Microbiology 81: 6915-6925. doi: 10.1128/aem.01826-15.

Luo M, Purdy H, Avis TJ (2019) Compost bacteria provide antifungal activity against grey mold and *Alternaria* rot on bell pepper fruit. Botany 97: 221-230. doi: 10.1139/cjb-2018-0180.

Mabood F, Zhou X, Smith DL (2014) Microbial signaling and plant growth promotion. Canadian Journal of Plant Science 94: 1051-1063. doi: 10.4141/cjps2013-148.

Martinez C, Avis TJ, Simard J-N, Labonte J, Belanger RR, Tweddell RJ (2006) The role of antibiosis in the antagonism of different bacteria towards *Helminthosporium solani*, the causal agent of potato silver scurf. Phytoprotection 87: 69-75. doi: 10.7202/013975ar.

Michotey V, Blanco C (1994) Characterization of an endoserine protease secreted by *Arthrobacter aureus*. Applied and Environmental Microbiology 60: 341-343. doi: 10.1128/aem.60.1.341-343.1994.

Mohamed R, Groulx E, Defilippi S, Erak T, Tambong JT, Tweddell RJ, Tsopmo A, Avis TJ (2017) Physiological and molecular characterization of compost bacteria antagonistic to soil-borne plant pathogens. Canadian Journal of Microbiology 63: 411-426. doi: 10.1139/cjm-2016-0599.

Morrissey RF, Dugan EP, Koths JS (1976) Chitinase production by an *Arthrobacter* sp. lysing cells of *Fusarium roseum*. Soil Biology & Biochemistry 8: 23-28. doi: 10.1016/0038-0717(76)90016-x.

Ongena M, Jacques P (2008) *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. Trends in Microbiology 16: 115-125. doi: 10.1016/j.tim.2007.12.009.

Papasotiriou FG, Varypatakis KG, Christofi N, Tjamos SE, Paplomatas EJ (2013) Olive mill wastes: A source of resistance for plants against *Verticillium dahliae* and a reservoir of biocontrol agents. Biological Control 67: 51-60. doi: 10.1016/j.biocontrol.2013.07.008.

Pedras MSC, Okanga FI (2000) Metabolism of analogs of the phytoalexin brassinin by plant pathogenic fungi. Canadian Journal of Chemistry 78: 338-346. doi: 10.1139/cjc-78-3-338.

Pinzon-Espinosa A, Martinez-Matamoros D, Castellanos L, Duque C, Rodriguez J, Jimenez C, Ramos FA (2017) Cereusitin A, a cyclic tetrapeptide from a *Bacillus cereus* strain isolated from the soft coral Antillogorgia (syn. *Pseudopterogorgia*) elisabethae. Tetrahedron Letters 58: 634-637. doi: 10.1016/j.tetlet.2017.01.002.

Raaijmakers JM, Mazzola M (2012) Diversity and natural functions of antibiotics produced by beneficial and plant pathogenic bacteria. Annual Review of Phytopathology, Vol 50 50: 403-424. doi: 10.1146/annurev-phyto-081211-172908.

Rungprorn W, Siwu ERO, Lambert LK, Dechsakulwatana C, Barden MC, Kokpol U, Blanchfield JT, Kita M, Garson MJ (2008) Cyclic tetrapeptides from marine bacteria associated with the seaweed *Diginea* sp and the sponge *Halisarca ectofibrosa*. Tetrahedron 64: 3147-3152. doi: 10.1016/j.tet.2008.01.089.

Santos A, Nunez-Montero K, Lamilla C, Pavez M, Quezada-Solis D, Barrientos L (2020) Antifungal activity screening of Antarctic actinobacteria against phytopathogenic fungi. Acta Biologica Colombiana 25: 353-358. doi: 10.15446/abc.v25n2.76405.

Schonbichler A, Diaz-Moreno SM, Srivastava V, McKee LS (2020) Exploring the potential for fungal antagonism and cell wall attack by *Bacillus subtilis* natto. Frontiers in Microbiology 11. doi: 10.3389/fmicb.2020.00521.

