Evaluating the antagonistic potential of bacteria on *Sclerotinia sclerotiorum*, causal agent of white mould of carrots

by

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Abstract

Disease suppressive composts are known to contain beneficial bacteria that can be antagonistic to plant pathogens. This research evaluated whether *Sclerotinia sclerotiorum* — commonly known as white mould disease — can be managed using antagonistic bacteria from forestry compost. *In vitro* and *in vivo* experiments demonstrated that bacteria from the *Pseudomonas* and *Bacillus* genera can inhibit mycelial growth and reduce white mould disease. Extraction of culture filtrates and isolation procedures were performed to identify potential bioactive compounds. Three cyclodipeptides were purified and characterized from culture filtrates of *Pseudomonas arsenicoxydans* (F9-7). The three cyclodipeptide were L-Val-L-Pro, L-Leu-L-Pro, and L-Phe-L-Pro and they are though to be bioactive compounds responsible for the antagonistic against *S. sclerotiorum*. The most antagonistic effect was obtained with the cyclodipeptide L-Leu-L-Pro at a concentration of 100 mg/mL. These results indicated a potential for some bacteria to reduce the growth of the fungus and its associated disease on carrots.

Keywords: *Sclerotinia sclerotiorum, Pseudomonas* spp., *Bacillus* spp., antifungal compounds, white mould disease, cyclic dipeptides

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Glossary

Concept	Definition
Antagonistic effect	An organism, or substance, that shows opposition to another.
Ascospores	A spore (sexual) that is contained and produced inside the reproductive cell of a fungus (ascus).
Bacillus	A genus of Gram-positive bacteria that is rod-shaped and needs oxygen to produce energy. Yet, it can switch to fermentation metabolism when oxygen is absent.
Bacterium (sing.) Bacteria (plur.)	Among the simplest microorganisms that lacks internal membranes as well as a nucleus. Bacteria are very small single-celled microorganisms; they can be visualized with a microscope. Bacteria can be seen in different shapes such as a rod, a sphere or a spiral; they can thrive in many different types of environments such as in soil, in the ocean or in the human gut.
Eucaryote organism	A complex organism that comprises internal membranes, as well as a membrane-bounded nucleus.
Fungus	A multicellular filamentous organism that feed on organic and decaying matter such as plants and food.
Fungicides	A chemical compound that can inhibit fungal growth.
Genus (sing.) Genera (plur.)	Part of a scientific ranking system utilized to group species that share a common ancestor. The genera are at the lower end of the rank situated between family (above) and species (below) — the lower ranking give a more specific description of the life forms, whereas the higher ranking is more general.
In vitro	An experiment done in a laboratory setting, and in which the procedure is constrained to a test tube or a Petri dish.
In vivo	An experiment done in a laboratory setting, and in which the procedure in perform on a living organism such as a plant.
Mould	A fungus that typically grows on a damp surface and decomposing matter such as food and plant.
Mycelium	A mass that derives from the collective of all hyphae of a fungal colony/individual.

Concept	Definition
Necrotrophic fungus	A fungus that kills its host and feeds on its dead matter.
Pathogen	An organism that causes disease or an infection upon its host.
Phytopathogen	A pathogen to plants.
Post-harvest disease	A disease that occurs after the crop has been harvested (i.e., during storage or transportation).
Pseudomonas	A genus of Gram-negative bacteria that is rod-shaped and needs oxygen to produce energy. Yet, it may also be able to adapt to conditions where oxygen is absent.
Pyrrolnitrin	A secondary metabolite with antifungal properties that can be synthesized by several *Pseudomonas* species.
Saprotrophic fungus	A fungus that feeds on dead matter such as senescent leaves.
Sclerotinia sclerotiorum	An ascomycetous fungus that can cause plant infections (e.g., white mould disease) under certain conditions.
Spore	A microscopic reproductive structure of fungi that can produce a new fungus.

Chapter I: Introduction

Food loss is a complex global problem in urgent need of solutions. According to the United Nations, every year roughly one-third of the food produced for human consumption — some 1.3 billion tonnes — gets lost or wasted worldwide. The United Nations estimates that this amount of food could have otherwise fed roughly 870 million hungry people on an annual basis. Beyond the human toll, this waste further creates negative environmental impact that contributes to climate change, and economic impact that globally amount to $990 million in revenue losses. Amongst all food categories, fruits and vegetables have the highest worldwide wastage rates at roughly 40% to 50% (United Nations Environment Programme, 2021).

To help address one dimension to global food waste, this research evaluates whether bacteria can be used in novel ways to manage *Sclerotinia sclerotiorum* — a common phytopathogenic mould that causes irreversible harm to plants and significantly contributes to food spoilage in vegetables worldwide. Currently, the risk of *S. sclerotiorum* spoiling food is generally addressed by two management approaches — chemical and non-chemical. Chemical approaches tend to focus on the use of synthetic fungicides (Kolaei, Tweddell, & Avis, 2012) while non-chemical approaches tend to focus on ventilation and temperature controls to better manage food storage and transportation dynamics. However, Canadian growers and distributers require additional or alternative measures to control moulds such as *S. sclerotiorum* in order to minimize economic losses and more sustainably satisfy the demands of consumers who depend on the availability of high-quality produce (Canadian Horticultural Council, 2015).

To support the industry's need for more management options, this research explores how bacteria antagonistic to plant pathogens might offer a promising alternative to synthetic fungicides (Avis, 2007; Pershakova et al., 2018). Drawing from research by Mohamed et al. (2017), the antagonistic effects, as well as the methods of action and putative bioactive compounds involved in two bacteria genera — *Pseudomonas* and *Bacillus* — are explored to control *S. sclerotiorum*.

Chapter II: Literature Review

2.1 Defining Food Loss, Waste, and Spoilage: A Global Problem.

The need to address food waste is a growing global concern (Environment and Climate Change Canada, 2020). According to the *Food and Agricultural Organization of the United Nations* — an agency that leads international efforts to defeat hunger and achieve food security for all — there is a distinction to be made between "food loss" and "food waste" (Ishangulyyev, Kim, & Lee, 2019).

Food waste "refers to a decrease in the quantity or quality of food resulting from decisions and actions by retailers, food service providers and consumers" (FAO, 2021). For example, retailers who waste food by not rotating perishable items to the front of the shelf before their expiry; food service providers who waste food during meal preparation; and consumers who waste food from incompletely eaten meals. Alternatively, food loss "refers to a decrease in the quantity or quality of food resulting from decisions and actions by food suppliers in the food supply chain" (FAO, 2021). For example, food suppliers experiencing production and harvest losses due to bad weather, or post harvest losses due to inadequate storage facilities and techniques, as well as processing, packaging, and distribution techniques.

Another viewpoint includes the broader idea of "food spoilage". According to the Canadian Institute of Food Safety, food spoilage occurs when there is a "disagreeable change in the normal state of the food". For example, a disagreeable change could include a change in the smell, taste, texture, or sight of a food item. These changes are often caused by bacteria, mould, and yeast. While their effects do not necessarily make food unsafe to eat, when their effects do make food unsafe these changes are described as "pathogenic spoilage" (Canadian Institute of Food Safety, 2017).

2.2 *Sclerotinia sclerotiorum* and Food Spoilage: A Microbial Perspective.

Pathogens are undesirable microorganisms that cause disease and damage to the host they infect. Some pathogens — like *Listeria monocytogenes* — can cause microbial damage to humans and can be lethal (Farber & Peterkin, 1991). Other pathogens — like *Beauveria bassiana* — can cause microbial damage and can be lethal to insects (Bidochka, Menzies, & Kamp, 2002). *Sclerotinia sclerotiorum* is a pathogen that causes irreversible damage to plants that leads to plant disease and food spoilage. It is found worldwide and is broadly recognized as a problem that needs to be prevented with management techniques.

In the context of food, three main kinds of microorganisms can cause spoilage — bacteria, yeasts, and moulds (Katte, 2020), and understanding their presence and characteristics helps to situate this research. **Bacteria** are single-celled microorganisms that lack a nuclear membrane. Bacteria are most likely to cause food spoilage when pH is neutral (close to 7), moisture is high, and temperatures are warm to high (5-60°C). Some bacteria require the presence of oxygen to grow (e.g., *Pseudomonas* genera), while others do not (e.g., *Clostridium* genera). **Yeasts** are single-celled fungi, which are usually oval in shape and may cause the fermentation of food. Some yeast species do not need oxygen to grow and are more likely to be the causal agents of food spoilage when pH is low (4 to 6.5), and moisture is low. **Moulds** are multicellular fungi or oomycetes, which can produce a visible mass on the surface of foods called mycelium. Moulds are aerobic organisms and most likely to cause food spoilage when pH is slightly acidic (4.5 to 6.8), moisture is low, and temperature conditions are warm (20-40°C) (Jean, 2016).

2.2.1 Sclerotinia sclerotiorum: Taxonomy and Origin

Sclerotinia sclerotiorum is a eukaryote organism and is considered as a mould within the kingdom Fungi (Gould, 2009; Smith & Bakaitis, 2017). The genus *Sclerotinia* belongs to the family of Sclerotiniaceae The mode of sexual reproduction, which includes the shape and structure of the apothecium, is helpful for further classifying mould into divisions (Gould, 2009). From this vantage point, *S. sclerotiorum* is part of the Ascomycota division, which can be defined by the presence of asci, which is typically a pouch that contains spores produced via meiosis.

Table 1) where several species of *Sclerotinia* are considered plant pathogens (Lorenzini & Zapparoli, 2016). Within the genus of *Sclerotinia*, three species often attract the focus of research

due to their phytopathogenicity: *S. sclerotiorum* (Lib.) de Bary; *S. minor* Jagger; and *S. trifoliorum* Erikss. All three species have the ability to readily infect a wide range of potential hosts (such as carrots) in numerous areas of the world.

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Table 1: Overview of Sclerotinia taxa

Kingdom:	Fungi
Division:	Ascomycota
Class:	Leotiomycetes
Order:	Helotiales
Family:	Sclerotiniaceae
Genus:	*Sclerotinia*
Species:	*sclerotiorum*

Adapted from (Allan McKay and Soonchye Tan, 2017)

Marie-Anne Libert (1782-1865), Anton de Bary (1831-1888), and Laurence Henry Purdy (1926-2015) are recognized as the historical pioneers of plant pathology research on what is today known as *S. sclerotiorum* (Saharan, 2008). Marie-Anne Libert was one of the first women mycologists and was honoured with several *taxa* including *Sclerotinia libertiana*, but due to botanical nomenclature rules the name could not last (Purdy, 1979). In 1884, Anton de Bary published novel research on bean pathology, and in due course his name became synonymous with the phrase, "*S. sclerotiorum* (Lib.) de Barry" (Purdy, 1979). Today, *S. sclerotiorum* is regarded as the plant pathogen responsible for disease generally named white mould (Saharan, 2008).

2.2.2 Sclerotinia sclerotiorum: The Cause of "White Mould Disease"

Sclerotinia sclerotiorum is the causal agent behind white mould disease, and it can infect as many as 408 plant species (Aldrich-Wolfe, Travers, & Nelson, 2015; Boland & Hall, 1994), which

include edible plants such as beans, carrots, celery, lettuce, peanuts, potatoes, soybeans, sunflowers, and tomatoes, but not, for example, cereals and grasses (Government of Saskatchewan, 2018; Saharan, 2008). An extensive review on *S. sclerotiorum* literature shows that more than sixty names have been used to refer to the diseases it causes (Purdy, 1979). Popular names include: white mould disease, rot disease, cottony soft rot, and watery soft rot (Saharan, 2008).

2.2.3 Sclerotinia sclerotiorum and the Infection Context

Sclerotinia sclerotiorum is a phytopathogen that attacks the cells of its host and uses the content for nourishment and growth. It can also utilize dead plant matter as a source of nutrition (Kora, McDonald, & Boland, 2003). *Sclerotinia sclerotiorum* causes two types of infections: the first involves "aerial infections" while the second involves "crown rot and basal stem infections" (Heffer Link, 2007). *Sclerotinia sclerotiorum* germinates in two possible ways — myceliogenic and carpogenic — to support its spread and survival (Aldrich-Wolfe et al., 2015; Rollins, 2007).

An aerial infection can occur when the exposed part of a plant (above soil) is parasitized by travelling ascospores (Heffer Link, 2007). Ascospores germinate by attaching themselves to the cells of a plant and begin feeding on them saprophytically. This situation causes the production of a specialized hyphae (an appressorium) that is able to penetrate and infect its host rapidly, which results in the growth of mycelium (Figure 1) (Rollins, 2007). Crown rot and basal stem infections occur when the roots of a plant (buried within soil) are parasitized by mycelium that was produced by a nearby sclerotium (Figure 1) (Heffer Link, 2007). Sclerotia (sclerotium sing.) are hard survival structures that develop mostly when the fungus is faced with limited nutrient resources, or when the environmental conditions are unfavourable for mycelium growth (Saharan, 2008). In both contexts, plant infection starts in a localized area, which is evidenced by a soft and watery lesion that eventually degrades the flesh of the plant. Fungal enzymes, as well as oxalic acid secretion, are responsible for plant tissues loosing firmness and becoming soft and saggy (Heffer Link, 2007). When the fungus has completely degraded the surrounding tissues, and no more nutrients are available to assure its growth, its mycelium aggregates to form a sclerotium, which will stay dormant until another potential host is in proximity.

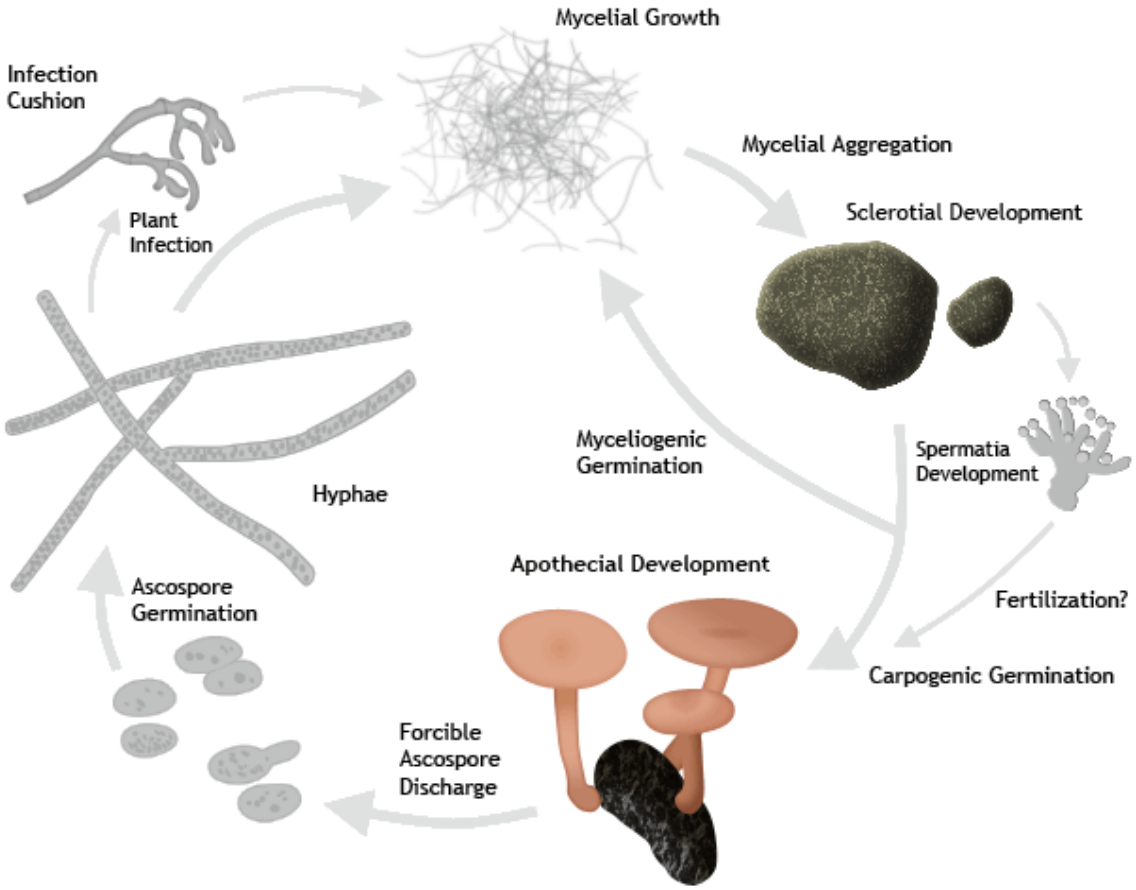


Figure 1: Sclerotinia life cycle (Rollins, 2007)

2.2.4 Sclerotinia sclerotiorum: The Role of Hyphae

Sclerotinia sclerotiorum growth includes the production of numerous hyphae, which are filament-like tubes that elongate to penetrate its host plant. As the growth evolves, branching hyphae emerge, and as its density increases, the branching hyphae connect to one another forming a visible thick fuzzy coat called mycelium, which can spread vastly. The hyphae play an important part in survival for two reasons: first it delivers nutrients to the fungi; and second it serves myceliogenic (asexual) reproduction purposes.

