STUDIES ON MYCOPARASITE INTERACTIONS WITH PLANT PATHOGENIC *FUSARIUM* SPP. AND THEIR MYCOTOXINS

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ABSTRACT

Sphaerodes mycoparasitica Vujan. SMCD 2220-01 is a mycoparasite that attacks major plant pathogenic *Fusarium* species such as *F. avenaceum* (Fr.) Sacc., *F. oxysporum* Schltdl., and *F. graminearum* Schwabe. The mycoparasite-host cell-to-cell replacement and interface changes in *F. graminearum*, as well as a decrease in production of mycotoxins in co-culture systems indicate a very complex fungus-fungus interrelationship in previous studies. The aim of this thesis is to investigate the interactions between the mycoparasite *S. mycoparasitica* and the host *Fusarium* spp., as well as the degradation of *Fusarium* mycotoxins by the mycoparasite. Host compatibility and adaptability of the mycoparasite with twelve *Fusarium* strains were examined using *Fusarium* filtrates through multiple paper disc assay and the results showed that the level of *S. mycoparasitica* adaptability strongly depends on types of *Fusarium* filtrates that indicate the degree of host compatibility ranging from biotrophic-attraction to antagonistic-inhibition relationships. The capacity of *S. mycoparasitica* to change the hydrophobicity of the host fungal surfaces during mycoparasitism was demonstrated by contact angles measurement using a dual culture assay. The results showed differential level of fungal surface hydrophobicity of *S. mycoparasitica*, *F. proliferatum* (Matsush.) Nirenberg SMCD 2246 (high hydrophobicity), and *F. redolens* Wollenw. SMCD 2401 (low hydrophobicity). They also change in hyphal surface hydrophobicity during mycoparasitism in different media. In terms of fungal surfaces, the isolates were assessed by atomic force microscopy (AFM). AFM results showed a shift in topography and physical properties of the hyphal surfaces affected by changes in nutrient and prolonged dry conditions. The potential for degradation of the mycotoxins by *S. mycoparasitica* and the metabolites were examined using thin layer chromatography and high performance liquid chromatography-electrospray ionization-high resolution mass spectrometry. The results showed a decrease in the mycotoxins such as zearalenone by 97%, 15-acetyl-deoxynivalenol by 72%, 3-acetyl-deoxynivalenol by 60%, and deoxynivalenol by 89%. Overall, *S. mycoparasitica* could be considered as a potential BCA specifically to reduce *Fusarium*-associated risks.

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
ADON	Acetyl-deoxynivalenol
AFM	Atomic force microscope
ANOVA	Analysis of variance
BCA	Biocontrol agent
d	Day
DON	Deoxynivalenol
EN	Enniatin
EtOAc	Ethyl acetate
FCRR	Fusarium crown and root rot
FHB	Fusarium Head Blight
HPLC-HR-ESI-MS	High performance liquid chromatography-high resolution-electrospray ionization-mass spectrometry
ICI	Minimal medium; Imperial Chemical Industries Ltd., UK
LSD	Least significance difference
MeOH	Methanol
MON	Moniliformin
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PKS	Polyketide synthase
ppm	Parts per million
SDW	Steilized distilled water
SMCD	Saskatchewan Microbial Collection and Database
TLC	Thin layer chromatography
WA	Water agar
ZEN	Zearalenone

1. INTRODUCTION

1.1 Overview

Sphaerodes mycoparasitica Vujan. SMCD 2220-01 was originally isolated and identified from wheat and asparagus fields in association with *F. oxysporum*, *F. avenaceum*, and *F. graminearum* (Vujanovic & Goh, 2009). *S. mycoparasitica* as a specific mycoparasite showed the suppression of *Fusarium* strains such as *F. avenaceum*, *F. oxysporum*, and *F. graminearum* during mycoparasitism and reduction in the production of mycotoxins such as DON, 3-ADON, 15-ADON, and ZEN produced by *F. graminearum* in the co-culture system (Vujanovic & Chau, 2012; Vujanovic & Goh, 2009; Vujanovic & Goh, 2010; Vujanovic & Goh, 2011b). Several research indicated that *S. mycoparasitica* possesses host specificity developed during fungus-fungus co-evolution processes (Goh & Vujanovic, 2010c; Vujanovic & Goh, 2010). It was also suggested that the fungal mycoparasite-fungal pathogenic host compatibility and mycoparasitism may be regulated by the mycoparasite resistance to mycotoxins and its ability to degrade or transform mycotoxins to non-toxic or less toxic metabolites. The compatibility referring to the ability of a mycoparasite to successfully parasitize a host might be resulted from the specific accommodation or adaptation of the mycoparasite to its several hosts (Manocha, 1981). Fungal surfaces or fungal cell walls as an initial barrier where antagonism and/or parasitism occur are known to play key roles in mechanical protection and aggressive or defensive mechanisms (Bowman & Free, 2006; Smits *et al.*, 2003). Thus, investigation on host compatibility and adaptability, as well as mycotoxin-degradating ability of the mycoparasite could be crucial evidence for the elucidation of the evolution and the mode of action, as well as mycotoxin resistance of the mycoparasite; the changes in fungal surfaces hydrophobicity during the mycoparasitism could explain the complex inter-relationship between the mycoparasite and different hosts in depth.

1.2 Hypotheses

Sphaerodes mycoparasitica is a specific mycoparasite as a safe and promising candidate for BCA of controlling plant pathogenic *Fusarium* species. Thus, the following hypotheses were investigated in this thesis to understand the mycoporasite's interaction with the host *Fusarium* strains and their mycotoxins: (1) *S. mycoparasitica* changes a pattern of hyphal (anamorphic stage) growth when exposed to different *Fusarium* filtrates. Adaptability of the mycoparasite's vegetative cells adapted over five generation on *Fusarium* filtrates differs from adaptability of teleomorphic cells measured by ascomata formation under the exposure to *Fusarium* filtrates. These hypotheses can offer key information about host compatibility and adaptability of the mycoparasite (chapter 3); and (2) *S. mycoparasitica* affects hyphal surface hydrophobicity and radial growth of host *Fusarium* strains such as *F. redolens* V and *F. proliferatum* during mycoparasitism. Hyphal surface topography/physical structure associated with hydrophobicity as well as radial growth of *Fusarium* hosts differ under the different environmental conditions. These hypotheses can reveal how the mycoparasite parasizes on the *Fusarium* hosts under different media conditions (chapter 4); and (3) *S. mycoparasitica* degrades mycotoxins such as deoxynivalenol, 3-acetyl-and 15-acetyl-deoxynivalenol, and zearalenone produced by *Fusarium* species. This hypothesis can demomstrate the resistance and biodegradability of the mycoparasite to the Fusarium mycotoxins (chapter 5).

1.3 Objectives

The objectives of this thesis are: (1) to examine host compatibility of *S. mycoparasitica* and its adaptability on multiple paper disc assay and microscopy, as well as to evaluate the effect of *Fusarium* filtrates on ascomata formation of *S. mycoparasitica* on modified slide culture assay; and (2) to measure radial growth and contact angles during mycoparasitism under different nutritional conditions using dual culture assay and to examine fungal surface differences such as hyphal surface topography and roughness by atomic force microscopy; and (3) to assess degradation ability of *S. mycoparasitica* through TLC and analyze transformant of mycotoxins by HPLC-HR-ESI-MS.

2. LITERATURE REVIEW

2.1 Preface

The aim of this review section is to provide new insight into distinct lifestyles of mycoparasites, particularly the mycoparasite-*Fusarium* relationship, which implies possible phytotoxic effects and/or mycotoxin contamination originating from mycoparasites on agricultural crops. Furthermore, this review section discusses the importance of seeking host-specific mycoparasites that are not harmful to beneficial fungal communities in ecological niches occupied by plant pathogens and do not pose a risk of mycotoxin production in environmental samples. Areas where further research is most urgently needed were also highlighted.

2.2 Biological control of plant pathogens

The global world population is expected to exceed 9.6 billion people by 2050, and food production needs to increase by 50% to 70% to enhance global food security (CEMA, 2015). In Western countries, industrial agriculture is based on an input-intensive and large-scale production system using synthetic pesticides to protect crop yield from pests, disease and insect outbreaks. Fusarium head blight (FHB) is one of the most important fungal diseases affecting major cereal crops including wheat, barley, and maize worldwide (Osborne & Stein, 2007). FHB is caused by plant pathogenic *Fusarium* spp., not limited to the red *F. avenaceum*, *F. culmorum*, and *F. graminearum* group of species. These *Fusarium* species produce toxic secondary metabolites, small molecular weight molecules called mycotoxins, such as deoxynivalenol (DON), enniatin (EN), moniliformin (MON) and zearalenone (ZEN) (Kokkonen *et al.*, 2010). DON and its type-B trichothecene derivatives (3-ADON/3-acetyl-deoxynivalenol and 15-ADON/15-acetyl-deoxynivalenol) are mainly produced by *F. graminearum* and *F. culmorum.* They act as inhibitors of cell protein synthesis and pose an important health risk to plants, humans and animals (Westerberg *et al.*, 1976).

Fungicides are a specific type of pesticides routinely used in commercial plantations to control fungal diseases by inhibiting or killing plant pathogens— a single major cause of economic losses in agriculture. However, the harmful effects of synthetic chemicals and natural toxic substances on agriculture, the environment, food quality and human health are attracting increased public and scientific concern (McNeil *et al.*, 2010). Biological control has been considered as a promising alternative to synthetic chemical pesticides over the last two decades (McNeil *et al.*, 2010; Paulitz & Bélanger, 2001). The term "biological control" can be abbreviated to "biocontrol" and defined as "the use of living organisms to curtail the growth and proliferation of other, undesirable ones" (Gnanamanickam *et al.*, 2002). The living organisms, particularly micro-organisms, used for the suppression, inhibition, and/or control of plant pathogens have been referred to as biological control agents (BCAs) (Pal & Gardener, 2006).

The mechanisms of biological control of pathogenic fungi include different types of interactions between fungal BCAs and their fungal hosts, such as direct, mixed-path, and indirect relationships (Vujanovic & Goh, 2011a). Direct interaction includes mycoparasitism or hyperparasitism and predation mechanisms. Mixed-path antagonism includes antibiotic secretion, lytic enzyme production, unregulated by-products, and physical-chemical influences. Indirect interaction includes competition, an intrinsic ability to utilize the nutrients released by the host plant, and host defense induction mechanisms. The most efficient BCAs simultaneously employ a combination of different types of interactions for controlling pathogens (Pal & Gardener, 2006).

There is increasing interest in the utilization of mycoparasites to control fungal plant pathogens. Recent discoveries highlighted a plethora of new mycoparasites with different lifestyles such as mycoparasitism, competition, and antagonism by the production of extracellular enzymes and/or secondary metabolites (Butt *et al.*, 2001). However, it is likely that the spectrum of studied mycoparasites is mostly limited to *Trichoderma* and *Clonostachys* fungal generalists. Despite the considerable research endeavor, the use of these mycoparasites has not yet achieved an economically sustainable *Fusarium* control perspective. Moreover, some *Trichoderma* and *Clonostachys* species or strains are known to contain trichothecene (*Tri*) genes (Tijerino *et al.*, 2011) that encode active molecules with high similarity to *Tri* proteins from *Fusarium* species (Cardoza *et al.*, 2011; Malmierca *et al.*, 2012). These molecules were also

reported as phytotoxic secondary metabolites and/or mycotoxins (Brian, 1944; Howell & Stipanovic, 1984).

2.3 Mycoparasitic lifestyles

Mycoparasitism or hyperparasitism is the parasitic interaction between one fungus and another as a direct biological control mechanism to control plant pathogenic fungi (Butler, 1957; Howell, 2003). Mycoparasitism can be largely categorized into two groups, biotrophic and necrotrophic parasitism (Boosalis, 1964). The classification of mycoparasitism is based on the mode of parasitism and its effect on the host fungi (Boosalis, 1964). Necrotrophic mycoparasites absorb nutrients from the killed host cells by means of enzymes or non-specific toxic compounds, whereas biotrophic mycoparasites derive nutrients from living cells through haustoria, which mediate intimate relationships with host cells (Barnett, 1963; Boosalis, 1964). Biotrophic mycoparasites can be subdivided into destructive and balanced mycoparasites (Boosalis, 1964). Balanced mycoparasites impose a low degree or no apparent harm to the host, whereas destructive mycoparasites may inflict damage or kill the host (Barnett, 1963; Jeffries, 1995). Biotrophic mycoparasites tend to have narrower host ranges than necrotrophic mycoparasites and often form specialized infection structures such as hook-like, braid-like, and clamp-like contact structures at host-parasite interfaces (Goh & Vujanovic, 2010b; Jeffries, 1995).

2.4 Generalists-nectorophic mycoparasites

The most well-studied necrotrophic mycoparasites are *Trichoderma* species, which are often predominant generalists in natural, plant and soil environments. *Trichoderma* species, including *T. harzianum* Rifai*, T. viride* Pers., *T. virens* (J.H. Mill., Giddens & A.A. Foster) Arx, *T. koningii* Oudem.*, T. gamsii* Samuels & Druzhin., and *T. atroviride* P. Karst (1892), represent a major proportion (95.22%) of the scientific articles on mycoparasites presented by the Web of Knowledge (Thomson Reuters) in the last decade, as shown in Figure 2.1.(a).

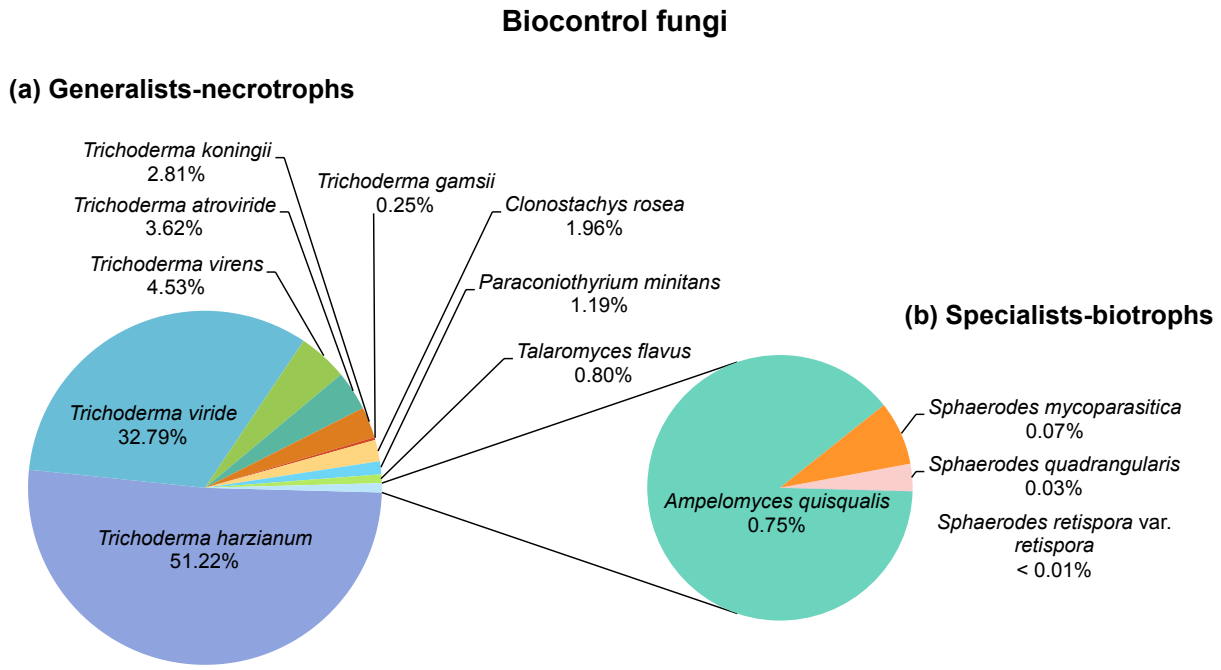


Figure 2.1 Scientific articles on mycoparasites published between 2005 and 2015. Data retrieved from Web of Knowledge (Thomson Reuters) on Sep 14, 2015. The figure shows the percentage of published articles for each species categorized by its biological control tactics or distinctive lifestyles. (**a**) ***Generalists-necrotrophs*** include *T. harzianum*, *T. viride*, *T. virens*, *T. atroviride*, *T. koningii*, *T. gamsii*, *C. rosea*, *P. minitans*, and *T. flavus*; (**b**) ***Specialists-biotrophs*** include *A. quisqualis* against the family *Erysiphaceae* (order *Erysiphales*), causing powdery mildew, *Sphaerodes* species such as *S. mycoparasitica*, *S. quadrangularis* and *S. retispora* var. *retispora* against *Fusarium* species.

2.4.1 *Trichoderma* species

Due to their multiple strategies such as mycoparasitism, antibiosis, competition for nutrients or space, and induced systemic resistance in plants against plant pathogens, *Trichoderma* spp. have been developed into commercially available BCAs to control a variety of plant pathogens such as *Pythium ultimum* Trow (oomycete), *Sclerotinia sclerotiorum* (Lib.) de Bary, *Rhizoctonia solani* J.G. Kühn [teleomorph: *Thanatephorus cucumeris* (A.B. Frank) Donk], *Botrytis cinerea* Pers., and *Fusarium oxysporum* Schltdl. (Table 2.1).

Although *Trichoderma* spp. are among the most effective known BCAs, environmental concerns related to the broad host range with non-target effects and non-specific toxic secondary metabolites have been extensively documented. In terms of a negative effect on beneficial fungal hosts, *T. harzianum* has shown mycoparasitism on arbuscular mycorrhizal fungi, e.g.,

Rhizophagus intraradices (N.C. Schenck & G.S. Sm.) C. Walker & A. Schüβler (synonym *Glomus intraradices* N.C. Schenck & G.S. Sm. 1982) by the penetration, dissolution, and rupture of host hyphae and spores (Rousseau *et al.*, 1996). At least three other *Trichoderma* species, *T. aggressivum* Samuels & W. Gams, *T. pleurotum* S.H. Yu & M.S. Park, and *T. pleuroticola* S.H. Yu & M.S. Park (syn. *T. fulvidum)*, have been reported to cause significant crop loss in the mushroom industry based on *Agaricus bisporus* (J.E. Lange) Imbach and *Pleurotus ostreatus* sensu Cooke (Hatvani *et al.*, 2007; Kredics *et al.*, 2009; Samuels *et al.*, 2002).

In addition to mycoparasitism against beneficial fungi, the gliotoxin and viridin produced by *T. harzianum*, *T. viride* and *T. virens* (synonym *Gliocladium virens*) affect crop plants through phytotoxicity by reducing seed germination in wheat (Vujanovic & Goh, 2012), cucumber, tomato, and pepper (Menzies, 1993) and by suppressing root growth and seed germination in mustard (Fravel, 1988; Lumsden & Beily, 1998; Lumsden *et al.*, 1992).

Gliotoxin, belonging to the class of diketopiperazines, was first discovered in *T. virens* (Weindling, 1934) and *T. viride* (Brian, 1944) and later in the grain mold *Aspergillus fumigatus* Fresen. (Glister & Williams, 1944). Gliotoxin was detected in soil inoculated with *T. virens* (Wright, 1952), implying the possibility of dispersion of this toxin in the environment or the contamination of cultivated plants. Recently, it was found that gliotoxin is involved in the mycoparasitism and biocontrol activity of *T. virens* against oomycetous *Pythium ultimum* Trow, infecting cotton seedlings, and plant pathogenic *Sclerotinia sclerotiorum* (Lib.) de Bary, through overexpression of the *gliP* cluster of genes responsible for the biosynthesis of gliotoxin (Vargas *et al.*, 2014).

Viridin was first discovered in *T. virens* as a steroidal antifungal compound and has since been found in *T. viride* and *T. koningii* (Singh *et al.*, 2005). Its antifungal activity prevented spore germination of *Botrytis allii* Munn, *Colletotrichum linicola* Pethybr. & Laff., *Fusarium caeruleum* Lib. ex Sacc., *Penicillium expansum* Link., *Aspergillus niger* Tiegh., and *Stachybotrys chartarum* (Ehrenb.) S. Hughes (synonym *S. atra* Corda) (Brian & McGowan, 1945; Reino *et al.*, 2008). The viridin produced by several *Trichoderma* spp. is easily converted to viridiol, which induces symptoms of necrosis in cotton seedlings (Howell & Stipanovic, 1984).

Other secondary metabolites such as 6-pentyl-*α*-pyrone (6PP), produced by *T. viride* (Collins & Halim, 1972), *T. harzianum* (Cutler *et al.*, 1986), and *T. koningii* (Benoni *et al.*, 1990), as well as koninginin A, produced by *T. koningii* (Cutler *et al.*, 1989) and *T. harzianum* (Almassi

et al., 1991), showed both antifungal activity and typical symptoms of phytotoxicity, namely decreasing growth of the coleoptile and the induction of etiolation in wheat (Cutler *et al.*, 1986; Cutler *et al.*, 1989).

Trichothecenes, mainly known as *Fusarium* mycotoxins, are also found in metabolites of *Trichoderma* species. Some trichothecenes produced by *Trichoderma* spp., including *T. viride* Pers. (1794) [synonym *T. lignorum* (Tode) Harz], *T. polysporum* (Link) Rifai [synonym *T. sporulosum* (Link) S. Hughes], and *T. harzianum* are followed by trichodermin, its deacetyl derivative trichodermol, T-2 toxin, and harzianum A, which possess a 12,13-epoxide ring that is essential for their toxicity (Roush & Russo-Rodriguez, 1987). Trichodermin isolated from *T. viride* (Godtfredsen & Vangedal, 1964) showed an inhibitory effect on the elongation and termination steps in the protein synthesis by stimulating the rate of synthesis of transfer RNA (Westerberg *et al.*, 1976). Trichodermin and trichodermol exhibited high and mild cytotoxicity against tumor cell lines, respectively (Choi *et al.*, 1996). T-2 toxin, produced by *T. lignorum* (Tode) Harz (Bamburg & Strong, 1969), is reported as a mycotoxin that poses a significant threat to human health by inhibiting protein synthesis (Ueno *et al.*, 1973) and suppressing the immune system (Jagadeesan *et al.*, 1982). Harzianum A produced by *T. harzianum* (Corley *et al.*, 1994) showed cytotoxicity against cancer cell lines (Lee *et al.*, 2005).

In addition, *Trichoderma* spp. may have undesired effects on host pathogens. It was demonstrated that the number of reproductive structures, apothecia, of *S. sclerotiorum* was increased when *Trichoderma* spp. were used as foliar sprays for controlling *S. sclerotiorum* (Gerlagh *et al.*, 1999).

2.4.2 *Clonostachys rosea*

Clonostachys rosea Link Schroers, Samuels, Seifert & W. Gams [synonym *Gliocladium roseum* Bainier], *Paraconiothyrium minitans* (W.A. Campb.) Verkley [synonym *Coniothyrium minitans* W.A. Campb.], and *Talaromyces flavus* (Klocker) Stolk & Samson are necrotrophic mycoparasites, with 3.95% contribution to the total number of published articles between 2005 and 2015 (Figure 2.1.a).

An ascomycetous *Clonostachys rosea* [teleomorph: *Bionectria ochroleuca* (Schwein.) Schroers & Samuels] has been reported as both a saprophyte and a necrotrophic mycoparasite on *Alternaria* spp. (causing black rot of carrot) (Jensen *et al.*, 2004), *Sclerotinia sclerotiorum* (Xue,

2003), *Verticillium dahliae* Kleb. (Keinath *et al.*, 1991), *Bipolaris sorokiniana* (Sacc.) Shoemaker (Knudsen *et al.*, 1995), *Fusarium culmorum* (Wm.G. Sm.) Sacc. (Jensen *et al.*, 2000), and *Botrytis cinerea* (Nobre *et al.*, 2005). Comparative genome analysis and phylogeny based on DNA sequences showed that *C. rosea* isolates formed a cluster of sister taxa to plant pathogenic *Fusarium* species. They have been evolved from saprotrophic and mycoparasitic *Trichoderma* spp. belonging to *T. reesei*, *T. virens*, *T. atroviride* clade (Karlsson *et al.*, 2015). Antagonism, competition, and mycoparasitism are the main tactics employed by *C. rosea* to control plant pathogens. In addition, the production of several metabolites such as gliotoxin and endochitinase, as well as their synergetic effects, lead to effective biological control for several plant diseases (Di Pietro *et al.*, 1993). However, gliotoxin may pose a risk to the immune systems of humans and animals through the ingestion of the toxin (Mullbacher *et al.*, 1985).

2.4.3 *Paraconiothyrium minitans*

Paraconiothyrium minitans (W.A. Campb.) Verkley [synonym *Coniothyrium minitans* W.A. Campb.] with teleomorph in *Paraphaeosphaeria* O.E. Erikss. has been reported to be an effective BCA of *Sclerotinia sclerotiorum* on canola (McLaren & Huang, 1996), bean (Gerlagh *et al.*, 1999), sunflower (McLaren *et al.*, 1994), and lettuce (Whipps & Gerlagh, 1992). There is no report of the detection of any harmful secondary metabolites or the evidence of phytotoxicity caused by this mycoparasite. However, *P. minitans* has limitations such as slow growth rate and growth preference for substrates based on liquid media, leading to difficulty in the mass production of fungal spores (De Vrije *et al.*, 2001).

2.4.4 *Talaromyces flavus*

Talaromyces flavus (Klocker) Stolk & Samson [synonym *T. vermiculatus* (P.A. Dang.) C.R. Benj.] is the most common species in the genus *Talaromyces*. It has shown mycoparasitic activity against *Sclerotinia sclerotiorum* (Lib.) de Bary on sunflower (McLaren *et al.*, 1994) as well as high biocontrol effectiveness *in vitro* against *Rhizopus oryzae* Went & Prins. Geerl., *Pythium graminicola* Subraman., (1928), and *Gibberella fujikuroi* (Sawada) Wollenw., causing rice seedling diseases (Miyake *et al.*, 2012). The mode of action of *T. flavus* is the result of demonstrated synergism between direct mycoparasitism and production of lytic enzymes (Madi *et al.*, 1997). In particular, talaron and glucose oxidase, produced by *T. flavus,* exhibit antibiotic

activity against the microsclerotia of *Verticillium dahliae* Kleb., which together with *V. albo-atrum* Reinke & Berthold is responsible for Verticillium wilt on approximately 400 plant species (Kim *et al.*, 1990; Murray *et al.*, 1997). However, *T. flavus*'s mycoparasitic action requires hydrogen peroxide generated from glucose by glucose oxidase (Kim *et al.*, 1988), which in turn may provoke phytotoxic effects in plants. Indeed, *T. flavus* induced the reduction of seedling height as well as suppression of seed germination and the formation of the lateral roots in cotton seedlings (Murray *et al.*, 1999), which may imply certain risks for the massive application of *T. flavus* on susceptible crop varieties.

