MOLECULAR AND MICROSCOPIC STUDIES OF A *FUSARIUM*-ASSOCIATED BIOTROPHIC MYCOPARASITE

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By

Yit Kheng Goh

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Head of the Department of Food and Bioproduct Sciences

University of Saskatchewan

51 Campus Drive, Saskatoon, Saskatchewan

Canada, S7N 5A8

ABSTRACT

Environmental hazards and health problems due to the application of chemical pesticides in agricultural sectors incite huge public concerns. Therefore, one of the better solutions is through introduction of biological control means to manage the outbreaks of plant diseases. To date, only small numbers of beneficial microorganisms - belonging to the category of hyperparasitic or mycoparasitic fungi have proven to keep plants or protect crops from plant pathogen infection. The objective of this study was to characterize a group of *Fusarium*-associated melanosporaceous biotrophic mycoparasitic fungal isolates, which were identified and pre-selected by Dr. Vladimir Vujanovic and deposited in the Saskatchewan Microbial Collection and Database (SMCD). Particular objectives were to examine spore germination of a biotrophic mycoparasite, to test effects of this fungus on seed germinations, to study interactions between the mycoparasite and *Fusarium* hosts, and to investigate relationships between the mycoparasite-the *Fusarium* host-wheat root under controlled conditions in the university Phytotron facilities.

Information related to this group of fungi is relatively limited. In order to identify and characterize potential biotrophic mycoparasitic fungal isolate(s), molecular and microscopy methods were performed to accomplish taxonomical, phylogenetical and morphological studies. Since, spore germination is a very crucial stage in fungal life cycle and growth, ascospores (sexual spores) of the biotrophic mycoparasite were isolated from a fungal colony. These spores were inoculated on media supplemented with different *Fusarium*-filtrates or suspended in different *Fusarium*-filtrates to examine spore germination rates and growth patterns. Together with other mycoparasitic fungi, this biotrophic mycoparasite was inoculated on spring wheat seeds, to test effects of these fungal inoculants on seedlings growth using *in vitro* assays. Dual-culture, slide

culture, and microscopy approaches were carried out to elucidate intimate and special relationship between the biotrophic mycoparasite and *Fusarium*-hosts. In order to study tritrophic interactions (biotrophic mycoparasite-*Fusarium* host-wheat root), spring wheat was grown in the phytotron with different treatments of fungal inoculations. Wheat roots were then subjected to genus-specific quantitative real-time PCR analyses.

One melanosporaceous biotrophic mycoparasitic strain was identified as a new species in the genus *Sphaerodes*. This biotrophic mycoparasite was isolated from *Fusarium*-infected fields in Saskatchewan and Quebec, and named *Sphaerodes mycoparasitica.* Germination of *S. mycoparasitica* sexual spores was improved when treated with filtrates or extracellular extracts from the *Fusarium*-host as compared to *Fusarium*-non-host filtrates. No pathogenic effects on wheat seeds were observed when inoculated with *S. mycoparasitica.* Furthermore, seedlings growth was enhanced with this biotrophic mycoparasite compared to other mycoparasitic fungi. Later, this biotrophic mycoparasitic strain was found to establish biotrophic fusion and haustorial contact relations with *F. avenaceum, F. oxysporum,* and two *F. graminearum* chemotypes. Since, 3-Acetyldeoxynivalenol-producing *F. graminearum* is one of the most highly toxigenic and aggressive wheat pathogens in Saskatchewan and North America, therefore, this pathogen strain was chosen for tritrophic interaction study. Under controlled conditions in the phytotron, *S. mycoparasitica* improved seedlings growth when these were challenged with *F. graminearum* as compared to seedlings only inoculated with the *Fusarium* pathogen. In conclusion, *S. mycoparasitica* could be a potential candidate for biological control of *Fusarium* diseases in wheat.

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LIST OF ABBREVIATIONS

ADON	Acetyldeoxynivalenol
AM	Arbuscular mycorrhizal
ANOVA	Analysis of variance
BCA	Biocontrol agent
BSA	Bovine serum albumin
CFU	Colony forming unit
CLA	Carnation leaves agar
Ct	Threshold cycles
DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
GFP	Green fluorescent protein
FDK	Fusarium damaged kernels
FHB	Fusarium Head Blight
ISR	Induced systemic resistance
LSU	Large-subunit
IDAC	International Depositary Authority of Canada
ITS	Internal transcribed spacer
LSD	Least significant difference
MBA	Myclobutanil agar
MEA	Malt extract agar
MLA	Modified Leonian's agar
MMT	Million metric tons
NIV	Nivalenol
PAUP	Phylogenetic analysis using parsimony

PCR	Polymerase chain reaction
PEA	Phenethyl alcohol
PDA	Potato dextrose agar
PDB	Potato dextrose broth
rDNA	Ribosomal deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
RPB2	RNA polymerase II
RT-PCR	Real-time polymerase chain reaction
SEM	Scanning electron microscope
SMCD	Saskatchewan Microbial Collection and Database
SSU	Small-subunit
TEM	Transmission electron microscope
WA	Water agar
ZEA	Zearalenone

1. INTRODUCTION

Canada is one of the major wheat (*Triticum* spp.) (spring, winter, and durum wheat) producers and exporters in the world (Food and Agricultural Organization of the United Nations 2009). Futhermore, wheat is the second largest category of field crops produced in Canada (Statistics Canada 2009). This crop can be grown and found throughout Canada, but major wheat production is located in Saskatchewan, Alberta, and Manitoba, i.e. the western Canadian provinces (USDA 2004). Among all different wheat varieties, spring wheat is one of the most important and highest yielding field crops in Saskatchewan. In year 2008, Saskatchewan produced around 12197.9 thousand tonnes of wheat; about 61% were spring wheat, 34.7% durum wheat, and 4.3% winter wheat (Saskatchewan Ministry of Agriculture 2009). The Saskatoon area was the second largest spring wheat production district in Saskatchewan with 461.1 thousands tonnes in 2007 (Saskatchewan Ministry of Agriculture 2008). Spring wheat, in all different phases of growth, is subjected to infection by fungi and other pathogens (Prescott et al. 1986). Fungal species are responsible for many severe diseases that can reduce grain quality and yield in wheat production.

Several previous surveys carried out throughout Saskatchewan and Alberta provinces indicated that *F. avenaceum* is the dominant species in common and durum wheat (Clear and Patrick 1990; Pearse et al. 2004; Pearse et al. 2005; Pearse et al. 2006; Turkington et al. 2006). First documentation of Fusarium Head Blight (FHB), caused by deoxynivalenol (DON)-producing *F. graminearum* reported in the Prairies – Manitoba, was in 1986 (Clear and Abramson 1986). More recent studies and surveys showed that *F. graminearum* strains expanded their territories from eastern Canada into the western Prairies (Celetti et al. 1998; Gilbert et al. 2008; Guo et al. 2008; Pearse et al. 2007; Pearse et al. 2008; Ward et al. 2008). In Western Europe, *F. graminearum* became more

dominant and replaced *F. culmorum* (Waalwijk et al. 2003). Ward and associates (2008) reported that the highly mycotoxigenic 3-ADON (3-acetyldeoxynivalenol) *F. graminearum* chemotype replaced the 15-ADON toxin-producing chemotype and other dominant FHB pathogenic fungi in North American wheat fields. Since pathogenic *F. graminearum* has became one of the most economically significant, rapid-spreading causal agents of FHB world wide (Goswami and Kistler 2004), it is important to control these pathogens. *Fusarium graminearum* was a highly aggressive and pathogenic to wheat grown in Canada, compared to other *Fusarium* strains (Xue et al. 2004).

Biological control was proposed as a promising environmental friendly approach (Cortes-Penagos et al. 2007; Pal and McSpadden 2006; Whipps 2001). Mycoparasitism is one of the biocontrol mechanisms used to control plant pathogenic fungi and defined as the parasitic interaction between one fungus with another fungus (Butler, 1957; Howell 2003; Paulitz and Bélanger 2001). According to Boosalis (1964), mycoparasitism interactions are categorized into two major catergories, biotrophic and necrotrophic parasitisms. The classification of the mycoparasitism interactions is based on the fungal parasitic bioactivity and the effects produced on the pathogenic host by the parasitism.

Biotrophic mycoparasites acquire nutrients from living host cells through haustoria or hyphae (which are useful for establishing intimate interactions with host cells or hyphae) (Boosalis 1964). Most of research is focuses on known and highly potential necrotrophs or biotrophs. However, relatively few investigations are studying novel biotrophic mycoparasites, which promise more efficiency in controlling *Fusarium* pathogens.

The objectives of this study were to identify a potential biocontrol biotrophic mycoparasitic fungus that is associated with pathogenic *Fusarium* species. For these

purposes, promising fungal isolates associated with *Fusarium* were pre-selected from Saskatchewan Microbial Collection and Database (SMCD). One biotrophic mycoparasitic fungus (Order Melanosporales) was identified as *S. mycoparasitica* (Appendix A). This biotrophic mycoparasite was subjected to ascospore germination tests to examine the ability of spore germination in different *Fusarium* extracellular filtrates. Under *in vitro* conditions, *S. mycoparasitica* was inoculated together with three *Fusarium* species (*F. avenaceum, F. oxypsorum,* and *F. graminearum* 3- and 15-ADON chemotypes) on slide culture, and using a dual-culture approach mycoparasite-*Fusarium* (host) interactions were investigated.

Wheat seedling assays were employed to test the effect of *S. mycoparasitica* on spring wheat seeds (CDC-TEAL 2001). This mycoparasite was reported to not have pathogenic effects on spring wheat. Since *F. graminearum* 3-ADON chemotype was reported to have spread rapidly into the western prairies and replacing other *Fusarium* species, the 3-ADON chemotype was chosen for a tritrophic interactions study. Under controlled conditions in the Phytotron service at the University of Saskatchewan, the potential biocontrol *S. mycoparasitica* candidate was used to examine the ability to suppress growth of *F. graminearum* in wheat root and mycoparasite-*Fusarium*-wheat root interactions were analyzed using genus-specific Real-Time Polymerase Chain Reaction (RT- PCR).

1.1 Study rationale

Saskatchewan is one of the major spring wheat producers in Canada. In Canada, Fusarium Head Blight and other *Fusarium* diseases are commonly found in association with spring and durum wheat. Outbreaks of *Fusarium* diseases are devastating to wheat production in both Saskatchewan and Canada. Therefore, it is important to control or manage *Fusarium* diseases properly. The environmental hazards of chemical pesticides

to control fungal diseases in agricultural sectors have raised a lot of debate and objections. Therefore, a safer alternative by biocontrol has been proposed. However, only small amounts of beneficial microorganisms, hyperparasites or mycoparasites, have proved to keep plants free of pathogens. Information related to the impact of biotrophic mycoparasites on *Fusarium* fungal pathogens in relation to wheat plants is relatively limited.

Sphaerodes mycoparasitica a potential mycoparasite candidate was identified and characterized to be associated with *Fusarium* species. It was isolated and discovered by Dr. Vujanovic from asparagus (Quebec in 2003) and wheat (Saskatchewan in 2005) using *Fusarium* selective myclobutanil agar (MBA) medium. Dr. Vujanovic is *S. mycoparastica*'s descriptor; he has deposited the new species in the Saskatchewan Microbial Collection and Database (SMCD) and in the International Depository Authority Canada collections as holotypus: SMCD 2220-01 and isotypus: IDAC301008 strains, Canada, Sept. 10, 2005, V. Vujanovic. Fungus description was publicly available in 2008 via the MycoBank (MB500012) and GenBank (FJ748916 and GQ354530). It was subsequently published as full length in Mycological Research (2009, 113: 1172-1180) and accepted by the Index Fungorum, the international fungal database. The description of *S. mycoparasitica* is provided under Appendices A. The text is the same as the original copy published in Mycological research. It was recuperated in its integrity for two principal reasons: (1) To familiarize the scientific audience with the new species as it is a core subject of the thesis or one on which the readers should have good grasp from the early start. It provides to readers the necessary and specific vocabulary or terminology surrounding the species and which is used later on for references; and (2) Although, Dr. Vujanovic is the descriptor or owner of the microorganism (i.e. the one who performed the taxonomy, phylogeny and systematics),

I was also involved by providing minor technical assistance while learning DNA extraction, PCR amplification, microscopy technique and fungal *in vitro* culturing that was necessary for species isolation and identification or the steps leading to new species discoveries. Also, while Dr. Vujanovic developed the key to *Sphaerodes* species, he taught me the components of a proper description for a new species. Therefore, although I am not co-descriptor of the new *Spaerodes mycoparasitica* species, I was granted the co-authorships on this article published in Mycological Research based on my assistantship contribution.

Germination of ascospores is a pivotal stage in the fungal life cycle and less information is available related to ascospore germination in the *Sphaerodes* mycoparasite, Chapter 3 is dedicated to study *S. mycoparasitica* ascospore germination patterns and rates in different *Fusarium* extracellular filtrate suspensions. Both germination patterns and rates could be one of the indications to show *Fusarium* hosts specificity for this biotrophic mycoparasite. *Sphaerodes mycoparasitica* was first isolated from different *Fusarium* species, however, there was no too much information related to biotrophic mycoparasitic interactions available for this *Sphaerodes* mycoparasite. In order to confirm that *S. mycoparasitica* as a biotrophic mycoparasite, Chapter 4 is provided to examine the interactions between *S. mycoparasitica* and *Fusarium* hosts, in particular *F. avenaceum* (most dominant in Saskatchewan), *F. oxysporum* (well-known fungal host for melanosporales biotrophic mycoparasites) and two *F. graminearum* chemotypes (3- and 15-ADON) (highly aggressive to the wheat). *Sphaerodes-Fusarium* interactions will reveal different types of biotrophic mycoparasitic structures produced by *S. mycoparasitica* on or inside *Fusarium* hosts but not in the non-host *Fusarium* species. Both ascospore germination and *Sphaerodes-*

Fusarium interaction studies will elucidate host specificity of *S. mycoparasitica* towards particular *Fusarium* hosts.

Information related to the effects of this *Sphaerodes* biotrophic mycoparasite on wheat is unknown. Potential mycoparasite might show beneficial effects on germination and growth of spring wheat seeds under *in vitro* assays. Furthermore, *Fusarium*-associated biotrophic mycoparasitic *S. mycoparasitica* could also improve seeds germination and growth, as well as reduce the amount of *Fusarium* DNA in the roots under controlled conditions. Hence, Chapter 5 is dedicated to examine beneficial effects posed by *S. mycoparasitica* on wheat under *in vitro* conditions and ability of this biotrophic mycoparasite in providing protection to wheat from *Fusarium* infection in tritrophic interactions.

Due to the specificity nature of biotrophic mycoparasite, *Sphaerodes mycoparasitica* could be applied on the wheat for biological control of *Fusarium* diseases caused by specific groups of *Fusarium* species.

2. LITERATURE REVIEW

2.1 Wheat diseases

Wheat is one of the principal cereal grains or crops produced for the world's food supplies. Between 1986 and 1990, wheat was the major cereal grain grown and produced, with an average of 533 million metric tons (MMT), followed by corn (451 MMT), are rice (331 MMT) (Oleson 1994). Barley was the fourth largest grain being produced at 178 MMT. Moreover, Canada is one of the major wheat producers as well as exporters in the world (Food and Agricultural Organization of the United Nations 2009; Statistics Canada 2009). However, wheat and other crops are vulnerable to a huge number of plant pathogens or pests causing economically significant problems. Wheat crops, at all different phases of growth, can be subjected to infection by several different groups of microbes or pests. These pests or pathogens associated with wheat can be grouped into fungi, bacteria, viruses, insects, and nematodes (Wiese 1977; Wiese 1987; Prescott et al. 1986). Fungal diseases that are commonly found on wheat crops are black kernel smudge, common bunt, root rot, crown root, FHB, rust, powdery mildew, blotch, take-all, tan spot, smut and wilt (Agrios 2005; Bridges 1995; Howard 2003; Prescott et al. 1986; Wiese et al. 2000).

2.1.1 *Fusarium* pathogens

Most *Fusarium* species are pathogenic to plants; these phytopathogens are responsible for major economically important crops diseases (Bai and Shaner 2004; Goh et al. 2009; Sutton 1982). *Fusarium* spp. can produce mycotoxins, which can cause animal diseases, affect animal feeds, and also harmful to humans (Fernando et al. 2000; Miller et al. 2007; Mills 1990). *Fusarium* diseases are not only found in Canadian wheat fields. Pathogenic *Fusarium* spp. can also infect barley, canola, asparagus, and other crops important to the Canadian bioeconomy (Calman et al. 1986; Fernandez et al.

2007b; Fernando et al. 2000; Vujanovic et al. 2002; Yergeau et al. 2005). In cereals, *Fusarium* species are commonly reported to cause crown and root rot diseases, FHB, Fusarium damaged kernels (FDK) and Fusarium wilt (Gilbert et al. 2001; Mavragani 2008). Crown and root rot diseases are frequently caused by *F. graminearum, F. pseudograminearum, F. culmorum, F. acuminatum, F. avenaceum, F. oxysporum, F. crookwellense,* and *F. equiseti* in wheat (Fernandez et al. 2007a; Fernandez and Jefferson 2004; Smiley and Patterson 1996; William et al. 2002). These pathogenic fungi showed high capability in reducing yields of wheat production, inhibiting seed germination, and affecting grain quality (Mavragani 2008). Therefore, it is important to control the outbreaks of *Fusarium* diseases or reduce the effects of *Fusarium* inoculants on crops and increase the plant yields. Biological control has been proposed as promising environmental solution (Pal and McSpadden 2006) and practical option against *Fusarium* pathogens (Vujanovic 2008).

2.2 Mycotoxin contamination in wheat and wheat products

Mycotoxins are toxic secondary metabolites generated by micro-organisms. These secondary metabolites are reported to cause disease and death in humans or mammals and other animals (Bennett and Klich 2003; Peraica et al. 1999). Accumulation of mycotoxins in stored grains was also suggested to be affecting the grain quality and reducing the shelf life (Magan et al. 2003). The genera of *Aspergillus, Penicillium, Fusarium,* and *Alternaria* harbour most of the world renowned mycotoxigenic and pathogenic fungi. These groups of mycotoxigenic fungi are able to produce more than 20 catagories of mycotoxins, such as aflatoxins, ochratoxins, cyclopiazonic acids, citrinin, fumonisins, trichothecenes, zearalenone, patulin, moniliformin, alternariol, tenuazonic acid, altenuene, fusaproliferin, beauvericin, and many others. These mycotoxins are causing problems to crops production, animal feeds, animals, human,

grains, plant-based products, and so on (D'Mello 2002; Logrieco et al. 2003; Pero et al. 1973). However, the top five most significant mycotoxins that could be found to appear naturally on agricultural products or foodstuffs are produced by: *Aspergillus flavus* – aflatoxin, *A. ochraceus* and *Penicillium verrucosum* – ochratoxin, *F. graminearum* – Deoxynivalenol (DON) and Zearalenone (ZEA), and *F. verticillioides* – fumonisin (Marasas et al. 2008).

2.2.1 *Fusarium* mycotoxins

Mycotoxins generated by *Fusarium* species are considered as the world's most problematic toxins. These toxins were found to contaminate domestic and imported cereal grains or cereal products (Chelkowski 1998). *Fusarium* species are able to produce four different types of trichothecenes: types A, B, C, and D (Foroud and Eudes 2009). Type A trichothecenes, such as T-2 and HT-2 toxins, are highly toxigenic to mammals compared to other trichothecenes (Mirocha et al. 2003). However, type B trichothecenes, such as DON, nivalenol (NIV), 3- and 15-Acetyldeoxynivalenol (ADON), are common toxins reported with FHB and also more phytotoxigenic (Foroud and Eudes 2009; Sudakin 2003). Type C and D are less toxicity and not found to associate with FHB (Desjardins and Proctor 2007; Foroud and Eudes 2009; Liddell 2003; Ueno 1983). Common fusarial mycotoxins reported as contaminants on food and feed items/products are NIV, ZEA, DON, and T-2 toxins (Bilgrami and Choudhary 1998). More than ten kinds of fusarial trichothecene mycotoxins were found to be positive on cereal-based foods (such as wheat, oat, and corn), vegetables, fruits, and foods of other plant origin marketed in Germany (Schollenberger et al. 2005). In Canada, ZEA, DON, and fumonisins were detected to be highest in soy-based infant cereals products (Lombaert et al. 2003). In another study, five different types of mycotoxins, such as DON, ZEA, T-2, HT-2, and diacetoxyscirpenol were found to be present on the

wheat and barley grains harvested from Atlantic Canada (Stratton et al. 1993). In the study by Roscoe et al. (2007), more Canadian breakfast cereals were monitored to contain DON, fumonisins, and ZEA, compared to NIV and HT-2 toxin. *Fusarium* secondary metabolites not only found as important contaminants on wheat and wheat-based products. However, some mycotoxins, such as DON produced by *F. graminearum* were observed to increase the level of virulence and pathogenesis ability in pathogenic *Fusarium* strains to infect wheat or other plants (Bai et al. 2001; Harris et al. 1999). In addition, mycotoxigenic and DON-producing *Fusarium* species were demonstrated to suppress the expression of a chitinase gene in *Trichoderma atroviride* P1 (biocontrol agent). This could be reducing the efficiency of this biocontrol agent in controlling *Fusarium* strains (Lutz et al. 2003).

2.3 Biological control

2.3.1 Introduction

Biological control is one of the promising alternatives to chemical pesticides and has received increasing attention over the last 20 years (Paulitz and Bélanger 2001). Environmental hazards due to the effects of excessive doses of chemical pesticides in agricultural sectors over past hundred years have prompted debates. Therefore, more and more pest management and biocontrol scientists/researchers are working on developing new alternatives for managing pests and controlling plant/crop diseases (Pal and McSpadden Gardener 2006).

2.3.2 Definition

The term biological control (biocontrol) bears several definitions, which cover various fields. The commonly used definition for biological control is "*the use of living organisms to curtail the growth and proliferation of other, undesirable ones*" (Gnanamanickam et. al. 2002). According to Pal and McSpadden Gardener (2006), a

narrower definition of biological control is "*the purposeful utilization of introduced or resident living organisms, other than disease resistant host plants, to suppress the activities and populations of one or more plant pathogens*". The living organisms and micro-organisms, which are showing suppression, inhibition, or control of the pathogens, have been named as the biological agents (BCA) (Pal and McSpadden Gardener 2006) (Table 2.1).

2.3.3 Mechanisms of biological control

Pal and McSpadden Gardener (2006) and Cortes-Penagos and associates (2007) suggested that the antagonisms between microbes are categorized into three major types: *direct*, *mixed-path*, and *indirect interactions*. Moreover, each type of interaction is sub-divided into various biological control mechanisms. *Mycoparasitism* or *hyperparasitism* and predation mechanisms belong to direct interaction. Mixed-path antagonism included four different mechanisms, such as antibiotics secretion, lytic enzyme production, unregulated by-products, and physical-chemical influences. Lastly, indirect interaction includes competition and host defenses induction mechanisms (Figure 2.1).

According to Pal and McSpadden Gardener (2006), the most efficient biocontrol agents are those with multiple mechanisms to suppress the pathogens. The meaning of multiple mechanisms refers to the engagement of more than one kind of biocontrol mechanisms. For instance, certain bacteria were reported to generate antibiotic compounds, and also involve the induction of host defenses towards pathogens (Iavicoli et al. 2003). Similarly, *Pythium oligandrum*, the mycoparasite of *Fusarium oxysporum*, also has the potential to trigger plant defenses (Benhamou et al. 1997).

2.3.4 Direct and Indirect mechanisms

Plant diseases, due to various phytopathogenic fungi, such as, *Fusarium* spp., *Pythium* spp., *Rhizoctonia solani*, and *Sclerotium rolfsii*, can cause serious yield losses

in agricultural sectors (Cortes-Penagos et al. 2007; Gnanamanickam et al. 2002; Xue et al. 2006). Therefore, plant diseases have to be controlled to reduce the level of yield losses and maintain the quality and quantity of food supplies (Fravel et al. 2003; Pal and McSpadden Gardener 2006).

2.3.4.1 Direct mechanism

Mycoparasitism or hyperparasitism is a direct biological control mechanism to control plant pathogenic fungi (Howell 2003; Paulitz and Bélanger 2001). Mycoparasitism is the parasitic interaction between one fungus with another fungus (Butler 1957). Hyperparasitism or direct parasitism is considered a synonym for mycoparasitism (Boosalis 1964; Kranz 1981). Indeed, parasites or fungi that parasitize on other fungi (host) are defined as mycoparasites or hyperparasites (Boosalis 1964). The first hyperparasite, *Darluca filum*, was described in 1813 (Kranz 1981), whereas more in-depth study into parasitic interactions between fungi were initiated in 1865 (Barnett 1964). Since then, more and more mycoparasites have been reported and in depth studies were established, such as taxonomy, morphological, cytological, histological, nutritional, interaction studies, molecular, and enzymes characterization and synthesis (Almeida et al. 2007; Assante et al. 2004; Barnett 1964; Fravel et al. 2003; Jeffries and Young 1994; Kranz 1981).

2.3.4.2 Indirect mechanism

Another mean of biocontrol mechanism is to utilize the indirect methods, which consist of two interactions – competition and host defense induction. In this chapter, we will briefly discuss the host defense induction. Plants are not only showing responses to environmental stresses, however, they also do respond to signals generated by non-pathogenic microbial agents. The signals produced by non-pathogenic microbial agents will trigger the increment of tolerance/resistance towards pathogens. This defense

Table 2.1 Biocontrol agents (BCA) agents against plant pathogens.

Biocontrol agent (BCA)	Pathogens	Reference
Bacteria	**Bacteria**	
Nonpathogenic	*Pseudomonas syringae*	Wilson et. al. 2002.
Antagonistic	*P. solanacearum*	Anuratha and Gnanamanickam 1990.
Fungi & Bacteria	**Fungi**	
Serratia plymuthica	*Verticillium dahlia*	Kurze et al. 2001.
S. plymuthica	*Phytophora cactorum*	Kurze et al. 2001.
Trichoderma lignorum	*Rhizoctonia solani*	Howell 2003; Weindling 1932.
Antagonistic fungi	*Fusarium culmorum*	Knudsen et al. 1995.
T. harzianum	*F. graminearum*	Dal Bello et al. 2002.
Bacillus cereus	*F. graminearum*	
Fungi & Bacteria	**Nematodes**	
Hirsutella spp. (fungi)	*Heterodera glycines*	Chen et al. 2000.
Rhizosphere antagonistic bacteria	*Meloidogyne incognita*	Kloepper et al. 2004.

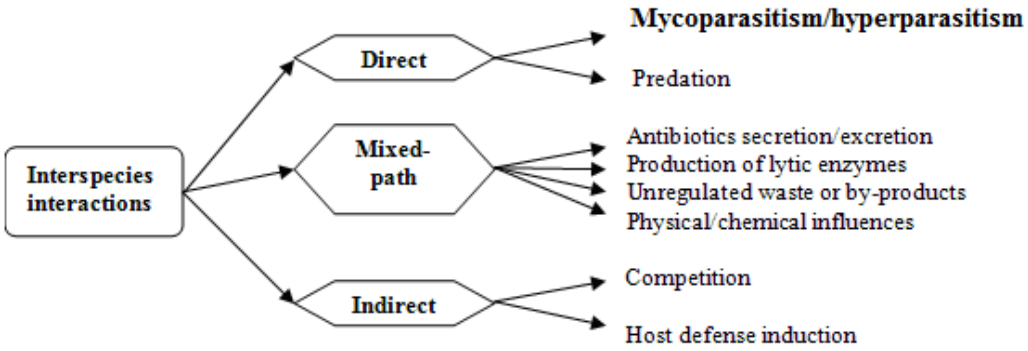


Figure 2.1 Types of interactions/anatagonisms between organisms/microbes and mechanisms involved in the biological control. Source: Adapted from Pal and McSpadden Gardener 2006; Cortes-Penagos et al. 2007.

induction is an induced systemic resistance (ISR) response (Fravel et al. 2003; Gnanamanickam et al. 2002; Pal and McSpadden Gardener 2006).

2.4 Mycoparasitism/hyperparasitism

According to Boosalis (1964), mycoparasitism interactions are categorized into two groups, biotrophic and necrotrophic parasitism. The classification of the mycoparasitism interactions is based on the fungal parasitic bioactivity and the effects produced on the pathogenic host by the parasitism.