Shin J, Seo Y, Lee HS, Rho JR, Mo SJ (2003) A new cyclic peptide from a marine-derived bacterium of the genus *Nocardiopsis*. Journal of Natural Products 66: 883-884. doi: 10.1021/np030075r.

Silva TL, Toffano L, Fernandes JB, da Silva MFdGF, de Sousa LRF, Vieira PC (2020) Mycotoxins from *Fusarium proliferatum*: new inhibitors of papain-like cysteine proteases. Brazilian Journal of Microbiology 51: 1169-1175. doi: 10.1007/s42770-020-00256-7.

Spadaro D, Droby S (2016) Development of biocontrol products for postharvest diseases of fruit: The importance of elucidating the mechanisms of action of yeast antagonists. Trends in Food Science & Technology 47: 39-49. doi: 10.1016/j.tifs.2015.11.003.

Sui Y, Wisniewski M, Droby S, Norelli J, Liu J (2016) Recent advances and current status of the use of heat treatments in postharvest disease management systems: Is it time to turn up the heat? Trends in Food Science & Technology 51: 34-40. doi: 10.1016/j.tifs.2016.03.004.

Tekbas OF, Ogur R, Korkmaz A, Kilic A, Reiter RJ (2008) Melatonin as an antibiotic: new insights into the actions of this ubiquitous molecule. Journal of Pineal Research 44: 222-226. doi: 10.1111/j.1600-079X.2007.00516.x.

Thornton CR, Wills OE (2015) Immunodetection of fungal and oomycete pathogens: Established and emerging threats to human health, animal welfare and global food security. Critical Reviews in Microbiology 41: 27-51. doi: 10.3109/1040841x.2013.788995.

Van Nam V, Huong DTM, Dao PT, Mai Anh N, Minh LTH, Van Cuong P (2019) Secondary metabolites produced by marine actinomycete *Micromonospora* sp. A258. Vietnam Journal of Chemistry 57:707-711. doi: 10.1002/vjch.201900098.

Velazquez-Becerra C, Macias-Rodriguez LI, Lopez-Bucio J, Flores-Cortez I, Santoyo G, Hernandez-Soberano C, Valencia-Cantero E (2013) The rhizobacterium *Arthrobacter agilis* produces dimethylhexadecylamine, a compound that inhibits growth of phytopathogenic fungi in vitro. Protoplasma 250: 1251-1262. doi: 10.1007/s00709-013-0506-y.

Wall MM, Biles CL (1993) Alternaria fruit rot of ripening chile peppers. Phytopathology 83: 324-328. doi: 10.1094/Phyto-83-324.

Wang Z-F, Zhang W, Xiao L, Zhou Y-M, Du F-Y (2020) Characterization and bioactive potentials of secondary metabolites from *Fusarium chlamydosporum*. Natural Product Research 34: 889-892. doi: 10.1080/14786419.2018.1508142.

Xie C-L, Niu S, Xia J-M, Peng K, Zhang G-Y, Yang X-W (2018) Saccharopolytide A, a new cyclic tetrapeptide with rare 4-hydroxy-proline moieties from the deep-sea derived actinomycete *Saccharopolyspora cebuensis* MCCC 1A09850. Natural Product Research 32: 1627-1631. doi: 10.1080/14786419.2017.1392956.

Xu H, Wang Q, Yang W-B (2010) Antifungal activities of some indole derivatives. Zeitschrift Fur Naturforschung Section C - A Journal of Biosciences 65: 437-439.

Zhang W, Wei S, Zhang J, Wu W (2013) Antibacterial activity composition of the fermentation broth of *Streptomyces djakartensis* NW35. Molecules 18: 2763-2768. doi: 10.3390/molecules18032763.