2.2.5 Sclerotia and the Germination Context

Sclerotia are capable of germinating in two possible ways. The first is myceliogenic germination, which directly leads to mycelium (Figure 1). The second is carpogenic germination, which will also lead to mycelium, but a few extra steps are involved beforehand. As depicted in Figure 1, sclerotia first develops into apothecia (apothecium, sing.), which can be defined as distinctive concave discoid receptacles — often referred to as a mushroom (Figure 2) — that hold millions of ascospores. Ascospores can survive up to seven months when environmental conditions are favourable (Saharan, 2008). In the case of *S. sclerotiorum*, an ascospore is a hyaline, ellipsoid and binucleate spore meant to be released into the air as a cloudy discharge to spread to a potential host (Aldrich-Wolfe et al., 2015; Rollins, 2007). When an ascospore reaches a host, it germinates to form hyphae, which then extends into forming mycelium. Both gemination processes help support the survival of *S. sclerotiorum* (Aldrich-Wolfe et al., 2015; Rollins, 2007).

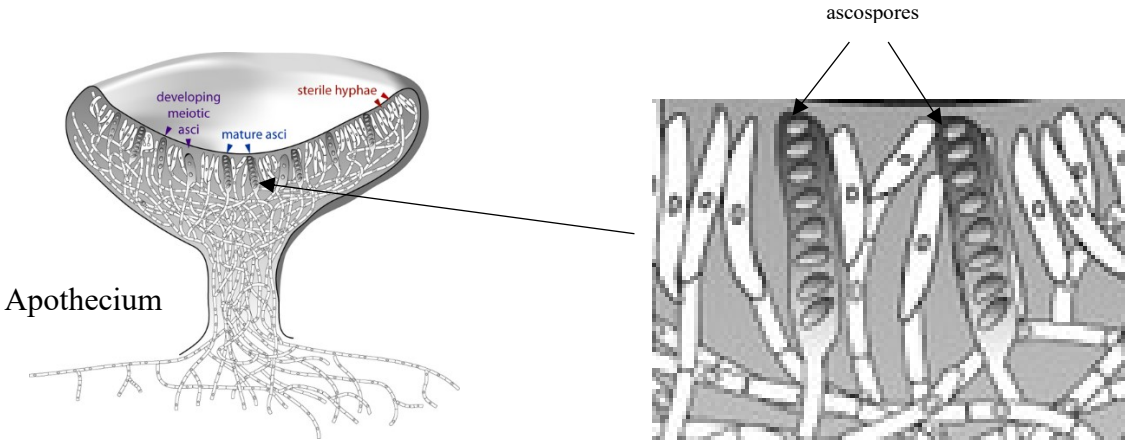


Figure 2: Apothecium showing developing asci and ascospores (Smith & Bakaitis, 2017)

2.2.6 Sclerotia formation

On a medium such as potato dextrose agar (PDA), the *S. sclerotiorum* mycelium covers the entire Petri plate within 4 to 5 days at 22°C (Figure 3). Without further space to expand, the mycelium thickens, which in turn forces the hyphae to merge with one another (Vinodkumar, Nakkeeran, Renukadevi, & Malathi, 2017). Small droplets arise, which appear to be an important source of nutrients to support mycelium growth, as evidenced by darker and more condense hyphae aggregate — a sclerotium (Government of Saskatchewan, 2018). The sclerotium eventually turns dark brown, or black, while the droplets fade away.

Mature sclerotia are composed of an outer melanized layer, also referred to as “the ring”, which is a high-molecular-weight polymer responsible for the dark-coloured pigmentation of the aggregate (Butler & Day, 1998). Melanin acts as a protective shield that can resist many difficult situations such as chemical exposure, harmful weather conditions, or antagonistic microorganisms, making sclerotia a long-term dormant structure that survives until environmental conditions conducive to germination and growth return (Butler & Day, 1998).

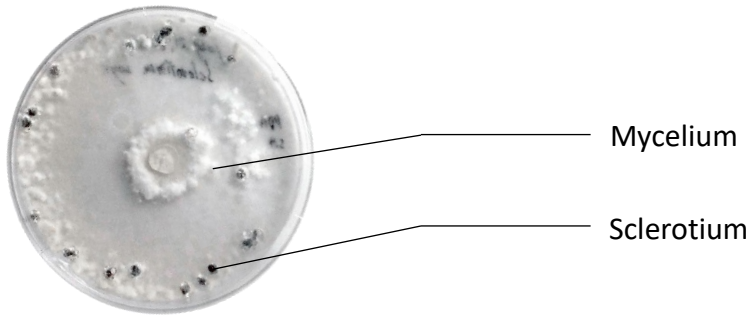


Figure 3: Sclerotinia sclerotiorum on PDA, Day 5

Studies have shown that sclerotia can survive in soil for up to a decade when environmental conditions are favourable and two key elements are present. The first key element is the depth of sclerotia in the soil, while the second is the moisture content of the soil (Smolińska & Kowalska, 2018). Studies show that sclerotia thrives in cool, moist, dark soil. When exposed to heat (30-33°C) and persistent water, sclerotia can be destroyed in as little as two weeks, which further stresses the importance of soil depth (Matheron & Porchas, 2005). Soil depth thus helps to keep sclerotia cool and moist without being exposed to extended periods of heavy rain.

The size of sclerotia is variable, and largely depends on the host it parasitized. The bigger the host, the bigger the possible sclerotia structure, yet in general it can range from 2 mm (on a bean) to 3.5 cm (on a sunflower) (Bolton, Thomma, & Nelson, 2006; Saharan, 2008). Another factor that influences the size of sclerotia is light exposure. Although results are somewhat conflicting, researchers seem to agree that a darker environment will produce a scant number of large sclerotia while greater light exposure will increase the number of smaller sclerotia (Tourneau, 1979). With respect to temperature, optimal growth has been shown to be 15°C, but evidence suggests that

sclerotia formation can occur at temperatures as low as 0°C and as high as 30°C when pH is maintained between 2.5 and 9 (Tourneau, 1979), which corresponds to most agricultural soils. Research shows that buried sclerotia remain a potential threat to plants because sclerotia that are produced over one growing season can survive to infect future plantations (Kora et al., 2003; Zeng, Kirk, & Hao, 2012).

2.2.7 Sclerotinia sclerotiorum: Ideal Environmental Conditions for Growth

Available research shows that *S. sclerotiorum* is largely present in temperate climates where temperatures oscillate between 15°C and 25°C, which also happens to correspond with the optimal range for mycelium growth, as well as carpogenic germination (Saharan, 2008). However, the presence of *S. sclerotiorum* has been reported around the world, and even in relatively hot and dry areas such as California in the United States (Purdy, 1979). Yet, when conditions are too dry and too warm, studies show that carpogenic gemination is completely inhibited (in as little as ten days) and apothecia are unable to arise when they have less than 70% water saturation (Smolińska & Kowalska, 2018). Therefore, the presence of water is a key element for survival of the fungus, and maximum expansion for infection have been reported when free water is present on the plant surface (Smolińska & Kowalska, 2018).

Overall, *S. sclerotiorum* has been defined as a "perfect" fungus because of its capacity to reproduce both sexually and asexually through its aggregate body, the sclerotia (Heffer Link, 2007). While all fungi play an important part in the environment by being a key decomposers of organic matter as saprophytes, *S. sclerotiorum* also causes unwanted plant disease and food spoilage, which makes it undesirable. Although *S. sclerotiorum* is relatively well-researched, more knowledge is needed on how to reduce, or inhibit, the damage this pathogen causes on plants (Saharan, 2008).

2.2.8 Sclerotinia sclerotiorum and Economic Loss

According to Purdy (1979), worldwide economic loss from *S. sclerotiorum* can be several hundred million dollars. In Canada, crop losses due to *S. sclerotiorum* are estimated between 30% and 50% (Finlayson, Rimmer, & Pritchard, 1989; Government of Manitoba, 2011). In Manitoba, losses in carrot crops due to white mould disease represents as much as one-third of annual yield productions (Finlayson et al., 1989), whereas in Saskatchewan, *S. sclerotiorum* can cause production losses greater than 50% (Government of Saskatchewan, 2018). Losses caused by

S. sclerotiorum not only reduce yields, but also reduce quality (or loss in grade) during storage (Purdy, 1979).

Over the past two decades, researchers have noted a lack of controlled measures to effectively prevent the occurrence of white mould disease in the field (McDonald et al. 2001) as cited in (Kora, McDonald, & Boland, 2005a). Moreover, research studies further note that some growers have decided to address *S. sclerotiorum* matters by either completely abandoning the problematic field, or by opting to grow another type of crop that is not susceptible to *S. sclerotiorum,* such as onions or beets (Government of Saskatchewan, 2020). For Canadian growers, the economic impact caused by crop losses due to white mould disease is a significant matter, which is why in 2003 the Government of Canada put forth a reduced-risk management strategy and program for numerous affected crops, including carrots (Health Canada, 2019).

2.3 Three Management Approaches for Preventing Food Spoilage

As most foods are highly perishable, managing food spoilage in an effective and efficient manner is a constant struggle. This management is often practiced through intervention approaches and prevention approaches. The former tends to prioritise management efforts that target already spoiled food from being consumed, or from further cross-contaminating other foods and surfaces. The latter tends to prioritise management efforts on avoiding food from becoming spoiled. It is generally recognized that prevention is the only true option available because food contamination is a non-reversible process. Once microorganisms invade the flesh of a food, deterioration begins, and deteriorated product eventually becomes unacceptable for human consumption. Within the broader realm of prevention, post-harvest management approaches can further involve the application of three techniques: physical, chemical, and biological. Although each method can minimize mould infections, none of them alone are able to effectively inhibit white mould disease post-harvest (Kora et al., 2003; Saharan, 2008). Indeed, after a carrot is harvested and removed from soil, its transportation and storage conditions become two critical quality control variables for influencing white mould disease (Tesfaendrias, McDonald, & Warland, 2010) and shaping physical, chemical, and biological techniques to prevent food spoilage. The following sections describe management strategies of carrot white mould.

2.3.1 Managing Post-Harvest Disease: Physical Approaches

Prevention-oriented approaches for physically managing the risk of food spoilage often involve a focus on appropriate food handling practices; segregation efforts; cleaning and sanitization practices; air circulation; rapid cooling through refrigeration and freezing; and maintaining proper humidity and atmospheric control. For example, in the case of handling carrots and their physical movement, experts advise that harvesting should be done in a manner that preserves the integrity of the periderm (Figure 4), which acts as a natural barrier to microbes (Garrod & Lewis, 1979; Government of Saskatchewan, 2020). Handling practices are viewed as particularly important because carrots that have been bruised or cut during handling are more susceptible to *S. sclerotiorum* infection, as their open flesh offers an entry point for the fungus, where the periderm would have otherwise acted as a protective layer. Alternatively, chemical approaches (discussed

in the next section) also tend to value the integrity of a periderm, as contact fungicides aim to operate much the same way by creating an added protective coat.

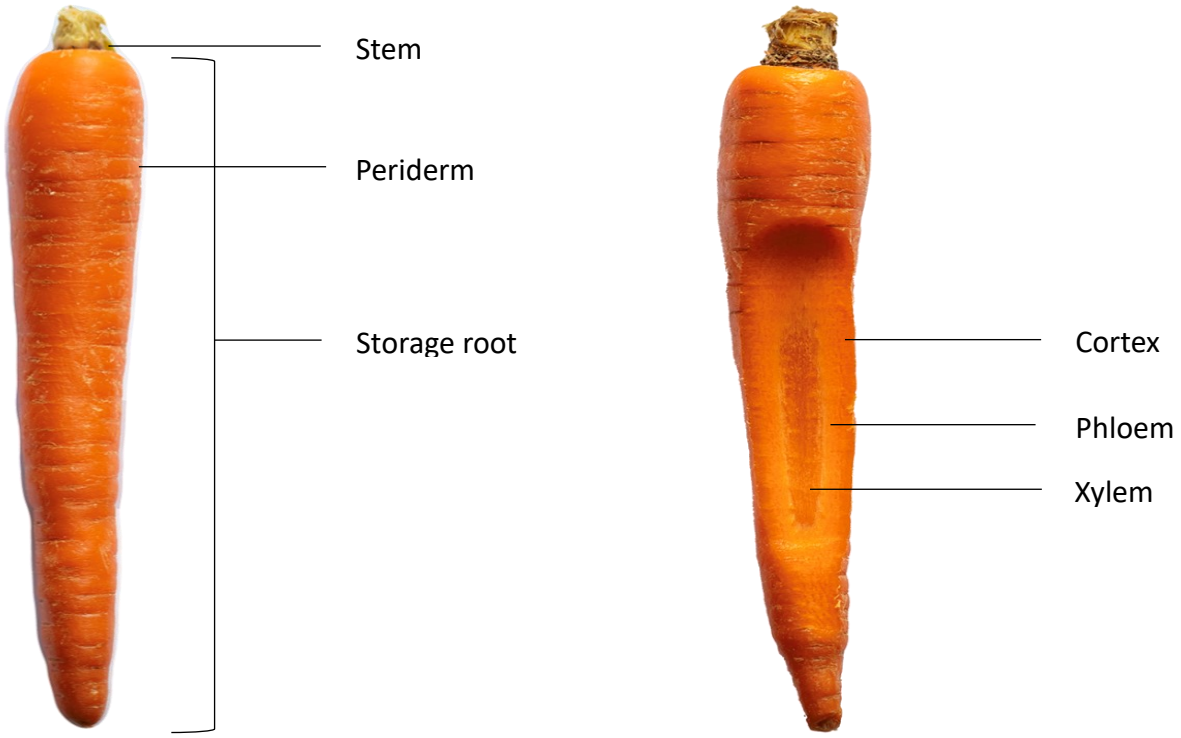


Figure 4: Carrot morphology

In the case of physical segregation practices, experts recommend that carrots with clearly apparent damage, or visibly diseased roots, ought to be removed and discarded from those that are not, as the post-harvest propagation of white mould disease is often attributed to earlier in-field infections that have gone unnoticed, or where infected carrot roots are stored alongside uninfected carrot roots (Snowdon, 1991). However, it is also recognized that current physical sorting practices are not sufficient to eliminate all infected carrots because it is practically impossible to visibly differentiate a healthy carrot from an infected one when the disease is at an early stage - a major limitation that requires other management approaches such as chemical and biological control. Studies show that the introduction of infected roots and field debris into a warehouse causes significant crop losses (Rader, 1952; Snowdon, 1992) as cited in (Kora, McDonald, & Boland, 2005b) as root crops, such as carrots, often carry soil when harvested, thereby potentially

harbouring *S. sclerotiorum*. Undetected sclerotia on carrots from the field can transfer to storage containers, thus impacting future carrots added to a contaminated container (Saharan, 2008).

