Analysis of three biological control agents and naturally-occurring fungal colonizers on the survival of sclerotia of *Botrytis squamosa, Sclerotinia sclerotiorum* and *Sclerotium cepivorum*

by

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A Thesis
presented to
The University of Guelph

In partial fulfillment of requirements
for the degree of
Master of Science
in
Environmental Sciences

Guelph, Ontario, Canada

ABSTRACT

ANALYSIS OF THREE BIOLOGICAL CONTROL AGENTS AND NATURALLY-OCCURRING FUNGAL COLONIZERS ON THE SURVIVAL OF SCLEROTIA OF *BOTRYTIS SQUAMOSA, SCLEROTINIA SCLEROTIORUM* AND *SCLEROTIUM CEPIVORUM*

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University of Guelph, 2014

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Management of sclerotia-forming phytopathogenic fungi is difficult due to the resilience of their sclerotia within soil. This thesis assessed the efficacy of three biological control agents (BCAs), *Coniothyrium minitans, Microsphaeropsis ochracea*, and *Trichoderma atroviride*, on laboratory-produced (LP) sclerotia of *Botrytis squamosa*, *Sclerotinia sclerotiorum* and *Sclerotium cepivorum* under field conditions for 20 months. *Trichoderma atroviride* was most consistent, reducing survival of *B. squamosa* and *S. sclerotiorum* to 19 and 23%. *S. cepivorum* did not vary. Further investigations compared the survivability of LP and field-produced (FP) sclerotia of *S. sclerotiorum*. Survival of LP sclerotia of *S. sclerotiorum* was greater than that of FP, which were heavily colonized with other fungi. Colonizers were isolated, identified, and evaluated for antagonistic and pathogenic abilities. 80% of isolates demonstrated antagonistism, and two isolates demonstrated pathogenicity. It is evident that sclerotia are not sterile propagules, and that antagonistic soil-dwelling microorganisms are important influential factors on sclerotial survival.

ACKNOWLEDGEMENTS

I would like to take this opportunity to express my sincere gratitude to the individuals and groups that have supported me throughout my continued education and completion of this degree at the University of Guelph. Firstly, my deepest appreciation to my co-advisors Dr. Greg Boland and Dr. Mary Ruth McDonald for providing me with this opportunity, and for their continuous support, assistance, and guidance. Your insightful encouragement has allowed me to develop my analytical and communication skills which will forever be an asset as I move forward in life. I would also like to extend my gratitude to the final member of my committee, Dr. Katerina Serlemitsos Jordan. Your insightful support throughout the program has broadened my view and further developed my understanding of plant epidemiology.

The assistance of several colleagues was invaluable for the completion of this thesis research. Much appreciation to the team at the Muck Crops Research Station for their field assistance, especially Michael Tesfaendrias for his technical guidance and expertise. To Dr. Monica Parker for her assistance with molecular laboratory techniques. Your innate ability to relay concepts and information has been of great benefit to me, and your continuous offer of guidance has been greatly appreciated. Thanks to Sara Stricker for her assistance with field work preparations. To Melody Melzer, although I did not have the joy of working with you during my graduate degree, your guidance during my time as a graduate student was essential for my success throughout this degree - thank you. Furthermore, much appreciation to Dr. Brian Allen and Michelle Edwards for their expert assistance with the statistical analyses.

I am very thankful for the generous funding agencies that provided financial assistance both for this research and for my academic studies. Specifically, the Ontario Ministry of Agriculture, Food & Rural Affairs, University of Guelph, and the Ontario Processing Vegetable Growers. The contributions from both Plant Products Co. Ltd. and ProPhyta GmbH are also much appreciated.

I must also recognize my family and the invaluable support that they provide me with each and every day. To my parents, your unwavering encouragement ever since I was young has brought me to challenge myself, and has allowed me to achieve many things that would not have been possible without you. Finally, these acknowledgements are far from complete without recognizing my close friends, both in and outside of the graduate program. Every ounce of support that I have received throughout this process has been greatly valued. Thanks to every one of you!

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LIST OF ABBREVIATIONS

Abbreviation - used throughout test and listed in alphabetical order.

APDA	acidified potato dextrose agar
AUDPC	area under the disease progress curve
AUDPS	area under the disease progress stairs
AUPSS	area under the percent survival stairs
BCA	biological control agent
BLAST	basic local alignment search tool
bp	base pairs
C	Celsius
cm	centimetre
DNA	deoxyribonucleic acid
dsRNA	double-stranded ribonucleic acid
FP	field-produced
g	grams
ITS	internal transcribed spacer
kPA	kilopascal
L	litre
LP	laboratory-produced
Max ID	max identification
min	minute
mL	millilitre
mm	millimetre
PCR	polymerase chain reaction

Abbreviation - used throughout test and listed in alphabetical order.

PDA	potato dextrose agar
QC	query cover
QTL	quantitative trait loci
RFLP	restriction fragment length polymorphism
sp./spp.	species
UV	ultraviolet
µm	micrometre

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Sclerotia-forming fungi are globally widespread and have the potential to cause considerable crop losses. Much like other soil-borne plant pathogens, these sclerotial pathogens can be difficult to control and chemical fungicides are heavily relied upon for management. The presence of sclerotia in the life cycle makes disease management more difficult because sclerotia are vegetative, melanised resting bodies that can tolerate an array of unfavourable conditions within the soil profile for potentially long periods of time (Willetts, 1972; Willetts & Bullock, 1992). Recent research has focused on the development and implementation of improved cultural, biological, mechanical and nutritional methods to assist disease control as alternatives to fungicidal use. This has been largely in response to the rising issue of plant pathogen resistance to chemical pesticides, and the poor performance of currently available fungicides. Of these alternative control methods, the most commonly studied is the potential use of biological control agents (BCAs).

1.2 Sclerotia-Forming Fungi

The formation of hard, resting structures from aggregates of hyphae, known as sclerotia, is found intermittently throughout the fungal phyla of Ascomycota, Basidiomycota and Deuteromycota (Willetts & Bullock, 1992). The filamentous fungal pathogens of interest within this thesis are members of the Sclerotiniaceae family within the Ascomycete fungi.

The primary role of sclerotia within the life cycle of fungal plant pathogens is to ensure contact between the pathogen and a susceptible organ of a host plant, and may do so long after its formation (Coley-Smith & Cooke, 1971). Sclerotia are capable of doing so over multiple growing seasons by remaining dormant during periods of unfavourable environmental conditions. This reduces the amount of endogenous energy necessary for maintenance, while promoting survival within highly competitive natural soil environment (Mondal & Hyakumachi, 2000).

Each sclerotium is morphologically variable, although the general structure consists of an outer rind layer formed by a continuous layer of pseudoparenchymatous, melanised cells. This layer encases a medulla of interwoven hyphae covered by a cortex of close-fitting hyphae just under the rind (Willetts & Bullock, 1992). These layers are distinguished by differing densities of mycelium and varying degrees of agglutination and pigmentation (Corner, 1950).

Formation of sclerotia occurs over three developmental stages; initiation, growth and development, and maturation (Townsend & Willetts, 1954). All stages are completed within 72 hours of initiation in *in vitro* conditions, and each has unique nutrient requirements (Townsend, 1957). The initiation of sclerotia is influenced by a multifaceted array of internal and external triggers and responses. Previous reviews addressing the factors that influence sclerotia initiation have noted that environmental factors such as light, temperature, aeration, pH, and interaction with chemical barriers or other microorganisms all elicit a response to produce sclerotia to some degree (Willetts, 1971; Chet & Henis, 1975; Willetts & Bullock, 1992). A number of chemical compounds have also been linked to the initiation of sclerotia formation, including phenolics and

polyphenoloxidases (Willetts, 1972; Wong & Willetts, 1974). More recently, Georgiou *et al*. (2006) has attributed previous results and the overall initiation of sclerotia formation to oxidative stress.

Sclerotia formation may take place in one of three ways: terminal, loose, or lateral (Willetts, 1972). Sclerotia of most economically-important pathogens develop terminally. This means that each sclerotium is formed by a well-defined pattern of hyphal tip branching to form a knot of hyphae held together by anastomoses. A number of hyphal initials then coalesce to form a sclerotium (Townsend & Willetts, 1954; Willetts & Wong, 1971).

Distinctions between the sizes and shapes observed among plant pathogenic sclerotia are based on their germination and infection methods. Garrett (1970) noted that sclerotia of root-infecting fungi, such as *Sclerotium cepivorum*, are small, spherical and regular in shape, acting directly as infective propagules. Alternatively, sclerotia of airborne pathogens, such as *Botrytis squamosa* and *Sclerotinia sclerotiorum*, are variable in size and shape, and produce infective propagules via fruiting bodies (Garrett, 1970).

1.3 Sclerotia Survival

There are a multitude of factors that influence the survival of sclerotia in natural settings, including the structural aspects of the sclerotium and environmental conditions surrounding the sclerotium. Both of these factors influence the quiescent state that sclerotia experience within the soil. There are two types of dormancy that sclerotia may enter; exogenous and endogenous. Exogenous dormancy is imposed by unfavourable environmental conditions. The return of favourable conditions interrupts this dormancy and promotes germination (McLean *et al*., 2005a). Endogenous, or constitutive,

dormancy is imposed by an inherent inability to germinate. This type of dormancy is interrupted by specific stimuli that are not necessarily required for vegetative growth. Sclerotia under constitutive dormancy may require a form of conditioning that involves a period of time in non-sterile soil, prior to stimulated germination (Coley-Smith *et al.*, 1987).

Sclerotia that are sensitive to fungistasis generally enter exogenous dormancy following formation. This prevents germination when sclerotia are under unfavourable conditions (Lockwood, 1977). Fungistasis is widely known as the restriction or inhibition of fungal growth or germination within soils due to characteristics other than temperature and moisture (Lockwood, 1977; de Boer, 2003; Garbeva, 2011). This nonspecific suppression has been attributed to a combination of the production of inhibitory compounds (Dobbs & Hinson, 1953, Watson & Ford, 1972) and the withdrawal of nutrients from fungal propagules (Lockwood & Lingappa, 1963; Lockwood, 1977) by the surrounding soil microbe community. The diversity, structure, and interaction of these communities are determinants of fungistasis, which can also be influenced by structural aspects of the sclerotium and environmental surroundings. The community composition of fungal suppressive soils have been identified to include a range of bacterial (e.g. *Pseudomonas, Bacillus, Streptomyces*) and fungal (e.g. *Trichoderma, Penicillium,* non-pathogenic *Fusarium* spp.) genera with antagonistic and saprophytic characteristics (Rothrock & Gottlieb, 1984; Berg *et al.*, 2002; Cotxarrera *et al*., 2002; Garbeva *et al*., 2004). Despite sclerotial dormancy, these communities are instrumental in the degradation of sclerotia over time via colonization (Papavizas & Collins, 1990; Benhamou & Chet, 1996).

1.3.1 Structural Aspects

One structural feature that aids in the survival of sclerotia is the rind. Although rindless sclerotia can have long survival periods, the quality of the rind, when present, does influence survival (Coley-Smith & Cooke, 1971). The sclerotial rind owes its resilience to melanin which protects against exogenous factors such as heat-induced desiccation, ultraviolet (UV) and visible light irradiation, and attack by lytic enzymes produced by soil microbes (Kou & Alexander, 1967, Rehnstrom & Free, 1996). This dark, poly-aromatic complex polymer of resonance-stabilized cyclic molecules can also be found within the cell walls of hyphae, spore bodies, and is an essential component in appressorium-based penetration (Butler *et al*., 2005). The rind also provides a barrier to germination and contributes to sclerotial dormancy. Sclerotia that experience physical damage to their rind germinate more rapidly compared to those with an intact rind (Coley-Smith, 1959). When the rind is compromised, there is a greater exposure to the surrounding microbial community and an increased risk of colonization and degradation (Coley-Smith & Cooke, 1971; Adams, 1975; Lockwood, 1977; Duncan *et al.*, 2006). Despite this, damage to the rind does not always result in sclerotial death as some pathogens have demonstrated the ability to regenerate rind tissue (Makkonen & Pohjakallio, 1960). Conversely, even well-developed rinds are permeable to water and gases, allow the diffusion of molecules such as carbohydrates, and are not entirely resistant to penetration by mycoparasites (Coley-Smith & Cooke, 1971).

Exogenous conditions experienced during growth and development have been observed to influence the resilience of sclerotia. This effect is frequently debated when considering the use of laboratory- (LP) or field-produced (FP) sclerotia for experimental

use as FP sclerotia are not always accessible or abundant. Producing sclerotia in culture allows for the mass production of uniformly shaped and sized sclerotia free of microbial contamination (Alexander & Stewart, 1994). Reports have demonstrated that LP sclerotia degrade more rapidly in natural settings than naturally-produced sclerotia (Coley-Smith *et al*., 1990; Mitchell & Wheeler, 1990), while others have demonstrated the contrary (Merriman, 1976; Alexander & Stewart, 1994). Despite this, sclerotia formed under natural conditions can have thinner or imperfect rind layers due to adverse environmental conditions (Merriman, 1976). LP sclerotia experience optimal conditions during growth and development and are likely to have a complete rind and thicker cortex, enhancing nutrient storage (Alexander & Stewart, 1994).

1.3.2 Environmental Conditions

Many abiotic factors of the exogenous environment, including temperature, moisture, soil organic content, and soil pH, influence the survival of sclerotia. Optimal environmental conditions for sclerotial survival differ among pathogens, although these conditions have little direct impact on sclerotial survival (Adams & Ayers, 1979). Exogenous factors usually have an indirect role on sclerotial survival by influencing surrounding antagonists and parasites. Certain conditions may increase the activity of these organisms, or damage the sclerotial rind sufficiently to increase the vulnerability to these microbes (Makkonen & Pohjakallio, 1960). For instance, drying of sclerotia can greatly enhance the exudation of nutrients, encouraging colonization and the further withdrawal of nutrients (Lockwood, 1977). Activity of antagonistic soil fungi has also been linked to soil pH, as well as increased soil temperature and moisture content, and is

variable depending on species (Makkonen & Pohjakallio, 1960; de Boer *et al.*, 1998b; Garbeva *et al*., 2011).

1.3.3 Evaluating Survival

Viability analyses are employed to evaluate the impact of exogenous factors on the viability of sclerotia survival and germination. One of the most commonly used methods is a simple test of germination where sclerotia are placed in environments favourable to germination, and viability is confirmed if they germinate within a given time frame (Coley-Smith & Javed, 1970). The germination test is based on the premise that a viable sclerotium is able to germinate and grow given optimal conditions. Furthermore, the continued growth and formation of secondary sclerotia indicates that a sclerotium is fully viable and capable of producing structures vital to its life cycle. The formation of secondary sclerotia also allows for positive identification of the pathogen since distinguishing between species via mycelial identification is not always possible. Prior to a germination test, sclerotia are surface-disinfested with alcohol, followed by sodium hypochlorite to reduce the growth of other microorganisms (Coley-Smith & Javed, 1970).

One limitation to this method is the inability to distinguish between viable non-germinated sclerotium and non-viable sclerotium (Schanding *et al.*, 1995). This uncertainty has been overcome by re-disinfesting ungerminated sclerotia, cutting in half, re-plating on favourable media, and re-examining for germination (Alexander & Stewart, 1994).

1.4 Pathogens of Interest

Each of the three soil-borne pathogens of interest in this thesis cause disease on either field carrots (*Daucus carota* L.) or dry onions (*Allium cepa* L.) grown in muck soil. In Canada, carrots and dry onions are two of the three most valuable field-grown vegetable crops, and are considered low acreage, high value crops. In 2011, 20,452 acres of carrots and 12,538 acres of dry onions were produced in Canada with farm gate values of $95 million and $73 million, respectively (Statistics Canada, 2012).

In the Bradford Marsh region of Ontario, the production of these crops requires friable, highly organic soils with good moisture-holding capacity, such as muck soils (Swaider & Ware, 2001). Muck soils contain 20-90% organic material that is highly decomposed to the point where original plant parts are not recognizable (Steila & Pond, 1989). Carrots and dry onions are generally rotated within the same fields, permitting the presence of sclerotia of all three pathogens within the same soils.

1.4.1 *Botrytis squamosa*

One of the most important fungal pathogens affecting onion is the Ascomycete *Botrytis squamosa* Walker. Commonly referred to as Botrytis leaf blight of onion, this disease was first documented in the United Kingdom and is now present throughout North America, Europe and China (Elad *et al*., 2007). The host range of this pathogen includes onion, garlic, shallots, chives, leeks and other *Allium* species (Jarvis, 1977).

Botrytis squamosa was once thought to be a neck rot pathogen; however, in recent years molecular sequence analysis of several onion neck rot pathogens have revised the taxonomy of this group, and it is now accepted that *Botrytis allii, Botrytis aclada,* and *Botrytis byssoidea* are distinct species causing onion neck rot (Walker, 1926; Nielsen &

Yohalem, 2001; Chilvers *et al*., 2004). The initial association of *B. squamosa* with neck rot was not wrong, as *B. squamosa* sclerotia do develop on the leaves, bulbs and outer neck regions of onion plants following infection, similar to neck rot (Ellerbrock & Lorbeer, 1997a). *Botrytis squamosa* is also capable of reducing seed production by infecting onion flower heads, causing flower blight (Ellerbrock & Lorbeer, 1977a).

Over-wintering sclerotia of *B. squamosa* are the primary source of initial inoculum (Carisse *et al*., 2006, Ellerbrock & Lorbeer, 1977b. Lorbeer *et al*., 2007). These sclerotia can be found within the soil, on bulb scales and blighted leaves not removed by mechanical harvesters, cull piles, and the scales and necks of bulbs planted for seed production (Ellerbrock & Lorbeer, 1977a).

1.4.1.1 Biology of *B. squamosa*

Symptoms tend to form early in the growing season when weather conditions are favourable and develop approximately 5-12 days after infection by conidia (Lorbeer *et al*., 2007). During this leaf-spotting period, distinct necrotic lesions form on the portion of leaves exposed to sunlight. These lesions are typically 2 mm in diameter, initially appear whitish in colour and develop into water-soaked lesions surrounded by a characteristic light green halo. Botrytis leaf blight is commonly misdiagnosed as other diseases caused by fungi within the *Botrytis* genus or even as herbicide injury; however, this halo is a unique diagnostic feature of *B. squamosa*. Within 2-4 days, lesions lose moisture, become straw-coloured and sunken, and will usually split in the center. Some mature lesions will continue to expand if the duration of leaf wetness exceeds 12 hours (Alderman & Lacy, 1983). As the disease progresses, the lesions coalesce and the leaves

die back from the tips. The masses of senesced tissue created are required for production of conidia (Clarkson *et al*., 2000).

Disease progression to the dieback stage is also mediated by leaf wetness duration and temperature. Conidia formation occurs at night when temperatures are between 8-22°C following at least 12 hours of leaf wetness (Sutton *et al*., 1983, Alderman & Lacy, 1983). The release of these conidia occurs in diurnal cycles and is greatest at daylight periods of increasing temperature and decreasing humidity; most commonly observed between 09:00 and 12:00 (Lorbeer *et al*., 2007, Sutton *et al*., 1983). Germination of these secondary conidia on *Allium* spp. tissue occurs most rapidly at temperatures between 18° and 20°C and as leaf wetness duration increased to 48 hours (Sutton *et al*., 1983). The continuous cycling between these two stages of the disease allows for rapid and prolonged conidial production under favourable conditions and can result in severe outbreaks within a field and epidemics within a region. Despite this, the occurrence and severity of disease varies from year to year due to the reliance on suitable environmental conditions (Clarkson *et al*., 2000).

The polycyclic nature of this disease is slowed when either nutrients become limited or environmental conditions are no longer suitable. In commercial onion crops, these conditions generally develop prior to harvest, and sclerotia are formed on the leaves, necks and upper portions of the bulb before or after harvest. In the field, sclerotia of *B. squamosa* are found late in the growing season on inner and outer leaf sheaths of the bulb neck, onion flower stocks, necrotic leaves in contact with the soil, and most commonly in matted clumps of blighted onion leaves where severe outbreaks of the disease are present (Ellerbrock & Lorbeer, 1977a). Sclerotia formation is greater during

periods of increased moisture. Ellerbrock & Lorbeer (1977b) evaluated the effect of temperature on sclerotia production *in vitro* and observed that sclerotia formation occurred between 3°-27°C, but was most rapid at 15°C for a period of 3 weeks. The size and shape of sclerotia can vary greatly based on the location of its formation, ranging from 2 mm to >1 cm in diameter. Sclerotia formed on leaf sheaths and bulb scales are larger and narrow in size, while those formed on blighted leaves appear spindle-shaped.

Sclerotia of *B. squamosa* have been observed to germinate up to four times in one growing season and can do so via carpogenic, sporogenic and myceliogenic germination (Clarkson *et al*., 2000; Ellerbrock & Lorbeer, 1977a,b). The carpogenic germination of *B. squamosa* produces apothecia and sexual ascospores. Apothecia-bearing sclerotia were first observed in nature by Ellerbrock and Lorbeer (1977b) on onion debris in fields previously cropped with onions in the spring. Naturally occurring apothecia were not present in the field late in the growing season, and were never found during epidemics. Consequently, this teleomorphic reproduction of *B. squamosa* appears to be solely utilized to give rise to new strains of the pathogen. This form of sexual reproduction increases genetic diversity and the likelihood of fungicide resistance (Tremblay *et al*., 2003). There are two mating types, and both are required within the same region to allow for sexual reproduction.

Of the three means of germination utilized by *B. squamosa*, the sporogenic production of asexual conidia is most commonly found throughout the growing season and has been recognized as the primary source of inoculum (Ellerbrock & Lorbeer, 1977b). Sporogenic germination has been observed on sclerotia found within cull piles, on mother bulbs in seed fields, and on the soil surface of commercial fields. When cull

piles and seed fields are not present, sclerotia within the soil and on infected volunteer onions provide initial conidial inoculum.

Ellerbrock & Lorbeer (1977b) observed that *B. squamosa* sclerotia germinated at a wide range of temperatures *in vitro* and that soil moisture was important for conidial germination. Clarkson *et al*. (2000) later confirmed that sclerotia germinated within 6 days at temperatures of 10° to 20°C and that low soil moisture limited the rate of sporogenic germination, as well as the number of conidia produced. Sclerotia of *B. squamosa* require UV light for sporogenic germination (Balis & Lorbeer, 1992). As a result, sclerotia must be near the soil surface where temperature and moisture conditions can change rapidly. These requirements contribute to variation in disease severity among years; however, even a small portion of viable sclerotia can result in severe disease since one sclerotia can produce up to 5×10^4 conidia (Clarkson *et al*., 2000).

Botrytis squamosa sclerotia have been observed to remain viable in soil for 21 months, allowing the pathogen to maintain a level of initial inoculum within the field until host crops are present (Ellerbrock & Lorbeer, 1977a, b). As a result, eradication or minimization of sclerotia will decrease sporulation and disease build-up.

1.4.1.2 Management of *B. squamosa*

Management of *B. squamosa* is dependent on cultivar selection and fungicides, although there has been extensive research on biocontrols of *Botrytis* spp. over the past 50 years. In North America, there are currently no resistant onion cultivars commercially available, although some cultivars are less susceptible than others (McDonald *et al.*, 2004). The most common and effective form of control is through the regular use of fungicide applications (Carisse *et al*., 2006). Under endemic levels of disease, protectant

fungicides, such as ethylene bisdithiocarbamates, are used from mid-June to harvest on a 7 -10 day schedule. However, when disease pressure is high, protectant fungicides are replaced with dicarboximide fungicides (Tremblay *et al*., 2003). The number of required spray applications can be effectively reduced with the use of disease forecasting systems such as BOTCAST which predicts sporulation and infection of the pathogen based on weather variables such as leaf wetness, temperature and relative humidity (Sutton *et al*., 1986). Disease severity values are accumulated each day until a cumulative disease severity index (CSDI) threshold is reached, triggering a fungicide application. The current Ontario Ministry of Agriculture and Food recommended threshold is 20 CSDI (OMAF, 2009).