Chapter 4: Overall Conclusions

Biocontrol agents have shown promise in mitigating risks associated with synthetic chemicals (e.g., health and environmental impacts, resistant pathogens) while decrease food loss caused by phytopathogens. Bacteria isolated from disease suppressive composts have demonstrated antagonistic properties reducing phytopathogens *in vitro* and disease *in vivo* (Cloutier et al. 2020; Luo et al. 2019; Mohamed et al. 2017). This study evaluated the antimicrobial properties of well researched antagonistic bacteria, namely *Bacillus* and *Pseudomonas* spp., as well as some less studied bacteria, such as *Arthrobacter* spp. Furthermore, *Arthrobacter* spp. were studied to gain insight into their mode of action for their possible future use as biocontrol agents.

Bacterial antagonists such as *Bacillus* spp. and related *Firmicutes*, *Pseudomonas* spp., and *Arthrobacter* spp. have previously been reported to suppress various plant pathogens mycelial growth and spore germination and to suppress their associated diseases (Cloutier et al. 2020; Luo et al. 2019; Mohamed et al. 2017). In this study, these bacterial isolates were further investigated to determine their antifungal activity against *F. sambucinum* and their effect on post-harvest loss caused by Fusarium dry rot in potato tubers. This study reported that all 32 tested bacterial isolates reduced the mycelial growth of *F. sambucinum in vitro*, albeit to varying degrees. When testing the antagonists against Fusarium dry rot, all antagonistic bacteria suppressed the internal dry rot lesions whereas, all but *B. subtilis* F9-2 and M9-7 suppressed the dry rot surface lesions at the highest concentration tested. The most inhibitory bacteria observed to suppress *F. sambucinum* and its symptoms on potato tubers were *P. moraviensis* F9-6, *P. koreensis* F9-9, *P. gessardii* M9-16, and *B. subtilis* B9-8. The bacterial extracts of *P. gessardii* M9-16, *P. koreensis* F9-9 and *P. moraviensis* F9-6 suppressed the mycelial growth of *F. sambucinum* whereas, *B. subtilis* B9-8, *P.*

koreensis F9-9 and *P. moraviensis* F9-6 inhibited conidial germination of *F. sambucinum*, which indicated antibiosis as a potential mode of action for these bacteria.

It was noted during *F. sambucinum*/dry rot work that some lesser studied bacteria, specifically *Arthrobacter* spp. were occasionally among the most effective in inhibiting *F. sambucinum* growth and suppressing dry rot. Limited research has been performed with *Arthrobacter* spp. and specifically with regard to their antifungal mode of action. Previous work reported some *Arthrobacter* spp. producing antimicrobial compounds, specifically arthroamide and dimethylhexadecylamine (Igarashi et al. 2015; Velazquez-Becerra et al. 2013), but little else was known. In the present work, *Arthrobacter* spp., namely *A. humicola* M9-1A, M9-2 and M9-8 and *A. psychrophenolicus* M9-17, were assayed against various plant pathogens to determine their antimicrobial potential and to gain insight into a potential mode of action. The *in vitro* results indicated that all *Arthrobacter* spp. or their cell-free filtrates can inhibit the growth of multiple plant pathogens. All bacterial isolates revealed the production of extracellular proteases, with *A.* humicola M9-1A showing the higher proteolytic activity. The cell-free filtrates showed antifungal activity against black mold disease on tomato fruit. *Arthrobacter psychrophenolicus* M9-17 was further investigated, as it was the most efficient bacteria *in vitro* (most pathogens inhibited) and its bacterial extract showed direct antimicrobial activity. Finally, two antifungal compounds were isolated from this bacterial isolate. Compound 1 was identified as *N*-acetyltryptamine, which has previously been isolated from a different marine actinomycete, *Micromonospora* sp. A258 (Van Nam et al. 2019). Compound 2 was identified as a novel cyclic tetrapeptide (Pro-Leu-Hyp-Tyr), which to the best of our knowledge is the first report of this compound.