These possible conditions bring into play the need for cleaning and sanitization practices. Prior to harvest, experts recommend that the storage chamber, as well as the handling equipment and the storage containers, ought to be sanitized and carefully dried. This step helps to minimize the transfer of possible existing microorganisms from the surface of the equipment to the carrot root, and helps to avoid damp areas, which creates a favourable growing environment for white mould disease. Wooden boxes are the primary container option used by Canadian farmers to transport and store carrots, and wooden containers have been identified as being a potential source of cross-contamination (Figure 5). While current guidelines recommend that boxes be carefully sanitized with disinfectants such as hypochlorite, quaternary ammonium, or phenol application to reduce the possibility of mycelium transfer, such practices are often not a common practice among vegetable farmers. Accordingly, the preparatory practice of cleaning surfaces and bins should be part of the "storage ritual" to minimize the potential transfer of mycelium from wood to carrot roots (Kora et al., 2005b).

Figure 5: Carrot crops contaminated by Sclerotinia sclerotiorum in storage (adapted from OMAF, 2016)

Washing carrots with recycled water that has been treated to reduce microbial levels to meet potable standards (*Canadian Agricultural Products Act, 1985*, Fresh Fruit and Vegetable Regulations C.R.C., c285) does not seem to cause microbial issues, but the water used for the cooling system, or for washing and rinsing handling equipment, could potentially contain spores, which could be transferred onto carrots (Sholberg & Haag, 1996). Thus, adequate filters for ventilation in storage is another paramount and physical factor to prevent microbial spores from

travelling from the cooling chamber to the cold storage, and potable water should be used for cleaning and rinsing to minimized cross-contamination (Visser et al., 2017).

In the case of temperature control, removing heat from food generally helps to reduce, or suppress, microbial metabolism and multiplication. The lower the temperature, the more effective the method. However, lower temperatures can also change the quality of a food product as cells may be sensitive to a cold environment. In the case of carrots, they should be rapidly cooled to a temperature as close as possible to it storage temperature, which is 0°C (the freezing point for carrots is -1°C). Keeping carrots moist without being damp (RH ≥ 90%) (Engineering ToolBox, 2004) is important to maintain quality (Tesfaendrias et al., 2010), as browning of the root is known to be a condition of water loss (Government of Saskatchewan, 2020). However, storage conditions can also be favourable to *S. sclerotiorum* growth even when temperatures are maintained as low as 0°C as the fungus is still capable (although to a lesser extent) of infecting hosts so long as the temperature does not drop below the freezing point (Purdy, 1979).

Another important factor to be considered is that the pH of carrots (5.88-6.40) (FDA, 2004) is favourable for *S. sclerotiorum* infection since it thrives at a pH between 4 and 5.5 (Saharan, 2008). Another encouraged practice is to rotate storage boxes among plant species that do not share the same disease susceptibility, such as sweet corn, beets, onions and spinach (Government of Saskatchewan, 2020), but this practice is also not common in most grower routines (Kora et al., 2005b).

Another physical method often utilized to manage undesired microorganisms in food is to modify the atmosphere around the food by, for example, controlling the type and concentration of gases in a room or food package. In some cases, it is possible to significantly reduce gases, and in turn food spoilage, by vacuum packing food (Jean, 2016). However, as illustrated above, physical prevention-oriented approaches can be labour intensive, costly, and never completely adequate when performed alone.

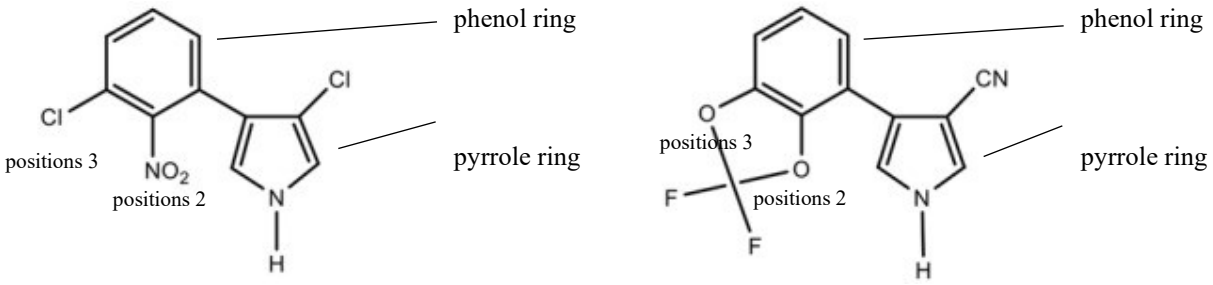
2.3.2 Managing Post-Harvest Disease: Chemical Approaches

Managing food spoilage with chemical approaches often involves targeting the biochemical processes that are unique to fungi and using chemical agents to supress fungal invasions. Chemical agents can be applied to food in several ways, but overall, the most applied method involves an

agent being sprayed over fields during production. Yet, it is also possible, and sometimes only permissible (as is the case with carrots) to dip post harvested food in a solution containing a chemical agent, or to add a chemical agent to the food packaging (Health Canada, 2019). Regardless of the application method, the use of chemical agents in Canada is governed by the *Food and Drug Act* and its *Regulations*. Prior to a chemical agent being approved, or used, it must meet certain criteria. For example, chemical agents must be harmless to animals and humans, be chemically stable, be able to rapidly inactivate the targeted microorganism, and be easy enough to distribute evenly on a food or crop (Jean, 2016).

The chemical agent for addressing post-harvest white mould disease on carrots in Canada is Scholar® 230SC — the only available and registered fungicide (since 2013) approved for minor-use (Health Canada, 2019). In addition to carrots, Scholar® 230SC is also a registered product for other crops such as apples, pears, cherries and stone fruits (Syngenta, 2019). Scholar® 230SC is a suspension of the active ingredient fludioxonil (phenylpyrrole class of chemistry) at a concentration of 230 g/L (Syngenta, 2019). Although fludioxonil is partially present in other fungicide formulations such as Cannonball, Maxim, Medallion, and Stadium (APS, 2020), Scholar® 230SC is the only fungicide prepared exclusively from this compound.

Fludioxonil is the common name for the synthetic analogue (phenylpyrrole) of a natural antifungal compound (pyrrolnitrin), which is produced by bacteria of the genus *Pseudomonas*. The difference between natural and synthetic compounds can be seen on the phenol ring (positions 2 and 3), and the pyrrole ring (position 3) (Figure 6) (Kilani & Fillinger, 2016). Pyrrolnitrin is a secondary metabolite produced when bacteria are confronted with a competitor (Williams, Stone, Hauck, & Rahman, 1989). Although pyrrolnitrin has showed good inhibitory results against fungal invasion (Howell & Stipanovic, 1979), this natural compound is sensitive to light (Sako et al., 2002) and light appears to limit its effectiveness over time. However, in the 1980s, Swiss chemicals' pioneers (Ciba-Geigy AG) developed a synthetic analogue to address the problem of light, and it became a worldwide success (Kilani & Fillinger, 2016).



Pyrrolnitrin ($C_{10}H_6Cl_2N_2O_2$) Fludioxonil ($C_{12}H_6F_2N_2O_2$)

Figure 6: Chemical structure of pyrrolnitrin & synthetic analogue fludioxonil (Leroux, 1996)

Scholar® 230 SC is a contact fungicide. Its active ingredient (fludioxonil) is applied to the surface of the plant, such as the leaf and root, which is the case of carrots. As a contact fungicide, it adheres to a plant's surface (it does not enter the plant) and consequently creates a protective coating (McGrath, 2004). In the case of carrots, once they are cleaned and washed, they are further drenched or dipped into a fungicide solution composed of 496 millilitres of fludioxonil (Scholar® 230SC) and 378 litres of water for 30 seconds, which can be performed once only (OMAFRA, 2021; Syngenta, 2019; Visser et al., 2017). Then, excess moisture is removed by moving the carrots over a conveyor. Thus, Scholar® 230 SC is meant to provide a protective coat that can help keep healthy carrots from being contaminated by fungi such as *S. sclerotiorum* (Syngenta, 2019).

Regarding the mechanism of action, fludioxonil is thought to block the HOG pathway (High Osmolarity Glycerol) by inhibiting key enzyme activities (protein kinase), which specifically target glycerol synthesis (Figure 7). This specifically causes an overproduction of glycerol, which in turn causes the cells of the fungus to burst (Lew, 2010; Sibirny, 2017; Takagi & Kitagaki, 2015). The inability to adapt to an increasing amount of environmental solute also causes interruptions of fungal mitochondrial respiration, and consequently inhibit spore gemination as well as mycelial growth, thus preventing the mould from spreading (Kilani & Fillinger, 2016; Leroux, 1996). On account the early stages of fungal contamination cannot be seen, and growers are unable to effectively sort healthy carrots from contaminated ones (Kora et al., 2003), Scholar® 230 SC can serve to protect healthy carrots that have not yet been contaminated.

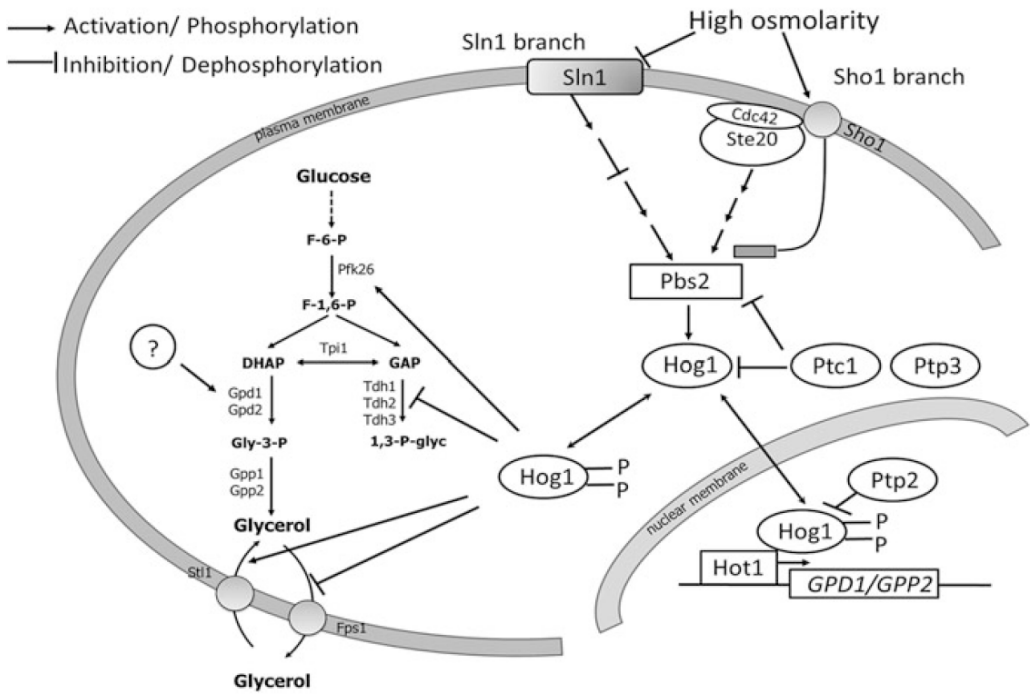


Figure 7: Overview of glycerol pathway metabolism in fungi (Sibirny, 2017)

2.3.2.1 Fungicide Concerns

While chemical agents do offer a viable approach for managing post-harvest diseases, some research studies have shown that their use over time can create disadvantages by becoming toxic to the environment, the consumer, or less effective by promoting microorganism resistance (Avis, Rioux, Simard, Michaud, & Tweddell, 2009). For example, fungicides can be toxic to the plant, and some research suggests that fungicides can leave a residual coat that remains on the root vegetable even after washing and peeling (Boyd, 2006). Thus, when applying Scholar® 230SC on carrot roots prior to storage, fludioxonil could potentially leave a residue, which in turn could be consumed and give rise to potential toxicity issues. For example, public health research on the impacts of fludioxonil has found evidence of fludioxonil causing adverse health effects to the endocrine system (Brandhorst, Kean, Lawry, Wiesner, & Klein, 2019). Consumers are becoming increasingly aware of potential health risks related to chemical residues left on the food they eat, which could explain the increasing market demand for organic produce (Macey, 2004).

Another drawback of fungicides is that their continuous use can increase the risk of fungus resistance. It is now well-known that repeated uses of the same class of chemistry fungicides promotes fungus adaptation, and in turn, leads to reduced efficacy. To help address this issue, and prevent the development of resistant pathogens, a Fungicide Resistance Action Committee (FRAC) was formed in the early 1980s whereby experts seek to find solutions for resistance preoccupations (https://www.frac.info/). Indeed, Scholar® 230SC has been reported to cause resistance of *S. sclerotiorum* (Health Canada, 2019; Kilani & Fillinger, 2016), which is why experts have prohibited its use in fields and exclusively require it be applied to only post-harvested carrot roots (Health Canada, 2019). As a reduced risk management approach, if growers wish to prevent fungicide resistance in field, then they are often advised to rotate their crops on a regular basis to minimize, but ultimately not prevent, fungicide resistance.

2.3.3 Managing Post-Harvest Disease: Biological Approaches

Managing food spoilage with biological approaches involves using microorganisms, or their metabolic products, in a manner that reduces the occurrence of other (undesirable) microorganisms. For example, this research involves using bacteria from the genera *Pseudomonas* and *Bacillus,* and in turn their specific natural antifungal properties, as a potential microbial biological control agent (MBCA) to limit the spread of moulds, such as *S. sclerotiorum.* However, many possibilities exist and as of 2018 there are no less than 200 known microbial strains from 94 different species that are commercially available around the world (Van Lenteren, Bolckmans, Köhl, Ravensberg, & Urbaneja, 2018). While chemical and biological approaches can both target biochemical processes that are unique to fungus, biological approaches are generally viewed as the more sustainable and nascent risk management approach to avoid the known concerns of chemical agent use.