2.5 Specialist-biotrophic mycoparasites

In addition to the described necrotrophic BCAs, little is known regarding biotrophic mycoparasites. Data presented by the Web of Knowledge indicate that although fungal genera *Ampelomyces* Ces. ex Schltdl. and *Sphaerodes* Clem. have been gaining importance since 2005 in terms of the number of publications, only 0.85% of articles published between 2005 and 2015 focused on biotrophic mycoparasites (Figure. 2.1.b). At the same time, the first results highlighted promising avenues for biotrophic mycoparasites, which calls for more research to uncover their diversity and translational biocontrol functions. Industries are rightfully demanding timely plant care against mycotoxigenic *Fusarium* species, but this critical knowledge for developing efficient biocontrol products remains in its infancy.

2.5.1 *Ampelomyces quisqualis*

Ampelomyces quisqualis Ces. is an obligate biotrophic mycoparasite that naturally colonizes powdery mildew (*Erysiphales*) on angiosperms. This BCA attacks more than 60 phytopathogenic hosts on most economically important glasshouse crops, including cucumber, strawberry, grape, and tomato. *A. quisquialis* demonstrated efficient biotrophic mycoparasitism through direct contact, invasion, and penetration of the host cell wall, provoking both mechanical and enzymatic cell destruction during the process of appressorium formation within the cell of its fungal host (Sundheim & Krekling, 1982). The final result of its action is host cell degeneration and cytoplasm decomposition, resulting in host death (Hashioka & Nakai, 1980). The production of toxic secondary metabolites with phytotoxic properties by *A. quisquialis* are not reported. In greenhouse experiments, *A. quisqualis* showed a particularly high level of biocontrol efficacy on

Podosphaera fuliginea (Schltdl.) U. Braun & S. Takam. [synonym *Sphaerotheca fuliginea* (Schltdl.) Pollacci] and *Podosphaera aphanis* (Wallr.) U. Braun & S. Takam [synonym *Sphaerotheca macularis* sensu auct. NZ] on cucumber (Sundheim, 1982) and strawberry (Pertot *et al.*, 2004), respectively. However, the presence of a fungal host and high relative humidity are required for the growth of *A. quisqualis*, implying limitations of applying this BCA under field conditions.

2.5.2 *Sphaerodes* spp.

Three species of *Sphaerodes, S. quadrangularis* Dania García, Stchigel & Guarro (2004), *S. mycoparasitica* Vujan. (2009), and *S. retispora* var. *retispora* (Udagawa and Cain) P.F. Canon & D. Hawksw. (1982) are recently discovered biotrophic mycoparasites. Although the initial information on their biocontrol applications is promising, the number of published articles is still very low, only 0.1% of all published data on biotrophic mycoparasites (Figure 2.1.b). *Sphaerodes* mycoparasites are known as specific BCAs for *Fusarium* hosts, although *S. mycoparasitica* exhibits a polyphagous lifestyle, attacking more than one *Fusarium* species. *S. quadrangularis* is a facultative contact biotrophic mycoparasite that only parasitizes *Fusarium avenaceum* (Fr.) Sacc. (teleomorph: *Gibberella avenacea* Cooke) (Goh & Vujanovic, 2010a). In contrast, *S. retispora* var. *retispora* is not a powerful competitor due to its nutritional dependency on its unique natural host *Fusarium oxysporum* f. sp. *nuveum* (E.F. Sm.) W.C. Snyder & H.N. Hansen (Harveson *et al.*, 2002). Thus, further research is needed to better understand the diversity of biotrophic mycoparasitism and the relevant control strategies, including tri-trophic fungus-fungus-plant relationships, in order to advance discovery for the future development of this type of BCA product.

S. mycoparasitica was initially isolated from wheat and asparagus fields in association with *F. oxysporum*, *F. avenaceum*, and *F. graminearum* (Vujanovic & Goh, 2009). Its biotrophic mycoparasitism has been described, its host-specificity evaluated, its compatibility tested, and the stimulatory effect of *Fusarium* filtrates on ascospore germination measured (Goh & Vujanovic, 2010c). The mycoparasitism of *S. mycoparasitica* is characterized by direct contact with *Fusarium* hyphae and the formation of haustoria-like parasitic structures inside *Fusarium* cells. It was found that during this colonization process, *S. mycoparasitica* degraded and absorbed aurofusarin, a constitutive mycotoxin in the form of a red pigment within the *F.*

graminearum cell wall. This BCA showed the capacity to transform, absorb or eliminate toxic secondary metabolites produced by *Fusarium* species (Vujanovic & Goh, 2011b). Moreover, *S. mycoparasitica* may reduce the quantity of DNA in its host and down-regulate *Tri 5* gene expression in controlling the biosynthesis of trichothecenes by *F. graminearum* 3-ADON and 15-ADON chemotypes (Vujanovic & Goh, 2011b). In addition, *S. mycoparasitica* suppressed the production of mycotoxins by Fusaria in a co-culture system, making this BCA attractive for applications in agriculture and food processing (Vujanovic & Chau, 2012). As a host-specific organism and a non-producer of mycotoxin or phytotoxin, this mycoparasite might be translated into an environmentally friendly BCA product with commercial potential.

2.6 Factors affecting mycoparasitism

The effectiveness of mycoparasitism is closely related to fungus-fungus compatibility, growth dynamic, pattern of colonization, and interaction between the mycoparasite and the plant pathogenic host at the cellular and molecular level. Mycoparasitism is often regulated by a combination of intrinsic and extrinsic factors. Intrinsic or genetic factors include susceptibility or characteristics of the plant pathogen/host that may relate to the developmental stage of the host. For example, the anamorph of the host was susceptible to parasites or parasitism, whereas the teleomorph of the host was resistant to parasites or parasitism (Slifkin, 1961). Extrinsic or environmental factors include nutrition, temperature, pH, light, and other organisms. Nutrition, one of the most important factors in fungal growth, affects the physiological and biochemical susceptibility of the host defense system and immunity. The high level of dextrose can positively influence the degree of mycoparasitism (Boosalis, 1964), whereas fluctuation in nutrient and carbon-nitrogen ratios may negatively affect the degree of mycoparasitism (Ayers, 1935; Butler, 1957).

2.7 Host-plant pathogenic *Fusarium* species

Fusarium species are common pathogens and cause disease symptoms such as Fusarium head blight (FHB), Fusarium crown and root rot (FCRR), and Fusarium wilt on economically important crops including wheat, barley, maize, and asparagus. Each particular symptom can be associated with more than one mycotoxigenic *Fusarium* species. For instance, FHB or scab of wheat and barley is mainly associated with *Fusarium graminearum* Schwabe, *F. avenaceum*

(Fr.) Sacc., *F. culmorum* (Wm.G. Sm) Sacc., and *F. poae* (Peck) Wollenw., (Parry *et al.*, 1995). Some other *Fusaria* are sporadically present, including *F. equiseti* (Corda) Sacc., *F. sporotrichioides* Sherb., *F. acuminatum* Ellis & Everh., *F. oxysporum* Schltdl., *F. incarnatum* (Desm.) Sacc. [synonym *F. semitectum* Berk. & Ravenel], *F. moniliforme* J. Sheld. [synonym *F. subglutinans* (Wollenw. & Reinking) P.E. Nelson, Toussoun & Marasas], *F. proliferatum* (Matsush.) Nirenberg ex Gerlach & Nirenberg, *F. sambucinum* Fuckel, *F. tricinctum* (Corda) Sacc., and *F. crookwellense* L.W. Burgess, P.E. Nelson & Toussoun (Wilcoxson *et al.*, 1988). Fusarium crown and root rot (FCRR) of wheat is caused by *F. acuminatum*, *F. avenaceum*, *F. culmorum*, *F. equiseti*, *F. graminearum* (Fernandez *et al.*, 2007), *F. pseudograminearum* O'Donnell & T. Aoki, and *F. oxysporum* (Fernandez & Jefferson, 2004). FCRR of asparagus is caused by another species complex: *F. oxysporum*, *F. proliferatum*, *F. solani* (Mart.) Sacc., *F. acuminatum*, and *F. redolens* Wollenw. (Borrego-Benjumea *et al.*, 2014; Elmer *et al.*, 1999). Fusarium ear rot of maize is mostly caused by *F. verticillioides* (Sacc.) Nirenberg, *F. proliferatum*, *F. subglutinans*, and *F. graminearum* (Sewram *et al.*, 2005). The diseased plants result in economic loss due to the reduced quantity and quality of crops, which is also due to the presence of mycotoxins.

2.8 Mycotoxins produced by *Fusarium* spp.

Plant pathogenic *Fusarium* spp. not only reduce crop yield but also contaminate grains by producing a variety of mycotoxins, known as fungal toxic secondary metabolites. The dominant mycotoxins in cereal production worldwide are trichothecenes, zearalenone, and fumonisins, whereas more emerging mycotoxins are moniliformin, fusaproliferin, beavericins, and eniatins. The contamination and accumulation of mycotoxins on crops are closely related to health risks through leading to carcinogenicity, neurotoxicity, and reproductive and developmental toxicity to humans and animals (Doi & Uetsuka, 2011; Gelderblom *et al.*, 1991; Malir *et al.*, 2013). *Fusarium graminearum* and *F. culmorum*, the two most aggressive and predominant plant pathogens among diverse *Fusarium* species on cereals and maize, are the main producers of trichothecenes and zearalenone.

Trichothecenes can be divided into two types such as Type A trichothecenes, including T-2 toxin, HT-2 toxin, diacetoxyscirpenol and Type B trichothecenes, including deoxynivalenol (DON), nivalenol, 3-acetyl-deoxynivalenol (3ADON), and 15-acetyl-deoxynivalenol (15ADON)

(McCormick *et al.*, 2011). These mycotoxins, biosynthesized by trichodiene synthase encoded on *Tri 5,* mainly affect metabolic mechanisms related to the inhibition of protein synthesis (Ehrlich & Daigle, 1987; Middlebrook & Leatherman, 1989). In animals, direct contact or oral ingestion of trichothecenes causes rapid irritation to the skin or intestinal mucosa (Yazar & Omurtag, 2008). Diarrhea, vomiting, leukocytosis, gastrointestinal hemorrhage related to acute toxicosis and anorexia, reduced weight gain, altered nutritional efficiency, and immunotoxicity related to chronic toxicosis are also reported as major impacts of trichothecens (Pestka & Smolinski, 2005). The most important trichothecenes are DON and T-2 toxin, which are responsible for alimentary toxic aleukia in humans and animals. Structurally, the 12,13-epoxide of the trichothecenes is essential to their toxicity (Desjardins *et al.*, 1993). Deoxynivalenol, also known as vomitoxin, is one of the most abundant and significant trichothecenes in food and feed (Awad *et al.*, 2010). As a mode of action, deoxynivalenol disrupts normal cell function by inhibiting protein synthesis via binding to the ribosome and by activating critical cellular kinases involved in signal transduction related to proliferation, differentiation, and apoptosis (Pestka & Smolinski, 2005).

Zearalenone (ZEN), a member of the resorcyclic acid lactone family, is an estrogenic toxin, an endocrine disruptor with estrogenic potency that causes reproductive problems in animals, particularly in swine (Katzenellenbogen *et al.*, 1979; Shier *et al.*, 2001). ZEN is mainly produced by *F. graminearum* and *F. culmorum* (Caldwell *et al.*, 1970; Katzenellenbogen *et al.*, 1979) and biosynthesized through the polyketide synthase (PKS) pathway (Gaffoor & Trail, 2006; Kim *et al.*, 2005; Lysøe *et al.*, 2006). Structurally, zearalenone displaces estradiol (a major estrogen) from its uterine binding protein due to its similar chemical structure (a resorcinol moiety fused to a 14-member macrocyclic lactone ring) (Kuiper *et al.*, 1997) to mammalian estrogen (Iqbal *et al.*, 2014; Shier *et al.*, 2001). The estrogenic effects of zearalenone, including infertility and reduced incidence of pregnancy, were observed in swine (Kordic *et al.*, 1992; Shier *et al.*, 2001). ZEN contamination, frequently found in maize, wheat, and even food commodities for human consumption, may cause potential reproductive problems in other animals and humans.

Fumonisins are widely found in maize and asparagus infected by *F. verticillioides* and *F. proliferatum*, known as the highest fumonisin producers (Liu *et al.*, 2007; Sewram *et al.*, 2005). Fumonisins can be classified into four main groups, FA, FB, FC, and FP. The FB group,

including FB_1, FB_2, and FB_3, occurs more frequently than the others. Due to their structural similarity to sphinganine, fumonisins disrupt the metabolism of sphingolipids by inhibiting shinganine and shingosine *N*-aceyltransferase, leading to the accumulation of free sphinganine and modification of the sphinganine to sphingosine ratio, which may result in cell death or proliferation and may be involved in carcinogenesis (Merrill Jr *et al.*, 2001; Riley *et al.*, 2001; Soriano *et al.*, 2005). Fumonisin B_1, the most abundant fumonisin, is known to cause leukoencephalomalacia (Kellerman *et al.*, 1990), pulmonary edema syndrome, hydrothorax (Harrison *et al.*, 1990), liver cancer (Gelderblom *et al.*, 1996), hypercholesterolemia, immunological alterations (Voss *et al.*, 2007), and death in animals (Sandmeyer *et al.*, 2015).

Moniliformin, an emerging mycotoxin, is frequently found on infected maize and is known to be biosynthesized by *F. redolens*, *F. oxysporum*, and *F. proliferatum* (Schütt *et al.*, 1998) through the polyketide pathway (Franck & Breipohl, 1984). Chemically, moniliformin is extremely soluble in water and is stable in acidic conditions. The mode of moniliformin action is known to competitively inhibit the activity of pyruvate dehydrogenase complex in respiratory reactions by preventing the conversion of pyruvic acid to acetyl CoA (Burka *et al.*, 1982; Gathercole *et al.*, 1986). The acute toxicity of moniliformin is higher than in other emerging mycotoxins and causes muscular weakness, respiratory stress, and myocardial degeneration, resulting in coma and death (Jestoi, 2008; Kriek *et al.*, 1977).

The reduction of these major mycotoxin contaminants in crops and other food commodities requires the application of a multifaceted approach to both pre- and post-harvest agriculture (Leslie & Logrieco, 2014). Among integrated control measures, biocontrol is gaining importance (Tsitsigiannis *et al.*, 2012) for its adaptability to both agriculture production systems and food processing, both focusing on the reduction of mycotoxins in the food chain.

2.9 Reduction of *Fusarium* mycotoxins

Approximately 25% of the world grain supply is contaminated by mycotoxins, according to the UN Food and Agriculture Organization. Many breeding and management efforts to address the mycotoxin problem in grain have generated limited effects (Leslie & Logrieco, 2014). The major difficulty associated with the possible control of mycotoxins is that a single *Fusarium* species can produce many different mycotoxins, and a single grain may contain more than one mycotoxigenic *Fusarium* species (Covarelli *et al.*, 2015; Jurado *et al.*, 2006). Climate change

only aggravates an already difficult situation with increasing mycotoxin contamination worldwide (Paterson & Lima, 2010). In addition, the detoxification of mycotoxins is a slow process, as they are highly stable compounds that are resistant to heat, radiation and other physical and chemical treatments (Kabak, 2010). Although some biological treatments using bacteria and fungi have shown promising results (McCormick, 2013), the detoxification or degradation of mycotoxins by BCAs has been poorly understood. Recently, the efficient biocontrol of mycotoxins by mycoparasites has been reported. Among necrotrophic mycoparasites, *Clonostachys rosea* showed a feasible capacity to degrade zearalenone. An enzyme, zearalenone lactonohydrolase (ZHD101) of *C. rosea,* can detoxify zearalenone in zearalenone-producing *F. graminearum* (Kosawang *et al.*, 2014). The transformant 1-(3,5-dihydroxyphenyl)-10´-hydroxy-1´-undecen-6´-one of zearalenone, converted by zearalenone lactonohydrolase (Takahashi-Ando *et al.*, 2002), was found to be a far less estrogenic compound than ZEN (El-Sharkawy & Abul-Hajj, 1988a). In addition to the necrotrophic mycoparasite *C. rosea*, the biotrophic mycoparasite *Sphaerodes mycoparasitica* was reported to reduce the quantity of the *Tri* gene involved in the biosynthesis pathway of trichothecenes in *F. graminearum* 3-ADON and 15-ADON chemotypes (Vujanovic & Goh, 2011b). This mycoparasite effectively suppresses the production of several mycotoxins, such as DON, 3-ADON, 15-ADON, and ZEN, in the same sample under co-culture system (Vujanovic & Chau, 2012). The mechanisms of the degradation or detoxification of mycotoxins by *S. mycoparasitica* remain unknown. A better understanding of the biocontrol mechanism of *S. mycoparasitica* against *Fusarium* species is warranted, as it is a *Fusarium*-specific mycoparasite, polyphagous on *Fusarium* spp., which means that this BCA can control a complex of species and spectrum of mycotoxins. The biocontrol and biodegradation of mycotoxins by mycoparasites are promising fields of biotechnology that open several opportunities through research and innovation on the functional diversity of mycoparasites and their lifestyles. Future studies using omics on mycoparasites to study the control of the *Fusarium* species complex and mycotoxins, the mechanism of down-regulation of the expression of *Tri* and *PKS* genes, and the suppression and/or biodegradation of multiple mycotoxins by *S. mycoparasitica* is merited.

Table 2.1 Different types of mycoparasites related with their lifestyles or mode of actions, secondary metabolites produced or genes responsible for biosynthesis of secondary metabolites as well as its toxicity to host plant pathogens and/or crops.

Lifestyle	Mycoparasite	Mode of action of mycoparasite	Secondary metabolites or gene responsible for biosynthesis	Toxicity of secondary metabolite	Host plant pathogens	Crops	References
Necrotrophic mycoparasite	*Trichoderma harzianum*	Necrotrophism Production of lytic enzymes and secondary metabolites Plant defense system	Gliotoxin	Reduction of fungal growth	*Sclerotium cepivorum*	Onion	(Haggag & Mohamed, 2002)
			Viridin	Reduction of fungal growth	*Sclerotium cepivorum*	Onion	(Haggag & Mohamed, 2002)
			6-Pentyl- α –pyrone (6PP)	Inhibition of plant growth and induction of etiolation	*Chaetomium spp.* *Aspergillus flavus*	Wheat coleoptiles	(Cutler *et al.*, 1986)
			6PP	Inhibition of plant growth and induction of etiolation	*Rhizoctonia solani*	Lettuce seedlings	(Claydon *et al.*, 1987)
			6PP		*Botrytis cinerea*		(Cooney & Lauren, 1998)
			Koninginin A				(Almassi *et al.*, 1991)
			Trichodermin	Reduction of fungal growth	*Sclerotium cepivorum*	Onion	(Haggag & Mohamed, 2002)
			Harzianum A (Type A trichothecene)	Antifungal activity	*Candida albicans* *Saccharomyces cerevisiae*		(Corley *et al.*, 1994)
			Harzianum A	Cytotoxicity to cancer cell lines			(Lee *et al.*, 2005)
			**Tri5* required for biosynthesis of trichothecene				(Gallo *et al.*, 2004)

**Tri5* gene encodes trichodiene synthase for production of trichothecenes.

Table 2.1 Different types of mycoparasites related with their lifestyles or mode of actions, secondary metabolites produced or genes responsible for biosynthesis of secondary metabolites as well as its toxicity to host plant pathogens and/or crops (continued).

Lifestyle	Mycoparasite	Mode of action of mycoparasite	Secondary metabolites or gene responsible for biosynthesis	Toxicity of secondary metabolite	Host plant pathogens	Crops	References
Necrotrophic mycoparasite	*Trichoderma viride* (synonym *T. lignorum*)	Necrotrophism Production of lytic enzymes and secondary metabolites Plant defense system	Gliotoxin				(Brian, 1944)
			Viridin (easily converted to viridiol)	Inhibition of germination of conidia	*Botrytis allii* *Fusarium spp.* *Trichothecium roseum* *Cephalosporium spp.* *Penicillium spp.* *Aspergillus spp.*		(Brian & McGowan, 1945)
			6PP				(Collins & Halim, 1972)
			T-2 toxin (Type A trichothecene)				(Bamburg & Strong, 1969)
			T-2 toxin	Inhibition of protein synthesis in animal cells			(Ueno *et al.*, 1973)
			T-2 toxin	Suppression of immune system in animal			(Jagadeesan *et al.*, 1982)
Necrotrophic mycoparasite	*Trichoderma virens* (synonym *Gliocladium virens*)	Necrotrophism Production of lytic enzymes and secondary metabolites Plant defense system	Gliotoxin		*Pythium ultimum* *Sclerotinia sclerotiorum*	Cotton seedlings	(Vargas *et al.*, 2014)
			Gliotoxin		*Botrytis cinerea*		(Lorito *et al.*, 1994)
			Gliotoxin	Antifungal activity	*Rhizoctonia bataticola* *Macrophomina phaseolina* *Pythium deharyanum* *Pythium aphanidermatum* *Sclerotium rolfsii* *Rhizoctonia solani*		(Singh *et al.*, 2005)
			Viridin				(Singh *et al.*, 2005)
			Viridiol				(Singh *et al.*, 2005)
			Viridiol	Necrosis of radicles		Cotton seedlings	(Howell & Stipanovic, 1984)
			Trichodermin (Type A trichothecene)	High cytoxicity on tumor cell lines			(Choi *et al.*, 1996)
			Trichodermol	Mild cytoxicity on tumor cell lines			(Choi *et al.*, 1996)

Table 2.1 Different types of mycoparasites related with their lifestyles or mode of actions, secondary metabolites produced or genes responsible for biosynthesis of secondary metabolites as well as its toxicity to host plant pathogens and/or crops (continued).

Lifestyle	Mycoparasite	Mode of action of mycoparasite	Secondary metabolites or gene responsible for biosynthesis	Toxicity of secondary metabolite	Host plant pathogens	Crops	References
Necrotrophic mycoparasite	*Trichoderma koningii*	Necrotrophism Production of lytic enzymes and secondary metabolites	Gliotoxin	Reduction of fungal growth	*Sclerotium cepivorum*	Onion	(Haggag & Mohamed, 2002)
			Viridin	Reduction of fungal growth	*Sclerotium cepivorum*	Onion	(Haggag & Mohamed, 2002)
			6PP		*Phytophthora cinnamomi*		(Benoni *et al.*, 1990)
			Koninginin A	Inhibition of plant growth and induction of etiolation		Wheat coleoptiles	(Cutler *et al.*, 1989)
			Trichodermin	Reduction of fungal growth	*Sclerotium cepivorum*	Onion	(Haggag & Mohamed, 2002)
Necrotrophic mycoparasite	*Clonostachys rosea* (synonym *Gliocladium roseum*)	Necrotrophism Production of lytic enzymes and secondary metabolites	Gliotoxin				(Papavizas, 1985)
				Reduction in viability of microsclerotia	*Verticillium dahliae*		(Keinath *et al.*, 1991)
					Bipolaris sorokiana	Barley	(Knudsen *et al.*, 1995)
					Fusarium culmorum	Barley Wheat	(Knudsen *et al.*, 1995)
					Fusarium culmorum	Barley Wheat	(Jensen *et al.*, 2000)
					Sclerotinia sclerotiorum	Pea	(Xue, 2003)
					Alternaria spp.	Carrot	(Jensen *et al.*, 2004)
				Reduction in sporulation	*Botrytis cinerea*	Rose Strawberry Eucalyptus globulus Tomato	(Nobre *et al.*, 2005)
			Peptaibols	Antifungal activity	*Sclerotinia sclerotiorum*	Lettuce Soybean	(Rodríguez *et al.*, 2011)
			Polyketide synthases **Tri5*				(Karlsson *et al.*, 2015)

**Tri5* gene encodes trichodiene synthase for production of trichothecenes.

Table 2.1 Different types of mycoparasites related with their lifestyles or mode of actions, secondary metabolites produced or genes responsible for biosynthesis of secondary metabolites as well as its toxicity to host plant pathogens and/or crops (continued).

Lifestyle	Mycoparasite	Mode of action of mycoparasite	Secondary metabolites or gene responsible for biosynthesis	Toxicity of secondary metabolite	Host plant pathogens	Crops	References
Necrotrophic mycoparasite	*Paraconiothyrium minitans* (synonym *Coniothyrium minitans*)	Necrotrophism Production of lytic enzymes	Unknown		*Sclerotinia sclerotiorum*	Lettuce	(Whipps & Gerlagh, 1992)
					Sclerotinia sclerotiorum	Sunflower	(McLaren *et al.*, 1994)
					Sclerotinia sclerotiorum	Canola	(McLaren & Huang, 1996)
					Sclerotinia sclerotiorum	Bean	(Gerlagh *et al.*, 1999)
Necrotrophic mycoparasite	*Talaromyces flavus* (synonym *T. vermiculatus*)	Necrotrophism Production of lytic enzymes and secondary metabolites			*Sclerotinia sclerotiorum*	Sunflower	(McLaren *et al.*, 1994)
			Talaron Hydrogen peroxide generated by glucose oxidase	Reduction in height, seed set, seedling germination, and lateral root formation	*Verticillium dahliae*	Cotton Tobacco	(Kim *et al.*, 1990) (Murray *et al.*, 1999)
					Rhizopus oryzae *Pythium graminicola* *Gibberella fujikuroi*	Rice seedling	(Miyake *et al.*, 2012)
Biotrophic mycoparasite	*Ampelomyces quisqualis*	Biotrophism Production of lytic enzymes	Unknown		*Sphaerotheca fuliginea*	Cucumber	(Sundheim, 1982)
					Sphaerotheca macularis	Strawberry	(Pertot *et al.*, 2004)

Table 2.1 Different types of mycoparasites related with their lifestyles or mode of actions, secondary metabolites produced or genes responsible for biosynthesis of secondary metabolites as well as its toxicity to host plant pathogens and/or crops (continued).