2.4.1 Necrotrophic mycoparasitism

Usually, necrotrophic parasites kill the host cells (contact cells or nearby regions) before occupying them, thus deriving nutrients from dead cells. Necrotrophic parasites commonly have broader host ranges compared to biotrophic parasites because necrotrophs produce nonspecific toxic compounds (Barnett 1963; Boosalis 1964). Weindling (1932) pioneered initial reports of *Trichoderma lignorum* (necrotroph) mycoparasitism interactions. Following these first reports on necrotrophic mycoparasitism, further reviews were made by Warren, Boosalis, Backus and Stowell, Howell, and so on (reviewed in Barnett 1963). According to Butler (1957) and Barnett (1963), the level of destruction might be due to several factors such as host resistance, characteristics of the host, the mycoparasite nutritional requirement, and environmental factors. *Trichoderma* species are the famous necrotrophic mycoparasites with their rapid growth capacities, which play a vital role in biological control. Howell (2003) described mechanisms that were utilized by *Trichoderma* spp. to control plant pathogens (mycoparasitism, antibiotic production, competition, enzymes generation, and plants defense induction).

2.4.2 Biotrophic mycoparasitism

Generally, biotrophic mycoparasites acquire nutrients from living host cells through haustoria (which allow intimate interactions with host cells). There are two types of biotrophic parasites: *destructive* and *balanced* parasites (Boosalis 1964). Balanced parasites are those that impose a low degree or no apparent harm to the host, whereas destructive parasites are named after those that may kill or inflict partial damage to the host (during development or growth of parasites) (Barnett 1963; Barnett 1964; Jeffries and Young 1994; Kranz 1981). Examples of biotrophic mycoparasites include *Cosmospora, Melanospora, Nitschkia,* and *Anthostomella.* These mycoparasites confer control of plant pathogenic *Fusarium* causing FHB and root rots, *Hypoxylon*-bark canker, *Nectria*-bark canker, *Cryptodiaporthe*-bark canker, and *Naemacyclus*-coniferous needle diseases (Vujanovic et al. 1999; Vujanovic 2002; Vujanovic et al. 2003; Vujanovic and Labrecque 2007).

2.4.2.1 Mode of contact

In order to secure nutrients from the living host cells, there are several contact modes for biotrophic mycoparasites to parasitize the host. Table 2.2 illustrates a few common contact modes for the biotrophic mycoparasites to absorb nutrients from the host or launch host-parasite interactions (Barnett, 1964; Boosalis, 1964; Jeffries and Young, 1994).

2.4.2.2 Factors affecting mycoparasitism

Growth of the parasite and degree of mycoparasitism are generally affected by intrinsic factors, and extrinsic factors. Intrinsic or genetic factors refer to susceptibility of the host to resist mycoparasitism, or characteristics of the host. For instance, certain stages of the host life cycle might inhibit the parasite's growth. When the host has become vegetative, the invasion of mycoparasite will become prominent (Boosalis 1964; Slifkin 1961). Berry (1959) reported that the parasitic activity of biotrophic

mycoparasite, *Piptocephalis virginiana,* was occasionally found on mature hyphae (Barnett 1963). In addition, some of the *in vitro* studies showed that the host may form a mechanical barrier to stop or reduce the invasion by mycoparasites (Boosalis 1964).

Extrinsic factors, such as, nutrition, temperature, light, pH, and other organisms, play a crucial role in determining the degree of parasitism. Nutrient quantity, quality, or both, are having great influence on physiological and biochemical aspects of the host. This might also affect the parasitic activity. For example, Boosalis (1964) proposed that the degree of parasitism is higher when the medium is supplemented with higher amount of dextrose; this was due to increasing the susceptibility in the host. Moreover, Butler (1957) and Ayres (1935) illustrated that different kinds of carbohydrates affected the degree of parasitism. In addition, carbon, nitrogen, and the carbon-nitrogen ratio affected the susceptibility of the host, as well as changing the degree of parasitism (Boosalis 1964). Deficiency of a growth factor in some biotrophic parasites, such as *Calcarisporium parasiticum* and *Gonatobotrys simplex*, could inhibit the growth of mycoparasites. Therefore, they need to absorb nutrients from living host cells/substrate with growth factor supplements (Barnett 1964).

Temperature, light, and pH are extrinsic factors, which also influence the degree of parasitism. For instance, *Thamnidum elegans* was severely parasitized by *P.virginiana* at 25°C but not at 15°C and 20°C (Boosalis 1964). Moreover, the optimum temperature for other balanced and destructive mycoparasites to parasitize the host was around 25°C and 30°C (Barnett and Lilly 1958; Butler 1957; Boosalis 1964). Shigo and associates (1960) stated that parasitic activity was better under dark conditions. In addition, the pH range for necrotrophic and obligate mycoparasites showed some degree of differences – obligate mycoparasites were torelating a wider range of pHs. Additionally, effects due to other organisms, which are around or growing together with

the mycoparasites will affect the level of mycoparasitic activity on the host. For example, some phytopathogenic mycoparasite might be a host to the other hyperparasite or could be suppressed or killed by other mycoparasites (Boosalis 1964).

2.4.2.3 Effects of biotrophic mycoparasites on the host

According to Jeffries and Young (1994), effects caused by necrotrophic mycoparasites are more harmful compared to biotrophic mycoparasites. One of the effects caused by biotrophs is a reduction of the growth rate and sporulation capacity of the host. For instance, growth rate and sporulation were reduced in *Pilaira anomala* by *Piptocephalis* spp. in culture (Jeffries and Young 1994; Wood and Cooke 1986). Berry (1959) reported suppression in mycelial growth of the host *Mycotypha microspora* when challenged with *P. virginiana.* In some studies, experimental results show that hyphal apical tips/regions of the host are more susceptible to mycoparasite attack (England 1969; Jeffries and Young 1994). England (1969) and Wood and Cooke (1986) described the formation of unusual morphology in host hyphae, such as hyphal swelling and abnormal branching due to a mycoparasite. In some cases, the life cycle of the host was affected by mycoparasites (Jeffries and Young 1994).

2.4.2.4 *Fusarium*-associated mycoparasites

Several *Fusarium* species, such as *F. oxysporum* and *F. culmorum,* were reported as the hosts for biotrophic and necrotrophic mycoparasites. Table 2.3 shows the phytopathogenic diseases caused by *Fusarium* spp. as well as *Fusarium* hosts for mycoparasites (references included). *Pythium* spp. were described as both biotrophic and necrotrophic mycoparasites (Hoch and Fuller 1977).

2.5 Phylogeny, bitrophic and tritrophic interactions

2.5.1 Phylogeny

Table 2.2 Typical structures in biotrophic mycoparasitic interactions, which are used to improve host cells attachment, hosts colonization and nutrients acquisition from host cells.

Mycoparasite	Mycoparasitic contact structures	Reference
Piptocephalis virginiana	- Formation of appressorium-like swelling - Haustorium production - Germ tubes tropism towards host hyphae	Berry and Barnett 1957
Calcarisporium parasiticum	- Host hyphae tropism towards germinated spores - Small buffer cell formation	Barnett and Lilly 1958
Gonatobotrys simplex	- Formation of special contact branches - Globose to finger-like shape - Partial gripping the host hyphae	Whaley and Barnett 1963
Gonatobotrys fuscum	- Two-celled, thin, and with flattened apex structure formation	Shiga 1960
Sphaerodes retispora	- Formation of hook-shape cells	Harveson and Kimbrough 2001b
Melanospora zamiae	- Formation of hook-shape cells	Jordan and Barnett 1978
Sphaerodes quadrangularis	- Formation of hook- and clamp-like cells	Goh and Vujanovic 2010

Different levels of relationship or diversity or evolution can be studied by phylogenetic analyses through employing different rRNA genes. Phylogenetic relationship analyses over a broad range of fungal taxonomic levels could be achieved by using comparative studies on the nucleotides sequences acquired from ribosomal RNA (rRNA) genes (Vujanovic et al. 2007; White et al. 1990). For instance, nuclear small-subunit rDNA sequences are useful for studying distantly related microorganisms (White et al. 1990). Large subunit rDNA sequences were used by Rehner and Samuels (1995) to show the molecular and phylogenetic relationship in the Hypocreales. Further, nuclear encoded small and large subunit ribosomal DNA (SSU and LSU nrDNA), and RNA polymerase II (RPB2) genes were sequenced for Sordariales related fungi in phylogenetic studies (Tang et al. 2006; Zhang and Blackwell 2002).

2.5.2 Bitrophic (fungal-fungal) interactions

Fungal-host or fungal-fungal interactions can be studied by examining the dual culture or *in vitro* antagonism test (Carisse et al. 2001; Li et al. 2003). Parasite and the host fungi were inoculated together (a few cm apart from each other) on potato dextrose agar (PDA)-coated microscopic slides (Hoch 1974; Hoch and Fuller 1977), or sterile glass cover slips coated with molten water agar (Laing and Deacon 1991), so, it would be easier to observe the host-parasite interactions under the microscope. Moreover, Chet and associates (1981) proposed that agar-coated slides with a cellophane membrane layer could provide better microscopic resolution. Microscopic techniques (light microscope, scanning electron microscope (SEM), transmission electron microscope (TEM), video microscopic methods, and fluorescent microscope) were employed to study host-parasite interactions in detail (Bélanger and Deacon 1996; Chet et al. 1981; Hoch and Fuller 1977; Jeffries and Young 1994; Laing and Deacon 1991; Jacobs et al. 2005; Lorang et al. 2001). More recently, Green fluorescent protein (GFP) was proposed

Table 2.3 Some *Fusarium*-associated mycoparasites.

Mycoparasite	Host	*Fusarium* disease	Reference
Biotrophic			
Nitschkia parasitans	*F. aquaeductum*	Fusarium canker	Vujanovic 2002
Persiciospora moreaui	*F. oxysporum*	Fusarium wilt	Harveson and Kimbrough 2000
Gliocephalis hyalina	*Fusarium* spp.	Fusarium diseases	Jacobs et al. 2005
Stephanoma phaeospora	*Fusarium* sp.	Fusarium diseases	Hoch 1978
Sphaerodes retispora	*F.oxysporum*	Watermelon wilt	Harveson and Kimbrough 2001b;
Persiciospora moreaui	*F.oxysporum*	Watermelon wilt	Harveson and
Melanospora zamie	*F.oxysporum*	Watermelon wilt	Kimbrough 2002
Sphaerodes quadragularis	*F. avenaceum*	Fusarium diseases	Goh and Vujanovic 2010
Necrotrophic			
Trichoderma harzianum	*Fusarium* spp.	Crown and root rot of tomatoes	Paulitz and Bélanger, 2001
Biotrophic/ Necrotrophic			
Pythium spp.	*F.culmorum*	Brown root rot of barley	Davanlou et. al. 1999

to be useful for studying the interactions between parasite and host (in bitrophic system) and parasite-host-plant (in tritrophic system) (Neveu et al. 2007; Lorang et al. 2001).

2.5.3 Tritrophic (parasite-host-plant) interactions

Other than bitrophic interactions, tritrophic interactions are also very interesting topics to explore. Tritrophic interactions allow us to assess plant response and determine whether mycoparasites are directly or indirectly beneficial to the plants. In the paper by Neveu and his colleagues (2007), GFP technology was utilized to study tritrophic interactions (between plant-powdery mildew pathogen-*Pseudozyma flocculosa*). However, other kinds of tritrophic interactions, such as parasite-host-insect, were reported by Posada and his associates (2004) on the topic of tritrophic interaction between *Syspastospora parasitica* (mycoparasite), *Beauveria bassiana* (host), and *Leptinotarsa decemlineata* (insect). Tritrophic interactions can also be analyzed through quantitative real-time PCR approaches. For instance, Filion and associates (2003) proposed the real-time PCR quantification method to quantify amounts of *Fusarium* pathogen and *Glomus* mycorrhizae DNAs in bean plants as well as surrounding mycorrhizosphere. Quantitative real-time PCR technique was also used to detect and quantify colonization of pathogenic *Sclerotinia* sp. sclerotia resting structures by potential biocontrol *Trichoderma* strains in soils (Kim and Knudsen 2008). This method was also employed to analyze colonization of arbuscular mycorrhizal (AM) fungus in plant roots (Isayenkov et al. 2004). In order to study tritrophic interaction between spruce, rust fungus, and opportunistic ascomycetes, quantitative real-time PCR was applied to study the relationship among these three organisms under natural conditions (Hietala et al. 2008). Furthermore, real-time quantitative PCR techniques were used to study phytopathogenic and antagonistic fungi in association with plants (Schena et al. 2004).

3. SPORE GERMINATION OF ASCOMYCETOUS BIOTROPHIC MYCOPARASITE IN RESPONSE TO SPECIFIC *FUSARIUM* HOSTS

3.1 Abstract

Sphaerodes mycoparasitica is an ascomycetous mycoparasite commonly found growing and sporulating on *Fusarium* pathogens in Canada. Ascospores failed to germinate under different standard laboratory conditions (sterile distilled water, on water agar and commercially available media), even after heat- and cold-shock treatments. In contrast, ascospore germination was obtained on general potato dextrose agar medium amended with *Fusarium*-filtrates. Significant improvement in percentage of spore germination was obtained for the ascospores suspended in *Fusarium*-filtrates. *Fusarium avenaceum* and *F. oxysporum* filtrates induced highest germination, followed by two *F. graminearum* chemotypes, then *F. sporotrichioides* and *F. proliferatum.* Filtrates of beneficial fungal inoculants: *Trichoderma harzianum* (RootShield®), *Penicillium bilaii* (JumpStart™), and *Chaetomium globosum* had no impact on germination. Ascospores suspensions showed a double-polar germination pattern in *F. avenaceum*-filtrate and a single-polar germination, when challenged with *F. oxysporum*-filtrate. No different between the amounts for single- and two-polar germination in filtrates of both *F. graminearum* chemotypes.

3.2 Introduction

Ascospore germination is a crucial developmental point in the Ascomycota life cycle. In filamentous fungi, it was proposed that sexual spore germination follows three important stages: activation, isotropic growth and polarized growth. These stages allow transformation of dormant ascospores into actively developing germ tubes and formation of rapid growing mycelia, which assist in colonization or infection processes by newly germinated spores (d'Enfert 1997). Ascospores of *Neurospora, Talaromyces,*

Lobaria, Aleuria, members of Sordariaceae and fungi from many other groups were found to remain in a dormant stage, not readily germinating, or germinating at an extremely low amount, when inoculated on common laboratory media (Denison 2003; Dijksterhuis and Teunissen 2004; Furuya and Naito 1980; Lingappa et al. 1970; Ogawa et al. 2000). In order to work with ascospores that hardly germinate, Sussman (1981) speculated that laboratory parameters used should imitate natural conditions of the fungal isolates for inducing ascospore germination.

Stimulations of ascospore germination or breakage of dormancy was reported as in ascospores exposed to several chemicals, temperatures and media. *Neurospora* species, such as *N. crassa* and *N. tetrasperma,* are a well-studied fungal group with respect to ascospore germination. Indeed, heat treatments in *Neurospora* were among the first to show breakage of ascospore dormancy and initiation of ascospore germination (Goddard 1935; Lindegren 1932; Shear and Dodge 1927). Later, Emerson (1948) suggested that furfural was an effective chemical compound that induced ascospore germination in *N. crassa.* Sussman and associates (1959) proposed that several organic solvents and furans were found to activate ascospore germination in *N. tetrasperma.* Involvement of proteins and lipids during heat activation, as well as an association of several categories of activators in ascospore germination, were discussed and reviewed by Sussman (1976). Other chemical agents, such as low concentrations of phenethyl alcohol (PEA) and phenolic compounds, were efficient in triggering ascospore germination in *Neurospora* species and in more than eight fungal taxa from the Sordariaceae, respectively (Furuya and Naito 1980; Lingappa et al. 1970). Not only heat activation and chemical compounds were capable of breaking the dormancy of ascospores and enhancing spore germination in ascomycetes, but cold treatments induced ascospore germination as well. In paper by Ogawa and colleagues (2000)

showed that even cold treatments at 4°C and –20°C for 3 months augmented the *Aleuria aurantia* spore germination. In the Peltigerales, a few fungal strains were proposed to germinate in higher number on the media supplemented with an adsorbant and combined with either a sugar-alcohol ribitol compound or sugar (Denison 2003).

The genera *Sphaerodes, Melanospora, Persiciospora, Microthecium,* and *Syspastospora* of the Family Melanosporaceae (Melanosporales), Ceratostomataceae (Hypocreales), or Hypocreaceae (Hypocreales) are morphologically very similar to each other (Bessey 1950; Cannon and Hawksworth 1982; García et al. 2004). Many species in these taxa were identified as mycoparasitic fungi of the economically important pathogenic *Fusarium* species and other fungal taxa (Harveson and Kimbrough 2001a; Harveson et al. 2002; Posada et al. 2004). *Fusarium* species are well-known fungal pathogens, which cause FHB, FDK, Fusarium-wilt, and Fusarium root rot on many plant hosts. *Fusarium* species are also considered economically important pathogenic fungi since they are causal agents of several crop diseases in North America (Bai and Shaner 2004, Goh et al. 2009; Vujanovic et al. 2006). Biotrophic mycoparasitic *Sphaerodes* and *Melanospora* were described to reduce *F. oxysporum* growth. Later, a *Sphaerodes* isolate was proposed as a biocontrol agent for managing *Fusarium* wilt in watermelon (Harveson and Kimbrough 2001b; Harveson et al. 2002).

Relatively numerous numbers of studies were carried out on the subject of spore germination in various other pathogenic or biotrophic pathogenic fungi. However, information based on ascospore germination and patterns of spore germination for *Sphaerodes* species and closely related species are limited. *In vitro* ascospore germination of *Sphaerodes* mycoparasitic fungal strains (Ascomycota; Melanosporales)

is pivotal for the study of these biotrophic mycoparasites and provides the basis idea on colonization pattern and host preference.

3.3 Objective and hypothesis

We hypothesized that ascospore germination patterns and rates could be one of the indications to illustrate *Fusarium* hosts specificity for this biotrophic mycoparasite. The objective of this study was to examine *S. mycoparasitica* ascospore germination patterns and rates in response to different *Fusarium* (host and non-host) extracellular filtrates or exudates.

3.4 Materials and methods

3.4.1 Media, Reagents, and chemicals

Potato dextrose agar (PDA), Potato dextrose broth (PDB), Yeast extract, Malt extract agar (MEA), Agar, and Peptone were from Difco, Becton Dickinson Diagnostics, Sparks, Maryland. Streptomycin sulphate, Neomycin sulphate, and other reagents of analytic grade were from Sigma-Aldrich, St Louis, Missouri.

3.4.2 Fungal strains and growth conditions

Four phytopathogenic *Fusarium* species (*F. avenaceum* SMCD 2241*, F. sporotrichioides* SMCD 2245*, F. oxysporum* SMCD 2242*,* and *F. proliferatum* SMCD 2246); two toxigenic *F. graminearum* chemotypes SMCD 2243 and 2244 (3- and 15-ADON); three beneficial fungal inoculants: *Trichoderma harzianum* SMCD 2166 (RootShield®, BioWorks, Inc., NY)*, Penicillium bilaii* SMCD 2301 (JumpStart™, PhilomBios, Inc., SK)*,* and *Chaetomium globosum* SMCD 2302*;* and one mycoparasitic fungal strain (*Sphaerodes mycoparasitica* SMCD 2220) - isolated from *F. oxysporum* host, were maintained on PDA amended with antibiotics (100 mg/L streptomycin sulphate and 12 mg/L neomycin sulphate) and used throughout this study. *Sphaerodes mycoparasitica* mycoparasite was separated from their *F. oxysporum* host, and

monosporic cultures were obtained according to methods described by Harveson and Kimbrough (2001a), with few modifications. Mature perithecia were picked up, suspended, and shaked in 2-mL sterilized water blanks. The ascospores-suspension was spread on PDA plates. After a few minutes, with the assistance of a Carl Zeiss Stemi 1000 dissecting microscope, individual ascospores were removed and transferred to PDA supplemented with 100 mL/L of *Fusarium* filtrate. All fungal isolates were maintained in the culture collection of Saskatchewan Microbial Collection and Database, Canada (SMCD).

3.4.3 Ascopores production

Mycelial plugs from the margin of actively growing monosporic *Sphaerodes* culture were cut and placed on Modified Leonian's Agar (MLA: maltose, 6.25 g; malt extract, 6.25 g; KH_2PO_4, 1.25 g; yeast extract, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.625 g; peptone, 0.625 g; agar, 20 g, and 1 L of dH_20) (Malloch and Cain 1971). Inoculated MLA plates were incubated at room temperature (23°C) in dark condition for a month before collecting the ascospores for germination assays. With the assistance of dissecting microscope, mature ascospores exuded from the ascomata and located on the ostiolar opening were harvested by picking up carefully with sterile needles and added to 2 mL tubes in 1 mL sterilized distillated water. The suspension was then filtered through four thin layers of cheesecloth to remove the remaining vegetative cells or mycelia. Density of the ascospore suspension was counted with haemocytometer and adjusted to approximately 5 - 6 x 10^5 ascospores per mL in sterilized water. Only freshly prepared aqueous ascospore suspensions were used for all the experiments.

3.4.4 Preparation of the fungal filtrates

Four pathogenic *Fusarium* species, two toxigenic *F. graminearum* chemotypes and three beneficial fungal strains were grown in shake cultures (250 rpm) for 7 days at

room temperature (23°C) in 500 mL flasks, each with 100 mL PDB medium. After incubation, the mycelia were removed by filtering through filter paper and the filtrates were then filter-sterilized (0.2 μm). Only fresh preparations of fungal filtrates were employed for the ascospore germination test in this study.

3.4.5 Effects of heat and cold treatments on *Sphaerodes* spore germination

As proposed by Lingappa and Sussman (1959), aqueous ascospore suspensions were heat-shocked at 60 and 65°C for 20 min; and cold-treated at 4, -20, and -70°C for 5 min and 20 min. Heat- and cold-shocked ascospore suspensions were then transferred and inoculated onto WA and PDA for 1 and 3 days. The readings of ascospore germination were checked daily. Ascospores not subjected to heat and cold treatments were used as controls.

3.4.6 Germination on various media

Aqueous ascospore suspensions were transferred and inoculated onto the surface of these media: 1.5% water agar (WA), PDA, MLA, MEA, carnation leaves agar (CLA) (Tschanz et al. 1976), 1.5% water agar plus 100 mL/L of the *Fusarium* filtrate, and PDA with 100 mL/L of *Fusarium* filtrate. *Fusarium* species utilized are *F. avenaceum, F. oxysporum, F. proliferatum,* and *F. sporotrichioides.* Ascospore-inoculated plates were then incubated at room temperature (23°C) for 3 days, and ascospore germination was examined daily.

3.4.7 Effects of fungal filtrates on ascospore germination

The aqueous ascospore suspensions were suspended in the filtrates of four separate pathogenic *Fusarium* species, two different *F. graminearum* chemotypes and three beneficial fungal strains for 1 and 3 days in the ratio of 1:2 (1 part of aqueous ascospore suspension: 2 parts of fungal filtrate)(Figure 3.1). The filtrate-treated

ascospores were then transferred and inoculated onto PDA for an additional day (Figure 3.1). Control treatments were suspended with sterilized distilled water or PDB.

3.4.8 Ascopore germination

Microscopic assessments of ascospore germination were conducted after incubation for 1 and 3 days. Percentage of germinated ascospores was obtained by obtained by scoring the ascospores on the Petri dish through utilizing the 200x and 400x objectives of the Carl Zeiss Axioskop2 microscope and systematically choosing 50 ascospores, starting at the top right corner and continuing to count until 50. Each drop of the ascospore suspension on a growth medium plate was considered as a subunit, and there were three subunits per plate. Three replicates per treatments. The experiments were repeated twice. An ascospore was considered germinated only when the length of germ tube exceeded the width of the ascospore (~ 10 µm). Germinated ascospores were counted and recorded as a percentage of the total ascospore number.

3.4.9 Statistical analyses

Ascospore germination for *S. mycoparasitica* were not normally distributed. Therefore, differences in germination between control ascospores (incubated on PDA and WA only) and ascospores inoculated on *Fusarium*-filtrate media (PDA and WA amended with fungal filtrate) in two different days (1st and 3rd day) were analyzed with Kruskal-Wallis test followed by Mann-Whitney U test. Differences among percentages of ascospore germination in different filtrates suspensions and between ascospore germination patterns were analyzed with a Kruskal-Wallis test followed by Mann-Whitney U test (SPSS 1990).

3.5 Results

Sphaerodes mycoparasitica SMCD 2220 was found to sporulate on or when inoculated together with *Fusarium* species, such as *F. avenaceum* and *F. oxysporum. Sphaerodes*

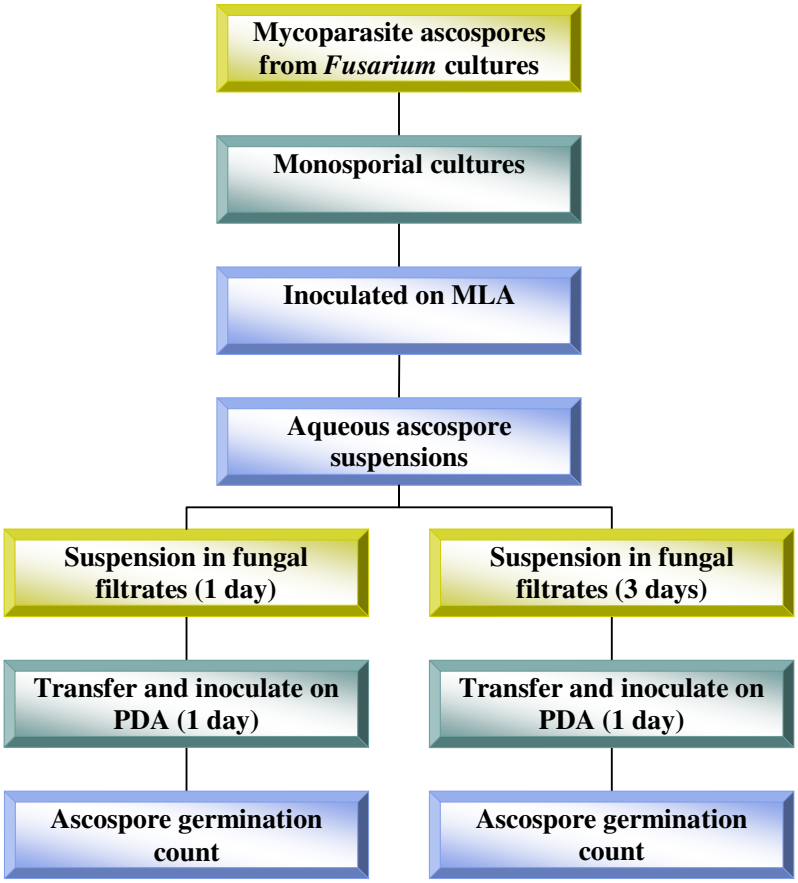


Figure 3.1 Flow diagram of *Sphaerodes mycoparasitica* ascospore germination assay. Assays established for the treatments of filtrates from four *Fusarium* species and three biocontrol agents.

mycoparasitica was observed to produce approximately the same amount of ascomata on both *F. oxysporum* and *F. avenaceum* (Data not shown). However, most of the perithecia were formed on the edge of *F. avenaceum* colony and scattered uniformly on *F. oxysporum* colony (Figure 3.2A, B). Furthermore, perithecia were also produced at the contact zone when challenged with *F. graminearum* chemotype 3 and 15. This biotrophic mycoparasitic fungus was not found to produce fruiting bodies on other *Fusarium* species and fungal strains such as *F. proliferatum, F. sporotrichioides, P. bilaai, T. harzianum,* and *C. globosum.*

3.5.1 Effects of temperature treatments and various media on ascospore germination

Heat activation and cold-shock treatments were performed to investigate ascospore germination in *S. mycoparasitica*. The parameters assessed in this experiment were 20 min at 60 and 65°C for heat-shock and in cold treatments – at 4, -20, and -70°C for 5 and 20 min. Control was ascospore suspension incubated at room temperature (23°C). There was no germination observed in all the heat and cold treatments for three days. No germination was observed in all the heat and cold treatments over a 7 days period.

In the ascospore germination experiment, untreated aqueous spore suspensions were inoculated onto different types of media (1.5% WA, PDA, MLA, MEA, CLA, and *Fusarium*-filtrate supplemented media). There was no spore germination on 1.5% WA, PDA, MLA, MEA, and CLA. The effects of 1.5% WA and PDA media amended with 100 mL/L of *Fusarium*-filtrates on spore germination were examined and are summarized in Table 3.1. No germination was recorded up to 7 days on WA or PDA alone and WA or PDA with either *F. proliferatum* or *F. sporotrichioides.* However, when either *F. avenaceum* or *F. oxysporum* filtrates were added to WA and PDA, the

percentage of ascospore germination in *S. mycoparasitica* drastically increased after 3 days of incubation. For instance, after 3 days of inoculation on *F. avenaceum*-PDA media, approximately 63.2% of the *Sphaerodes* ascospores germinated. On day 3, germination increased on the PDA amended with *F. oxysporum*-filtrate, compared to inoculation on WA supplemented with *F. oxysporum*-filtrate for *S. mycoparasitica* (Table 3.1).