Investigations into potential biocontrols of *B. squamosa* have identified key microbial genera that possess antagonistic potential, *Trichoderma, Gliocladium, Ulocladium, Pseudomonas* and *Bacillus* (Elad & Stewart, 2007). Similar to other *Botrytis* spp. pathogens, *B. squamosa* is susceptible to competition due to the heavy reliance on exogenous energy inputs for pathogenesis (Elad & Stewart, 2007). Saprophytic microorganisms are capable of limiting nutrients available to *B. squamosa,* as well as limiting available tissue for sporulation by feeding on necrotic tissue within an infected field (Kohl *et al.,* 1995). Other biocontrol modes of action effective at reducing *Botrytis* spp. diseases include the modification of plant host surface to reduce host adhesion by *Pseudomonas* spp. (Bunster *et al.,* 1986), direct parasitism through cell wall degrading enzymes (Kohl & Schlosser, 1989), and antibiosis through inhibitory compounds (Dobus, 1987; Schirmbock *et al.,* 1994).

Conidia and germ tubes are susceptible to direct antagonism by antibiotics and lytic enzymes; however, the time frame that these structures are active in the phylloplane is short and limited to a few hours (Elad & Stewart, 2007). As a result, parasitism of quiescent infections and competitive exclusion are the most effective modes of action for biocontrols. A number of *Trichoderma* spp. have demonstrated an ability to parasitize and reduce survival of sclerotia of *B. cinerea* under a range of temperatures (Kohl & Schlosser, 1989). Globally, various *Trichoderma* spp. have been commercially produced for the suppression of *Botrytis* diseases, including Binab (Binab Bio-Innovation AB, Sweden), Plantshield (Bioworks Inc., USA), and Trichodex (Makhteshim, Israel) (Elad & Stewart, 2007). Currently, none of these products are available for use in Canada.

Cultural control methods can also be utilized to reduce the build up of inoculum in areas surrounding production fields. These methods include the removal of cull piles, locating onion seed fields distant from onion production fields, and removal of volunteer onions early in the growing season (Lorbeer *et al*., 2007). Postharvest, all leaf debris should be removed from the field and destroyed with cull piles (Ellerbrock & Lorbeer, 1977b).

1.4.2 *Sclerotinia sclerotiorum*

Sclerotinia sclerotiorum (Lib.) de Bary is one of the most widely distributed plant pathogens throughout the world. Its presence is most common in, but not limited to, cool and moist regions (Purdy, 1979). *Sclerotinia sclerotiorum* has also been observed in semi-arid regions of the world when microclimatic conditions are suitable for disease development (Purdy, 1979). This pathogen can cause disease in more than 400 host plant species within 64 families, including many economically important crops such as dry

bean (*Phaseolus vulgaris* L.), soybean (*Glycine max* L.), carrot (*Daucus carota* L.), lettuce (*Lactuca sativa* L.), peanuts (*Arachis hypogaea* L.) and sunflower (*Helianthus annuus* L.) (Boland and Hall, 1994). This extensive host range and wide distribution is accompanied by an ability to cause excessive crop damage, with losses reaching 100% annually in certain instances, resulting in economic losses of millions of dollars (Purdy, 1979; McDonald & Boland, 2004). The success of this pathogen can be attributed to the ability to overcome unfavourable environmental conditions by producing sclerotia. These overwintering bodies are a main source of primary inoculum and make disease management and eradication difficult (Bolton *et al.*, 2006).

1.4.2.1 Biology of *S. sclerotiorum*

Primary inoculum originate from sclerotia present within the soil, which can remain viable for up to eight years (Adams & Ayers, 1979). The primary inoculum may be asexual vegetative hyphae or sexual ascospores (Bolton *et al*., 2006). *Sclerotinia sclerotiorum* does not produce asexual conidia, yet microconidia have been observed to form on hyphae or alongside ascospores on the apothecial hymenium, but do not germinate or infect (Kohn, 1979). Both forms of inoculum utilize necrotic plant tissue as an exogenous nutrient source to produce mycelium. This mycelium then invades adjacent healthy plant tissue and further branches, initiating colonization and disease development (Purdy, 1979). Ascospore-initiated infection relies on senescing flower parts as an initial source of necrotic tissue, resulting in common above-ground tissue diseases, while hyphal-initiated infection targets susceptible roots with necrotic tissue, causing few root and crown diseases (Inglis & Boland, 1990; Bardin & Huang, 2001; Bolton *et al*., 2006). As a result, different host crops are subject to different modes of infection.

Similarly, symptoms of disease development are expressed differently among host species, leading to the more than 60 common names used to describe this disease (Saharan & Mehta, 2008). One common name is cottony soft rot, due to the production of thick, fluffy mycelial mats that develop on the tissue of almost all plant hosts, although here it will be referred to as sclerotinia rot (Bolton *et al*., 2006). Following infection by *S. sclerotiorum*, symptoms generally first appear on leaves or young stems of some hosts as water-soaked spots that enlarge to become a watery soft rot. In other hosts, distinct lesions develop on stems, stalks, branches, or twigs and appear dry (Purdy, 1979). These dry lesions enlarge until they eventually girdle the plant, resulting in the yellowing and wilting of distal leaves. In all instances, *S. sclerotiorum* spreads rapidly throughout the plant either internally in the pith of the stem or externally on collapsed wilted foliar and stem tissue (Saharan & Mehta, 2008). Eventually, these lesions give way to thick white, fluffy, mycelial growth, either within the field or during storage of particular hosts. Under moist conditions, this mycelial growth may cover the entire plant. It is in these mats of mycelia where numerous sclerotia, 2 mm - 35 cm in size, are produced (Bolton *et al.*, 2006). Sclerotia of *S. sclerotiorum* first appear white, but soon develop a black outer rind. The shape and size of the sclerotia is dependent upon the host as in certain instances, sclerotia will form internally and mimic the shape of seeds, as is the case with dry beans (Purdy, 1979; Saharan & Mehta, 2008).

After sclerotia are produced, they enter dormancy and must undergo a conditioning period before germination may occur. Bardin and Huang (2001) describe this conditioning as a combination of wetting and drying events, low temperatures and time required to break dormancy. The conditioning of sclerotia typically requires 1-2

weeks of soil water potentials of 100 kPa and temperatures of 10-25°C, and can be mimicked within a laboratory setting (Clarkson *et al*., 2004b). Following conditioning, sclerotia of *S. sclerotiorum* can germinate carpogenically or myceliogenically. Carpogenic germination involves the production of apothecia and subsequent ascospores, while myceliogenic germination is the eruptive production of vegetative hyphae (Purdy, 1979; Bolton *et al.*, 2006).

Carpogenic germination is most prevalent and has the greatest disease potential as apothecia are capable of aerial dispersal of up to 30 million ascospores via multiple expulsions that occur over periods greater than 10 days (Schwartz & Steadman, 1978; Clarkson *et al*., 2003). This form of germination is dependent on a number of abiotic factors, including temperature, moisture, light, soil depth and exogenous nutrition. Soil moisture and temperature are arguably the most important factors in apothecial production (Clarkson *et al*., 2004b). Following the development of a crop canopy, these conditions are more consistent within the microclimate that sclerotia experience, causing carpogenic germination to occur more readily (Bolton *et al*., 2006).

An apothecium first emerges from a sclerotium as a stipe which forms a flat to concave hymenial layer at the tip, 2-10 mm in diameter. Multiple rows of asci form on the hymenial layer, each containing eight hyaline, binucleate ascospores (Bolton *et al*., 2006). Ascospores are simultaneously released from the asci through puffing of the apothecium which is initiated by changes in humidity or physical disturbance (Hartill & Underhill, 1976). Once expelled, ascospores disperse within a 100-150 m radius and may remain viable for a period of 2 weeks depending on exposure to relative humidity and UV light (Ben-Yephet & Bitton, 1985; Clarkson *et al*., 2003). Ascospores are able to

adhere to host plant tissue with the help of sticky mucilage, and will germinate if an exogenous nutrient source and a film of water are available (Abawi & Grogan, 1979).

Myceliogenic germination is exclusive to only a few crops with susceptible root tissues, such as sunflowers (Bardin & Huang, 2001). Germination of *S. sclerotiorum* sclerotia in this manner occurs more readily if exogenous nutrients are not present, or if the rind is damaged or incompletely melanised (Huang, 1985; Huang & Kozub, 1994). Other factors affecting which type of germination occurs include freezing and desiccation (Abawi & Gordon, 1979; Huang, 1991). In any case, single sclerotia have been observed to germinate both carpogenically and myceliogenically (Huang, 1991).

1.4.2.2 Management of *S. sclerotiorum*

Since the source of primary inoculum resides within the soil, eradication of the pathogen is not easy. There is no single control method that is successful at completely controlling the disease; however, a number of cultural, chemical and biological management strategies may be used in conjunction to effectively manage disease outbreaks during favourable environmental conditions.

There are a number of cultural controls that target different stages of the pathogen's life cycle. These include reduced or no-tillage, increased width of rows, and crop rotation, among others. By reducing tillage, it is believed that surface sclerotia are exposed to more variable climatic conditions and likely to be less viable (Tu, 1997). In contrast, sclerotia buried at depths greater than 8-10 cm are considered to be ineffective since stipes are unlikely to reach the soil surface (Merriman *et al*., 1979). Deep ploughing may be used to bury 90% of surface debris below 10 cm; however subsequent ploughing or tillage can re-surface previously-buried sclerotia. Another means of reducing

germination of sclerotia is to increase row width to reduce the appropriate microclimate conditions present when canopy cover occurs (Peachey *et al*., 2006). In crops affected by foliar rots, such as carrots, this may also be achieved by lateral trimming of canopy foliage (Kora *et al.*, 2005). Finally, crop rotation has been used in attempts to avoid disease when the pathogen becomes present in a field. This practice has had minimal success due to the extensive host range of *S. sclerotiorum* and the long term survival of sclerotia in soil (Schwartz & Steadman, 1978).

Identification of host resistance in the field based on genotype is challenging due to the variable nature of disease development in time and space (Bolton *et al*., 2006). Currently, partial resistance in host species is relied upon to maintain economic disease control (Kim & Diers, 2000). Host crops that have demonstrated some resistance include dry bean, soybean, sunflower and peanut (Willetts & Wong, 1980; Kim & Diers, 2000). The majority of resistance is based on disease escape mechanisms such as narrow canopy architecture, upright growth and flowering date (Bardin & Huang, 2001, Nelson *et al*., 1991). Recent genetic work has focused on quantitative trait loci (QTL) which allows for screening of resistance based on genotypes and phenotypes (Kim & Diers, 2000). In addition, transgenic research has demonstrated the effective use of wheat germ genes capable of degrading the oxalic acid produced by *S. sclerotiorum* (Lane *et al*., 1993; Peng & Kuc, 1992). Conversely, the topic of transgenic produce is still highly debated globally due to the threat of gene escape, and the future for this form of host resistance is unclear.

The use of chemical fungicides is a prime method of control for *S. sclerotiorum*; however, the degree of control is dependent upon preventative application preceding disease development (Mueller *et al*., 1999). Unlike *B. squamosa*, forecasting systems for

S. sclerotiorum are not extensively used in Canada, making it difficult to meet management demands while keeping unnecessary spray applications to a minimum (McDonald & Boland, 2004). Fungicide efficacy may be inconsistent because of high disease severity or problems in achieving adequate spray coverage (Mueller *et al*., 2002). Presently, there are few chemical fungicides registered for use in Canada against *S. sclerotiorum* diseases and only on a limited number of crop hosts (McDonald & Boland, 2004). Of those available, fluazinam and fludioxonil are effective at reducing the growth of *S. sclerotiorum* to a greater degree than that of vinclozolin, a previously registered fungicide (Matheron & Porchas, 2004). Despite the success of these new fungicides, a number of applications should be replaced with cultural or biocontrol methods to avoid pathogen resistance (Gossen *et al*., 2001).

Due to the extensive host range and associated epidemiology of *S. sclerotiorum*, finding a single biocontrol agent to effectively manage every disease caused by this pathogen is difficult. Current literature suggests that different biocontrol agents demonstrate varying degrees of antagonistic success based on the host crop and disease (Bardin & Huang, 2001). The main biocontrol modes of action that have been researched focus on hypovirulent strains of the pathogen (Boland, 1992), soil amendments (Huang & Huang, 1993), and sprays and soil treatments with either mycoparasitic fungi or insects (Anas & Reeleder, 1988; Gracia-Garza *et al*., 1997). Hypovirulence has been reported in both *S. sclerotiorum* and *S. minor*, although there is little future for the hypovirulent strains due to the large number of mycelial compatibility groups (Boland, 1992).The basis for incorporating organic and inorganic soil amendments is to create soil conditions that are suppressive to *S. sclerotiorum* and favourable to beneficial microorganisms and

plant growth (Huang & Huang, 1993). In one successful study, Huang *et al*. (1997) demonstrated a reduction in carpogenic germination of *S. sclerotiorum* and a promotion in growth of *Trichoderma* spp., following the application of fermented agricultural wastes.

The most effective parasitic insect of *S. sclerotiorum* is the fungus gnat, *Bradysia coprophila* (Anas & Reeleder, 1988). The feeding of *B. coprophila* larvae on the large sclerotia of *S. sclerotiorum* reduces sclerotia germination and promotes sclerotial colonization by other microorganisms such as *Trichoderma viride* (Gracia-Garza *et al*., 1997). The majority of fungi tested for antagonistic activity are competitors of *S. sclerotiorum* within soils or on the phylloplane of hosts. One of the most extensively studied fungal mycoparasites of *S. sclerotiorum* is the mycoparasite *Coniothyrium minitans* Campbell. This microorganism is effective at parasitizing sclerotia of *S. sclerotiorum* and providing protection to the progression of disease by colonizing senescent tissue of host crops (Bremer *et al.*, 2000). Consequently, *C. minitans* is used commercially for the suppression of *S. sclerotiorum* and *S. minor*, and will be discussed later in this review. Other competitive microorganisms include *Fusarium graminearum* and *Epicoccum purpurascens*. Each demonstrated inconsistent disease management within the field (Boland & Inglis, 1989).

1.4.3 *Sclerotium cepivorum*

Another disease threatening global *Allium* spp. production is white rot, caused by the Ascomycete *Sclerotium cepivorum* Berk. Much like *B. squamosa*, this soil-borne fungus is limited to regions where *Allium* spp., such as onion, garlic, shallot, and leek are commercially produced (Utkhede *et al*., 1982). Worldwide, *S. cepivorum* is distributed

via the transportation of infected planting material and is present in Europe, Asia, Africa, Australia and New Zealand, as well as throughout North and South America (Ulacio-Osorio *et al*., 2006). Sclerotia of *S. cepivorum* are the source of primary inoculum and are capable of causing disease wherever crops of *Allium* spp. are grown. Colonized tissues of *Allium* spp. become so heavily infected that they senesce, resulting in unmarketable bulbs. Crop losses up to 100% can occur based on the presence of inoculum and weather conditions (Ulacio-Osorio *et al*., 2006). Even one sclerotia per gram of soil can result in crop failure (Crowe *et al*., 1980). White rot outbreaks can occur in fields that were not planted with susceptible hosts for many years. These outbreaks have been attributed to the long term survival of *S. cepivorum* sclerotia (Coley-Smith *et al*., 1990). Due to this characteristic, infested fields are usually abandoned for *Allium* spp. production, making white rot one of the most economically important and limiting diseases of global *Allium* spp. crop production (Coley-Smith & King, 1969; McLean *et al*., 2005a).

1.4.3.1 Biology of *S. cepivorum*

Sclerotia within the soil germinate to produce mycelia which must come into contact with plant tissue 10-14 days after germination before they begin to decay (Coley-Smith, 1960). The teleomorph of *S. cepivorum* has not been observed, and the pathogen survives as an anamorph and does not produce spores in nature (Brix & Zinkernagel, 1992). Microconidia have been observed on mycelia emerging from sclerotia in aseptic conditions; however, they only germinated in high moisture conditions (Coley-Smith, 1960; Gindro & L'Hoste, 2008). Although believed to be a rare occurrence in nature, the infectious potential of microconidia was demonstrated cytochemically (Gindro & L'Hoste, 2008).

Infection of host plants occurs at the roots and base of the bulb. Colonization can occur pre- or post-emergence and is mediated by soil temperature and moisture. Disease development is most active at cool temperatures (10°-18°C) and at moderate soil moisture levels (-45 bars to -3 bars), and may occur during only a portion of the growing season (Crowe & Hall, 1980). Pre-emergence colonization results in symptoms resembling those caused by damping-off fungi when young plants become weak and decayed (Entwistle, 1990). Above-ground symptoms become visible within 20 days of initial infection and include wilting and yellowing of older leaves, along with die-back of leaf tips (Tims, 1948). These symptoms progress down the leaf blades until they eventually collapse and decay. Root infection results in gradual, watery decay that develops into mats of fluffy, white mycelial growth at the base of the bulbs. It is within these mycelial mats where thousands of sclerotia (200-500 µm in diameter) are produced. Disease progression at this region can completely rot the root and bulb portions of the plant and even low levels of infection can produce damage sufficient to reduce marketable value of the crop (Tims, 1948). Secondary infection involves the growth of hyphae from an infected root or bulb to the roots, basal plate, or bulb of an adjacent plant. Due to this method of dissemination, disease usually develops as patches within a field. Disease within infested fields soon becomes more evenly distributed due to mechanical distribution of infested soil throughout the field. Distribution may also occur through wind, water and equipment (Crowe *et al*., 1980).

Sclerotia of *S. cepivorum* are among the smallest naturally-produced sclerotia, with an average diameter of 300 µm. Since *S. cepivorum* cannot survive in nature saprotrophically, there is limited mycelial survival in the absence of a host. As a result,

these melanised structures serve as the primary means of survival and dissemination. Sclerotia of *S. cepivorum* are able to survive and remain viable for extensive periods of time within the field either in the presence or absence of host crops (Coley-Smith, 1960; Coley-Smith *et al*., 1990). Coley-Smith *et al.* (1990) has reported that sclerotia of *S. cepivorum* may remain viable within soil for at least 20 years. Much of this impressive survival is attributed to the fact that germination only occurs in the presence of exudates of *Allium* spp. (Coley-Smith & King, 1969; Somerville & Hall, 1987).

Following formation, sclerotia of *S. cepivorum* are subject to two forms of dormancy, exogenous and constitutive. In natural conditions, newly-formed sclerotia undergo a state of dormancy that restricts germination for a period of 1-3 months even in the presence of host *Allium* spp. (Gerbrandy, 1989). This initial state of dormancy is better known as constitutive dormancy which involves the restraint of biochemical processes required for normal germination (Coley-Smith *et al*., 1960; McLean *et al*., 2005a). During this time, sclerotia are conditioned under cool temperatures. Once this constitutive dormancy subsides, sclerotia are then subject to exogenous dormancy which is brought about by unfavourable environmental conditions (McLean *et al*., 2005a). In the absence of suitable host plants, sclerotia remain in this state of dormancy and may do so for a number of years (Gerbrandy, 1989). It is only when *Allium* spp. are present that this dormancy can be broken. *Allium* species contain S-alk(en)yl-L-cysteine sulphoxides which give them their general taste and odour. When these compounds are exuded into the soil, they are metabolized by soil microflora to form volatile sulphur compounds, diallyl disulphides (4,5-dithia-1,7-octadiene), which stimulate the germination of dormant *S. cepivorum* sclerotia (Coley-Smith & King, 1969; McLean *et al*., 2005a).

When germination eventually occurs, sclerotia of *S. cepivorum* undergo myceliogenic germination with the use of eruptive mycelial plugs (Coley-Smith & Cooke, 1971; Willetts & Bullock, 1992). Initial external signs include a bulge on the sclerotium surface, followed by a rupturing of the rind. From this rupture point, one or more large, dense plugs of mycelium are extruded (Coley-Smith, 1960). These mycelial plugs first originated in the sclerotia medulla and eventually produce branching hyphae capable of infecting plant hosts and producing microconidia (Coley-Smith & Cooke, 1971).

1.4.3.2 Management of *S. cepivorum*

Due to the soil-borne nature of *S. cepivorum,* the most effective form of management is an integrated approach that incorporates the use of cultivars demonstrating a high tolerance to white rot, sclerotia germination stimulants, and a chemical fungicide. In general, leeks (*Allium ampeloprasum* L.) are the most resistant *Allium* spp., followed by onion (*Allium cepa* L.) and garlic (*Allium sativum* L.) (Coley-Smith & Esler, 1983). More specifically, the most commonly used cultivar of onion to display consistently reduced disease incidence of white rot is Fortress (McDonald *et al*., 2004). To reduce the level of initial inoculum within the soil, an effective practice is to use germination stimulants post-harvest and pre-seeding (Hovius & McDonald, 2002). These stimulants mimic the presence of *Allium* spp., resulting in the untimely germination of sclerotia. One of the most effective germination stimulants is diallyl disulphide (DADs) which is a synthate of a metabolized *Allium* exudate (McLean *et al*., 2005a). Coley-Smith & Parfitt (1986) demonstrated that when applied at 0.1-0.2 g/L in the absence of suitable hosts, germination of 90-100% was achieved.

Finally, chemical fungicides are used to provide host plant protection in the field. Dicarboximide systemic fungicides such as iprodione and vinclozolin, previously mentioned for use against *B. squamosa,* are unfit for use against *S. cepivorum* as they demonstrate enhanced degradation in previously-treated soils due to enhance microbial activity (Slade *et al*., 1992). This is not an issue in the case of *B. squamosa* as they are used as foliar sprays. In Canada, one of the most effective chemical fungicides for white rot is the active ingredient tebuconazole which has demonstrated a reduction in the incidence of disease by up to 50% (McDonald *et al*., 2004). Unfortunately, tebuconazole has exhibited high levels of phytotoxicity in certain instances (Fullerton *et al*., 1995).There are no chemical fungicides currently registered in Canada to control *S. cepivorum.*

Other management techniques that may be utilized for management of white rot include avoidance, crop rotation, soil solarization, and biocontrol agents. If a field has a history of white rot, it should be avoided for the production of *Allium* spp. crops as there is a high likelihood that sclerotia continue to dwell within the soil. An additional method for reducing the number of viable sclerotia within the soil is soil solarization. This technique involves an increase in soil temperature by wetting and mulching soil, and covering with thin clear polyethylene sheeting during the hottest months of the year (Mahrer *et al*., 1984). The tarps used prevent heat loss from the soil and trap long-wave radiation, creating a greenhouse effect which raises soil temperatures well above those required to kill soil-borne pathogens, including sclerotia of *S. cepivorum* (Porter & Merriman, 1983; Porter & Merriman, 1985). In many cases, this temperature threshold occurred between 20-55°C (Porter & Merriman, 1983; Porter & Merriman, 1985;

McLean *et al*., 2001). Consequently, the high day time temperatures required for the success of this technique does not allow for its use in some temperate regions within the United Kingdom and Canada where *Allium* spp. are produced.

Finally, research into the use of biocontrol agents has been quite extensive in recent years and has resulted in promising candidates. One of the most effective groups of antagonistic microorganisms studied are the *Glomus* spp. of vesicular arbuscular mycorrhizae fungi (VAM) which colonize root tissue of *Allium* spp. and are able to provide significant protection against *S. cepivorum* for a number of weeks (Torres-Barragan *et al*., 1996, Jaime *et al.*, 2008). The *S. sclerotiorum* mycoparasite, *Coniothyrium minitans*, has been isolated from sclerotia of *S. cepivorum*, and is said to parisitize these sclerotia through breaks in the rind surface (Turner & Tribe, 1976; Harrison & Stewart, 1988; Stewart & Harrison, 1988). Conversely, success in an agricultural setting is variable and some reports claim *C. minitans* to be ineffective at reducing *Allium* white rot (DeOliveira *et al.*,1984). A number of other antagonistic fungi and bacteria have been studied for activity, including *Chaetomium globosum*, *Bacillus* spp., and *Trichoderma* spp., which utilize antibiotics and competitive saphrophytic activity, ultimately reducing viable sclerotia within the soil post-harvest and pre-seeding (McLean & Stewart, 2000; Clarkson *et al.*, 2004a).