Applying biological control generally involves two approaches (Table 2). The first entails a direct approach, whereby an MBCA directly interacts with the pathogen to cause parasitism and/or antibiosis. The second entails an indirect approach, whereby an MBCA does not have direct interaction with a pathogen, but causes induced resistance or competition for nutrients and space (Köhl, Kolnaar, & Ravensberg, 2019).

Table 2: Overview of biological control approaches

Direct mechanisms		Indirect mechanisms	
parasitism	antibiosis	induced resistance	competition for nutrients and space

Direct mechanisms

Parasitism involves one organism becoming dependent on another for its survival, and often to the detriment of the organism that is parasitized (the host). If the host is also a parasite, the relationship is defined as hyperparasitism. For example, hyperparasitism could occur when MBCAs are incorporated on a carrot for their potential antagonistic activities against a mould, such as *S.sclerotiorum*. In this example, the carrot is being parasitized by *S. sclerotiorum*, and the mould is being parasitized by MBCA. The MBCA would invade and degrade the structure of the pathogen (*S. sclerotiorum*) such as its mycelium, spores or its resting structure (sclerotia) to a point of fatality in order to obtain its nutrients (Ghorbanpour, Omidvari, Abbaszadeh-Dahaji, Omidvar, & Kariman, 2018). Ultimately, the carrot benefits from the death of *S. sclerotiorum* as it is no longer a threat.

Antibiosis involves at least two organisms where the interaction between the two favours one organism and harms the other. Habitually, the biological interaction involves the production of antimicrobial secondary metabolites that provide harmful effects to the growth or metabolic activities of a particular pathogen, which in turn inhibits its propagation (Köhl et al., 2019; Raaijmakers & Mazzola, 2012). Secondary metabolites are only produced in the presence of a stressor because it is used as a defence mechanism only (Köhl et al., 2019). For example, when confronting a MBCA with *S. sclerotiorum*, the bacterium would be stressed in the presence of the mould, which would then trigger the bacterium's internal defence mechanism, thereby favouring the production of secondary metabolites with antagonistic activities against the pathogen.

Indirect mechanisms

Induced resistance stimulates the plant to express its own modes of defense against a pathogen. This is done through a cascading series of events triggered by a relatively small amount of stimulus released into the nearby environment (Köhl et al., 2019). Therefore, in the case where bacteria are a biocontrol agent, these bacteria would release a substance in the environment close to the plant,

which would have the effect of triggering plant defence mechanisms generating a cascade of reactions, which in turn induces resistance against the pathogen in question (*S. sclerotiorum*). Some examples of induced resistance mechanisms may include chemical mechanisms, such as the production of phenolic compounds or a highly reactive chemical molecule containing oxygen, which chemically reacts with the pathogen causing physiological damage. In such cases, physical mechanisms could include changes in the cell wall or cuticle, which act as a physical barrier to pathogen invasion (Köhl et al., 2019).

Organisms such as plants, bacteria and mould need nutrients and space to germinate and grow. In this respect, **competition** for nutrients and space is a key factor. For example, *S. sclerotiorum*, as a necrotrophic microorganism that relies on dead matter to feed itself, would first cling to its host and then kill its cells so that it can feed on its contents. *Sclerotinia sclerotiorum* is also a saprophytic fungus, which means that it will also feed on senescent tissues, which could be found on bruise or wounded tissues. Consequently, using MBCAs can be helpful in the presence of necrotic host tissues where the antagonistic bacterium would outcompete the fungus for nutrients and space, which would in turn limit the pathogen population, although likely not eliminating it completely (Köhl et al., 2019).

In Canada, biological agents are regulated under the *Pest Control Products Act,* as well as the *Food and Drug Act* and its *Regulations*. Accordingly, biological agents must prove their efficacity and meet safety criteria before their use can be authorized (Chandler et al., 2011; Health Canada, 2001; Jean, 2016). An understanding of the various MBCA mechanisms involved is thus necessary for obtaining regulatory approvals that not only focus on matters of efficacity, but also consumer safety. For this reason, it is not only important to establish whether biological approaches are effective against *S. sclerotiorum*, but also to understand their mechanisms of activity and if bioactive compounds might be responsible for the observed effects.

2.4 Using Bacteria as a Biocontrol Agent: *Pseudomonas* and *Bacillus* Genera as a Case Study.

2.4.1 Pseudomonas spp.

Pseudomonas is a genus of Gram-negative bacteria that have been highly studied since the late nineteenth century (L.-P. Gross, & Kole,, 2014). The genus is characterized as a small rod (straight or slightly curved) with sizes ranging between 0.5-1.0 × 1.5-5.0 µm (2005). *Pseudomonas* spp. can be found in many natural niches such as water and soil, so long as oxygen remains available and pH is above 4.5 (Brenner et al., 2005). Members of this genus do not directly depend on another organism for survival (Özen & Ussery, 2012) as they are capable of feeding on decaying organic matter, such as senescent leaves (Ramos, 2007). *Pseudomonas* spp. are motile thanks to one or more flagella (Brenner et al., 2005). *Pseudomonas* spp. are easy to grow on media, as they require minimal nutrients and only a source of carbon for energy. They can grow over a range of temperatures (4°C-45°C), but growth is optimal at 28°C (Brenner et al., 2005).

While the *Pseudomonas* genus contains no less than 128 known species (Peix, Ramírez-Bahena, & Velázquez, 2009), a special interest has been directed to the *P. fluorescens* group due to its potentially beneficial effects against pathogens such as fungi, and the fact that this group is not known to contain human pathogens (Scales, Dickson, LiPuma, & Huffnagle, 2014). Scales et al. (2014) were able to compile an extensive list of secondary metabolites produced by *P. fluorescens*, which allows the bacteria to compete with other microorganisms for survival. Some of the secondary metabolites identified with antibiotic, antiviral, and antifungal activities include: phenazine, a pigmented compound (Mavrodi, Blankenfeldt, & Thomashow, 2006), hydrogen cyanide (HCN) (H. Gross & Loper, 2009), 2,4-diacetylphloroglucinol (DAPG) (Weller et al., 2007), rhizoxin (H. Gross & Loper, 2009), pyoluteorin (Kraus & Loper, 1995), and pyoverdine, which is a siderophore (an iron chelating compound utilized for iron acquisition by the bacterium) with a fluorescent pigment under UV light (H. Gross & Loper, 2009).

In this study, all bacteria of the genus *Pseudomonas* are from the *P. fluorescens* group (Mulet, Lalucat, & García-Valdés, 2010) and were isolated from a disease suppressive compost prepared from forestry residues. Six strains were used throughout this study: two *P. moraviensis* (F9-6 and

F9-11), *P. arsenicoxydans* (F9-7), *P. koreensis* (F9-9), and two *P. brenneri* (F9-10 and F9-13) (Mohamed et al., 2017).

2.4.2 Bacillus spp.

The *Bacillus* genus is a well-studied group of Gram-positive bacteria, which comprises 142 species where most have little or no pathogenic potential, and are rarely associated with disease in humans or other animals with the exception of *Bacillus anthracis* (Whitman, 2009) and *Bacillus cereus* (2018). This genus is well known for its antagonistic activity against plant pathogenic bacteria, fungi and viruses (Fira, Dimkić, Berić, Lozo, & Stanković, 2018). *Bacillus* spp. are classified as aerobic and facultative anaerobic, which can grow over a range of temperatures between 10-60°C, and can tolerate NaCl up to a concentration 20% (Whitman, 2009). They are easy to grow *in vitro* on nutrient agar, as well as blood agar, and are characterized as a rod-shaped cell, which can be straight or slightly curved with sizes of 0.4 to 1.8 μm in diameter and 0.9 to10.0 μm in length (Whitman, 2009). The *Bacillus* cells may be seen individually or in pairs, others may be seen in chains of different lengths, while some will form filaments. As ubiquitous bacteria, *Bacillus* spp. can be found in a variety of ecological niches, which includes soil, water, and air, but it is also found in food and in clinical specimens such in the gastrointestinal tract of animals (Whitman, 2009). With their well-organized flagella, *Bacillus* spp. are motile. In addition, this genus can survive difficult conditions because of their endospores, which are dormant and non-reproductive structures produced when nutrients become less available or environmental conditions are otherwise unfavourable (Whitman, 2009). From this point of view, endospores could be seen as an advantage when considering biocontrol agent because this structure allows the bacteria to remain viable for a longer period.

Special interest has been shown to the *Bacillus* genera for its ability to produce a variety of structurally different antagonistic substances, which can be beneficial when trying to control plant pathogenic bacteria, fungi and viruses. The active compounds produced by the genus have been identified as peptides and lipopeptides, polyketides, bacteriocins, siderophores, and other compounds (Chen, Zhu, Ding, & Shen, 2011; Rückert, Blom, Chen, Reva, & Borriss, 2011; Stein, 2005).

Bacillus subtilis is of a great importance in microbiology and has been utilized as a model to understand the Gram-positive endospore-forming bacteria (Moszer, Jones, Moreira, Fabry, &

Danchin, 2002). A study by Stein et al., (2002) has demonstrated that this species has the ability to produce over two dozen antimicrobial compounds with a wide array of structures, with peptide antimicrobial compounds being predominant. Some strains of this species have been commercialized as biocontrol agents (e.g., strain QST 713) (Pérez-García, Romero, & de Vicente, 2010).

In this study, the bacteria from the genus *Bacillus* belong to the *B. subtilis* species, which research has previously shown to be effective in controlling white mould disease on carrots during storage (Pershakova et al., 2018). All *B. subtilis* species were isolated from the same forestry disease suppressive compost as the *P. fluorescens* group strains and are comprised of three novel strains: F9-2, F9-8, and F9-12 (Mohamed et al., 2017).

Chapter III: Research Objectives and Hypothesis

3.1 Research Objectives

The ultimate objective of this research is to establish whether *S. sclerotiorum* — commonly referred to as white mold disease — can be managed in a post-harvest context by using bacteria as biocontrol agents. Accordingly, the specific research objectives were:

1. To perform *in vitro* and *in vivo* studies to identify whether bacteria — from the *Pseudomonas* and *Bacillus* genera — have an antagonistic effect against *S. sclerotiorum* and carrot white mould.
2. To identify potential mechanisms of action for the antagonistic effects observed.
3. To evaluate the efficacy of bacterial culture filtrates on the growth of *S. sclerotiorum*.
4. To purify and characterize bioactive compounds that are responsible for antagonistic effects.

3.2 Research Hypotheses

The hypotheses of this study were:

1. Bacteria from these genera *Pseudomonas* and *Bacillus* can inhibit the growth of *S. sclerotiorum*.
2. Bacteria from the genera *Pseudomonas* and *Bacillus* can reduce the incidence and/or severity of post-harvest carrot white mould disease.
3. Cultures filtrates from antagonistic bacteria inhibit the growth of *S. sclerotiorum*.
4. Extracts from culture filtrates containing antimicrobial compounds against *S. sclerotiorum*.

Chapter IV: Material and Methods

4.1 Microbial Material

Sclerotinia sclerotiorum (*Ascomycota*) was isolated from soybean residues and maintained on a potato dextrose agar (PDA, Becton Dickinson, Sparks, MD, USA).

Nine bacteria strains from the genera *Pseudomonas* and *Bacillus* (see Table 3) were isolated from a disease suppressive forestry residue compost, using a process described by Mohamed et al. (2017). Bacteria were maintained on a tryptic soy agar (TSA, Becton Dickinson).

Table 3: The nine antagonistic bacteria utilized in this research

Reference code	Bacterium name	GenBank acc. No.
F9-2	*Bacillus subtilis* (Ehrenberg and Cohn)	KT382234
F9-6	*Pseudomonas moraviensis* (Tvrzová et al.)	KT382235
F9-7	*Pseudomonas arsenicoxydans* (Campos et al.)	KT382236
F9-8	*Bacillus subtilis*	KT382237
F9-9	*Pseudomonas koreensis* (Kwon et al.)	KT382238
F9-10	*Pseudomonas brenneri* (Baïda et al.)	KT382239
F9-11	*Pseudomonas moraviensis*	KT382240
F9-12	*Bacillus subtilis*	KT382241
F9-13	*Pseudomonas brenneri*	KT382242

4.2 Antagonistic Effects of Bacteria on the Mycelial Growth of *S. sclerotiorum In Vitro*

To determine the inhibitory effect of the bacteria on the mycelial growth of *S. sclerotiorum*, an *in vitro* experiment was conducted in accordance with the procedures outlined by Kurniawan et al. (2018). These bioassays were performed by aseptically placing a 0.5 cm diameter agar plug from a three-day-old *S. sclerotiorum* thallus (face down) in the centre of a 10-cm diameter Petri plate containing PDA. The Petri plates were aseptically inoculated with 1 cm streaks of a two-day-old bacterial culture placed at the four cardinal points at a distance of 3.5 cm around the fungal plug (see Figure 8:). The control was *S. sclerotiorum* without bacterial inoculation. Experiments were incubated in the dark at a constant room temperature (21 ± 1°C) for three days. Following incubation, the diameter of *S. sclerotiorum* was measured in centimetres (cm) and recorded, as the average of two perpendicular diameters (see line in blue in Figure 8) of the fungal mycelium growth. The experiment was conducted as a randomized complete block design with nine repetitions.

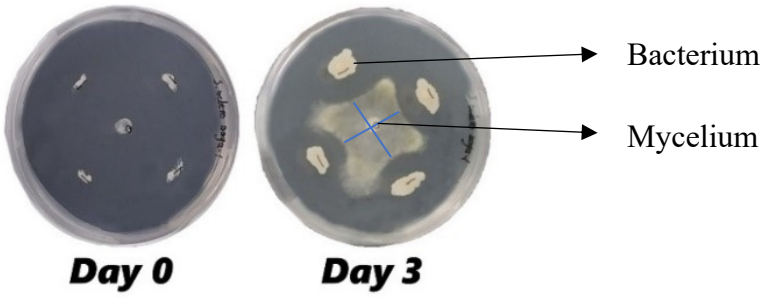


Figure 8: In vitro experiment of Sclerotinia sclerotiorum

4.3 Antagonistic Effects of Bacteria on Carrot White Mould *In Vivo*

To prepare the *in vivo* experiment, carrot roots (*Daucus carota* L.) were surface sterilized by soaking them into a sodium hypochlorite solution at a concentration of 0.5% for five minutes, followed by a 5 min bath of 70% ethanol. Finally, carrot roots were rinsed with sterilized distilled water and air dried under a biosafety cabinet for thirty minutes. Carrots were cut to uniform lengths of 8 cm to fit in an extra deep Petri plate (10 cm diameter; 2.6 cm deep). An active three-day-old fungal agar plug (0.5 cm) was aseptically placed upside down at one end of a carrot root. A two-day-old bacteria streak was aseptically recovered from one of the nine different strains and inoculated at a distance of 3.5 cm from the agar plug using an inoculation loop. This procedure was repeated for all nine bacteria. *Sclerotinia sclerotium* without bacteria inoculation served as the control. A high relative humidity was maintained by using a cotton ball towel saturated with sterile distilled water. All Petri plates were sealed and stored in the dark at 17 ± 1 °C. The distance of *S. sclerotiorum* coverage was compared with the control on day five (~120 h), day six (~144 h) and day seven (~168 h) according to equation 1. The experiment was conducted as a randomized complete block design with nine repetitions.