Lifestyle	Mycoparasite	Mode of action of mycoparasite	Secondary metabolites or gene responsible for biosynthesis	Toxicity of secondary metabolite	Host plant pathogens	Crops	References
Biotrophic mycoparasite	*Sphaerodes mycoparasitica*	(Polyspecific) Biotrophism through hook-shaped contact	Unknown		*F. avenaceum* *F. oxysporum* *F. culmorum*	Wheat Asparagus	(Vujanovic & Goh, 2009) (Vujanovic & Goh, 2010)
		Biotrophism through contact and intracellular penetration			*F. equiseti*		(Vujanovic & Goh, 2010)
		Biotrophism through clamp- and hook-like contact and penetration Removal of red pigment of *F. graminearum* 3-ADON Significant decrease in **Tri5* gene expression of *F. graminearum* 3-ADON and 15-ADON			*F. graminearum* 3-ADON *F. graminearum* 15-ADON	Wheat	(Vujanovic & Goh, 2009) (Vujanovic & Goh, 2011b)
Biotrophic mycoparasite	*Sphaerodes quadrangularis*	(Monospecific) Biotrophism through hook-shaped and clamp-like contact	Unknown		*F. avenaceum*	Wheat	(Goh & Vujanovic, 2010a)
Biotrophic mycoparasite	*Sphaerodes retispora* var. *retispora*	(Monospecific) Biotrophism	Unknown		*F. oxysporum* f. sp. *niveum*	Watermelon	(Harveson & Kimbrough, 2001) (Harveson *et al.*, 2002)

**Tri5* gene encodes trichodiene synthase for production of trichothecenes.

3. ADAPTABILITY OF ANAMORPHIC AND TELEOMORPHIC STAGES IN *SPHAERODES MYCOPARASITICA* TOWARDS ITS MYCOPARASITIC-POLYPHAGOUS LIFESTYLE

3.1 Abstract

Sphaerodes mycoparasitica Vujan. is a newly discovered *Fusarium*-specific mycoparasite. Some recent discoveries recognize its biotrophic polyphagous lifestyle as an interesting biocontrol property against a broad spectrum of mycotoxigenic *Fusarium*-hosts. Secondary metabolites such as mycotoxins produced by *Fusarium* spp. may play an important role in the signaling process, allowing an early mycoparasite-host recognition. A multiple paper disc assay has been conducted to test *S. mycoparasitica* hyphal adaptability to filtrates of *Fusarium* strains. This study shows that shifts of adapted and non-adapted hyphal migration towards different *Fusarium*-hosts may partly explain *S. mycoparasitica* polyphagous lifestyle. This implies that the mycoparasite could possibly use a group of mycotoxins produced by specific *Fusarium* spp. as an adaptive selective mechanism which facilitates a parasite-host recognition. In particular, relative polarity or hydrophilicity/hydrophobicity of mycotoxins may be related to solubility and absorption property into hyphae of the mycoparasite. In addition to the anamorphic aspect of the mycoparasite, the effect of *Fusarium* filtrates on ascomata (teleomorph) formation of the mycoparasite was measured by the number of ascomata produced in the presence of *Fusarium* filtrates compared with sterilized distilled water (SDW) through the modified slide culture assay.

3.2 Introduction

Wheat is one of the major cereal grains or crops to satisfy demand for food and feed worldwide. Canada is one of the largest wheat producers and exporters in the world. The majority of Canadian wheat is grown in Saskatchewan, with 46% of total Canadian wheat production (AAFC, 2010). *Fusarium* spp, are the major cause of Fusarium head blight (FHB),

also known as scab disease, of small grain cereals including wheat, barley, oats, rye, and corn. FHB results in reduction of crop production and grain quality (Chen *et al.*, 2013; Osborne & Stein, 2007; Waśkiewicz *et al.*, 2013). Several *Fusarium* species detected in cereals and maize, such as *F. avenaceum*, *F. acuminatum*, *F. culmorum*, *F. graminearum*, *F. oxysporum*, *F. equiseti, F. proliferatum,* and *F. redolens,* are responsible for crop diseases and mycotoxin-related economic loss. Biological control agents are seen as socialy acceptable or eco-friendly solutions in controlling plant pathogens (Pal & Gardener, 2006). Indeed, mycoparasitic fungus *Sphaerodes mycoparasitica* was isolated from wheat and asparagus associated with *F. avenaceum*, *F. oxysporum*, and *F. graminearum* (Vujanovic & Goh, 2009). The host-range is restricted to a single host species, *Fusarium* species, indicating important biocontrol traits to be considered for preventing or reducing *Fusarium* outbreaks in crops. It was found that the anamorphic stage of the mycoparasite suppressed the growth of *Fusarium*-host species. The shift in the ascospore germination pattern has been detected in the presence of various *Fusarium* filtrates (Goh & Vujanovic, 2010c) as an indicator of the parasite-host compatibility (Vujanovic & Goh, 2011b). Thus, investigation on the possible parasite-host compatibility based on the adaptability of the mitosporic mycoparasite (asexual/somatic cell) growth pattern to different *Fusarium* filtrates can be crucial evidence for the elucidation of the co-evolution between mycoparasite's holomorph (sexual and asexual stages) with its host(s). The changes of the mode of action of the mycoparasite when exposed to different filtrates (Manocha, 1981) may be also result of the adaptation of the mycoparasite to its specific host towards efficient biocontrol of *Fusarium* spp., including their respective mycotoxins.

3.3 Hypotheses and objectives

In gerenall, a specific mycoparasite do parasitize only one host species reffereing to host-specificity. The host-specificity of the mycoparasite leads performance of the mycoparasite to parasitize original or fundamental host group; the host-specificity of *Sphaerodes mycoparasitica* might be affected by genetic foundation, *Fusarium*-host's metabolites and environment. It was hypothesized that *S. mycoparasitica* will change a pattern of hyphal (anamorphic, asexual, or mitosporic stage) growth when exposed to different *Fusarium* filtrates. Thus, it will indicate the level of parasite-host compatibility, preference or specificity. Furthermore, adaptation of *S. mycoparasitica* to host components might indicate adaptability or plasticity of the mycoparasite.

It was speculated that the mycoparasite's vegetative cells adapted over five generations on *Fusarium* filtrates will differ from sexual (reproductive stage) cells' adaptability measured by ascomata or teleomorph formation under exposure to *Fusarium* filtrates. The first may be epigenetically regulated as opposed to the second found to be genetic regulatory mechanisms (Goh & Vujanovic, 2010c). The objectives of this study were: (1) to examine host compatibility of *S. mycoparasitica* and its mitosporic adaptability by applying a multiple paper disc assay and assessing by microscopy; and (2) to evaluate the effect of *Fusarium* filtrates on ascomata formation of *S. mycoparasitica* on modified slide culture assay.

3.4 Materials and Methods

3.4.1 Fungal isolates and culture conditions

Mycoparasite *Sphaerodes mycoparasitica* Vujan. SMCD 2220-01 and twelve plant pathogenic *Fusarium* strains, such as *Fusarium oxysporum* Schltdl. SMCD 2242, *Fusarium avenaceum* (Fr.) Sacc SMCD 2241, *Fusarium torulosum* (Berk. & M.A. Curtis) Nirenberg SMCD 2139, *Fusarium graminearum* Schwabe 3-ADON chemotype SMCD 2243, *Fusarium graminearum* Schwabe 15-ADON chemotype SMCD 2244, *Fusarium graminearum* Schwabe 14A SMCD 2910, *Fusarium culmorum* (Wm.G. Sm.) Sacc. SMCD 2248, *Fusarium equiseti* (Corda) Sacc. SMCD 2134, *Fusarium acuminatum* Ellis & Everh. SMCD 2423, *Fusarium proliferatum* (Matsush.) Nirenberg SMCD 2246, *Fusarium redolens* Wollenw. V OTU 18 SMCD 2401, and *Fusarium redolens* Wollenw. W OTU 27 SMCD 2402 were retrieved from Saskatchewan Microbial Collection and Database (SMCD) and used in this study. Plugs of actively growing fungal cultures were inoculated and maintained on potato dextrose agar (PDA) (Difco) medium at 23 °C in the dark. Potato dextrose broth (PDB) was used for fungal growth in liquid to obtain *Fusarium* cultures followed by *Fusarium* filtrates (Goh & Vujanovic, 2010c).

3.4.2 Adaptation of *Sphaerodes mycoparasitica* to twelve *Fusarium* filtrates

Fusarium fungal isolates were inoculated in 30 mL of PDB medium and then incubated at 23 °C in the dark for 14 d. *Fusarium* cultures from twelve different types of *Fusarium* isolates were filtered through four thin layers of cheesecloth and sterilized by 0.2 µm filter. The prepared *Fusarium* filtrates were diluted with PDB medium (1:1, v/v) and used as nutrient sources for adaptation of *S. mycoparasitica* to each of twelve *Fusarium* filtrates. Adaptation of *S.*

mycoparasitica to twelve *Fusarium* filtrates was conducted on 96 wells plates. Plugs of *S. mycoparasitica* (5/5 mm) which was grown on PDA were incubated with 200 µL of the diluted twelve *Fusarium* filtrates, respectively. After incubation at 23 °C in the dark for 3 d on rotary shaker (130 rpm), plugs were washed using sterilized distilled water and then transferred into new wells of plates. Fresh *Fuarium* filtrates were supplied to allow *S. mycoparasitica* to adapt each of twelve *Fusarium* filtrates. After five times transferring, the plugs adapted to *Fusarium* filtrates were used for the multiple paper disc assay as an adapted mycoparasite to each of twelve *Fusarium* filtrates. The plugs incubated in PDB medium diluted with SDW (1:1, v/v) without *Fusarium* filtrates were used as a non-adapted mycoparasite for the multiple paper disc assay (Garrett & Robinson, 1969).

3.4.3 Multiple paper disc assay

Host compatibility and adaptability of the mycoparasite were investigated on a multiple paper disc assay. The non-adapted mycoparasite and the mycoparasite adapted to each of *Fusarium* filtrates were placed on the center of PDA plates. Two hundreds microlitre of *Fusarium* filtrates was spotted on filter paper discs around the mycoparasite and 200 µL of PDB was used as a control. The experiments were conducted in four combinations: SMCD 2242, 2241, 2139, and PDB; SMCD 2243, 2244, 2910, and PDB; SMCD 2248, 2134, 2423, and PDB; and SMCD 2246, 2401, 2402, and PDB. The combinations were decided by taxonomical sections and produced mycotoxins as well as the morphology of *Fusarium* strains. The plates were incubated at 23 °C in the dark for 7 d. The hyphal migration of mycoparasite was observed and the radial growth of the mycoparasite toward each of *Fusarium* filtrates and control was recorded (Ouimet *et al.*, 1997; R. M. Harveson & J. W. Kimbrough, 2001). The relative radial growth of the non-adapted mycoparasite toward each *Fusarium* filtrate was calculated by following formula:

$$Mean\ percentage\ of\ relative\ radial\ growth = Mean\left[\frac{(Radial\ growth\ toward\ each\ Fusarium\ filtrate - Radial\ growth\ toward\ PDB\ as\ a\ control)}{Radial\ growth\ toward\ PDB\ as\ a\ control} \times 100\right] \quad \text{(Equation 3.1)}$$

3.4.4 Modified slide culture assay

The effect of *Fusarium* filtrates on ascomata formation of the mycoparasite was investigated on the modified slide culture assay as shown in Figure 3.1. The slide culture assay allows one to observe hyphal migration directly under microscope without further treatment as well as to count ascomata easily. Mycelia patterns of the mycoparasite were observed under a Carl Zeiss Axioskop2 microscope equipped with Carl Zeiss AxioCam ICc1 camera with the 20x, 40x, and 100x objectives. For the preparation of slide cultures, 2 mL of the sterilized water agar was spread uniformly on the sterilized microscope slides (76.2 mm × 25.4 mm). After cooling and hardening the medium on a slide, the mycoparasite was placed on the center of the slide medium. The capillary tubes including 5 µL of *Fusarium* filtrates were loaded around the mycoparasite. The sterilized distilled water (SDW) was used as a control. The modification of the slide culture assay by placing the capillary tubes enables the mycoparasite to absorb *Fusarium* filtrates slowly (Cole *et al.*, 1969). The combinations of SMCD were the same as in multiple paper disc assay with SDW as the reference instead of PDB. The inoculated slide cultures were incubated in petri dishes (200 mm × 20 mm) at 23 °C in the dark for one month. After one month of incubation, the number of ascomata was counted. Ascomata formation or teleomorph sporulation, as well as relative ascomata formation, were calculated by the following formulas (Goh & Vujanovic, 2010a).

$$Mean\ percentage\ of\ ascomata\ formation = \frac{Number\ of\ produced\ ascomata\ for\ each\ Fusarium\ filtrate}{Total\ number\ of\ produced\ ascomata\ on\ the\ slide\ culture} \times 100 \quad \text{(Equation 3.2)}$$

$$Mean\ percentage\ of\ relative\ ascomata\ formation = Mean\left[\frac{(Number\ of\ produced\ ascomata\ for\ each\ Fusarium\ filtrate - Number\ of\ produced\ ascomata\ for\ SDW\ as\ a\ control)}{Number\ of\ produced\ ascomata\ for\ SDW\ as\ a\ control} \times 100\right] \quad \text{(Equation 3.3)}$$

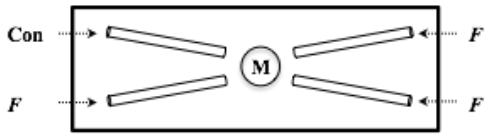


Figure 3.1 Illustration for the modified slide culture assay. M, Con, and F indicate mycoparasite, sterilized distilled water, and *Fusarium* filtrates, respectively.

3.4.1 Statistical Analysis

Mean percentage of relative radial growth of the non-adapted mycoparasite toward *Fusarium* filtrates was analyzed by one-way analysis of variance (ANOVA) with Tukey's honest significant difference test (Tukey's HSD) at *p*-value 0.05 to evaluate host compatibility. Radial growth between the non-adapted mycoparasite and adapted mycoparasite toward twelve *Fusarium* filtrates were analyzed by a two-factor factorial design-two way ANOVA with Least Significant Difference (LSD) test to assess if interaction between adaptation and types of *Fusarium* filtrates is detected. When there is a significant interaction, differences among treatments were tested with LSD method. Data are reported as means and standard errors of three replicates ($p < 0.05$). The effect of *Fusarium* filtrates on ascomata formation of the mycoparasite through the modifed slide culture assay was analyzed by one-way ANOVA (SPSS, 1990).

3.5 Results

3.5.1 Host compatibility of mycoparasite

Host compatibility or host preference of mycoparasite *S. mycoparasitica* was represented by the percentage of relative radial growth of the non-adapted mycoparasite toward each of the *Fusarium* filtrates through the multiple paper disc assay as shown in Figure 3.2. Mean percentage of relative radial growth of the non-adapted mycoparasite toward *Fusarium* filtrates was significantly differerent in twelve *Fusarium* filtrates since the *p*-value was less than 0.05 based on one-way ANOVA analysis (Figure 3.3).

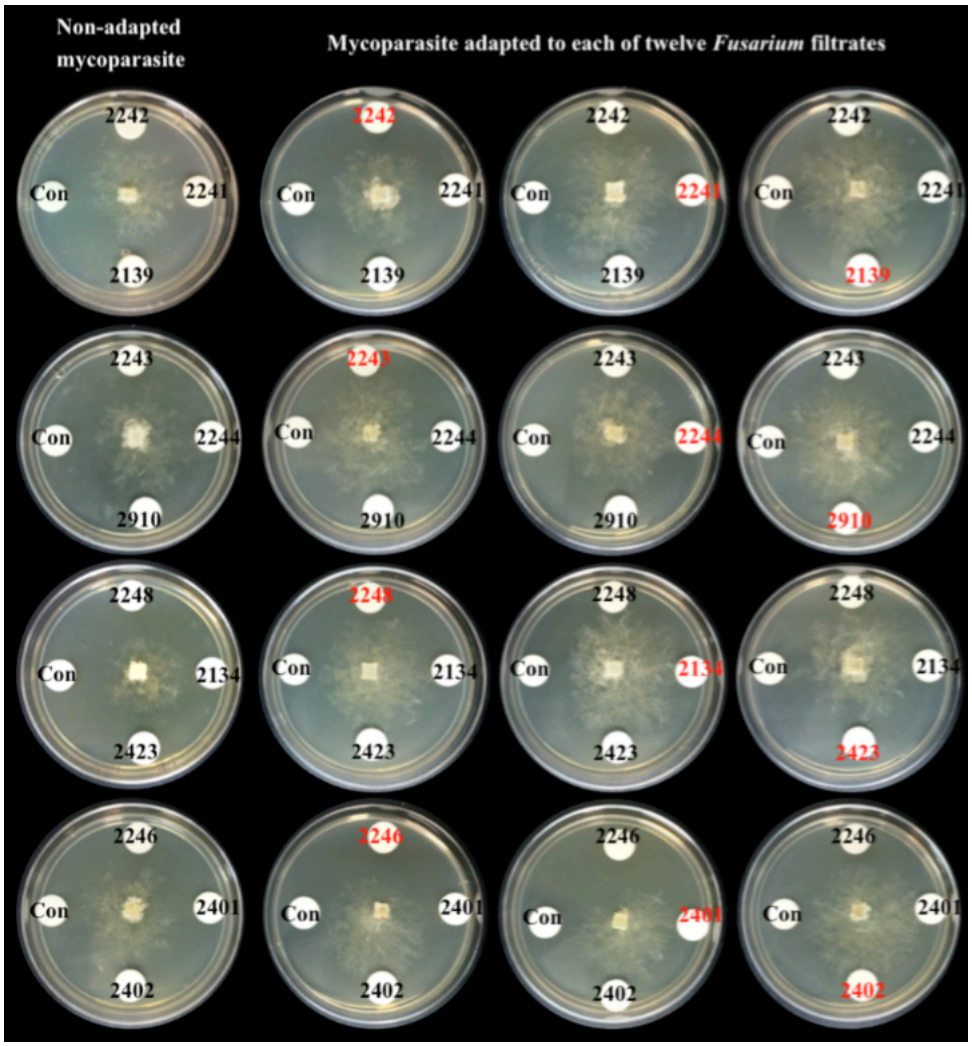


Figure 3.2 The hyphal growth of non-adapted mycoparasite and mycoparasite adapted to each *Fusarium* filtrate on the multiple paper disc assay. From left to right side, the first column shows the hyphal growth of non-adapted mycoparasite toward each *Fusarium* filtrate. The second, third, and last columns show the hyphal growth of mycoparasite adapted to each *Fusarium* filtrate. The red-colored letters indicate the adaptation to the selected *Fusarium* isolate on each combination. The first row shows the first combination of filtrates (*F. oxysporum* SMCD 2242, *F. avenaceum* SMCD 2241, and *F. torulosum* SMCD 2139). The second row shows the second combination of filtrates (*F. graminearum* 3-ADON SMCD 2243, *F. graminearum* 15-ADON SMCD 2244, and *F. graminearum* 14A SMCD 2910). The third row shows the third combination of filtrates (*F. culmorum* SMCD 2248, *F. equiseti* SMCD 2134, and *F. acuminatum* SMCD 2423). The last row shows the fourth combination of filtrates (*F. proliferatum* SMCD 2246, *F. redolens* V SMCD 2401, and *F. redolens* W SMCD 2402). PDB medium was used as a control for all the combinations.

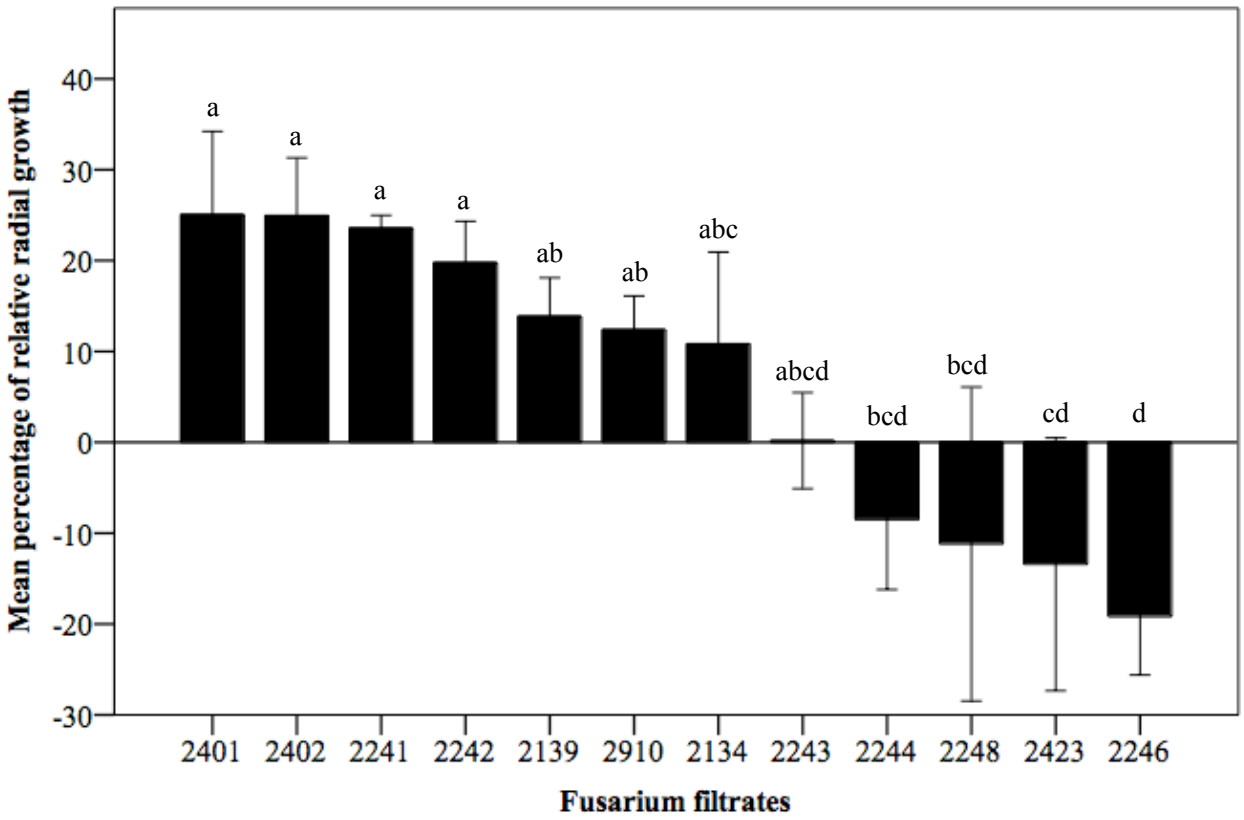


Figure 3.3 The relative host compatibility or host preference of the mycoparasite based on mean percentage of relative radial growth of mycoparasites toward each of twelve *Fusarium* filtrates. Data are means and standard errors (n=3) analyzed by ANOVA with Tukey's HSD (p-value < 0.05). Different letters indicate significant differences between treatments. The zero line indicates the radial growth of mycoparasite toward PDB as a control.

3.5.2 Adaptability of mycoparasite to *Fusarium* filtrates

The hyphal adaptability based on the somatic cell behaviour of the mycoparasite was assessed by comparisions of radial growth between the non-adapted mycoparasite and adapted mycoparasite toward twelve *Fusarium* filtrates using four types of combinations through the multiple paper disc assay as shown in Figure 3.4, 3.5, 3.6, and 3.7 in sequence. Statistically, a two-factor factorial design-two way ANOVA was performed to test hyphal adaptability. There are three assumptions; 1) there is no interaction between adaptation and types of *Fusarium* filtrates. 2) there is no effect of adaptation. 3) there is no effect of types of *Fusarium* filtrates. Interpretation of all the output was described as follows.

First combination of Fusarium filtrates

The output of the first combination of *Fusarium* filtrates was obtained and interpreted based on three assumptions. The first assumption was rejected since the *p*-value was less than 0.05 from the table of tests of between-subjects effects. It was concluded that there is a significant interaction between adaptation and types of *Fusarium* filtrates. The second assumption was rejected since the *p*-value was less than 0.05 from the table of tests of between-subjects effects. It was concluded that there is a significant effect of adaptation. The last assumption was rejected since the *p*-value was less than 0.05 from the table of tests of between-subjects effects. It was concluded that there is a significant effect of *Fusarium* filtrates. Our data indicate that the effect of adaptation depends on whether *Fusarium* filtrates are used or not.

All the pairwise comparisons were performed and described as follows, since the interaction between adaptation and *Fusarium* filtrates was significant. As shown in Figure 3.4, the mean radial growth of the non-adapted mycoparasite toward SMCD 2242 filtrate is significantly higher than that of the non-adapted mycoparasite toward PDB (p-value < 0.05). The mean radial growth of the non-adapted mycoparasite toward SMCD 2241 filtrate is significantly higher than that of the non-adapted mycoparasite toward PDB (p-value < 0.05). It can be concluded that mycoparasites without adaptation showed host preference for SMCD 2242 and 2241 filtrates. This result may be related with the origin of the mycoparasite, which was isolated from wheat fields affected by *F. oxysporum* and *F. avenaceum*; adaptation of parasites to their local hosts is a common phenomenon (Kaltz & Shykoff, 1998).

The mean radial growth of the mycoparasite adapted to SMCD 2242 filtrate toward 2242 filtrate is significantly higher than that of the mycoparasite adapted to 2242 filtrate toward PDB (p-value < 0.05). The mean radial growth of the mycoparasite adapted to SMCD 2242 filtrate toward 2242 filtrate is significantly higher than that of the mycoparasite adapted to 2242 filtrate toward 2241 filtrate (p-value < 0.05). The mean radial growth of the mycoparasite adapted to SMCD 2242 filtrate toward 2242 filtrate is significantly higher than that of the mycoparasite adapted to 2242 filtrate toward 2139 filtrate (p-value < 0.05). It was clear that mycoparasite adapted to SMCD 2242 filtrate showed significant radial growth toward 2242 filtrate compared to 2241 and 2139 filtrate as well as PDB as a control.

The mean radial growth of the mycoparasite adapted to SMCD 2241 filtrate toward 2241 filtrate is significantly higher than that of the mycoparasite adapted to 2241 filtrate toward PDB (p-value < 0.05). The mean radial growth of the mycoparasite adapted to SMCD 2241 filtrate toward 2241 filtrate is significantly higher than that of the mycoparasite adapted to 2241 filtrate toward 2139 filtrate (p-value < 0.05). The mycoparasite adapted to SMCD 2241 filtrate showed significant radial growth toward 2241 filtrate compared with 2139 filtrate and PDB.

The pairwise comparisons for mycoparasites adapted to SMCD 2139 filtrate on the first combination were not significantly different. It seems that mycoparasites adapted to SMCD 2139 filtrate do show the broad spectrum of *Fusarium* filtrates or nutrient absorption.

It is evident that mycoparasites adapted to filtrate show selective and strong migration to SMCD 2242 filtrate. Mycoparasite adapted to 2241 filtrate show less strong migration to 2241 filtrate compared with mycoparasite adapted to 2242 filtrate.

There is the significant effect of adaptation (Table 3.1). Pairwise comparisons for adaptation are described as follows. The mean radial growth of the non-adapted mycoparasite was significantly higher than that of the mycoparasite adapted to SMCD 2242 filtrate (p-value < 0.05). The mean radial growth of the mycoparasite adapted to SMCD 2241 filtrate was significantly higher than that of the mycoparasite adapted 2242 filtrate (p-value < 0.05). The mean radial growth of the mycoparasite adapted to SMCD 2139 filtrate was significantly higher than that of non-adapted mycoparasites and mycoparasites adapted to 2242 filtrate and 2241 filtrate (p-value < 0.05).

It seems that mycoparasites show different responses to adaptation depending on types of *Fusarium* filtrates. For example, mycoparasites adapted to highly comparable hosts such as

SMCD 2242 increase the selectivity to the particular host used for the adaptation but decrease the range of host spectrum and radial growth, whereas mycoparasites adapted to host such as SMCD 2139 represent an increase in the radial growth and decrease in selectivity to the particular host used for adaptation.

There is the significant effect of *Fusarium* filtrates. Pairwise comparisons for types of *Fusarium* filtrates are as follows. The mean radial growth for SMCD 2242, 2241, and 2139 filtrates was significantly higher than the mean radial growth for PDB (p-value < 0.05). It is clear that *Fusarium* filtrates indicate positive effect of radial growth of the mycoparasite.