1.5 Biocontrol Agents

In an agricultural context, biocontrol can be loosely described as the manipulation of beneficial microorganisms for the suppression or management of plant diseases. In recent decades, alternatives to traditional chemical controls of plant pathogens have been explored. This is in part due to reduced registration of chemical fungicides (Gullino &

Kuijpers, 1994; Ragsdale & Sisler, 1994), increased costs of developing new fungicide chemistries, increased risks in pathogen resistance (Gossen *et al.*, 2001; Tremblay *et al.*, 2003, van den Bosch & Gilligan, 2008) and consumers' negative perception of fungicides (Boland, 1990; Schneider & Dickert, 1994; Cunniffe & Gilligan 2011). Consequently, biocontrol agents, where effective, are an ideal alternative. Their use can be combined with chemical fungicides to provide other modes of action, ultimately reducing the possible development of pathogen resistance (Fravel, 2005).They may also be used in situations where no other controls are available.

A number of soil-dwelling microbes have demonstrated mycoparasitic activity towards sclerotia-forming fungi. These mycoparasites can either be facultative, unspecialized species that survive in soils on a variety of organic matter, or specialized to target specific pathogens (Kaur *et al*., 2005). This review will focus on two obligate mycoparasites, *Coniothyrium minitans* Campbell and *Microsphaeropsis ochracea* Carisse & Bernier, and one facultative mycoparasite, *Trichoderma atroviride* Karsten.

1.5.1 Biocontrol Agents of Interest

1.5.1.1 *Coniothyrium minitans*

Coniothyrium minitans Campbell is a fungus classified within the fungal Class Coelomycetes. It was originally isolated from a sclerotium of *S. sclerotiorum* in the United States in 1947, but has since been isolated all over the world (Whipps & Gerlagh, 1992a; Whipps *et al*., 2008). The natural host range of this fungus is limited to *S. sclerotiorum, Sclerotinia trifoliorum* Erikss and *S. cepivorum*, although *in vitro* studies have demonstrated parasitism on other melanised fungi such as *B. cinerea*, and *S. minor* (Turner & Tribe, 1976; Whipps & Gerlagh, 1992a). Antagonistic activities of *C. minitans*

provide control of mycelial growth, prevention of ascospore infection and disease development, and reduced sclerotia production (Gerlagh *et al*., 1999). *C. minitans* is currently produced as a biocontrol product by the company Prophyta GmbH (Malchow/Poel, Germany) under the name "Contans" and is registered in several countries, including Canada.

Mycoparasitism by *C. minitans* is primarily achieved via direct parasitic antagonism of host species; however, other research has demonstrated the production of the antibiotic compound, macrosphelide A, *in vitro* that acts indirectly to reduce fungal growth via antibiosis (McQuilken *et al*., 2003; Tomprefa *et al*., 2011). Mycelial contact with sclerotia or host hyphae is the most common mode of mycoparasitic attack (Whipps & Gerlagh, 1992a), followed by competition between mycelia of *C. minitans* and a host on infected plant tissue (Li *et al*., 2002). *Coniothyrium minitans* does not infect healthy plant tissue, only tissue that has been colonized by a phytopathogenic fungus. In soil, only two conidia of *C. minitans* are required to infect a sclerotium under ideal conditions (Gerlagh *et al*., 2003). Conditions favouring sclerotial infection are between 2°-24°C, with substantial decay occurring between 15-24°C (Trutman *et al*., 1980). Optimum conditions for germination, growth and infection have been reported to be 20°C and above 95% relative humidity (Trutman *et al*., 1980). Following infection and colonization, the inner medulla of infected sclerotia is converted to pycnidia of *C. minitans*, while droplets of conidia are exuded onto the surface of the remaining intact rind (Bennett *et al*., 2006). *Coniothyrium minitans* produces cell wall-degrading enzymes such as chitinase and glucanases (Giczey *et al*., 2001). *In vitro*, *C. minitans* has demonstrated the ability to produce a wide spectrum of cell-wall degrading enzymes, as

well as antimicrobial compounds (Giczey *et al*., 2001; McQuilken *et al*., 2003; Kaur *et al*., 2005), although sufficient information is lacking on how these compounds are used in mycoparasitic or competitive interactions. Although *C. minitans* is a poor competitor within the soil environment and cannot grow through bulk soil, conidia are capable of remaining viable for long periods of time and over winter conditions within parasitized sclerotia (Bennett *et al*., 2006; Huang & Erikson, 2002).

1.5.1.2 *Microsphaeropsis ochracea*

The fungus, *Microsphaeropsis ochracea* Carisse & Bernier, is also classified within the fungal Class Coelomycetes. It was first isolated from leaf litter of an apple orchard in Quebec in 1996 (Carisse & Bernier, 2002b). Initial identification of this fungus described it as *Microsphaeropsis arundinis* Ahmad based on the production of small, smooth-walled, cylindrical conidia; however, recent physiological and biochemical characterization have led to the description of the new species, *M. ochracea*. Attention was given to this fungus due to its strong antagonistic activity against the melanin component of the pathogen *Venturia inaequalis* (El Bassam *et al*., 2002; Carisse & Bernier, 2002). Subsequent research demonstrated that the application of *M. ochracea* conidia inhibited the growth of *in vitro* and field-produced ascospores of *V. inaequalis*, as well as *in vitro*-produced ascospores of *Gibberella zeae* (Bujold *et al*., 2001; Carisse *et al.*, 2000). Since both of these pathogens produce ascospores in melanised structures, further research focused on mycoparasitic activity on the production and/or germination of other fungal structures that contained melanin. *Microsphaeropsis ochracea* proved to be capable of reducing the production and viability of sclerotia of *Rhizoctonia solani*

Kuhn on potato tubers in storage, and the production of sclerotia-borne conidia of *B. squamosa* in onion plots (Carisse *et al*., 2001; Carisse *et al*., 2006).

The mycoparasitic activity of *M. ochracea* has been attributed to a complex synergy among hydrolytic enzymes, mainly chitinase and glucanases, and antibiotics (Benyagoub *et al*., 1998; Carisse *et al*., 2001; El Bassam *et al.,* 2002). Enzymatic activity of *M. ochracea* allows for direct penetration and active growth within host hyphae or sclerotia (Benyagoub *et al*., 1998). The growth of *M. ochracea* hyphae within host cells results in severe damage, leaving empty hyphal shells or sclerotia vulnerable to various biotic and abiotic pressures (Benyagoub *et al*., 1998). The use of antibiotics by *M. ochracea* has yet to be extensively studied; however, some evidence has supported its presence and possible activity. Prior to penetration by *M. ochracea*, hyphae of *V. inaequalis* underwent drastic cytoplasmic disorganization, similar to other antagonistic interactions that employ antibiosis (Belanger *et al.*, 1995; Carisse *et al*., 2006).

As with all potential BCAs, environmental conditions can be a limiting factor to success. In the case of *M. ochracea*¸ optimum temperature for mycelial growth was 25°C and 15°-25°C for pycnidial growth which requires a minimum of 8 hours of light per day (Carisse & Bernier, 2002a). Spore germination occurred at temperatures above 15°C and was optimal at pH 4 (Carisse & Bernier, 2002a).

1.5.1.3 *Trichoderma atroviride*

Trichoderma atroviride Karsten was first isolated in Europe in 1892 (Dodd *et al*., 2003), and is classified within the fungal Class Hyphomycetes. Initially overlooked in Rifai's (1969) monograph of *Trichoderma*, this fungus was later reintroduced and described by Bissett in 1992. For many years, *T. atroviride* has been confused as another

Trichoderma species with similar morphology, *T. harzianum* (Bisset, 1992; Dodd *et al*., 2003). Both species produce green, smooth, globose to subglobose conidia; however, *T. atroviride* may be distinguished by the production of an antibiotic compound that has a distinct sweet, coconut odour (Bisset, 1992; Dodd *et al*., 2003). *T. harzianum* biotype "Th 3" is now recognized to be *T. atroviride*, suggesting that *T. atroviride* has been largely misrepresented within previous research.

Since its rediscovery, *T. atroviride* has received attention due to its diverse biochemical and biological activities. *Trichoderma atroviride* is a ubiquitous, facultative, necrotrophic mycoparasite that demonstrates antagonism on a wide range of phytopathogenic fungi (Olmedo-Monfil *et al*., 2002). Among these hosts are a number of melanised fungi, including *B. cinerea, R. solani, S. sclerotiorum, V. inaequalis,* and *Verticillium dahliae* Kleb (Bolar *et al*., 2001; Harman *et al*., 2004; Kaur *et al*., 2005; Reithner *et al*., 2005). In soil, *T. atroviride* is highly competitive towards other microflora for nutrients, such as decaying organic matter, and space (Kaur *et al*., 2005). Competitive advantage is maintained with the production and use of non-enzyme antifungal metabolites, including α-pyrones and peptaibols (Dennis & Webster, 1971; Reithner *et al*., 2005). Upon interfungal contact, *T. atroviride* recognizes and responds to signals from the host by coiling around the host hyphae, initiating direct mycoparasitism (Kullnig *et al*., 2000; Rocha-Ramirez *et al*., 2002). During the next phase, *T. atroviride* produces lytic enzymes such as glucanases, chitinases, and proteases, which cause cell-wall degradation, cell lysing and cytoplasmic leaching, utilizing the cellular content as a nutrient source (Reithner *et al*., 2005). Once host nutrients are depleted, *T. atroviride* diverts its remaining resources to the production of asexual conidia (Rocha-Ramirez *et*

al., 2002). *Trichoderma atroviride* is active in temperatures between 4°-33°C and is best adapted to acidic soils (Benitez *et al*., 2004).

Trichoderma spp. have developed the ability to interact not only with other fungi, but also with plants, and can do so simultaneously (Marra *et al*., 2006). *T. atroviride* can also induce plant host resistance and plant growth promotion (Harmen *et al*., 2004). When *Trichoderma* spp. colonize the root surfaces of host plants, an interaction zone is established where bioactive molecules that elicit resistance are released by certain isolates. Some of these bioactive molecules include homologues of avirulence proteins, proteins with enzymatic functions and oligosaccharide compounds with low-molecular-weight (Harmen *et al*., 2004). Current research within this area of biocontrol research involves utilizing proteomic analysis to identify differential proteins involved in the complex communication system (Marra *et al*., 2006).

Trichoderma spp. have also been associated with improved plant growth, making their use as biocontrol agents even more beneficial. In particular, the presence of *Trichoderma* spp. within the rhizosphere can increase the uptake and concentration of a variety of nutrients, including phosphorus, iron, and magnesium (Altomare *et al*., 1999; Yedidia *et al*., 2001). These species are also capable of reducing the effects of deleterious root microflora that can reduce plant growth in the absence of plant pathogens. Ezzi & Lynch (2002) demonstrated that *Trichoderma* spp. can degrade harmful cyanide, emitted from the microflora within the rhizosphere, by producing the enzymes cyanide hydratase and rhodanese. In both instances, the presence of *Trichoderma* spp. increased host plant efficiency and these effects may be most noticeable when growing conditions are unfavourable (Harman *et al.*, 2004).

1.5.2 Factors Affecting BCA Efficacy

One of the major drawbacks of BCAs is the variability of control in comparison to chemical pesticides. Since BCAs are living entities, their survival and activity may be heavily influenced by the environment that surrounds them, threatening the reproducibility of control (McQuilken *et al*., 1997). Conditions such as temperature, relative humidity, and soil moisture can vary within and between growing seasons, and at times may be more conducive to the plant pathogen than the mycoparasite (Boland, 1990; Hannusch & Boland, 1996). Soil organic matter can also influence the success of a BCA due to the increased presence of microbial competitors (Papavizas, 1985). Although *T. atroviride* is a highly competitive saprotroph, its success can be compromised by other microbes with antibiotic characteristics such as *Pseudomonas* spp. which produce a metabolite toxic to *Trichoderma* spp. (Hubbard et al 1983). Conversely, obligate mycoparasites such as *C. minitans* and *M. ochracea* are highly adapted to infect sclerotia and have low competitive saprophytic ability (Gerlagh *et al*., 1996). Competitive ability is an important attribute when considering the distribution of sclerotia within the soil. If BCA spores are unable to exist as saprophytes, they may experience fungistasis and enter a stage of dormancy (Papavizas & Lumsden, 1980).

1.6 Conclusions

When looking for novel approaches to effectively manage plant pathogens, it is critical that all ecological and epidemiological processes are fully understood. This is especially true when it comes to the use of BCAs to ensure that they are utilized most efficiently. It is evident that ecological aspects of sclerotia and the plant pathogens that produce them are still not understood in their entirety. One issue that requires attention is

the conflicting evidence regarding the long-term survival potential of sclerotia of *B. squamosa, S. sclerotiorum,* and *S. cepivorum*. It is important to establish a baseline survival period to be able to effectively evaluate the efficiency of biocontrol agents. Clarification is also required as to whether laboratory-produced (LP) sclerotia are able to effectively represent field-produced (FP) sclerotia in experimental designs.

Numerous studies have evaluated various antagonistic and/or parasitic microorganisms as potential BCAs against sclerotia of phytopathogenic fungi. *Coniothyrium minitans* was discovered to be a successful mycoparasite against a number of sclerotia-forming fungi and is currently marketed as a biocontrol agent. As a result, this organism may be considered an established and commercially-used BCA, and may act as a positive control. Alternatively, research surrounding the effectiveness of *T. atroviride* as a mycoparasite is uncertain due to previous discrepancies regarding identification. Research based on this microorganism may potentially be largely misrepresented within the literature and clarification as to its antagonistic ability is required. Regardless, the abundance of successful biocontrol research involving the fungal genera *Trichoderma,* and their well documented ability as competitive excluders, suggests that this organism will be an effective biocontrol agent. Furthermore, the novel *M. ochracea* has received minimal attention since its recent discovery. This microorganism demonstrates strong antagonistic action against melanised fungal structures, yet a deeper knowledge of this organism's antagonistic abilities and survival within natural soils is required.

One of the common downfalls of biocontrols is that many have a limited host range of phytopathogens that each are able to antagonize and effectively manage. This

limitation makes them an expensive option for growers. Despite the varying epidemiological processes of *B. squamosa, S. cepivorum* and *S. sclerotiorum*, their common life stage as sclerotia, and their pathogenesis to host crops commonly used in rotation in the same soils, makes them ideal candidates for a multi-host biocontrol agent. They are not all easily controlled by foliar fungicides and an alternative to fungicide sprays would be of great benefit within an integrated pest management program. *Botrytis squamosa* is quite easily managed with foliar fungicides due to the visible and predictable development of spores from lesions. It is more difficult to control *S. sclerotiorum*, and very difficult to control *S. cepivorum* with foliar fungicides due to limited points of contact with the pathogen. Each of the biocontrol agents used within this study have demonstrated some form of antagonism on melanised structures, similar to the melanised rind of sclerotia. It is hypothesized that these three biocontrol agents will demonstrate antagonistic activity towards the three pathogens of interest.

1.7 Thesis Objectives

The objectives of this thesis were to:

1. Evaluate the long-term survival of LP sclerotia in a natural environment;
2. Evaluate the ability of *C. minitans, M. ochracea* and *T. atroviride* to inhibit the germination of sclerotia of *B. squamosa*, *S. sclerotiorum,* and *S. cepivorum*;
3. Evaluate the ability of LP sclerotia of *S. sclerotiorum* to effectively represent FP sclerotia of *S. sclerotiorum*;
4. Identify natural colonizers of FP sclerotia of *S. sclerotiorum*;

5. Qualitatively evaluate the naturally-occurring fungal colonizers of sclerotia of *S. sclerotiorum* for antagonistic and/or parasitic interactions with *S. sclerotiorum*; and
6. Qualitatively evaluate the naturally-occurring fungal colonizers of sclerotia of *S. sclerotiorum* for pathogenic interactions with host seedlings.

CHAPTER 2

LONG-TERM SURVIVAL OF SCLEROTIA OF *BOTRYTIS SQUAMOSA, SCLEROTINIA SCLEROTIORUM,* AND *SCLEROTIUM CEPIVORUM* TREATED WITH THREE BIOLOGICAL CONTROLS UNDER FIELD CONDITIONS

2.1 Abstract

The primary overwintering form of *B. squamosa*, *S. sclerotiorum* and *S. cepivorum* are sclerotia. Management of these pathogens is difficult due to the resilience of sclerotia within the soil; however, this common life stage is an ideal target for a multi-host BCA. The objective of this research was to determine the impact of three BCAs, *C. minitans*, *M. ochracea* and *T. atroviride*, on the survival of sclerotia of *B. squamosa*, *S. sclerotiorum* and *S cepivorum* under field conditions within a two year time span. Laboratory-produced sclerotia of *B. squamosa*, *S. sclerotiorum,* and *S cepivorum* were treated with spore suspensions of the individual BCAs. Sclerotia were buried 5-10 cm deep in pots containing muck soil and placed outdoors at the Muck Crops Research Station in December 2011. Percent survival was assessed at one to three month intervals post-burial. Once recovered, sclerotia were surface-disinfested, plated onto acidified PDA, and observed for germination and formation of daughter sclerotia. Fourteen months post-burial, percent survival of untreated control sclerotia decreased by 32, 78, and 1% for *B. squamosa*, *S. sclerotiorum*, and *S. cepivorum*, respectively, compared to one month post-burial. Sclerotia of all three pathogens treated with *T. atroviride* demonstrated the lowest percent survival at this time. Percent survival of *B. squamosa* and *S. sclerotiorum* reduced to 19 and 23, while *S. cepivorum* did not vary.

2.2 Introduction

Sclerotia-forming fungi are among the most destructive soil-borne plant pathogens affecting agricultural crops. Control of these pathogens is considered difficult compared to foliar pathogens due to the formation of persistent sclerotia that are distributed in an unpredictable pattern within the soil (Becker and Schwinn, 1993; Georgiou *et al.,* 2006). Sclerotia are asexual, multicellular, melanised resting structures that allow pathogens to tolerate an array of unfavourable conditions within the soil matrix for potentially long periods of time (Willets, 1972; Willets & Bullock, 1992). Control measures available to growers are limited and generally pathogen. Ultimately, the number of effective chemical solutions for sclerotial pathogens is low, leaving a great potential for BCAs such as mycoparasitic fungi.

Three plant pathogens that produce sclerotia and are of economic importance to vegetable production in Canada are *B. squamosa, S. sclerotiorum* and *S. cepivorum*. Botrytis leaf blight of onion is caused by the necrotrophic pathogen, *B. squamosa*. Symptoms can be seen as distinct white water-soaked lesions that form on leaves of infected onion plants (Clarkson *et al*., 2000). These lesions further produce infectious conidia, resulting in polycyclic disease development. Sclerotia form on the inner and outer leaf sheaths of onion bulbs and can be variable in shape and size, ranging from 2 mm to 10 mm in diameter (Ellerbrock & Lorbeer, 1977b). The saprophytic pathogen *S. sclerotiorum* produces sclerotia that germinate to form apothecia that release air-borne ascospores that target petals and, in carrots, the above-ground foliage. Sclerotia may also germinate to infect the host tissue directly in some pathosystems (Purdy, 1979). Progression of the disease results in the formation of water-soaked lesions that enlarge

and give way to the formation of fluffy, white mycelial mats. It is within these mats that numerous circular sclerotia, 2mm - 35 cm in size, are then formed (Bolton *et al.*, 2006). Finally, *S. cepivorum* is a necrotrophic, root-infecting pathogen that causes white rot on *Allium* spp., such as dry onions. Sclerotia of *S. cepivorum* only germinate in the presence of *Allium* spp. and produce mycelia. The mycelia infects onion roots and bulb bases, and colonization can occur pre- or post-host plant emergence (Coley-Smith, 1960). Infected bulbs gradually decay and develop mats of fluffy, white mycelial growth that produces thousands of tightly-packed sclerotia, 200-500 µm in diameter (Tims, 1948).

It is evident that despite the common life cycle stage of dormant sclerotia, the epidemiology of these pathogens vary greatly, resulting in variation among the sclerotia they produce (Chet & Henis, 1975). The morphological variation of these sclerotia has been associated with variations in natural sclerotial survival within soil (Ellerbrock & Lorbeer, 1977a,b; Adams & Ayers, 1979; Coley-Smith *et al.*, 1990). Conflicting evidence regarding the length of long-term survival of sclerotia of *B. squamosa, S. sclerotiorum*, and *S. cepivorum* demonstrates that a baseline survival period should be established. This will assist in effectively evaluating the efficiency of potential biocontrol agents.

These three pathogens of focus are considered important plant pathogens within the muck soils of the Bradford Marsh, Ontario. This is due to the presence of susceptible hosts, carrots and dry onions, which are both considered high value, low acreage crops. The production of these crops takes place in friable, high organic matter soils with good moisture-holding capacity, such as muck soils (Swaider & Ware, 2001). Consequently, carrots and dry onions are generally rotated within the same fields, resulting in the presence of sclerotia of all three pathogens within the same soils. Although these

pathogens have varying life cycles, their common formation of melanised sclerotia and likely presence within the same soils make them ideal candidates for a non-specific, ecologically facultative mycoparasite that targets survival structures such as sclerotia.

In recent decades, alternatives to traditional chemical controls of plant pathogens have been extensively explored, including the use of mycoparasitic fungi as biological control agents (BCA) (Gullino & Kuijpers, 1994; Ragsdale & Sisler, 1994). This thesis will focus on two obligate parasite species, *C. minitans* and *M. ochracea*, and one facultative parasite species, *T. atroviride*. The well known mycoparasite, *C. minitans* is the active ingredient in the biocontrol product, Contans (Prophyta GmbH, Malchow/Poel, Germany), registered for use on sclerotia of *S. sclerotiorum* and *S. minor*. This naturally-occurring, necrotrophic fungus provides control of mycelial growth, prevents ascospore infection and disease development, as well as reduces sclerotial production (Gerlagh *et al.*, 1999; Li *et al.*, 2001; McQuilken *et al.*, 2003; Tomprefa *et al.*, 2011). Despite being a poor competitor within the soil environment, conidia of *C. minitans* are able to remain viable for long periods of time and through harsh winter conditions within host sclerotia (Huang & Erikson, 2002; Bennett *et al.*, 2006). This mycoparasite is considered an established BCA that produces acceptable levels of control of sclerotia of *S. sclerotiorum*.

Over the past decade, attention has been given to *M. ochracea*, as a potential biocontrol agent, due to its ability to inhibit the production of melanised reproductive structures in a number of economically-important plant pathogens (Benyagoub *et al.*, 1998; Carisse *et al.*, 2000; Bujold *et al.*, 2001). The antagonistic effect of this saprophyte on the survival of sclerotia has not been evaluated prior to this study. Despite this, mycoparasitic capabilities of this organism have been attributed to a complex synergy

among hydrolytic enzymes, mainly chitinase and glucanase, and antibiotics (Benyagoub *et al.,* 1998, Carisse *et al.,* 2001; Bassam *et al.,* 2002).

The fungal genus *Trichoderma* is globally distributed and known for broad range of antagonistic capabilities. Since its rediscovery in 1992, *T. atroviride* has received attention due to its diverse biochemical and biological activities. *Trichoderma atroviride* is a ubiquitous, facultative, necrotrophic mycoparasite that demonstrates both direct and indirect antagonism on a wide range of phytopathogenic fungi (Olmedo-Monfil *et al.,* 2002). Although the success of many biocontrol agents is limited by environmental factors, *T. atroviride* is a promising candidate which is active at a temperature range between 4-33ºC (Benitez *et al.,* 2004). Research surrounding the mycoparasitic abilities of this species on sclerotia is lacking and should be explored, especially within a natural setting.