3.5.2 Effects of fungal filtrates on ascospore germination

Fungal filtrates from four different phytopathogenic *Fusarium* species, two *F. graminearum* chemotypes and three beneficial fungal isolates were employed to study ascospore germination of *S. mycoparasitica*, and host specificity response, from an ascospore germination perspective. There were no ascospore germination observed in the treatments with *P. bilaai* and *C. globosum* fungal filtrates for the whole experiments. Both water and potato dextrose broth (PDB) suspension controls triggered approximately 1.8-3.8% spore germination for *S. mycoparasitica*, on 3rd day with one additional day on PDA only (Appendix B). Ascospores of biotrophic mycoparasite suspended in filtrates of pathogenic *F. sporotrichioides* and beneficial *T. harzianum* showed no germination on day 1 in suspension, but low number of ascospore germination was observed after 1 day in suspension followed by an additional day on PDA, and in the other incubation treatments (Figure 3.3 and Appendix B). Ascospores suspended in *F. proliferatum*-filtrate were observed to germinate in low abundance after 1 day, 1 day with an addition on PDA, 3 days, and 3 days plus an additional on PDA. *Sphaerodes* ascospores showed highest number of germinated ascospores in *F. avenaceum*-filtrate suspension. In the day 1 suspension treatments, ascospore germination of *S. mycoparasitica* in *F. avenaceum*-filtrate was significantly higher with 89.2% than in other treatments (Figure 3.3).

When ascospores of the biotrophic mycoparasite were suspended in *F. oxysporum*-filtrate, ascospore germination was found to be relatively high (Figure 3.3). There was no germination recorded for the 1 day suspension in *F. oxysporum*-filtrate treatment (Figure 3.2C). However, a low number of ascospores was stimulated in *F. oxysporum*-filtrate suspension on the second day (Data not shown). Based on the ascospore germination response, *Sphaerodes* biotrophic mycoparasite showed high specificity to the *Fusarium* genus, especially *F. avenaceum* and *F. oxysporum*.

Ascospore germination of *Sphaerodes mycoparasitica* in both *F. graminearum* chemotype 3 and 15 filtrates was significantly lower as compared to incubation in filtrates of *F. avenaceum* (in all incubation treatments) and in *F. oxysporum* filtrates (for three incubation treatments other than 1 day suspension in the filtrate) ($P < 0.05$) (with Mann-Whitney Test) (Figure 3.3). We could conclude that the primary hosts for *S. mycoparasitica* might be *F. avenaceum* and *F. oxysporum.* However, *Sphaerodes mycoparasitica* could also responded to *F. graminearum* chemotypes as the more distant hosts.

3.5.3 Germination patterns

Ascopores from *S. mycoparasitica* demonstrated two kinds of germination patterns. Single polar germination was more prevalent in *F. oxysporum*-filtrate suspension (Figure 3.2D, E, 3.4). Small numbers of ascospores from *S. mycoparasitica* were found to produce shorter double-polar germination in the treatment with *F. oxyporum*-filtrate after 3 days of suspension followed by an additional day on PDA (Figure 3.2F). In *F. avenaceum*-filtrate suspension, ascospores of *S. mycoparasitica* showed higher preference for two-polar germination (Figure 3.2G, 3.4). Single polar germination was also found in *F. avenaceum*-filtrate suspension; however it was lower than in *F. oxysporum*-filtrate treatment. Commonly, these single-polar germinated

Table 3.1 Germination of ascospores (in percentage) of *Sphaerodes mycoparasitica* SMCD 2220 on various types of *Fusarium*-filtrate supplemented media.

Medium	*S. mycoparasitica*	
	Day 1	Day 3
Water agar (WA)	0 [d]	0 [d]
F. avenaceum-WA	11.1 ± 1.7 [b]	35.2 ± 2.6 [b]
F. oxysporum-WA	1.6 ± 1.3 [c]	18 ± 1.4 [c]
F. proliferatum-WA	0 [d]	0 [d]
F. sporotrichioides-WA	0 [d]	0 [d]
Potato dextrose agar (PDA)	0 [d]	0 [d]
F. avenaceum-PDA	21.5 ± 1.4 [a]	63.2 ± 2.4 [a]
F. oxysporum-PDA	2.5 ± 2 [c]	37.5 ± 2 [b]
F. proliferatum-PDA	0 [d]	0 [d]
F. sporotrichioides-PDA	0 [d]	0 [d]

* Numbers in each column represented mean of ascospore germination (in %) ± standard deviation.

ǂ Each incubation day for *Sphaerodes* was analyzed separately. Means within each column for each medium treatment followed by the same letter (in superscript) are not significantly different at $P < 0.05$ after Mann-Whitney U test.

ascospores were observed to produce larger web-like organizations and longer hyphal formation (in *F. avenaceum*-filtrate suspension), which was rarely found in *F. oxysporum*-filtrate suspended asspores (Figure 3.2H, I). No significant different bewteen the amounts of single- and two-polar germination in *S. mycoparasitica'*s ascospores when suspended in the filtrates of *F. graminearum* 3 and 15 chemotypes (Data not shown). The majority of the germinated ascospores in this pyrenomycetous biotrophic mycoparasite, which was activated through suspension in *F. oxysporum*-filtrate were detected to be either at an angle of 90^{o} (Figure 3.2E, F) or between 90^{o} and 180^{o} (Figure 3.2F) at the tip of the polar germ pores. However, very few ascospores illustrated germination at an angle of 180^{o} (angle between the ascospore and the germ tube) (Figure 3.2D). Most activated ascospores (with *F. avenaceum*-filtrate treatment) showed germination at an angle of 180^{o} (Figure 3.2G).

3.6 Discussion

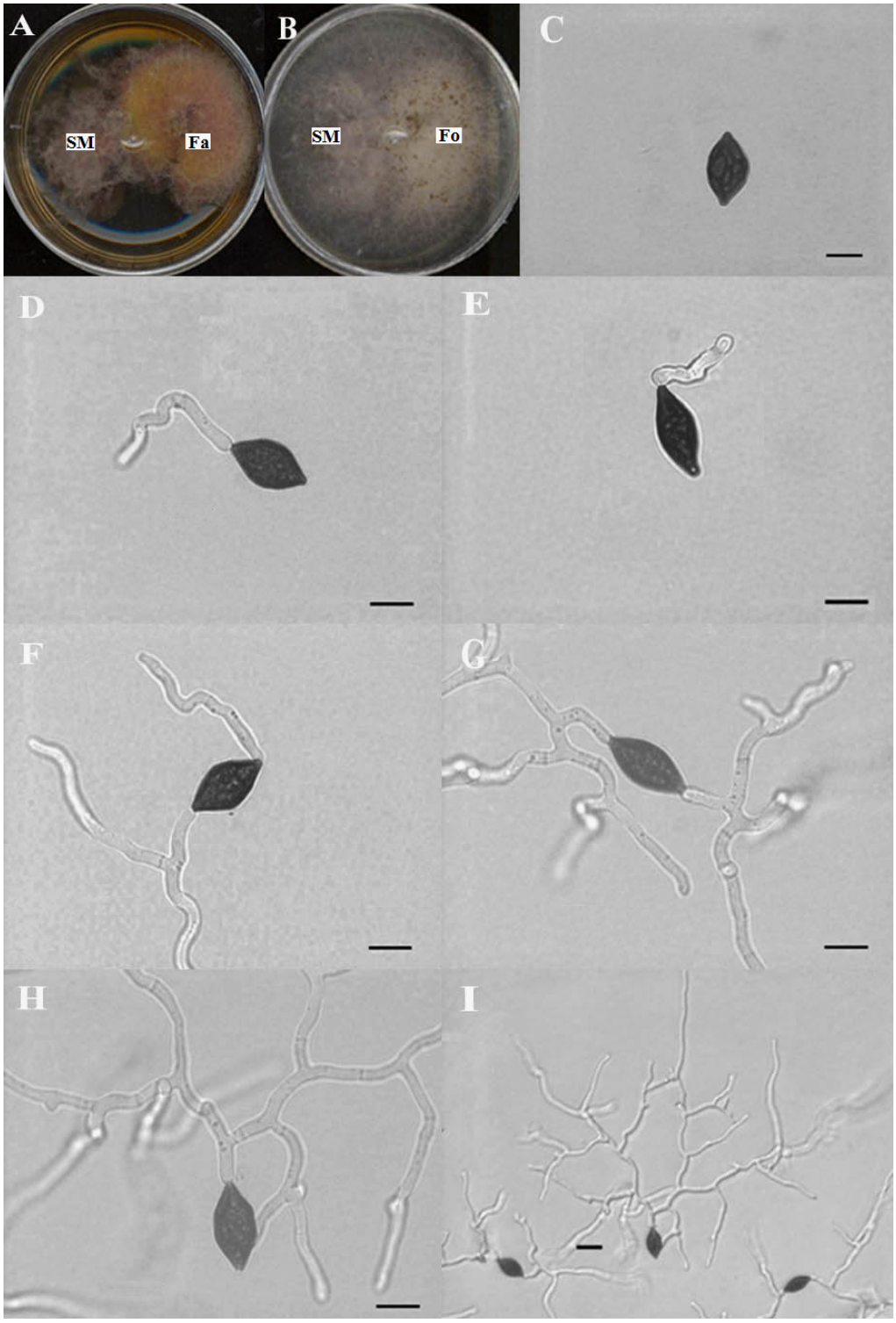
Previous studies demonstrated that *Sphaerodes retispora, Melanospora zamiae, M. pampeana, Gliocephalis hyaline,* and *Persiciospora* species were able to sporulate in the presence of certain *Fusarium* strains and several other fungal groups (Harveson & Kimbrough 2001a; Harveson & Kimbrough 2001b; Jacobs *et al.* 2005; Jordan and Barnett 1978; Robbins and Kavanagh 1942). Several *Melanospora, Persiciospora*, *Olpitrichum* species and one *Sphaerodes* strain were described as biotrophic mycoparasitic fungi of *Fusarium* species and other fungi. These biotrophs were also reported to produce hook-shaped structures and contact cells during the mycoparasitism interactions (Harveson and Kimbrough 2000; Jeffries and Young 1994; Jordan and Barnett 1978; Li and Shen 1996). Harveson and associates (2001a, 2001b, 2002) were the first team who suggested that *Sphaerodes* species were reported as biotrophic mycoparasitic fungi.

3.6.1 Effects of temperature treatments on ascospore germination

Effects of temperature treatments on ascospore germination were previously examined in *Neurospora* spp., *A. aurantia*, and *Monilinia vaccinii-corymbosi.* Cold-treated and heat-shocked spores were found to enhance spore germination in a certain group of ascomycetes compared to the non-treated ascospores (Goddard 1935; Lindegren 1932; Lingappa and Sussman 1959; Ogawa et al. 2000; Wharton and Schilder 2005). However, in our studies, there was no germination in different temperatures treatments. This might be due to the spores from this group of fungi not being responsive towards temperature changes. They may need certain host factors to trigger the spore germination. Sussman (1981) suggested that the relationship between fungi and their ecological settings are the keys for initiation of spore germination. Different fungi could have different factors or stimuli that of spore germination. This might be the reason why this biotrophic mycoparasitic fungus only responded to exudates from a specific group of *Fusarium.*

3.6.2 Effects of different media on ascospore germination

Effects of different media on ascospore germination were also investigated. No germination was detected on the media without *Fusarium*-filtrate supplements. CLA, MLA, and MEA were observed to induce the formation of sexual fruiting bodies in this biotrophic mycoparasite with highest amounts in MLA and followed by MEA and CLA (Data not shown). CLA was also suggested by Leslie and Summerell (2006) as one of the media that can trigger ascospore formation in *Fusarium* species. MLA was proposed to be able to enhance perithicia formation in ascomycetes (Malloch and Cain 1971). Therefore, these media were chosen to examine their ability to trigger ascospore germination in *Sphaerodes*. In addition, MLA was selected because this medium was found to be efficient in promoting a high number of perithecia and increased


A
SM
Fa
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SM
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E
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I

Figure 3.2 *Sphaerodes mycoparasitica* ascomata and ascospores. A. Formation of *Sphaerodes* ascomata on *F. avenaceum* colony (Arrows illustrated ascomata were produced near or surrounding *Fusarium* culture). B. Production of numerous *Sphaerodes* ostiolated perithecia on *F. oxysporum* colony (Arrows demonstrated pericthecia were formed on *Fusarium* isolate). C. Ungerminated dark-brownish reticulated *Sphaerodes* ascospore. D. Germinating ascospore of *Sphaerodes* in *F. oxysporum*-filtrate suspension showing one polar germ pore. E. Single polar germinating spore of *Sphaerodes* for 3d suspension in *F. oxysporum*-filtrate. F. Ascospore of *Sphaerodes* illustrating two polar germination in *F. oxysporum*-filtrate suspension for 3d with additional 1d on PDA. G. Pattern of *Sphaerodes* two polar germination in *F. avenaceum*-filtrate suspension with additional 1d on PDA. H. Single polar germination in *Sphaerodes* spore suspended 3d in *F. avenaceum*-filtrate with 1d incubation on PDA. I. Single and double polar germinations demonstrated by *Sphaerodes* ascospores from 3d *F. avenaceum*-filtrate suspension plus 1d additional incubation on PDA. Scale bars for C to H are 10μm and for I is 20μm. SM, Fa, and Fo represents *S. mycoparasitica, F. avenaceum,* and *F. oxysporum,* respectively.

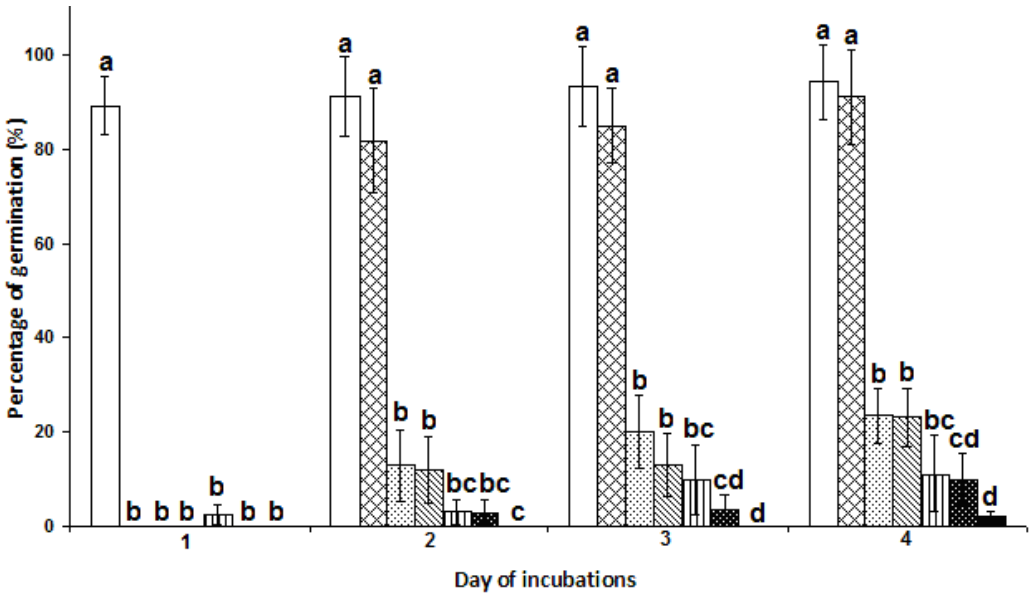

Figure 3.3 Germination of *Sphaerodes mycoparasitica* ascospores in filtrates of six *Fusarium* strains and water suspension treatments at four different incubation days. Means of spore germination (%) within each day of incubations were analyzed separately and using Kruskall-Wallis followed by Mann-Whitney Test at $P = 0.05$ (within each incubation days). Suspensions in different treatments were: *F. avenaceum*-filtrate (□); *F. oxysporum*-filtrate (▨); *F. graminearum* chemotype 3 filtrate (▣); *F. graminearum* chemotype 15 filtrate (▧); *F. proliferatum* filtrate (◫); *F. sporotrichioides* filtrate (◙); and water = control (■). Day of incubations were indicated as: 1 = spores suspended for 1d in different suspension treatments; 2 = spores suspended for 1d in suspension treatment were then inoculated onto PDA medium for an additional day; 3 = spores suspended for 3d in different suspension treatments; and 4 = spores suspended for 3d in suspension treatment were then inoculated onto PDA medium for an additional day.

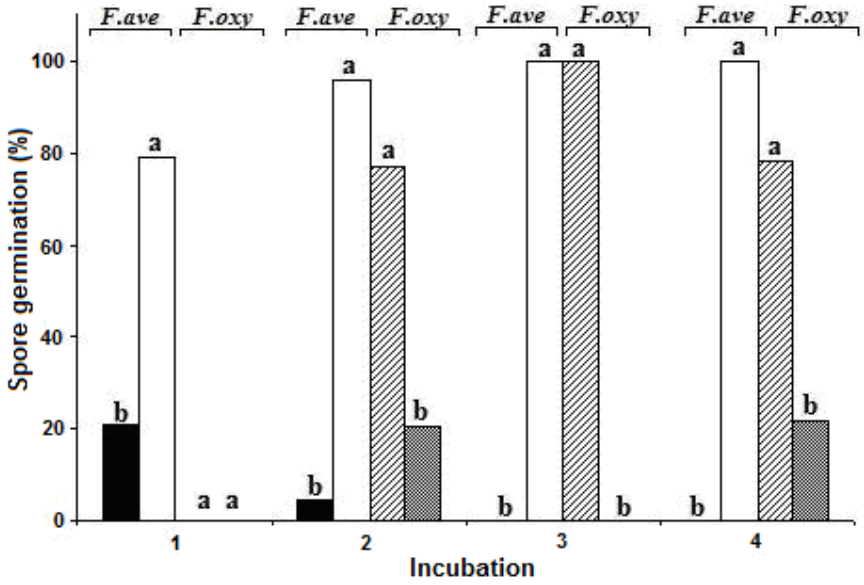

Figure 3.4 Spore germination patterns of *Sphaerodes* biotrophic mycoparasitic fungus with spores isolated from *F. oxysporum* colony. Single (■) and two (□) polar germination in *F. avenaceum*-filtrate suspension (*F.ave*); and Single (▨) and two (▩) polar germination in *F. oxysporum*-filtrate suspension (*F.oxy*). 1 = 1d suspension, 2 = 1d suspension plus 1d on PDA incubation, 3 = 3d suspension, and 4 = 3d suspension plus 1d on PDA incubation.

* Each incubation day for both *Sphaerodes* was analyzed separately. Means within each *Fusarium*-filtrate treatments followed by the same letter are not significantly different at $P < 0.05$ after Mann-Whitney U test.

sporulation rate in *S. mycoparasitica.* Futhermore, MLA was proposed to be more suitable for ascomycetous fungi as well (Malloch and Cain 1971). Aerial growth of this biotrophic mycoparasite was suppressed by this medium, which then allowed us to collect perithecia more easily.

Media supplemented with host substrates or filtrates were proposed to improve the ascomata formation in ascomycetous fungi (Baker et al. 1977, Jacobs et al. 2005, Lilly and Barnett 1951). Later, Harveson and Kimbrough (2001a) suggested that media amended with *F. oxysporum*-filtrate could induce greater ascospore germination in *Sphaerodes* species, which corresponded to our findings. However, water agar (WA) supplemented with *Fusarium* filtrates showed no ascospore germination, compared to PDA amended with *Fusarium* filtrates. This might due to a lack of glucose or other nutrients in the medium, which was also reported for ascospore germination for *Chaetomium globosum* (Chapman and Fergus 1975). In the paper by Chapman and Fergus (1975), they described that less ascospore germination was found in media that lacked glucose.

In our study, *Fusarium* exudates were observed to confer significant improvement on ascospore germination of *S. mycoparasitica*, especially exudates from *F. avenaceum* and *F. oxysporum* hosts. In other fungal taxa, exudates of *Cronartium quercuum* spores enhanced germination of basidiospores (Spaine and Kaneko 1993). Furthermore, germination and growth of asexual spores also improved when treated with extracts from the *Fusarium* hosts. Li and Shen (1996) showed that conidia of *Olpitrichum tenellum,* a contact biotrophic mycoparasite, germinated and developed better on media amended with a water extract of *F. moniliforme*. Jacobs and associates (2005) illustrated that spores of *Gliocephalis hyalina* contact biotrophic mycoparasitic fungus were found to germinate only in the presence *Fusarium* mycelia. Boogert van den and

Deacon (1994) proposed that in the presence of *Rhizoctonia solani* and other fungal hosts, spore germination was stimulated in *Verticillium biguttatum.* Other natural products or compounds or metabolites were also found to activate or enhance spore germination in fungi as well. For instance, volatiles from actinomycetes were noted to enhance spore germination of an arbuscular mycorrhizal fungus, *Gigaspora margarita* (Carpenter-Boggs et al. 1995). In addition, arbuscular mycorrhizal fungi (AMF) spores were recorded to germinate in response to plant host root exudates (Steinkellner et al. 2007). Furthermore, conidiospores from *Fusarium solani* and *Alternaria alternata* were triggered by exposing to volatiles from aged Alaska pea seeds (Harman et al. 1978). Surface wax isolated from Avocado fruits were suggested as chemical signals to induce fungal spore germination and appressorium formation in *Colletotrichum gloeosporioides* (Podila et al. 1993). However, some natural metabolites were discovered to inhibit germination of fungal spores. For example, suspension of *Curvularia lunata, Alternaria solani,* and *Colletotrichum capsici* in some fungal exudates was observed to inhibit spore germination for these fungi (Siradhana and Muralia 1974). Extracellular metabolites from biocontrol agent, *Gliocladium virens* were investigated and reported to show inhibitory effects on *Pythium ultimum* (Roberts and Lumsden 1990).

3.6.3 Effects of different fungal filtrates on ascospore germination

Due to the specificity, *Sphaerodes mycoparasitica* biotrophic mycoparasite only responded to factors from certain *Fusarium* species, such as *F. avenaceum, F. oxysporum, F. graminearum* chemotype 3- and 15-ADON strains. Previous studies on biotrophic mycoparasitic fungi showed that biotrophs commonly have a smaller range of hosts. In this study, exudates from *P. bilaai* and *C. globosum* were not able to trigger spore germination in *S. mycoparasitica,* which might be due to *Penicllium* and

Chaetomium species not being the hosts for *S. mycoparasitica*. In a literature by Jordan and Barnett (1978), *P. clavariforme* and *C. globosum* (non-hosts) were observed not to induce formation of contact branches from *M. zamiae.* Additionally, *C. globosum* and *P. bilaai* were located far from the order of Hypocreales in the order of Sordariales and Eurotiales, respectively; therefore, they might not be the hosts for these two biotrophs. On the other hand, *T. harzianum* belongs to the order of Hypocreales, which is in same order as *Fusarium* species. Therefore, *Trichoderma* species might be able to pose some degree of stimulation effects on *Sphaerodes*. *Sphaerodes* was reported to show parasitism activity towards a handful of *Fusarium* species and other closely related fungi (Harveson and Kimbrough 2001b; Jordan and Barnett 1978). Therefore, we could hypothesize the closer the fungi to the order of Hypocreales, which includes *Fusarium* species, the higher the possibility they are to be hosts of *Sphaerodes* and *Melanospora.* Based on the observations related to host-specific spore germination, Degawa and Gams (2004) suggested specificity of biotrophic mycoparasites to particular groups of host species – ascospores of mycoparasites only germinate when challenged with certain host species.

Furthermore, the numbers of *S. mycoparasitica* germinated ascospores in the filtrates of *F. graminearum* chemotype 3 and 15 were observed to be significantly lower than in *F. avenaceum* and *F. oxysporum* filtrates. Ascospore germination was significantly higher than in *F. sporotrichioides* filtrate on the 3rd day of suspension and or after 3 days in suspension with an additional day on a PDA plate (Figure 3.3). This could be due to the phylogenetical distance of *F. graminearum* strains from the primary hosts, *F. avenaceum* and *F. oxysporum.* In the study by Paavanen-Huhtala et al. (1999), both *F. avenaceum* and *F. oxysporum* were suggested to form one clade that originated from *F. graminearum* (separate clade). Guadet et al. (1989) also observed that *F.*

graminearum formed a separate clade from *F. avenaceum. F. proliferatum* and *F. sporotrichioides* also formed a separate clade and were distant from *F. oxysporum* (Waalwijk et al. 1996, Yergeau et al. 2005).

3.6.4 Ascospore germination patterns

We found that there were two different germination patterns, single- and double-polar germinations. Filtrate from *F. avenaceum* tended to stimulate two germ tubes formation. However, *F. oxysporum* exudates showed higher tendency to induce single-polar spore germination. Although, single- or double-polar germination was observed in *Neurospora, Sordaria, Sphaerodes,* and *Melanospora* species, there are no studies on the effects of different *Fusarium* exudates on spore germination patterns. The actual stimuli for stimulating single- and double-polar germination for *Sphaerodes* ascospores are not known. Yarden and Russo (1999) proposed that the regulations of fungal developmental switches are different from one fungus to the other. They also suggested that these switches are influenced by the interrelationship between genetic and environmental mechanisms. Therefore, we can conclude that there might be different genetic switches in *Sphaerodes* which responsed or interact with environments and metabolites from hosts and react differently. This could be explained through germ tubes tropism towards the host hyphae (Barnett and Lilly 1958; Jacobs et al. 2005). In a previous study, Spaine and Kaneko (1993) discussed that exudate factors were observed to trigger morphological alterations at the actively growing tips. However, the role of these exudate factors in spore germination has not been determined. These exudates were hypothesized to protect spores from being triggered and germinated directly on the plant surface of non-hosts. In addition, results from our study showed that the length of germ tube was longer in *F. avenaceum*-filtrate treatment as compared to *F. oxysporum*-filtrate. In few previous studies, measurement on the length of germ tube

formation was proposed to be useful in determining the vigor of spores' germination (Bega 1960; Spaine and Kaneko 1993). Therefore, we can conclude that *S. mycoparasitica* is more aggressive towards *F. avenaceum* and this *Fusarium* could be the major host for these biotrophic mycoparasites. *F. avenaceum,* one of the most important FHB pathogenic fungi was also isolated from residues and living roots of noncereal crops (Fernandez et al. 2005). *F. avenaceum* was found to be one of the most dominant species isolated from barley in Saskatchewan (Fernandez et al. 2007). Therefore, this biotrophic mycoparasite could be a potential biocontrol agent to control *F. avenaceum, F. oxysporum* and *F. graminearum* (a more distant) pathogens.

4. *SPHAERODES MYCOPARASITICA* AS BIOTROPHIC MYCOPARASITIC FUNGUS OF PHYTOPATHOGENIC *FUSARIUM* SPECIES

4.1 Abstract

Macroscopic and microscopic techniques were important in determining the host specificity of *S. mycoparasitica* to with five *Fusarium* species, such as *F. oxysporum, F. avenaceum, F. proliferatum, F. sporotrichioides,* and two *F. graminaerum* chemotypes. Results suggested that *S. mycoparasitica* established contact and haustorial-like biotrophic mycoparasitic relationships with *F. avenaceum, F. oxysporum* and two *F. graminearum* chemotypes, while *F. proliferatum* and *F. sporotricioides* were not the hosts for *S. mycoparasitica.* Data obtained from dual-culture assay and parasitism interactions revealed that this newly described contact biotrophic mycoparasitic fungus was able to reduce linear growth of *F. avenaceum, F. oxysporum* (in co-inoculation assay) and *F. graminearum* chemotypes (in pre-inculation assay). Furthermore, this mycoparasite significantly affected the diameter of *Fusarium* host hyphal cells through infection and penetration.

4.2 Introduction

Mycoparasitism or hyperparasitism refers to the parasitic interactions between one fungus (parasite) with another fungus (host) (Butler 1957). Parasitic fungi are defined as mycoparasites and can be categorized into two major groups, necrotrophic and biotrophic (Boosalis 1964). Necrotrophic and biotrophic mycoparasites can also be separated into several sub-categories. For instance, necrotrophic mycoparasitic fungi were grouped into contact and invasive necrotrophs; and biotrophic mycoparasites were divided into intracellular, haustorial, and fusion biotrophic fungi based on host-parasite interactions (Hoch 1977; Jeffries 1995). Differences between these five categories of mycoparasitic fungi were reviewed and highlighted in Jeffries and Young (1994).