Future work should include further investigation on the mode of action between bacteria and potato tubers defense mechanism, the identification and characterization of antimicrobial

compounds from the most inhibitory bacterial isolate against Fusarium dry rot and investigation of the antimicrobial potential of bacterial isolates in combination with cultural control methods. Additionally, future studies should characterize specific proteases produced by *Arthrobacter* spp. and determine their activity against fungal and oomycetal proteins, establish mechanisms of action for both isolated compounds, and evaluate the toxicity on humans and the environment of the isolated compounds. Further studies will help elucidate the precise mode of action, which will facilitate an eventual registration of these antagonistic bacteria as biocontrol agents.

Overall, these studies indicated that the most effective bacterial antagonists used antibiosis i.e., production of antimicrobial compounds, as a main mode of action. This study strengthened the understanding of *Bacillus* and *Pseudomonas* spp. used as pathogen antagonists and supported antibiosis as a primary mechanism of action in *Arthrobacter* spp. and specifically *A. psychrophenolicus*. Therefore, the present work suggests that these bacterial antagonists may find use as alternative control methods in the fight against plant disease and food loss.

4.1 References

Cloutier A, Tran S, Avis TJ (2020) Suppressive effect of compost bacteria against grey mould and *Rhizopus* rot on strawberry fruit. Biocontrol Science and Technology 30: 143-159. doi: 10.1080/09583157.2019.1695745.

Igarashi Y, Yamamoto K, Fukuda T, Shojima A, Nakayama J, Carro L, Trujillo ME (2015) Arthroamide, a cyclic depsipeptide with quorum sensing inhibitory activity from *Arthrobacter* sp. Journal of Natural Products 78: 2827-2831. doi: 10.1021/acs.jnatprod.5b00540.

Luo M, Purdy H, Avis TJ (2019) Compost bacteria provide antifungal activity against grey mold and *Alternaria* rot on bell pepper fruit. Botany 97: 221-230. doi: 10.1139/cjb-2018-0180.

Mohamed R, Groulx E, Defilippi S, Erak T, Tambong JT, Tweddell RJ, Tsopmo A, Avis TJ (2017) Physiological and molecular characterization of compost bacteria antagonistic to soil-borne plant pathogens. Canadian Journal of Microbiology 63: 411-426. doi: 10.1139/cjm-2016-0599.

Van Nam V, Huong DTM, Dao PT, Mai Anh N, Minh LTH, Van Cuong P (2019) Secondary metabolites produced by marine actinomycete *Micromonospora* sp. A258. Vietnam Journal of Chemistry 57:707-711. doi: 10.1002/vjch.201900098.

Velazquez-Becerra C, Macias-Rodriguez LI, Lopez-Bucio J, Flores-Cortez I, Santoyo G, Hernandez-Soberano C, Valencia-Cantero E (2013) The rhizobacterium *Arthrobacter agilis* produces dimethylhexadecylamine, a compound that inhibits growth of phytopathogenic fungi in vitro. Protoplasma 250: 1251-1262. doi: 10.1007/s00709-013-0506-y.