The objectives of this study were to (1) evaluate the long-term survival of LP sclerotia in a natural environment; and (2) evaluate the ability of *C. minitans, M. ochracea* and *T. atroviride* to inhibit the germination of sclerotia of *B. squamosa, S. sclerotiorum,* and *S. cepivorum.*

2.3 Materials and Methods

2.3.1 Production and collection of sclerotia

In September 2011, sclerotia of *S. sclerotiorum* and *S. cepivorum* were collected from naturally-infected dry bean plants and dry onions, respectively, surrounding the University of Guelph Muck Crops Research Station in the Bradford Marsh of Ontario. Plants showing extensive symptoms of disease and sclerotia production were harvested and sclerotia were collected. Some plants were placed in moist chambers for 1-2 days to

allow for completion of any ongoing sclerotial formation. Lids of chambers were then removed to promote drying of plant tissue and mycelia. After 3-4 days, sclerotia were harvested and stored at 4°C. Pure cultures of *B. squamosa* were obtained from a previous trial where the pathogen was originally isolated from onion plants from Ontario.

Mass production of sclerotia within the laboratory began with 10-20 sclerotia of *S. sclerotiorum* and *S. cepivorum*, and mycelial plugs from pure cultures of *B. squamosa*. These were individually plated onto 10 cm diameter plates of Difco™ potato dextrose agar (PDA) (Becton, Dickinson and Company, Sparks, MD). After 4-5 days incubation at room temperature (20-22°C), 2 mm mycelial plugs were obtained from plates of *B. squamosa* and *S. sclerotiorum,* and transferred to hundreds of PDA plates for production of sclerotia. Inoculated plates were wrapped with Parafilm (Bemis Flexible Packaging, Neenah, WI) to prevent desiccation of media, and incubated in a growth room (19-22°C) with a 16:8 light:dark period for two weeks. After this time, Parafilm was removed and each plate lid was slightly offset for 1 day to promote desiccation of media and mycelia, and to increase the ease of sclerotial recovery. For *S. cepivorum*, initial colonies grown from field-produced sclerotia were used for sclerotia production. After 10-12 days, plate lids were displaced for a few hours to promote desiccation of media and mycelia. Sclerotia of all pathogens were recovered by tapping plates to dislodge sclerotia from surrounding mycelia and media. LP sclerotia were stored at 4°C for two to three weeks prior to use.

Following production of sclerotia, initial survival was evaluated for each pathogen. Five replicates of 25, 12 and 25 sclerotia of *B. squamosa, S. sclerotiorum*, and *S. cepivorum*, respectively, were plated on acidified (pH 3.5) PDA (APDA) and

incubated at room temperature (20-22 ºC). Plates were monitored for germination over a three week period. Survival was determined based on the percent of sclerotia that germinated and produced daughter sclerotia (Table A.1).

2.3.2 Long-term survival of sclerotia untreated and treated with three biocontrols in a natural setting

LP sclerotia of *B. squamosa, S. sclerotiorum,* and *S. cepivorum* were separated into groups of 50, 25, and 50, respectively. Each group was replicated five times for each of four treatments, which were prepared eight times to fulfill eight destructive sampling dates. Each group of sclerotia was placed in a 5 cm x 6 cm mesh (158 meshcount, SaatiPrint Canada, Mississauga, ON) nylon bag and incorporated with either 10 mL (*B. squamosa, S. sclerotiorum*) or 5 mL (*S. cepivorum*) of muck soil (pH 6.3, 74% organic matter) collected from the Muck Crops Research Station. Variations of sclerotia group sizes and accompanying soil amounts were to account for differences in sclerotial sizes among pathogens. Mesh bags of each pathogen were either left untreated or treated with one of three biocontrol treatments. Spore suspensions of the BCAs were composed of viable granular formulations provided by Prophyta GmbH (Malchow/Poel, Germany) and sterile deionised water at rates of 6.2 x 10^7 spores/mL for Contans (*C. minitans)*, 1.2 x 10^8 spores/mL for *M. ochracea*, and 1.1 x 10^8 spores/mL for *T. atroviride* strain B, based on recommended rates and previous experimental rates (Carisse *et al.,* 2006). Biocontrol solutions were applied as a drench of 2 mL/bag to *B. squamosa* and *S. sclerotiorum*, and 1 mL/bag to *S. cepivorum*. Once treated, nylon mesh bags were sealed with a heat sealer (Model: AIE-400P, American International Electric Inc, City of Industry, CA). Bags were buried 5-10 cm deep in 1 L pots of muck soil and arranged in a completely

randomized design in each of eight destructive sampling dates. Replicate bags were buried in separate pots. Pots were placed outside in a fenced enclosure at the Muck Crops Research Station in December 2011. Recovery dates took place every 2-3 months for 20 months.

At each recovery date, one block of pots was removed from the Muck Station and bags were recovered from each pot. Sclerotia were separated from the soil by dry sieving with soil screens, sizes 10 (2 mm) and 14 (1.4 mm) (Fisher Scientific International Inc., Hampton, NH). Sclerotia were placed back into mesh bags and subjected to surface disinfestation by submerging bags in 70% ethanol for 1 min, followed by 4 mins in 5% sodium hypochlorite, and rinsed three times with sterile deionised water. Sclerotia were then removed from bags, blotted dry with sterile paper towel and plated onto 10 cm quarter-sectioned Petri plates (Fischer Scientific International Inc., Hampton, NH) containing APDA. Plates were incubated at room temperature and monitored for up to 4 weeks. Initial counts of sclerotia recovered were recorded. The number of sclerotia germinated and number producing secondary sclerotia were assessed every 4-7 days. After 4 weeks, percent survival was calculated for each replication with the following formula (Legget *et al.*, 1983):

$$\frac{(\#\ \text{of sclerotia recovered} \times \%\ \text{of recovered sclerotia germinated and producing sclerotia})}{\#\ \text{of sclerotia originally in bag}} \times 100$$

2.3.3 Statistical analysis

Percent survival of sclerotia were arcsine-transformed to improve normality of distribution and expressed as Area Under the Percent Survival Stairs (AUPSS) to compare survival between untreated and treated sclerotia for each pathogen. AUPSS is based on the revised version of Area Under the Disease Progress Curve (AUDPC),

known as the Area Under the Disease Progress Stairs (AUDPS) (Simko & Piepho, 2012) that aims at improving the estimation of disease progress by incorporating the first and last observation points. AUPSS data were calculated from the arcsine transformed percent survival data at eight recovery dates over the 20 month observation period, with the following equation:

$$AUDPS = \left[\sum_{i=1}^{n-1} \frac{y_i + y_{i+1}}{2} \times (t_{i+1} - t_i) \right] + \left[\frac{y_1 + y_n}{2} \times \frac{D}{n-1} \right]$$

where y_i is the percent survival at the i^{th} observation, t_i is the time at the i^{th} observation, n is the total number of observations, and D is t_n - t_1. To determine differences among the 20 month survival of untreated sclerotia of the pathogens, analyses of variance were performed on the AUPSS of the untreated control of the pathogens, and means were separated using Tukey's adjustment. The same analyses were completed for each treatment by pathogen to evaluate the efficacy of each biocontrol. To assess the survival over the total observation period, number of sclerotia recovered, percent of recovered sclerotia that germinated, and percent survival data were analyzed by pathogen. To improve normality, square root transformations were used on numbered data and arcsine transformations were used on percent data. Analysis of variance were performed for each rating and for each treatment/pathogen combination, and means were separated using Tukey's adjustment. Statistical analyses were performed using the PROC GLM procedures of SAS (v.9.3) with a type III error of 0.05.

2.4 Results

2.4.1 Survival of untreated sclerotia in a natural setting

At the end of the 20 month observation period, only the percent survival of untreated sclerotia of *S. sclerotiorum* had decreased compared to that at one month post-burial. Differences in percent survival of sclerotia of *S. cepivorum* were observed among recovery dates throughout the observation period, but not between one and 20 months post-burial. No differences in percent survival were observed for sclerotia of *B. squamosa* (Table 2.1). The AUPSS based on arcsine transformed percent survival data were different among pathogens with the highest for *B. squamosa* (23.5) , followed by *S. sclerotiorum* (21.1), and *S. cepivorum* (1.7) (Figure 2.1). The low survival of *S. cepivorum* was established just one month post-burial. At this time, percent survival and percent germination of *S. cepivorum* were both 1%. This increased to 9% at 3 months post-burial, but then returned to low percent survival for the remainder of the observation period (Figure 2.1, Table 2.1). The germination of *S. cepivorum* sclerotia was 98% prior to the start of the experiment (Table A.1). The number of sclerotia recovered fell from 13 at one month post-burial to 2 at 20 months post-burial (Table 2.1).

All aspects of survival of *B. squamosa* remained consistent over the observation period. No differences in number of sclerotia recovered, percent germination, or percent survival were found among recovery dates (Table 2.1). Percent germination prior to burial and one month post-burial were high at 100% and 95%, respectively (Table A.1). After 20 months of burial, percent survival was 63%. *Botrytis squamosa* also had the highest AUPSS of all three pathogens (Figure 2.1).

Table 2.1. Number recovered, percent germinated and percent survival of laboratory-produced sclerotia of *Botrytis squamosa*, *Sclerotinia sclerotiorum* and *Sclerotium cepivorum*, treated with three biological control treatments and recovered at two to three month intervals post-burial from December 2011 to August 2013.

Months Post Burial	Untreated Control			*Coniothyrium minitans*			*Microsphaeropsis ochracea*			*Trichoderma atroviride*		
B. squamosa (50)[a]	# Recovered[b]	% Germinated[c]	% Survival[b]	# Recovered	% Germinated	% Survival	# Recovered	% Germinated	% Survival	# Recovered	% Germinated	% Survival
1	48 ns	98 ns	95 ns	47 ns	92 ns	90 *ab*	48 *abc*	98 *ab*	94 *a*	47 ns	76 *ab*	72 *ab*
3	47	95	95	47	94	97 *a*	49 *a*	99 *a*	94 *a*	48	97 *a*	85 *a*
5	49	94	93	46	98	92 *ab*	48 *ab*	95 *abc*	92 *ab*	48	34 *b*	33 *bc*
8	46	78	73	40	72	72 *ab*	49 *ab*	85 *bc*	84 *ab*	41	32 *b*	32 *bc*
11	44	66	58	42	79	68 *ab*	39 *bc*	94 *abc*	74 *ab*	38	31 *b*	23 *bc*
14	33	65	54	29	57	32 *b*	42 *abc*	83 *c*	71 *b*	33	36 *b*	23 *bc*
17	43	96	84	37	92	70 *ab*	43 *abc*	99 *a*	92 *ab*	44	59 *ab*	44 *abc*
20	36	71	63	30	81	48 *ab*	38 *c*	92 *abc*	71 *b*	34	28 *b*	19 *c*
SE	0.509	0.133	0.123	0.659	0.137	0.149	0.132	0.0556	0.0612	0.420	0.119	0.110

Months Post Burial	Untreated Control			*Coniothyrium minitans*			*Microsphaeropsis ochracea*			*Trichoderma atroviride*		
S. sclerotiorum (25)	# Recovered	% Germinated	% Survival	# Recovered	% Germinated	% Survival	# Recovered	% Germinated	% Survival	# Recovered	% Germinated	% Survival
1	25 ns	98 *ab*	98 *a*	23 *a*	61 ns	64 *b*	25 ns	95 ns	95 ns	24 *a*	40 *ab*	40 ns
3	24	96 *abc*	99 *a*	24 *a*	99	100 *a*	24	97	99	23 *a*	95 *a*	41
5	23	88 *abc*	84 *ab*	23 *a*	29	27 *c*	24	96	93	23 *a*	32 *ab*	31
8	17	99 *a*	69 *ab*	7 *b*	60	18 *c*	20	70	64	23 *a*	31 *b*	30
11	10	39 *bc*	34 *b*	3 *b*	71	12 *c*	19	80	74	4 *b*	6 *b*	4
14	22	92 *abc*	81 *ab*	2 *b*	63	8 *c*	19	76	72	15 *ab*	40 *ab*	31
17	12	100 *a*	48 *ab*	4 *b*	67	12 *c*	22	99	89	16 *ab*	34 *b*	28
20	10	35 *c*	20 *b*	3 *b*	86	9 *c*	14	92	52	13 *ab*	41 *ab*	23
SE	0.471	0.169	0.169	0.243	0.180	0.0621	0.365	0.189	0.164	0.442	0.149	0.135

Months Post Burial	Untreated Control			*Coniothyrium minitans*			*Microsphaeropsis ochracea*			*Trichoderma atroviride*		
S. cepivorum (50)	# Recovered	% Germinated	% Survival	# Recovered	% Germinated	% Survival	# Recovered	% Germinated	% Survival	# Recovered	% Germinated	% Survival
1	13 *a*	1 ns	1 *b*	9 ns	3 ns	1 ns	7 ns	16 ns	2 ns	6 ns	0 ns	0 ns
3	14 *a*	28	9 *a*	8	17	3	6	13	2	7	15	2
5	6 *ab*	0	0 *b*	4	0	0	6	50	5	8	22	6
8	7 *ab*	25	3 *ab*	3	13	1	5	0	0	8	16	3
11	4 *b*	0	0 *b*	3	0	0	3	3	0	5	0	0
14	5 *ab*	0	0 *b*	3	20	2	3	0	0	4	0	0
17	5 *ab*	18	5 *ab*	2	0	0	4	8	1	5	13	1
20	2 *b*	0	0 *b*	3	16	1	4	8	1	3	0	0
SE	0.281	0.114	0.0825	0.358	0.122	0.0879	0.277	0.154	0.0832	0.295	0.113	0.0727

[a] Number of sclerotia initially in mesh bag at start of experiment
[b] Original data displayed, analysis of variance performed on arcsine transformed data
[c] Original data displayed, analysis of variance performed on log transformed data
a, *b*, *c* Means in a column, for a pathogen followed by the same letter are not significantly different at P=0.05 according to Tukey`s HSD
ns = no significant differences in column
% Survival = [(# of sclerotia recovered x % of recovered sclerotia germinated and producing sclerotia) / # of sclerotia originally in bag] x 100

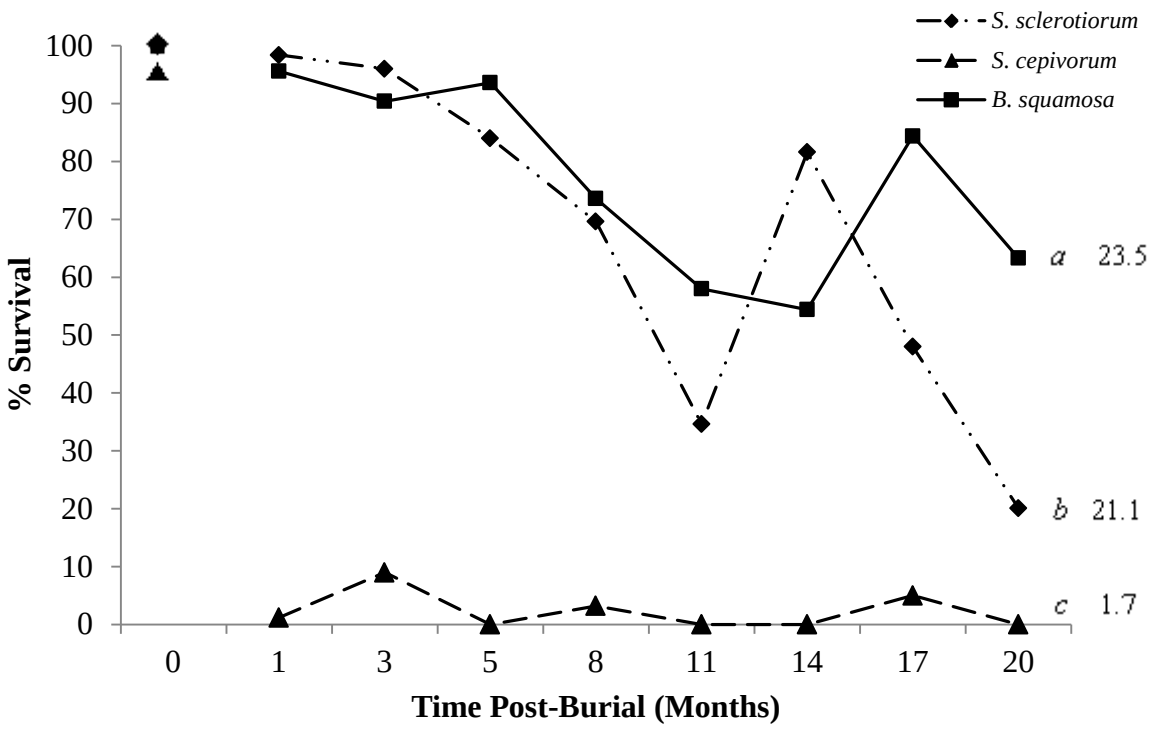


Figure 2.1. Percent survival of laboratory-produced sclerotia of *Botrytis squamosa, Sclerotinia sclerotiorum* and *Sclerotium cepivorum,* recovered at two to three monthly intervals post-burial (December 2011). Values on far right represent AUPSS values of untransformed data. Independent data points at time zero represent % germination prior to burial (Table A.1). Different letters denote significant differences at P=0.05 according to Tukey's HSD among arcsine-transformed Area Under the Percent Survival Stairs (AUPSS) data. Original % survival data displayed. (SE = 0.4733).

Sclerotia of *S. sclerotiorum* demonstrated the least consistent trends in survival characteristics over the 20 month observation period. Although there were no differences among number of sclerotia recovered, differences were seen in percent germination and percent survival (Table 2.1). After 20 months, percent germination had decreased to 35% compared to 100% prior to burial, and 98% one month post-burial. Percent survival at 11 (32%) and 20 (20%) months post-burial were different than that at one month post-burial (98%) (Table 2.1). Throughout the experimental period, there was a general decrease in percent survival, with the exception of observations at 14 months post-burial. Overall, sclerotia of *S. sclerotiorum* demonstrated the greatest reduction in survival over the observation period (Figure 2.1).

2.4.2 Survival of treated sclerotia of *B. squamosa* in a natural setting

Sclerotia of *B. squamosa* treated with *T. atroviride* had a reduced AUPSS compared to that of untreated sclerotia over the observation period (Figure 2.2). Sclerotia treated with *C. minitans* and *M. ochracea* did not differ in survival from untreated sclerotia (Figure 2.2). At the final recovery date, 20 months post-burial, sclerotia treated with *T. atroviride* and *M. ochracea* had reduced survival when compared to that at one month post-burial (Table 2.1). At this time, percent survival of *T. atroviride*-treated sclerotia had been reduced to 19%, while survival of untreated and *C. minitans*-treated sclerotia were 63% and 48%, respectively. Percent survival of sclerotia treated with *M. ochracea* was 71%. Over the observation period, differences in percent survival among recovery dates were observed for all treated sclerotia. Percent survival curves of each treatment demonstrated a relatively steady decline over the 20 month observation period,

with the exception of an increase in survival at 17 months post-burial (May 2013). This isolated increase in survival was not significant and did not surpass percent survival at one month-post burial (Table 2.1, Figure 2.2). Variations among recovery dates over the observation period were also seen in number of sclerotia recovered and percent germination for those treated with *M. ochracea*, and percent germination of those treated with *T. atroviride.* Much like percent survival, an increase in these survival characteristics was observed at 17 months post-burial.

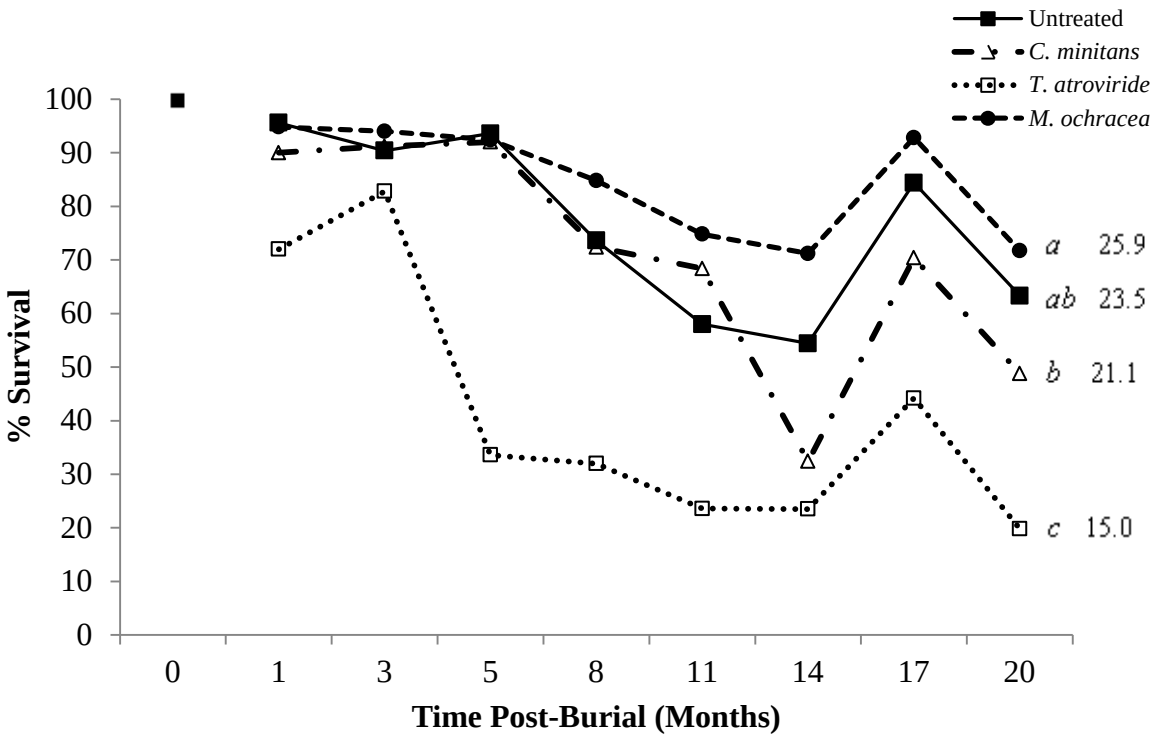


Figure 2.2. Percent survival of laboratory-produced sclerotia of *Botrytis squamosa*, treated with three biological control agents and recovered at two to three monthly intervals post-burial (December 2011). Values on far right represent AUPSS values of untransformed data. Independent data point at time zero represents % germination prior to burial (Table A.1). Different letters denote significant differences at P=0.05 according to Tukey's HSD among arcsine-transformed Area Under the Percent Survival Stairs (AUPSS) data. Original % survival data displayed. (SE = 0.8416).

2.4.3 Survival of treated sclerotia of *S. sclerotiorum* in a natural setting

Treatment of sclerotia of *S. sclerotiorum* with *C. minitans* and *T. atroviride* resulted in lower AUPSS values than that of untreated sclerotia (Figure 2.3). Sclerotia treated with *M. ochracea* had the highest AUPSS value, not different from untreated sclerotia, demonstrating the least antagonistic activity. Reductions in percent survival 20 months post-burial were observed only for untreated sclerotia (20%) and sclerotia treated with *C. minitans* (9%), when compared to that at one month post-burial (Table 2.1). However, treatment with *T. atroviride* reduced percent survival considerably, to 40% within one month of treatment, even though there was no change over the remainder of the time period. Sclerotia treated with *M. ochracea* had the highest percent survival (52%) at 20 months post-burial. There was considerable variation in survival characteristics of sclerotia of *S. sclerotiorum* among recovery dates, with the exception of those treated with *M. ochracea*. Variations in number of sclerotia recovered were observed for sclerotia treated with *C. minitans* and *T. atroviride*, while variations in percent germination were also observed in sclerotia untreated and treated with *T. atroviride* (Table 2.1). Variation was also seen among percent survival as these curves did not demonstrate steady declines (Figure 2.3). Both untreated sclerotia and sclerotia treated with *T. atroviride* experienced a dip in survival at 11 months post-burial (November 2012), followed by an increase at 14 months, similar to that observed at 8 months post-burial (Figure 2.3). Percent survival of sclerotia treated with *C. minitans* increased in survival up to 100% 3 months post-burial, followed by a plunge at 5 months to 27% and a steady decline to 9% 20 months post-burial.