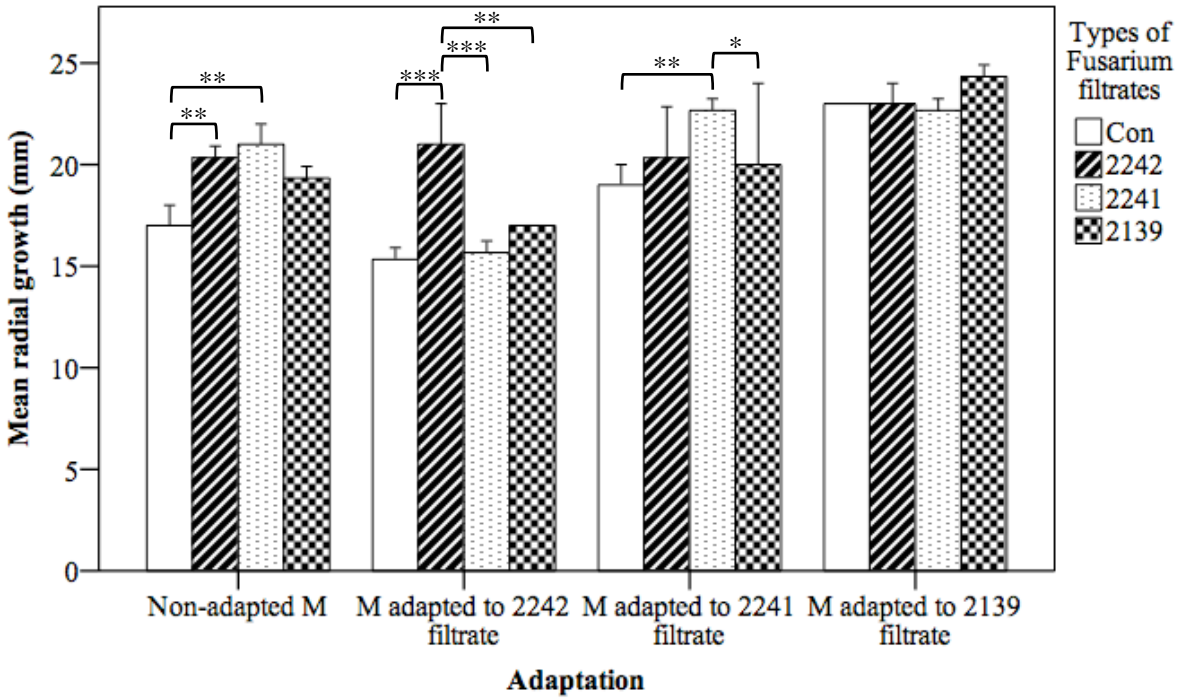


Figure 3.4 Difference between means and siginificance of pairwise comparisons of the radial growth between non-adapted mycoparasites and mycoparasites adapted to filtrates of *Fusarium oxysporum* SMCD 2242, *F. avenaceum* SMCD 2241, and *F. torulosum* SMCD 2139 on 1 week-old cultures. PDB was used as a control. Data are means and standard errors of three replicates (Two-way ANOVA with LSD test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Table 3.1 Relations between adaptation and radial growth of the mycoparasite in the first combination of *Fusarium* filtrates.

Adaptation	Radial growth
2242	↓*
2241	↑
2139	↑*

The asterisk indicates significant increase or decrease in radial growth of the mycoparasite after adaptation to each *Fusarium* filtrate (ANOVA, followed by LSD test, $p < 0.05$).

Second combination of Fusarium filtrates

The output of the second combination of *Fusarium* filtrates was obtained and interpreted based on three assumptions. The first assumption was not rejected since the *p*-value was more than 0.05 from the table of tests of between-subjects effects. It was concluded that there is no significant interaction between adaptation and types of *Fusarium* filtrates. Therefore, pairwise comparisons for the adaptation and *Fusarium* filtrates were not performed. The second assumption was rejected since the *p*-value was less than 0.05 from the table of tests of between-subjects effects. It was concluded that there is a significant effect of adaptation. The last assumption was rejected since the *p*-value was less than 0.05 from the table of tests of between-subjects effects. It was concluded that there is a significant effect of *Fusarium* filtrates. Our data indicate that adaptation and types of *Fusarium* filtrates independently affect radial growth of the mycoparasite.

There is the significant effect of adaptation (Table 3.2). The pairwise comparisons for adaptation are described as follows. As shown in Figure 3.5, the mean radial growth of the mycoparasite adapted to SMCD 2243 filtrate is significantly higher than that of the non-adapted mycoparasite and the mycoparasite adapted to SMCD 2244 and 2910 filtrates (p-value < 0.05). The mean radial growth of the mycoparasite adapted to SMCD 2244 filtrate is significantly higher than that of the non-adapted mycoparasite (p-value < 0.05). It can be concluded that SMCD 2243 filtrate is the most powerful source for adaptation to increase radial growth of the mycoparasite among other *Fusarium graminearum* filtrates.

There is the significant effect of *Fusarium* filtrates. The pairwise comparisons for types of *Fusarium* filtrates are as follows. The mean radial growth of the mycoparasite for SMCD 2910 filtrate is significantly higher than that of the mycoparasite for SMCD 2243 and 2244 filtrates as well as PDB as a control (p-value < 0.05). Our data indicate that SMCD 2910 filtrate is the most attractive source to increase the radial growth of the mycoparasite among other *F. graminearum* filtrates.

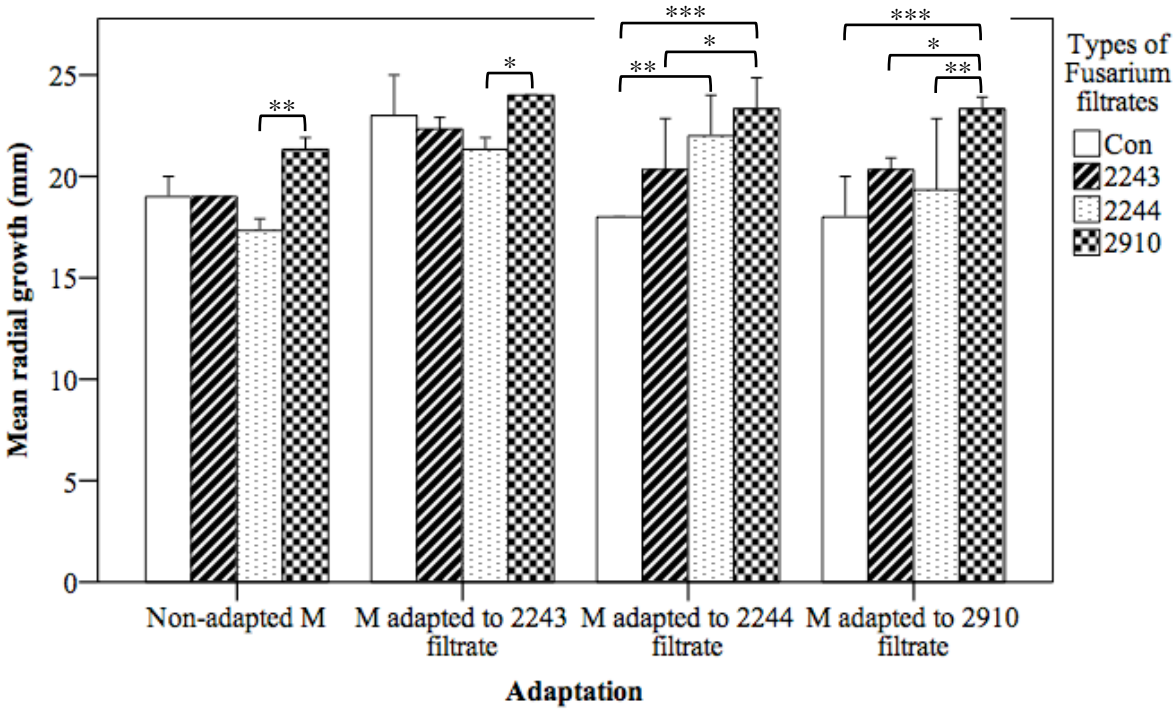


Figure 3.5 Difference between means and siginificance of pairwise comparisons of the radial growth between non-adapted mycoparasites and mycoparasites adapted to filtrates of *Fusarium graminearum* 3-ADON SMCD 2243, *F. graminearum* 15-ADON SMCD 2244, and *F. graminearum* 14A SMCD 2910 on 1 week-old cultures. PDB was used as a control. Data are means and standard errors of three replicates (Two-way ANOVA with LSD test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Table 3.2 Relations between adaptation and radial growth of the mycoparasite in the second combination of *Fusarium* filtrates.

Adaptation	Radial growth
2243	↑*
2244	↑*
2910	↑

The asterisk indicates significant increase in radial growth of the mycoparasite after adaptation to each *Fusarium* filtrate (ANOVA, followed by LSD test, $p < 0.05$).

Third combination of Fusarium filtrates

The output of the third combination of *Fusarium* filtrates was obtained and interpreted based on three assumptions. The first assumption was not rejected since the *p*-value was more than 0.05 from the table of tests of between-subjects effects. It was concluded that there is no significant interaction between adaptation and types of *Fusarium* filtrates. Therefore, pairwise comparisons for the adaptation and *Fusarium* filtrates were not performed. The second assumption was rejected since the *p*-value was less than 0.05 from the table of tests of between-subjects effects. It was concluded that there is a significant effect of adaptation. The last assumption was not rejected since the *p*-value was more than 0.05 from the table of tests of between-subjects effects. It was concluded that there is no significant effect of *Fusarium* filtrates. Our data indicate is that only adapation is effective to radial growth of the mycoparasite.

There is the significant effect of adaptation (Table 3.3). The pairwise comparisons for adaptation are described as follows. As shown in Figure 3.6, the mean radial growth of the mycoparasite adapted to SMCD 2134 filtrate is significantly higher than that of the mycoparasite adapted to 2248 filtrate and the non-adapted mycoparasite (*p*-value < 0.05). The mean radial growth of the mycoparasite adapted to SMCD 2423 filtrate is significantly higher than that of the mycoparasite adapted to 2248 filtrate and the non-adapted mycoparasite (*p*-value < 0.05). Our data indicate that SMCD 2134 and 2423 filtrates are effective sources for adaptation of mycoparasites to increase radial growth of the mycoparasite.

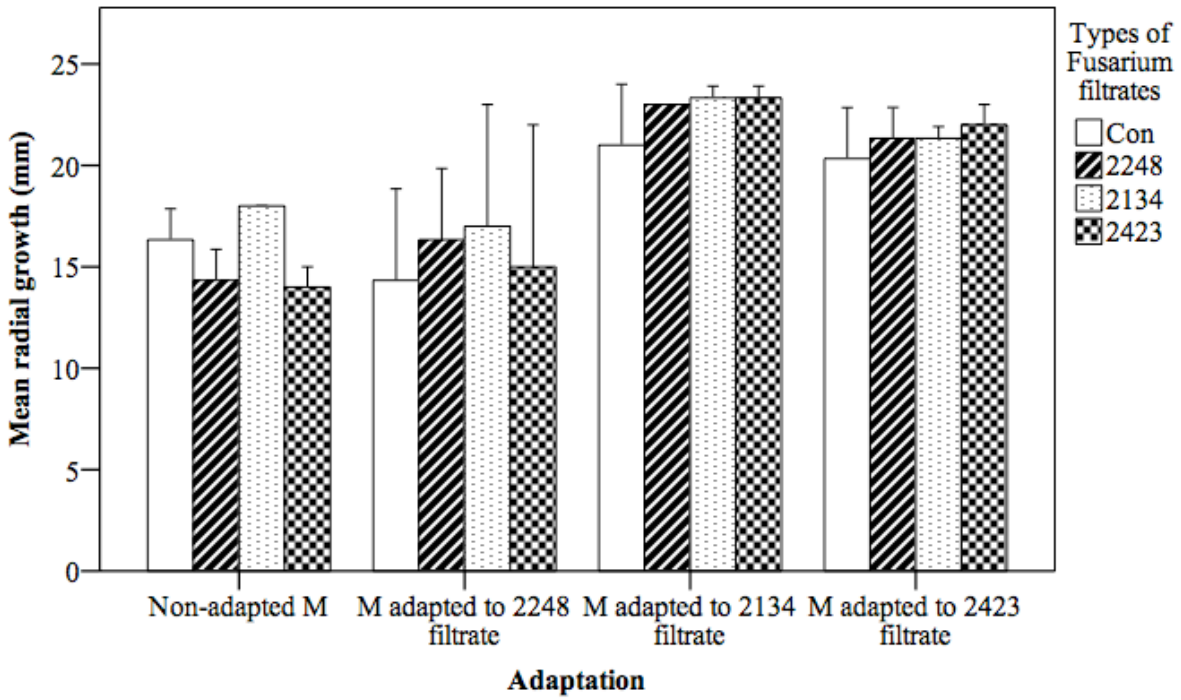


Figure 3.6 Difference between means and siginificance of pairwise comparisons of the radial growth between non-adapted mycoparasites and mycoparasites adapted to filtrates of *Fusarium culmorum* SMCD 2248, *F. equiseti* SMCD 2134, and *F. acuminatum* SMCD 2423 on 1 week-old cultures. PDB was used as a control. Data are means and standard errors of three replicates (Two-way ANOVA with LSD test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Table 3.3 Relations between adaptation and radial growth of the mycoparasite in the third combination of *Fusarium* filtrates.

Adaptation	Radial growth
2248	No change
2134	↑*
2423	↑*

The asterisk indicates significant increase in radial growth of the mycoparasite after adaptation to each *Fusarium* filtrate (ANOVA, followed by LSD test, $p < 0.05$).

Last combination of Fusarium filtrates

The output of the last combination of *Fusarium* filtrates was obtained and interpreted based on three assumptions. The first assumption was not rejected since the *p*-value was more than 0.05 from the table of tests of between-subjects effects. It was concluded that there is no significant interaction between adaptation and types of *Fusarium* filtrates. Therefore, pairwise comparisons for the adaptation and *Fusarium* filtrates were not performed. The second assumption was not rejected since the *p*-value was less than 0.05 from the table of tests of between-subjects effects. It was concluded that there is no significant effect of adaptation (Table 3.4). The last assumption was rejected since the *p*-value was less than 0.05 from the table of tests of between-subjects effects. It was concluded that there is a significant effect of *Fusarium* filtrate. Our data indicate that *Fusarium* filtrates only affect radial growth of the mycoparasite.

There is the significant effect of *Fusarium* filtrates. The pairwise comparisosns for types of *Fusarium* filtrates are described as follows. As shown in Figure 3.7, the mean radial growth of the mycoparasite for PDB is significantly higher than that of the mycoparasite for SMCD 2246 filtrate (p-value < 0.05). The mean radial growth of the mycoparasite for SMCD 2401 is significantly higher than that of the mycoparasite for PDB and SMCD 2246 filtrate (p-value < 0.05). The mean radial growth of the mycoparasite for SMCD 2402 is significantly higher than that of the mycoparasite for PDB and SMCD 2246 filtrate (p-value < 0.05). It can be concluded that SMCD 2401 and 2402 filtrates positively affect radial growth of the mycoparasite, whereas SMCD 2246 filtrate negatively affects radial growth of the mycoparasite.

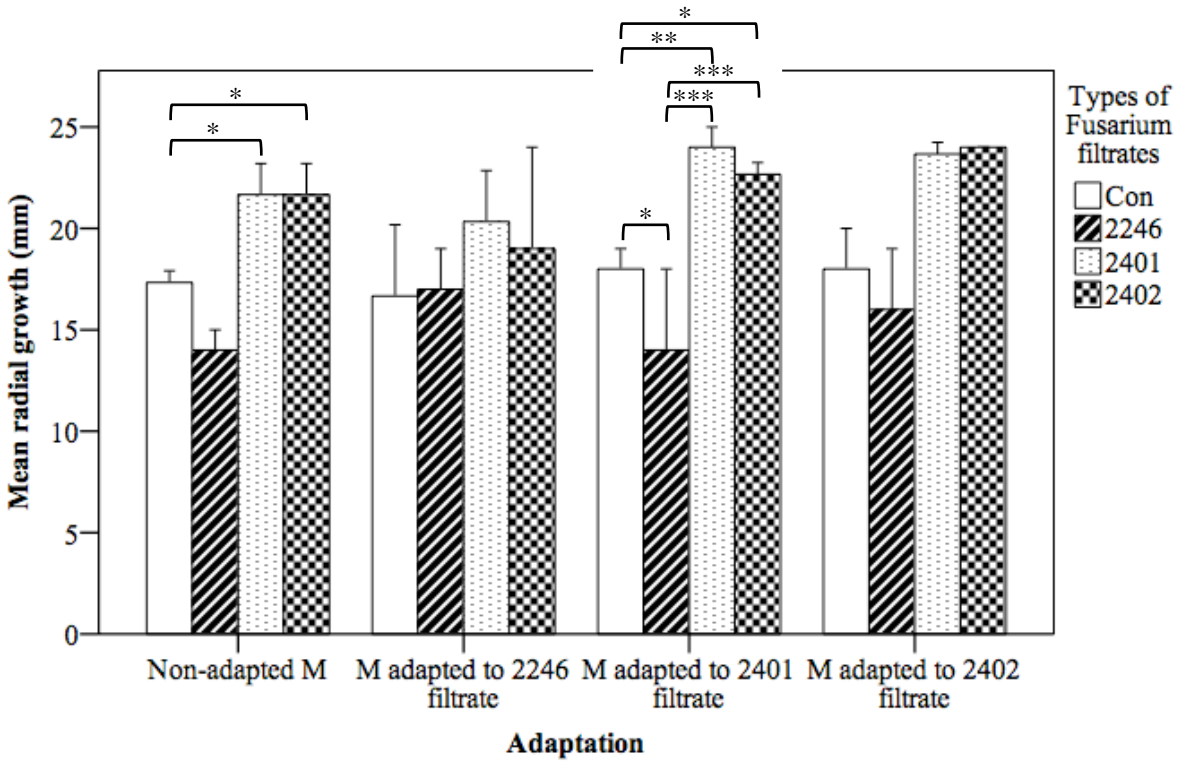


Figure 3.7 Difference between means and siginificance of pairwise comparisons of the radial growth between non-adapted mycoparasites and mycoparasites adapted to filtrates of *Fusarium proliferatum* SMCD 2246, *F. redolens* V SMCD 2401, and *F. redolens* W SMCD 2402 on 1 week-old cultures. PDB was used as a control. Data are means and standard errors of three replicates (Two-way ANOVA with LSD test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Table 3.4 Relations between adaptation and radial growth of the mycoparasite in the last combination of *Fusarium* filtrates.

Adaptation	Radial growth
2246	↓
2401	↑
2402	↑

The asterisk indicates significant increase in radial growth of the mycoparasite after adaptation to each *Fusarium* filtrate (ANOVA, followed by LSD test, $p < 0.05$).

To summarize all the four combinations of *Fusarium* filtrates, the degree of hyphal migration as a somatic cell behaviour of the mycoparasite varies in types of *Fusarium* filtrates. Furthermore, the degree of adaptation could be dependent on types of *Fusarium* filtrates. These hyphal migration after adaptaion of a mycoparasite imply that an adapted mycoparasite could recognize a compatible host selectively among hosts or could increase the range of host spectrum depending on which *Fusarium* filtrate was used for adaptation.

3.5.3 Effect of *Fusarium* filtrates on ascomata formation of mycoparasite

The effect of *Fusarium* filtrates on ascomata formation of the mycoparasite in the teleomorphic stage was indicated by comparisons of the percentage of ascomata formation between *Fusarium* filtrates and sterilized distilled water (SDW) through the modified slide culture assay. There was no significant difference between four combinations of *Fusarium* filtrates (*p*-value 0.05). However, the results are biologically important and can be useful indication to compare the effect of different *Fusarium* filtrates on ascomata formation.

On the first combination of *Fusarium* filtrates (SMCD 2242, 2241, 2139, and SDW), it seems that ascomata formation of the mycoparasite in co-culture with or exposed to filtrates of SMCD 2242, 2241, and 2139 was slightly lower than that of SDW, as shown in Figure 3.8. On the second combination of *Fusarium* filtrates (SMCD 2243, 2244, 2910, and SDW), it seems that ascomata formation of the mycoparasite in co-culture with or exposed to filtrates of SMCD 2243, 2244, and 2910 was higher and considerably higher than that of SDW, as shown in Figure 3.9. On the third combination of *Fusarium* filtrates (SMCD 2248, 2134, 2423, and SDW), it seems that ascomata formation of the mycoparasite in co-culture with or exposed to filtrates of SMCD 2248, 2134, and 2423 was higher than that of SDW, as shown in Figure 3.10. On the last combination of *Fusarium* filtrates (SMCD 2246, 2401, 2402, and SDW), it seems that ascomata formation of the mycoparasite in co-culture with or exposed to filtrates of SMCD 2246 and 2401 was higher than that of SDW, whereas ascomata formation of the mycoparasite in co-culture with or exposed to filtrates of SMCD 2402 was slightly lower than that of SDW as shown in Figure 3.11.

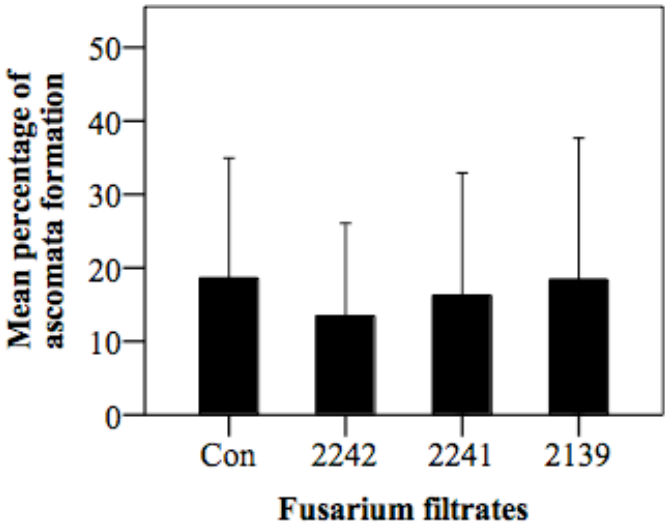


Figure 3.8 The percentage of ascomata formation of mycoparasites around filtrates of *Fusarium oxysporum* SMCD 2242, *F. avenaceum* SMCD 2241, and *F. torulosum* SMCD 2139 on 1 month-old cultures. Sterilized distilled water was used as a control. Data are means and standard errors of three replicates (One-way ANOVA, $p < 0.05$).

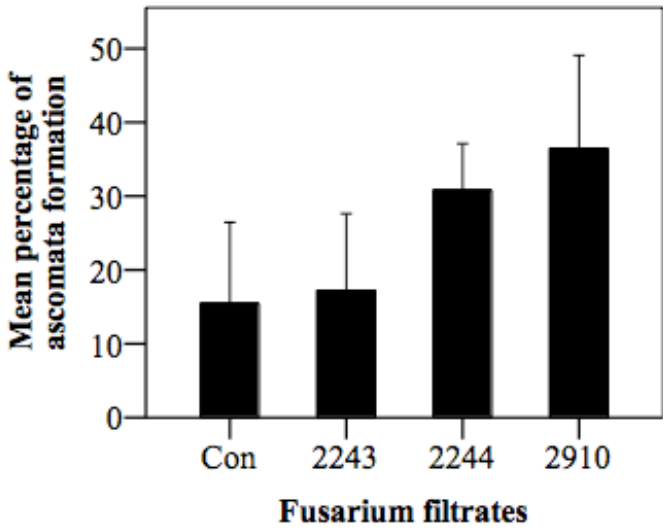


Figure 3.9 The percentage of ascomata formation of mycoparasites around filtrates of *Fusarium graminearum* 3-ADON SMCD 2243, *F. graminearum* 15-ADON SMCD 2244, and *F. graminearum* 14A SMCD 2910 on 1 month-old cultures. Sterilized distilled water was used as a control. Data are means and standard errors of three replicates (One-way ANOVA, $p < 0.05$).

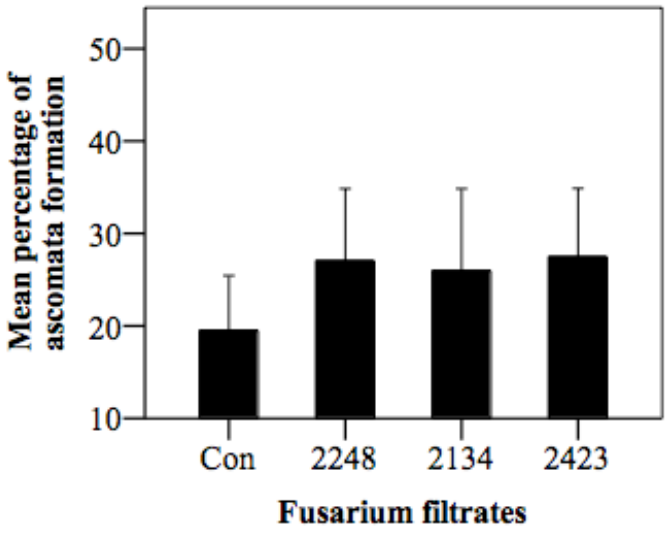


Figure 3.10 The percentage of ascomata formation of mycoparasites around filtrates of *Fusarium culmorum* SMCD 2248, *F. equiseti* SMCD 2134, and *F. acuminatum* SMCD 2423 on 1 month-old cultures. Sterilized distilled water was used as a control. Data are means and standard errors of three replicates (One-way ANOVA, $p < 0.05$).

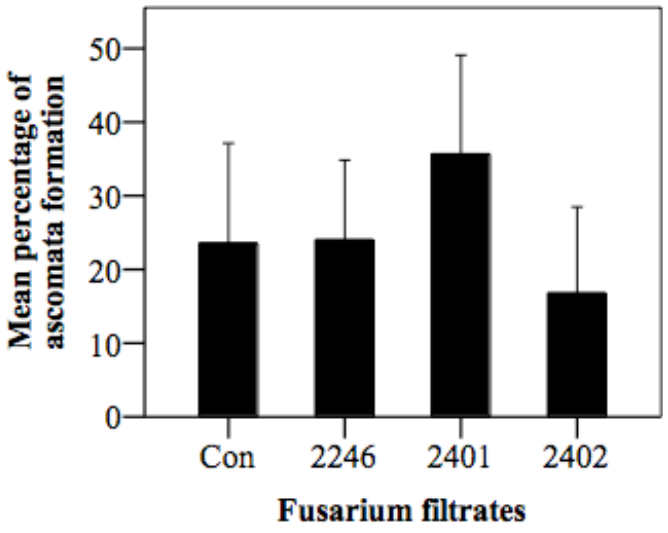


Figure 3.11 The percentage of ascomata formation of mycoparasites around filtrates of *Fusarium proliferatum* SMCD 2246, *F. redolens* V SMCD 2401, and *F. redolens* W SMCD 2402 on 1 month-old cultures. Sterilized distilled water was used as a control. Data are means and standard errors of three replicates (One-way ANOVA, $p < 0.05$).