Biotrophic mycoparasitic fungi are characterized by the formation of intimate contact, with or without penetration, the generation of short haustoria or appressoria or absorptive cells, and the most importantly is the cytoplasm of the host remains in a healthy stage; at least during certain phase(s) of the mycoparasitic interactions (Jeffries 1995). Fusion biotrophic mycoparasites are characterized by the formation of micropore(s) in the interface of parasite-host interactions, and this group of parasites include *Melanospora zamiae* (Jordan and Barnett 1978), *M. damnosa* (teleomorph of *Gonatobotrys simplex*) (Harveson et al. 2002; Hoch 1977), *Olpitrichum tenellum* (Li and Shen 1996), *Persiciospora moreaui* (Harveson and Kimbrough 2000), *Sphaerodes retispora* (Harveson and Kimbrough 2001a,b), and *Gliocephalis hyalina* (Jacobs et al. 2005). Fusion biotrophs were described and proposed to show mycoparasitic characteristics towards *Fusarium*. However, the term haustorial biotroph refers to penetration of the host hypha by haustorial branch generated from the hypha of the mycoparasitic fungi (Jeffries 1995). Most of the known literature on this subject pertains to Zygomycetous haustorial biotrophic mycoparasites and little information is available on haustorial biotrophs from Basidiomycota and Ascomycota. Ascomycetous haustorial biotrophic mycoparasitic fungi include species of *Sporidesmium* and *Teratosperma* that are well-known sclerotial biotrophs (Adams and Ayers 1983; Jeffries and Young 1994; Mischke 1998).

Few studies have identified ascomycetous mycoparasitic fungi capable of sporulating on on *Fusarium* species, in particular *F. oxysporum.* Investigations into the newly identified *S. mycoparasitica* suggests that it could be a potential candidate for controlling or suppressing the phytopathogenic and toxigenic *Fusarium* species, which are considered economically important crop pathogens as well as responsible for multi-billion dollars lost to agriculture field throughout Canada, North America and world

(Bai and Shaner 2004; Fernandez et al. 2001; Goh et al. 2009; Osborne and Stein 2007; Uhlig et al. 2007; Vujanovic et al. 2006).

4.3 Objectives and hypothesis

We hyphothesized: (1) *Sphaerodes mycoparasitica* is able to establish intimate and biotrophic relationship with *Fusarium* hosts by producing special organs or structures to acquire nutrients from hosts. However, this mycoparasite will not generate any special organs or contact structures on non-host *Fusarium* species. (2) *Sphaerodes mycoparasitica* can control or suppress *Fusarium* (host) mycelial growth under *in vitro* conditions and will not inhibit the growth of non-host *Fusarium* species. The objectives of this study were: (1) to examine *S. mycoparasitica* as a contact biotrophic mycoparasite on slide culture assays and (2) to evaluate *S. mycoparasitica* as a potential candidate for controlling *Fusarium* pathogens under *in vitro* conditions.

4.4 Materials and methods

4.4.1 Fungal isolates and growth

Four phytopathogenic *Fusarium* species (*F. avenaceum* SMCD 2241*, F. oxysporum* SMCD 2242*, F. proliferatum* SMCD 2244*,* and *F. sporotrichioides* SMCD 2243), two different mycotoxigenic *F. graminearum* chemotypes (SMCD 2243 and 2244, producing 3- and 15-ADON, respectively) and one biotrophic mycoparasitic fungus (*Sphaerodes mycoparasitica* SMCD 2220) were maintained on PDA (Difco, Becton Dickinson Diagnostics, Sparks, Maryland) amended with antibiotics (100 mg/L streptomycin sulphate and 12 mg/L neomycin sulphate) (Sigma-Aldrich, St Louis, Missouri) and used throughout this study.

4.4.2 Dual-culture assays

The purpose of the dual-culture assay was to assess the degree of hyphal growth inhibition or damage to *Fusarium* species in *in vitro* system. Five mm diameter

mycelial plugs of *Fusarium* species (*F. avenaceum, F. oxysporum* and two *F. graminearum* chemotypes) and *S. mycoparasitica* were taken from the actively growing 3-day-old culture and placed approximately 2 cm apart on PDA plates. Since, both *F. graminearum* chemotype 3 and 15 are fast-growing fungi as compared to *S. mycoparasitica* and other *Fusarium* species. For instance, linear mycelia growth of *F. graminearum* chemotypes were observed to be approximately 0.72±0.05 cm per day (on PDA) and *S. mycoparasitica* was 0.50±0.07 cm per day (on PDA). Therefore, *S. mycoparasitica* was pre-inoculated onto the PDA plates for 2 days prior to inoculation of *F. graminearum* mycelial plug. Assays were repeated twice with three replicates per treatment. These dual-culture assay plates were then compared to *Fusarium* isolates growing singly on PDA medium and which were chosen as control. Linear mycelial growth of *Fusarium* species for all treatments indicated above was measured and recorded daily for 5 days and observations were taken up to a week (Harveson and Kimbrough 2000; Quimet et al. 1997).

4.4.3 Establishment of mycoparasitism

Four different methods were described by Harveson and Kimbrough (2001a) to determine parasitism of mycoparasitic pyrenomycetous fungi on *F. oxysporum*. One of the approaches was related to exploitation of slide culture. Hyphal interactions between the mycoparasite and *Fusarium* species were observed by utilizing the slide culture method (Anthony and Walkes 1962; Cole et al. 1969). Slide cultures were prepared according to Jacobs et al. (2005) with slight modifications. Pre-cleaned slides were sterilized by heating with a flame. Approximately one mL of 15% water agar was spread onto the sterilized slides and allowed to solidify at room temperature. Small mycelial plugs from pure cultures of the *Fusarium* and *Sphaerodes* isolates were cut and transferred onto the slides. *Fusarium* species and *Sphaerodes* mycelial plugs were

kept ~ 1 cm apart from one another and allowed to grow for 7 days. Hyphal interactions between mycoparasitic *Sphaerodes* and five *Fusarium* species were examined on the slide cultures and observations were made daily under 20x, 40x, and 100x objectives of the light microscope (Carl Zeiss Axioskop2) attached to Carl Zeiss AxioCam ICc1 for up to 10 days. Diameters of infected (penetration) and non-infected (without penetration) *Fusarium* mycelia were measured and recorded.

4.4.4 Formation of intracellular parasitism

On the culture slides, *Fusarium* mycelia infected or penetrated with *Sphaerodes* haustoria or hyphae were stained with a drop of 0.01% lactofuchsin (85% Acid lactic: Fisher Scientific Co. and Acid Fuchsin: Acros Organics, NJ, USA) for 5 minutes (Carmichael 1955). Stained hyphae or mycelia of both the *Fusarium* host and the *Sphaerodes* mycoparasitic fungus on the culture slides were examined under the Carl Zeiss Axioskop2 fluorescent microscope attached to Carl Zeiss AxioCam ICc1 with 40x and 100x objectives.

4.4.5 Statistical analysis

T-test was used to determine the differences in linear growth of four treated and non-treated *Fusarium* strains (*F. avenaceum, F. oxysporum, F. proliferatum,* and *F. sporotrichioides*) in dual-culture assays over 5 days of incubations. Differences between linear mycelial growth of *F. graminearum* (for chemotype 3 and 15) in control, *Sphaerodes mycoparasitica* co-inoculated, and *S. mycoparasitica* pre-inoculated treatments for 5 days of incubation were analyzed using ANOVA(analysis of variance)-LSD(least significant different) (SPSS 1990). Differences between the diameters of infected (with penetration) (n = 6) and non-infected (without penetration) (n = 6) all *Fusarium* mycelia were analyzed using T-test (SPSS 1990).

4.5 Results

4.5.1 Dual-culture assays

Dual-culture assay was utilized to examine the linear mycelial growth of six *Fusarium* species: *F. oxysporum, F. avenaceum, F. proliferatum*, *F. sporotrichioides*, and both *F. graminearum* chemotypes. When challenged with the biotrophic mycoparasitic *S. mycoparasitica*, there were no significant inhibition or suppression of linear mycelial growth of *F. proliferatum* and *F. sporotrichioides* (non-host *Fusarium* species) compared to the control (without *Sphaerodes* biotroph) ($P \geq 0.05$, with T-test) (Figure 4.1A, B). On the other hand, in *F. avenaceum* and *F. oxysporum* dual-culture assays, there were significant difference between the linear mycelial growths between singly inoculated isolates on PDA and co-cultured isolates with *S. mycoparasitica* on same medium, starting at day 3 for *F. avenaceum* ($P < 0.05$, with T-test) and on day 7 for *F. oxysporum* ($P < 0.05$, with T-test) (Figure 4.2A, B, Appendix C). *Fusarium proliferatum* and *F. oxysporum* were observed to grow faster and cover the 5 cm plates in a week compared to *F. avenaceum* and *F. sporotrichioides*, which were slow growing and which took approximately 8 days to cover the whole plate (Figure 4.1, 4.2). In the *F. oxysporum – S. mcyoparasitica* co-culture plates, aerial mycelial growths decreased (Data not shown).

Since, *Sphaerodes mycoparasitica* (0.56 cm per day) was observed to have slower mycelial growth as compared to both *F. graminearum* chemotypes (0.72 cm per day), we decided to assess linear growth of *F. graminearum* mycelia in dual-culture by using a pre-inoculation method. *Sphaerodes mycoparasitica* was pre-inoculated initially on PDA for 2 days and followed by both *F. graminearum* chemotypes. This pre-inoculation approach demonstrated significant differences (starting on day 3) in suppressing linear growth of *F. graminearum* chemotype 3 and 15 as compared to a co-inoculation approach, where both the mycoparasite and *F. graminearum* were

inoculated together at the same time (Figure 4.3A, B). There was no inhibition on *F. graminearum* chemotypes in co-inoculation assay (both inoculated at the same time) and control (Figure 4.3A, B).

4.5.2 Establishment of parasitism

Morphological details of the *Fusarium-Sphaerodes* interactions were examined by growing both the *Fusarium* host and the *Sphaerodes* mycoparasite on the slide cultures. Microscopy slide cultures of *Fusarium* species and *S. mycoparasitica* showed that as the hyphae of the two different fungi interacted at the contact zone, short hook-shaped contact structures (Figure 4.4A, B, C, 4.5E) were generated in higher frequency as compared to clamp-like contact structures (Figure 4.4E, F). These different contact branches or structures were only produced at the contact zones with *F. avenaceum, F. oxysporum,* and two *F. graminearum* chemotypes (Figure 4.4, 4.5). The hook-shaped contact cells were observed to form singly (Figure 4.4B, 4.5E) and doubly at the same point of contact (Figure 4.4E). Most regularly hook-shaped structures were formed when hyphae of the host and mycoparasite were in parallel or side by side or at an acute angle (Figure 4.4A, B, C, G, 4.5E, F). Formation of clamps was normally observed when hyphae of the *Fusarium* host and parasite were at a right angle (Figure 4.4F). Infrequently, clamps were detected to form in series of two or more (Figure 4.4E). There was no formation of hook-, clamp-like, or any other contact structures in the interactions with *F. proliferatum* and *F. sporotrichioides.*

In addition to hook- and clamp-like structures, braid-like structures were observed in the host-parasite hyphal interactions. However, these structures appeared to be in extremely low abundance and were produced from two different hyphal cells (Figure 4.4D). Normally, each hyphal cell was found to be capable of generating one or two absorptive cells or hook-/clamp-like structures. In rare cases, the hyphae of *S.*

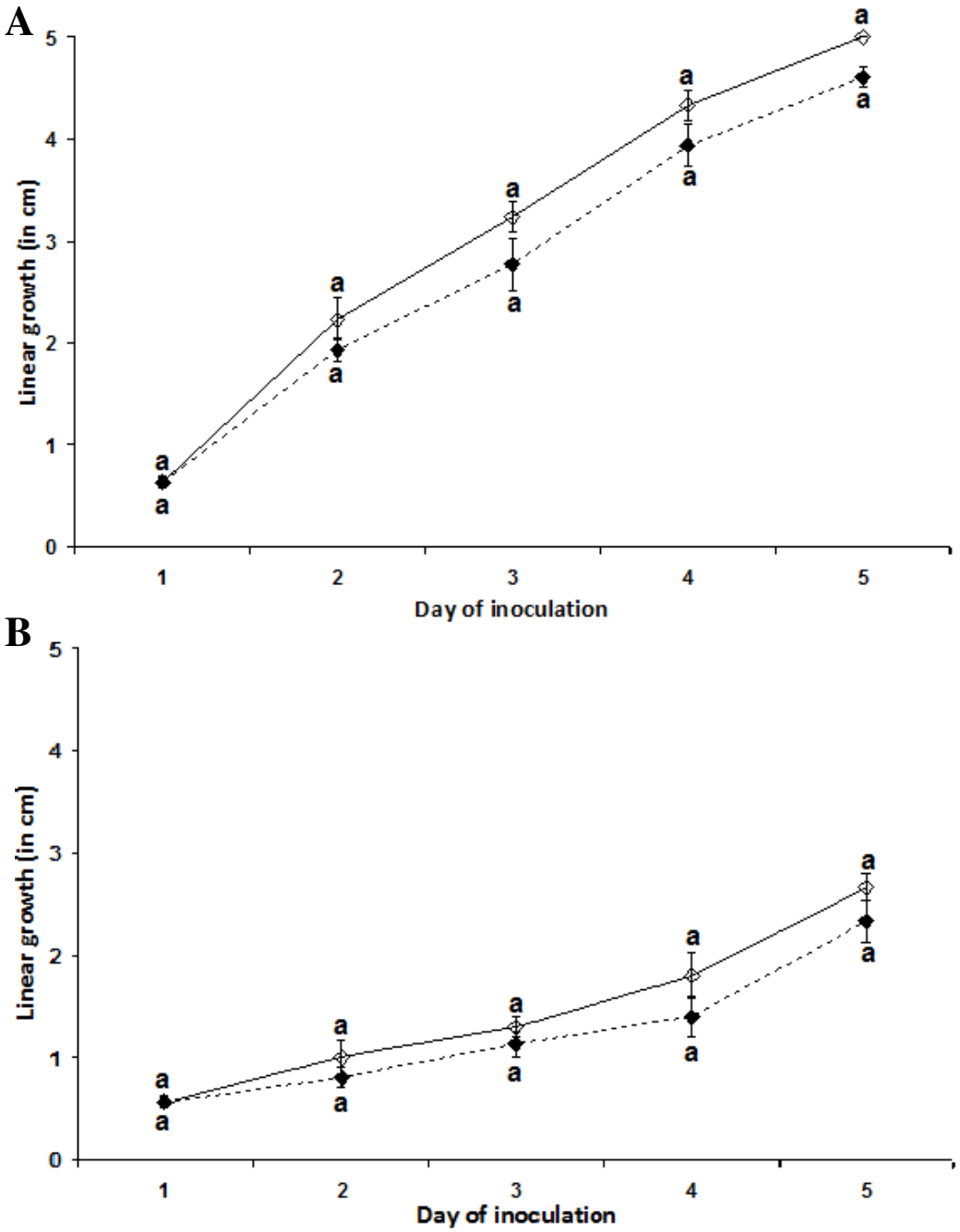

Figure 4.1 Dual-culture assays of two non-host *Fusarium* species for measuring linear mycelial growth. (A) *F. proliferatum* and (B) *F. sporotrichioides* on PDA medium challenged with *S. mycoparasitica* (--◆--) and control - without biotrophic mycoparasitic fungus (—◇—). Data are means and standard deviations. Dots with the same lowercase letter indicate no significant diffference between the control and dual-culture with *S. mycoparasitica* after 5 days of inoculation (T-test at P = 0.05).

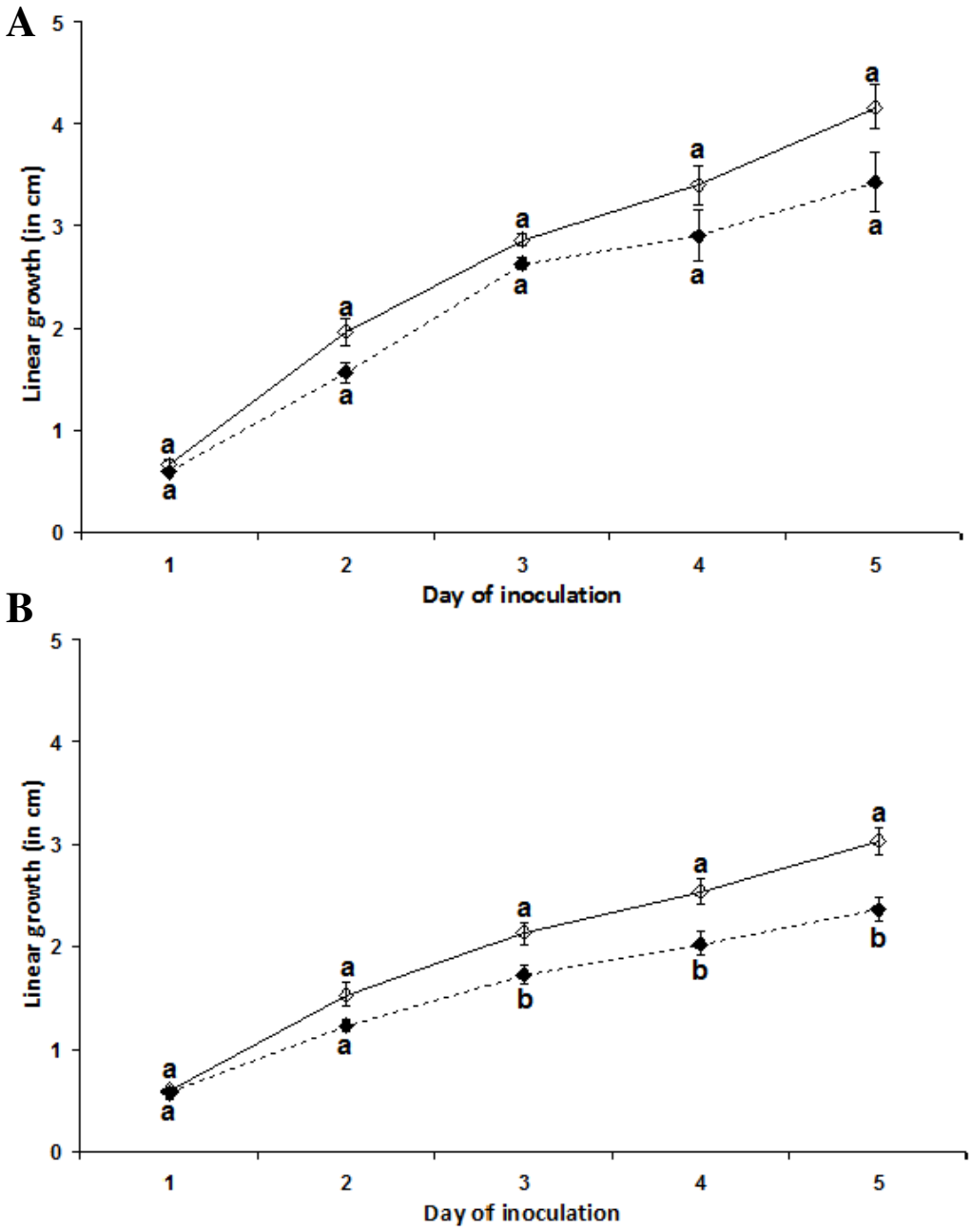

Figure 4.2 Dual-culture assays of two host *Fusarium* species for measuring linear mycelial growth. (A) *F. oxysporum* and (B) *F. avenaceum* on PDA medium challenged with *S. mycoparasitica* (--◆--) and control - without biotrophic mycoparasitic fungus (—◇—).Data are means and standard deviations. Dots with the same lowercase letter indicate no significant diffference between the control and dual-culture with *S. mycoparasitica* after 5 days of inoculation (T-test at P = 0.05).

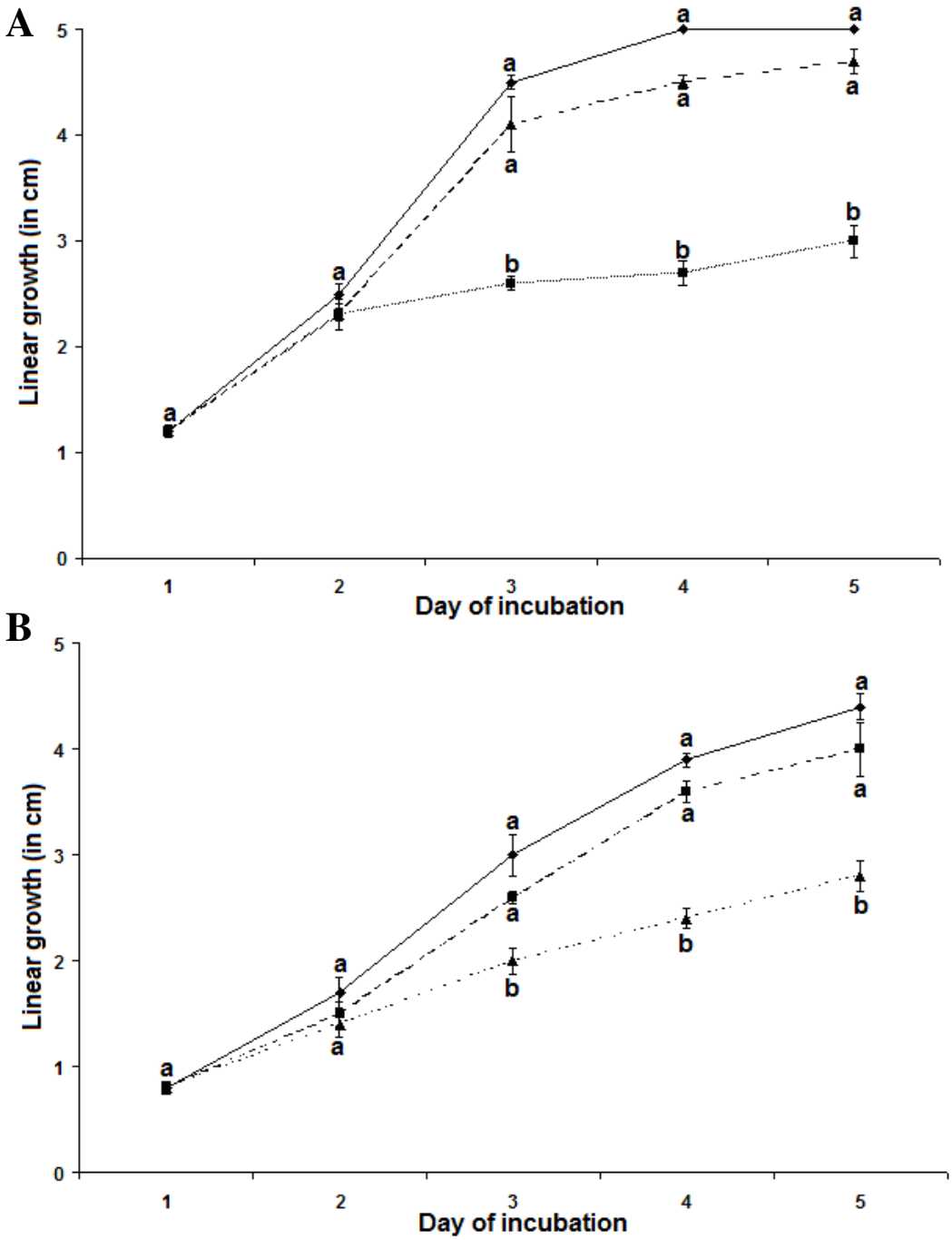

Figure 4.3 Linear mycelial growths of *F. graminearum* chemotypes. *Fusarium graminearum* chemotype 3 (A) and 15 (B) in the dual-culture assays challenged with *Sphaerodes mycoparasitica* in treatments of co-inoculation (same day) (--▲--), pre-inoculation (2 days prior to *Fusarium* inoculation) (···■···) and control (without mycoparasite) (—◆—) for 5 days. With ANOVA – LSD at $P = 0.05$ (comparison within each incubation days).

mycoparasitica were monitored to form contact relationship at the distance, approximately 11.2 to 43 μm, from the *Fusarium* host hyphal cells (Figure 4.4H).

After 3 days of inoculation on PDA culture slides with *F. graminearum* 3 and 15, there were no clamp- or hook-like structures generated by *S. mycoparasitica* on these two *F. graminearum* chemotypes. On the fifth day of inoculation, clamp- and hook-like contact structures as well as *Fusarium* hyphal cell penetration (with haustorium-like structure) were observed to form (Figure 4.5E to I). Formation of these structures and penetration by *S. mycoparasitica* were found to be 2 days slower as in *S. mycoparasitica – F. avenaceum* or *F. oxysporum* interactions (Figure 4.3, 4.4). However, on the *F. graminearum* chemotype 3 and *S. mycoparasitica* slide cultures, *S. mycoparasitica* could be absorbing red pigment/phenolic compounds/mycotoxins from the mycelia of *F. graminearum* on day 3 (Figure 4.5A to D). This gave mycelia of *S. mycoparasitic* a redish colour (Figure 4.5C). Around day 4 to 5, formation of reddish colour crystal-like pellets were detected on the mycelia of the mycoparasite (Figure 4.5D). For *F. graminearum* chemotype 15, there was no intake of the red complex and no red crystal-like structures formed on *S. mycoparasitica.* However, there was some kind of abnormal branching, which might be the indication of inhibitions (Figure 4.5J).

4.5.3 Haustorial-like parasitism

Haustorial-like mycoparasitism started with the formation of appressorium-like cells at the contact points for attachments and penetrations, the haustorial-like organs were then produced inside the host hyphae. Frequently, formation of appressorium-like and haustoria were observed on *Fusarium* host hyphae, which were larger in size as compared to the hyphae of *Sphaerodes* mycoparasite (Figure 4.5F to I, 4.6). On the other hand, hook- or clamp-shaped structures were more prominent on the smaller size *Fusarium* hyphae. In some cases, hyphae of the parasite in *Fusarium* host cells were

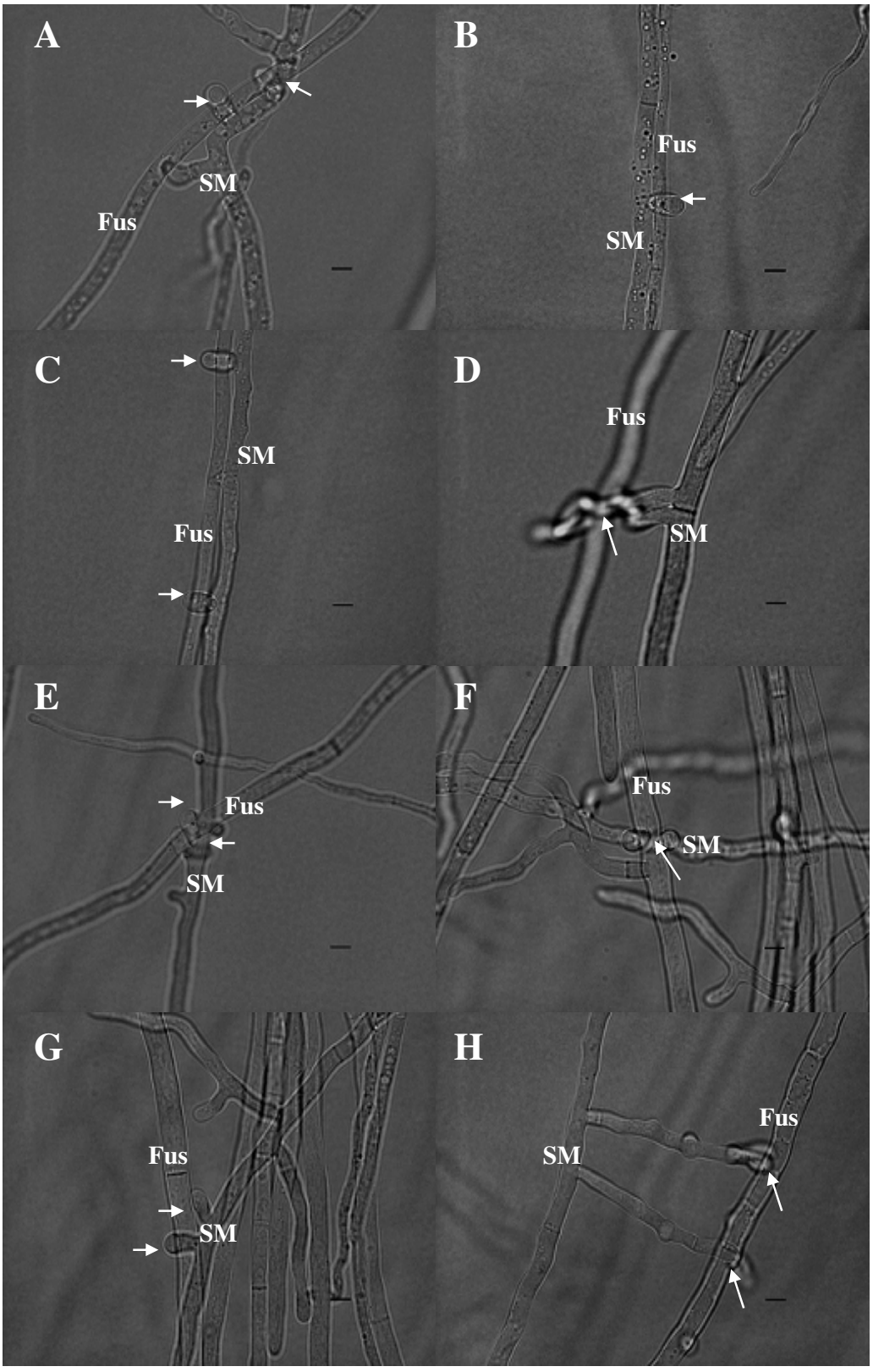
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Figure 4.4 Formation of different contact structures by *Sphaerodes mycoparasitica* (SM) on *Fusarium avenaceum* and *F. oxysporum* (Fus). A. Hook-like (arrows), B. Single hook-shaped (arrow), C. Double hook-shaped in series (arrows), D. Braid-like (arrow), E. Clamp-like in series of two (arrows), F. Clamp-shaped (arrow), G. Hook-like formed in doubly (arrows), and H. Elongated contact structures (arrows). All bar scales were 5µm.