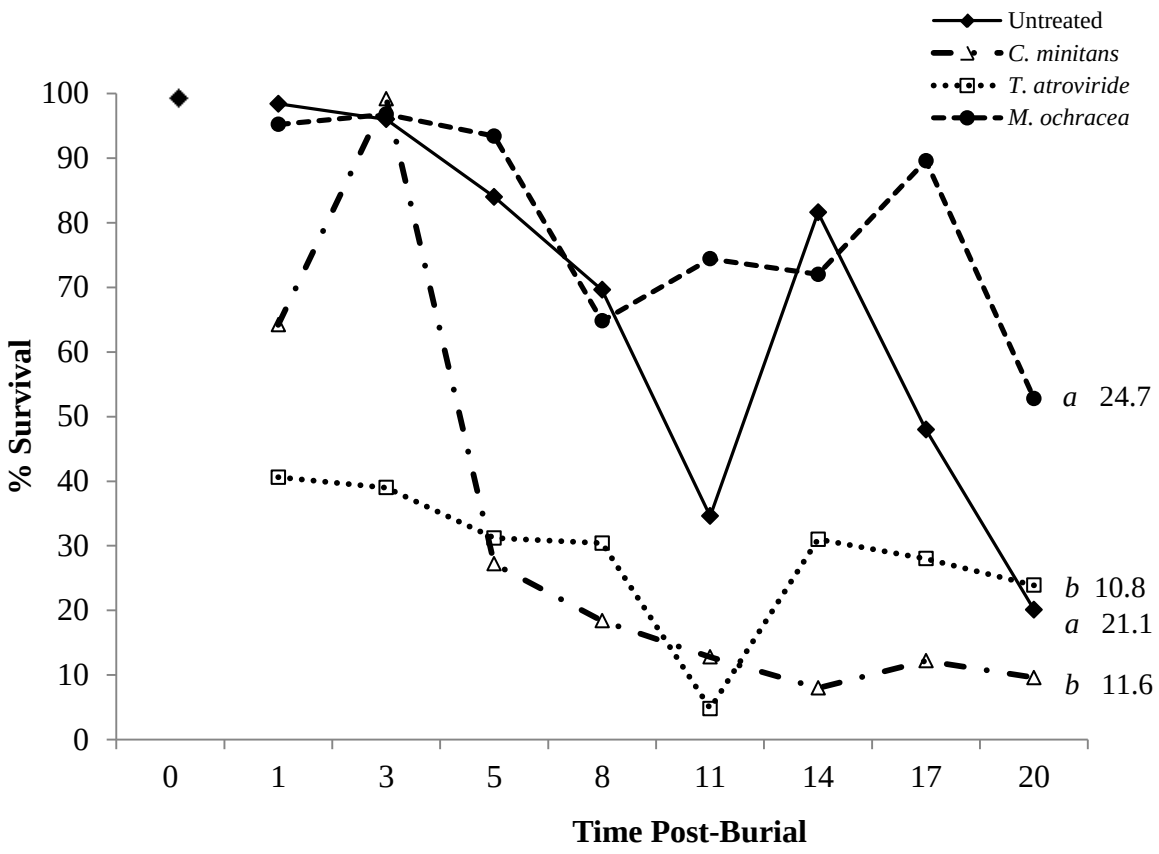


Figure 2.3. Percent survival of laboratory-produced sclerotia of *Sclerotinia sclerotiorum*, treated with three biological control agents and recovered at two to three monthly intervals post-burial (December 2011). Values on far right represent AUPSS values of untransformed data. Independent data point at time zero represents % germination prior to burial (Table A.1). Different letters denote significant differences at P=0.05 according to Tukey's HSD among arcsine-transformed Area Under the Percent Survival Stairs (AUPSS) data. Original % survival data displayed. (SE = 0.6885).

2.4.4 Survival of treated sclerotia of *S. cepivorum* in a natural setting

The AUPSS values among treated sclerotia of *S. cepivorum* where not significantly different from that of untreated sclerotia (Figure 2.4). Throughout the observation period, percent survival remained extremely low and no significant declines were observed between one month and 20 months post-burial. After one month, percent survival of treated and untreated sclerotia were all lower than 2%, much lower than that of the pre-burial percent germination of untreated sclerotia (98%) (Table 2.1, Figure 2.4, Table A.1). Over the 20 month observation period, percent survival did not exceed 9%, which was observed 3 months post-burial for the untreated control. The percent survival curves of sclerotia were variable, although significant differences were only detected with untreated sclerotia at the 3 month recovery, compared to one and 20 months post-burial (Figure 2.4). Variations in survival characters among recovery dates were only observed for untreated sclerotia. After 20 months post-burial, the number of untreated sclerotia recovered was down to 2, compared to 13 recovered at one month post-burial (Table 2.1).

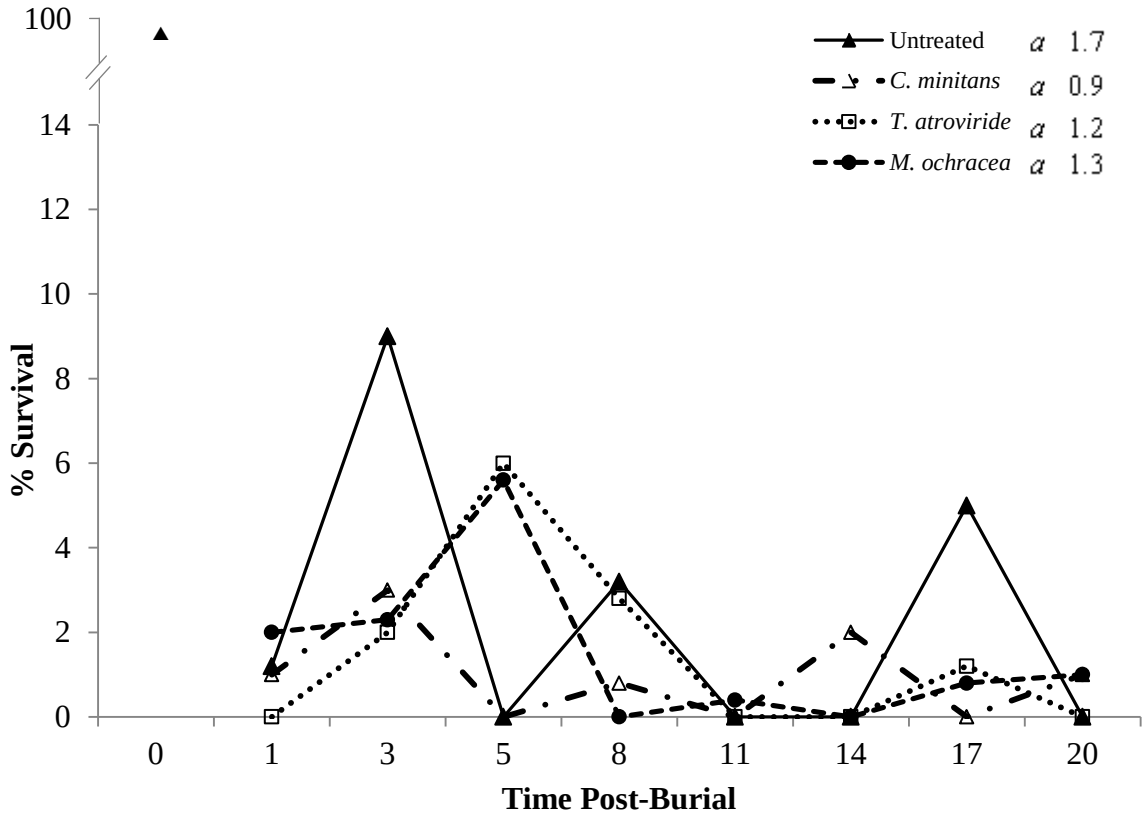


Figure 2.4. Percent survival of laboratory-produced sclerotia of *Sclerotium cepivorum*, treated with three biological control agents and recovered at two to three monthly intervals post-burial (December 2011). Values on far right represent AUPSS values of untransformed data. Independent data point at time zero represents % germination prior to burial (Table A.1). Different letters denote significant differences at P=0.05 according to Tukey's HSD among arcsine-transformed Area Under the Percent Survival Stairs (AUPSS) data. Original % survival data displayed. (SE = 0.2651).

2.5 Discussion

This is the first study to examine interactions of three BCAs and three plant pathogens. The results confirm that select BCAs, such as *Trichoderma* spp., can suppress sclerotial survival of more than one pathogen in the same soil environment. In this report, *T. atroviride* was the most effective BCA evaluated, capable of suppressing the percent survival of sclerotia of *B. squamosa* and *S. sclerotiorum* to 19% and 23%, 20 months post-burial. Survival of sclerotia of *S. cepivorum* did not differ compared to that at one month post-burial. Among all pathogens, *T. atroviride* was capable of reducing sclerotial survival to a similar or greater level than that of a registered BCA, *C. minitans.* Although these results are based on differing concentrations of each BCA, application rates were based on previous literature and recommended spore suspension concentrations.

Results of this study demonstrate that the efficacy of the BCAs were relatively consistent with the reported range of pathogens affected by these BCAs. *Trichoderma atroviride* was antagonistic toward *B. squamosa* and *S. sclerotiorum*, and *C. minitans* reduced survival of *S. sclerotiorum*. Previous studies of *T. atroviride* have reported antagonism towards *S. sclerotium* and *S. cepivorum*, while there are no reports involving *B. squamosa* (Li *et al.*, 2005; McLean *et al*., 2005b; Matroudi *et al.*, 2009; McLean *et al.*, 2012). The antagonistic capabilities of *C. minitans* towards *S. sclerotiorum* and *S. cepivorum* have been reported, although the current results cannot confirm the previous reports of efficacy on *S. cepivorum* (Ahmed & Tribe, 1977; Gerlagh *et al.*, 1996; Bremer *et al.*, 2000; Gerlagh *et al.*, 2003). This is due, in part, to the very low survival of untreated sclerotia of *S. cepivorum*, which prevented evaluation of BCA efficacy. The reduction in survival of sclerotia of *S. sclerotiorum* by *C. minitans* was not observed until

5 months post burial. This reduced activity during winter months supports previous finding demonstrating comparatively slow rates of germination and infection of sclerotia of *S. sclerotiorum* by *C. minitans* (Trutmann *et al.,* 1980). Finally, reports on the efficacy of *M. ochracea* as a BCA suggest antagonism towards *B. squamosa* and *S. sclerotiorum* (Carisse, 2001; Carisse *et al.*, 2006), but there was no evidence of antagonistic activity against any of the three pathogens in the current study. The inactivity of *M. ochracea* may be due to an incompatibility with the target pathogens. Analysis of the antagonistic processes of *M. ochracea* includes the binding and incorporation of lytic compounds into the melanin of susceptible hosts, such as *V. inaequalis* (El Bassam *et al.*, 2002). This mode of action may not be successful against sclerotia due to variations among the binding properties of fungal melanins (Wheeler, 1983; El Bassam *et al.*, 2002; Butler *et al.*, 2005; Butler *et al.*, 2009). For instance, sclerotial melanin of *S. sclerotiorum* is believed to contain admixed or bound materials that prevent bonding and proper wetting (Butler & Lachane, 1986; Butler *et al.*, 2005). It is likely that sclerotial rind melanin may also prevent the binding of lytic compounds from *M. ochracea*, inhibiting antagonistic activity.

Relatively little attention has been given to biological control options for the management of *B. squamosa*. Investigations into potential BCAs have demonstrated key microbial genera that possess antagonistic potential, namely *Trichoderma, Gliocladium, Ulocladium, Pseudomonas* and *Bacillus* (Elad & Stewart, 2007). *Trichoderma* spp. have been commercialized in many parts of the world for the suppression of *Botrytis* diseases. This is the first report of the long-term antagonistic effects of *T. atroviride* on sclerotia of *B. squamosa*. In this study, *T. atroviride* was the only BCA to reduce the survival of

sclerotia of *B. squamosa* compared to the untreated sclerotia. There are conflicting reports on the ability of *C. minitans* and *M. ochracea* to reduce survival of sclerotia of *B. squamosa*. Carisse *et al*. (2006) reported that treating sclerotia of *B. squamosa* with *M. ochracea* reduced conidia production by 75% compared to the untreated control, while conidial production on sclerotia treated with *C. minitans* did not differ from that of the untreated control sclerotia. The results of the current study and of Carisse *et al.* (2006) are consistent with other reports of the lack of efficacy of *C. minitans* on other *Botrytis* spp. (Turner & Tribes, 1976; Gerlagh *et al.*, 1996).

There has been extensive research on biological control options for the management of *S. sclerotiorum* as a result of the wide host range, global distribution and economic importance of this pathogen (Anas & Reeleder 1988; Huang & Huang, 1993; Garcia-Garza *et al*., 1997; Zhou & Boland, 1998). Although only two of the three BCAs in this study were able to reduce the survival of sclerotia of *S. sclerotiorum*, all three of these BCAs have been previously reported to demonstrate antagonism against *S. sclerotiorum*. Unfortunately, due to variation in experimental methodologies, strict comparisons among studies are difficult to make. As *C. minitans* is the active ingredient of Contans, a registered product for use against *S. sclerotiorum*, there are numerous reports demonstrating the antagonistic activity and survival potential of this mycoparasite (Trutmann *et al.*, 1980; Gerlagh *et al.*, 1996; Bennett *et al.*, 2006; Huang & Erickson, 2002;Whipps *et al.*, 2008). There are minimal studies on the use of *T. atroviride* against sclerotia of *S. sclerotiorum*. In one study, Jones & Stewart (2000) observed that 21% and 0% of sclerotia of *S. sclerotiorum* treated with *T. atroviride* remained viable following 4 and 8 weeks incubation on sand plates. The ineffective antagonism of *M. ochracea*

observed in the current study conflicts with previous observations by Carisse (2001) where *M. ochracea* was capable of significantly reducing percent germination of sclerotia of *S. sclerotiorum* following 14 days incubation at room temperature. Variation between those results and results of the current study is likely due to the added environmental stressors experienced by *M. ochracea* in the current study, suggesting that this BCA is a weak competitor in soil.

There have also been an array of studies investigating potential BCAs for the control of *S. cepivorum*. The mycoparasite *C. minitans* has been isolated from sclerotia of *S. cepivorum* and is believed to naturally parasitize this pathogen through holes in the sclerotial rind. Various studies have observed the inhibition of *S. cepivorum* by *C. minitans* (Ghaffar, 1969; Turner & Tribe, 1976; DeOliveira *et al.*, 1984; Harrison & Stewart, 1988). Although the current study could not demonstrate this, control of *S. cepivorum* by *T. atroviride* was first identified in 1994 (Kay & Stewart, 1994). More recently, McLean *et al.* (2005b) observed up to 80% disease control in glasshouse and field trials, depending on formulation and disease pressure. No previous studies have evaluated the antagonistic potential of *M. ochracea* on sclerotia of *S. cepivorum*. In the current study, it is difficult to evaluate the efficacy of the three BCAs against untreated sclerotia due to the very low survival of the untreated control.

The natural long-term survival of sclerotia has been extensively studied for only some sclerotia-forming fungi. This is one of the few studies to observe the survival of sclerotia of *B. squamosa in situ* for a period of 20 months. Over this observation period, survival was reduced from 95% to 63%, while germination was reduced from 98% to 35%. These results are similar to the few reports in the literature which observed rates of

66% survival after 21 months of burial at a depth of 15 cm (Ellerbrock & Lorbeer, 1977 a, b). In that same study, only 3% of sclerotia survived when buried at a depth of 3 cm, demonstrating the impact that burial depth may have on sclerotia survival (Ellerbrock & Lorbeer, 1977b). Conversely, the survival of sclerotia of *S. sclerotiorum* has been extensively studied due to the extent of disease that this pathogen can cause globally. This study demonstrated that the survival of sclerotia of *S. sclerotiorum* was reduced to 20% following 20 months burial. These observations are comparable to other studies conducted over multiple growing seasons. Early records of survival were based on grower experiences and ranged from 1-10 years (Davis, 1925; Young & Morris, 1927; Brown & Butler, 1936; Starr *et al.*, 1953; Adams & Ayers, 1979). When considering the proportion of an original population that survives for these lengths of time, Cosic *et al.*, (2012) observed the recovery of 0-6 sclerotia, out of 40, 2 years post-burial. This is similar to the recovery of 10 sclerotia, 20 months post-burial, seen in this study. Stipe formation prior to recovery has been recorded and was also observed in this study. The germination of sclerotia during burial suggests that secondary sclerotia may be present within sample populations. Williams & Western (1965) noted this occurrence and attributed spikes in recovery in later months due to the production of secondary sclerotia which may have compensated for earlier losses.

Reports on the survival of sclerotia of *S. cepivorum* are quite variable and conflict with the results in this study. Here, pre-burial results demonstrated a 98% germination rate, while survival and percent germination at one month post-burial was 1%, and was 0% 20 months post-burial. These results are unique and do not support previous findings; however, similar observations have been reported in the Bradford

Marsh at the Muck Crop Research Station (Tesfaendrias & McDonald, 2012). One of the most widely cited studies evaluating the long-term survival of sclerotia of *S. cepivorum* is that of Coley-Smith *et al.* (1990) who observed survival of sclerotia 20 years post-burial. After this time, approximately 72-88% of sclerotia, depending on burial depth, were collected from nylon bags that did not contain soil (Coley-Smith *et al.*, 1990). Similar studies observed recovery after 15 and 20 years post-burial with survival values of 90% and 88%, respectively (Croxall *et al.*, 1953; Entwistle & Munasinghe, 1975; Crowe *et al.*, 1980; Coley-Smith & Sansford, 1986). Much like *S. sclerotiorum*, observations of secondary sclerotia have been made both within and outside of sample bags while buried, prior to recovery, influencing results of number of sclerotia collected (Coley-Smith *et al.*, 1987; Entwistle & Munasinghe, 1981). More recent results from Leggett *et al.*, (1983) demonstrated the viability of only 23.6% of sclerotia 16 months post-burial in muck soil. Declines in survival were attributed to heavy rains and flooding occurring during the winter months within the Fraser Valley, BC (Leggett *et al.*, 1983).

Throughout these studies, it is evident that a number of factors can affect the survival of sclerotia within the soil profile, including depth of burial and water flow through the soil, both of which may be a contributing factor to the very low survival of sclerotia of *S. cepivorum* observed in this study. Furthermore, flooding of soils containing sclerotia of *S. cepivorum* has been considered a cultural control method for white rot, and is capable of reducing survival to 1% at 12 weeks after field flooding (Leggett & Rahe, 1990; Crowe & Debons, 1992). Such flooding is likely to have occurred in our sample pots due to snow melt and reduced ease of water flow. Another environmental factor that may contribute to reductions in sclerotial survival is the presence of crop residues of

Brassica spp. residues. The soil used within this study was collected from the Muck Crop Research Station where a variety of *Cruciferous* spp. are grown to facilitate research on the club root pathogen, *Plasmodiophora brassicae* Woronin. This soil characteristic has been shown to reduce survival of sclerotia of *S. cepivorum* to 1.1% following 60 days of incubation in soil containing *Brassica napus* residues (Smolinska, 2000). The process behind this antagonism is based on the presence of glucosinolates within *Brassica* spp., which are broken down to produce volatile isothiocyanates following enzymatic degradation by soil microbes (Smolinska & Horbowica, 1999; Coventry *et al.*, 2005). The presence of these toxins weakens the sclerotial rind, while encouraging bacterial and fungal activity, predisposing the pathogen to further degradation (Smolinska, 2000).

These various soil factors may have contributed to a reduction in survival of *S. cepivorum* over time; however, they do not explain the extreme reduction in recovery and survival just one month post-burial. One factor that might attribute to this loss is the effects that freezing can have on sclerotia (Table D.1). Rapid freezing rates, which would have been experienced directly following experimental set-up during December 2011, are known to promote the formation of intracellular ice crystals that damage cell membranes of living tissue (Morris *et al.*, 1988; Tan & van Ingen, 2004; Lehto *et al.*, 2008). Aside from these conditions, the susceptibility of fungi to detrimental environmental effects can vary within a genus and even among populations of the same pathogen due to genetic variation. The presence of dsRNA has been reported in sclerotia of *S. cepivorum* from the Bradford Marsh, suggesting the presence of mycoviruses or virus-like agents, and genetic heterogeneity within populations of this pathogen (Earnshaw, 1994).

It is evident that both sclerotial survival and antagonistic activity of BCAs are mediated by factors beyond the controllable parameters of a field study, and are likely to differ among environments and pathogenic populations. Each of these factors increase the difficulty of bringing a BCA through production to commercial use. Regardless, this study was able to demonstrate the success of *T. atroviride* at reducing the survival of three pathogenic fungi to a similar or lower level than that of a commercially available biocontrol product.

CHAPTER 3

EVALUATION OF LONG-TERM SURVIVAL AND NATURALLY-OCCURRING FUNGAL COLONIZERS OF FIELD-PRODUCED SCLEROTIA OF *SCLEROTINIA SCLEROTIORUM*

3.1 Abstract

Sclerotia of *Sclerotinia sclerotiorum*, collected from the Bradford Marsh, Ontario, were observed to be heavily colonized (e.g. up to 84% of sclerotia) with various other fungi. It was uncertain whether these fungi were opportunistic saprotrophs or mycoparasites of *S. sclerotiorum*. Thirty-six fungal cultures were isolated from these colonized sclerotia over a five month period, and single-spore cultures were prepared. Fungal DNA was extracted from each isolate, and PCR products from the internal transcribed spacer (ITS) region were sequenced and subjected to a BLAST search via GenBank. Results demonstrated the majority of these fungi belonged to the genus *Fusarium*, with 36% being *Fusarium oxysporum* Schlecht. emend. Snyd. & Hans. Isolates were grouped on sequence alignments and species identifications, and 19 representative isolates were selected for antagonism testing. These isolates were subjected to an interaction bioassay with *S. sclerotiorum*. Each dual culture was rated on five qualitative interactions that developed between the two colonies after 7 days. Of the 20 isolates tested, 16 demonstrated antagonistic or parasitic interactions with *S. sclerotiorum*. Ten isolates were tested for pathogenicity on three potentially susceptible crop species, corn, soybean, wheat. Two of these 10 isolates, both identified as *Gibberella* sp., were pathogenic to all three hosts, resulting in 0% emergence, which was lower than the control treatments.

3.2 Introduction

Sclerotinia sclerotiorum is a globally distributed, highly-nonspecific fungal plant pathogen (Purdy, 1979; Boland & Hall, 1994). The necrotrophic nature of *S. sclerotiorum* allows it to infect a range of host tissues, making it capable of causing up to 100% crop loss in a number of economically important crops, including carrots and dry beans (Purdy, 1979). Its global distribution is most common, but not limited to, temperate regions, and is attributed to the ability to withstanding unfavourable environmental conditions (Bolton *et al.*, 2006). *Sclerotinia sclerotiorum* achieves this through the production of sclerotia, which play an important role in the disease cycle. These overwintering bodies provide initial inoculum by germinating either carpogenically, producing airborne sexual ascospores, or myceliogenically, producing hyphae that target host roots (Coley-Smith & Cooke, 1971). Sclerotia are the pathogen's primary long-term survival structure that can remain dormant and viable within the soil profile for up to eight years, making disease management or eradication difficult (Coley-Smith & Cooke, 1971; Bolton *et al.*, 2006). Microbial degradation of sclerotia is instrumental in reducing the number of these propagules within the soil (Smith, 1972; Duncan *et al.*, 2006).