Equation 1: Inhibition percentage of bacteria compared with control

$$Inhibition\ (\%) = \left(\frac{\bar{x}\ control - \bar{x}\ sample}{\bar{x}\ control}\right) * 100$$

$\bar{x}\ represents\ the\ average$

This same experiment was conducted in the absence of *S. sclerotiorum* in order to determine if the bacteria alone had any potential spoilage effects on the carrots. The experiment was conducted as a randomized complete block design with three repetitions.

4.4 Antifungal Culture Filtrates from Compost Bacteria

To determine the inhibitory effect of the compounds released by the bacteria in culture media on the mycelial growth of *S. sclerotiorum*, an *in vitro* experiment was conducted in accordance with the procedures outlined by Ramlawi et al. (2021) using the nine forestry compost bacteria listed in Table 3.

Briefly, each of the nine bacteria was aseptically inoculated into a medium optimized for lipopeptide production (MOLP) (Table 4; Akpa et al. (2001)). One inoculation loop of each bacterium was transferred into a sterile centrifuge tube containing approximately 10 mL of sterile distilled water. The suspension was vortexed, and 1 mL of the bacterial suspension was aseptically transferred into ten individual Erlenmeyer flask each containing 100 mL of sterile MOLP medium. Flasks were incubated in a controlled environment where the temperature was set at 30 °C and agitation at 120 RPM. After 72h of incubation time, cultures were transferred into two 50 mL sterile centrifuge tubes. The bacterium suspensions were centrifuged at 10,000 RPM for 30 minutes at 4 °C. The pellet was discarded, and the residual bacterial biomass was removed through filtration using a sterile 150 mL bottle top vacuum with a 0.22 µm polyethersulfone (PES) filter (Corning Incorporated, NY, USA). The cell-free filtrates were stored at 4 °C until used.

The antifungal activity of the obtained filtrates was tested at concentrations of 0 (control), 5, 10, and 15% (v/v) in potato dextrose agar (PDA). Briefly, to obtain a filtrate concentration of 5%, 5 mL of cell-free filtrate was added to 95 mL of sterile PDA cooled at 55 °C. Both liquids were swirled to obtain a homogeneous medium prior to being poured into 10 cm diameter Petri plates and cooled for an hour. Lastly, an active three-day-old fungus agar plug (3 mm in diameter) was aseptically placed upside down in the center of the Petri plate. This procedure was repeated for all nine bacteria, and for each tested concentration. Each Petri plate was then sealed and carefully placed in a controlled environment — protected from light by storing in the dark at a constant temperature (17 ± 1 °C). After three days of growth, the *in vitro* diameter of *S. sclerotiorum* was measured in centimetres (cm) and recorded as the average of the two perpendicular diameters of the fungal mycelium growth. The experiment was conducted as a randomized complete block design for a total of three repetitions.

Table 4: Recipe for 1.0 L of MOLP

Substrate	Mass or volume required for 1.0 L of MOLP
Sucrose	20 g
Peptone	30 g
Yeast extract	7 g
KH_2PO_4	1.9 g
$MgSO_4$	0.45 g
TES*	9 mL
Citric acid solution (10 g/L)	9 mL
Distilled H_2O	982 mL

*Trace Element Solution (TES) was prepared by adding $CuSO_4$ (0.001 g), $FeCl_3$ (0.005 g), Na_2MoO_4 (0.004 g), KI (0.002 g), $ZnSO_4$ (0.014 g), H_3BO_3 (0.01 g), and $MnSO_4$ (0.0036 g) to 1 L of sterile ultra-pure water (DeFilippi, Groulx, Megalla, Mohamed, & Avis, 2018).

4.5 Effects of *Pseudomonas arsenicoxydans* Extracts on the Mycelial Growth of *S. sclerotiorum*

4.5.1 *Extraction of Antifungal Compounds from P. arsenicoxydans (F9-7)*

Using the procedure outlined in the previous section (3.4), 12 L of cell-free filtrates was prepared with the bacterium of interest (*P. arsenicoxydans* (F9-7). Liquid-liquid extractions were performed in order to recover potential antimicrobial compounds.

Briefly, each litre of cell-free filtrate was saturated with sodium chloride (NaCl) and extracted with 600 mL of ethyl acetate (EtOAc). The EtOAc fraction was recovered, and the aqueous phase was re-extracted with 400 mL of EtOAc. Organic phases were combined and filtered through anhydrous sodium sulfate (Na_2SO_4) using Whatman No. 1 filter paper into a round-bottom flask. The combined organic phases were dried using a Heidolph 4000 rotatory evaporator (Schwabach, Germany). The mass of the dried extract was recorded prior to further analysis.

4.5.2 *Disk Bioassays*

The dried extract was re-suspended in EtOAc in order to obtain a concentration of 500 mg/mL. The solution was vortexed and 20 µL of the homogenized solution was transferred to a 0.5 cm sterile Whatman No. 1 filter paper disk. EtOAc served as the control. The inoculated disks were dried under aseptic condition. An active three-day-old fungal agar plug (3 mm in diameter) was aseptically placed upside down in the centre of the Petri plate. Dried disks were placed on PDA at a distance of 2.5 cm from *S. sclerotiorum*. Each Petri plate was sealed and carefully placed in a controlled environment — isolated from light by storing them in a dark at 21 ± 1 °C. Following two days of growth, the *in vitro* radius of the area between the fungus plugs and the disk was measured in centimetres (cm) and recorded. The experiment was conducted as a randomized complete block design for a total of three repetitions.

4.6 Isolation and Purification of Antimicrobial Compounds from *P. arsenicoxydans* Strain (F9-7)

4.6.1 *Preparative Flash Column Chromatography*

The crude cell-free EtOAc soluble culture filtrate extract (1,005.2 mg) was resuspended in methanol and mixed with diatomaceous silica (Celite® S), which was then dried using a Heidolph 4000 rotatory evaporator (Schwabach, Germany). The dried compound-silica mixture was applied to the top of the prepared normal phase silica column (Silicycle; 40–63µm) between two layers of sand.

The column was first conditioned with hexanes:EtOAc (9:1, v/v), followed by six sequential elutions as described in Table 5 using airflow. All eluted solvent was collected in 12 mL disposable glass tubes (102 total tubes collected) and examined using thin layer chromatography to visualize compounds as described below.

Table 5: Elution details for flash chromatography

	Mobile phase composition in mL		
Elution	**Hexane**	**Ethyl acetate**	**Methanol**
Conditioning	225	25	—
1	225	25	—
2	175	75	—
3	125	125	—
4	75	175	—
5	25	225	—
6	—	225	25

4.6.2 *Analytical Thin Layer Chromatography (TLC)*

Aluminum foil backed silica gel coated TLC (Silicycle; 40–63µm) were cut to size prior to spotting and migration. Every fifth tube was spotted using a capillary tube. TLCs were migrated in 9:1 chloroform:methanol (v/v) and compounds were visualized under UV light (254 and 365 nm).

Samples that yielded similar TLC profiles were combined, resulting in five fractions. Fractions were dried using a rotary evaporator, transferred to amber vials, dried under a stream of nitrogen gas and the mass of each fraction was recorded (Table 6).

Table 6: Mass of each fraction after flash chromatography

Fractions	Mass of dried compounds (mg)
1	31.3
2	12.7
3	15.9
4	12.0
5	1005.2

4.6.3 *Preparative Thin Layer Chromatography (TLC)*

Fraction 5 was the largest fraction and was studied further. Other fractions were not pursued in this work. Fraction 5 was subsequently subjected to preparative thin-layer chromatography on 20 × 20-cm glass back 0.5-mm silica gel TLC plates (Analtech). The compounds were resolved using 9:1 chloroform:methanol (v/v). Following chromatographic migration and drying of the TLC plate, each of the seven visualized bands (254 nm) was removed using a razor blade. Compounds were extracted from the silica by suspending in 150 mL of 2:1 ethyl acetate:methanol (v/v) for 3 h with shaking (150 rpm) at room temperature. The suspensions were then filtered by gravity using Whatman No. 1 filter papers. Extracts were dried and prepared as previously described.

4.6.4 *Preparative High Performance Liquid Chromatography (HPLC)*

Band 6 was the most abundant fraction from preparative thin layer chromatography—was further analysed by analytical HPLC and peaks were 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 1 mL/min in analytical mode and 4 mL/min in semi-preparative mode.

Linear HPLC elution profiles to obtain purified compounds were metabolite specific. For metabolite peaks 1, 2, and 3, the solvent gradient began at 5% ACN and held for 2 minutes, increased to 36% ACN over 16 minutes, then further increased to 100% ACN over 30 seconds and held for 1.5 minutes and finally, decreased back to 5% ACN. The total chromatographic run was 23.5 minutes.

Peak 4 contained compounds which were purified further using a solvent gradient system that began with 2.5% ACN that was held for 2 minutes, increased to 20% ACN over 16 minutes and was held for 2 minutes, and finally, decreased to 2.5% ACN. The run time was 25 minutes long.

4.7 General Experimental Procedures

4.7.1 Nuclear Magnetic Resonance (NMR) Analysis

NMR spectra (^{1}H, ^{13}C, COSY, HSQC, HMBC) of purified compounds were recorded with a JEOL ECZS 400 MHz NMR spectrometer (Akishima, Tokyo, Japan) using an auto-tuning broadband probe. Samples were dissolved in acetonitrile-d_3 (CDN Isotopes, Pointe-Claire, Quebec). The spectra were referenced to the appropriate solvent peak: δH 1.94 and δC 118.7 for acetonitrile-d_3.

4.7.2 Other Procedures

Optical rotations were acquired with an Autopol IV polarimeter (Rudolph Analytical, Hackettown, NJ). UV spectra were recorded on a Varian Cary 3 UV−Vis spectrophotometer scanning from 190 to 800 nm. Metabolites were purified by semi-preparative HPLC utilizing an Agilent 110 HPLC system equipped with a diode array detector (190-400 nm), a Phenomenex Kinetex C_{18} column (250 × 10.0 mm, 5 µm) maintained at 25 °C and a mobile phase consisting of MeCN−ddH_2O. Linear gradients were programmed according for each metabolite with a flow rate of 4 mL min^{-1}.

4.7.3 Determination of Antimicrobial Activity of Isolated Compounds through Disk Diffusion Bioassay

To quantify the antimicrobial activity of the identified compounds, concentrations of 50 mg/mL and 100 mg/mL of compound in methanol was tested. In addition, a combined 50:50 treatment with two of the compounds was assayed. Methanol alone served as the control. Disk assay was performed as described above

4.8 Statistical Analysis

An analysis of variance (ANOVA) was performed for all *in vitro* and *in vivo* experiments. When the p-value was less than 0.05, a means comparison was performed using Fisher's protected Least Significant Difference (LSD) test at an α of 0.05. Statistical analysis was performed using SAS 9.4 (SAS Institute, Cary, NC).

Chapter V: Results

5.1 The Antagonistic Effects of Bacteria on the Mycelial Growth of *S. sclerotiorum – In Vitro*

Several *in vitro* results (Figure 9) were observed by directly confronting *S. sclerotiorum* with nine bacteria from the *Pseudomonas* and *Bacillus* genera using a potato dextrose agar (PDA). Six bacteria — *P. arsenicoxydans* (F9-7); *B. subtilis* (F9-2); *B. subtilis* (F9-12); *P. koreensis* (F9-9); *P. moraviensis* (F9-11) and *P. brenneri* (F9-10) significantly inhibited mycelial growth when compared to the control. More specifically, *P. arsenicoxydans* (F9-7), *B. subtilis* (F9-2) and *B. subtilis* (F9-12) were shown to be the most inhibitory, reducing mycelial growth by an average of 54%, relative to the control. Conversely, three bacteria — *P. brenneri* (F9-13); *P. moraviensis* (F9-6) and *B. subtilis* (F9-8) — did not significantly inhibit mycelial growth under our experimental conditions when compared to the control.

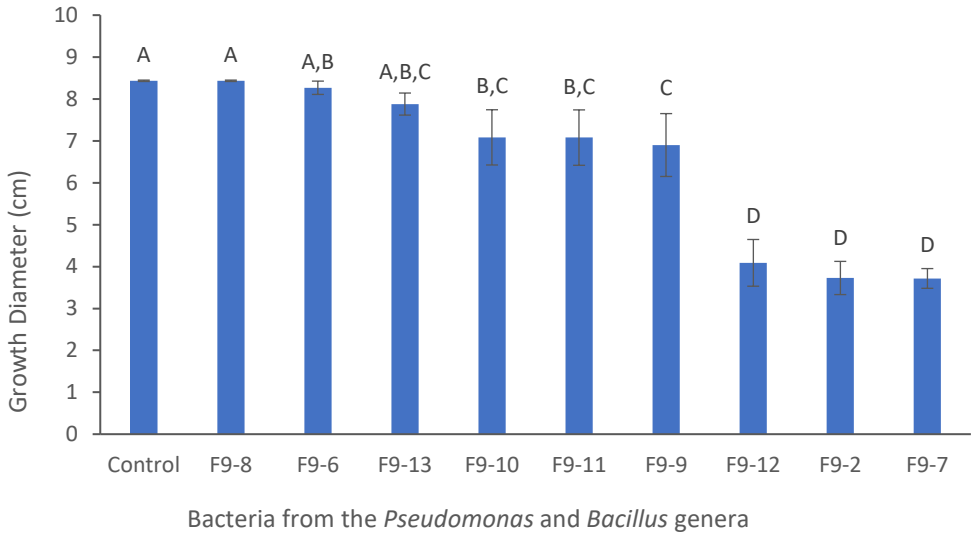


Figure 9: Compost bacteria effects on S. sclerotiorum, after three days of growth on PDA. Growth with the same letters is not significantly different according to Fisher's protected LSD test (P = 0.05).

5.2 The Antagonistic Effects of Bacteria on White Mould – *In Vivo*

Several *in vivo* results (Figure 10; Figure 11; Figure 12) were observed by directly confronting *S. sclerotiorum* with nine bacteria from the *Pseudomonas* and *Bacillus* genera on the periderm of carrot roots. All results were compiled over two-time periods (six days and seven days) for each of nine bacteria.

Six days following inoculation, two bacteria — *P. arsenicoxydans* (F9-7) and *B. subtilis* (F9-12) — had significantly suppressed white mould by an average of 31% when compared to the control. Conversely, seven bacteria — *P. moraviensis* (F9-6); *B. subtilis* (F9-2); *P. moraviensis* (F9-11); *P. koreensis* (F9-9); *P. brenneri* (F9-10); *P. brenneri* (F9-13) and *B. subtilis* (F9-8) — did not significantly affect white mould disease under our experimental conditions.