3.6 Discussion

Generally, mycoparasites are categorized as biotrophic and necrotrophic mycoparasites based on their mode of parasitism and effect on the host fungi (Boosalis, 1964). Biotrophs derive nutrients from living host cells by haustoria mediating intimate relationships with host cells, whereas necrotrophs acquire nutrients from the killed host cells by the production of lytic enzymes and toxic secondary metabolites (Barnett, 1963; Boosalis, 1964). *Gliocephalis hyalina* Matr., (1899), *Melanospora zamiae* Corda (1837), *Persiciospora moreaui* P.F. Cannon & D. Hawksw., (1982), *Sphaerodes retispora* var. *retispora*, and *S. quadrangularis* Dania Garcia, Stchigel & Guarro (2004) are known as biotrophic mycoparasites. These biotrophic mycoparasites form intimate contact and infection structures on hosts during mycoparasitism; they are able to produce spores in the presence of certain *Fusarium* strains, indicating a narrow host range, mostly limited to *F. oxysporum* and *F. avenaceum* (Goh & Vujanovic, 2010a; Harveson & Kimbrough, 2001; Jacobs *et al.*, 2005). In contrast, *T. harzianum* Rifai*, T. koningii* Oudem.*, T. viride* Pers., *T. virens* (J.H. Mill., Giddens & A.A. Foster) Arx, and *Clonostachys rosea* (Link) Schroers, Samuels, Seifert & W. Gams (1999) are known as necrotrophic mycoparasites against a broad range of hosts, including *Fusarium* spp., *Penicillium* spp. (Brian & McGowan, 1945), *Aspergillus* spp., *Rhizotonia solani* [*Thanatephorus cucumeris* (A.B. Frank) Donk, (1956)], *Sclerotium rolfsii* [*Athelia rolfsii* (Curzi) C.C. Tu & Kimbr., (1978)], (Inbar & Chet, 1995) *S. cepivorum* [*Stromatinia cepivora* (Berk.) Whetzel (1945)], (Metcalf & Wilson, 2001) and antagonism is a major mechanism of these mycopathogenic necrotrophs (Ojha & Chatterjee, 2011; Rodríguez *et al.*, 2011; Singh *et al.*, 2005).

In previous research, *F. avenaceum*, *F. oxysporum*, *F. culmorum*, *F. equiseti*, and *F. graminearum* 3-ADON and 15-ADON were determined to be hosts of *Sphaerodes mycoparasitica*. *S. mycoparasitica* showed to be specific to its hosts, by attaking through contact and intracellular penetration as typical traits of the biotrophic mycoparasitism. However, in this study, we demonstrated for the first time the diphasic lifestyle of *S. mycoparasitica* going from biotrophism via fungus-fungus attraction to antagonism or mycopathogenic inhibition zone formed in the presence of particular *Fusarium* filtrates. This plasticity or adaptive lifestyle in *S. mycoparasitica* may be related to the various type of secondary metabolites dissolved in those filtrates, such as specific pigments and mycotoxins produced by each particular *Fusarium* species.

3.6.1 Host compatibility of mycoparasite

S. mycoparasitica indicated the polyphagous trophic relation with a dozen of *Fusarium* taxa as demonstrated by testing *Fusarium* spp. filtrates in the multiple paper disc assay (Figure 3.2). The host compatibility was measured by radial growth of this mycoparasite indicating diphasic fungus-fungus interactions such as biotrophic-attraction and antagonistic-inhibition relationships. The three *S. mycoparasitica*-host compatibility groups were recognized related to the spectrum of mycotoxins produced within each taxonomical section of tested *Fusarium* strains according to Leslie & Summerell (2006). The relative *S. mycoparasitica*-host compatibility level or host preference measured by percentage of relative radial growth of mycoparasite toward each of twelve *Fusarium* filtrates depicts those three groups: Group 1; SMCD 2401 (25.1%), 2402 (24.9%), 2241 (23.6%), 2242 (19.8%), and 2139 (13.9%) as non-trichothecene producers; and Group 2; (2) SMCD 2910 (12.4%), 2134 (10.8%), 2243 (0.2%), 2244 (-8.5%), 2248 (-11.2%), and 2423 (-13.4%) as trichothecene *Fusaria* producers; and Group 3; SMCD 2246 (-19.2%) as Fumonisin B_1 producer.

In group 1, SMCD 2401, 2402, and 2242 belong to the *Elegans*; SMCD 2241 belong to the *Roseum*; SMCD 2139 belong to the *Discolor* sections (Bosch *et al.*, 1989; Christ *et al.*, 2011; Mirocha *et al.*, 1989; Sørensen & Giese, 2013; Stepien, 2013; Zain *et al.*, 2012). Particularly, SMCD 2139, belonging to the *Discolor* section, is known to produce wortmannin (Ryley *et al.*, 2007). Group 1 can be considered as biotrophic-attraction relationships between mycoparasite-*Fusarium* taxa due to the stable and positive relative radial mycoparasitic growth over *Fusarium* mycelia. In group 2, SMCD 2910, 2243, and 2244 belong to the *Discolor*; SMCD 2134, 2248, and 2423 belong to the *Gibbosum* sections (Langseth *et al.*, 1998; Tan *et al.*, 2012).; Particularly, *S. mycoparasitica* produced the largest inhibition zone triggered by SMCD 2246, belonging to the *Liseola* section (Stepien, 2013; Thiel *et al.*, 1991). Group 2 and 3 could be considered as antagonistic-inhibition relationships between mycoparasite-*Fusarium* taxa due to the suppressive effect of *S. mycoparasitica* on the growth of *Fusarium* mycelia. Assessment of the type and amount of mycotoxins produced by *Fusarium* strains exposed to *S. mycoparasitica* will be a beneficial information to further elucidate mycoparasite-host compatability mechanisms and eventually prevent mycotoxins production.

3.6.2 Adaptability of mycoparasite to *Fusarium* filtrates

Moreover, investigation of the hyphal adaptability based on somatic cell behaviour of mycoparasites is a particular scientific approach proposed by Little (2006) (Little *et al.*, 2006). In this study, it was assessed that radial growth of non-adapted and adapted mycoparasites toward twelve *Fusarium* filtrates using four types of combinations through the multiple paper disc assay (Figure 3.2). The combinations were decided by taxonomical sections and produced mycotoxins as well as the morphology of *Fusarium* strains.

On the first combination (SMCD 2242, 2241, 2139, and PDB), the significant effect of *Fusarium* filtrates on mycoparasite adaptation has been observed. Interestingly, the mycoparasite showed different responses to adaptation depending on types of *Fusarium* filtrates. For example, mycoparasites adapted to highly comparable host such as SMCD 2242 increase the selectivity to the particular host used for the adaptation but decrease the range of host spectrum and radial growth, whereas mycoparasites adapted to host such as SMCD 2139 represent an increase in the radial growth and a decrease in the selectivity to the particular host used for adaptation. Furthermore, there is the significant interaction between adaptation and types of *Fusarium* filtrates. Specifically, the mycoparasite without adaptation showed host preference for SMCD 2242 and 2241 filtrates. This result may be related with the origin of SMCD 2220-01 which was isolated from wheat field affected by *F. avenaceum*, as well as the ancestor host *F. oxysporum* for all *Sphaerodes* species (Goh & Vujanovic, 2010c; Vujanovic & Goh, 2010).

On the second combination of *Fusarium* filtrates (SMCD 2243, 2244, 2910, and PDB), there is the significant effect of adaptation. It was concluded that SMCD 2243 filtrate is the most powerful source for adaptation to increase radial growth of the mycoparasite among other *F. graminearum* filtrates. There is also the significant effect of *Fusarium* filtrates. It was concluded that SMCD 2910 filtrate is the most attractive source to increase the radial growth of the mycoparasite among other *F. graminearum* filtrates. However, it was concluded that there is no significant interaction between adaptation and types of *Fusarium* filtrates. Our data indicate that adaptation and types of *Fusarium* filtrates independently affect radial growth of the mycoparasite.

On the third combination of *Fusarium* filtrates (SMCD 2248, 2134, 2423, and PDB), only adapation is effective to radial growth of the mycoparasite. It can be concluded that SMCD 2134 and 2423 filtrates are effective sources for adaptation of the mycoparasite to increase radial growth of the mycoparasite.

On the last combination of *Fusarium* filtrates (SMCD 2246, 2401, 2402, and PDB), *Fusarium* filtrates only affect radial growth of the mycoparasite. It can be concluded that SMCD 2401 and 2402 filtrates positively affect radial growth of the mycoparasite, whereas SMCD 2246 filtrate negatively affects radial growth of the mycoparasite.

To summarize all the four combinations of *Fusarium* filtrates, the degree of hyphal migration as a somatic cell behaviour of the mycoparasite varies in types of *Fusarium* filtrates. Furthermore, the degree of adaptation could be dependent on types of *Fusarium* filtrates. These hyphal migration after adaptation of a mycoparasite imply that an adapted mycoparasite could recognize a compatible host selectively among various hosts or could increase the range of host spectrum depending on which *Fusarium* filtrate was used for adaptation. The host compatibility of the mycoparasite could be enhanced by the adaptation procedure since the mycoparasite adapted to each of *Fusarium* filtrates showed higher radial growth compared with non-adapted mycoparasites, generally. To the best of our knowledge, this is a first attempt to better understand a somatic cell by using the approach previously applied by Goh and Vujanovic (2010) on sexual spores germination patterns.

Resistance of mycoparasites to mycotoxins might be driven by compatibility and adaptability of the mycoparasite to different hosts. In that regards, the type of mycotoxin produced by each *Fusarium*-host has particular significance because of the different chemical composition, structure, and bioactivity of these molecules (Bennett & Klich, 2003). Relative hydrophilicity or hydrophobicity of mycotoxins may be an additional factor related to solubility and absorption of mycotoxins into a mycoparasite (Cole, 2012). The molecular polar surface area (PSA), a sum of surface of polar atoms (e.g. oxygens, nitrogens, and attached hydrogens) in a molecule can be easily calculated by using cheminformatics, a free online software on the website, http://www.molinspiration.com (Hansch *et al.*, 1995; Remko *et al.*, 2006), as a useful and indicative value for relative hydrophilicity or hydrophobicity as shown in Table 3.5.

Table 3.5 Hydrophilic and hydrophobic properties of mycotoxins related with tested *Fusarium* species.

Major group of mycotoxins	**Mycotoxins**	**Polar Surface Area (Å^2) of mycotoxins**	**Tested *Fusarium* species for host compatibility**
Non-trichothecenes	Fusaric acid	50.19	*F. redolens* V SMCD 2401
			F. redolens W SMCD 2402
	Moniliformin	54.37	*F. avenaceum* SMCD 2241
			F. oxysporum SMCD 2242
			F. torulosum SMCD 2139
Trichothecenes type B	Zearalenone	83.83	*F. graminearum* 14A SMCD 2910
	Deoxynivalenol	99.52	*F. graminearum* 3-ADON SMCD 2243
	3-Acetyl-deoxynivalenol	105.59	*F. graminearum* 15-ADON SMCD 2244
	15-Acetyl-deoxynivalenol	105.59	*F. equiseti* SMCD 2134
Trichothecenes type A	HT-2 toxin	114.83	*F. culmorum* SMCD 2248
	T-2 toxin	120.91	*F. acuminatum* SMCD 2423
Fumonisins	Fumonisin B_1	288.51	*F. proliferatum* SMCD 2246

hydrophilic

hydrophobic

3.6.3 Effect of *Fusarium* filtrates on ascomata formation of mycoparasite

Several studies on *Neurospora crassa* as a model organism (Perkins & Davis, 2000) reported that ascomata (fruit bodies) formation requires particular environmental factors, such as light, and varies in natural substrata, such as different species of wood (Lee, 2012), and is also regulated by particular genes, such as NCU06316 and NCU07508 (Lehr *et al.*, 2014). Particularly, synthetic media combined with host substrates or filtrates have been proposed to improve ascomata formation in ascomycetous fungi (Baker *et al.*, 1977; Lilly, 1951). Furthermore, a recent study conducted by Goh and Vujanovic showed *S. mycoparasitica* sporulated when inoculated with *Fusarium avenaceum* and *F. oxysporum* (Goh & Vujanovic, 2010c). In this study, in addition to the anamorphic aspect of the mycoparasite, the effect of *Fusarium* filtrates on ascomata formation of the mycoparasite in the teleomorphic stage was indicated by comparisons of the percentage of ascomata formation exposed to different *Fusarium* filtrates and sterilized distilled water (SDW) through the modified slide culture assay. As shown on Figure 3.12, the tested twelve *Fusarium* strains could be categorized by mycotoxin production into two groups: non-trichothecene and trichothecene producers. The first group, including SMCD 2242, 2241, 2139, 2246, 2402, and 2401 is a non-trichothecene producer, whereas the second group, including SMCD 2248, 2423, 2134, 2243, 2244, and 2910 is a trichothecene producer. The two groups of *Fusarium* strains differently influence on ascomata production. It seems that the *Fusarium* filtrates of the second group are higher inducers of ascomata formation in the mycoparasite compared with that of the first group. Future identification of the types and amounts of secondary metabolites produced by *Fusarium* strains will lead us to elucidate the range of host *Fusarium* strains and differences among *Fusarium* strains responsible for host compatibility and ascomata formation of the mycoparasite.

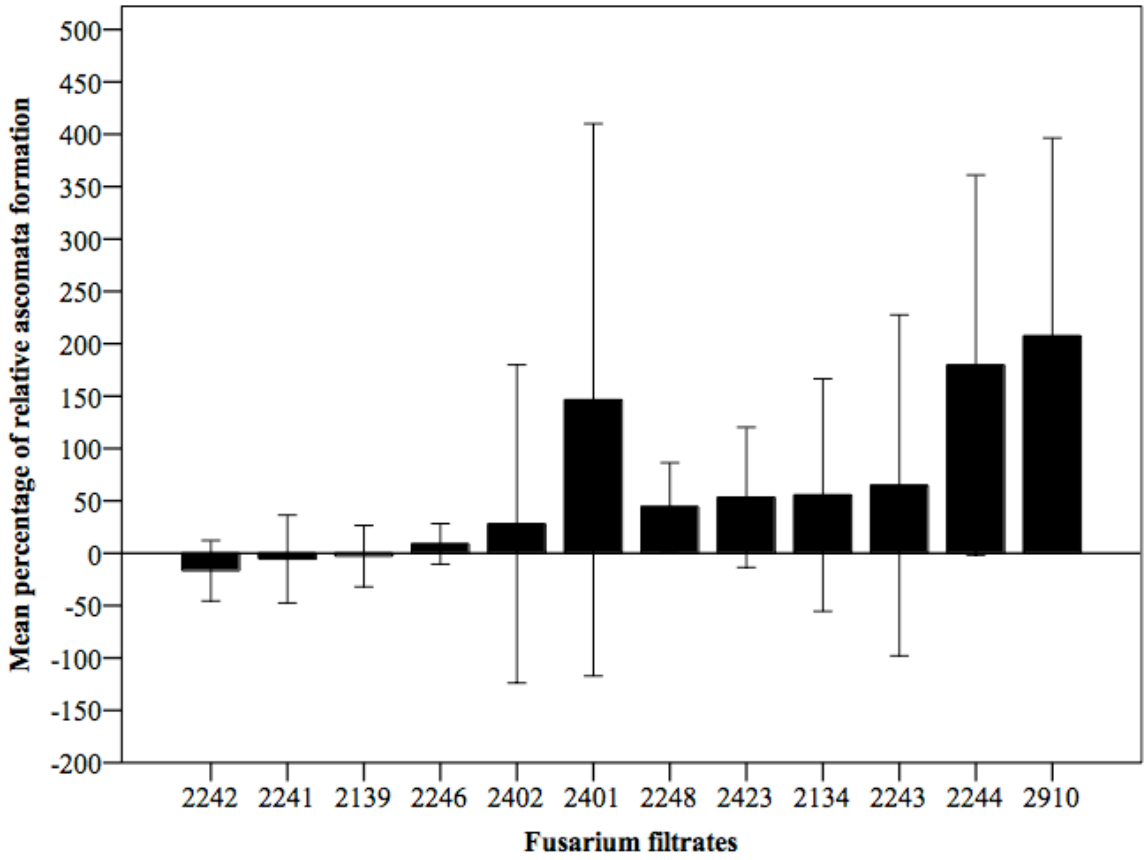


Figure 3.12 The relative ascomata formation of mycoparasites affected by twelve different *Fusarium* filtrates based on the modified slide culture assay. Normalized data include means and standard errors of three replicates analyzed by ANOVA (zero line, SDW).

3.7 Conclusions

Sphaerodes mycoparasitica Vujan. SMCD 2220-01 showed the broad host compatibility with twelve *Fusarium* strains and the level of host compatibility using *Fusarium* filtrates through the multiple paper disc assay. The host compatibility of the mycoparasite can be expressed by diphasic interactions such as biotrophic-attraction and antagonistic-inhibition relationships based on relative radial growth. The level of host compatibility may result from the variety and attractiveness of secondary metabolites, such as mycotoxins and pigments produced by *Fusarium* species. Moreover, host compatibility and adaptability of the mycoparasite implicated the presence of a defense or resistance mechanism to toxic secondary metabolites (mycotoxins) by host fungi. In addition to characterization of host compatibility, the host compatibility of the mycoparasite could be enhanced by an adaptation procedure since the mycoparasite adapted to

each of *Fusarium* filtrates showed higher radial growth compared with the non-adapted mycoparasite.

3.8 Connection to the next study

In this study (chapter 3), mycoparasite *S. mycoparasitica* showed a broad host compatibility and different level of adaptability depending on *Fusarium* strains. Moreover, based on relative radial growth of the mycoparasite, host compatibility could be categorized by biotrophic-attraction and antagonistic-inhibition relationships. In particular, it was assumed that *S. mycoparasitica* may perform a biotrophic-attraction relationship with SMCD 2401 and an antagonistic-inhibition relationship with SMCD 2246 since both were representatives of the two described relationships. These two different interactions may also be related or result of different properties occurring on mycoparasite vs. *Fusarium* hyphal surfaces.

4. INVESTIGATION OF FUNGAL SURFACE HYDROPHOBICITY RELATED TO MYCOPARASITISM UNDER DIFFERENT MEDIA CONDITIONS

4.1 Abstract

Mycoparasitism is the parasitic interaction between a fungal parasite and a fungal host. Fungus-fungus interfaces mediated by fungal cell wall interactions play an imporatant role in defining mycoparasitism. Fungal surface hydrophobicity, as one of the physicochemical properties contributed by fungal cell wall components and/or secondary metabolites, are responsible for the contact attachment and colonization of mycoparasites. The main objective of this study was to measure fungal surface hydrophobicity of host *Fusarium* strains and the mycoparasite during mycoparasitism under different media conditions through contact angles measurements. Our results from contact angles measurment showed differential expression of fungal surface hydrophobicity of *Sphaerodes mycoparasitica* SMCD 2220-01, *Fusarium proliferatum* (Matsush.) Nirenberg SMCD 2246, and *Fusarium redolens* Wollenw. V OTU 18 SMCD 2401 as well as changes in hyphal surface hydrophobicity of host *Fusarium* strains during mycoparasitism under PDA and ICI media conditions. Additionally, observation of all the fungal hyphal surfaces under atomic force microscopy (AFM) indicated differential topography and physical properties of the hyphal surface. The differences in hyphal surfaces were noticeable under different media conditions. These findings suggest that *S. mycoparasitica* might contribute to changes in host fungal surface hydrophobicity and also that mycoparasitism might be influenced by growth and environmental conditions.

4.2 Introduction

The fungal cell wall as an initial barrier faced with hostile environments, have a protective fuction that provides mechanical strength for maintaining cell shape and intergrity, as well as an aggressive function that releases proteins and toxic molecules resulting from

interaction with biotic and/or abiotic stresses from environments (Bowman & Free, 2006). The fungal cell wall varies in fungal species; the structure and synthesis of the fungal cell wall are affected by changes in environmental conditions (Latge, 2007). Glycoproteins and polysaccharides, such as glucans, chitin, chitosan, mannans, and/or galactomannans, are known as the main components of fungal cell walls. These components form a complex network to provide the structural basis of the fungal cell wall (Bowman & Free, 2006).

To understand the functions of fungal cell walls, studies on their structural and physical properties have been conducted using atomic force microscopy (AFM). AFM is an evolving three-dimensional imaging and measurement tool in real time at a high resolution based on the interaction between a probe or tip and a sample surface (Vahabi *et al.*, 2013). Unlike electron microscopy including scanning electron microscopy (SEM) and transmission electron microscopy (TEM), AFM requires little or no sample manipulation (staining and drying procedures) prior to examination, which leaves a microbial cell surface intact (Binnig *et al.*, 1986). As a measurement tool, AFM can provide evidence for the mechanical and physical properties of the sample surface, such as roughness and even molecular interactions.

The fungal surface or fungal cell wall play a crucial role in fungus-fungus interactions including antagonism and/or parasitism since the fungal surface on the physicochemical aspect is known to control fungi and their interactions at the interfaces (Smits *et al.*, 2003). Cell surface hydrophobicity due to the presence of hydrophobic moieties as one of the surface properties was reported to influence microbial adhesion, pathogenesis, and surface tension (Bayry *et al.*, 2012; Glee *et al.*, 1995; van Loosdrecht *et al.*, 1987). Fungal surface hydrophobicity was reported to originate from hydrophobins as a class of cysteine rich proteins (Wosten, 2001). In addition to hydrophobins, fungal secondary metabolites such as cordycepsidone A and B, known as antifungal compounds belonging to a class of depsidones, were also reported to contribute to surface hydrophobicity (Varughese *et al.*, 2012). Thus, the changes in fungal surface hydrophobicity during the mycoparasitism need to be investigated for appreciation of the complex interrelationship between the mycoparasite and different hosts under different media conditions.

4.3 Hypotheses and objectives

We hypothesized that *Sphaerodes mycoparasitica* affects hyphal surface hydrophobicity and radial growth of host *Fusarium* strains such as *F. redolens* V as a representative of a highly related to biotrophic-attraction relationship and *F. proliferatum* as a representative of a highly related to antagonistic-inhibition relationship with the mycoparasite. Furthermore, we speculated that hyphal surface topography and physical structure associated with hydrophobicity as well as radial growth of the representative *Fusarium* hosts during mycoparasitism differ under the different environmental conditions. The first objective of this study was to measure radial growth and contact angles during mycoparasitism under the different nutrient media condition using dual culture assays. The second objective of this study was to analyze hyphal surface topography and roughness by atomic force microscopy for examination of hyphal surface differences.

4.4 Materials and Methods

4.4.1 Fungal isolates

Mycoparasite *Sphaerodes mycoparasitica* Vujan. SMCD 2220-01 and two plant pathogenic fungi such as *Fusarium proliferatum* (Matsush.) Nirenberg SMCD 2246 belonging to the antagonistic-inhibition relationship and *Fusarium redolens* Wollenw. V OTU 18 SMCD 2401 belonging to the biotrophic-attraction relationship were used in this study. Fungal strains were maintained on PDA at 23 °C in the dark. The plugs of an actively growing culture of fungal strains were used in this experiment.

4.4.2 Different media conditions

In order to examine fungal surface hydrophobicity during mycoparasitism between the mycoparasite SMCD 2220-01 and hosts such as *F. proliferatum* SMCD 2246 and *F. redolens* V SMCD 2401 under different media conditions, potato dextrose broth (PDB) and nitrogen rich ICI-glucose (ICI) medium at pH 6 were used in this study. ICI medium is a chemically defined solution containing the following, in ppm: NH_4NO_3, 5,000; KH_2PO_4, 1,000; $MgSO_4 \cdot 7H_2O$, 5,000; glucose, 80,000; and 2 mL of microelement solution. A microelement solution contains following, in ppm: $NaNO_3$, 848; KCl, 300; $MgSO_4 \cdot 7H_2O$, 165; NaH_2PO_4, 100; $CaC1_2 \cdot 2H_2O$, 40; H_3BO_3, 5.7; $FeSO_4 \cdot 7H_2O$, 5.0; $ZnSO_4 \cdot 7H_2O$, 4.4; $MnSO_4 \cdot H_2O$, 3.1; $Na_2MoO_4 \cdot 2H_2O$, 2.5; and $CuSO_4 \cdot 5H_2O$, 0.4 (Geissman *et al.*, 1966). ICI medium is commonly used as to define nitrogen

source for production of secondary metabolites (Wiemann *et al.*, 2009). In this study, ICI was used for supplying minimal nutrients for fungal growth compared with PDB. PDB medium is a common medium for fungal growth in liquid made from potato infusion and dextrose with unknown nitrogen content.

4.4.3 Dual culture assay

Mycoparasitism between the mycoparasite SMCD 2220-01 and *Fusarium* strains such as SMCD 2246 and 2401 was investigated through dual culture assays. The mycoparasite was 3 d pre-inoculated on PDA and ICI agar plates. SMCD 2246 and 2401 were inoculated 2 cm apart from the mycoparasite and incubated at 23 °C in the dark for 4 d, respectively. Single cultures of the mycoparasite and *Fusarium* strains were used as controls (Carisse *et al.*, 2001; Li *et al.*, 2003; Vujanovic & Goh, 2009). The radial growth and contact angles of cultures were measured on 7 d incubation.

4.4.4 Optical microscopy and contact angles measurement

Mycoparasitism was observed under a Carl Zeiss Axiokop2 microscope equipped with Carl Zeiss AxioCam ICc1 camera with 20x, 40x, and 100x objectives. In order to investigate hydrophobicity of fungal surfaces, contact angles measurement was employed as a direct and simple method. The small plugs (5 mm × 5 mm) were taken from dual cultures and single cultures. The fungal samples taken were transferred on slide glasses and 2 µL of water drops were added on the fungal samples on the slide glasses. Images of contact angles taken from the fungal samples were viewed and acquired by the modified stereomicroscope with a horizontal light path. The used apparatus consisting of a Zeiss SV 6 Stereomicroscope and a Nikon Coolpix 8400 camera was designed (Chau *et al.*, 2009). The contact angles of the fungal surface images were obtained by using the Low Bond Axisymmetric Drop Shape Analysis Model of Drop Shape Analysis (LB_ADSA) plug-in coupled with ImageJ software. ImageJ, the open source multi-platform java image processing program, is available at http://rsb.info.nih.gov/ij/. LB_ADSA plug in is also available online at http://bigwww.epfl.ch/demo/dropanalysis/. This LB_ADSA approach, suggested by Stalder et al. (2006), provides high-precision contact angle measurements by using image gradient energy and cubic spine interpolation and the whole drop

profile for measuring axis-symmetric drops by utilizing first-order perturbation solution of the Laplace equation.

4.4.5 Atomic force microscopy

SMCD 2220-01, 2246, and 2401 were grown on PDA and ICI plates at 23 °C in the dark. The plugs of actively grown cultures were transferred onto sterilized slide glasses and then PDB and ICI broth were supplied instead of solid media to avoid impurities from agar compositions. Hyphal growth was continued onto the glass coverslips to reduce the density of mycelia since the dense mycelia cause difficulty in observing individual hyphae under atomic force microscopy (AFM). The grown hyphae on the glass coverslips were rinsed with sterilized distilled water (SDW) three times to remove extra salts mediated by media composition before AFM. The slide glasses and glass coverslips were cleaned with ethanol and allowed to dry in air in the laminar hood before using them. The hyphal surface on the tip of hypha was used for AFM since the tip is crucial part of the fungal growth, development, and interaction with biotic and abiotic environments.