The longevity of sclerotia within soil is influenced by a number of biotic and abiotic variables. One structural aspect of sclerotia that is essential to survival is the outer rind. This layer of melanised cells protects against exogenous factors, provides a barrier to germination in unfavourable conditions, and contributes to dormancy (Chet, 1969). Successful formation of the rind is influenced by the surrounding environment of a sclerotium. Sclerotia are generally produced when a nutrient-limited environment is encountered (Christias & Lockwood, 1973). In natural pathogenic conditions, this occurs

in the presence of other microorganisms, such as mycoparasites and opportunistic saprotrophs, as well as abiotic environmental stressors. These conditions are not experienced in laboratory settings, resulting in differences in rind formation between the two settings. Currently, it is common practice is to use LP sclerotia for experimental purposes due to ease of mass production of sclerotia of uniform shape and size, and freedom from microbial contaminants (Alexander & Stewart, 1994). Previous studies have evaluated the impact that variable rind formation in these two environments has on sclerotia survival and colonization by other microorganisms. Scanning electron micrographs of sclerotial rinds of *S. sclerotiorum,* recovered after 245 days in or on soil, have illustrated distinct differences between sclerotia collected from naturally-infested beans and sclerotia produced in culture (Merriman, 1976). Sclerotia collected from the field had collapsed or perforated rind cells, while cultured sclerotia still had intact outer rind cells. Comparative studies that have looked at the influence of sclerotia origin on survival have done so only over relatively short time periods, and have resulted in conflicting findings as to whether LP or FP sclerotia are more resilient (Cook *et al.,* 1975; Hoes & Huang, 1975; Merriman 1976; Gladders & Coley-Smith *et al.,* 1980; Coley-Smith *et al.,* 1990; Mitchell & Wheeler, 1990; Alexander & Stewart; 1994).

The integrity of the sclerotial rind can mediate how exogenous influences affect the sclerotial survival (Smith, 1972). When the rind is incomplete or physically damaged, nutrients leak from the sclerotia, and these nutrients can promote microbial activity and sclerotial degradation (Adams, 1975; Lockwood, 1977; Duncan *et al.,* 2006). Damaged rinds can also stimulate germination, ultimately reducing the longevity of the sclerotia (Smith, 1972). In recent decades, researchers have utilized this knowledge and developed

a technique known as sclerotia baiting to determine the presence of natural parasites or predators of sclerotia-forming pathogenic fungi (Anas & Reeleder, 1987; Whipps *et al.*, 1993). This method is based on the hypothesis that existing functional antagonists *in situ* would colonize sclerotia present in the system (Mew *et al.*, 2001). A number of these studies have identified the colonization capabilities of fungi in the genera *Trichoderma, Penicillium, Fusarium,* and *Gliocladium* on sclerotia of *S. sclerotiorum* (Zazzerini & Tosi, 1985; Anas & Reeleder, 1987; Alexander & Stewart, 1994). What these studies fail to acknowledge is the colonization of sclerotia by various naturally-occurring microorganisms during formation, when nutrient exudation occurs (Willetts & Bullock, 1992). Further information is required on the ability of phylloplane microorganisms to colonize premature sclerotia, potentially acting as fungal entophytes, latent pathogens, or beneficial symbionts (Xing *et al.*, 2012).

The objectives of this study were to (1) evaluate the ability of LP sclerotia of *S. sclerotiorum* to effectively represent FP sclerotia of *S. sclerotiorum*; (2) identify natural colonizers of FP sclerotia of *S. sclerotiorum*; (3) qualitatively evaluate the naturally-occurring fungal colonizers of sclerotia of *S. sclerotiorum* for antagonistic and/or parasitic interactions with *S. sclerotiorum*; and (4) qualitatively evaluate the naturally-occurring fungal colonizers of sclerotia of *S. sclerotiorum* for pathogenicity on host seedlings.

3.3 Materials and Methods

3.3.1 Production and collection of sclerotia

Sclerotia of *S. sclerotiorum* were collected in September 2011 from naturally-infected dry bean plants surrounding the University of Guelph Muck Crops Research

Station in the Holland Marsh of Bradford, Ontario. Plants showing extensive symptoms of sclerotinia rot and sclerotia production were harvested and sclerotia were collected. Some plants were placed in moist chambers for 1-2 days to allow for completion of any ongoing sclerotial formation. Lids of chambers were then removed to promote drying of plant tissue and mycelia. After 3-4 days, FP sclerotia were harvested and stored at 4°C.

A subsample of 10-20 FP sclerotia were individually plated onto 10 cm diameter Petri plates containing Difco™ PDA (Becton, Dickinson and Company, Sparks, MD). After 4-5 days incubation at room temperature (20-22°C), 2 mm mycelial plugs were cut from developing colonies of *S. sclerotiorum* and transferred to hundreds of PDA plates for production of sclerotia. Inoculated plates were wrapped with Parafilm™ (Bemis Flexible Packaging, Neenah, WI) to prevent desiccation of media, and incubated in a growth room (19-22°C) with a 16:8 light:dark period for two weeks. After this time, Parafilm was removed and plate lids were slightly offset for one day to promote desiccation of PDA medium and mycelia, and to increase the ease of sclerotial recovery. LP sclerotia were recovered by tapping plates to dislodge sclerotia from surrounding mycelia and media. LP and FP sclerotia were stored at 4°C prior to use.

Initial survival of LP sclerotia was evaluated by plating five replications of 12 sclerotia on APDA, incubated at room temperature (20-22 ºC), and monitored for germination over a three week period. Survival was determined based on the percent of sclerotia that germinated and produced daughter sclerotia (Table A.1).

3.3.2 Comparative long-term survival of sclerotia of *S. sclerotiorum* in a natural setting

LP and FP sclerotia of *S. sclerotiorum* were each separated into five replicate groups of 25 sclerotia. Each group of sclerotia was placed in a 5 cm x 6 cm mesh nylon (158 meshcount, SaatiPrint Canada, Mississauga, ON) bag and incorporated with 10 mL of soil collected from the Muck Crops Research Station. The mesh bags were then sealed with a heat sealer (Model: AIE-400P, American International Electric Inc, City of Industry, CA). One bag each of LP and FP sclerotia/soil were buried 5-10 cm deep in a 1 L pot of muck soil, with five replicated pots for each of eight recovery dates. Pots were placed outside in a fenced enclosure at the Muck Crops Research Station in December 2011 and arranged in a completely randomized design in each of eight destructive sampling dates. These dates took place every 2-3 months for 20 months.

At each recovery date, one block of pots were removed from the Muck Crops Research Station and bags were recovered from each pot. Sclerotia were separated from the soil by dry sieving with soil screens, sizes 10 (2 mm) and 14 (1.4 mm) (Fisher Scientific International Inc., Hampton, NH). Sclerotia were placed back into mesh bags and subjected to surface-disinfestation by submerging bags in 70% ethanol for 1 min, followed by 4 mins in 5% sodium hypochlorite, and rinsed three times with sterile deionised water. Sclerotia were then removed from bags, blotted dry with sterile paper towel, and plated onto 10 cm quarter-sectioned plates of APDA. Plates were incubated at room temperature and monitored for up to four weeks. Initial counts of the number of sclerotia recovered were recorded, as well as number of sclerotia germinated and number producing secondary sclerotia every 4-7 days for the 4 week period. After 4 weeks,

percent survival was calculated for each replication with the following formula (Legget *et al.*, 1983):

$$\frac{(\#\ \text{of sclerotia recovered} \times \%\ \text{of recovered sclerotia germinated and producing sclerotia})}{\#\ \text{of sclerotia originally in bag}} \times 100$$

3.3.3 Identification of natural colonizers of FP sclerotia of *S. sclerotiorum*

At each recovery date, plates of sclerotia of *S. sclerotiorum* being monitored for germination were noted to be growing fungi visibly different from *S. sclerotiorum* (e.g. sclerotial colonizers). These colonies were isolated and single-spore cultured on APDA at each recovery date, and stored at 4ºC. In June 2012, representative colonies of colonizers from the first three recovery dates were reisolated and fungal deoxyribonucleic acid (DNA) was extracted from their mycelia using the MoBio Power Soil® DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA). Samples were stored at -20°C.

Polymerase chain reaction (PCR) amplification was completed using the internal transcribed spacer (ITS) region primers F1 and 4A (University of Guelph Genomics Facility). Amplified products were sequenced with the BigDye version 3.1 Ready Reaction Kit (Applied BioSystems, Streetsville, ON) on an ABI 3730 DNA Analyzer (Applied BioSystems) at the University of Guelph Genomics Facility. Forward and reverse sequences were assembled and aligned using Geneious Pro software (v.5.5.7 ; http://www.geneious.com) and subject to a baseline local alignment search via GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Isolates were grouped based on sequence alignment and BLAST Analysis species results (selected for high query cover [QC %] and maximum identification [Max ID %]).

Table 3.1. Nineteen isolates of naturally-occurring fungal colonizers of field-produced sclerotia of *S. sclerotiorum*, collected from a dry bean field in the Bradford Marsh in October 2011, identified by the internal transcribed spacer (ITS) region, and used in a bioassay to evaluate antagonistic activity against *S. sclerotiorum*.

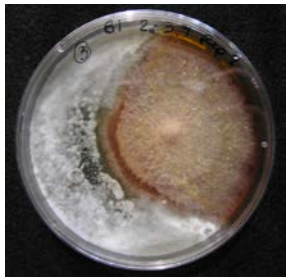
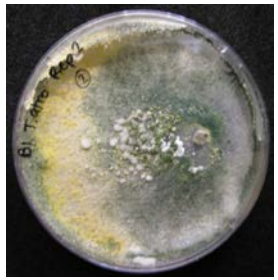
Isolate[a]	PCR Fragment Length	GenBank Identification	GenBank Accession Code	QC (%)[b]	Max ID (%)[c]
1-1.13*	575	*Gibberella* sp.	FJ466715.1[d]	100	97
1-1.23*	592	*Fusarium equiseti*	EU595566.1	99	99
1-2.20*	586	*Fusarium oxysporum*	DQ535184.1	98	100
2-1.1*	604	*Fusarium oxysporum*	FJ466709.1	99	99
2-1.7	595	*Fusarium equiseti*	EU595566.1	99	99
2-1.14	497	*Cylindrocarpon* sp.	JF735315.1[e]	100	99
2-1.19*	564	*Fusarium equiseti*	EU595566.1	100	99
2-2.22*	568	*Fusarium acuminatum*	HM068320.1	99	99
2-2.25	536	*Penicillium citrinum*	FJ765031.1	100	99
2-3.7	569	*Fusarium oxysporum*	DQ535184.1	99	99
2-3.9	537	*Epicoccum nigrum*	JN689342.1	100	99
2-4.15*	562	*Gibberella* sp.	FJ466715.1[d]	99	100
3-1.2*	502	*Fusarium oxysporum*	JF311936.1	100	99
3-1.3*	513	*Fusarium equiseti*	EU595566.1	99	99
3-2.15	560	*Trichoderma hamatum*	JQ040347.1	100	99
3-2.17	511	*Fusarium oxysporum*	JQ340086.1	99	99
3-3.2*	538	*Fusarium solani*	HQ439150.1	99	100
3-4.14	504	*Fusarium oxysporum*	DQ535184.1	98	99
3-5.10	514	*Penicillium citrinum*	FJ765031.1	99	99

[a] First number in isolate code indicates which sample date it was isolated from (1 = January 2012; 2 = March 2012; 3 = May 2013)
[b] QC (%) = Estimated Query Cover; the extent to which the query sequence is covered by the target sequence
[c] Max ID (%) = Maximum Identification; the maximum percentage of identical nucleotides within the overlap between query and target sequences
[d] Synonymous to the organism *Fusarium* sp. UFMGCB_536
[e] Anamorph form *Cylindrocarpon* sp. AC-2011a, associated with *Ilyonectria radicicola* species complex (Cabral *et al.*, 2012)
* Isolates selected for use in growth room trial to evaluate pathogenicity activity against susceptible host crop seedlings

3.3.4 Evaluation of sclerotial colonizers for antagonistic and parasitic activity

Nineteen representative isolates of the identified sclerotial colonizers were selected for an interaction bioassay with *S. sclerotiorum* to assess their antagonistic and mycoparasitic activity towards actively-growing mycelia of *S. sclerotiorum* (Table 3.1). One isolate of *Trichoderma atroviride* strain B, provided by ProPhyta GmbH (Poel, Germany) was included as a positive mycoparasitic control, as well as a blank PDA plug as a negative control. Dual cultures were prepared on 10 cm diameter plates of PDA, each with a 5 mm plug of *S. sclerotiorum* and a 5 mm plug of an individual isolate, spaced 4-5 cm apart. Each dual culture was replicated three times and incubated at room temperature for 7 days. After this time, cultures were rated based on five qualitative interactions that developed in the area between the two colonies; modified from Boyd-Wilson *et al.* (2000) (Table 3.2). Isolates that were completely overgrown by mycelial growth of *S. sclerotiorum* received a Rating A. Intergrowth between mycelia of *S. sclerotiorum* and the isolate received a Rating B. Antagonistic interactions were the growth of each culture towards one another with no interaction, and the presence of an inhibition zone between cultures, which received Rating C and D, respectively. A mycoparasitic interaction was given a Rating E and was observed when the isolate mycelium overgrew that of *S. sclerotiorum.*

Table 3.2. Five point rating scale to qualitatively evaluate antagonistic and/or parasitic abilities of naturally-occurring fungal colonizers of field-produced sclerotia of *S. sclerotiorum*, collected from a dry bean field in the Bradford Marsh in October 2011.

Rating[a]	A	B	C	D	E
Activity of Isolate	Isolate completely over-grown by pathogen	Mycelial intergrowth between cultures	Mycelia of cultures grow up to each other and stop	Inhibition zone present between cultures	Isolate over-grows pathogen with collapse of pathogen mycelia
Visual Representation					

[a] Rating value C, D classified as an antagonistic interaction by isolate; rating value E classified as a parasitic interaction by isolate

3.3.5 Evaluation of sclerotial colonizers for pathogenic activity

Ten isolates that demonstrated antagonistic/mycoparasitic activity towards actively growing mycelia of *S. sclerotiorum* were selected to assess their pathogenicity to the potential host crops: corn (*Zea maize* L.), soybean (*Glycine max* L.), and wheat (*Triticum aestivum* L.) (Table 3.1). This selection was based on association with crop diseases and/or the re-occurrence of these isolates at least twice over the three recovery dates. Equal volumes of water and untreated rye seed (700 mL) were autoclaved for one hour at 121ºC in bags with a gas exchange patch (#14 Patch PP, Western Biologicals Ltd., Aldergrove BC). Once cooled, each bag was inoculated with agar plugs cut from one-half of a 9 cm diameter pure culture of an individual isolate. One bag was left uncolonized to represent a control. Bags were sealed and agitated by hand to ensure mixing of inoculum and rye, then left to colonize the rye at room temperature for 5-7 days. Colonized rye was then dried on screens for 72 hours in a growth room (19-22°C) with a 16:8 light:dark period. Once dried, inoculated rye was ground in a grain grinder (FGM Enterprises Inc., Daytona Beach FL) and stored at 4-8°C.

To infest soil, 8 g of rye inoculum of each isolate were incorporated into 10 L of soilless mix (LC1 Mix, Sun Gro Horticulture, AB). Each 10 L of inoculated mix were used to fill twelve 12 x 12 cm square pots (JVK, Saint Catherines, ON) to provide four repetitions for each host. Pots were seeded with either five corn seeds, five soybean seeds, or 10 wheat seeds, and maintained in a growth room (19-22°C; 16:8 light:dark period). This trial was established as a completely randomized design, and repeated once. For the first 5-7 days, pots were incubated in plastic bags to promote disease development. The percent of emerged seedlings in each pot was recorded 7, 14 and 28

days after seeding. Plants, including roots, were harvested after 28 days and rated for disease incidence (percent of plants showing disease symptoms), and disease severity by rating severity of disease for each shoot/root (with the exception of wheat) on a 0-10 rating scale, where 0 represents no disease, 5 represents disease symptoms on 50% of the shoot and roots, and 10 represents full discolouration and death of the plant. Fresh shoot weight per pot was also recorded.

3.3.6 Statistical analysis

Percent survival of sclerotia were arcsine-transformed to improve normality of distribution and homogeneity of variances (Little & Hills, 1978), and expressed as Area Under the Percent Survival Stairs (AUPSS) to compare survival between LP and FP sclerotia. AUPSS is based on a revised calculation of Area Under the Disease Progress Curve (AUDPC), known as the Area Under the Disease Progress Stairs (AUDPS) (Simko & Piepho, 2012) that aims at improving the estimation of disease progress by incorporating the first and last observation points. AUPSS data were calculated from the arcsine-transformed percent survival data at eight recovery dates over the 20 month observation period, with the following equation:

$$AUDPS = \left[\sum_{i=1}^{n-1} \frac{y_i + y_{i+1}}{2} \times (t_{i+1} - t_i) \right] + \left[\frac{y_1 + y_n}{2} \times \frac{D}{n-1} \right]$$

where y_i is the percent survival at the i^{th} observation, t_i is the time at the i^{th} observation, n is the total number of observations, and D is t_n - t_1. Analysis of variance was performed on the AUPSS of each source of sclerotia (lab, field) and means were separated using Tukey's adjustment.

To evaluate variation among characteristics of survival over the observation period, number recovered, percent germinated, and percent survival data were analyzed. To improve normality, square root transformations were used on numbered data and arcsine transformations were used on percent data. Analysis of variance was performed for each rating and means were separated using Tukey's adjustment. Statistical analyses were performed using the PROC GLM procedures of SAS (v.9.3) with a type III error of 0.05.

To evaluate pathogenicity of sclerotia-colonizing isolates on potentially susceptible host seedlings, arcsine-transformed 28 day emergence and disease incidence, and log-transformed disease severity data were analyzed. Analysis of variance of isolate means was performed separately for each assessment criteria and means were separated using Tukey's adjustment. Statistical analyses were performed using the PROC MIXED procedures of SAS (v.9.3) with a type III error of 0.05.

3.4 Results

3.4.1 Comparative long-term survival of sclerotia of *S. sclerotiorum* in a natural setting

All sclerotia were tested for viability *in vitro* prior to establishment of field experiments. LP sclerotia demonstrated 100% germination, but FP sclerotia only demonstrated 18% germination, with 82% contamination (Table A.2). Following 20 months of exposure in field settings, the AUPSS based on the arcsine-transformed percent survival of LP sclerotia (20.9) was greater than that of FP sclerotia (4.7) (Figure 3.1). This greater percent survival of LP was observed even after one month post-burial when survival of FP was 95% lower than that of LP. Despite this, the percent survival of

LP was only 16% greater than that of FP sclerotia at the final observation point and had an overall 78% decrease, compared to survival one month post-burial (Table 3.3). The percent survival curves of both LP and FP sclerotia differed from one another over the observation period, both in variation among recovery dates and in overall reduction over time. The curve of LP sclerotia followed a general reduction in survival, with a sudden spike at the 14 month recovery time point. The number of LP sclerotia recovered did not differ among recovery dates, but percent germination of recovered sclerotia did (Table 3.3). There was a 64% decrease in percent germination of recovered LP sclerotia at 20 months, compared to that at one month post-burial. The percent survival curve of FP sclerotia demonstrated a slight spike at the 3 month recovery point, followed by a general decline for the remainder of the observation period (Figure 3.1). The only significant reduction in percent survival was seen between 3 months and 17 months post-burial. Unlike the LP sclerotia, the number of FP sclerotia recovered differed among recovery dates, but percent germination of recovered sclerotia did not (Table 3.3). Reductions in number of FP sclerotia recovered were observed 5 months post-burial, and again at 8 months post-burial. After 20 months, the number of FP sclerotia recovered was reduced from 24 to 1. At each recovery date, FP sclerotia were heavily colonized with other microorganisms which were isolated at one, three, and five months post-burial, and used for further research within this study. No colonization was observed on LP sclerotia.

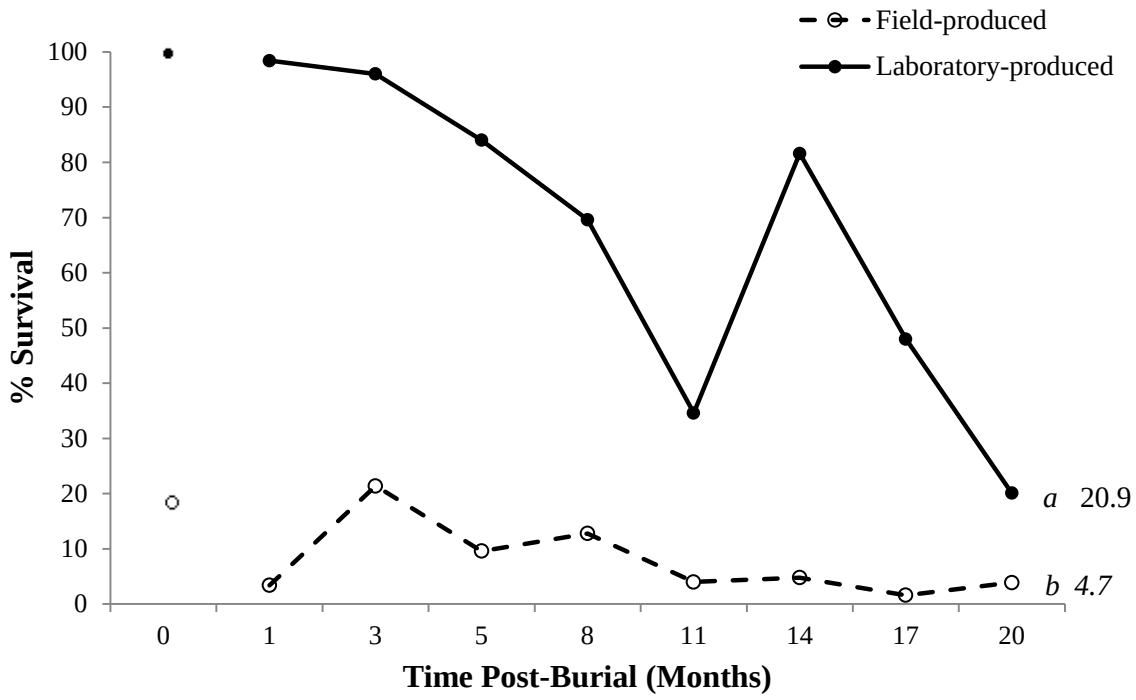


Figure 3.1. Percent survival of laboratory-produced and field-produced sclerotia of *Sclerotinia sclerotiorum*, recovered at two to three monthly intervals post-burial (December 2011). Values on far right represent AUPSS values of untransformed data. Independent data points at time zero represent *in vitro* % germination following sclerotia production (Table B.1). Different letters denote significant differences at P=0.05 according to Tukey's HSD among arcsine-transformed Area Under the Percent Survival Stairs (AUPSS) data. Original % survival data displayed. (SE = 0.6067).

Table 3.3. Number recovered, percent germinated and percent survival of laboratory-produced and field-produced sclerotia of *Sclerotinia sclerotiorum*, recovered at two to three month intervals post-burial from December 2011 to August 2013.