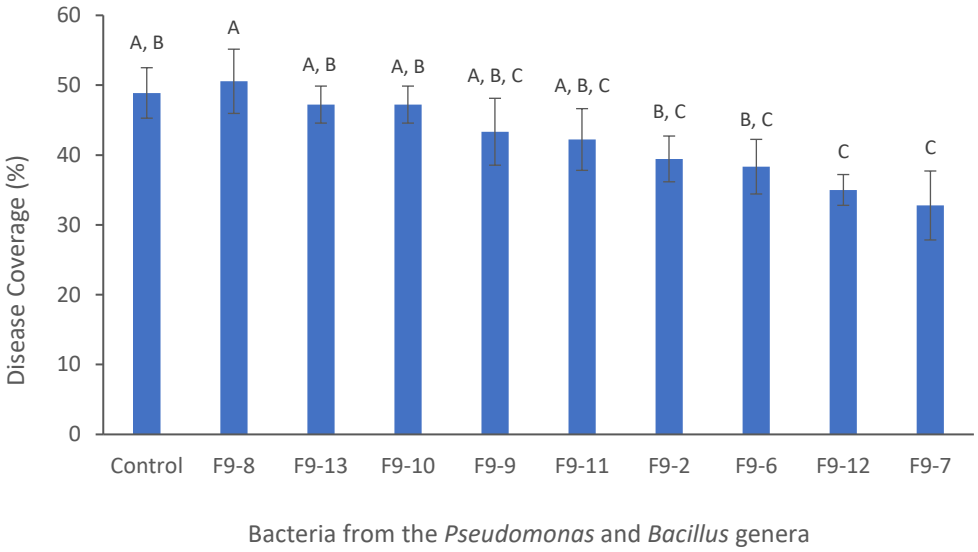


Figure 10: Effect of compost bacteria on white mould after six days of growth. Disease coverages with the same letter are not significantly different according to Fisher's protected LSD test (P = 0.05).

Seven days following inoculation, six bacteria — *P. arsenicoxydans* (F9-7); *B. subtilis* (F9-12); *B. subtilis* (F9-2); *P. moraviensis* (F9-6); *P. koreensis* (F9-9) and *P. brenneri* (F9-10) — had significantly inhibited mycelial growth when compared to the control. *P. arsenicoxydans* (F9-7); *B. subtilis* (F9-12) and *B. subtilis* (F9-2) were the most effective, suppressing white mould by an average of 36%, relative to the control. Conversely, three bacteria — *P. brenneri* (F9-13); *P. moraviensis* (F9-11) and *B. subtilis* (F9-8) — did not significantly affect white mould disease under our experimental conditions.

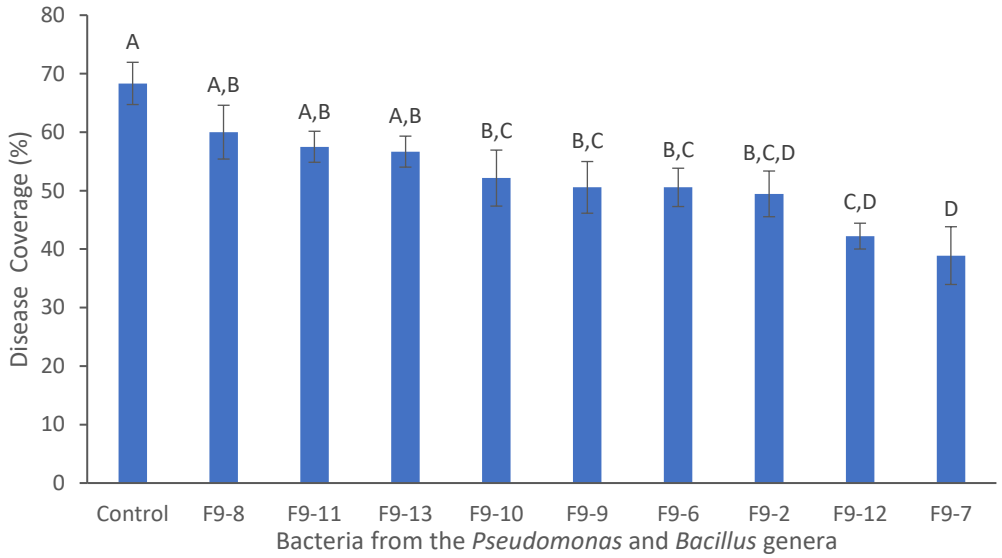


Figure 11: Effect of compost bacteria on white mould after seven days of growth. Disease coverages with the same letter are not significantly different according to Fisher's protected LSD test (P = 0.05).

Treatment	Day 6	Day 7
S. sclerotiorum control		
S. sclerotiorum and F9-2		
S. sclerotiorum and F9-6		
S. sclerotiorum and F9-7		
S. sclerotiorum and F9-8		
S. sclerotiorum and F9-9		
S. sclerotiorum and F9-10		
S. sclerotiorum and F9-11		
S. sclerotiorum and F9-12		
S. sclerotiorum and F9-13		

Figure 12: In vivo results after 6 and 7 days of incubation at 17 ± 1 °C

5.3 The Antagonistic Effects of Bacteria on the Mycelial Growth of *S. sclerotiorum* - Antifungal Culture Filtrates

Two clear results were observed from the cell-free filtrate bioassay performed at a concentration level of 15% (Figure 13). First, all culture filtrates were significantly antifungal except for the filtrates of *P. brenneri* (F9-10). Secondly, *B. subtilis* (F9-2), *P. brenneri* (F9-13), *P. arsenicoxydans* (F9-7) and *P. moraviensis* (F9-6) were observed as inhibiting mycelial growth the most by an average of 29% relative to the control.

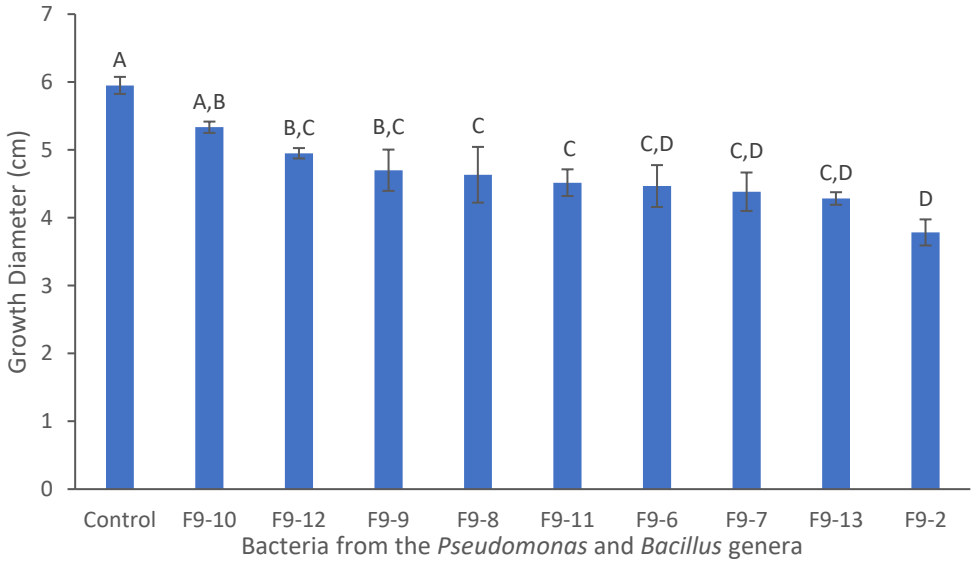


Figure 13: Effect of antifungal culture filtrate compost bacteria at a concentration of 15% on white mould after two days of growth. Growth diameters with the same letter are not significantly different according to Fisher's protected LSD test (P = 0.05).

Several cell-free filtrate bioassay results (Figure 14) were also observed by directly confronting *S. sclerotiorum* with nine antifungal culture filtrates from *Pseudomonas* and *Bacillus* genera at three different concentration levels (5%, 10% and 15%) and comparing them to a control of 0%. More specifically, only two bacteria were able to inhibit mycelial growth across all concentration levels — *B. subtilis* (F9-2) and *P. moraviensis* (F9-11). At a minimum concentration level of 10%, six bacteria were able to inhibit mycelial growth — *P. brenneri* (F9-13), *P. arsenicoxydans* (F9-7), *P. moraviensis* (F9-6), *P. koreensis* (F9-9), *B. subtilis* (F9-12), and *P. brenneri* (F9-10). Conversely, *B. subtilis* (F9-8) was only able to inhibit mycelial growth at a concentration level of 15%.

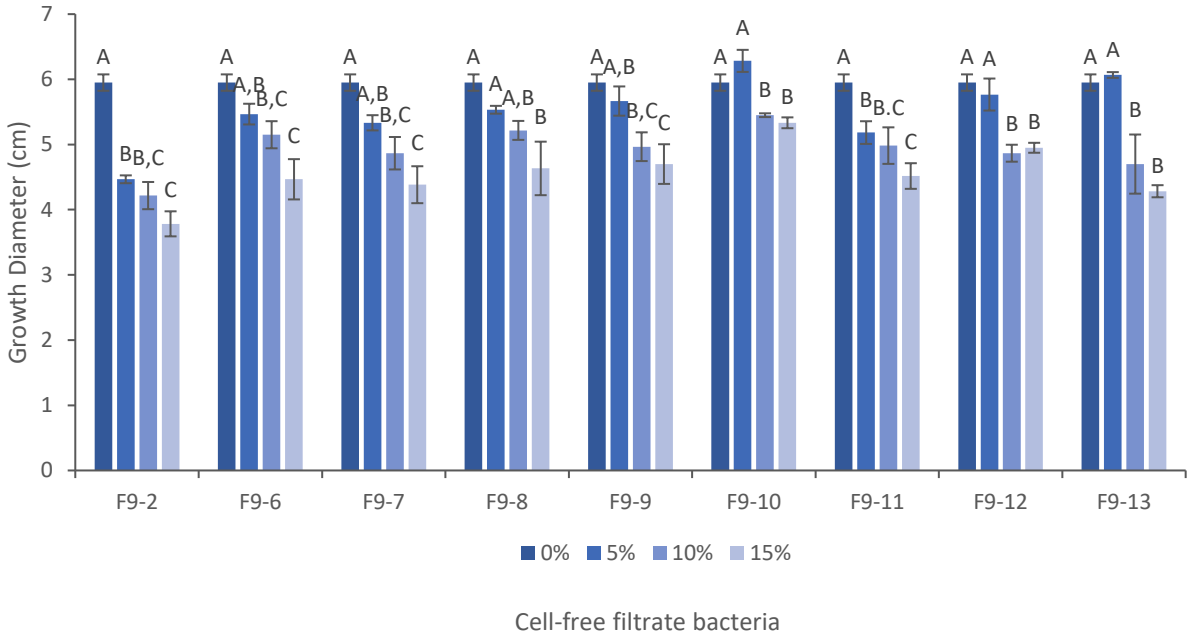


Figure 14: Effect of antifungal culture filtrates on mycelial growth after two days at three different concentration levels (5%, 10%, and 15%) relative to a control (0%). Growth diameters with the same letter are not significantly different according to Fisher's protected LSD test (P = 0.05).

5.4 The Antagonistic Effects of *P. arsenicoxydans* (F9-7) on the Mycelial Growth of *S. sclerotiorum*

When compared to the ethyl acetate control, the disk bioassays with the crude extract from *P. arsenicoxydans* (F9-7) revealed antimicrobial activity against *S. sclerotiorum* after a 48-hour incubation period. The extract was therefore fractionated in an attempt to purify and characterize antimicrobial compounds responsible for the observed activity.

5.5 The Bioactive Compounds of *P. arsenicoxydans* (F9-7)

5.5.1 Flash Column Chromatography and Thin Layer Chromatography on Fraction 5

One hundred and two eluates were obtained from normal phase silica gel flash column chromatography. A combination of analytical TLC and HPLC allowed pooling of eluates with similar compound profiles, resulting in five fractions. Fraction 5 was selected for subsequent analysis, because its mass was highest and therefore the most likely to afford sufficient material for spectroscopic characterization and bioassay assessment.

Preparative TLC of fraction 5 (1,005.2 mg) allowed isolation of multiple bands. As it was the most abundant, Band 6 was selected for further study as follows. Band 6 was subjected to semi-preparative HPLC which revealed the presence of six major peaks (Figure 15). Four of the peaks were of interest because of their apparent abundance, namely those eluting at 12 min, 15 min, 15.8 min, and 17.2 min (Figure 15). Thus, the four peaks of interest were purified using preparative HPLC. A mass of 12.6 mg, 13.9 mg, 20.7 mg, and 22.3 mg were obtained for the four peaks collected, respectively.

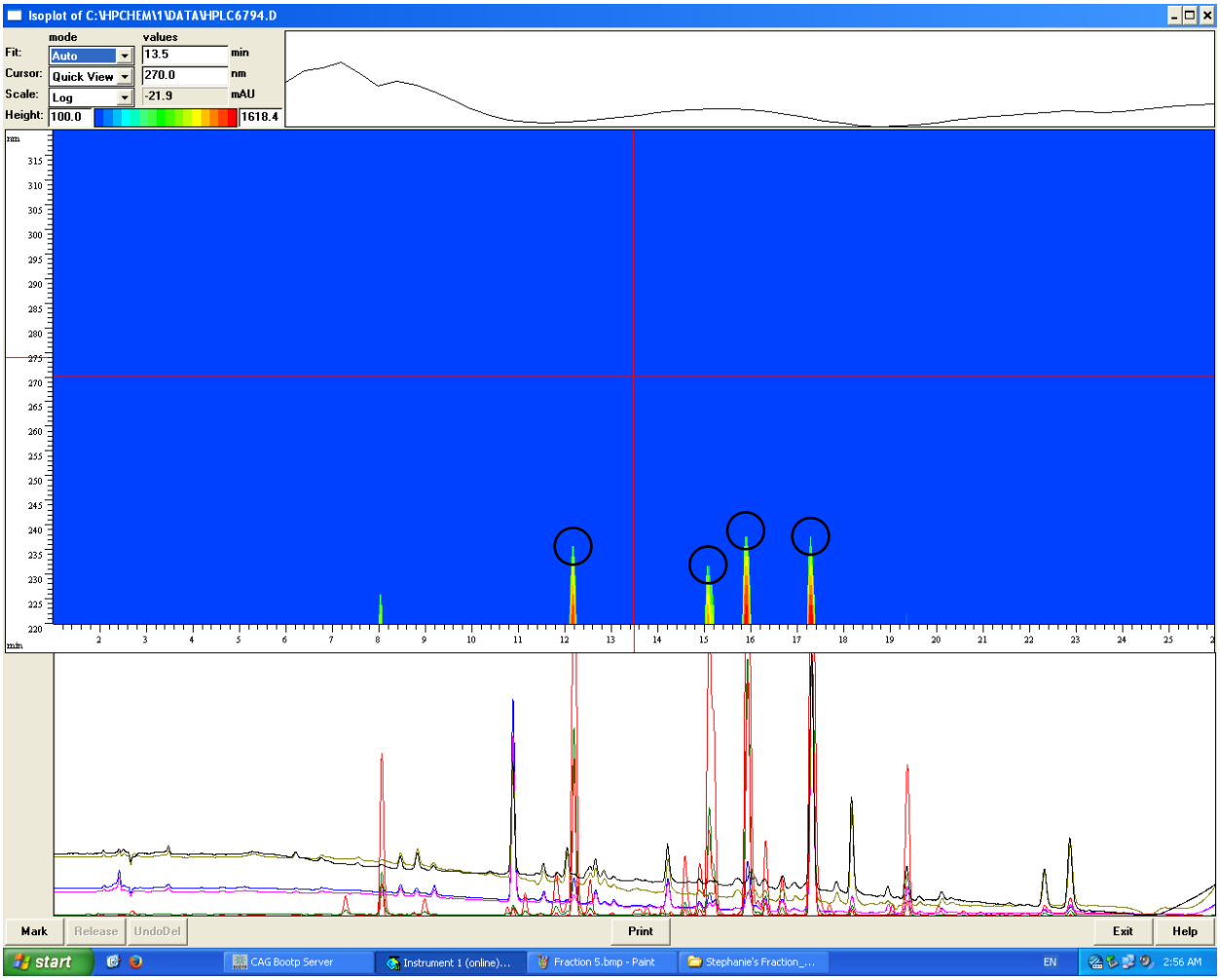


Figure 15: Band 6 analyzed with HPLC

5.5.2 Identification of Compounds

Nuclear Magnetic Resonance (NMR), mass spectrometry (MS), and optical rotation analysis allowed the identification of peaks 1, 3, and 4 as diketopiperazines as described below. Peak 2 contained insufficient material (9.8 mg) to enable a structural characterization.