All experiments were conducted in air, using tapping-mode atomic force microscope (Molecular imaging Inc, Nanoscope IIIa; Digital Instruments, Santa Barbara, Calif.) equipped with a J-type piezo scanner, at Saskatchewan Structural Sciences Centre. An Olympia Inverted Optical Microscope was used to locate the sample or the sample plate under the AFM tip for optimal setup. Aspire Conical Tapping mode Silicon Probes (CT; Nanoscience Instruments, Inc) were used for the imaging. Amplitude and height images were obtained in the tapping mode with a scan speed of 1ln/s (line/second) and an integral gain of 3 to 5. The tapping force was adjusted by changing the set point voltage until high-resolution images were obtained in minimal tapping force. All images were recorded at 23 °C. To perform force measurements, a hypha was scanned in tapping mode to obtain a high-magnification image and to locate a position on the hypha for force measurements. The cantilever deflection was calibrated by taking force curves on bare coverslips. To avoid large variation of spring constants of individual cantilevers, only one type of cantilever was used. The specifications of the used cantilever are as follow: spring constant, 50 N/m; resonance frequency, 170 kHz. The height of conical tip was 15 μm and the radius of curvature was 8 nm.

The obtained AFM images were visualized and analyzed using Gwyddion which is a modular program for scanning probe microscopy data and an open software on http://gwyddion.net/download.php.

4.4.6 Statistical analysis

To compare means between single and dual cultures, the radial growth and contact angles of fungal cultures based on the dual culture assay were analyzed by independent-samples T-test at *p*-value 0.05.

4.5 Results

4.5.1 Fungal radial growth

The radial growth of SMCD 2401 was significantly reduced on dual cultures compared with that of single cultures, whereas the radial growth of SMCD 2246 was not significantly different between single culture and dual cultures with the mycoparasite SMCD 2220-01 on both PDA and ICIA as shown in Figure 4.1. Based on the morphology of cultures, an inhibition or suppression zone between SMCD 2246 and 2220-01 was observed when SMCD 2246 was challenging against SMCD 2220-01 (Figure 4.2), which could be explained with mycoparasitism through antagonism. As shown in Figure 4.3, contact mode of action and intracellular penetration between the mycoparasite and the host SMCD 2246 were observed; decomposition process between the mycoparasite and the host SMCD 2401 was observed. Those results showed that the mycoparasite can suppress hyphal growth of host *Fusarium* strains, indicating the different degree of suppression forced by the mycoparasite. Mycoparasitism could be affected by media conditions and the type of *Fusarium* strain.

4.5.2 Fungal surface hydrophobicity

SMCD 2246 showed significantly reduced contact angles on PDA dual culture compared to that of 2246 on the single culture, while SMCD 2401 showed significantly increased contact angles compared to 2401 on PDA dual culture compared to that of the single culture (Figure 4.4.a). To be specific, the single culture of SMCD 2246 as a control illustrated high hydrophobic surface property due to contact angles (128° ± 5) > 90° on PDA culture. SMCD 2246 on dual cultures of 2246 and 2220-01 had contact angles (100° ± 10). In contrast, the single culture of

SMCD 2401 as a control illustrated hydrophilic surface property due to contact angles (25° ± 1) < 90°. SMCD 2401 on dual cultures of 2401 and 2220-01 had contact angles (99° ± 13). SMCD 2220-01 showed a hydrophlilic surface property due to contact angles (50° ± 3) < 90°. In this PDA culture system, SMCD 2401 showed more noticeable changes in contact angles than 2246 by 2220-01.

However, SMCD 2246 did not show significantly reduced contact angles on ICIA dual culture compared to that of 2246 on the single culture. SMCD 2401 showed no considerable change in contact angles between dual and single cultures on ICIA (Figure 4.4.b). In detail, the single culture of SMCD 2246 as a control illustrated high hydrophobic surface property due to contact angles (130° ± 3) > 90° on ICI culture. SMCD 2246 on dual culture of 2246 and 2220-01 had contact angles (82° ± 36). Interestingly, the single culture of SMCD 2401 as a control illustrated hydrophobic surface property due to contact angles (90° ± 29) > 90°, which was significantly different in value from single culture of SMCD 2401 on the PDA condition. SMCD 2401 on dual cultures of 2401 and 2220-01 had contact angles (96° ± 27). SMCD 2220-01 showed a hydrophlilic surface property due to contact angles (25° ± 5) < 90°. In this ICI culture, there was no significant change in contact angles for both 2246 and 2401.

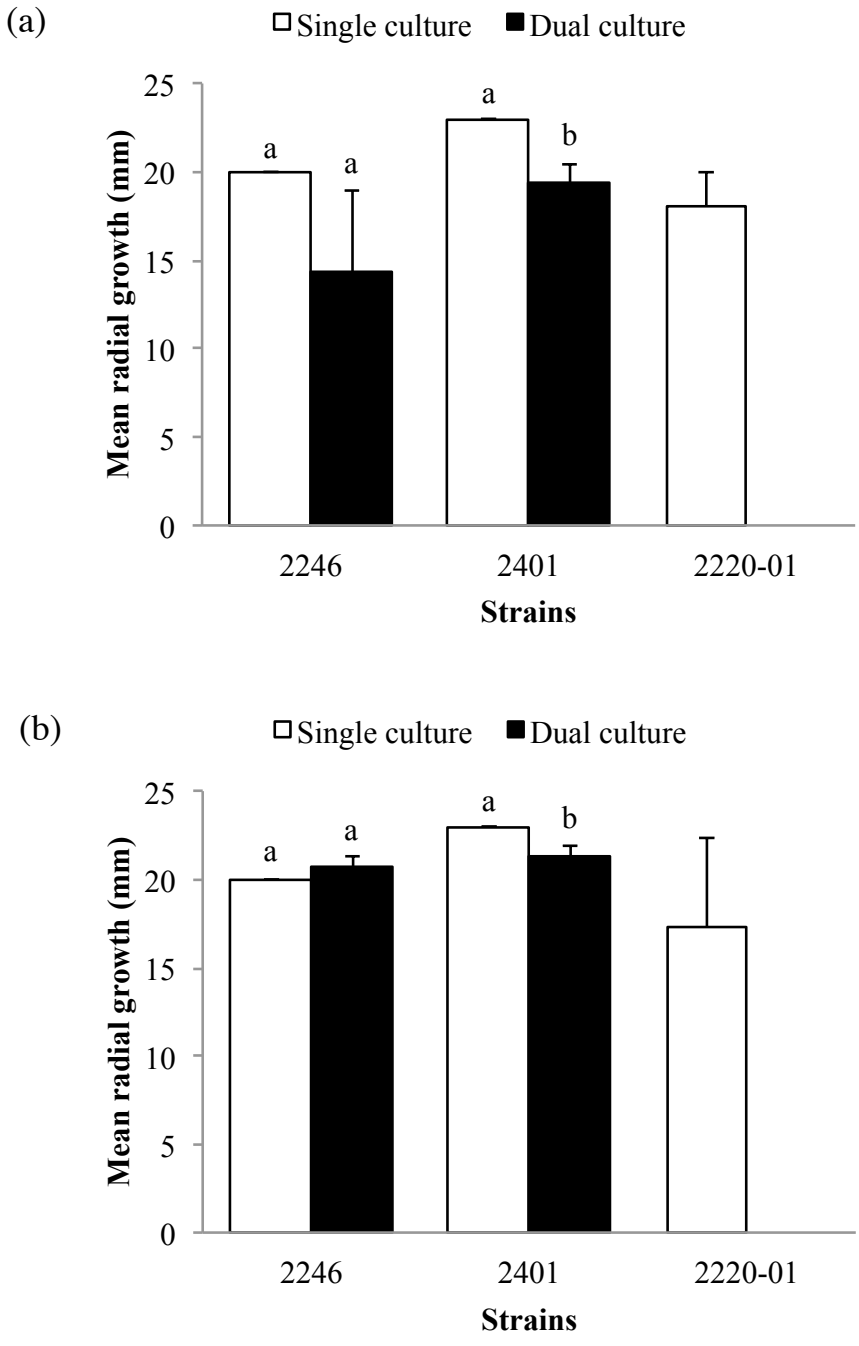


Figure 4.1 The fungal radial growth of SMCD 2246, 2401, and 2220-01 on PDA (a) and ICIA (b). The radial growth of single cultures (□) and dual cultures (■) between the mycoparasite and host *Fusarium* strains on PDA for 7 d incubation. Data are means and standard deviations of three replicates. Bars with the different lowercase letters are significantly different between mean radial growth of single and dual cultures at *p*-value 0.05, with independent-samples T-test. Each *Fusarium* species was analyzed separately.

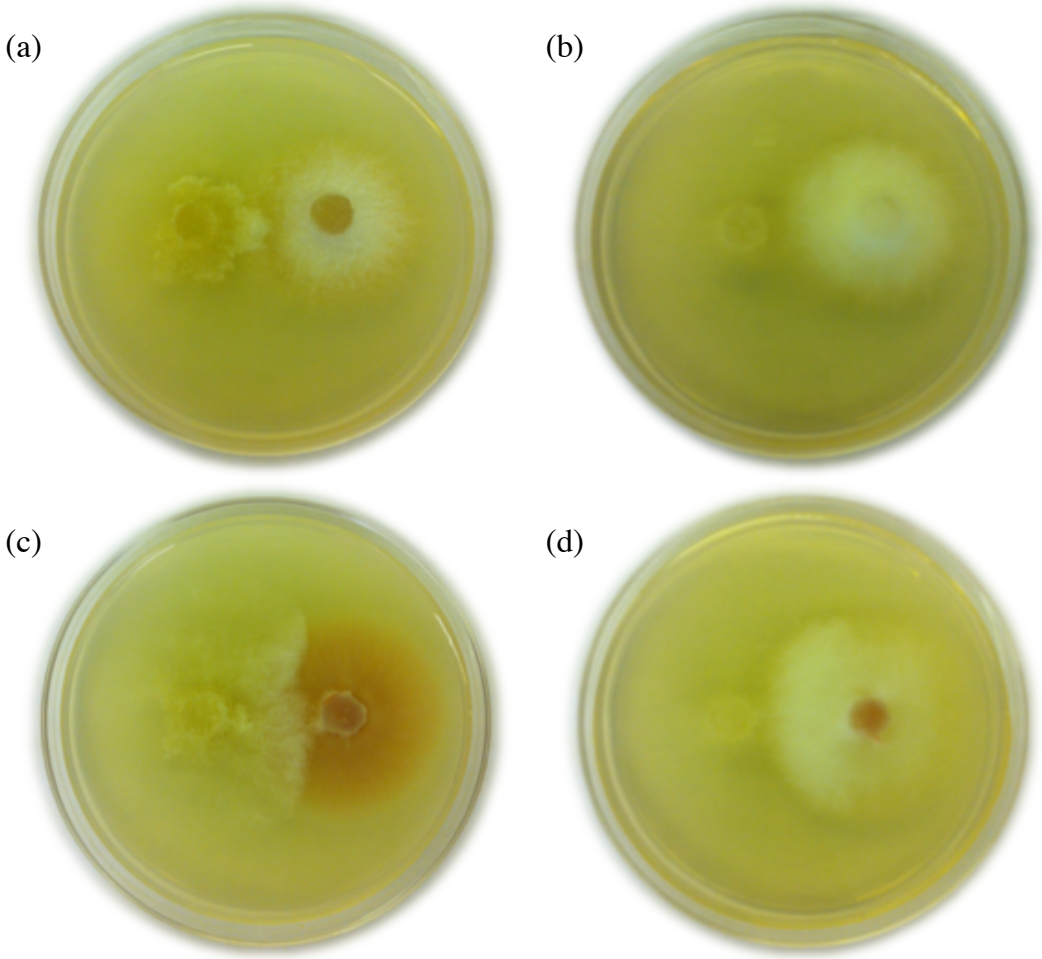


Figure 4.2 Macroscopic images indicating the diphasic interactions between the mycoparasite and *Fusarium* hosts. Antagonistic interaction in dual cultures of the mycoparasite SMCD 2220-01 (left side) and the host SMCD 2246 (right side) for 7 d incubation on PDA and ICIA (a and b). Biotrophic interaction in dual cultures of the mycoparasite SMCD 2220-01 (left side) and the host SMCD 2401 (right side) for 7 d incubation on PDA and ICIA (c and d).

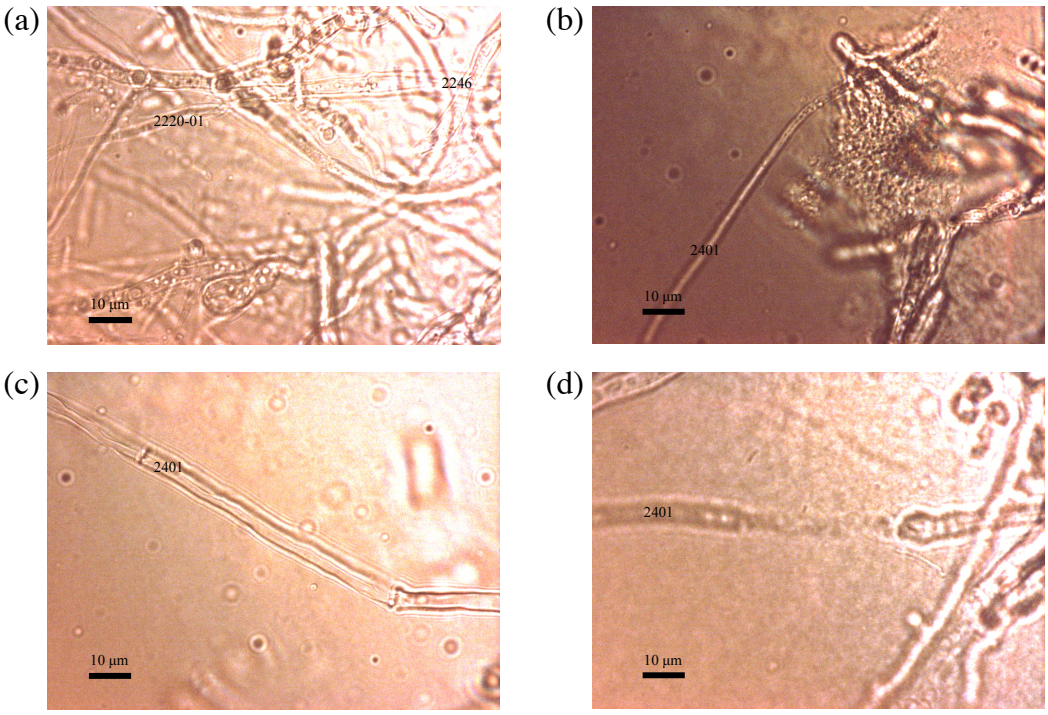


Figure 4.3 Microscopic images indicating the different mode of action of the mycoparasite to *Fusarium* hosts. Contact mode of action and intracellular penetration (a) on dual cultures of the mycoparasite SMCD 2220-01 and host SMCD 2246 for 7 d incubation on PDA. Decomposition process on dual cultures of the mycoparasite SMCD 2220-01 and host SMCD 2401 for 7 d incubation on PDA (b and c) and ICIA (d).

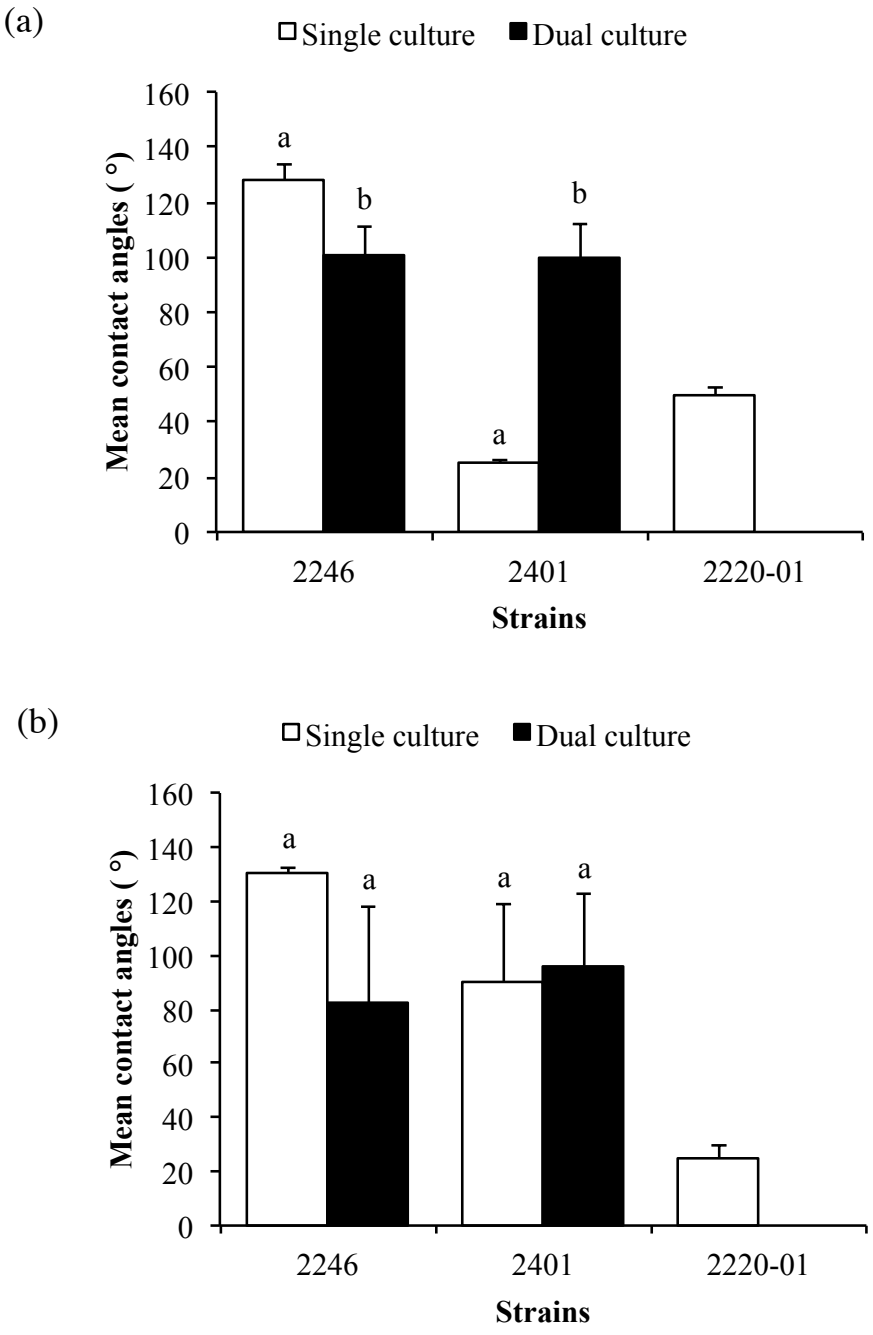


Figure 4.4 The contact angles of SMCD 2246, 2401, and 2220-01 on PDA (a) and ICIA (b). The contact angles of single cultures (□) and dual cultures (■) between the mycoparasite and host *Fusarium* strains on PDA for 7 d incubation. Data are means and standard deviations of three replicates. Bars with the different lowercase letters are significantly different between mean contact angles of single and dual cultures at *p*-value 0.05, with independent-samples T-test. Each *Fusarium* species was analyzed separately.

4.5.3 Fungal surfaces analyzed by atomic force microscopy

SMCD 2220-01 incubated in PDB showed different surface topology at 0, 1, and 2 hrs exposed on the general dryness or desiccation (Figure 4.5). The hyphal surface at 0 hrs of SMCD 2220-01 showed the tendency to have a broad and slightly convex surface (approximately 533 nm width) and few concavities. The range of roughness was from -0.04 to 0.04 V. After 1 hr, it seemed that the area of the broad convex surface was changed to the small parts with increasing the number of convexity. The range of roughness was from -0.3 to 0.3 V. Then, the small convexity was likely to move downward at 2 hrs. The range of the roughness was from -0.4 to 0.3 V. In the case of ICI medium condition, the hyphal surface of SMCD 2220-01 incubated indicated the tendency to have a small convexity similar to that of SMCD 2220-01 incubated in PDB at 2 hrs exposed on desiccation. The range of the roughness was from -0.2 to 0.2 V.

SMCD 2246 incubated in PDB showed the particular structure on the hyphal surface (Figure 4.6). The hyphal surface at 0 hrs of SMCD 2246 showed an unique and long concave as well as characteristic rodlet structures or layers, which were reported on the hyphal surface of the fruit body of *Agaricus bisporus* and the spore surface of *Aspergillus nidulans*. The range of roughness was from -0.5 to 0.5 V. Particularly, the hyphal surface at 1 hr of SMCD 2246 showed different shapes of protrusions with the strong roughness ranging from -6 to 2 V. At 2 hrs exposure, the hyphal surface of SMCD 2246 was observed to show irregular particles. The range of roughness was from -1 to 1 V. In the case of ICI medium condition, the hyphal surface of SMCD 2246 indicated the tendency to have small convexity different from that of SMCD 2246 incubated in PDB. The range of roughness at 0 hrs was from -0.08 to 0.05 V. The convexity seemed to be moving downward at 1 hr. The range of roughness was from -0.1 to 0.1 V. It seemed that the hyphal surface of SMCD 2246 incubated in ICI was softer than that of SMCD 2246 incubated in PDB, according to the range of the roughness.

SMCD 2401 incubated in PDB showed the different hyphal surface morphology at 0, 1, and 2 hrs exposed on the desiccation (Figure 4.7). The hyphal surface at 0 hrs of SMCD 2401 showed the tendency to have an uneven surface. The range of roughness was from -0.7 to 0.7 V. After 1 hr, the uneven surface is likely to be concave. The range of roughness was from -0.4 to 0.3 V. Then, the number of convexity was increased at 2 hr. The range of the roughness was from -0.3 to 0.3 V. In the case of ICI medium condition, SMCD 2401 indicated the cloudiness on the hyphal surface at 0 hrs and 1 hr, exposed on the desiccation. The range of roughness at 0 hrs

is from -0.1 to 0.1 V. The range of roughness at 1 hr is from -0.04 to 0.06 V. It seemed that the hyphal surface of SMCD 2401 incubated in ICI was softer than that of *F. redolens* incubated in PDB, according to the range of the roughness.

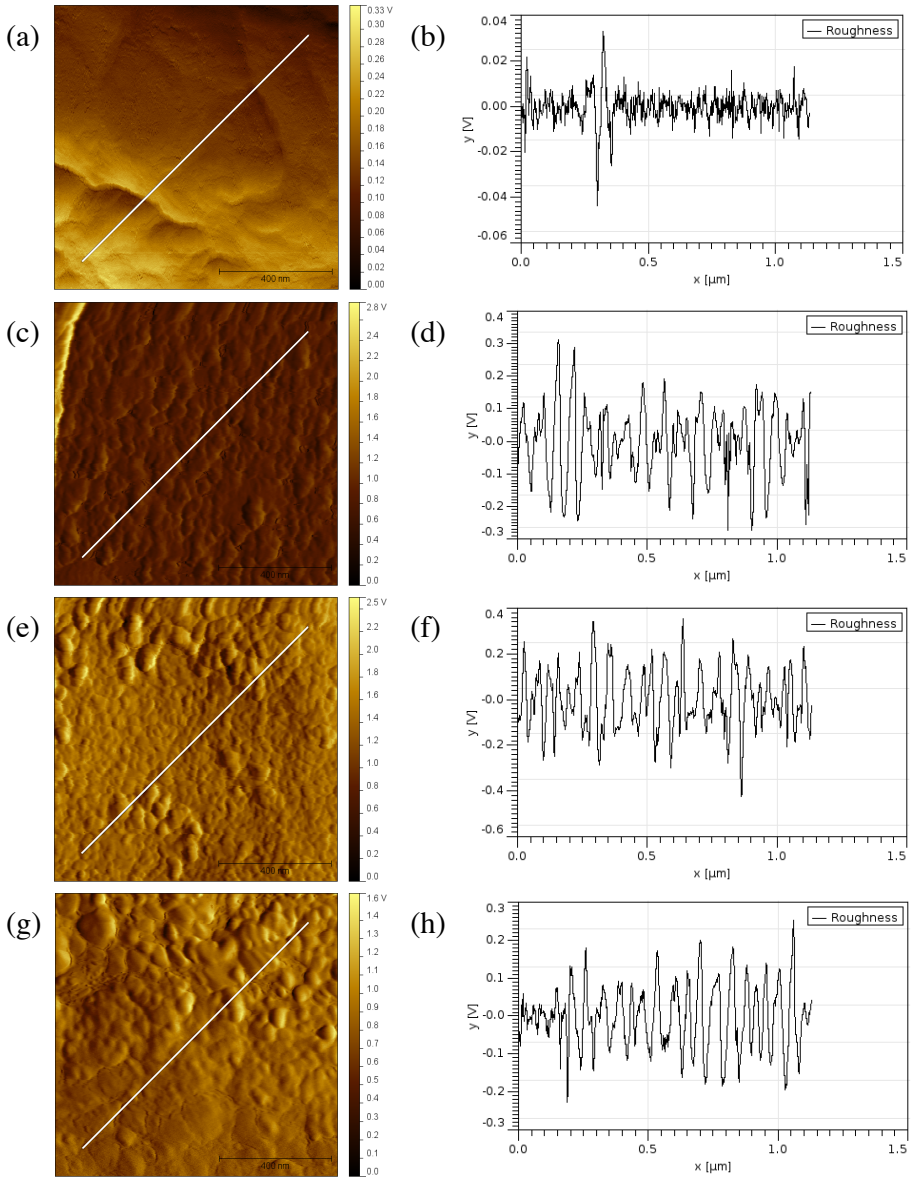


Figure 4.5 Images generated by AFM in the tapping mode of the hyphal surfaces of SMCD 2220-01 and cross sections along the lines in panels (a, c, e, and g), indicating roughness of fungal hyphae (b, d, f, and h). SMCD 2220-01 incubated in PDB at 0 hrs (a), 1 hr (c), and 2 hrs (e) and incubated in ICI at 0 hrs (g), exposed on desiccation. Bar length, 400 nm.