Months Post Burial	Laboratory-Produced (25)[a]			Field-Produced (25)		
	# Recovered[b]	% Germinated[c]	% Survival[b]	# Recovered	% Germinated	% Survival
1	25 ns	98 *a*	98 *a*	24 *a*	3 ns	3 *ab*
3	24	99 *a*	96 *a*	25 *a*	22	21 *a*
5	23	88 *ab*	84 *ab*	13 *b*	24	9 *ab*
8	18	99 *a*	70 *ab*	5 *c*	59	13 *ab*
11	10	39 *b*	35 *b*	2 *c*	48	4 *ab*
14	22	92 *ab*	82 *ab*	3 *c*	40	5 *ab*
17	12	100 *a*	48 *ab*	2 *c*	17	2 *b*
20	11	36 *b*	20 *b*	1 *c*	67	4 *ab*
Standard Error	0.4714	0.1677	0.1703	0.2396	0.2474	0.06252

[a] Number of sclerotia initially in mesh bag at start of experiment
[b] Original data displayed, analysis of variance performed on arcsine transformed data
[c] Original data displayed, analysis of variance performed on log transformed data
a, *b*, *c* Means followed by the same letter are not significantly different at P=0.05 according to Tukey`s HSD
ns = no significant differences in column
% Survival = [(# of sclerotia recovered x % of recovered sclerotia germinated and producing sclerotia) / # of sclerotia originally in bag] x 100

3.4.2 Identification of natural colonizers of FP sclerotia of *S. sclerotiorum*

Following single-spore isolation and DNA extraction, sclerotia-colonizing fungi were subjected to a GenBank BLAST search, resulting in the identification of plant pathogenic, soil dwelling, and antagonistic fungal species. Identifications suggested by GenBank were based on an average of query coverage (QC) and maximum identification (Max ID) scores higher than 98 (Table 3.1). The QC is an estimated percentage of the extent to which the query sequence is covered by, or overlaps with, the target sequence, and the Max ID is the maximum percentage of identical nucleotides within the noted alignment length between the query sequence and the target sequence. Results demonstrated that of the 19 representative isolates recovered from sclerotial of *S. sclerotiorum*, 12 belonged to the genus *Fusarium*, with six being *Fusarium oxysporum*

(isolates 1-2.20, 2-1.1, 2-3.7, 3-1.2, 3-2.17, 3-4.14) . Three of the six isolates of *F. oxysporum* were collected at the third recovery date, 5 months post-burial. Other species included, *F. equiseti* (1-1.23, 2-1.7, 2-1.19, 3-1.3), *F. acuminatum* (2-2.22), and *F. solani* (3-3.2). The query sequences of three isolates only allowed the identification of a genus rather than a species, which included *Gibberella* sp. (1-1.13, 2-4.15) and *Cylindrocarpon* sp. (2-1.14). The accession code for the isolates identified as *Gibberella* sp. UFMGCB_536 was originally associated with the *Fusarium* sp. UFMGCB_536, originally described as an "endophytic fungi associated with ethno-pharmacological plants from Brazilian biomes" (NCBI, 2008). The accession code for the isolate identified as *Cylindrocarpon* sp. AC-2011a was associated with a fungus submitted to GenBank as an anamorph associated with *Ilyonectria radicicola* species complex (Cabral *et al.*, 2012). The majority of these listed species and genera are associated with root rot and/or stem diseases of high value crops, including wheat, corn, and soybean. Finally, four isolates were identified as non-pathogenic, *Epicoccum nigrum* (2-3.9), *Penicillium citrinum* (2-2.25, 3-5.10) and *Trichoderma hamatum* (3-2.15). Although not a plant pathogen, *P. citrinum* is an economically important species due to its production of the mycotoxin citrinin in plants harbouring the fungus, including grains.

Isolates of *F. oxysporum* (1-2.20, 2-3.7, 3-4.14; DQ535184.1) and *F. equisiti* (1-1.23, 2-1.7, 3-1.3; EU595566.1) were the only two species re-isolated at each of the three sampling dates with the same accession code among all three dates. An accession code is assigned to a particular sequence when it is submitted to GenBank and is a unique identifier for a complete sequence record. This suggests that the same formae speciales or individual of these species were re-isolated at all three sampling dates. Isolates recovered

with the same accession code at two sampling dates included *Gibberella* sp. (1-1.13, 2-4.15; FJ466715.1) and *P. citrinum* (2-2.25, 3-5.10; FJ65031.1).

3.4.3 Evaluation of sclerotial colonizers for antagonistic and parasitic activity

Sixteen of the isolates demonstrated antagonistic (rated C, D) or parasitic (rated E) activity towards mycelia of *S. sclerotiorum* (Table 3.4). The single isolate of *T. hamatum* was the only isolate that demonstrated parasitic activity towards *S. sclerotiorum* mycelia, aside from the positive control isolate of *T. atroviride*. Both isolates of the other non-pathogenic species, *P. citrinum*, demonstrated antagonistic activity towards *S. sclerotiorum*, as did the single isolates of *Cylindrocarpon* sp., *F. acuminatum*, and *F. solani*. Of the reoccurring accession code isolates, those of *F. equisiti* maintained a consistent antagonistic interaction with *S. sclerotiorum*; however, only the *F. oxysporum* isolate from the first recovery date demonstrated an antagonistic interaction (Table 3.4). Isolates that demonstrated benign activity (rating A, B) towards *S. sclerotiorum* were *E. nigrum* (2-3.9), one isolate of *Gibberella* sp. (2-4.15), and two isolates of *F. oxysporum* (2-3.7, 3-4.14).

Table 3.4. Observed interactions among dual cultures of *Sclerotinia sclerotiorum* and isolates of naturally-occurring fungal colonizers of field-produced sclerotia of *Sclerotinia sclerotiorum* throughout 2012.

Isolate	Fungal Name [GenBank Designation]	Rating[a]				
		A	B	C	D	E
Blank PDA Plug		xxx[b]				
Trichoderma atroviride						xxx
1-1.13	*Gibberella* sp.			x	xx	
1-1.23	*Fusarium equiseti*				xxx	
1-2.20	*Fusarium oxysporum*		x	xx		
2-1.1	*Fusarium oxysporum*			xxx		
2-1.7	*Fusarium equiseti*				xxx	
2-1.14	*Cylindrocarpon* sp.				xxx	
2-1.19	*Fusarium equiseti*				xxx	
2-2.22	*Fusarium acuminatum*				xxx	
2-2.25	*Penicillium citrinum*			x	xx	
2-3.7	*Fusarium oxysporum*		xxx			
2-3.9	*Epicoccum nigrum*		xxx			
2-4.15	*Gibberella* sp.		xx	x		
3-1.2	*Fusarium oxysporum*				xxx	
3-1.3	*Fusarium equiseti*				xxx	
3-2.15	*Trichoderma hamatum*					xxx
3-2.17	*Fusarium oxysporum*			xxx		
3-3.2	*Fusarium solani*			xxx		
3-4.14	*Fusarium oxysporum*		xxx			
3-5.10	*Penicillium citrinum*			xxx		

[a] Rating value C, D classified as an antagonistic interaction by isolate; rating value E classified as a parasitic interaction by isolate

[b] X represents one replication of each isolate

3.4.4 Evaluation of sclerotial colonizers for pathogenic activity

Pathogenicity of sclerotial colonizers on selected crop plants varied among isolates and hosts, although disease symptoms were observed for every isolate, and also control treatments. As a result, focus was placed on 28 day emergence counts as an indicator of pathogenic ability. Analysis of variance did not identify an effect of trial for either 28 day emergence, disease incidence or disease severity, therefore, data from each of the two trials were pooled. Effects of host, isolate, and an isolate*host interaction were identified for 28 day emergence, and an effect of host on disease incidence. No effects were identified for disease severity. *Gibberella* sp. isolates 1-1.13 and 2-4.15 reduced emergence of corn and wheat compared to the control treatments, with 0% emergence for both (Table 3.5). In soybean, *F. oxysporum* isolates 1-1.13, 2-4.15, and 2-1.1 all resulted in 0% emergence, different from that of the control treatment. Other isolates to reduce emergence compared to the soybean control were 1-1.23 (*F. equiseti*), 1-2.20 (*F. oxysporum*), 2-1.19 (*F. equiseti*), 3-1.2 (*F. oxysporum*), and 3-3.2 (*F. solani*) (Table 3.5). All isolates produced disease symptoms, including the control treatments, and no significant differences were observed for disease incidence or disease severity in any host. Disease incidence ranged from 38-100% among all hosts. Disease severity remained low with the highest rating of 6.1 for isolate 2-1.1 on wheat.

At 28 days, emergence was lowest in soybean, with the exception of the untreated control. Isolates 1-1.13 (*Gibberella* sp.) and 2-4.25 (*Gibberella* sp), resulted in zero emergence for all hosts (Table 3.6). One isolate, *F. equiseti* (2-1.19), reduced emergence of wheat as compared to corn. The control treatment had high emergence with no differences among hosts.

Table 3.5. Mean emergence 28 days after seeding, disease incidence and disease severity of corn, soybean, and wheat exposed to 10 isolates of naturally-occurring fungal colonizers of field-produced sclerotia of *Sclerotinia sclerotiorum*.

Corn (5)[a]	Identification	28 Day Emergence[b]		Disease Incidence[b]		Disease Severity[c]	
Control		90.0	*ab*	79.4	ns	1.8	ns
1-1.13	*Gibberella* sp.	0.0	*c*	--		--	
1-1.23	*Fusarium equiseti*	70.0	*b*	92.8		5.7	
1-2.20	*Fusarium oxysporum*	100.0	*a*	100.0		2.9	
2-1.1	*Fusarium oxysporum*	87.5	*ab*	100.0		5.4	
2-1.19	*Fusarium equiseti*	90.0	*ab*	100.0		3.7	
2-2.22	*Fusarium acuminatum*	100.0	*a*	95.0		3.8	
2-4.15	*Gibberella* sp.	0.0	*c*	--		--	
3-1.2	*Fusarium oxysporum*	97.5	*ab*	100.0		4.2	
3-1.3	*Fusarium equiseti*	97.5	*ab*	87.5		2.2	
3-3.2	*Fusarium solani*	100.0	*a*	75.0		4.2	
Standard Error		0.1563		0.3126		0.1657	
Soybean (5)	Identification	28 Day Emergence		Disease Incidence		Disease Severity	
Control		85.0	*a*	100	ns	3.3	ns
1-1.13	*Gibberella* sp.	0.0	*c*	--		--	
1-1.23	*Fusarium equiseti*	25.0	*bc*	62.5		3.2	
1-2.20	*Fusarium oxysporum*	22.5	*bc*	87.5		4.1	
2-1.1	*Fusarium oxysporum*	0.0	*c*	--		--	
2-1.19	*Fusarium equiseti*	15.0	*c*	50.0		2.4	
2-2.22	*Fusarium acuminatum*	60.0	*ab*	96.9		3.9	
2-4.15	*Gibberella* sp.	0.0	*c*	--		--	
3-1.2	*Fusarium oxysporum*	10.0	*c*	50.0		3.3	
3-1.3	*Fusarium equiseti*	55.0	*ab*	87.5		3.4	
3-3.2	*Fusarium solani*	32.5	*bc*	75.0		3.6	
Standard Error		0.1563		0.3126		0.1657	
Wheat (10)	Identification	28 Day Emergence		Disease Incidence		Disease Severity	
Control		81.3	*a*	38.0	ns	2.3	ns
1-1.13	*Gibberella* sp.	0.00	*b*	--		--	
1-1.23	*Fusarium equiseti*	68.8	*a*	20.3		2.6	
1-2.20	*Fusarium oxysporum*	82.5	*a*	11.6		1.3	
2-1.1	*Fusarium oxysporum*	80.0	*a*	66.9		6.1	
2-1.19	*Fusarium equiseti*	71.3	*a*	55.6		4.3	
2-2.22	*Fusarium acuminatum*	96.3	*a*	48.0		4.6	
2-4.15	*Gibberella* sp.	0.0	*b*	--		--	
3-1.2	*Fusarium oxysporum*	90.0	*a*	57.4		5.9	
3-1.3	*Fusarium equiseti*	82.5	*a*	46.5		3.4	
3-3.2	*Fusarium solani*	96.3	*a*	25.1		3.1	
Standard Error		0.1563		0.3126		0.1657	

[a] Number of seeds planted

[b] Original data presented, analysis of variance performed on arcsine-transformed data

[c.] Original data presented, analysis of variance performed on square-root transformed data

a, b, c Means in a column for each crop following the same letter are not significantly different at *P*=0.05 according to Tukey's HSD

ns = no significant differences in column

Table 3.6. Mean emergence 28 day after seeding corn, soybean, and wheat inoculated with 10 isolates of naturally-occurring fungal colonizers of field produced sclerotia of *Sclerotinia sclerotiorum* as a bioassay for pathogenicity. Means separations performed per isolate across three hosts.

Isolate	Identification	Corn[a] (5)[b]		Soybean (5)		Wheat (10)	
Control		90.0	ns	85.0		81.3	
1-1.13	*Gibberella* sp.	0.0	ns	0.0		0.00	
1-1.23	*Fusarium equiseti*	70.0	*a*	25.0	*b*	68.8	*a*
1-2.20	*Fusarium oxysporum*	100.0	*a*	22.5	*b*	82.5	*a*
2-1.1	*Fusarium oxysporum*	87.5	*a*	0.0	*b*	80.0	*a*
2-1.19	*Fusarium equiseti*	90.0	*a*	15.0	*c*	71.3	*b*
2-2.22	*Fusarium acuminatum*	100.0	*a*	60.0	*b*	96.3	*a*
2-4.15	*Gibberella* sp.	0.0	ns	0.0		0.0	
3-1.2	*Fusarium oxysporum*	97.5	*a*	10.0	*b*	90.0	*a*
3-1.3	*Fusarium equiseti*	97.5	*a*	55.0	*b*	82.5	*ab*
3-3.2	*Fusarium solani*	100.0	*a*	32.5	*b*	96.3	*a*
Standard Error				0.1563			

[a] Original data presented, analysis of variance performed on arcsine-transformed data
[b] Number of seeds planted
a, b, c Means in a row followed by the same letter are not significantly different at P=0.05 according to Tukey`s HSD
ns = no significant differences

3.5 Discussion

This is the study to evaluate the long-term survival of sclerotia of *S. sclerotiorum* in a natural setting over 20 months. The comparison of LP and FP sclerotia demonstrated major differences that are possibly the results of effects of exposure to variable environments during rind formation can have on sclerotial survival and colonization by other microorganisms. The greater AUPSS of LP sclerotia of *S. sclerotiorum* over 20 months observed in this study agrees with previous suggestions that sclerotia produced in culture or laboratory settings are more resilient to harsh environmental conditions, compared to FP sclerotia (Merriman, 1976; Smith, 1989, Alexander & Stewart, 1994). After eight months of burial, Merriman (1976) observed lower recovery (50%) and

viability (48%) of sclerotia formed via natural infestation compared to that of sclerotia produced in culture (95% and 62%, respectively). The survival of LP sclerotia in particular were very similar to the current work (70%) at 8 months post-burial. Sclerotia formed in the field also had a higher incidence rate, up to 75%, of *Fusarium* spp., *Mucor* spp. and *Trichoderma* spp. contamination (Merriman, 1976). These findings are comparable to the results of this study in which survival of FP sclerotia (4%) was lower than LP sclerotia (20%), and contamination was only seen in FP sclerotia.

There are several factors known to influence the way abiotic environmental factors affect sclerotial survival. Some of these are genotypic variation within a population (Bolton *et al.*, 2006), nutrient limitations (Wong & Willets, 1974), location within the soil profile (Leggett *et al.*, 1983; Duncan *et al.*, 2006), and untimely germination (Coley-Smith & Cooke, 1971). In this study, the integrity of the sclerotial rind may be an important factor affecting sclerotial survival.

Many sclerotia longevity studies have used LP sclerotia due to ease of mass production, possibly resulting in misleading findings. Sclerotia produced in culture are formed in ideal environmental conditions with abundant nutrient resources that promote complete and uniform rind formation. The absence of competition, hyperparasites and abiotic stressors in a laboratory setting allows for the efficient use of available resources, resulting in a greater amount of stored food reserves (lipids and/or carbohydrates) to be utilized during dormancy (Wong & Willets, 1974). In a natural environment, sclerotia production occurs in the presence of stressors, and under greater nutrient limitations due to the presence of competitive phylloplane microorganisms (Leggett *et al.*, 1983). As a result, formation of FP sclerotia is likely to result in imperfect rind formation that may

leave cracks and exposed underlying tissue. Such perforations lead to a hastening of resource depletion and promotion of degradation by surrounding microflora.

Once in the soil, there are a number of environmental factors that can limit the survival of sclerotia. The quality of sclerotial rind can influence these impacts and either make the sclerotium more resilient or vulnerable. Abiotic environmental factors generally act complementary to one another in complex interactions with sclerotia. Some of these factors include soil type and pH, temperature, moisture, tillage and aeration (Coley-Smith & Cooke, 1971; Kurle *et al.,* 2001; Hao *et al.,* 2003). While Leggett & Rahe (1985) and Alexander & Stewart (1994) observed rapid declines in sclerotia survival during winter months due to flooding effects, an increase in the survival of LP sclerotia was seen in this study during winter months or 14 months post burial. This may have been a result of variation typical in field trials. During the entire observation period, water fluctuations within pots of soil, as well as other abiotic environmental conditions, likely occurred. During collection, it was evident that soil moisture and bag depth were variable among pots, as well as recovery dates. According to previous studies, a decrease in the depth of burial would have likely increased sclerotial decay as a result of increased germination with subsequent decay, and exposure to harsh environmental conditions (Cook *et al.,* 1975; Merriman, 1979; Leggett *et al.,* 1983; Mitchell & Wheeler, 1990; Duncan *et al.,* 2006). Some pots may have experienced this to a greater degree than others, influencing the number of sclerotia collected, as well as their germination rate following collection.

The most important factor affecting sclerotial survival within soil is the activity of soil microorganisms surrounding these resting bodies, including potentially antagonistic bacterial, fungal and nematode populations (Adams, 1990; Duncan *et al.*, 2006). Similar

to previous reports by Merriman (1976) and others, recovery and viability were lower, and incidence of saprophytic fungi much greater in FP sclerotia compared to LP (Cook *et al.,* 1975; Zazzerini & Tosi, 1985; Smith *et al.* 1989, Alexander & Stewart, 1994; Jones & Stewart, 2000; Cosic *et al.,* 2012). Positive correlations between the colonization of sclerotia with antagonists and the decrease in sclerotial survival have also been previously reported, suggesting that naturally-occurring soil microbial activity is instrumental in the degradation of sclerotia over time (Papavizas & Collins, 1990; Jones & Stewart, 2000). Early observations of low numbers of sclerotia in soils with histories of Sclerotinia rot led to the search for natural antagonists of sclerotia that may be associated with sclerotial decay (Anas & Reeleder, 1987; Whipps *et al.,* 1993; Jones & Stewart, 2000, Duncan *et al.,* 2006).

It is possible that the unsterilized soil used in this study contained natural antagonists of sclerotia of *S. sclerotiorum,* and colonized sclerotia following the experiment set-up. FP sclerotia may have been colonized to a greater degree than LP sclerotia due to the susceptibility imposed by an incomplete rind, or a nutrient limitation followed by germination. Of the sclerotial colonizers identified in this study, many have been previously identified on buried sclerotia and associated with decay, especially *Trichoderma* spp., *Fusarium* spp., and *Penicillium* sp. (Merriman, 1976; Anas & Reeleder, 1987; Jones & Stewart, 2000). The identification of *T. hamatum* within this study, which has demonstrated parasitic abilities against *S. sclerotiorum,* agrees with previous reports that identify this species of *Trichoderma* as an antagonist to *Sclerotinia* spp. and other sclerotia-forming phytopathogenic fungi (Chet *et al.,* 1981; Lewis & Papavizas, 1987; Rabeendran *et al.,* 2006; El-Hassan *et al.,* 2013). Rabeendran *et al.*

(2006) observed successful control of *S. minor* on lettuce by *T. hamatum*, and found a reduction in disease pressure up to 45%, comparable to that of *C. minitans* (24-64%), when incorporated into plant potting mix. These results were equivalent to, or greater than, those provided by a standard carbencazim fungicide treatment (Rabeendran *et al.*, 2006). Accordingly, the presence of *T. hamatum* on sclerotia of *S. sclerotiorum* in this study is likely because it is a functional mycoparasite that colonized sclerotia within the soil following experimental set up. The two isolates identified as *P. citrinum* in this study demonstrated antagonistic activity towards *S. sclerotiorum* and are also likely to be functional mycoparasites. Rai & Saxena (1975) isolated *P. citrinum* from sclerotia of *S. cepivorum* and observed strong mycoparastic activity towards *S. sclerotiorum*, including inhibition of mycelial growth and complete disintegration of sclerotia (Rai & Saxena, 1975).

The colonization of sclerotia by various *Fusarium* spp. has also been previously reported (Merriman, 1967; Adams, 1990; Zazzerini & Tosi, 1985; Jones & Stewart, 2000; Cosic *et al.*, 2012). These common soil dwelling saprophytes comprised 63% of the isolates from sclerotia in this study. They have been associated with various root and shoot rots, as well as suppressive soils against *Fusarium* rots (Clark, 1980; Rodriguez *et al.*, 2006; Goswami *et al.*, 2010; Kaur *et al.*, 2010). Bioassay results from this report echo this dichotomy; every *Fusarium* spp. that demonstrated antagonistic activity against mycelia of *S. sclerotiorum* also demonstrated some level of pathogenicity to the tested crop species.

Many *Fusarium* spp. are recognized as weak antagonists to surrounding microorganisms within the soil profile and general secondary colonizers of substrates

(Kaur *et al.*, 2010). Their presence on FP sclerotia may have been opportunistic, either as a result of imperfect rind formation, or following colonization by a more aggressive antagonist, such as *Trichoderma* spp. or *Penicillium* spp. This case may also be made for other soil dwelling saprophytes identified in this study, including *E. nigrum*, and *Cylindrocarpon* sp. Both species have been reported to colonize and inhabit tissue or sclerotia of *S. sclerotiorum* (Gladders & Coley-Smith, 1980; Brown *et al.*, 1987; Larena *et al.*, 2003; Larena *et al.*, 2005). The *Cylindrocarpon* sp. could only be identified to the genus level, making it difficult to determine the mechanisms behind this isolate`s presence on sclerotia, especially since *C. destructans* is a known phytopathogen of a number of plant hosts, including ginseng in Ontario (Reeleder & Brammall, 1994).

In this study, pathogenicity testing resulted in symptoms of disease in all treatments, including non-inoculated controls of each crop. This may be attributed to a contamination of control inoculum, or more likely, the effects of *Pythium* spp. within the potting soil mix. *Pythium* spp. are capable of parasitizing seeds and roots of a wide range of plants, and are most commonly associated with damping off of seedlings (Robertson, 1973). Consequently, focus was placed on 28 day emergence to determine the pathogenicity of an isolate.

The colonization of sclerotia by phytopathogenic species of fungi is not widely reported. One known symbiotic relationship is that between sclerotia of *Polyporus umbellatus* and the forest pathogenic fungi *Armillariella mellea*, where colonization by *A. mellea* is necessary for sclerotial development by *P. umbellatus* (Guo & Xu, 1992; Xing *et al.*, 2012). In this study, sclerotial colonizers that caused reduced emergence of seedlings in comparison to a control treatment (i.e. were pathogenic) in corn and wheat

include isolates 1-1.13 and 2-4.15, which were both identified as *Gibberella* sp. All isolates were pathogenic to soybean, with the exception of isolates identified as *F. acuminatum* (2-2.22) and *F. equiseti* (3-1.3). Each of the species that caused disease are causal agents of root and shoot rots, head blights, or wilts on a number of global economically important crops. The frequent presence of these and other colonizers on FP but not LP sclerotia both prior to, and following, experimental set up may be explained by the possibility that these fungi colonized sclerotia of *S. sclerotiorum* during formation prior to collection, rather than post burial. Although these pathogens are soil dwelling fungi, they have been previously isolated from symptom-free plant stems (Ogawa & Kamada, 1984; Postma & Ratting, 1992). These pathogens are able to take spatial and structural refuge within an antagonist-free space, such as a sclerotium, to avoid influence of antagonists (Johnson, 2010). Jeffries & Lawton (1984) suggested that pathogens may compete for, or evolve to, acquire enemy-free space and that the ability to acquire this space may be an important determination of species survival. Consequently, plant pathogens have likely been under selection pressure to achieve enemy-free space prior to host invasion. This may explain the antagonistic abilities of these plant pathogenic colonizers towards mycelia of *S. sclerotiorum* observed in this study which would ultimately allow them to utilize the protection and nutrients within the sclerotial rind.