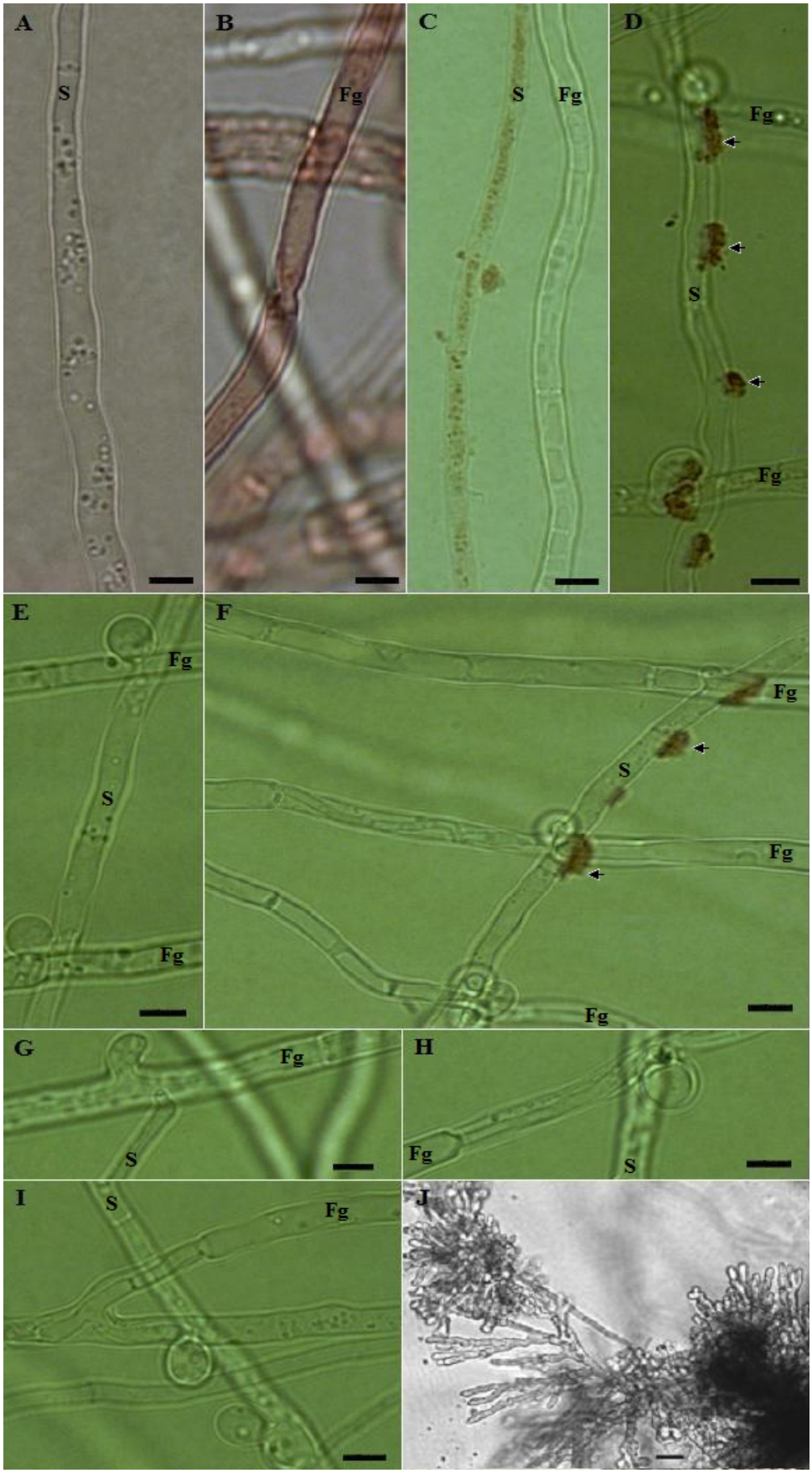
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Figure 4.5 Microscopic interactions between *F. graminearum* 3 chemotype (Fg) and chemotype 15 on the slide culture assays *with S. mycoparasitica* (S) biotrophic mycoparasitic fungus. A. Single mycelium of *S. mycoparasitica*, B. *F. graminearum* mycelium with *Fusarium* red complex or pigment, C. Absorption of red complex from *F. graminearum* by *S. mycoparasitica* (arrow), D. Excretion of red complex in crystal-forms (arrows) by *S. mycoparasitica* from mycelium in the interactions with *F. graminearum* chemotype 3 only, E. Formation of series hook-like structures by *S. mycoparasitica,* F. Parasitism of *F. graminearum* mycelium by *S. mycoparasitica* with formation of hook-shaped structures, excretion of crystal-like compounds (arrows), and internal haustorium, G. Initiation of penetration-peg formation by *S. mycoparasitica* on *F. graminearum,* H. Infected or penetrated and non-infected myclial cells, I. Branching of haustorium inside *Fusarium* host, J. Formation of extensive short branching structures by *F. graminearum* chemotype 15 only at the contact zone with *S. mycoparasitica.* Bar scales: A to I - in 5μm and J - in 20μm.

found to be in uneven or coiling forms (Figure 4.6B, C, E) and these uneven hyphae were only observed to remain within the host hyphae. At the penetration sites of *Fusarium* host by mycoparasite, haustorium-like cells were detected to grow inside the host hyphae (Figure 4.6A, D). The haustorium-like cells could be reported to extend in uni- or bi-directionally inside the hyphae of *Fusarium* hosts (Figure 4.6D). The haustorium-like structures developed in the host had become septated. In most cases, the penetrated and occupied host hyphal cells were recorded to be reduced in size (in term of diameter) as compared to the adjacent non-infected hyphal cells (Figure 4.7). The infected or penetrated host cells were lacking of cytoplasm contents (Figure 4.5H, I, 4.6E to H). There were significant differences between the diameter size of infected and non-infected *Fusarium* host hyphal cells, for *F. avenaceum, F. oxysporum* and both *F. graminearum* chemotypes (P <0.05, with T-test) (Figure 4.7). Furthermore, *Fusarium* host cells penetrated b *S. mycoparasitica* were not illustrating high intensity of fluorescent property (Figure 6F, H). The penetration and haustorial formation was not observed in the hyphal cells of *F. sporotrichioides* and *F. proliferatum.*

4.6 Discussion

4.6.1 Dual-culture assays

During the dual-culture study, the *Sphaedes mycoparasitica* was also observed to sporulate on *F. avenaceum, F. oxysporum* and both *F. graminearum* chemotypes, but not on *F. sporotrichioides* and *F. proliferatum.* The ability of pyrenomycetous fungi that are closely related to *Melanospora, Sphaerodes* and *Persiciospora* genera to sporulate on *Fusarium* or other hosts was described to be one of the main criteria in determining host ranges (Harveson and Kimbrough 2001). In several other contact biotrophic mycoparasitic fungi, such as *Gonatobotrys simplex, Dicyma parasitica, Stephanoma phaeospora, Melanospora destruens,* and *Piptocephalis unispora,* it was reported that

they absorb or acquire certain nutrients or growth substances, such as mycotrophein, biotin, or aneurin from the specific hosts to grow and generate sexual fruiting bodies (Hawker 1938, Jeffries and Young 1994; Rakvidhyasastra and Butler 1973, Whaley and Barnett 1963). In addition, there was significant difference between linear growth of *F. avenaceum* and *F. oxysporum* challenged with *Sphaeroes* and only *F. avenaceum* and *F. oxysporum* alone (Figure 4.2A, B). This represents the first report on *S. mycoparasitica* suppressing linear growth of and sporulating on *F. avenaceum* host. On the other hand, there was no significant difference between treated and non-treated *F. sporotrihioides* and *F. proliferatum* (Figure 4.1A, B). Effects of mycelial growth inhibition and aerial growth reduction of *F. oxysporum* were also found to be less in two out of three *S. retispora* strains (Harveson and Kimbrough 2001). Linear growth of *F. oxysporum* challenged with *Persiciospora moreaui* was reported to be not significantly different from that of *F. oxypsporum* grown singly (Harveson and Kimbrough 2000).

Since, *F. graminearum* chemotypes are fast-growing and highly virulence pathogens (when inoculated on PDA, linear mycelial growth of *F. graminearum* chemotypes were approximately 0.72±0.05 cm per day and *S. mycoparasitica* was 0.50±0.07 cm per day), therefore, we have decided to pre-inoculate the PDA plates with *S. mycoparasitica* for two days before inoculating *F. graminearum* mycelial plug. To date, there is no biotrophic mycoparasitic fungi reported to suppress on *F. graminearum.* Therefore, this is the first observations of *S. mycoparasitica* was able to parasitize and suppress *F. graminearum.*

4.6.2 Establishment of parasitism

Hook-shape and clamp-like contact structures are very common in contact biotrophic mycoparasites (Jeffries 1995; Jeffries and Young 1994). Formation of hook-like contact structures in *M. zamiae* were seen occurring on *Fusarium* and *Tritirachium*

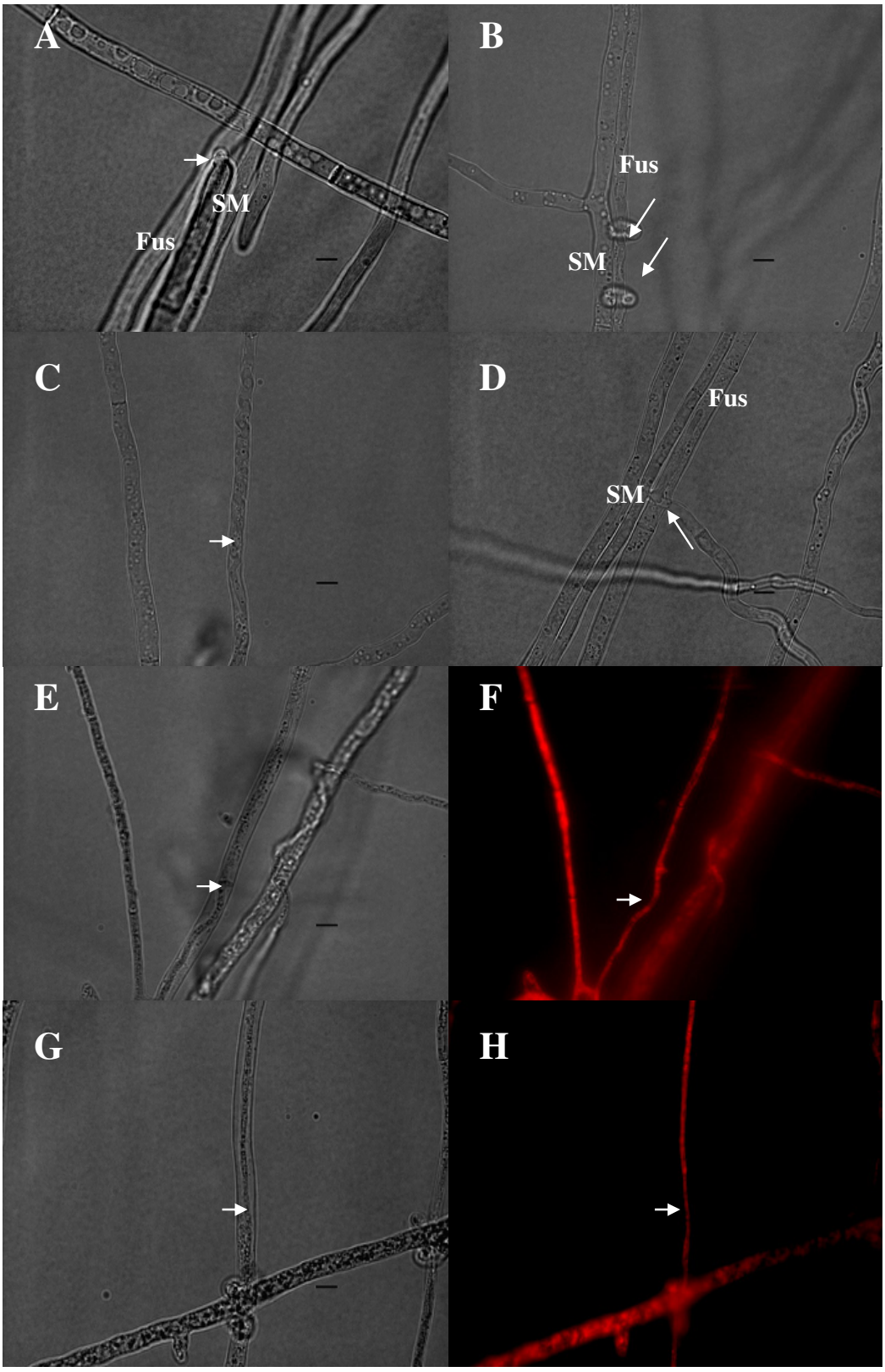
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Figure 4.6 Penetration and haustorial-like parasitism by *Sphaerodes mycoparasitica* (SM) on and inside *Fusarium* hyphal cells (*F. avenaceum* and *F. oxysporum*) (Fus) with light and fluorescent microscopic approaches. A. Beginning of penetration (arrow), B. Formation of serial clamp-like structures with intracellular haustorium (arrows), C. with coiling haustorium (arrow), D. with penetration site and beginning of haustorial growth (arrow), E and F, comparison between light and fluorescent microscopy on intracellular haustorium (arrow), G and H, comparison between light and fluorescent microscopy on intracellular parasitism and clamp-like structure (arrow). All bar scales were 5μm.

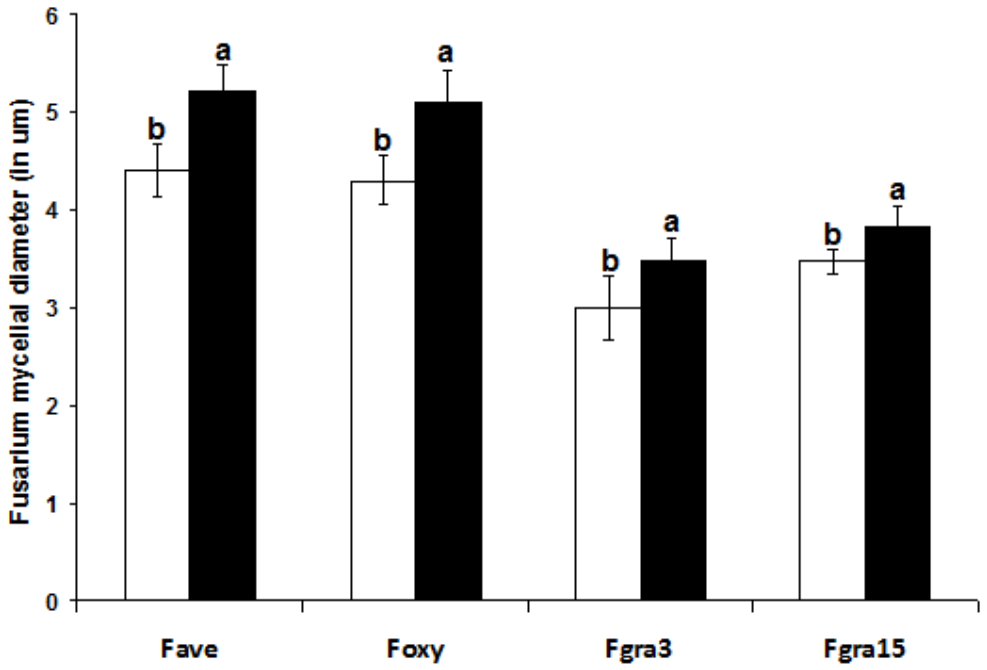

Figure 4.7 Mycelial diameters of *F. avenaceum* (Fave), *F. oxysporum* (Foxy*), F. graminearum* chemotype 3-ADON (Fgra3) and 15-ADON (Fgra15). Mycelial diameters of *Fusarium* species on slide cultures (co-culture between *Fusarium* and *Sphaerodes*) with (□) and without (■) infection/penetration of *Sphaerodes* biotrophic mycoparasite. Data are means and standard deviations. Bars with the same lowercase letter are not significant different between the sizes of infected (□) and non-infected (■) mycelia for two different *Fusarium* strains at $P = 0.05$, with T-test. All *Fusarium* species were analyzed separately.

species (Jordan and Barnett 1978), as were the both hook- and clamp-like structures in *Stephanoma phaeospora* on *Fusarium, Cladosporium* and other ascomycetes (Rakvidhyasastra and Butler 1973), and hook-shaped contact cells in *Olpitrichum tenellum* on *Fusarium, Alternaria* and *Cladosporium* species (Li and Shen 1996). These structures were also observed in this study, but only by *S. mycoparasitica* on *F. oxysporum, F. avenaceum,* and two *F. graminearum* hosts (Figure 4.4, 4.5, 4.6). Furthermore, we also found braid-like structures as parts of the interactions, for which the function is uncertain (Figure 4.4D). With all these structures and contact cells that were corresponding to the previous findings, we concluded that *S. mycoparasitica* is a contact biotrophic mycoparasite of *F. avenaceum, F. oxysporum* and *F. graminearum*.

In addition, during the interactions with *F. graminearum* chemotype 3, *Sphaerodes mycoparasitica* was observed to remove red-complexes or compounds from *Fusarium* cells and released as crystal-like red coloured substances. The real mechanisms are unknown. This could be due to the capacity of *S. mycoparasitica* to neutrilize compounds produced by *F. graminearum* with fungitoxic effects. A zearalenone mycotoxin biodegradation mechanism was found in mycoparasitic *Gliocladium roseum.* Detoxification of zearalenone is achieved through the production of a lactonase catalytic enzyme that involved in zearalenone hydrolysis (Utermark and Karlovsky 2007). This enzyme was also proposed to protect *Gliocladium* mycoparasite from toxigenic effects of zearalenone. *Fusarium graminearum* chemotype 15 was observed to form irregular mycelia and morphological alteration at the contact zone when challenged with *S. mycoparasitica* (Figure 4.5J). These phenomena were reported by Maddau et al. (2005) in the interactions between beneficial endophytes and Oak pathogenic fungi. As similar phenomenon – the deformation of mycelia and hyphae

was also observed in *F. oxysporum* pathogens when challenged with antagonistic bacteria (Chaurasia et al. 2005).

4.6.3 Haustorial-like mycoparasitism

Haustorial biotrophic mycoparasitism was first reported in *Piptocephalis fresiniana* by Brefeld in 1872, which was also given the name balanced mycoparasitim (Boosalis 1964; de Bary 1887). Most of the research related to haustorial biotrophs has been concentrated on Zygomycetous fungi and Basidiomycetes. There is less information about haustorial biotrophic mycoparasitism in the phylum of Ascomycota. For instance, ascomycetous *Sporidesmium sclerotivorum* with haustorium-like infection organs produced on *Sclerotinia minor* (Bullock et al. 1986) and *Teratosperma oligocladium* with haustorial formation on sclerotial fungal hosts (Ayers and Adams 1981). Frequently, *Fusarium* host hyphal cells infected by *S. mycoparasitica* were found to reduce hyphal diameter, and induce cytoplasm degeneration (Figure 4.5, 4.6, 4.7). These characteristics are similar to haustorial biotrophic mycoparasitic *P. virginiana* with *Mycotypha microspora* (Armentrout and Wilson 1969). Penetration of host by biotrophic mycoparasite was proposed to be caused by mechanical and enzymatic mechanisms. Furthermore, strict regulation control of the chitinase and chitosanases lytic enzymes was reported to be characteristic of biotrophs (Manocha 1987). These enzymes are very crucial in degrading and playing important roles in penetration of the fungal host by biotrophic mycoparasites (Jollès and Muzzarelli 1999). The cell wall chitins might be degraded by the chitinases or lytic enzymes generated by *Sphaerodes* mycoparasite. Therefore, this might be the potential explanations to why penetrated *Fusarium* host cells were not illustrating high intensity of fluorescent property. In addition, lactofuchsin, cotton blue, and other dyes are commonly used in staining fungal samples for microscopy examinations and also useful in staining or

binding on fungal cell walls and chitin that found in cell walls (Shivas and Beasley 2005). This was concord to the descriptions in paper by Bushnell (1972). Bushnell (1972) also described that some haustorial hyphae were able to form coiling inside the host cells. Therefore, *Sphaerodes mycoparasitica* could be biotrophic mycoparasite that is able to establish haustorial-like parasitic relationship with *F. avenaceum, F. oxysporum* and *F. graminearum.*

5. QUANTIFICATION OF *SPHAERODES MYCOPARASITICA* BIOTROPHIC MYCOPARASITE USING GENUS SPECIFIC REAL-TIME PCR FOR BIOCONTROL OF *FUSARIUM GRAMINEARUM*

5.1 Abstract

Sphaerodes mycoparasitica was observed to improve wheat seedling and growth significantly compared to necrotrophic mycoparasites under *in vitro* assays. *Trichoderma* species were found to suppress seed germination and growth. During the Phytotron studies, both *S. mycoparasitica* and *T. harzianum* were able to improve wheat growth. In real-time polymerase chain reaction (RT-PCR) with genus-specific primer sets, both *S. mycoparasitica* and *T. harzianum* were found to reduce the amount of *F. graminearum* DNA in the roots when challenged together with *F. graminearum* 3-ADON chemotype. The amount of *S. mycoparasitica* DNA was relatively stable in the roots; however, the amount of *T. harzianum* DNA was reduced in the presence of *F. graminearum. Sphaerodes mycoparasitica* was able to promote wheat growth and protect plant from *F. graminearum* under *in vitro* and controlled conditions.

5.2 Introduction

Most of the *Fusarium* species are pathogenic to plants and these phytopathogens are responsible for major economically important crops diseases (Bai and Shaner 2004; Sutton 1982). *Fusarium* diseases are not only found in Canadian wheat fields, this group of pathogens can also infect barley, canola, asparagus, and some other crops in Canada (Calman et al. 1986; Fernandez et al. 2007b; Vujanovic et al. 2002; Yergeau et al. 2005). *Fusarium* species are commonly reported to cause crown and root rot diseases, Fusarium Head Blight (FHB), Fusarium damaged kernels (FDK), and Fusarium wilt in wheat (Mavragani 2008). FHB is one of the most threatening diseases caused by *Fusarium* species, especially *F. graminearum, F. culmorum,* and *F.*

pseudograminearum (Fernandez et al. 2007a; Fernandez and Jefferson 2004). Recently, *F. graminearum* strains are replacing other *Fusarium* species and becoming more dominant in Canada, particularly in the western praires (Ward et al. 2008). In a recent study by Ward and associates (2008), they proposed that *F. graminearum* chemotype 3 (3-ADON-producer) is the most aggressive and virulent strain in Canada, as well as replacing other dominant *Fusariums* species. These pathogenic fungi are observed to show a high capability in reducing yields of wheat production, inhibiting seed germination, and affecting the quality of grains (Mavragani 2008; Sutton 1982; Ward et al. 2008). Besides affecting the quality of crops and grains, the trichothecene mycotoxins are also reported to be a threat to humans and animals health concerns – neurotoxicity, severe toxiocoses, vomiting, and immunosuppression (Edwards et al. 2001; Leslie and Summerell 2006; Lutz et al. 2003; Vasada and Hsieh 1987). Therefore, it is important to control the outbreaks of *Fusarium* diseases or reduce the effects of *Fusarium* inoculum on crops, especially wheat.

Biological control is proposed as a promising, environmentally solution (Pal and McSpadden 2006) and practical option against *Fusarium* pathogens (Vujanovic 2008). Mycoparasites have been reported and suggested to be potential and useful biocontrol agents or candidates for managing many different fungal pathogens (Cortes-Penagos et al. 2007; Jeffries and Young 1994; Viterbo et al. 2007). However, there are very few biotrophic mycoparasitic biocontrol agents available for controlling *Fusarium* pathogens (Cortes-Penagos et al. 2007; Howell 2003; Jeffries 1995; Paulitz and Bélanger 2001). Recently, *Sphaerodes, Melanospora* and *Persiciospora* biotrophic mycoparasitic strains have been proposed to be potential biocontrol candidates for plant pathogenic fungi, in particular *F. oxysporum* (Harveson and Kimbrough 2000; 2001a; 2001b; Zhang and Blackwell 2002). Later, *Sphaerodes* species were described to pose greater inhibition

effects on *F. oxysporum* under field studies and were suggested as better biocontrol candidates compared to other pyrenomycetous biotrophs (Harveson and Kimbrough 2001b; 2002). *Sphaerodes mycoparasitica* was identified and described as new *Sphaerodes* biotrophic mycoparasite which is able to colonize and control various pathogenic *Fusarium* species (Appendix A, Chapter 4). It is known that *Sphaerodes* biotrophic and *Trichoderma* necrotrophic mycoparasitic fungi are able to parasitize on *Fusarium* pathogenic strains and proposed to be potential biocontrol agents. However, effects of *S. mycoparasitica* and *T. harzianum* on spring wheat seedlings under *in vitro* assays as well as under controlled conditions have never been tested. Therefore, *S. mycoparasitica* was selected and included in the mycoparasitic *Sphaerodes-F. graminearum*-spring wheat tritrophic interactions studies.

The quantitative real-time Polymerase Chain Reaction (PCR) approach has been employed to study fungal-fungal-plant, fungal-plant or fungal-fungal interactions and is considered as a very useful and fast quantitative method for quantifying the amounts of specific fungal DNAs in plants or substrates (Gachon et al. 2004). For instance, RT-PCR was utilized to quantify amounts of *Fusarium* pathogen and mycorrhizae in bean plants for studying their relations with plants (Filion et al. 2003) and this technique was used to assess colonization of arbuscular mycorrhizal (AM) fungus in plant roots (Isayenkov et al. 2004). Since the tritrophic *Sphaerodes-Fusarium*-wheat interactions are very important to explore, RT-PCR was used to analyze amounts of pathogenic and beneficial fungi in spring wheat roots under controlled conditions. Furthermore, the effects of these fungi on spring wheat growth were also evaluated.

5.3 Objectives and hypothesis

First hypothesis was that potential biocontrol agents – both biotrophic and necrotrophic might show beneficial effects on spring wheat seedlings under *in vitro*

assay. The second hypothesis was that both biotrophic (*S. mycoparasitica*) and necrotrophic (*T. harzianum*) are able to improve wheat seeds germination and growth when inoculated together with *Fusarium* pathogen under controlled conditions. The third hypothesis was these potential biocontrol agents are able to reduce amounts of *Fusarium* DNA in wheat through suppressing the growth or colonization of *Fusarium* on wheat roots.

The objectives of this study were to study: (1) effects of biotrophic and necrotrophic mycoparasites as well as *Fusarium* pathogens on wheat seedlings; (2) interactions between *Sphaerodes*-*Fusarium*-wheat roots; (3) ability of *Sphaerodes* to control *Fusarium* pathogen from infecting wheat. In order to understand these, quantitative real-time PCR was used to quantify amounts of *Sphaerodes, Trichoderma,* and *Fusarium* DNAs in spring wheat under controlled conditions.

5.4 Materials and methods

5.4.1 Fungal strains and growth conditions

Two biotrophic mycoparasitic fungi (*Sphaerodes mycoparasitica* SMCD 2220 and *Melanospora* sp. SMCD 2021); two necrotrophic mycoparasitic *Trichoderma* strains (*T. harzianum* T-22 SMCD 2166 and *T. viride* SMCD 2300); and six phytopathogenic *Fusarium* species (*F. graminearum* chemotype 3 SMCD 2243, *F. graminearum* chemotype 15 SMCD 2244, *F. sporotrichioides* SMCD 2245, *F. avenaceum* SMCD 2241, *F. oxysporum* SMCD 2242, and *F. proliferatum* SMCD 2246) were obtained from the Saskatchewan Microbial Collection and Database (SMCD). These fungal cultures were grown on potato dextrose agar (PDA) supplemented with antibiotics (Reisher et al. 2004) prior to the study. Only *S. mycoparasitica, T. harzianum* T-22 and *F. graminearum* chemotype 3 were selected for tritrophic interactions later in the study.