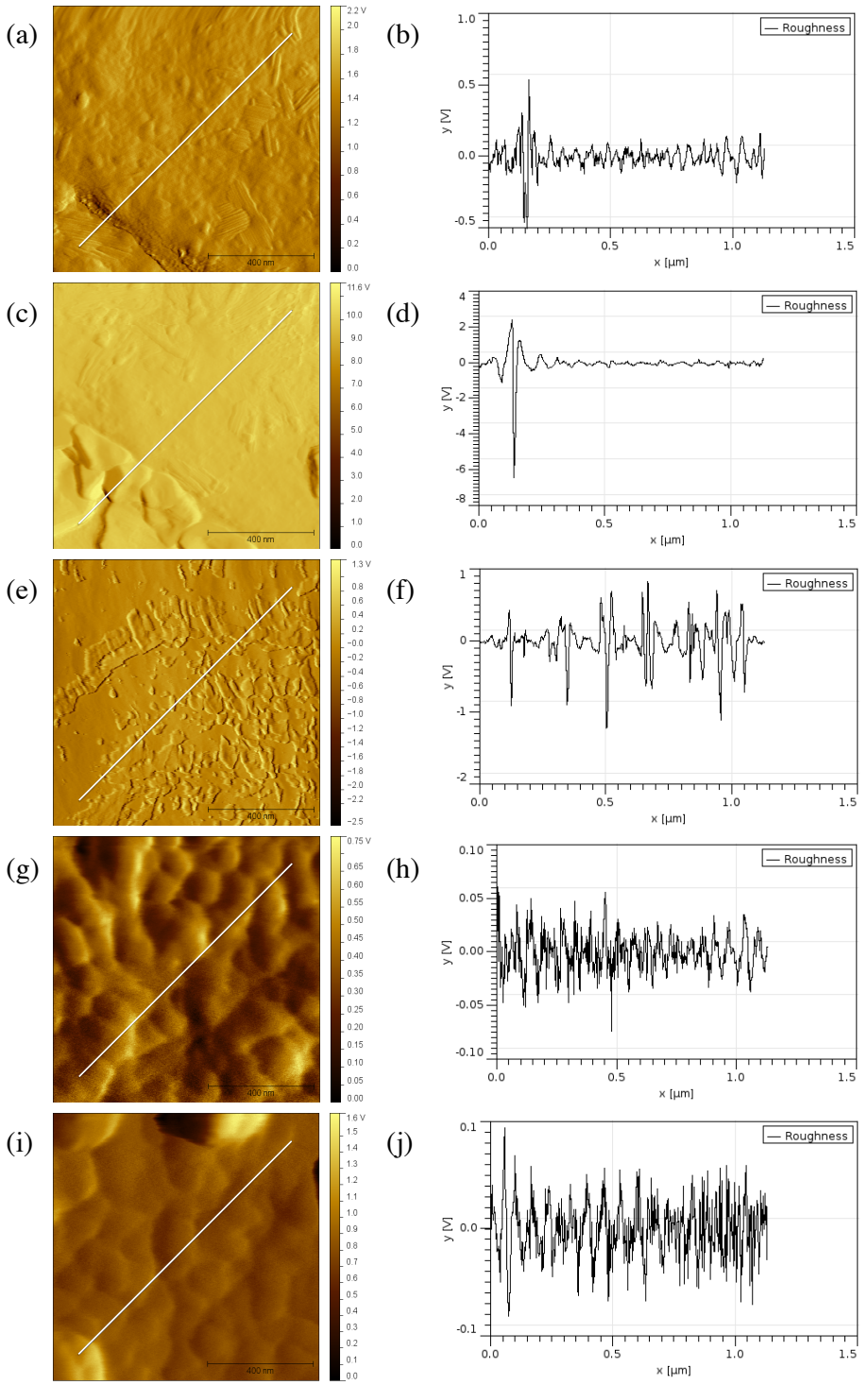


Figure 4.6 Images generated by AFM in the tapping mode of the hyphal surfaces SMCD 2246 and cross sections along the lines in panels (a, c, e, g and i), indicating roughness of fungal hyphae (b, d, f, h and j). SMCD 2246 incubated in PDB at 0 hrs (a), 1 hr (c), and 2 hrs (e) and incubated in ICI 0 hrs (g) and 1 hr (i), exposed on desiccation. Bar length, 400 nm.

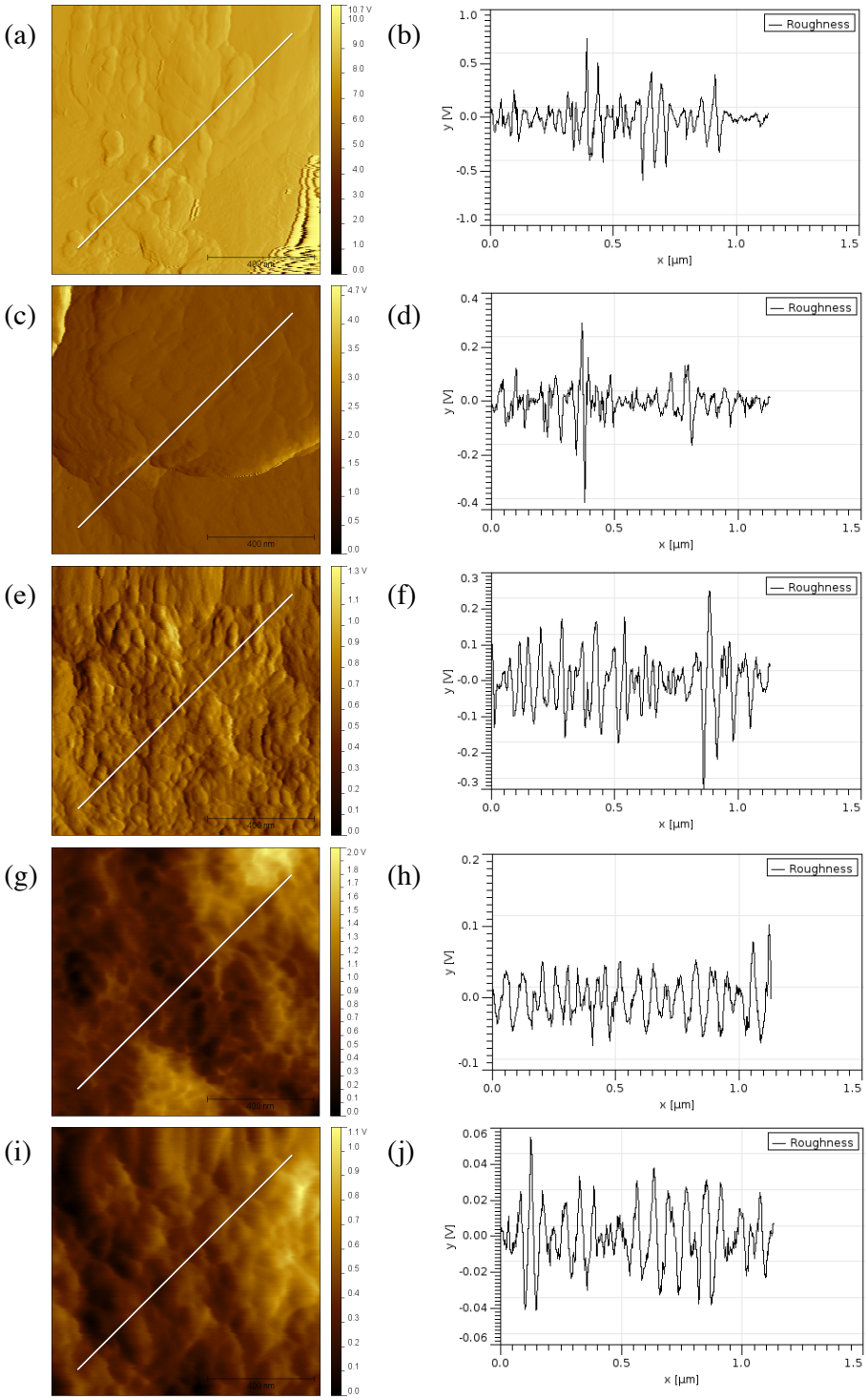


Figure 4.7 Images generated by AFM in the tapping mode of the hyphal surfaces SMCD 2401 and cross sections along the lines in panels (a, c, e, g and i), indicating roughness of fungal hyphae (b, d, f, h and j). SMCD 2401 incubated in PDB at 0 hrs (a), 1 hr (c), and 2 hrs (e) and incubated in 0 hrs (g) and 1 hr (i), exposed on desiccation. Bar length, 400 nm.

4.6 Discussion

4.6.1 Fungal radial growth

The degree of decrease in radial growth of SMCD 2401 and 2246 was different on the used media when the hosts were challenging against the mycoparasite SMCD 2220-01 (Figure 4.1). It seems that the efficacy of the mycoparasite is higher on PDA than ICIA. This might be driven by the type of the medium, especially the amount of carbon and nitrogen in the medium (Persson & Bååth, 1992). Also, the mycoparasite showed different mode of mycoparasitism, such as antagonistic interaction with the host SMCD 2246 and biotrophic interaction with the host SMCD 2401 (Figure 4.2). The phenomenon may occur according to the hosts, in particular their cell wall (Latge, 2007). Different types of cell wall of the hosts could influence on the action of the mycoparasite (Ojha & Chatterjee, 2011). Moreover, as shown in Figure 4.3, the decomposition process may be the result of the mycoparasite's production of lytic enzyme, which is a key mechanism frequently found in mycoparasitism of *Trichoderma* spp. (Gajera & Vakharia, 2012).

4.6.2 Fungal surface hydrophobicity

The values of contact angles on PDA and ICIA for single cultures of SMCD 2246, 2401, 2220-01 and dual cultures between the host and the mycoparasite were shown in Figure 4.4. On the common fungal medium (PDA), each single culture of SMCD 2246, 2401, 2220-01 as a control illustrated a high hydrophobic surface property, evidenced with contact angles (128° ± 5) > 90° and a hydrophilic surface property, shown by contact angles (25° ± 1) < 90 and (50° ± 3) < 90°, respectively. On minimal medium (ICI), each single culture of SMCD 2246, 2401, 2220-01 as a control illustrated a high hydrophobic surface property shown by contact angles (130° ± 3) > 90°, a hydrophobic surface property evidenced with contact angles (90° ± 29) > 90°, and a hydrophilic surface property shown by contact angles (25° ± 5) < 90°. Previously, Smits et al. (2003) showed the differences of contact angles depending on fungi such as *Fusarium oxysporum* and *Trichoderma harzianum* and the different media (Smits *et al.*, 2003). More interestingly, our results indicated that the growth medium considerably affect the fungal surface properties of the tested fungi. Furthermore, it was observed that contact angles during mycoparasitism between SMCD 2246 and 2220-01 were significantly reduced (100° ± 10), whereas contact angles during mycoparasitism between SMCD 2401 and 2220-01 were

significantly increased (99° ± 13) compared with the single culture of the hosts on PDA. These shifts in fungal surface properties might driven by the intra-penetration of the mycoparasite to the hosts. As a possible explanation, the mycoparasite may produce and accumulate particular substances on the former host cell wall and/or in the contact zone with the host cell wall. According to Vergara-Fernández et al (2011), volatile organic compounds (VOCs) could modify the morphology of *Fusarium solani* (Vergara-Fernández *et al.*, 2011). However, on ICIA, there was no significant differences of contact angles: between SMCD 2246 and 2220-01 (82° ± 36); between SMCD 2401 and 2220-01 (96° ± 27) compared to the single culture of the hosts. It seems that these huge variability on ICIA were caused by unstable growth of the mycoparasite, since the consistent and stable contact angles require stability of fungal growth (Chau *et al.*, 2009).

4.6.3 Fungal surfaces analyzed by atomic force microscopy

AFM in tapping-mode was used to assess fungal hyphal surface morphology or topology with roughness at different exposure time on the general dryness or dessication, as shown in Figure 4.5, 4.6, and 4.7. The hyphal surface of SMCD 2220-01 in PDB showed the change in the morphology from soft to hard surface and roughness from low to high. In ICI medium, the morphology of hyphal surface of SMCD 2220-01 was similar to that of 2220-01 in PDB at 2 hrs, and the roughness was slightly lower than that of 2220-01 in PDB at 2 hrs. The morphology of the hyphal surface of SMCD 2246 was completely different from that of 2220-01. In particular, SMCD 2246 showed a unique structure, which was reported on the hyphal surface of the fruit body of *Agaricus bisporus* (Lugones *et al.*, 1996) and the surfaces of the spore and hypha of *Aspergillus nidulans* as a rodlet structure or layer (Ma *et al.*, 2005). By increasing time, the morphology and roughness was changed. In ICI medium, the morphology of SMCD 2246 was quite different from that of SMCD 2246 in PDB and the roughness was considerably lower than that of 2246 in PDB. The morphology and the roughness of the hyphal surface of SMCD 2401 varied depending on the media condition and exposure time on the general dryness. The roughness of the hyphal surface of SMCD 2401 was changed from high to low roughness by increasing exposure time in both media condition; the hyphal surface of SMCD 2401 in ICI medium, was considerably softer than that of 2401 in PDB. These results imply that the fungi

can rapidly change the composition of their cell walls depending on environmental conditions and their stage of growth (Bowman & Free, 2006).

The changes or dynamic coordination in fungal cell walls could be regulated by particular genes, such as the velvet gene (ve A), known as an important regulator of asexual and sexual development, based on the fungus *Aspergillus nidulans* as a model system. It was shown that the contents of cell walls are strongly correlated with hyphal walls in *Aspergillus nidulans* (Alam *et al.*, 2014). Also, it was found that FvVe1 plays a crucial role in cell wall integrity and the cell surface hydrophobicity of *Fusarium verticillioides* (Li *et al.*, 2006).

4.7 Conclusions

The results of contact angles measurement demonstrated differential expression of fungal surface hydrophobicity of SMCD 2220-01, 2246, and 2401, as well as changes in hyphal surface hydrophobicity of host *Fusarium* species during mycoparasitism under PDA and ICI media conditions. In addition, observation of all the fungal hyphal surfaces under AFM indicated differential surface topology with roughness. The differences in hyphal surfaces were noticeable under different media conditions and exposure to desiccation.

4.8 Connection to the next study

In this study (chapter 4), *Sphaerodes mycoparasitica* SMCD 2220-01 as a host-specific mycoparasite showed the mycoparasitism including suppression of host *Fusarium* strains. Thus, this mycoparasite can be considered as a potential biocontrol agent for Fusaria. Many *Fusarium* species are not only plant pathogens but also mycotoxin producers. Contamination with mycotoxins and accumulation of mycotoxins in cereal grains have been threatening human and animal health. Therefore, it is suggested to evaluate effectiveness of *S. mycoparasitica* as a biodegrader of mycotoxins.

5. EFFICACY OF *SPHAERODES MYCOPARASITICA* IN BIODEGRADATION OF MYCOTOXINS ANALYZED BY HPLC-HR-ESI-MS

5.1 Abstract

The fungus *Sphaerodes mycoparasitica* Vujan. SMCD 2220-01 is a host specific mycoparasite against plant pathogenic *Fusarium* species, which produce mycotoxins such as zearalenone (ZEN), 3-acetyl-deoxynivalenol (3-ADON), 15-acetyl-deoxynivalenol (15-ADON), and deoxynivalenol (DON). *S. mycoparasitica* was reported to remove a constitutive mycotoxin, aurofusarin (also known as a red pigment), produced by *Fusarium graminearum* (Vujanovic & Goh, 2011b). Furthermore, SMCD 2220-01 showed the capacity to reduce the production of DON, 3-ADON, 15-ADON, and ZEN produced by *F. graminearum* in the co-culture system. In this study, biodegradation of mycotoxins by SMCD 2220-01 was conducted by shake culture technique. A culture broth of SMCD 2220-01 was extracted with ethyl acetate to qualify and quantify residual mycotoxins. Extracts were analyzed by thin-layer chromatography (TLC) and high performance liquid chromatography–electrospray ionization-mass spectrometry (HPLC–HR-ESI–MS). Furthermore, anlaysis of biotransformants of the mycotoxins in the culture extracts of SMCD 2220-01 was carried out using HPLC-HR-ESI-MS. TLC clearly showed that degradation of four mycotoxins occurred, compared to control. Natural decomposition of the mycotoxins was not observed. In HPLC–HR-ESI–MS analysis, a decrease in the amounts of each mycotoxin was observed and several biotransformants of each mycotoxin were detected. Particularly, the amount of ZEN was decreased by 97%, and zearalenone sulfate ($[M-H+SO_3]^-$ at *m/z* 397.1052 $C_{18}H_{21}O_8S_1$) was detected as a metabolite of ZEN by the mycoparasite in the culture extracts. In addition, SMCD 2220-01 appeared to degrade 15-ADON, 3-ADON, and DON by 72%, 60%, and 89%, but their metabolites were not characterized. These findings indicate that SMCD 2220-01 might metabolize mycotoxins to less toxic metabolites. Further

research will be focused on enzymes and genes particularly involved in detoxification of mycotoxins.

5.2 Introduction

Fusarium head blight (FHB) is one of the most important fungal diseases affecting crops such as wheat and barley in the world. The disease is caused by plant pathogenic *Fusarium* spp., including *Fusarium avenaceum*, *Fusarium culmorum*, and *Fusarium graminearum*. These *Fusarium* species produce toxic secondary metabolites, mycotoxins such as deoxynivalenol (DON), its derivatives, and zearalenone (ZEN). Zearalenone (ZEN), a member of the resorcyclic acid lactone family, is a known hydrophobic mycotoxin produced in particular by *F. graminearum* and *F. culmorum* (Caldwell *et al.*, 1970; Katzenellenbogen *et al.*, 1979). ZEN is biosynthesized through the Polyketide synthase (PKS) pathway (Gaffoor & Trail, 2006; Kim *et al.*, 2005; Lysøe *et al.*, 2006). ZEN contamination found in maize and wheat as well as wheat-derived products for human consumption, poses a threat to human health due to its similar chemical structure to estrogen (Iqbal *et al.*, 2014; Shier *et al.*, 2001). Deoxynivalenol, 3-acetyl-deoxynivalenol, and 15-acetyl-deoxynivalenol, belonging to Type B trichothecenes, have an effect on metabolic mechanisms related to inhibition of protein synthesis (Ehrlich & Daigle, 1987; Middlebrook & Leatherman, 1989).

Sphaerodes mycoparasitica Vujan. SMCD 2220-01 was originally isolated and identified from wheat and asparagus fields in association with *F. oxysporum*, *F. avenaceum*, and *F. graminearum* (Vujanovic & Goh, 2009). SMCD 2220-01 as a mycoparasite was shown to be a potential biological control agent against plant pathogenic *Fusarium* species (Vujanovic & Goh, 2010). In addition to the biocontrol of *Fusarium* species, SMCD 2220-01 showed the ability to reduce the production of DON, 3-ADON, 15-ADON, and ZEN produced by *F. graminearum* in the co-culture system (Vujanovic & Chau, 2012). It is necessary for mycoparasites to resist or tolerate toxic secondary metabolites produced by host fungi (Kosawang *et al.*, 2014).

5.3 Hypothesis and objective

We speculated that *Sphaerodes mycoparasitica* SMCD 2220-01 degrades mycotoxins produced by *Fusarium* species, since SMCD 2220-01 showed the successful mycoparasism with *Fusarium* species, implying its resistance to *Fusarium* mycotoxins. The main objective of this

study was to assess the ability of SMCD 2220-01 to degrade *Fusarium* mycotoxins such as ZEN, DON, 15-ADON, and 3-ADON through TLC. Furthermore, if SMCD 2220-01 possesses the capacity to degrade the mycotoxins, the transformants, or byproducts of the mycotoxins was investigated using HPLC-HR-ESI-MS.

5.4 Materials and Methods

5.4.1 Fungal cultures and media conditions

Sphaerodes mycoparasitica Vujan. SMCD 2220-01 was generally maintained on potato dextrose agar (PDA). In order to induce degradation capacity of SMCD 2220-01, SMCD 2220-01 was grown on the cellulose membrane placed on PDA amended with 1 ppm of each of mycotoxins as a final concentration. The induced SMCD 2220-01 was inoculated in 5 mL of potato dextrose broth (PDB) and incubated on a shaking incubator with 120 rpm at 23 °C for 3 d in the dark condition. The 3 d pre-cultured SMCD 2220-01 was used further experiments.

5.4.2 Chemicals

All the mycotoxins, such as deoxynivalenol (DON), 3-acetyl-deoxynivalenol (-3ADON), 15-acetyl-deoxynivalenol (15-ADON), and zearalenone (ZEN), as shown in Table 5.1 were purchased from Sigma-Aldrich Canada Ltd., Oakville, ON. HPLC grade organic solvents were purchased from Fisher Scientific. The stock solutions of each of mycotoxins were prepared by dissolving each mycotoxin in acetonitrile.

5.4.3 Biodegradation of mycotoxins

Biodegradation or biotransformation of mycotoxins was conducted by shake culture techniques. Two parts per million (ppm) of mycotoxins as a final concentration were added into the pre-cultures of SMCD 2220-01. In other words, SMCD 2220-01 was exposed in 10 µg of each of the mycotoxins, since 10 µl of 1000 ppm mycotoxin stock solution was added in 5 ml of the pre-cultures and PDB. A non-contaminated and inoculated medium (only SMCD 2220-01) was prepared to exclude metabolites of SMCD 2220-01. A contaminated but not inoculated medium (only each mycotoxin, a control) was prepared to check natural decomposition of mycotoxins. A non-contaminated and non-inoculated medium (PDB, a control) was used to exclude impurities from the medium itself. All the cultures were incubated on shaking incubator

with 120 rpm at 23 °C in the dark condition. Cultures were harvested at 1, 2, and 3 weeks after the addition of mycotoxins. The harvested cultures were filtered by Watman filter paper Grade 2 to remove mycelia. The culture filtrates were used for extraction of residual mycotoxins by liquid-liquid partition.

5.4.4 Extraction of fungal cultures

Qualification of the residual level of mycotoxins was performed by liquid-liquid partition using organic solvent. The culture filtrates were extracted by 5 mL of ethyl acetate (EtOAc). The obtained EtOAc phases were evaporated by rotary evaporator. The concentrated extracts were dissolved in 200 µl of chloroform for thin layer chromatograpy (TLC) (Bejaoui *et al.*, 2006; Garda-Buffon & Badiale-Furlong, 2010; Teniola *et al.*, 2005). Theoretically, extracts of mycotoxins as a control were supposed to contain 0.4 µg of mycotoxins since 8 µl of extracts of mycotoxins were used for TLC analysis.

5.4.5 Detection and quantification of mycotoxins

The extracts of culture filtrates dissolved in chloroform were applied as a small spot near the base of an aluminum TLC silica gel 60 F_{254} plate. The separation process was performed by using a combination of dichloromethane and methanol as a mobile liquid phase for developing the TLC plate (Abbas *et al.*, 1984). Then, the developed TLC plate was visualized by charring solutions after checking under ultra violet light if needed. To interpret TLC spots, the relative mobility or retention factor (R_f) was calculated by the following formula:

$$Retention\ factor = \frac{Distance\ from\ start\ to\ center\ of\ substance\ spot}{Distance\ from\ start\ to\ solvent\ front} \quad \text{(Equation 5.1)}$$

Quantification of TLC spots was achieved through densitometry analysis using Image J software. Image J software is available online at http://rsbweb.nih.gov/ij/plugins/index.html and provides an easy access to extract the area occupied by a specific color. Relative density of residual mycotoxin in culture was acquired by the following formula:

$$Relative\ density\ of\ residual\ mycotoxin\ in\ culture = \frac{Density\ of\ residual\ mycotoxin\ in\ culture}{Density\ of\ residual\ mycotoxin\ in\ control} \quad \text{(Equation 5.2)}$$

In order to generate a standard curve of mycotoxins, 2, 4, 8, and 10 µl of 100 ppm mycotoxins were used by indicating the presence of 0.2, 0.4, 0.8, and 10 µg of mycotoxins, which includes the theoretical amount of mycotoxins in a positive control.

In order to elucidate transformants of mycotoxins by SMCD 2220-01 as well as confirmation of quantifying residual mycotoxins, HPLC-HR-ESI-MS was performed on an Agilent 1100 series high-performance liquid chromatography (HPLC) system equipped with an automatic injector, quaternary pump, degasser, and a diode array detector (DAD, wavelength range 190-600 nm) connected to a Qstar XL systems Mass Spectrometer (Hybrid Quadrupole-TOF LC/MS/MS) with turbospray electrospray ionization (ESI) source. Chromatographic seperations were carried out using Eclipse XDB-C-18 column (5 µm particle size silica, 150 × 4.6 mm I.D.). All the extracted samples were dissolved in acetonitrile. The mobile phase consisted of a linear gradient of 0.1% formic acid in water and 0.1% formic acid in methanol (95:5 in 5 min, to 80:20 in 25 min, to 50:50 in 35 min, to 25:75 in 40 min, to 5:95 in 45 min) and a flow rate of 0.1 mL/min. Data acquisition was carried out either positive or negative polarity mode for LC run (DON, 3-ADON, and ZEN on negative mode and 15-ADON on positive mode). Data processing was conducted by Analyst QS Software. Percent degradation of the mycotoxins by SMCD 2220-01 was calculated by using the formula:

$$Percent\ degradation = \left[\frac{(Residual\ mycotoxin\ in\ control - Residual\ mycotoxin\ in\ culture)}{Residual\ mycotoxin\ in\ control} \times 100\right] \quad \text{(Equation 5.3)}$$

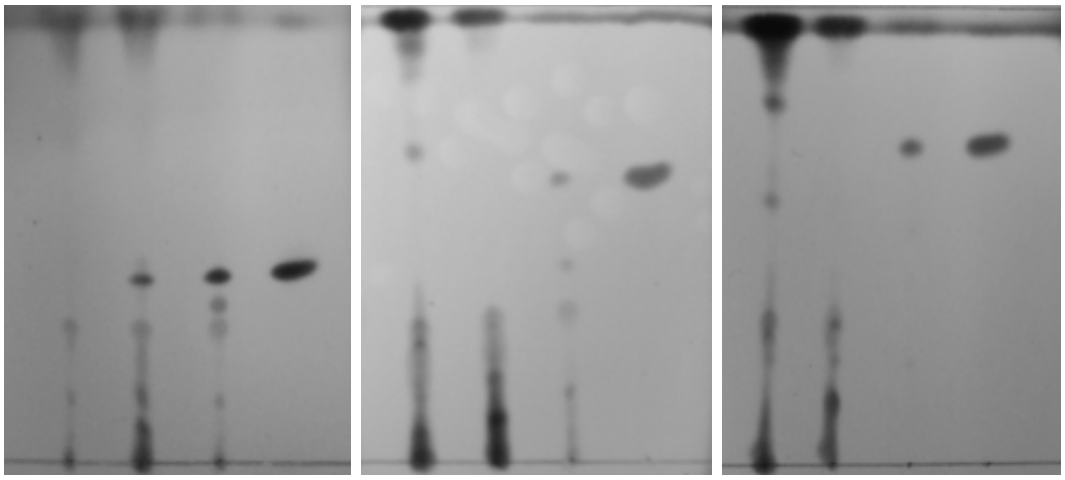
5.5 Results

5.5.1 Thin layer chromatography (TLC)

TLC analysis indicated the different level of residual mycotoxins at 1, 2, and 3 weeks old cultures after the addition of each mycotoxin, as shown in Figure 5.1, 5.3, 5.5, and 5.7. R_f value of ZEN was 0.36, 0.63, and 0.69 in the solvent system (95% dichloromethane and 5% methanol with developing 1, 1, and 2 times). R_f value of 15-ADON was 0.35, 0.6, and 0.58 in the solvent system (95% dichloromethane and 5% methanol with developing 3, 4, and 4 times). R_f value of 3-ADON was 0.48, 0.72, and 0.68 in the solvent system (95% dichloromethane and 5% methanol with developing 3, 4, and 4 times). R_f value of DON was 0.58, 0.6, and 0.63 in the solvent system (93% dichloromethane and 7% methanol with developing 3, 5, and 5 times). It

seemed that deoxynivalenol with the transformants by SMCD 2220-01 was masked due to the similar polarity between deoxynivalenol and the transformants.