Appendix A

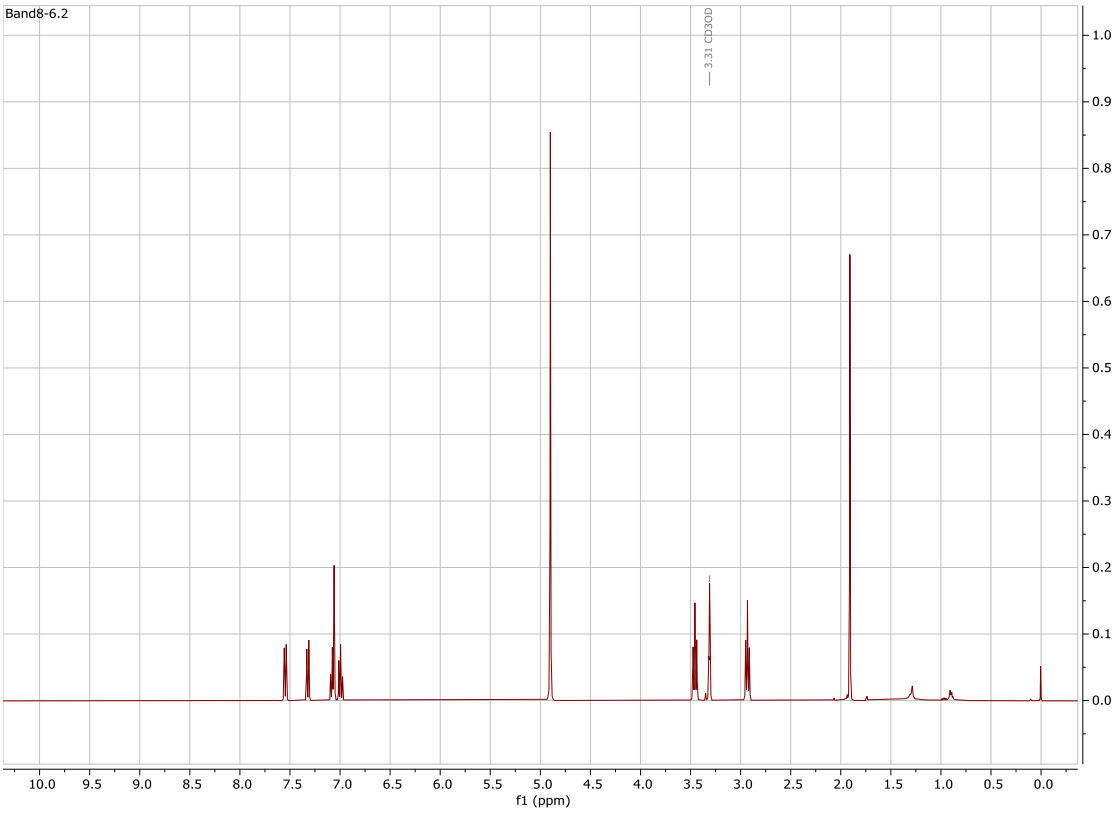


Figure A-1. ^{1}H (400 MHz) NMR spectrum for *N*-acetyltryptamine characterized from *Arthrobacter psychrophenolicus* M9-17 in methanol-d$_4$