Peak 1:

Compound **1** had a retention time of 12.0 minutes and was eluted with 26:74 ACN: ddH_2O from the semi-preparative HPLC. A total of 12.6 mg of compound **1** was purified, which had a visual appearance of a yellow oil. Compound **1** was identified as a dipeptide, more specifically as cyclo

(L-Pro-L-Val) (Figure 16) following the interpretation of NMR data (Table 7) and the supporting information included in Jiang et al. (2015). The optical rotation value ($[\alpha]_D$) was -159.67° (c 0.34, MeOH), ($[\alpha]_D$ = -122.4° (c 0.14, MeOH) (Jiang et al., 2015)), which confirmed L-configuration of both amino acid residues. The UV profile of the compound was also compared against a reference standard, which confirmed the identity of compound **1**: UV (MeOH) λ_{max} (log ε) 215 (4.68), 264 (4.21), 303 (3.82). For ^{1}H and ^{13}C NMR spectroscopic data, see Table 7.

Peak 3:

Compound **2** had a retention time of 15.8 minutes and was eluted with 35:65 ACN: ddH_2O from the semi-preparative HPLC. A total of 12.7 mg of compound **2** was purified, which had a visual appearance of a yellow oil. Compound 3 was identified as a dipeptide, more specifically as cyclo (L-Pro-L-Leu) (see Figure 16) following the interpretation of NMR data (Table 7) and the supporting information included in Jiang et al. (2015). The optical rotation value ($[\alpha]_D$) was -49.143° (c 0.34, MeOH), ($[\alpha]_D$ = -28.1° (c 0.17, MeOH) (Jiang et al., 2015)), which confirmed L-configuration of both amino acid residues. The UV profile of compound **2** was also compared against a reference standard, which confirmed the identity of compound **2**: UV (MeOH) λ_{max} (log ε) 215 (4.68), 264 (4.21), 303 (3.82); *m/z* 211.1436 $[M+H]^+$; (calc. for $[C_{11}H_{19}O_2N_2]^+$ 211.1441, -2.5 ppm).For ^{1}H and ^{13}C NMR spectroscopic data, see Table 7.

Peak 4:

Compound **3** had a retention time of 17.2 minutes and was eluted with 100:00 ACN: ddH_2O from the preparative HPLC. A total of 14.0 mg of compound **3** was purified, which had a visual appearance of a yellow oil. Compound **3** was identified as a dipeptide, more specifically as cyclo (L-Pro-L-Phe) (see Figure 16) following the interpretation of NMR data (Table 7) and the supporting information included in Jiang et al. (2015). The optical rotation value ($[\alpha]_D$) was -68.760° (c 0.34, MeOH), ($[\alpha]_D$ = -23.1° (c 0.51, MeOH) (Jiang et al., 2015)), which confirmed L-configuration of both amino acid residues. The UV profile of the compound was also compared against a reference standard, which confirmed the identity of compound **3**: UV (MeOH) λ_{max} (log ε) 215 (4.68), 264 (4.21), 303 (3.82); *m/z* 245.1280 $[M+H]^+$; (calc. for $[C_{14}H_{17}O_2N_2]^+$ 245.1285 -1.85 ppm). For ^{1}H and ^{13}C NMR spectroscopic data, see Table 7.

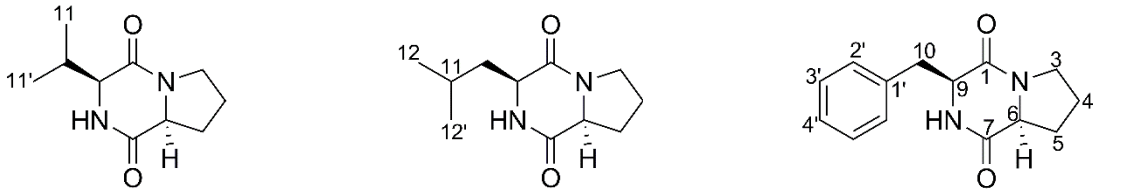


Figure 16: Chemical structures of diketopiperazines produced by P. arsenicoxydans (F9-7)

Table 7: ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data for diketopiperazines produced by P. arsenicoxydans (F9-7) in CD_3CN.

	Cyclo (L-Pro-L-Val) (1)		Cyclo (L-Pro-L-Leu) (2)		Cyclo (L-Pro-L-Phe) (3)	
Position	**δ_C, type**	**δ_H (*J* in Hz)**	**δ_C, type**	**δ_H (*J* in Hz)**	**δ_C, type**	**δ_H (*J* in Hz)**
1	166.0, C		166.0, C		167.4, C	
3	45.7, CH_2	3.49, dt (11.9, 8.1)	45.6, CH_2	3.48, m	45.9, CH_2	3.34, m
		3.36, ddd (11.9, 8.1, 4.2)		3.39, m		
4	22.9, CH_2	1.82, m	23.0, CH_2	1.89, m	23.4, CH_2	1.81, m
5	28.9, CH_2	2.11, m	29.1, CH_2	2.22, m	28.6, CH_2	2.11, m
		1.60, m		1.91, m		1.90, m
6	59.6, CH	4.04, m	59.4, CH	4.06, m	59.6, CH	4.04, m
7	170.2, C		171.2, C		171.4, C	
8		6.06, bs		6.33, bs		6.31, bs
9	57.9, CH	4.33, m	60.9, CH	3.92, m	54.0, CH	3.93, dd (8.6, 3.9)
10	36.9, CH_2	3.26, dd (14.4, 4.7)	29.2, CH	2.44, m	39.1, CH_2	1.80, m
		2.97, dd (14.4, 7.2)				1.35, m
11			18.9, CH_3	1.04, d (7.2)	25.2, CH	1.78, m
11’			16.5, CH_3	0.88, d (6.8)		
12					23.2, CH_3	0.87, d (6.5)
12’					21.7, CH_3	0.84, d (6.5)
1’	137.9, C					
2’	130.5, CH	7.28, m				
3’	129.4, CH	7.29, m				
4’	127.7, CH	7.25, m				

5.6 Determination of Antimicrobial Activity of Isolated Compounds Through Disk Diffusion Bioassay

To test the antifungal activity of the identified dipeptides, a disk bioassay was performed. However, it is important to note that only two of the three identified dipeptides were tested with disk bioassay, as unfortunately the amount of cyclo-(L-Phe-L-Pro) was not sufficient to perform the assay. First, the dipeptides were tested individually against *S. sclerotiorum*. The results showed that when the concentration of either dipeptide was 50 mg/mL, the antagonistic effect was not significantly different compared to the control under our experimental conditions (results not shown). However, when the concentration was increased to 100 mg/mL cyclo-(L-Pro-L-Leu) was able to significantly reduce fungal growth by 14%. On the other hand, cyclo-(L-Pro-L-Val) was not inhibitory (Figure 17). Finally, when the dipeptides were combined at an equal concentration (i.e., 50 mg/mL of each dipeptide tested), the result is significantly different from the control with an inhibition factor of 7% under our experimental conditions.

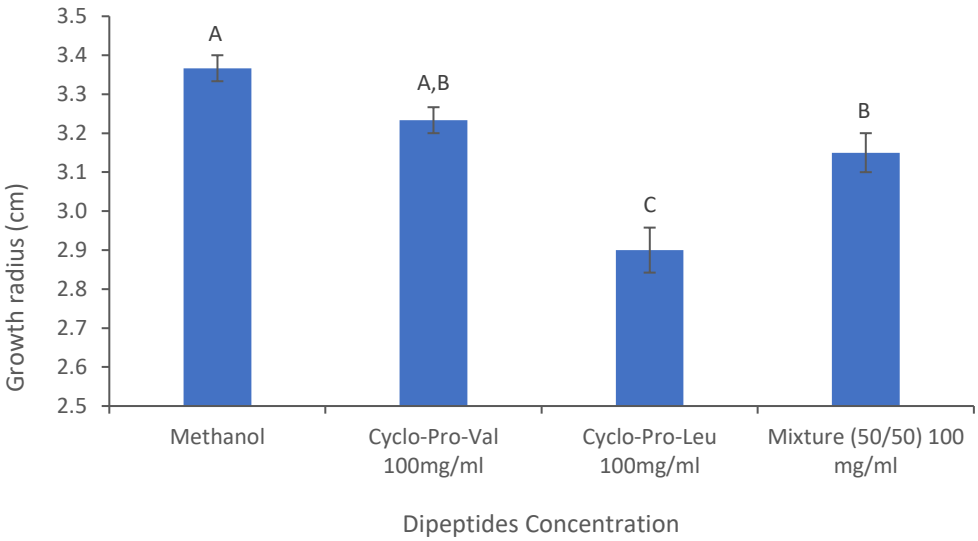


Figure 17: Effect of dipeptides (cyclo-Pro-Val, and cyclo-Pro-Leu) on mycelial growth after two days at a concentration of 100 mg/ mL relative to a control (MeOH). Growth radii with the same letter are not significantly different according to Fisher's protected LSD test (P = 0.05).

Chapter VI: Discussion

Food loss is recognized as a complex, global issue. Since the 1960s, the utilization of synthetic chemicals has been the dominant approach for managing the risk of food loss. Yet, despite their utility as a means to control microbes, the use of chemical agents has serious drawbacks. For example, it is now understood that the recurrent utilization of synthetic chemicals can present risks for humans and the environment. Moreover, the repeated utilization of synthetic chemicals is now known to increase microbial resistance (Avis et al., 2009), which means even more synthetic chemicals are called upon to achieve similar past efficacy or simply that chemical lose all efficacy. Therefore, alternatives are urgently required. The use of bacteria, as biocontrol agents, holds great promise in addressing the drawbacks of synthetic chemicals, and is increasingly viewed as a reliable method to help enhance crop quality with little to no phytotoxicity.

This research studied nine bacteria from the *Pseudomonas* and *Bacillus* genera as a biological approach for managing the ever-present risk of white mould disease. Research showed that *Pseudomonas* and *Bacillus* species have antagonistic properties, and within the marketplace, Bacilli has become recognized as one of the most commercialized biocontrol agents due to their effectiveness (Pérez-García et al., 2010). All bacteria utilized in this research are from recognized species or groups considered safe for the environment and cause no harm to human health. This research focused on *S. sclerotiorum* because it is one of the most widespread moulds responsible for irreversible crop loss, and it is regarded as a difficult mould to manage (Saharan, 2008). This research hypothesised that bacteria from the genius *Pseudomonas* and *Bacillus* can significantly inhibit growth of *S. sclerotiorum* and suppress carrot white mould disease in a post-harvest context, and consequently, serve as a viable management alternative to fungicides.

6.1 Antagonistic Effects of Bacteria on the Mycelial Growth of *S. sclerotiorum – In Vitro*

The mycelial growth of *S. sclerotiorum,* when directly confronted with nine bacteria on potato dextrose agar, showed clear results (Figure 9). Among the bacteria that did have a statistically significant effect in reducing mycelial growth, the greatest inhibiting effect observed with *P. arsenicoxydans* (F9-7), *B. subtilis* (F9-2) or *B. subtilis* (F9-12). While further research could be explored on whether these antifungal effects could be further enhanced, or amplified, the resulting impacts of *B. subtilis* observed in this research resonates well with the outcomes of several other recent studies. For example, the work of Vinodkumar et al. (2017) on 55 strains of *B. subtilis* was able to similarly demonstrate that *B. subtilis* was most effective in inhibiting *S. sclerotiorum in vitro*, and that the mycelial plug containing *S. sclerotiorum* failed to grow on PDA when exposed to some *B. subtilis*. The work of Sun et al. (2017) demonstrated that *B. subtilis* resulted in an inhibitory that was superior to 80%. The work of Pershakova et al. (2018) showed that *B. subtilis* caused a delay in *S. sclerotiorum* growing *in vitro*. The work of Kamal et al. (2015) also found that *B. subtilis* strongly inhibited mycelial growth with complete inhibition of sclerotial germination *in vitro*.

Regarding the *P. arsenicoxydans* (F9-7) strain, Mohamed et al. (2017) found that it was able to reduce the growth of both *Fusarium sambucinum* and *Verticillium dahliae* — two other mould phytopathogens. More broadly, Savchuk et al. (2002) tests on eight strains of *Pseudomonas* from *Pseudomonas* spp. and *P. chlororaphis,* which were able to all significantly inhibit *S. sclerotiorum in vitro* conditions with an inhibition factor of at least 50%. The work of Expert (1995) on nine strains from the *Pseudomonas fluorescens* group also showed significant reductions in mycelium growth against *S. sclerotiorum*. Also, the antagonistic bacteria were able to inhibit sclerotia production *in vitro*. The work of Fernando et al. (2007) on *Pseudomonas* spp. and *P. chlororaphis* further demonstrated significant reduction of mycelium growth against *S. sclerotiorum*, and further added that the treatment is less effective when ascospores are present before the inoculation of the antagonistic bacterium, but when the bacteria is inoculated prior or at the same time as *S. sclerotiorum*, growth is completely inhibited.

6.2 Antagonistic Effects of Bacteria on Carrot White Mould – *In Vivo*

The development of white mould disease, with application of nine bacteria on the periderm of a carrot, showed two clear *in vivo* results. First, the antagonistic potential of bacteria on mycelial growth became more evident with the passage of time. For example, on day six of the *in vivo* studies, only two bacteria (F9-12 and F9-7) demonstrated an influence in reducing mycelial growth, and they did so by an average of 31%. Yet, on day seven, the number of bacteria demonstrating an influence had increased to seven (F9-12; F9-7; F9-10; F9-9; F9-6; F9-2) and collectively they reduced mycelial growth by an average of 64%. Moreover, three of these bacteria (F9-10, F9-9, and F9-6) showed antagonistic results that were not clearly observed during *in vitro* studies. Second, the antagonistic potential of two bacteria — *B. subtilis* (F9-12) and *P. arsenicoxydans* (F9-7) — showed the greatest consistency in suppressing the disease over the least amount of time. For example, the F9-12 and F9-7 bacteria reduced mycelial growth by an average of 54% during the *in vitro* studies and an average of 41% during the *in vivo* studies.

The resulting impacts of *B. subtilis* observed in this *in vivo* research on white mould disease on carrots resonates well with the outcomes of several other studies. For example, Pershakova et al. (2018) studies on three strains of *B. subtilis* was equally able to cause *in vivo* delays in the growth of *S. sclerotiorum* on carrots. Furthermore, these bacteria also demonstrated that they could significantly reduce other pathogenic microorganisms such as the mould *Alternaria radicina* and the bacterium *Erwinia carotovora* on carrots. While not specific to carrots, the work of Kamal (2015) on canola plants equally demonstrated that *B. subtilis* is effective in reducing both sclerotia in soil and white mould disease on whole canola plants.