5.4.2 *In vitro* assays

Spring wheat seeds (*Fusarium* susceptible variety CDC-Teal 2001), kindly provided by Dr. PJ Hucl (Crop Development Centre, University of Saskatchewan) were surface sterilized according to the procedures outlined in Vujanovic et al (2009). An *in vitro* assay was carried out by placing 0.5-cm^2 agar plugs with mycelia of the *Fusarium* pathogens, the biotrophic and necrotrophic mycoparasitic fungi individually onto surface-sterlized spring wheat seeds placed on PDA. Non-inoculated surface-sterilized spring wheat seeds were used as positive control. Five seeds per Petri plate and three replicates per treatment were employed. All experiments were repeated twice. Inoculated and non-inoculated plates were incubated at 22^oC for one week in darkness. After 7 days of inoculation, percentages of seed germination, lengths of the shoot and root were measured and recorded.

5.4.3 PCR primers and primers designing

A specific PCR primer set (SmyITSF/R) was developed in this study to quantify *Sphaerodes mycoparasitica* in real-time PCR quantification assays (SmyITSF: 5'-TCA TGG CTC TGC CAA CCC TGT GAA-3' and SmyITSR: 5'-AAT GCA GGG CAC AGA GGA CAT CG-3'). *F. graminearum* (Fg16NF/R) and *T. harzianum* (TGP4-F/R) specific primer sets used in this study were proposed by Nicholson et al. (1998) and Kim and Knudsen (2008), respectively. The SmyITS primer set was designed based on ITS regions. A total of 20 ITS1F, 5.8S small-subunit rDNA, 28S large-subunit rDNA, and ITS4 *Fusarium* sequences were either obtained from GenBank or sequenced in this study. Twenty *Fusarium* sequences were aligned with sequences from *Sphaerodes* and *Trichoderma* species, and *Sphaerodes*-specific primer sets for PCR detection and real-time PCR assay were designed based on non-consensus regions of sequences using BioEdit (Hall 1999). Four candidate primer sets were designed and tested for specificity for *Sphaerodes mycoparasitica. Sphaerodes mycoparasitica, T. harzianum,* and *F.*

graminearum chemotype 3 were used to assess specificity for PCR amplification. Initial amplifications were performed in a 25 µl reaction mixture (as described Appendix A) by using Thermal Cycler epgradient S (Eppendorf, Mississauga, ON). PCR conditions for ITS primer set was (i) 3 min at 94ºC, (ii) 30 cycles of 30 s at 94ºC, 30 s at 55ºC and 1 min at 72ºC, and (iii) 10 min at 72ºC (Sokolski et al. 2004). Amplified PCR products were run on a 1.0% agarose gel electrophoresis containing ethidium bromide. One primer set (SmyITSF/R) was found to successfully amplify *Sphaerodes* genomic DNA. PCR products amplified with this primer set were purified and sent for sequencing as outlined in Appendix A. The sequences were aligned and checked with the original *Sphaerodes* sequences.

5.4.4 Validation of *Sphaerodes*-specific primer set

To evaluate *Sphaerodes*-genus specific primer set for conventional PCR detection, genomic DNAs from *S. mycoparasitica*, six *Fusarium* species (*F. graminearum* chemotype 3, chemotype 15, *F. avenaceum, F. oxysporum, F. proliferatum,* and *F. sporotrichioides*; nine different ascomycetous fungal isolates (*Cladosporium cladosporioides, C. minourae, Cenococcum geophilum, Penicillium aurantiogriseum, P. bilaai, Alternaria alternata, Alternaria* sp., *Trichoderma harzianum,* and *T. viride*); two zygomycete fungi (*Mortierella hyaline* and *Mortierella* sp.), and three basidiomycetous fungal strains (*Laccaria laccata, L. trichodermophora,* and *Suillus tomentosus*) were used.

5.4.5 Optimization of SmyITSF/R primer set for real-time PCR assay

For *Sphaerodes*-specific primer set, annealing temperatures tested ranged from 55 to 72ºC with genomic DNAs from *Sphaerodes, T. harzianum,* and two different *F. graminearum* chemotypes. At the end, 66ºC was chosen as the annealing temperature. Using real-time PCR, temperatures lower than 66ºC showed non-specific or unintended

binding towards DNA from *Fusarium* strains. The finalized real-time PCR conditions for SmyITSF/R primer set was (i) 3 min at 94ºC, (ii) 30 cycles of 30 s at 94ºC, 30 s at 66ºC and 1 min at 72ºC, and (iii) 10 min at 72ºC. For TGP4-F/R and Fg16NF/R primer sets; real-time PCR conditions were outlined in Kim and Knudsen (2008) and Nicholson et al. (1998), respectively. Real-time PCR amplifications of genomic DNAs (for *S. mycoparasitica, T. harzianum,* and *F. graminearum*), and total DNA extracted from spring wheat roots harvested at the mid-seedling growth (Zadok's growth stage 13) (Zadoks et al. 1974) were carried out in MiniOpticon (Bio-Rad). All real-time PCR reactions were performed using real-time PCR MJ white tubes (Bio-Rad) with a total volume of 25 µl. The reaction mixture for all real-time PCR assays included 12.5 µl of IQ Supermix (Bio-Rad), 1 µl of each 10 µM forward/reverse primers (Invitrogen), 3.4 µl of BSA (Bovine Serum Albumin) (1.47 µg/µl) (Ishii and Loynachan 2004), 6.1 µl of sterilized UltraPure Millipore water, and 1 µl of DNA template.

5.4.6 Standards

Standard curves for *Sphaerodes*-, *Trichoderma*-, and *F. graminearum*-specific primer sets were generated based on threshold cycles (Ct) using a series of 10-fold diluted genomic DNAs from *S. mycoparasitica* (spanning from 3.8×10^{2} to 3.8×10^{-2} ng/µl), *T. harzianum* (from 7×10^{2} to 7×10^{-2} ng/µl), and *F. graminearum* (from 2.7×10^{3} to 2.7×10^{-1} ng/µl). Negative control was run with autoclaved UltraPure Millipore water. Diluted genomic DNAs and negative controls were run in triplicate. Linear regression graphs, equations, and r^2 values were generated and calculated by plotting Ct values against log-transformed DNA amounts (in ng/µl) of each series of 10-fold diluted fungal DNA.

5.4.7 Growth conditions and fungal inoculation

Five mycelial plugs from *S. mycoparasitica, T. harzianum,* and *F. graminearum* were cut, transferred and grown in three separate 500 mL sterile flasks each with 100 mL of PDB (potato dextrose broth) shake cultures for 14 days at room temperature (23ºC). After 14 days of incubation in PDB medium, fungal cultures were filtered through a Whatman No. 1 filter paper to remove the liquid medium aseptically. Mycelia were transferred to 50 mL sterile Falcon tubes with 20 sterile glass beads and 40 mL of autoclaved distilled water. Subsequently, the Falcon tubes filled with mycelia were vortexed vigorously for 1 min to separate the mycelia into smaller pieces. Mycelial suspension was filtered through 2 layers of cheesecloth to remove the glassbeads and bigger mycelial clumps. The flow-through was then used as mycelial suspension stock (10^0) for serial dilution. Stock of mycelial suspension was further diluted into a series starting from 10^{-2} to 10^{-4}. These dilution series were plated on PDA using the pour plate method – serial diluted suspensions were loaded onto the plates, then PDA was poured into the plates and mixtures of both mycelial suspension and PDA was swirled. Number of CFU (colony forming unit) was counted and recorded. Mycelial suspensions were adjusted with sterile water to $10^5 – 10^6$ CFU/mL for *S. mycoparasitica* and *T. harzianum*, and to $10^4 – 10^5$ CFU/mL for *F. graminearum.*

Quantification of mycoparasite-pathogen-wheat root interactions was conducted on spring wheat CDC-TEAL 2001. Wheat plants were grown in pots (4 x 4 x 16 cm) with 10 g of different soil layers. All the seeds were surface-sterilized prior to planting as outlined in Vujanovic et al. (2009). The pots were lined with filter paper and packed with 6 g of ProMix soil (Sun Gro Horticulture, BC). This layer was then overlayed with Layer B (1g) (either ProMix soil homogenized with ~ 5 to 6 x 10^4 CFU of *F. graminearum* mycelial suspension or ProMix soil with water only). Layer B was followed by Layer A (1g) (either Sunshine® peat moss homogenized with ~ 5 to 6 x 10^5

CFU of *S. mycoparasite* or *T. harzianum* mycelial suspension or with water suspension only). Six spring wheat seeds were then sowed on Layer A and topped with 2 g of Sunshine® peat moss only (Figure 5.1). Spring wheat plants were grown under a 16-h photoperiod (22ºC day/15ºC night) with light intensity of 250 µmol m^{-2} s^{-1}, watered every 2 d, and fertilized every 14 d using 1300 ppm of NPK (20-20-20) fertilizer (Fernandez and Chen 2005; Lawrie et al. 2006). All treatments in the experiment were with three replicates and repeated twice.

At the mid- seedling growth (Zadok's growth stage 13) (Zadoks et al. 1974), wheat plants with their roots were removed from the pots and washed under running tap water to remove all soil particles. Washed roots were dried with filter papers. Number of germinated seeds, total biomass, root biomass, total length, and root length were counted and measured. Percentage of seed germination was calculated with the following formula: (Number of germinated seeds in particular treatment/number of germinated seeds in control treatment) x 100%. The roots were then subjected to total DNA extraction with DNeasy Plant Mini Kit (Qiagen Inc., Mississauga, ON). Extracted total DNAs from roots of different treatments were employed in real-time PCR quantification.

5.4.8 Statistical analyses

Percentages of seed germination, lengths of the leaves and roots under *in vitro* studies were analyzed using ANOVA – LSD test at $P = 0.05$. For Phytotron studies, root biomass (g), total biomass (g), root length (cm), total length (cm), seed germination (%); and *S. mycoparasitica, T. harzianum,* and *F. graminearum* genomic DNA quantification from the roots of spring wheat plants were analyzed using analysis of variance (ANOVA). Log_{10} transformations were carried out whenever required to meet the ANOVA requirements (Lehman 1975). Multiple comparisons for more than two samples were analyzed by Tukey's studentized range test at $P = 0.05$ (SPSS 1990)

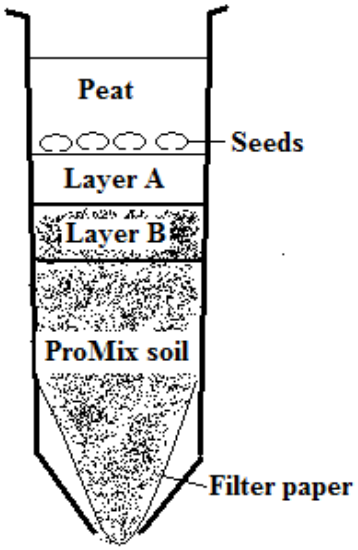

Figure 5.1 Diagram for growing wheat plants in a pot (4 x 4 x 16 cm) with different layers of soils.

(Filion et al. 2003).

5.5 Results

5.5.1 *In vitro* assays

Spring wheat seeds inoculated with *Sphaerodes mycoparasitica* were observed to have significant higher germination percentage (> 90%) and longer root and shoot (> 4.5 cm) compared to control and other potential biocontrol agents (Figure 5.2). *Trichoderma* species did show some degree of suppression in seed germination and growth (Figure 5.2). Percentages of seed germination, shoot, and root length were significantly lower for seeds inoculated with *Fusarium* spp. than control (Figure 5.3).

5.5.2 Validation of the *Sphaerodes*-specific primer set

SmyITSF/R primer set was tested with *S. mycoparasitica*, seven *Fusarium* species, night different ascomycetous fungal isolates two zygomycete fungi, and three basidiomycetous fungal strains. This primer set only amplified *S. mycoparasitica* (Figure 5.4).

5.5.3 Standard curves

The standard curves based on known diluted concentrations of DNAs from *S. mycoparasitica, F. graminearum,* and *T. harzianum* were constructed (Figure 5.5 and 5.6A, B). In current study, standard curves were achieved using a series of 10-fold diluted DNA spanning from 3.8×10^{2} to 3.8×10^{-2} ng for *S. mycoparasitica,* 2.7×10^{3} to 2.7×10^{-1} ng for *F. graminearum* chemotype 3, and 7.0×10^{2} to 7.0×10^{-2} ng for *T. harzianum.* Quantification demonstrated linear relation ($r^2 = 0.999$ for *S. mycoparasitica,* $r^2 = 0.998$ for *F. graminearum*, and $r^2 = 0.996$ for *T. harzianum*) between $\log_{10}$ of fungal genomic DNA (in ng/µl) and real-time PCR threshold cycles (Ct) (threshold fluorescence signal of 0.025 was used for all three fungal isolates) (Figure 5.5 and 5.6A, B). The sigmoidal curves for different diluted *S. mycoparasitica*

DNAs (3.8×10^{2} to 3.8×10^{-2} ng) were illustrated in Figure 5.7.

5.5.4 Wheat growth and fungal inoculation

Root biomass, total biomass, root length, total length, and seed germination of *F. graminearum* infected spring wheat were significantly increased with the treatments of *S. mycoparasitica* and *T. harzianum,* as compared to inoculation with *F. graminearum* alone (Table 5.1). These were further confirmed by using quantification real-time PCR to evaluate the quantity of *S. mycoparasitica, F. graminearum,* and *T. harzianum* DNAs in the roots of spring wheat challenged with different treatments (Figure 5.8-5.10). Amounts of *F. graminearum* DNA detected in the treatments with *S. mycoparasitica* and *T. harzianum* were significantly reduced (Figure 5.8). On the other hand, for the amount of *S. mycoparasitica* DNA detected there was no significantly difference between wheat inoculated with *F. graminearum* and without *Fusarium* (Figure 5.9). Amounts of *T. harzianum* DNA detected in the treatment inoculated with *F. graminearum* was observed to be reduced significantly, as compared to non-*Fusarium* treatment (Figure 5.10).

5.6 Discussion

During *in vitro* studies, *Trichoderma* species were observed to suppress seed germination. The result agreed with the findings of *T. viride* to be suppressive to cucumber, tomato, and pepper seedlings, and to reduce growth of these seeds (Menzies 1993). This could be due to gliotoxin and viridin produced by *Trichoderma* species. These mycotoxins or metabolites were found to inhibit root growth and seed germination of mustard seeds (Bailey and Lumsden 1998; Fravel 1988). 6-pentyl-α-pyrone, generated by *T. harzianum* and *T. viride,* and Koninginin A, produced by *T. koningii* were described to inhibit wheat coleoptile etiolation and growth (Cutler et al. 1986; Cutler et al. 1989). Apart from *Trichoderma* species, several other beneficial

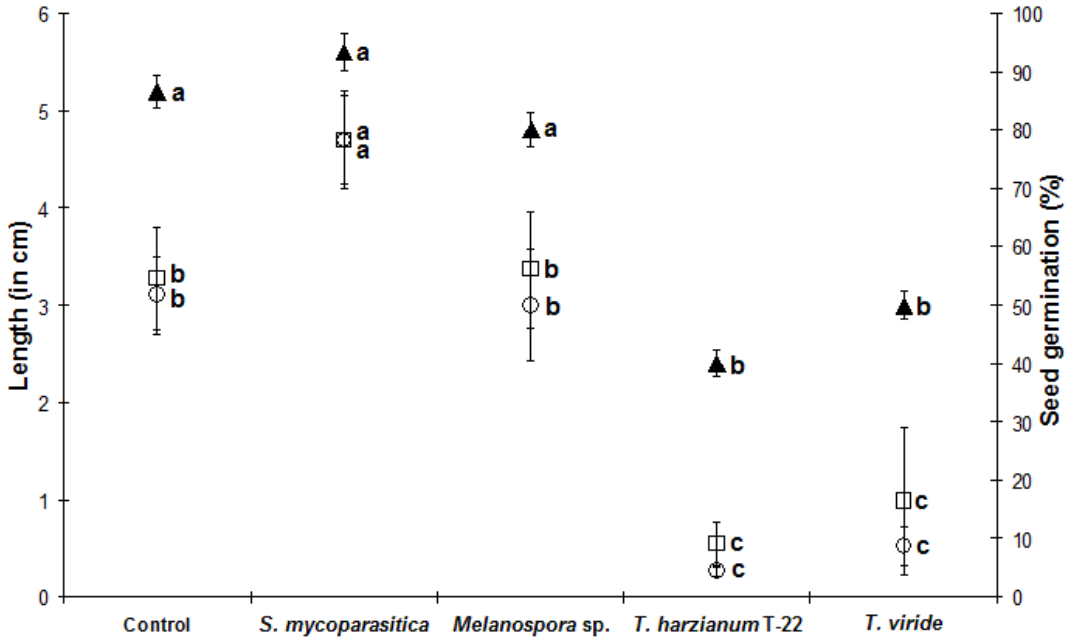

Figure 5.2 Seed inoculation assays for examining seed germination (▲) and growth – shoot (□) and root (○) to compare between biotrophic *Sphaerodes / Melanospora* and necrotrophic *Trichoderma* species. Means of seed germination (%), shoot and root length (in cm) were analyzed separately.

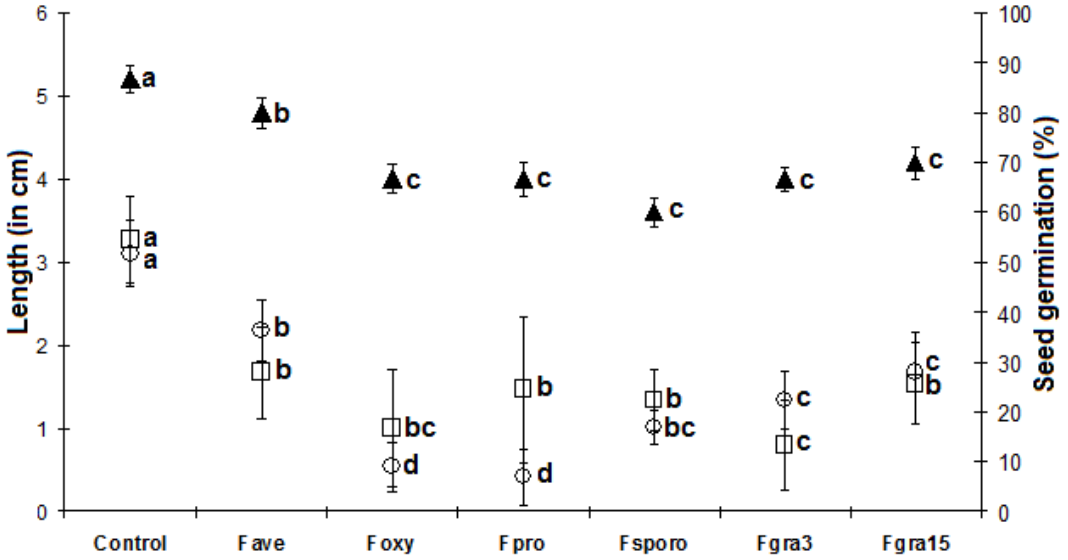

Figure 5.3 Seed inoculation assays for examining seed germination (▲) and growth – shoot (□) and root (○) to compare between pathogenic *Fusarium* species. Means of seed germination (%), shoot and root length (in cm) were analyzed separately. Legends: Fave – *F. avenaceum,* Foxy – *F. oxysporum,* Fpro – *F. proliferatum,* Fsporo – *F. sporotrichioides,* Fgra3 – *F. graminearum* 3-ADON chemotype, and Fgra15 – *F. graminearum* 15-ADON chemotype.

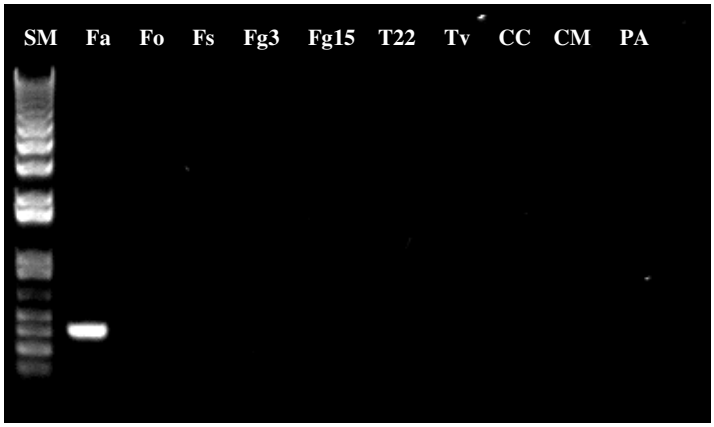

Figure 5.4 SmyITSF/R primers amplified PCR products for *S. mycoparasitica* (SM), five *Fusarium* strains (Fa = *F. avenaceum,* Fo = *F. oxysporum,* Fs = *F. sporotrichioides,* Fg3 = *F. graminearum* chemotype 3, and Fg15 = *F. graminearum* chemotype 15), two *Trichoderma* species (T22 = *T. harzianum* T22 and Tv = *T. viride*), two *Cladosporium* species (CC = *C. cladosporioides* and CM = *C. minourae*), and *Penicillium aurantiogriseum* (PA) were electrophoresed on 1% agarose gel at 100 V for 20 minutes. The size of the band is around 300 to 400 bp.

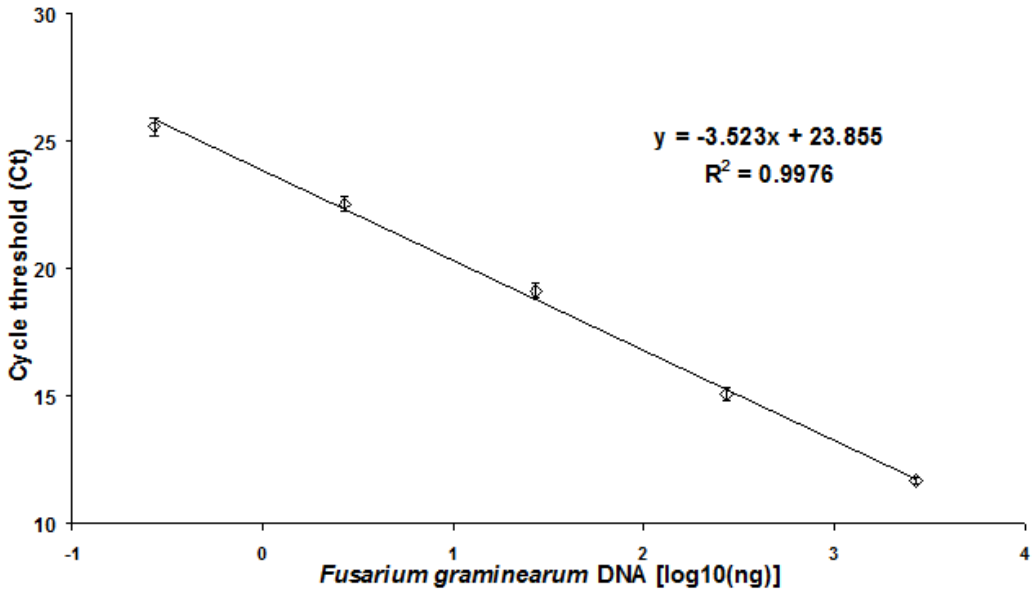

Figure 5.5 Standard curves for *Fusarium graminearum* chemotype 3 (in the range of 2.7 x 10^{3} to 2.7 x 10^{-1} ng in ten-hold decreasing manner)

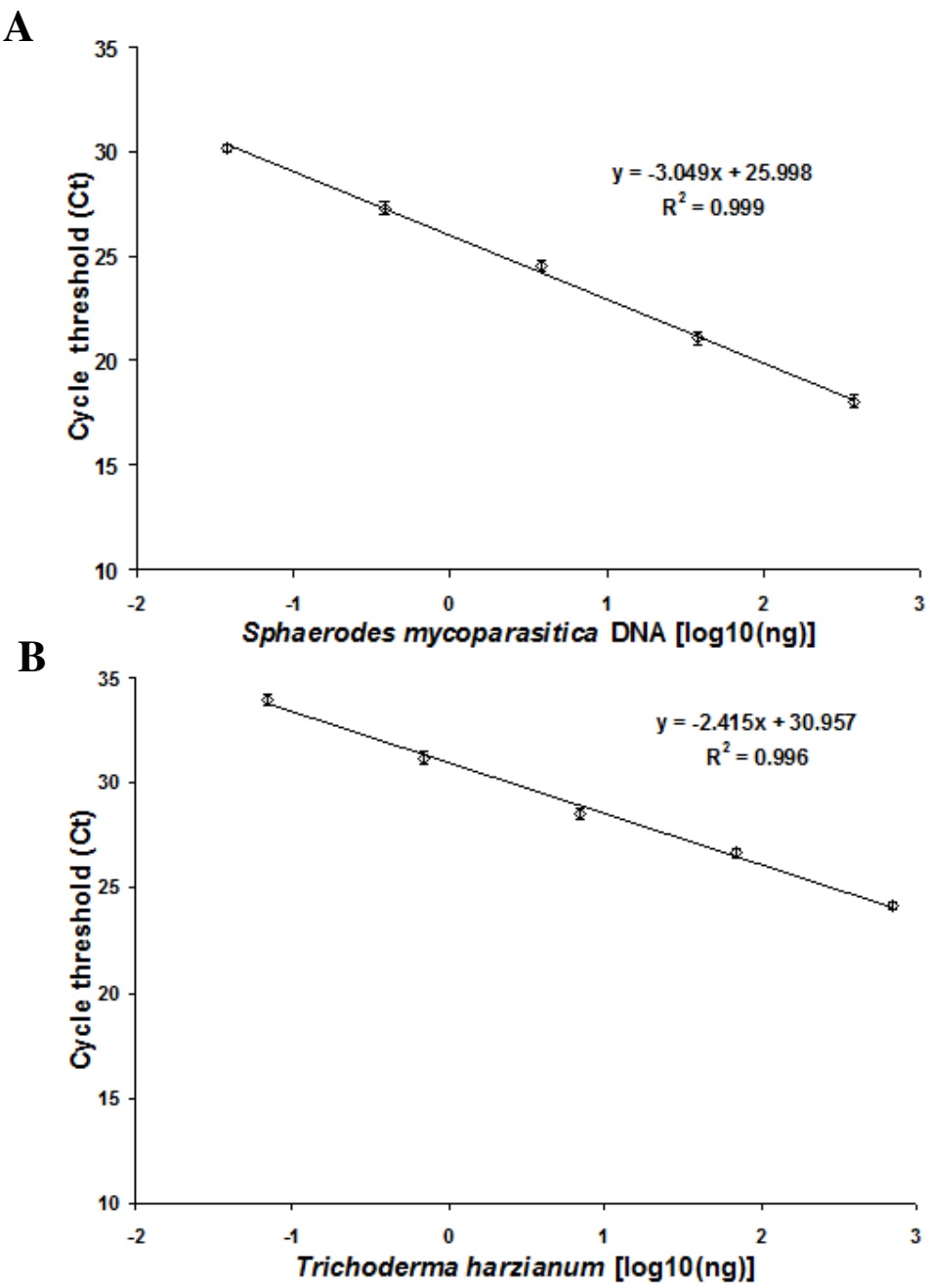

Figure 5.6 Standard curves for (A) *Sphaerodes mycoparasitica* (in the range of 3.8 x 102 to 3.8 x 10-2 ng in ten-hold decreasing manner) and (B) *Trichoderma harzianum* T-22 (in the range of 7.0 x 10^{2} to 7.0 x 10^{-2} ng in ten-hold decreasing manner).

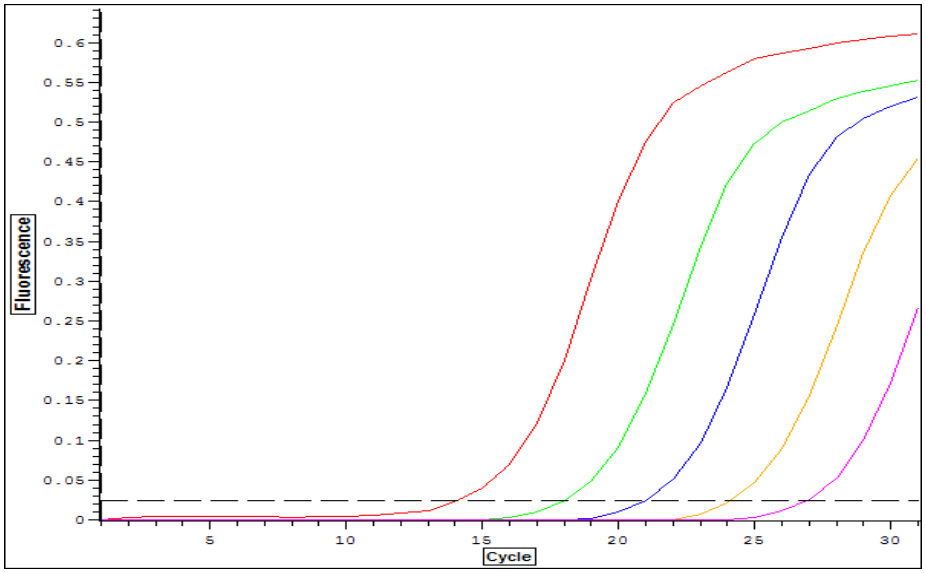

Figure 5.7 Sigmoidal curves for *Sphaerodes mycoparasitica* (with 0.025 fluorescence line) (in the range of 3.8 x 10^{2} to 3.8 x 10^{-2} ng in ten-hold decreasing manner).