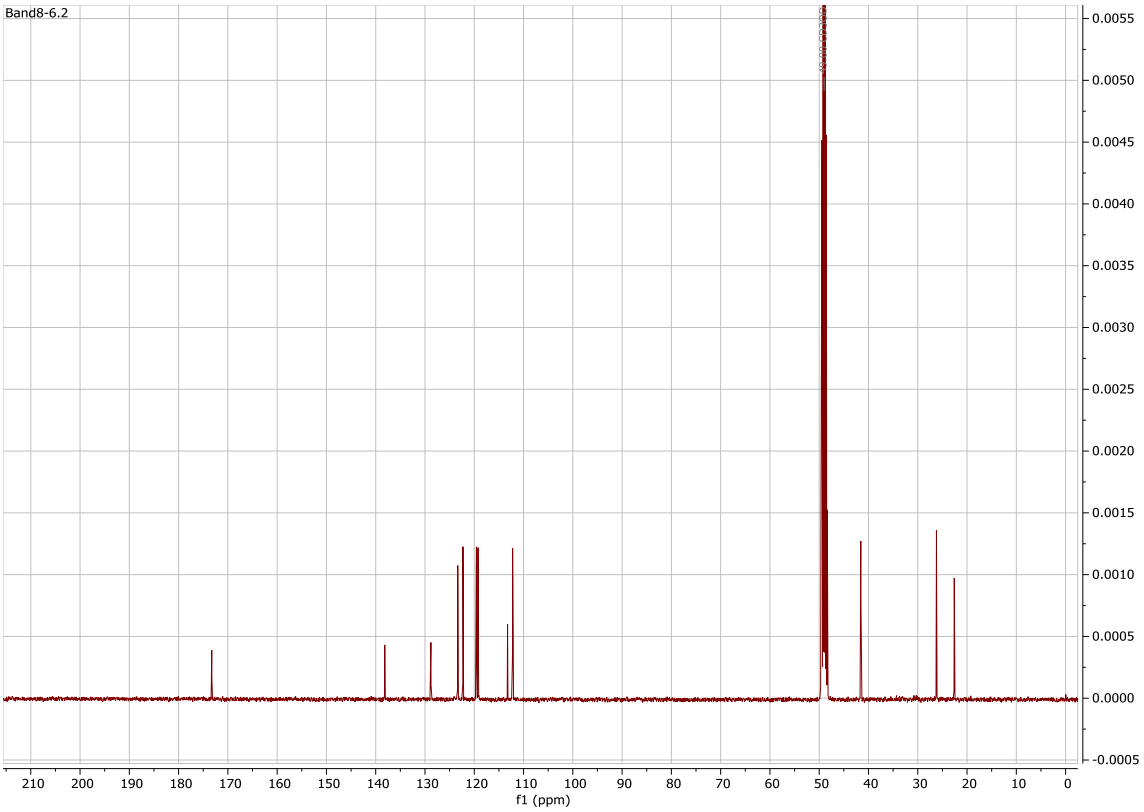


Figure A-2. ^{13}C (100 MHz) NMR spectrum for *N*-acetyltryptamine characterized from *Arthrobacter psychrophenolicus* M9-17 in methanol-d_4