More broadly, across the agricultural sector, *Bacillus* is increasingly being applied, and recognized, as a multi-faceted biocontrol agent with insecticidal, antifungal, and fertilizing properties. For example, in the field of (bio)pesticides, *B. thuringiensis* is being applied in bioinsecticide products to control pests like lepidopteran, dipteran and coleopteran larvae. In the field of (bio)fertilisers, *B. amyloliquefaciens* is being applied in fertilizing products to improve the bioavailability of essential compounds and stimulate root proliferation and nutrient uptake by supporting the production of phytohormones. In the field of (bio)fungicides, *B. licheniformis, B. pumilus* and *B. subtilis* species are being employed in products to control a variety of phytopathogens, such as *Rhizoctonia*, *Pythium*, *Fusarium*, *Phytophthora*, and *Sclerotinia*, which

was the specific focus of this research. Companies like Growth Products and AgraQuest, which are both based in the United States, have commercially applied *B. subtilis* as a biofungicide product, to address *Sclerotinia*. These products can be found under the trade names, Companion and Serenade (Pérez-García et al., 2010).

Regarding *Pseudomonas,* and more specifically *P. arsenicoxydans* on carrots, no explicit research on that relationship was found, which highlights the thrust of this research. Although not specific to carrot, research on *Pseudomonas* from the *fluorescens* group was present in the work of Fernando et al. (2007) who studied the efficacy of *P. chlororaphis* on canola petals. Their work concluded that *P. chlororaphis* was able to significantly reduce white mould disease caused by *S. sclerotiorum* under field conditions. Furthermore, they demonstrated that ascospore germination could be inhibited and plant defense mechanisms can be triggered when *P. chlororaphis* is applied to the petals. The work of Savchuk & Fernando (2004) on testing the efficacy of using *P. chlororaphis* against *S. sclerotiorum* on canola petals further concluded that *P. chlororaphis* was able to successfully colonize canola petals for several days, which in turn controlled the development of the white mould disease cause by *S. sclerotiorum*. They were able to demonstrate that bacteria can effectively compete for nutrients, even after growth and establishment of the pathogen. They also concluded that the treatment is more effective when applied prior to mould infestation. The work of Saharan (2008) further identified five *Pseudomonas* strains as potential biocontrol agents to control *S. sclerotiorum*, which are: *Pseudomonas* sp., *P. chlororaphis*, *P. cepacian*, *P. fluorescens*, and *P. putida*, which was supported by research on different types of crops such as sunflower (*P. fluorescens*, and *P. putida*) and canola (*P. chlororaphis*). To our knowledge, this research offers a first insight on the use of *Bacillus* and *Pseudomonas* against *S. sclerotiorum* on carrots.

6.3 Antifungal Culture Filtrates from Compost Bacteria

Building upon the *in vitro* and *in vivo* findings that showed the growth of *S. sclerotiorum* was most affected (i.e., inhibited) by the presence of *P. arsenicoxydans* (F9-7), *B. subtilis* (F9-12) and *B. subtilis* (F9-2), a deeper cell-free filtrate bioassay analysis at different concentration levels (5%, 10% and 15%) was performed to evaluate whether ~~s~~secondary metabolites with antifungal properties could be further identified. Ultimately, this approach showed that all tested bacteria with the one exception of *P. brenneri* (F9-10) were able to reduce mycelial growth with statistical significance. However, this desired effect mostly derived after confronting with higher concentration levels (i.e., 15%) than lower concentration levels (i.e., 5%). For example, at the higher concentration level of 15%, all bacteria strains except *P. brenneri* (F9-10) showed significant inhibition. At the lowest concentration level of 5%, only two bacteria strains — *B. subtilis* (F9-2) and *P. moraviensis* (F9-11) — showed significant inhibition. Together, these results suggest that antibiosis may be involved in the mechanism of suppression of *S. sclerotiorum* for all tested bacteria except possibly *P. brenneri* (F9-10). However, *P. brenneri* (F9-10) may require the presence of a competitor to begin antimicrobial compound production, which would not be determined in this study. In addition, because *B. subtilis* (F9-8) was unable to inhibit mycelial growth at either lower concentration levels, this could mean the compounds that promote growth in the filtrate, and the quantity of antifungal compounds that may be present, may be insufficient to mitigate this effect. *Bacillus subtilis* (F9-2), and *P. moraviensis* (F9-11) may produce a compound that is inhibitory at lower concentrations or that the combination of compounds in the filtrate were acting in an additive or synergistic manner.

Linking these results with research from Raaijmakers et al. (2012), it is plausible to assume that most of the tested bacteria (with the possible exception of *P. brenneri* (F9-10)) produce, to a certain level, secondary metabolites that have antifungal properties against *S. sclerotiorum*. If the antagonistic activity is only due to the secondary metabolite, then the antagonistic activity would involve antibiosis, as a direct mechanism of action. However, the antagonistic activity could also be caused by competition for nutrients and/or space, which involves an indirect mechanism of action (Köhl et al., 2019).

When comparing the cell-free filtrate bioassay with the confrontational bioassay for F9-7 and F9-12 some difference in the effectiveness of the antagonistic effect can be observed. In fact, for the confrontational bioassay an inhibition factor of 54% was obtained for the *in vitro* experiment, and 31% and 41% was obtained for the *in vivo* experiment at day 6 and day 7, respectively. Whereas for the cell-free bioassay, an inhibition factor of 26% was obtained at a concentration of 15% when considering only these two bacteria. The difference in the inhibition factors for all bioassays may be due to differences in the environmental setting. In fact, for the confrontational bioassays (*in vitro* and *in vivo*), *S. sclerotiorum* was directly confronted with the whole bacterium whereas for the cell-free filtrate bioassay *S. sclerotiorum* was confronted with each bacterium cell-free filtrate. While it is assumed that cell-free filtrate contained secreted secondary metabolites (and possibly other elements) with antifungal activity against *S. sclerotiorum*, research by DeFilippi et al. (2018) showed that the presence of the stressor agent matters in the production of secondary metabolites. Briefly, the presence of the pathogen may have an important role in the specificity of the antagonistic activity because by being directly confronted with the opponent the production of secondary metabolites may be more specific to target the opponent. Also, its production could vary substantially temporally depending on the targeted fungus (Martinez et al., 2006). Hence, although MOLP contain stressor agents that activate the production of secondary metabolites, it is not possible to know if they are in sufficient amount, and which one are triggered, nor whether these secondary metabolites are necessarily effective against *S. sclerotiorum*.

Another difference that may have influenced the outcome of the confrontational bioassays, the bacteria were grown and maintained on TSA, which is a solid medium, whereas for the cell-free filtrate bioassay the bacteria were grown in a liquid MOLP medium. Research by Séveno et al. (2001) showed that the state of the culture (liquid or solid) matters. In this study, the production of secondary metabolites may have been influenced by the culture media (MOLP) when compared to the growth media TSA where bacteria were grown and maintained for the confrontational bioassay.

This study contributes novel insights on the use of cell-free filtrates from *P. arsenicoxydans* (F9-7) against *S. sclerotiorum*, which addresses a knowledge gap on the effect of cell-free filtrates from *P. arsenicoxydans* (F9-7) against *S. sclerotiorum*.

6.4 Isolation and Purification of Antimicrobial Compounds from *P. arsenicoxydans* strain (F9-7)

Within this experiment, three dipeptides (diketopiperazines) were identified from *P. arsenicoxydans* (F9-7). They were cyclo-(L-Pro-L-Leu), cyclo-(L-Pro-L-Val) and cyclo-(L-Pro-L-Phe). This is an important finding, as the production of secondary metabolites, like dipeptides, is irrefutably linked to biocontrol efficacy (L.-P. Gross, & Kole,, 2014), and the presence of these bioactive compounds may have been helpful in producing the antagonistic effects observed against *S. sclerotiorum*. Their application is also promising, as dipeptides are relatively simple molecules that can be easily found in nature (Prasad, 1995) and are known to support many biological activities associated with antibiotics, toxins, hormones, and ion transport regulation (Ovchinnikov & Ivanov, 1975). In addition, dipeptides can degrade rapidly to their respected amino acids, and have no human toxicity (Loffet, 2002). Further bioactivity information on each of the identified dipeptides is below.

Cyclo-(L-Pro-L-Leu)

Research by Rhee (2003) demonstrates that cyclo-(L-Pro-L-Leu) has antifungal activity against rice blast fungus — *Magnaporthe oryzae* — when the concentration of the dipeptide is 0.0025 mg/mL. Research by S. Nishanth Kumar (2012) showed that cyclo-(L-Pro-L-Leu) was antifungal against five different fungi, yet at varying concentrations. For example, a concentration of 0.004 mg/mL was needed for inhibiting the fungus *Penicilium expansum*; 0.008 mg/ mL was needed inhibiting *Rhizoctonia solani*; 0.16 mg/ mL was needed for inhibiting *Aspergillus flavus* and *Fusarium oxysporum;* and 0.5 mg/ mL was needed for inhibiting *Candida albicans.* In this study, a concentration of 100 mg/mL of cyclo-(L-Pro-L-Leu) was needed to significantly inhibit *S. sclerotiorum* (Figure 17). When comparing our findings with the literature, it is assumed that the inhibitory effect of this dipeptide is minimal, and thus not a good alternative for post-harvest white mould disease.

Cyclo-(L-Pro-L-Val)

Research by Sansinenea et al. (2016) demonstrates that cyclo-(L-Pro-L-Val) showed antifungal activity against *Fusarium oxysporum* and *Penicillium* when the concentration of the dipeptide is 75 mg/mL. Research by Tezuka et al. (1994) demonstrates that 0.03 mg/mL of cyclo-(L-Pro-L-

Val) or 0.03 mg/ mL of cyclo-(L-Pro-L-Leu) against *Ganoderma lucidum* (a fungus that grows on hardwood trees) had no inhibitory effect. However, while these two dipeptides had no inhibitory effects against *G. lucidum,* they nevertheless might still be useful on other moulds, as secondary metabolites with a 5-formyl group (which none of these dipeptides have) may play an important role for inhibitory effects to occur. In this study, no significant inhibition of *S. sclerotiorum* could be observed when confronted with cyclo-(L-Pro-L-Val) at a concentration of 100 mg/mL (Figure 17). When comparing our findings with the literature, it is presumed that this dipeptide has no inhibitory effect against *S. sclerotiorum*, and thus not a good alternative for post-harvest white mould disease. Furthermore, a mixture of cyclo-(L-Pro-L-Leu) and cyclo-(L-Pro-L-Val) at a concentration of 50 mg/mL each was tested against *S. sclerotiorum* to see if by combining the two dipeptides we could have a cumulative or a synergic effect. The results show that when combined the two dipeptides, although we observe a significant reduction of *S. sclerotiorum* when compared to the control, the inhibition is less than when cyclo-(L-Pro-L-Leu) is tested alone. This suggested an additive rather than a synergistic effect of this treatments.

Cyclo-(L-Pro-L-Phe)

Research by Holden et al. (1999) identified cyclo-(L-Pro-L-Phe) in *P. fluorescens* and *P. alcaligenes,* and further found that it was involved in the activation of a biosensor involved in quorum-sensing in *E. coli*. Research by Strom (2002) found that cyclo-(L-Pro-L-Phe) was effective against *Fusarium sporotrichioides* and *Aspergillus fumigatus* at a concentration of 20 mg/mL. Cyclo-(L-Pro-L-Phe) was also assayed against *Penicillium roqueforti*, but no inhibitory effect was observed. In this research there was not enough cyclo-(L-Pro-L-Phe) available to test it against *S. sclerotiorum*.

To our knowledge, the production of cyclo-(L-Pro-L-Leu), cyclo-(L-Pro-L-Val), and cyclo-(L-Pro-L-Phe) by *P. arsenicoxydans* (F9-7) is reported here for the first time Although little work has been performed on the potential mechanisms of antifungal activity of cyclic dipeptides, a possible mechanism could be the inhibition of chitin synthesis, which would negatively affect fungal cell wall synthesis (Martins & Carvalho, 2007).

Chapter VII: Conclusion

Nine bacteria strains from the genera *Pseudomonas* and *Bacillus* were tested against *S. sclerotiorum*. In a controlled laboratory setting, *P. fluorescens* (F9-7) and *B. subtilis* (F9-2 and F9-12) inhibited the mycelial growth of *S. sclerotiorum* by up to 54%, which validates earlier *in vitro* research showing the potential of bacteria as an antagonistic agent (Expert & Digat, 1995; Pershakova et al., 2018). An inhibition of 36% (for day 7) was obtained when the experiment was conducted *in vivo* (on carrots), and when taking only the cell-free filtrate for the same three bacteria, the inhibition rate was 27% when considering a concentration of 15%. These results agree with the proposed hypotheses.

Taking to account the fact that the inhibition rate dropped when comparing the confrontational bioassays (*in vitro*) with the cell-free filtrate bioassay, it is plausible to assume that when the whole bacterium is confronted with *S. sclerotiorum*, competition for nutrients and/or space as well as antibiosis may play a role in the antagonistic activity. Whereas, for the cell-free bioassay, the only plausible mechanism of action would be antibiosis.

Three compounds from *P. arsenicoxydans* (F9-7) were purified and characterized as cyclodipeptides: (L-Val-L-Pro), (L-Leu-L-Pro), and (L-Phe-L-Pro). The most antifungal effect was obtained with the cyclodipeptide L-Leu-L-Pro at a concentration of 100 mg/mL. These results suggest that the extracts from culture filtrates may contain antimicrobial compounds against *S. sclerotiorum*, unfortunately, probably not in a sufficient concentration to be utilized as a reduced-risk management strategy. However, the use of the bacterium as the control measure could produce a more continuous supply of antifungal compounds, and might be useful to reduce disease progression, as seen in the *in vitro* assays.

An avenue for future research could explore whether the environmental conditions have an impact on the antifungal activity. For example, the assay could be done under conditions more similar to industrial conditions (e.g., in a refrigerator for a long storage period). Future research could also investigate whether white mould disease could be suppressed more effectively on another type of plant such as beans, celery, lettuce, peanuts, potatoes, soybeans, sunflowers, or tomatoes. Also, cell-free filtrate could be produced in greater quantity to maximize the production of antimicrobial metabolites at a higher concentration, making easier to characterize. Furthermore, MOLP media could be customized. For example, changing the carbon source (e.g., sucrose for lactose) or the

nitrogen source (e.g., animal peptones for plant or microbial peptides) of the media could potentially have a benefit on the production of microbial secondary metabolites. Moreover, isolating *B. subtills* secondary metabolite and assaying against white mould disease could be an avenue for future research. Lastly, trying different combinations of bacteria to investigate if an increase of the antifungal activity against white mould disease could be observed.

S. sclerotiorum is a common phytopathogenic mould that causes irreversible harm to plants and contributes significantly to food spoilage in vegetables around the world. The need for additional ways to control the risks of *S. sclerotiorum* spoilage post-harvest, and reduce food spoilage in general, is a global challenge in need of additional biological or biochemistry solutions.

Chapter VIII: References

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