Table 5.1 Effects of S. *mycoparasitica, T. harzianum,* and *F. graminearum* inoculation treatments on root biomass (g), total biomass (g), root length (cm), total length (cm) and seed germination (%) of spring wheat plants.

Treatment	Root biomass (g)	Total biomass (g)	Root length (cm)	Total length (cm)	Seed germination (%)
Control	0.27 ± 0.06 b	0.56 ± 0.07 b	9.63 ± 0.74 b	31.88 ± 2.39 c	NA
***S. mycoparasitica* (SM)**	0.32 ± 0.03 ab	0.69 ± 0.10 a	13.88 ± 1.11 a	38.13 ± 2.76 ab	101 ± 2 a
***T. harzianum* (T-22)**	0.33 ± 0.05 a	0.73 ± 0.04 a	14.13 ± 1.03 a	41.13 ± 2.23 a	103 ± 2.1 a
***F. graminearum* (Fgra)**	0.18 ± 0.03 c	0.38 ± 0.05 c	7.88 ± 1.89 c	23.75 ± 1.98 d	31.25 ± 1.4 c
SM-Fgra	0.27 ± 0.03 b	0.54 ± 0.05 b	12.13 ± 1.35 ab	32.13 ± 3.91 bc	87.25 ± 2 b
T22-Fgra	0.28 ± 0.04 b	0.57 ± 0.04 b	13.88 ± 1.73 a	35.63 ± 1.98 bc	87.5 ± 2 b

* Average of seed germination for the control treatment was used to compare with values obtained from other treatments.

ǂ Values are the means of six replicates ± standard deviation of the mean. Values followed by same letters within each column are not significantly different using Tukey's studentized range test ($P < 0.05$).

antagonistic fungi – *Penicillium* spp., *Chaetomium* sp. and *Aspergillus* sp. were also found to decrease capacity of plant seeds germination (Fravel 1988). However, *Sphaerodes* biotrophic mycoparasite was reported to improve plant dry weight and decrease plant mortality when infected with *Fusarium* pathogen (Harveson et al. 2002). In our study, we also found that *S. mycoparasitica* is able to enhance seed germination and growth (Figure 5.2). Under *in vitro* conditions, *S. mycoparasitica* was found to promote better seedlings growth compared to *Trichoderma* species. All *Fusarium* species were observed to be pathogenic to spring wheat seeds. Therefore, *S. mycoparasitica* was selected for further studies.

Pathogenic and toxigenic *Fusarium graminearum* strains are well-known causal agents for many economical important plant diseases (Bai and Shaner 2004). They were reported to cause seedling blight, pre- and post-emergence damping off, FHB, Fusarium crown and root rots, and Fusarium FDK (Dal Bello et al. 2002; Mavragani 2008; Leslie and Summerell 2006). These diseases are able to reduce seed germination, seedling growth, yield, and grain quality (Dal Bello et al. 2002; Sutton 1982). In this study, amounts of genomic DNA of *F. graminearum* were found to be significantly reduced in spring wheat roots when inoculated with *Sphaerodes* and *Trichoderma* (Figure 5.7). Furthermore, seed germination, root biomass, total biomass, and root length were significantly increased when treated with biotrophic or necrotrophic mycoparasite (Table 5.1). These results indicate that *S. mycoparasitica* provided protection through mycoparasitism and triggered changes in wheat plants for controlling and suppressing colonization or spread of *F. graminearum.* This is the first report on *Sphaerodes* taxon as potential biocontrol agent against mycotoxigenic *F. graminearum* under controlled conditions. In a previous study, *Sphaerodes retispora* was described to show an ability to reduce amounts of *F. oxysporum* in soil, increase plant dry weight, and protect

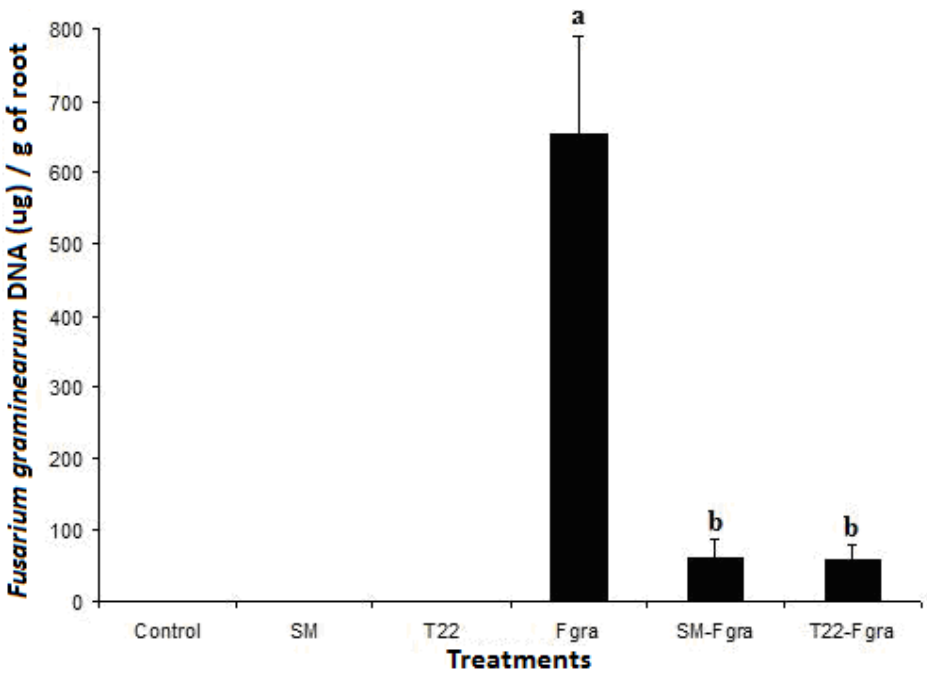

Figure 5.8 Effects of biotrophic *S. mycoparasitica* (SM), necrotrophic *T. harzianum* (T22) mycoparasites, and *F. graminearum* (Fgra) inoculation treatments on *F. graminearum* genomic DNA monitored in spring wheat roots using real-time PCR. Treatments were: Control = uninoculated control; SM = inoculated with *S. mycoparasitica* only; T22 = inoculated with *T. harzianum* only; Fgra = inoculated with *F. graminearum* only; SM-Fgra = inoculated with both *S. mycoparasitica* and *F. graminearum;* and T22-Fgra = inoculated with *T. harzianum* and *F. graminearum.* All values obtained were the means of six replicates. Error bars indicate standard deviation of the mean.

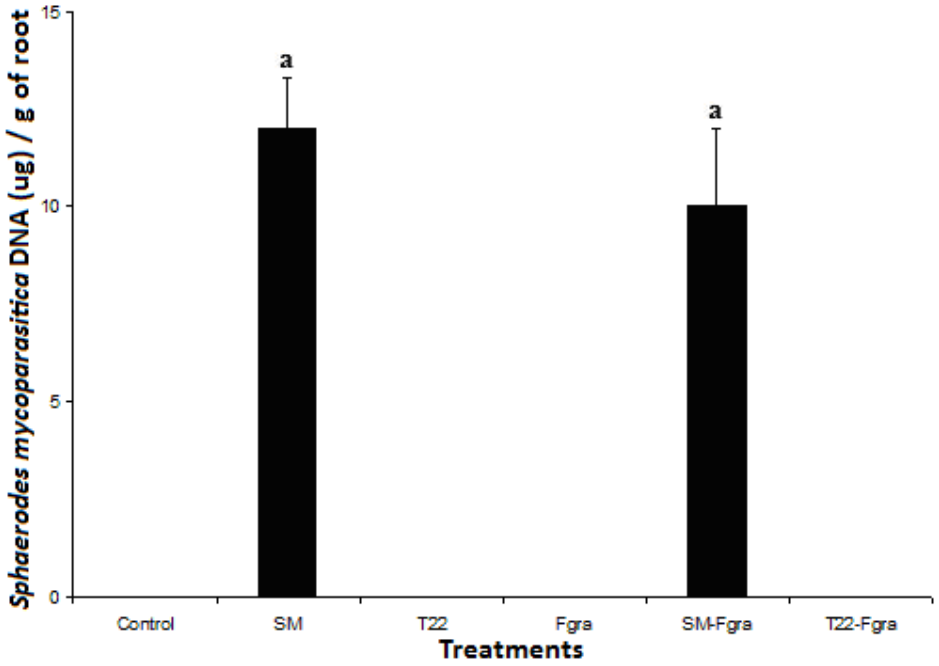

Figure 5.9 Effects of *F. graminearum* (Fgra) and *S. mycoparasitica* (SM) inoculation treatments on *S. mycoparasitica* genomic DNA detected in wheat roots employing RT-PCR. Treatments were: Control = uninoculated control; SM = inoculated with *S. mycoparasitica* only; T22 = inoculated with *T. harzianum* only; Fgra = inoculated with *F. graminearum* only; SM-Fgra = inoculated with both *S. mycoparasitica* and *F. graminearum;* and T22-Fgra = inoculated with *T. harzianum* and *F. graminearum.* All values obtained were the means of six replicates. Error bars indicate standard deviation of the mean.

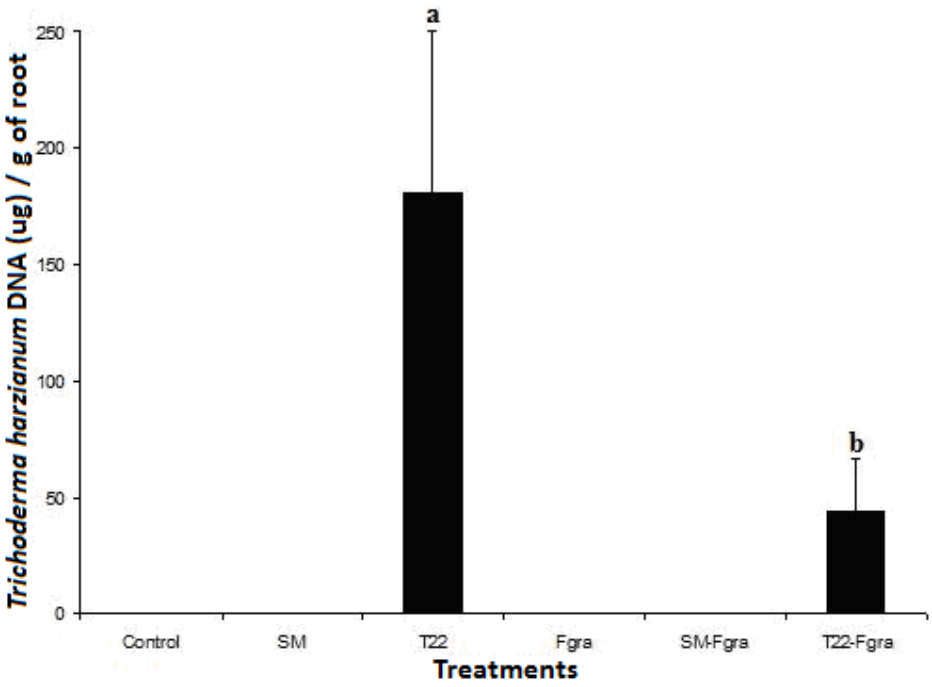

Figure 5.10 Effects of *F. graminearum* (Fgra) and *T. harzianum* (T22) inoculation treatments on *T. harzianum* genomic DNA monitored in wheat roots utilizing RT-PCR. Treatments were: Control = uninoculated control; SM = inoculated with *S. mycoparasitica* only; T22 = inoculated with *T. harzianum* only; Fgra = inoculated with *F. graminearum* only; SM-Fgra = inoculated with both *S. mycoparasitica* and *F. graminearum;* and T22-Fgra = inoculated with *T. harzianum* and *F. graminearum.* All values obtained were the means of six replicates. Error bars indicate standard deviation of the mean.

watermelon plants from *Fusarium* infection (Harveson et al. 2002).

Sphaerodes mycoparasitica was observed to have slower growth rate as compared to *F. graminearum* (Chapter 4). Therefore, we decided to mix *S. mycoparasitica* with peat and inoculate as shown in Figure 5.1 to prevent direct contact between seeds and *Fusarium* inoculants. Viterbo et al. (2007) proposed that normally biological control agents have to be applied prior to the onsets of the diseases. In other studies, seeds were coated or treated with a layer of biocontrol agents prior to the sowing or growing (Bardin et al. 2003; Mao et al. 1997). In the paper by Bailey et al. (2008), they described several different methods of pre-inoculating the biocontrol agents before challenged with pathogenic fungi. All these approaches are aimed to cover the seeds with fungal inoculants or propagules and try to minimize the contact between seeds and pathogenic fungal isolates.

Genomic DNA of *S. mycoparasitica* in wheat roots was detected to be relatively low and remained the same even after challenged with *F. graminearum* (Figure 5.8). This result is concords to the findings by Harveson and associates (2002). In their study, *S. retispora* was hardly recovered from watermelon roots in the culture with biotrophic mycoparasite alone. Furthermore, biotrophic *Melanospora, Sphaerodes,* and *Persiciospora* mycoparasitic strains were described to be isolated from *Fusarium* colonies or closely related to *Fusarium* isolates, not with plant roots (Harveson and Kimbrough 2001a, 2001b; Harveson et al. 2002). On the other hand, the amount of *T. harzianum* genomic DNA detected in the treatment inoculated with *F. graminearum* was observed to be reduced significantly as compared to non-*Fusarium* treatment (Figure 5.9). This is concords to the findings by Sivan and Chet (1989). They observed that the number of *T. harzianum* counts on root segments decreased as the

concentration of *Fusarium* pathogen in soil increased. In addition, they suggested that this is due to the competition between the beneficial fungus and the pathogen.

6. GENERAL SUMMARY AND DISCUSSIONS

The order Melanosporales harbours several genera of biotrophic mycoparasitic fungi that are associated with *Fusarium* species. Melanosporaceuos biotrophic mycoparasites commonly isolated from *Fusarium* colonies are from the genera of *Melanospora, Sphaerodes,* and *Persiciospora.* Moreover, other melanosporaceous biotrophs were also found to establish unique relationships with other groups of fungi. *Sphaerodes mycoparasitica* was isolated from *Fusarium* infected asparagus and wheat fields in Canada. This *Sphaerodes* species was described and characterized in 2009 as *S. mycoparasitica* by Dr. Vujanovic and published in Mycological Research (Appendix A). *Sphaerodes mycoparasitica* was then studied in more details on the aspects of ascospore germination, fungal-fungal and fungal-fungal-plant interactions to be shown as following.

Extracellular filatrates from *F. avenaceum* and *F. oxysporum* were observed to trigger *S. mycoparasitica* ascospore germination. Filtrates from other non-hosts (*F. proliferatum, F. sporotrichioides, T. harzianum, P. bilaii,* and *C. globosum*) or more distant hosts (two *F. graminearum* chemotypes) were reported to show less or no spore germination. This study has provided us with information related to host-specificity of *S. mycoparasitica* from spore germination aspect. Ascospores of *S. mycoparasitica* were found to be more responsive towards *F. avenaceum* and *F. oxysporum* filtrates.

Sphaerodes mycoparasitica was observed to show intimate relations with *F. avenaceum, F. oxysporun,* and two *F. graminearum* chemotypes. No biotrophic mycoparasitism was found between *S. mycoparasitica* and *F. sporotrichioides* or *F. proliferatum.* This newly described fungus was not only found to develop contact mycoparasitic property towards *Fusarium*, but also able to form haustorial-like parasitic structures inside these *Fusarium* hosts. Interestingly, *S. mycoparasitica* was observed to

absorb red-coloured complex or substance from *F. graminearum* chemotype 3 (3-ADON producer) and release it as red-coloured crystal-like compounds. The mechanism is unknown. Furthermore, *S. mycoparasitica* was detected to pose mycelial growth inhibition in *F. avenaceum, F. oxysporum,* and *F. graminearum* chemotypes (both 3- and 15-ADON producers) under *in vitro* assays. Based on the observations reported in this study and information from previously described melanosporaceous biotrophic mycoparasites, *S. mycoparasitica* was found to be a contact and haustorial-like biotrophic mycoparasite of three *Fusarium* species (*F. avenaceum, F. oxysporum* and two *F. graminearum* chemotypes), but not mycoparasite of *F. proliferatum* and *F. sporotrichioides.*

Melanosporaceous biotrophic mycoparasites have been described to affect growth of *Fusarium* pathogens, however there are no reports on whether this group of fungi is posing adverse effects on wheat plants or not. Therefore, we attempted to examine effects of *S. mycoparasitica, Melanospora* sp., and two necrotrophic *Trichoderma* on wheat seedlings under *in vitro* conditions. *Sphaerodes mycoparasitica* was observed to promote wheat seed germination and growth compared to other biotrophic or necrotrophic mycoparasites. Result showed that *S. mycoparasitica* is not harmful to the wheat seeds and no pre- or post-emergence symptoms were observed.

Fusarium species are caused economically important plant pathogens. In each and every stage of wheat growth, wheat plants are susceptible towards *Fusarium* infections. *Fusarium graminearum* is considered as one of the most aggressive and virulent *Fusarium* species among all others (Sutton 1982). In recent studies, *F. graminearum* chemotype 3 was detected to become more dominant in Canada. This species also reported to be replacing other dominant *Fusarium* species in Canada and expanding

from Eastern Canada into the western praires. Therefore, *F. graminearum* chemotype 3 was chosen as the pathogenic candidate for tri-trophic interactions study.

In the study of tritrophic (*Sphaerodes-Fusarium*-wheat) interactions, *S. mycoparasitica* was observed to improve seed germination and plant growth of spring wheat seeds when inoculated with toxigenic *F. graminearum* under controlled conditions in the Pytotron. *Sphaerodes mycoparasitica* was also detected to reduce amounts of *F. graminearum* genomic DNA in wheat roots. The efficiency of *S. mycoparasitica* to promote seedlings growth and protect wheat from *F. graminearum* was relatively similar to necrotrophic *T. harzianum.* Therefore, we proposed that this biotrophic mycoparasitic fungal species could be a potential biological control candidate for managing pathogenic *F. graminearum* infection in wheat.

7. FUTURE STUDIES

Further studies on this potential biocontrol biotrophic mycoparasitic fungal candidate under greenhouse and field conditions will be useful and helpful to determine the ability of this fungal strain to suppress *Fusarium* pathogens. Furthermore, green fluorescent protein (GFP) transformed *S. mycoparasitica* can be produced to study more in-depth on the aspects of *Sphaerodes-Fusarium, Sphaerodes*-plant root and *Sphaerodes-Fusarium*-plant root interactions. *Sphaerodes-Fusarium* interactions can also be examined by using higher magnification microscopic techniques, such as transmission electron microscopy (TEM) and scanning electron microscopy (SEM) to reveal the cytological information related to this unique relationship. More studies can be conducted on the domains of finding related factors or molecules that responsible *S. mycoparasitica* ascospore germination patterns. Examination into the effects of *S. mycoparasitica* on *Fusarium* species at the genetic levels through molecular biology fields will provide us with more information related to how this biotrophic can parasitize or control the *Fusarium* pathogens. Furthermore, investigations related to capability of this biotrophic mycoparasite to torelate or deal with the highly toxigenic 3-ADON mycotoxin produced by *F. graminearum* will give us some insight into how *S. mycoparasitic* survives or lives together with mycotoxigenic *Fusarium* species.

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APPENDIX A

Sphaerodes mycoparasitica sp. nov., a new biotrophic mycoparasite on *Fusarium avenaceum*, *F. graminearum* and *F. oxysporum*

Introduction

The study of biotrophic mycoparasites of *Fusarium* on wheat and asparagus crops throughout Canada resulted in the isolation of a fungus belonging to the genus *Sphaerodes*. Although, at first glance, the fungus resembles *Melanospora* species erected by Corda (1837), many of its taxonomical features differ from *Melanospora* when considering both the original *Melanospora* genus description by Clements (1909) and recent revisions of allied genera by Cannon & Hawksworth (1982). Cannon and Hawksworth (1982) provided the first comprehensive classification of these Melanosporales fungi. *Melanospora* species were noted to posses smooth, ellipsoidal to citriform ascospores and non-tuberculate germ pores, whereas those with wall ornamentations or ascospores of other shapes were segregated into *Persiciospora*, which contains species with a delicately pitted ascospore wall and *Sphaerodes* which have a complex raised tubercle-like germ pore and usually a coarsely reticulate ascospore wall (Cannon and Hawksworth 1982; Zhang and Blackwell 2002).

In early studies, *Melanospora* species were observed to establish intimate relationships with *Fusarium* and few other ascomycetous strains (de Bary 1887; Jordan and Barnett 1978). Recent studies also showed that *Melanospora*, *Persicispora* and *Sphaerodes* often in association with distinct *Fusarium* species, especially *F. oxysporum* (Harveson and Kimbrough 2000, 2001b).

The taxonomy of Melanosporales with mycoparasitic behaviour had been very conflicting due to the unique combination of their ecological and morphological features. They had been placed in Hypocreales, Ceratostomataceae of the Sordariales, or

Diaporthales. Previous phylogenetic studies based on rDNA sequences suggested that *Melanospora* is within or near Hypocreales (Spatafora and Blackwell 1994, Zhang and Blackwell 2002). When analyzed with more taxa of the Sordariomycetes, *Melanospora* formed a distinct clade outside Hypocreales (Castlebury et al. 2004). A more recent study based on a four-gene phylogeny (Zhang et al. 2006) supported the exclusion of *Melanospora* from Hypocreales (Hibbett et al. 2007). Furthermore, a close relationship between Coronophorales and Melanosporales was recognized (Zhang et al. 2006), although not yet approved (Huhndorf et al. 2004). Similar morphological and ecological features of the two orders include a pseudoparenchymatous ascomal wall, clavate, deliquescent asci, lack of paraphyses (with a few exceptions in Coronophorales) and, often, a mycoparasitic habit (Zhang et al. 2006). The fungus described in this study is unlike any of the known species of *Sphaerodes* in having a combination of different morphological features including ascospore shape and size. However, due to variations in morphological characteristics (Miller and Huhndorf, 2005), its inclusion in melanosporaceus genera was problematic (Table A-1). Therefore, we used ITS (internal transcribed spacer), LSU (large subunit) and SSU (small-subunit) ribosomal DNA molecular analyses to objectively assess the phylogenetic importance of distinctive morphological characters in the fungus.

Objective and hypothesis

The hypothesis was that this melanosporales biotrophic mycoparasite will be located in the order or family most members of which are mycoparasitic or hyperparasitic fungal strains by using phylogenetic studies. Moreover, this melanosporaceous fungus might show intimate relations or produce certain contact structures when inoculated together with particular *Fusarium* hosts, especially *F. oxysporum, F. avenaceum,* and *F. graminearum.*

The ultimate objective of this study was to identify and describe a melanosporaceous biotrophic mycoparasitic fungus isolated from *F. avenaceum* and *F. graminearum* pathogens originating from wheat fields in Saskatchewan and from *Fusarium oxysporum* originating from asparagus fields in Quebec, Canada. In order to have in-depth understanding of this melanosporaceous fungal isolate, phylogenetic and morphology studies were performed.

Materials and Methods

Sampling, fungal growth and microscopy

Sphaerodes was recovered occasionally from *F. graminearum* and abundantly from *F. avenaceum* isolates originating from wheat fields in Saskatchewan (2005); it was also isolated from *Fusarium oxysporum* from asparagus fields in Quebec (2003), Canada. This strain (*Sphaerodes* in association with *Fusarium* spp.) were maintained on potato dextrose agar (PDA) (Difco, Detroit, Mich.) amended with 100µg/L streptomycin sulphate and 13µg/L neomycin sulphate (Sigma-Aldrich, St. Louis, MO), and stored at 4ºC in the Saskatchewan Microbial Collection and Database (SMCD2220), and the International Depositary Authority of Canada (IDAC301008-01) collections. The pyrenomycetous mycoparasitic *Sphaerodes* isolate was separated from its *Fusarium* host, and a monosporic culture was obtained according to methods described by Harveson and Kimbrough (2001a), with a few modifications as outlined in Chapter 3. Fungal growth was assessed on MLA (modified Leonian's agar) and PDA media (Malloch and Cain 1971). Morphological studies of ascomata, ascospores, mycelia, and anamorphic structures were performed using Carl Zeiss Axioskop2 with attached Carl Zeiss AxioCam ICc1 camera. Fungal materials for microscopic observation were mounted in lactofuschin and lactophenol cotton blue dyes.

Table A-1 Synopsis of main characteristics of Melanosporaceous taxa.

Character	*Sphaerodes mycoparasitica*	*Sphaerodes*	*Melanospora*	*Persiciospora*	*Phaeostoma*	*Syspastospora*
Stroma	Absent	Absent	Absent (rarely present)	Absent	Present	Absent
Ascoma positon	Superficial to immersed	Superficial to immersed	Superficial	Immersed to superficial	Superficial	Superficial
Ostile	Usually absent	Usually absent	Usually present	Present	Present	Present
Perithecial neck	Absent to short cellular	Absent to very short, cellular	Absent to long cellular	Short, cellular	Long cellular	Long, hyphal
Ostiolar setae	Absent to rarely present	Absent to rarely present	Present	Absent to very short	Absent	Absent
Ascus shape	Clavate (65-100x17-25)	Clavate to ellipsoid	Clavate (rarely ellipsoid)	Clavate to cylindric	Ovoid	Clavate
Ascospore colour	Brown to dark brown	Dark brown to black	Brown	Brown	Brown	Brown
Ascospore shape	Fusiform	Citriform to fusiform	Citrioform to ellipsoid (to discoid)	Ellipsoid-fusiform	Ovoid -ellipsoid	+ / -cylindrical
Ascospore aperture	2 terminal strongly apiculate pores	2 terminal strongly apiculate pores	2 not or slightly apiculate pores	2 slightly apiculate pores	1 sunken pores	2 terminal crateriform pores
Ascospore ornamentation	Usually coarsely reticular	Usually coarsely reticular	Smooth	Weakly pitted	Smooth	Smooth

DNA extraction, amplification and sequencing

Fungal strains were inoculated on fresh PDA medium at 21ºC for a week prior to DNA extraction. Genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen Inc., Mississauga, ON). ITS, SSU (small subunit) and LSU (large subunit) rDNA fragments were amplified using primer sets ITS1F/ITS4 (White et al. 1990), NS1/NS6 (White et al. 1990) and LS1/LR5 (Hausner et al. 1993; Rehner and Samuels 1995; Zhang and Blackwell 2002). The target regions in fungal genomic DNA samples were amplified using polymerase chain reaction (PCR) in a 25 µl reaction mixture containing 2.5 µl of 10X buffer, 5 µl of Q buffer, 0.5 µl 10 mM dNTPs, 1 µl of each primer, 0.13 µl of 0.625 unit of Taq DNA Polymerase, 2 µl of extracted fungal DNA, and 13.87 µl of sterilized ultra-pure Millipore water. The Qiagen Taq PCR core kits were purchased from Qiagen Inc., Mississauga, ON. PCR conditions for ITS was outlined in Sokolski et al (2004). The PCR conditions for SSU was (i) 2 min at 95ºC, (ii) 14 cycles of 35 s at 96ºC, 55 s at 53ºC, and 35 s at 72º C, (iii) 11 cycles of 35 s at 96ºC, 55 s at 53ºC, and 2 min at 72ºC, (iv) 15 cycles of 35 s at 96ºC, 55 s at 53ºC, and 3 min at 72º C, and (v) 10 min at 72º C (Simon et al. 1992). PCR conditions for LSU were (i) 3 min at 94ºC, (ii) 35 cycles of 1 min at 94ºC, 1 min at 55ºC and 2 min at 72ºC, and (iii) 10 min at 72ºC (Hausner et al. 1993; Rehner and Samuels 1994). All PCR products were purified using the QIAquick PCR Purification Kit (Qiagen Inc., Mississauga, ON). Purified DNA PCR products were sent to Plant Biotechnology Institute (PBI), Saskatoon, SK for sequencing.

Sequence alignment and phylogenetic analyses

Sequences of SSU and LSU from this study and other fungal sequences were retrieved from GenBank, aligned using Clustal X software (version 1.82) (Thompson et al. 1997), and edited in Bioedit (Hall 1999). Distance trees were produced with PAUP

(phylogenetic analysis using parsimony) 4.0b10 software (Swofford 2000) using a neighbor-joining approach, and validated using a bootstrap analysis with 1,000 repetitions. A fungal distance tree was prepared with sequences that matched at 50% and higher. Trees were rooted with sequences: *Xylaria hypoxilon* U47841 for LSU and *Xylaria hypoxylon* AY544692 for SSU. Sequences were submitted to GenBank under numbers: SSU (FJ748919) and LSU (FJ748916).