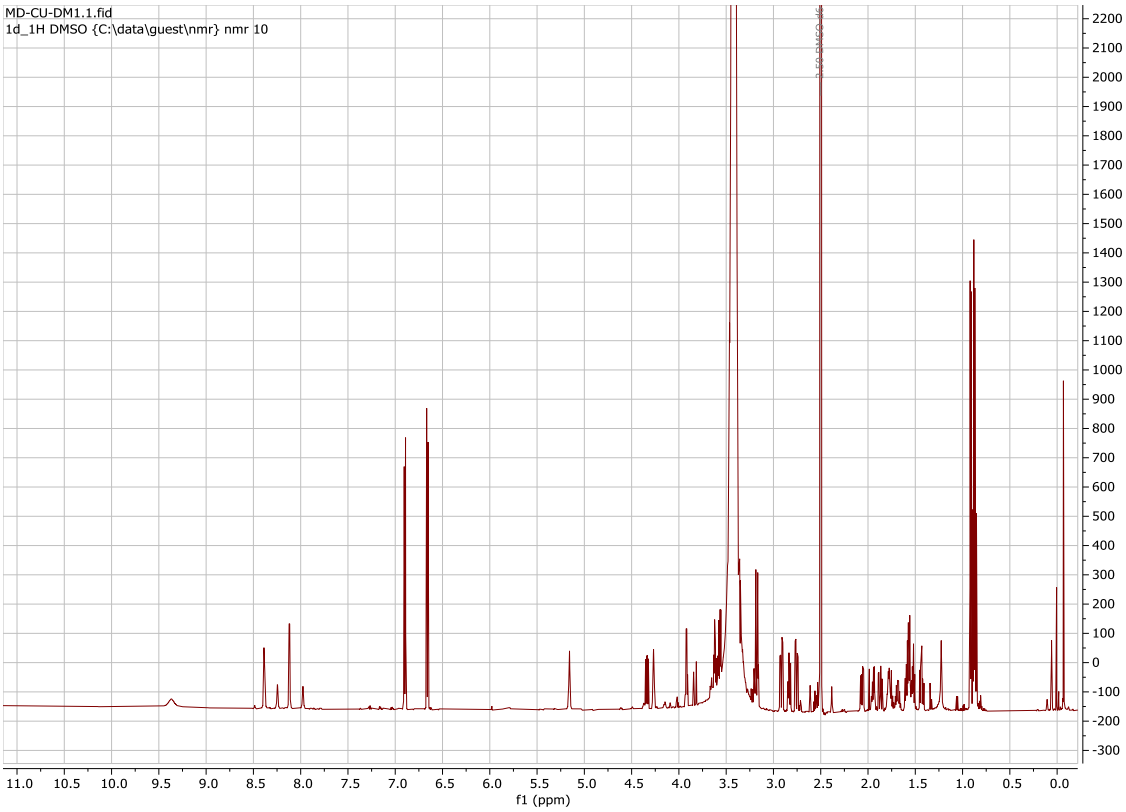


Figure A-3. ^{1}H (600 MHz) NMR spectrum for cyclo(Pro-Leu-Hyp-Tyr) characterized from *Arthrobacter psychrophenolicus* M9-17 in DMSO-d$_6$

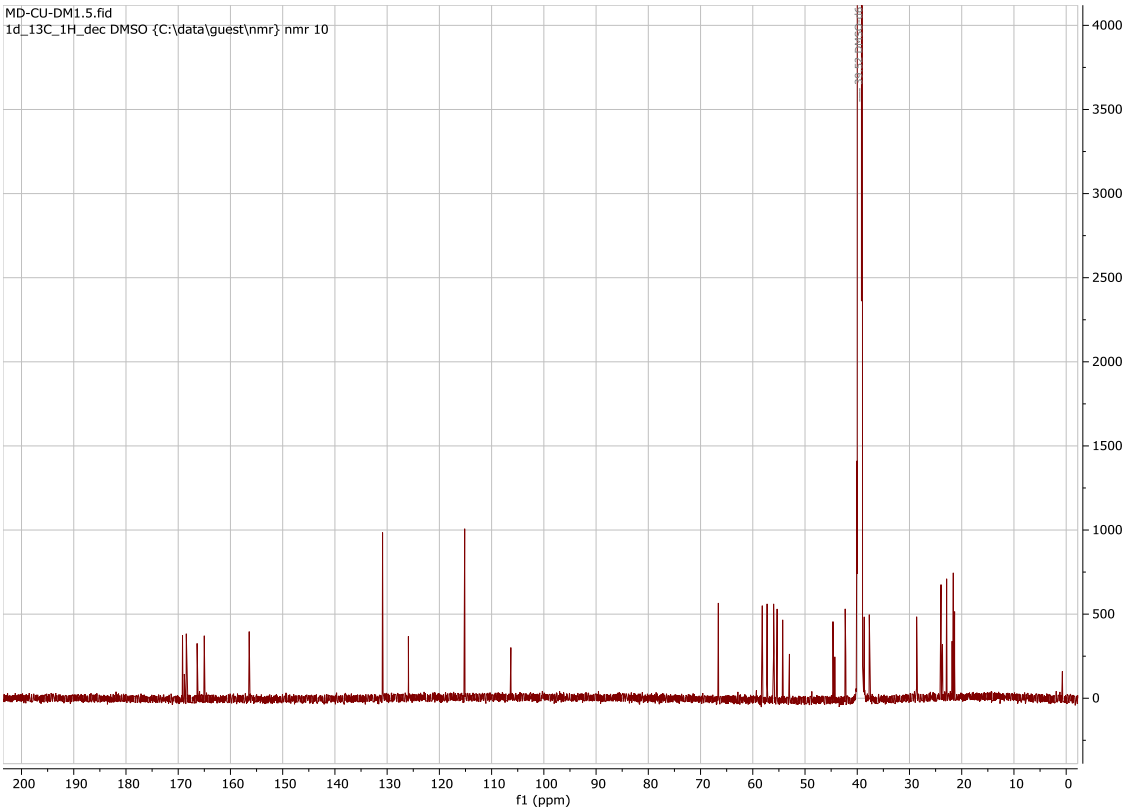


Figure A-4. ^{13}C (125 MHz) NMR spectrum for cyclo(Pro-Leu-Hyp-Tyr) characterized from *Arthrobacter psychrophenolicus* M9-17 in DMSO-d$_6$