The findings of the antagonism and pathogenicity bioassays within this study were based on observational results. Future studies should include microscopic examination of antagonistic hyphal interactions, antagonistic or parasitic activity against sclerotial rind tissue, and the identification of antibiotic production by isolates. Variation among antagonistic abilities of *Fusarium* spp. may be reconciled through

chromatographic techniques to determine metabolic responses, such as the production of cyclosporine A, a suppressive and antifungal compound produced by nonpathogenic *Fusarium oxysporum* (Rodriguez *et al.,* 2006). Similarly, PCR-based restriction fragment length polymorphism (RFLP) analyses of the intergenic spacer region of the ribosomal RNA operon has been designed to characterize the non-pathogenicity of *Fusarium* species (Nel *et al.,* 2006). Use of this and other molecular techniques would help to distinguish between pathogenic and non-pathogenic isolates, and rule out levels of uncertainty and inconsistency that may occur in growth room trials.

The results from this study indicate that the environment in which sclerotia are produced greatly impacts their survival and susceptibility to colonization by surrounding fungi. These sclerotial-colonizing fungi range in their purpose for invading sclerotial tissues but ultimately reduce disease caused by sclerotia forming fungi, making them an important part of natural pathogen control. The use of LP sclerotia within experimental research can effectively represent FP sclerotia, especially the most resilient individuals of natural populations. When testing the efficacy of possible control agents, both biological and chemical, it is suggested that LP sclerotia be used. If control measures prove to be effective on LP sclerotia, they will likely be even more effective on FP sclerotia, while reducing the survival of resilient members of the populations.

CHAPTER 4

DISCUSSION AND CONCLUSIONS

This study examined the survival of sclerotia of three economically important phytopathogenic fungi, untreated and treated with three BCAs, under natural field conditions and over an extensive period of time. For the first time, a multi-season analysis of survival of sclerotia treated with the BCA, *T. atroviride*, demonstrated reductions in percent survival of sclerotia of three pathogens greater than, or comparable to, that of an established BCA, *C. minitans*. The findings were in contrast to prevailing literature on the natural long-term survival of sclerotia of *S. cepivorum*, while results for *B. squamosa* and *S. sclerotiorum* support previous studies. New insights into the nature of microbial-sclerotial interactions were also observed, such as sclerotia harbouring other plant pathogenic fungi. This further demonstrates the importance of sclerotial eradication and the antagonistic potential of natural soil microbial communities.

There is a large collection of research on the antagonistic activity of *Trichoderma* spp., although only recently have studies focused on the activity of *T. atroviride*. This is largely due to the misidentification of this species as *T. harzianum* prior to 2003 (Bisset, 1992; Dodd *et al.* 2003). Studies that have examined *T. atroviride* have demonstrated diverse biochemical and biological activities, as well as antagonism towards a wide range of phytopathogenic fungi, suggesting the potential of this species as a broad-range BCA (Olamedo-Monfil *et al*., 2002). This study demonstrated that *T. atroviride* is capable of reducing the percent survival of sclerotia lower than (19%, *B. squamosa*) or similar to (23%, *S. sclerotiorum* and 0%, *S. cepivorum*) the reduction produced by the established BCA, *C. minitans*. *Trichoderma atroviride* was able to do so over two growing seasons,

including two winter seasons, supporting the previous reports that described *T. atroviride* as being a cold tolerant species that remains highly competitive within the soil profile (Dennis & Webster, 1971; Benitez *et al.*, 2004; Kaur *et al*., 2005; Reithner *et al.*, 2005).

Globally, *T. atroviride* has been commercialized as two biofungicide products in New Zealand, Tenet® and Sentinel®, for the management of onion white rot and botrytis in grapes and greenhouse tomatoes, respectively (McLean *et al.*, 2010). In Canada, numerous *Trichoderma*-based products are commercially available as biofungicides, including Rootshield® (*T virens* and *T. harzianum*), T34 Biocontrol® (*T. asperellum*), G-41® Technical (*T. virens*), and Bora® (*T. harzianum*). These products are registered for broad range use against various soil-borne pathogens causing root diseases and wilts (PMRA, 2013). The availability of these products, both globally and in Canada, along with the results of this study, suggest that there is potential for a commercialized *T. atroviride* biological fungicide to control a broad range of sclerotia-forming fungi in Canada.

While an overall reduction in sclerotial survival was observed following treatment by *T. atroviride*, fluctuations were present throughout the observation period, demonstrating that environmental conditions are an influential factor to the success of this BCA, as expected. Further studies towards a complete understanding of the response of this species to field conditions could reveal the full host and environment range in which this mycoparasite may thrive. Inclusion of various strains of sclerotia-forming soil-borne pathogens, soil types, cropping systems, and field locations are necessary to identify ideal environment-host-mycoparasite interactions that would promote successful disease control by *T. atroviride*. Furthermore, identifying genetic stability of this *T.*

atroviride isolate, and its compatibility with agrochemicals would both give insight into the potential success this BCA could provide within a fully operating agroecosystem.

This study has also contributed to a collection of literature that attempts to determine the long-term survival of sclerotia within the soil profile. The 63% survival of sclerotia of *B. squamosa* and the 10 sclerotia of *S. sclerotiorum* recovered, 20 months post-burial, support previous findings within literature (Ellerbrock & Lorbeer 1977 a,b; Cosic *et al.*, 2012). However, the 0% survival of *S. cepivorum* at 20 months post-burial contradicts commonly cited literature that these sclerotia persist for many years (Crowe *et al.*, 1980; Leggett *et al.*, 1983; Coley-Smith *et al.*, 1990). Although the percent survival of *S. cepivorum* did not exceed 9% throughout the observation period, it is accepted that even 1 sclerotia/g of soil can result in crop loss and failure (Crowe *et al.*, 1980). Similar sclerotial thresholds should be determined for diseases such as *B. squamosa* and *S. sclerotiorum*. Furthermore, these connections with previous literature have been made despite variations among experimental parameters and results, suggesting that a more standardized approach is necessary to evaluate the efficacy of sclerotia control products.

Results from Chapter 3 suggest that further studies to determine acceptable levels of sclerotia of *S. sclerotiorum* within the soil should do so with LP sclerotia. While FP sclerotia may provide a more representative level of sclerotia survival and subsequent disease, LP sclerotia may provide estimates of optimal survival, representing the more resilient members of natural populations. Similarly, variability is likely to be greater among FP survival due to differences in substrate nutrients, surrounding microbial communities, and inherent genetic traits within the population at each formation site.

It is evident that survival of sclerotia is greatly influenced by the surrounding biotic and abiotic environments during formation and dormancy, making generalizations of long-term survival difficult. This research exhibits how these environments are site-specific and how their characteristics, such as microbial community composition, are able to reduce the survivability of a population of sclerotia. This was observed through the colonization of FP sclerotia of *S. sclerotiorum* by a variety of fungi, including mycoparasitic (*T. hamatum*) and phytopathogenic (*Gibberella* sp.) isolates. Many reports have noted the colonization of sclerotia by ubiquitous soil-dwelling fungi, such as *Trichoderma* spp., *Fusarium* spp. and *Penicillium* spp. (Merriman, 1976; Anas & Reeleder, 1987; Jones & Stewart, 2000). The presence of these antagonistic species indicates that there is a natural antagonistic potential of the biotic environment where sclerotia are developed or eventually reside, as expected. The reduced survival of FP sclerotia compared to that of LP sclerotia further points towards a natural level of disease control at these sites. Testing for sclerotial colonizers of *S. sclerotiorum* at other locations throughout the Bradford Marsh, as well as within other crops and regions, would provide a better understanding of the extent of this antagonistic potential within agricultural settings.

Conversely, the presence of phytopathogenic fungi on sclerotia suggests that sclerotia may harbour other pathogens, providing them with spatial and structural refuge. Some of these pathogens also demonstrated antagonistic abilities towards *S. sclerotiorum*. The presence of contaminants within these sclerotia prior to burial demonstrated that at least some colonization occurred during sclerotial formation. Jeffries & Lawton (1984) proposed that pathogens may compete for enemy-free space and that the ability to acquire

this space may be an important determinant of species survival. This supports the antagonistic abilities of plant pathogenic colonizers observed in this study, ultimately allowing them to utilize the protection and nutrients within the sclerotium. This speculation requires the support of molecular evidence to qualitatively demonstrate both antagonistic and pathogenic abilities of such isolates, as well as further quantitative lab observations. Similarly, clarification as to the specific stage of formation that colonization occurred would provide a better understanding of this pathogen/antagonist-pathogen interaction. The findings of this study confirm that sclerotia remain an important stage in the life cycle of soil-borne pathogens that represent an ideal target to prevent possibly multiple disease carry-over between growing seasons. As a result sclerotial eradication is vital in disease control of *B. squamosa, S. sclerotiorum* and *S. cepivorum.*

In summary, this work is the first to demonstrate the capability of *T. atroviride* to reduce the survival of sclerotia of *S. sclerotiorum* and *B. squamosa* to similar or greater levels than that of an established BCA while in a natural setting for over 20 months. This work also demonstrated that LP sclerotia of *S. sclerotiorum* are more resilient and less likely to be colonized by surrounding microorganisms. Analysis of colonizers of FP sclerotia of *S. sclerotiorum* demonstrated the presence of plant pathogenic fungi, revealing that sclerotia of *S. sclerotiorum* can harbour other plant pathogens.

Future research to continue and build upon the work of this thesis should:

- re-evaluate the long-term survival of LP sclerotia of *B. squamosa, S. sclerotiorum* and *S. cepivorum* without the presence of soil in the mesh bags to improve recovery;

- evaluate the efficacy of *T. atroviride* in an agricultural setting to determine the potential of this formulated isolate to become a commercially available product;
- confirm the site specificity of the colonization of FP sclerotia of *S. sclerotiorum* by sampling sclerotia from other locations within the Bradford Marsh;
- evaluate the ability of sclerotia-colonizing isolates to inhibit germination of sclerotia of *S. sclerotiorum* to determine a range of antagonistic abilities; and
- quantitatively evaluate the pathogenicity of antagonistic *Fusarium* spp. isolates with the use of PCR techniques to determine their potential as BCAs.

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APPENDIX A - INITIAL SCLEROTIA SURVIVAL

Table A.1. Initial *in vitro* survival of sclerotia of *Botrytis squamosa, Sclerotinia sclerotiorum,* and *S. cepivorum* produced in laboratory.

Pathogen	% germination	% contamination
Botrytis squamosa	100	0
Sclerotinia sclerotiorum	100	0
Sclerotium cepivorum	98	0

Table A.2. Initial *in vitro* survival of sclerotia of *Sclerotinia sclerotiorum* produced in laboratory and collected from naturally infected bean plants surrounding the Bradford Marsh, Ontario in September 2011.

S. sclerotiorum	% germination	% contamination
Laboratory-produced	100	0
Field-produced	18	82

APPENDIX B - CHAPTER 2 ANOVA TABLES

Table B.1. Analysis of variance of area under the percent survival stairs (AUPSS) of arcsine-transformed percent survival of sclerotia of *Botrytis squamosa, Sclerotinia sclerotiorum* ,and *Sclerotium cepivorum*, recovered 20 months post-burial.

Source	DF	Type I SS	Mean Square	F value	Pr > F
Pathogen	2	1430.392	715.196	638.43	<0.0001
Error	12	13.443	1.120		

Table B.2. Analysis of variance of area under the percent survival stairs (AUPSS) of arcsine-transformed percent survival of sclerotia of *Botrytis squamosa* treated with three biological controls, recovered 20 months post-burial.

Source	DF	Type I SS	Mean Square	F value	Pr > F
Pathogen	3	288.189	96.063	27.13	<0.0001
Error	14	49.577	3.541		

Table B.3. Analysis of variance of area under the percent survival stairs (AUPSS) of arcsine-transformed percent survival of sclerotia of *Sclerotinia sclerotiorum* treated with three biological controls, recovered 20 months post-burial.

Source	DF	Type I SS	Mean Square	F value	Pr > F
Pathogen	3	677.941	225.980	95.35	<0.0001
Error	15	35.552	2.370		

Table B.4. Analysis of variance of area under the percent survival stairs (AUPSS) of arcsine-transformed percent survival of sclerotia of *Sclerotium cepivorum* treated with three biological controls, recovered 20 months post-burial.

Source	DF	Type I SS	Mean Square	F value	Pr > F
Pathogen	3	599.889	199.963	84.64	<0.0001
Error	14	33.074	2.362		

Table B.5. Analysis of variance of square root transformed number of sclerotia of *Botrytis squamosa* recovered, treated with three biological controls, recovered 20 months post-burial.

Source	DF	Type I SS	Mean Square	F value	Pr > F
Control					
Month	7	13.488	1.927	1.48	0.2090
Error	32	41.613	1.300		
C. minitans					
Month	7	18.097	2.585	1.19	0.3368
Error	31	67.301	2.171		
M. ochracea					
Month	7	3.860	0.551	6.34	0.0001
Error	32	2.783	0.0869		
T. atroviride					
Month	7	9.094	1.299	1.47	0.2140
Error	31	27.379	0.8831		

Table B.6. Analysis of variance of square root transformed number of sclerotia of *Sclerotinia sclerotiorum* recovered, treated with three biological controls, recovered 20 months post-burial.

Source	DF	Type I SS	Mean Square	F value	Pr > F
Control					
Month	7	27.994	3.999	3.60	0.0057
Error	32	35.551	1.111		
C. minitans					
Month	7	88.288	12.613	42.62	<0.0001
Error	32	9.469	0.296		
M. ochracea					
Month	7	7.529	1.076	1.61	0.1682
Error	32	21.369	0.668		
T. atroviride					
Month	7	49.814	7.116	7.29	<0.0001
Error	31	30.261	0.976		

Table B.7. Analysis of variance of square root-transformed number of sclerotia of *Sclerotium cepivorum* recovered, treated with three biological controls, recovered 20 months post-burial.

Source	DF	Type I SS	Mean Square	F value	Pr > F
Control					
Month	7	20.621	2.946	7.47	<0.0001
Error	32	12.628	0.395		
C. minitans					
Month	7	13.471	1.924	3.00	0.0159
Error	31	19.892	0.642		
M. ochracea					
Month	7	4.598	0.657	1.71	0.1413
Error	32	12.272	0.384		
T. atroviride					
Month	7	5.321	0.760	1.74	0.1354
Error	31	13.523	0.436		

Table B.8. Analysis of variance of arcsine-transformed percent germination of sclerotia of *Botrytis squamosa*, treated with three biological controls, recovered 20 months post-burial.

Source	DF	Type I SS	Mean Square	F value	Pr > F
Control					
Month	7	1.940	0.277	3.13	0.0124
Error	32	2.836	0.0886		
C. minitans					
Month	7	1.385	0.198	2.11	0.0722
Error	31	2.908	0.0938		
M. ochracea					
Month	7	0.659	0.0942	6.10	0.0001
Error	32	0.494	0.0154		
T. atroviride					
Month	7	3.799	0.543	7.62	<0.0001
Error	31	2.208	0.0712		

Table B.9. Analysis of variance of arcsine-transformed percent germination of sclerotia of *Sclerotinia sclerotiorum,* treated with three biological controls, recovered 20 months post-burial.

Source	DF	Type I SS	Mean Square	F value	Pr > F
Control					
Month	7	6.367	0.909	6.37	<0.0001
Error	32	4.567	0.143		
C. minitans					
Month	7	2.959	0.423	2.61	0.0301
Error	32	5.189	0.162		
M. ochracea					
Month	7	1.036	0.148	0.83	0.5702
Error	32	5.707	0.178		
T. atroviride					
Month	7	4.375	0.625	5.66	0.0003
Error	31	3.426	0.110		

Table B.10. Analysis of variance of arcsine-transformed percent germination of sclerotia of *Sclerotium cepivorum,* treated with three biological controls, recovered 20 months post-burial.

Source	DF	Type I SS	Mean Square	F value	Pr > F
Control					
Month	7	2.063	0.295	4.51	0.0014
Error	32	2.092	0.0654		
C. minitans					
Month	7	0.887	0.127	1.69	0.1481
Error	31	2.326	0.0750		
M. ochracea					
Month	7	2.409	0.344	2.90	0.0182
Error	32	3.793	0.119		
T. atroviride					
Month	7	1.027	0.147	2.31	0.0513
Error	31	1.970	0.0635		

Table B.11. Analysis of variance of arcsine-transformed percent survival of sclerotia of *Botrytis squamosa,* treated with three biological controls, recovered 20 months post-burial.

Source	DF	Type I SS	Mean Square	F value	Pr > F
Control					
Month	7	2.044	0.292	3.89	0.0036
Error	32	2.404	0.0751		
C. minitans					
Month	7	3.099	0.443	3.97	0.0033
Error	31	3.455	0.111		
M. ochracea					
Month	7	0.954	0.136	7.29	<0.0001
Error	32	0.598	0.0187		
T. atroviride					
Month	7	2.858	0.408	6.71	<0.0001
Error	31	1.886	0.0608		

Table B.12. Analysis of variance of arcsine-transformed percent survival of sclerotia of *Sclerotinia sclerotiorum,* treated with three biological controls, recovered 20 months post-burial.

Source	DF	Type I SS	Mean Square	F value	Pr > F
Control					
Month	7	6.868	0.981	6.86	<0.0001
Error	32	4.579	0.143		
C. minitans					
Month	7	7.156	1.022	53.08	<0.0001
Error	32	0.616	0.0193		
M. ochracea					
Month	7	2.262	0.323	2.39	0.0436
Error	32	4.322	0.135		
T. atroviride					
Month	7	1.182	0.169	1.84	0.1142
Error	31	2.842	0.0917		

Table B.13. Analysis of variance of arcsine-transformed percent survival of sclerotia of *Sclerotium cepivorum*, treated with three biological controls, recovered 20 months post-burial.

Source	DF	Type I SS	Mean Square	F value	Pr > F
Control					
Month	7	0.421	6.08×10^{-2}	8.02	<0.0001
Error	32	0.239	7.49×10^{-3}		
C. minitans					
Month	7	0.122	1.74×10^{-2}	3.34	0.0090
Error	31	0.161	5.20×10^{-3}		
M. ochracea					
Month	7	1.569	2.24×10^{-2}	2.59	0.0310
Error	32	0.277	8.66×10^{-3}		
T. atroviride					
Month	7	0.169	2.42×10^{-2}	2.30	0.0524
Error	31	0.327	1.05×10^{-3}		

APPENDIX C - CHAPTER 3 ANOVA TABLES

Table C.1. Analysis of variance of area under the percent survival stairs (AUPSS) of arcsine-transformed percent survival of sclerotia of *S. sclerotiorum,* LP and FP, recovered 20 months post-burial (see Figure 2.1).

Source	DF	Type I SS	Mean Square	F value	Pr > F
Treatment	1	656.111	656.111	356.54	<0.0001
Error	8	14.722	1.840		

Table C.2. Analysis of variance of square root-transformed number of sclerotia of *S. sclerotiorum,* LP and FP, recovered 20 months post-burial (see Table 2.3).

Source	DF	Type I SS	Mean Square	F value	Pr > F
Laboratory-produced					
Month	7	27.9936	3.999	3.60	0.0057
Error	32	35.5514	1.1109		
Field-produced					
Month	7	92.7906	13.2558	46.19	<0.0001
Error	32	9.1833	0.2869		

Table C.3. Analysis of variance of arcsine-transformed percent germination among recovered sclerotia of *S. sclerotiorum,* LP and FP, 20 months post-burial (see Table 2.3).

Source	DF	Type I SS	Mean Square	F value	Pr > F
Laboratory-produced					
Month	7	6.5472	0.9353	6.66	<0.0001
Error	32	4.4958	0.1405		
Field-produced					
Month	7	3.3489	0.4784	1.56	0.1821
Error	32	9.7912	0.3059		

Table C.4. Analysis of variance of arcsine-transformed percent survival among recovered sclerotia of *S. sclerotiorum*, LP and FP, 20 months post-burial (see Table 2.3).

Source	DF	Type I SS	Mean Square	F value	Pr > F
Laboratory-produced					
Month	7	6.3450	0.9064	6.25	0.0001
Error	32	4.6385	0.1449		
Field-produced					
Month	7	0.6127	0.08753	4.48	0.0014
Error	32	0.6254	0.01954		

Table C.5. Analysis of variance for arcsine-transformed 28 day emergence of three plant hosts (corn, soybean and wheat) exposed to 11 isolates of sclerotial colonizers of *S. sclerotiorum* in a growth room trial in 2013 (see Table 2.5 and Table 2.6).

Covariance Parameters	Estimate	Standard Error	Z value	Pr Z
Rep(Trial)	1.79×10^{-3}	1.61×10^{-3}	1.11	0.1337
Trial*Isolate*Host	1.62×10^{-2}	6.16×10^{-3}	2.63	0.0043
Residual	3.29×10^{-2}	3.36×10^{-3}	9.80	<0.0001
Effect	Num DF	Den DF	*F*-value	Pr > *F*
Trial	1	6	0.32	0.5939
Host	2	32	123.21	<0.0001
Isolate	10	32	52.44	<0.0001
Isolate*Host	20	32	6.19	<0.0001

Table C.6. Analysis of variance for arcsine-transformed disease incidence on three plant hosts (corn, soybean, wheat) exposed to 11 isolates of sclerotial colonizers of *S. sclerotiorum* in a growth room trial in 2013 (see Table 2.5).

Covariance Parameters	Estimate	Standard Error	Z value	Pr Z
Rep(Trial)	1.73×10^{-3}	4.71×10^{-3}	0.37	0.3565
Trial*Isolate*Host	5.66×10^{-2}	2.80×10^{-2}	2.02	0.0218
Residual	0.164	1.89×10^{-2}	8.66	<0.0001
Effect	Num DF	Den DF	*F*-value	Pr > *F*
Trial	1	6	1.99	0.2078
Host	2	25	27.41	<0.0001
Isolate	8	25	0.93	0.5118
Isolate*Host	15	25	1.37	0.2384

Table C.7. Analysis of variance for log-transformed disease severity on three plant hosts (corn, soybean, wheat) exposed to 11 isolates of sclerotial colonizers of *S. sclerotiorum* in a growth room trial in 2013 (see Table 2.5).

Covariance Parameters	Estimate	Standard Error	Z value	Pr Z
Rep(Trial)	1.81×10^{-3}	1.81×10^{-3}	1.00	0.1590
Trial*Isolate*Host	1.89×10^{-2}	7.83×10^{-3}	2.41	0.0079
Residual	3.43×10^{-2}	3.96×10^{-3}	8.66	<0.0001
Effect	Num DF	Den DF	*F*-value	Pr > *F*
Trial	1	6	0.13	0.7331
Host	2	25	0.49	0.6196
Isolate	8	25	1.60	0.1755
Isolate*Host	15	25	1.41	0.2150

APPENDIX D - WEATHER DATA

Table D.1. Monthly maximum, minimum and average temperatures, and average rain fall recorded at the University of Guelph Muck Crops Research Station between December 2011 and August 2013.

Sample Date	Year	Month	Temperature (° C)			Rainfall (mm)
			Max	Min	Average	
	2011	December	3.7	-3.9	-0.1	49
1	2012	January	1.3	-6.9	-2.8	39
	2012	February	2.7	-5.6	-1.5	15
2	2012	March	12.7	-0.1	6.3	30
	2012	April	12.5	0.0	6.3	51
3	2012	May	23.4	8.4	15.9	49
	2012	June	26.9	13.2	20.1	55
	2012	July	29.7	14.7	22.2	140
4	2012	August	27.0	13.1	20.1	69
	2012	September	21.7	8.0	14.9	94
	2012	October	14.6	4.8	9.7	123
5	2012	November	7.3	-1.4	3.0	32
	2012	December	3.8	-3.4	0.2	35
	2013	January	2.6	-1.8	0.4	33
6	2013	February	-0.6	-5.4	-3.0	36
	2013	March	4.0	-0.3	1.8	21
	2013	April	11.4	0.8	6.1	83
7	2013	May	21.9	7.6	14.9	113
	2013	June	24.2	12.8	18.5	94
	2013	July	27.5	15.1	21.3	104
8	2013	August	26.7	12.4	19.5	87