Results

TAXONOMY

Sphaerodes mycoparasitica Vujanovic sp. nov. Table A-1 and Figure A-1 to 6 *Coloniae* in agaro potato dextrosum lentior crescents, 4.0 cm ad 7d, floccose, pallido-brunneis. *Hyphis* septatis, ramosis, laevibus, palide fulvis, 2.5 – 5.0 µm diam. compositum. *Ascomata* superficialia vel immersa, pyriformia vel globosa, ostiolata, flavo-brunnea, 250–300µm longa, 200–280µm diam. *Collum* nul vel conicum, (0–) 30-75µm longum, (0-) 50-70µm latum ad basim. *Peridium* membranaceum, cellulis 8–15µm, e 3–6 stratis, 8–15µm crassum, textura angulari, compositum. *Setae* rectae vel parum curvae, hyalinae vel dilute flavae, crassitunicatae, 10-40µm longae, septatae. *Asci* 8-spori, ovoidei vel clavati, 50–75 x 17–25µm, superne late rotundati, brevistipitati, tenuitunicati, evanescentes. *Paraphysis* nullis. *Ascosporae* unicellulares, irregulariter biseriatae, primum hyalinae, deinde brunneae vel atrobrunneae, crassitunicatae, fusiformes, 18.5–24 x 9–12 µm, reticulatae, costis protrudentbus, e polo visae polygonales, utrinque umbonatae, foramine germinali praeditae. *Phyalidis* hyalinis, status conidialis.

Typus : **Canada**: Saskatchewan, on *Fusarium avenaceum* from wheat, Saskatoon, SK, Canada, 10 Sep. 2005, V. Vujanovic (*holotypus*: SMCD 2220-01; *isotypus*: IDAC301008-01).

Etymology: mycoparasitica; referring to fungus parasitic on another fungus.

Host range: Associated with *Fusarium avenaceum, F. graminearum* on wheat and *F. oxysporum* on asparagus.

Cultural characteristics: Colonies on Modified Leonian's agar grew more rapidly than on PDA, expending 1.1 cm versus 0.6 cm per day (21–22ºC) and consisting of slightly submerged mycelium and aerial hyphae, granulose due to ample number of ascomata. On PDA, a woolly mycelium adapted yellowish to pinky brownish color on both sides (Figure A-1). At 37ºC, no growth. *Hyphae* white to pale yellow, 2.5 – 5.0 µm diam., septate. Colonies on Potato dextrose agar (PDA) spread with abundant, white to pale yellow aerial mycelium and low number of ascomata. *Ascomata*, perithecial or cleistotecial, scattered or aggregated in small groups, superficial, pyriform to globose, ostiolate (when mature), light to dark yellowish brown, translucent, appearing black due to mass number of mature ascospores, 250–300 µm high, 200–280 µm diam. *Neck* absent to short conical or cylindrical (0–) 25–75 µm long, 0–70 µm wide at the base, surrounded with short upright crown of rare setae, (0–) 10–40 µm long. *Peridium* membranaceous, 3–6-layered, 8–10 µm thick, translucent, pale yellow to light brown, textura angularis, with cells 8–15 µm diam. *Asci* 8-spored, clavate, 50–75 x 17–25 µm, rounded at apex, without apical structures, thin-walled and evanescent at mature. *Paraphyses* absent. *Ascospores* irregularly arranged inside the asci, hyaline at young but becoming brown to dark brown on maturity, thick-walled, single-celled, fusiform to rarely triangular, 18.5–24 x 9–12 µm, reticulate to rarely smooth, with one transvere section, and strongly umbonate germ pore at each end. *Phyalides* hyaline directly attached to ascomata or produced on ascomata surrounding hyphae.

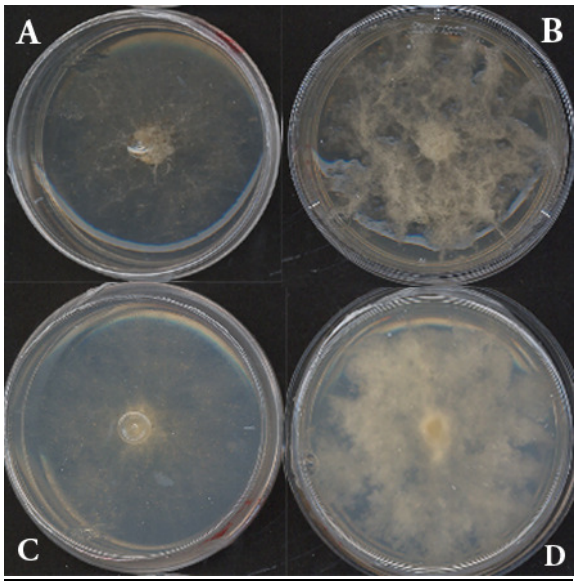


Figure A-1 *Sphaerodes mycoparasitica* on A) Modified Leonian's agar and B) PDA– upper sides, C) and D) - down sides.

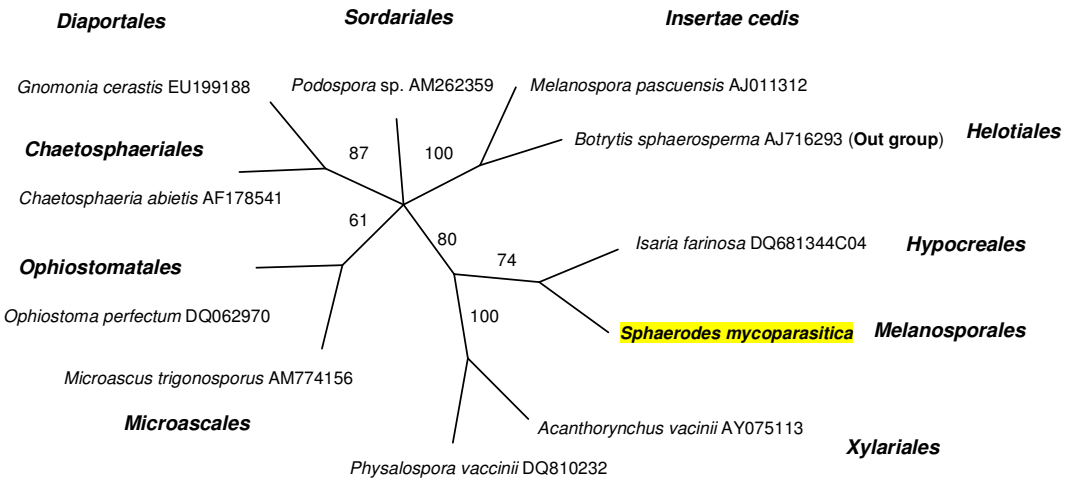


Figure A-2 Phylogenetic tree based on ITS rDNA sequences showing the position of the *Sphaerodes mycoparasitica* (in bold) colonizing *Fusarium*. Bootstrap values of 50% or greater from 100bootstrap replications are indicated for the corresponding branches.

3.5 Discussion

With ITS sequences *S. mycoparasitica* was located close to the order of Hypocreales, however there is very limited amount of ITS sequences available in Genbank (Figure A-2). Sequence alignments and parsimony analyses of the LSU (1266bp) and SSU (1223bp) sequences classify *S. mycoparasitica* within *Sphaerodes* in well-supported clade of *Melanosporales* (Figure A-3 and A-4). These results are in agreement with those of Zhang and Blackwell (2002). Furthermore, our species is typical of *Sphaerodes* showing such morphological features characterized by cleistothecial or ostiolate perithecia with an absent to short neck (Figure A-6A, G). Necks of species in *Sphaerodes-Melanospora* clade have a cellular rather than a hyphal structure and are fringed with hyaline setae (Figure A-6B) (Cannon and Hawksworth 1982). Furthermore, in Melanosporales taxa, *Melanospora* are classified by smooth, ellipsoidal to citriform ascospores and non-tuberculate germ pores, and those with wall ornamentations or ascospores of other shapes are segregated into other genera (Zhang and Blackwell 2002). For example, *Persiciospora* contains species with a delicately pitted ascospore wall and *Syspastospora* have smooth but cylindrical ascospores, whereas species in *Sphaerodes* have a complex raised tubercle-like germ pore and usually a coarsely reticulate ascospore wall (Cannon and Hawksworth 1982, Horie et al. 1986). Moreover, the raised rim around the germ pore is a feature of *Sphaerodes* and not Melanospora (Chaudhary et al. 2006). In this study, observed ascomata features, reticulate ascospores with an polygonal irregular transverse section, as well as raised rim around the germ pore placed *S. mycoparasitica* close to *S. quadrangularis* reported by Garcia et al. (2004) (Figure A-5, Table A-3). However, a unique combination of features - type of irregular transverse section (Figure A-5 and A-6C), spores reticulate 19–23 × 9–12µm (Figure A-6D), occasionally triangular to smooth (Figure A-6D, E),

and the asexual stage attached to ascomata (Figure A-6F) - differentiated *S. mycoparasitica* from *S. quadrangularis*. Morphology of its asexual stage (Figure A-6F) also differed from *Melanospora* anamorphs belonging to a wide range of genera, including *Acremonium*, *Chlamydomyces*, *Harzia*, *Papulospora, Paecilomyces* and *Proteophiala* (Kendrick and Di Cosmo 1979; Hawksworth et al. 1999; Davery et al. 2008). In view of its most important relationship to Sphaerodes, we establish our fungus as a new member of this genus.

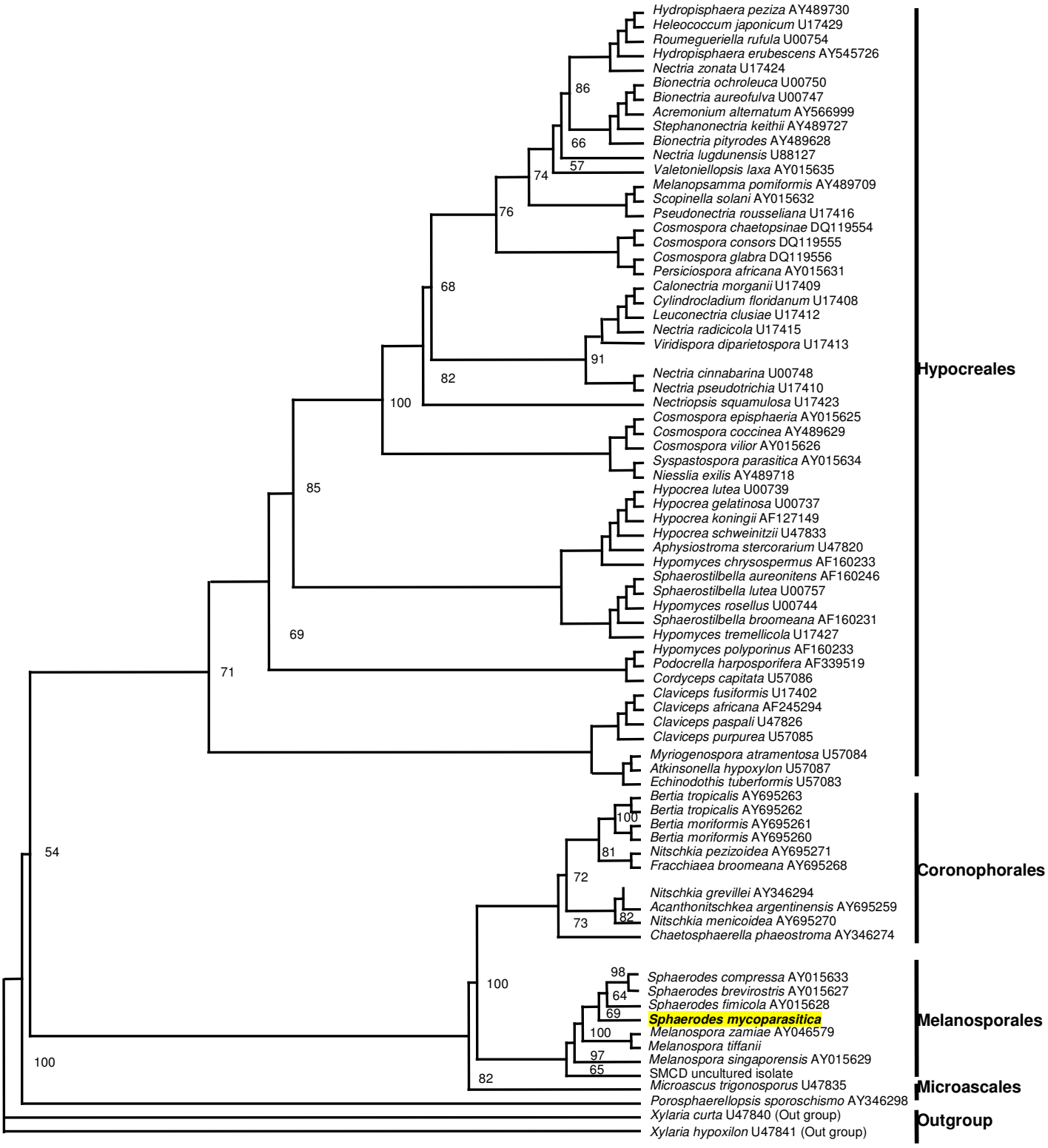


Figure A-3 Phylogenetic tree based on LSU rDNA sequences showing the position of *Sphaerodes mycoparasitica* (in bold) colonizing *Fusarium*. Bootstrap values of 50% or greater from 1000 bootstrap replications are indicated for the corresponding branches.

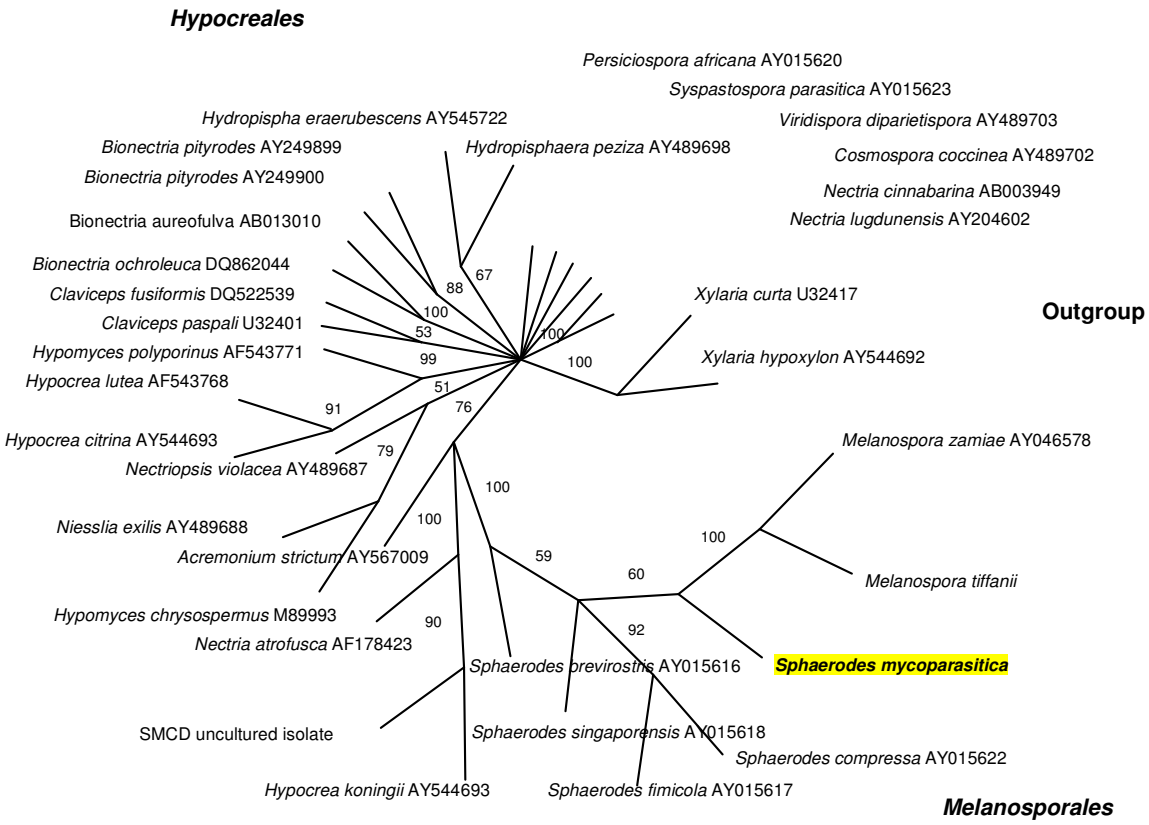


Figure A-4 Phylogenetic tree based on SSU rDNA sequences showing the position of the *Sphaerodes mycoparasitica* (in bold) colonizing Fusarium. Bootstrap values of 50% or greater from 1000 bootstrap replications are indicated for the corresponding branches.

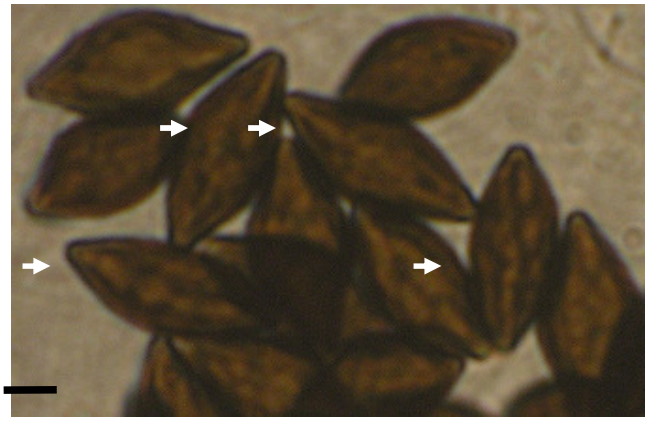


Figure A-5 *Sphaerodes mycoparasitica* ascospores showing irregular transverse sections (arrows). Bar scale 5 μm.

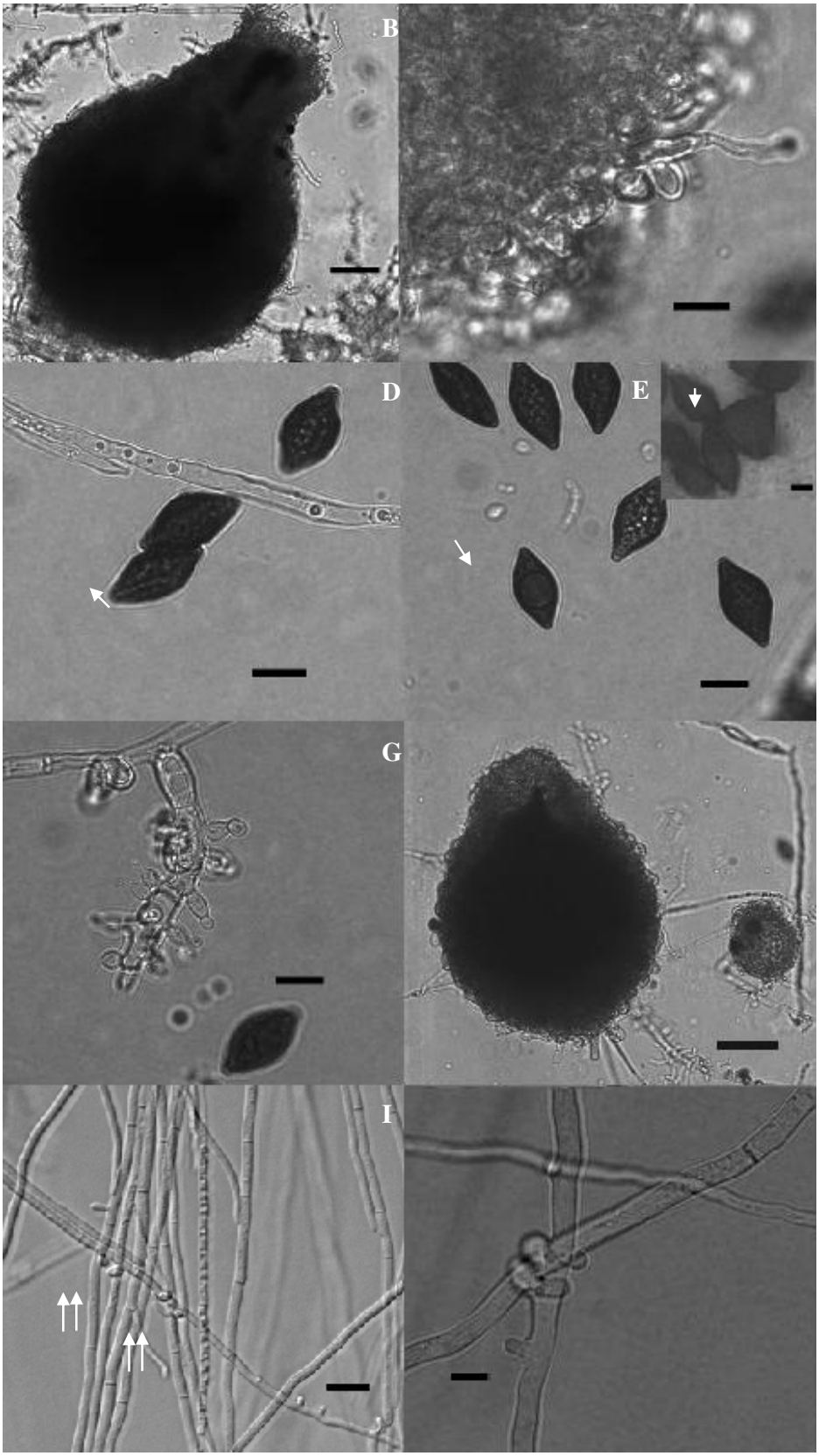
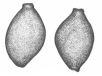
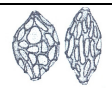
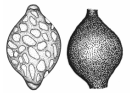
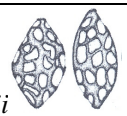
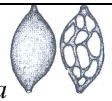
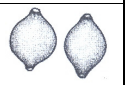
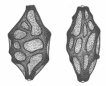
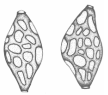

B
D
E
G
I

Figure A-6 *Sphaerodes mycoparasitica*: A) Ascoma, B) Neck surrounded with hyaline setae, C) Reticulate ascospores with transverse section (arrow), D) Smooth ascospore (arrow), E) Triangular ascospore (arrow), F) Phyalides produced on ascoma surrounding hyphae, G) Formation of mature and starting ascomata, H) Formation of hook-like structures by *S. mycoparasitica* parasiting on living hypha of *Fusarium oxysporum* (arrows) and I) Large view of hook-like structure on living hypha of *F. avenaceum*. Bar scales for A and G were 50 μm, for B, C, D, E, F, and I were 10 μm, for H was 25 μm.

Table A-2 Key to species of *Sphaerodes*

1. Ascomata ostiolate.....2
1. Ascomata non-ostiolate 5
2. Ascospores coarsely reticulate 3
2. Ascospores smooth-walled, strongly umbonate at both ends, 15–18 × 9–11 µm …….*S. singaporensis* 
3. Ascospores fusiform.....4
3. Ascospores citriform, 14–26 × 10–17 µm.....*S. fimicola* 
4. Ascospores delicately pitted, circular, transverse section, 18–22 × 9–11 × 8–9 µm…….. *S. micropertusa* 
4. Ascospores reticulate, polygonal transverse section, 23–28 × 10–12 × 8–10 µm…….....*S. quadrangularis* 
4. Ascospores reticulate, polygonal irregular transverse sections, 18–24 X 9–12µm, asexuale stage attached to ascomata…….....….***S. mycoparasitica*** 
5. Ascospores shorter than 20 µm 9
6. Asci 8-spored.....7

6. Asci 4-spored.....8
7. Ascospores coarsely reticulate, 25–34 × 12–18 µm.....*S. episphaeria* 
7. Ascospores with indistinct reticulation, 19–23 × 12–17 µm..... ...*S. tenuissima* 
8. Ascospores (25–)28–34 × 14–16(–18) µm; coarsely reticulate .....*S. beatonii* 
8. Ascospores 22–28 × 12–15 × 9–11 µm; both smooth and reticulate ascospores present…….. *S. perplexa* 
9. Ascospores smooth, umbonate, and with a tuberculate germ pore at each end.....10
9. Ascospores reticulate.....11
10. Ascospores citriform, 10–17 × 8–12 × 9–10 µm; phialidic anamorph present .....*S. levita* 
10. Ascospores fusiform, 16–18 × 6–8 µm; anamorph absent .....*S. ellipsospora* 
11. Ascospores reticulate.....12

11. Ascospores smooth and with a narrower sides reticulate .....*S. compressa*	
12. Ascospores prominently reticulated .....*S. retispora var. retispora*	
12. Ascospores inconspicuously reticulated.....*S. retispora var. inferior*	

Note: Except for *S. mycoparasitica* ascospore drawing, drawings of ascospores are used from STUDIES IN MYCOLOGY 50: 63–68. 2004 – permission granted by Dr. Josep Guarro.

APPENDIX B

Table B-1 Germination of ascospores for *S. mycoparasitica* (in percentage) challenged in filtrates of three biocontrol agents with sterilized water and PDB as control for four different chronosequences. 1d Suspension, 1d sus +1d PDA, 3d suspension and 3d sus + 1d PDA indicate spores were suspended for 1d, 1d with an additional day on PDA, 3d, and 3d with an additional 1d on PDA in control and different fungal filtrates.

S. mycoparasitica	Spore germination (in %)			
Treatments	1d Suspension	1d sus + 1d PDA	3d suspension	3d sus + 1d PDA
Water	0	0	0	2 ± 1.2
PDB	0	0	0	2.8 ± 1.9
P. bilaii-filtrate	0	0	0	0
T. harzianum-filtrate	0	9 ± 1.1	1.6 ± 1.2	10.4 ± 1.5
C. globosum-filtrate	0	0	0	0

* Numbers in each column represented mean of ascospore germination (in %) ± standard deviation.

APPENDIX C

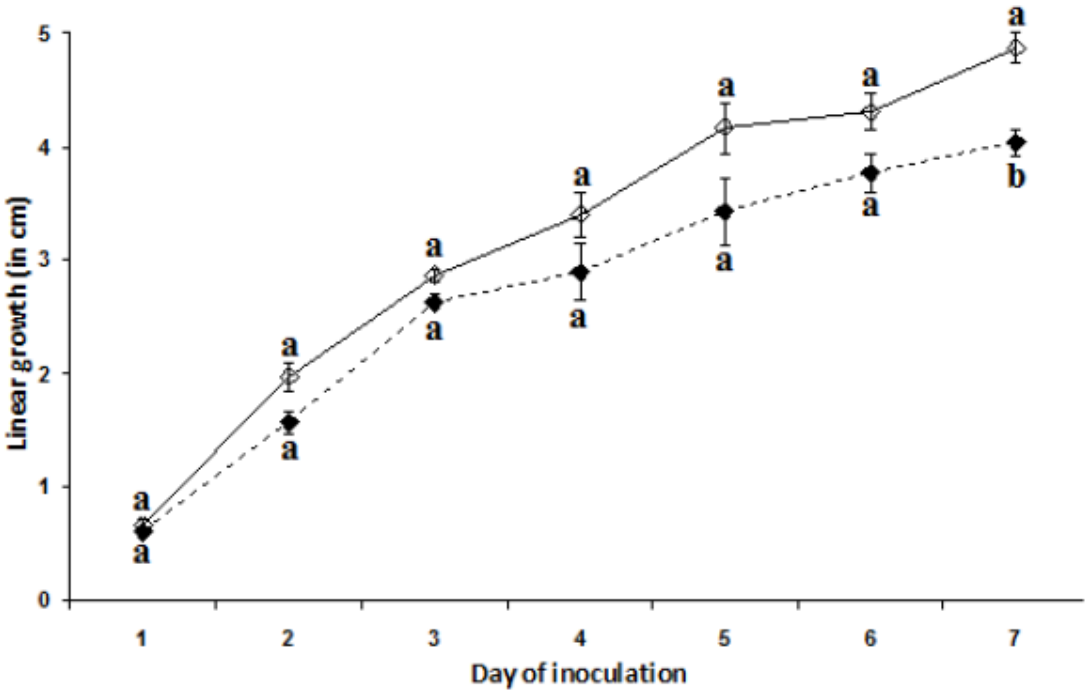


Figure C-1 Dual-culture assays of *F. oxysporum* for measuring linear mycelial growth on PDA medium challenged with *Sphaerodes* (--◆--) and control - without biotrophic mycoparasitic fungus (—◇—). Data are means and standard deviations. Dots with the same lowercase letter are not significant different between treatments with *Sphaerodes* and control for *F. oxysporum* up to 7 days of co-culturing at $P = 0.05$, with T-test.