The relative density of zearalenone (0.62 ± 0.12, 0, and 0) revealed that SMCD 2220-01 degrades 38%, 100%, 100% of zearalenone (ZEN) when grown in liquid shaking culture at 1, 2, and 3 weeks after the addition of ZEN, respectively (Figure 5.2). The relative density of 15-acetyl-deoxynivalenol (0.61 ± 0.19, 0.56 ± 0.29, and 0.27 ± 0.26) revealed that SMCD 2220-01 degrades 39%, 44%, 73% of 15-acetyl-deoxynivalenol (15-ADON) when grown in liquid shaking culture at 1, 2, and 3 weeks after the addition of 15-ADON, respectively (Figure 5.4). The relative density of 3-acetyl-deoxynivalenol (0.42 ± 0.15, 0.32 ± 0.28, and 0.53 ± 0.32) revealed that SMCD 2220-01 degrades 58%, 68%, 47% of 3-acetyl-deoxynivalenol (3-ADON) when grown in liquid shaking culture at 1, 2, and 3 weeks after the addition of 3-ADON, respectively (Figure 5.6). Particularly, the relative density of deoxynivalenol (2.58 ± 0.60, 2.17 ± 0.15, and 1.17 ± 0.19) revealed that deoxynivalenol was masked with its transformants or metabolites of SMCD 2220-01 due to the similar polarity between DON and the transformants or the metabolites (Figure 5.8). It seemed that SMCD 2220-01 showed the most effective degradability on ZEN among other mycotoxins (Figure 5.9). In case of DON, HPLC-HR-ESI-MS analysis was strongly recommended for examination of DON degradation due to difficulty to be analyzed by TLC. The standard curves of all the mycotoxins included the quantity of each mycotoxin in controls as shown in Appendix (Figure 9.1, 9.2, 9.3, and 9.4).



(a) (b) (c)

Figure 5.1 Thin layer chromatograms of 1, 2, and 3 week-old cultures (a, b, and c) of SMCD 2220-01 supplemented with 2 ppm of ZEN as a final concentration. From the left side, the first, second, third, and fourth lanes indicate the cultures of only SMCD 2220-01, the cultures of SMCD 2220-01 with ZEN, the cultures of ZEN, and standard ZEN as a reference.

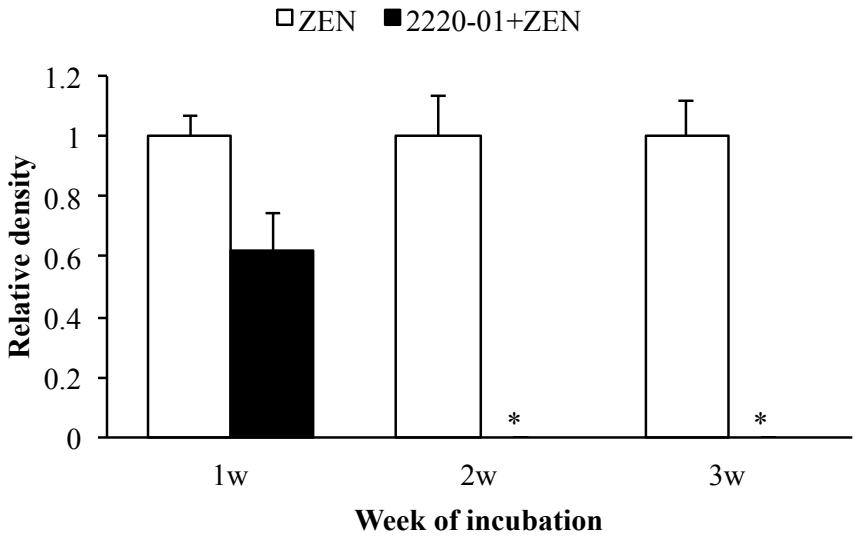
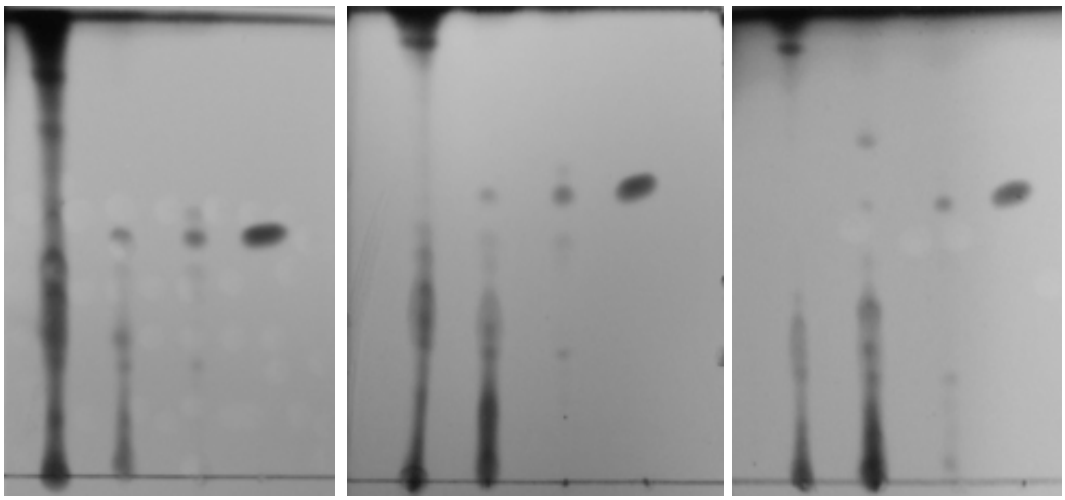


Figure 5.2 Relative density of residual zearalenone at 1, 2, and 3 weeks of the culture extracts (SMCD 2220-01 with ZEN, ■). Contaminated but not inoculated culture (only ZEN, □) was used as a control. Asterisks indicate 0 value. Data are means and standard deviations of three replicates.



(a) (b) (c)

Figure 5.3 Thin layer chromatograms of 1, 2, and 3 week-old cultures (a, b, and c) of SMCD 2220-01 supplemented with 2 ppm of 15-ADON as a final concentration. From the left side, the first, second, third, and fourth lanes indicate the cultures of only SMCD 2220-01, the cultures of SMCD 2220-01 with 15-ADON, the cultures of 15-ADON, and standard 15-ADON as a reference.

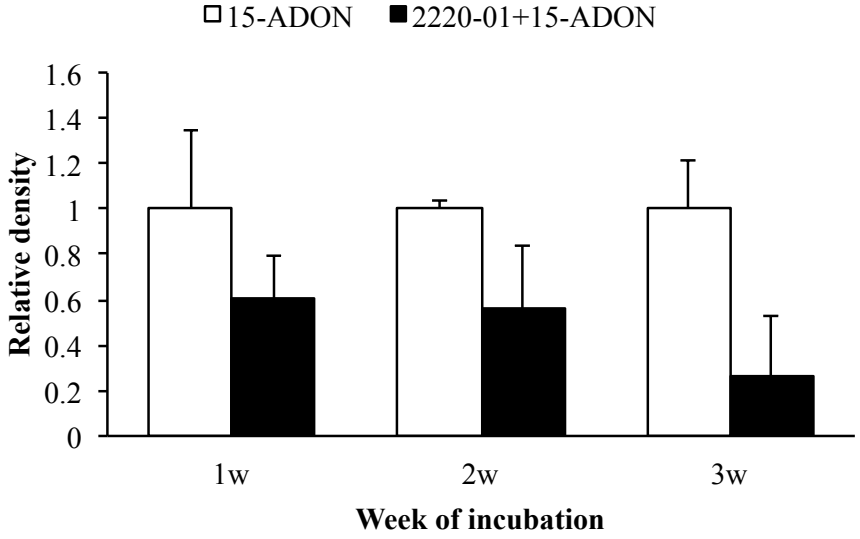
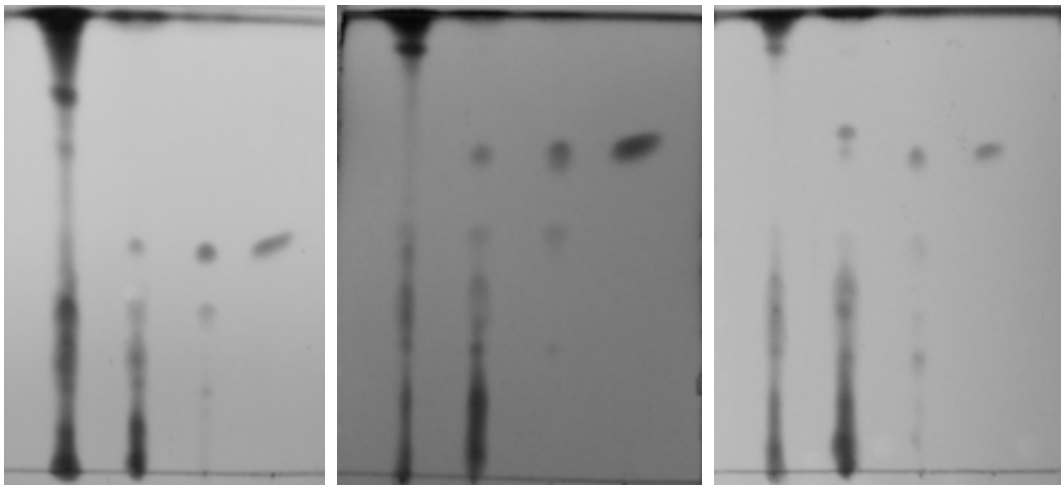


Figure 5.4 Relative density of residual 15-acetyl-deoxynivalenol at 1, 2, and 3 weeks of the culture extracts (SMCD 2220-01 with 15-ADON, ■). Contaminated but not inoculated culture (only 15-ADON, □) was used as a control. Data are means and standard deviations of three replicates.



(a) (b) (c)

Figure 5.5 Thin layer chromatograms of 1, 2, and 3 week-old cultures (a, b, and c) of SMCD 2220-01 supplemented with 2 ppm of 3-ADON as a final concentration. From the left side, the first, second, third, and fourth lanes indicate the cultures of only SMCD 2220-01, the cultures of SMCD 2220-01 with 3-ADON, the cultures of 3-ADON, and standard 3-ADON as a reference.

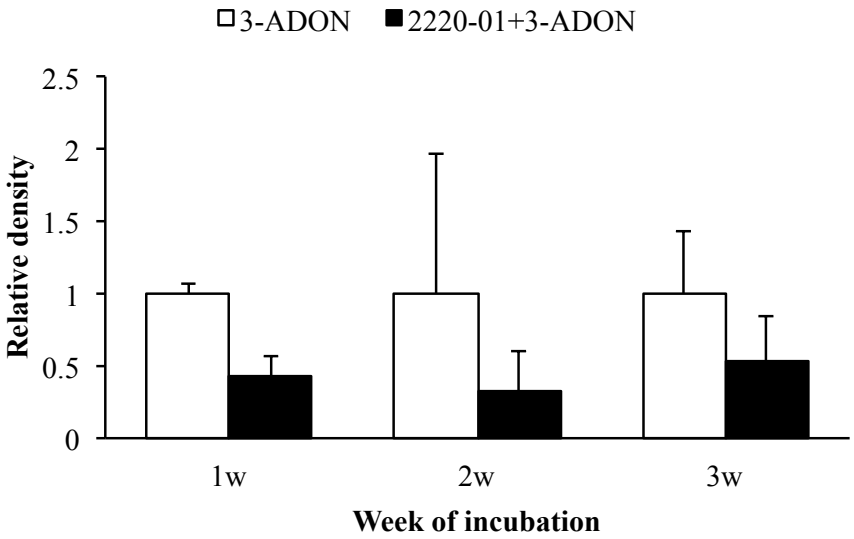
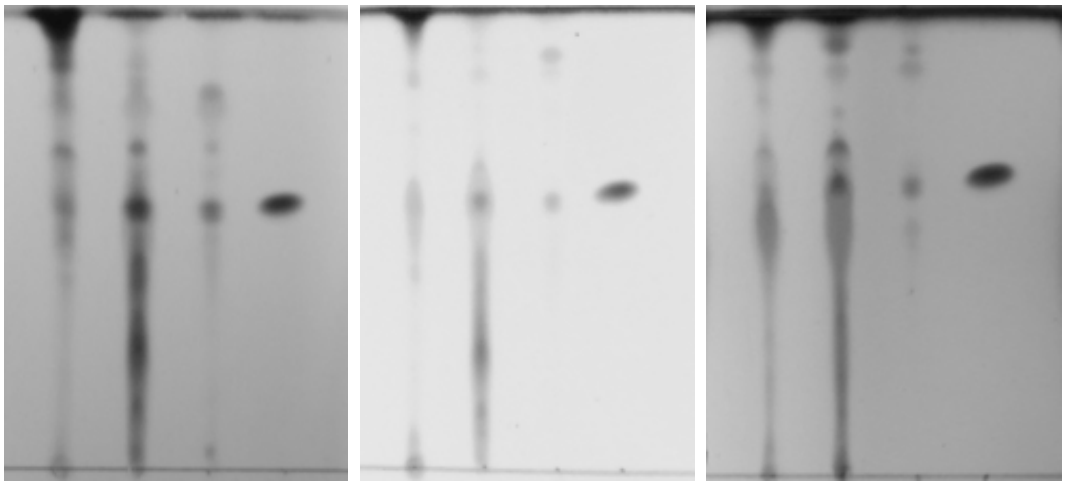


Figure 5.6 Relative density of residual 3-acetyl-deoxynivalenol at 1, 2, and 3 weeks of the culture extracts (SMCD 2220-01 with 3-ADON, ■). Contaminated but not inoculated culture (only 3-ADON, □) was used as a control. Data are means and standard deviations of three replicates.



(a) (b) (c)

Figure 5.7 Thin layer chromatograms of 1, 2, and 3 week-old cultures (a, b, and c) of SMCD 2220-01 supplemented with 2 ppm of DON as a final concentration. From the left side, the first, second, third, and fourth lanes indicate the cultures of only SMCD 2220-01, the cultures of SMCD 2220-01 with DON, the cultures of DON, and standard DON as a reference.

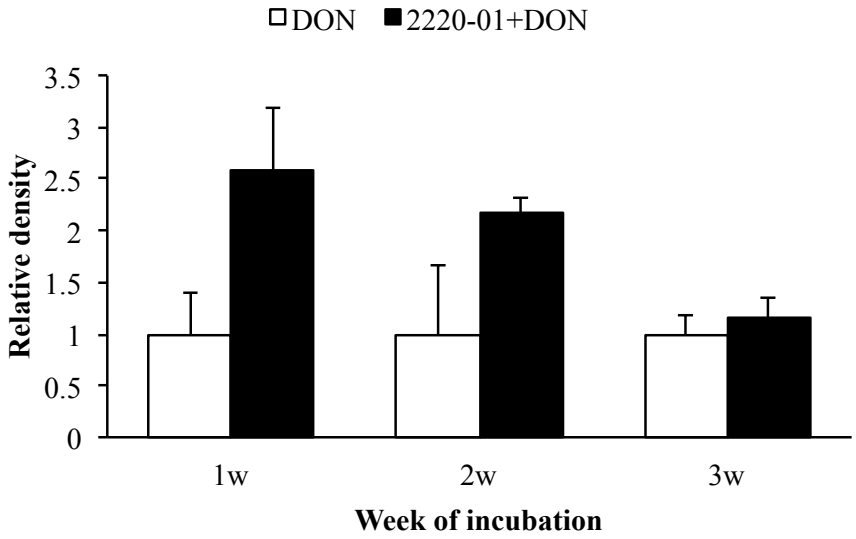


Figure 5.8 Relative density of residual deoxynivalenol at 1, 2, and 3 weeks of the culture extracts (SMCD 2220-01 with DON, ■). Contaminated but not inoculated culture (only DON, □) was used as a control. Data are means and standard deviations of three replicates.

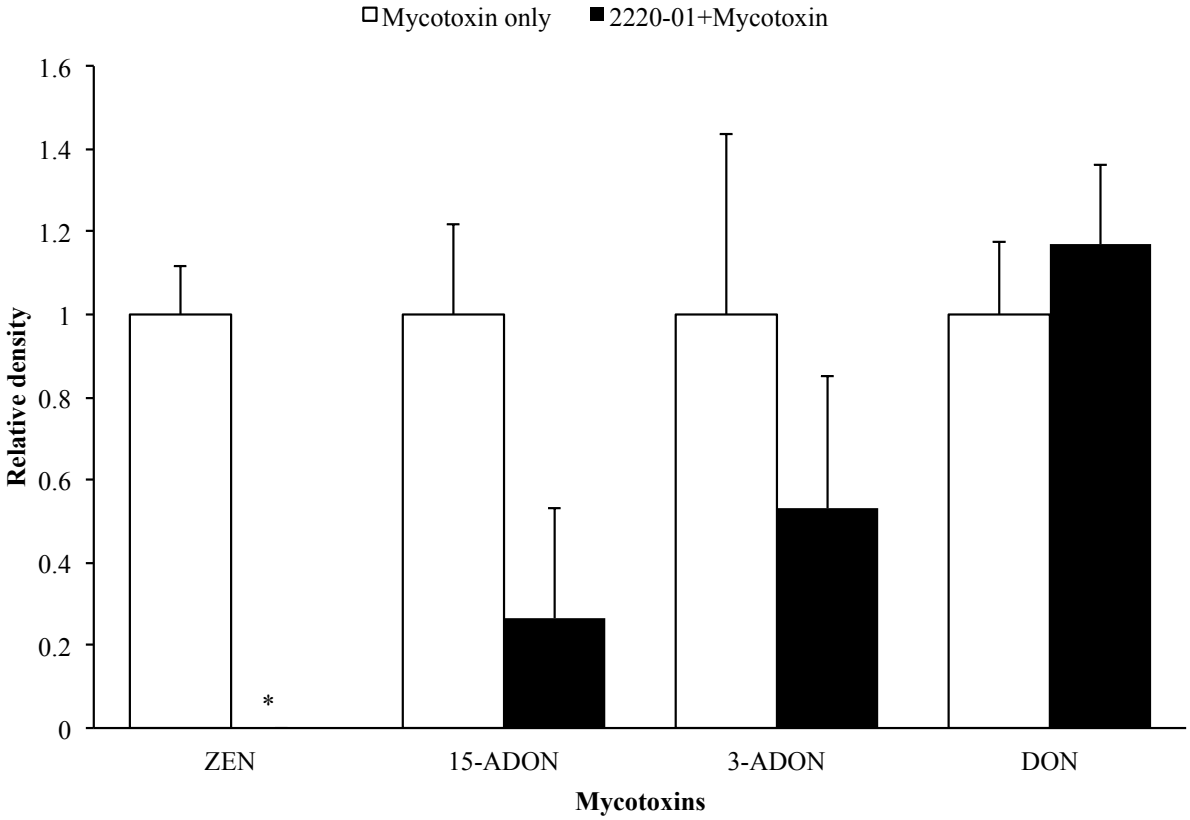


Figure 5.9 Comparison of the relative density of residual mycotoxins on 3 weeks incubation (SMCD 2220-01 with mycotoxins, ■). Contaminated but not inoculated culture (only mycotoxins, □) was used as a control. The asterisk indicates 0. Data are means and standard deviations of three replicates.

5.5.2 HPLC-HR-ESI-MS analysis

The quantification and the qualification of residual mycotoxins in culture broth of SMCD 2220-01 was confirmed by extracted ion chromatograms (XIC) derived from mycotoxins through HPLC-HR-ESI-MS analysis. It was shown that SMCD 2220-01 degrades 97%, 72%, 60%, and 89% of ZEN, 15-ADON, 3-ADON, and DON on 3 weeks incubation in PDB after the addition of the mycotoxins, respectively as shown in Figure 5.10. Mycotoxins degradation ability of SMCD 2220-01 indicated by HPLC–ESI analysis was consistent with the results of TLC analysis. In case of DON, XIC allowed us to calculate residual DON successfully. The determined concentration and analytic mode of the mycotoxins are shown in Appendix (Figure 9.5, 9.6, 9.7, 9.8, and 9.9).

The extract of zearalenone showed a signal of $[M - H]^-$ at *m*/*z* 317.1480 indicating ZEN, while the extract of SMCD 2220-01 with ZEN showed a signal of $[M - H + SO_3]^-$ at *m*/*z* 397.1052 in negative-ion mode (Figure 5.11). The empirical formula proposed for the compound was $(C_{18}H_{21}O_8S)^-$ corresponding to a calculated mass of 397.0962. The spectrum revealed the ion with an observed mass of 397.1052, which differs from the calculated empirical formula by only 9.0 millimass units (Plasencia & Mirocha, 1991). These difference of mass units may result from the electron-withdrawing effect of the sulfate group and by affected by dissolving solvent (Barron *et al.*, 1988). It is likely that two signals at *m*/*z* 195.05 and 117.02 in negative-ion mode are related with PDB compositions. The signals at *m*/*z* 137.02 and 165.05 seem to relate with metabolite of SMCD 2220-01.

The extract of deoxynivalenol and SMCD 2220-01 with DON showed the signal of $[M + HCOO]^-$ at *m*/*z* 341.1214 and 341.1358 in negative-ion mode (Figure 5.12), which indicated the presence of DON. It is likely that signals at *m*/*z* 151.02, 237.07, 273.17, and 345.23 are related with metabolite of DON by SMCD 2220-01 or metabolite of SMCD 2220-01 induced by DON. The signal at *m*/*z* 117.02 seems to relate with PDB compositions.

The extract of 3-acetyl-deoxynivalenol and SMCD 2220-01 with 3-ADON showed the signal of $[M + HCOO]^-$ at *m*/*z* 383.1345 in negative-ion mode (Figure 5.13), which indicated the presence of 3-ADON. It is likely that the signals at *m*/*z* 151.02, 273.17, and 345.23 are related with metabolite of 3-ADON by SMCD 2220-01 or metabolite of SMCD 2220-01 induced by 3-ADON. The signal at *m*/*z* 117.02 seems to relate with PDB compositions.

The extract of 15-acetyl-deoxynivalenol and SMCD 2220-01 with 15-ADON showed signals of $[M + H]^+$ at *m*/*z* 339.1603 and $[M + Na]^+$ at *m*/*z* 361.1453 in positive-ion mode (Figure 5.14), indicating the presence of 15-ADON. It is likely that signals at *m*/*z* 225.20, 226.20, and 321.15 are related with 15-ADON, which might be fragments of 15-ADON. The very weak signal at *m*/*z* 190.05 seems to relate with metabolite of 15-ADON by SMCD 2220-01. The signals at *m*/*z* 151.07, 204.07, and 214.92 seems to relate with metabolite of SMCD 2220-01.

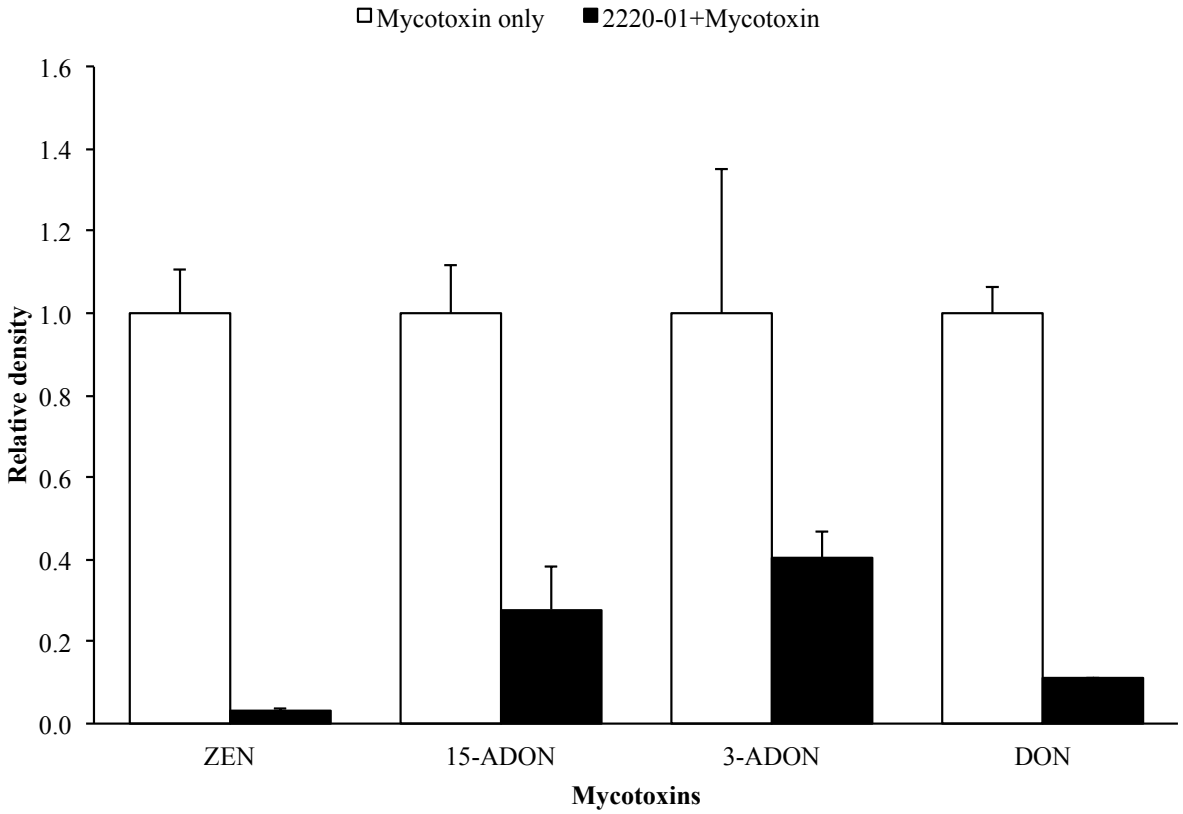
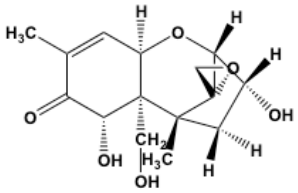
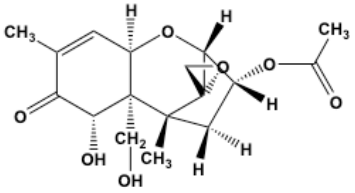
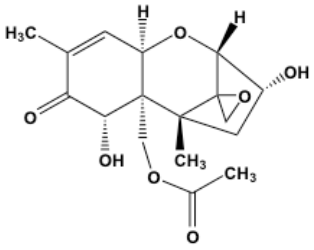
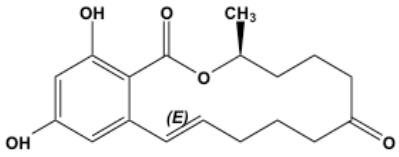


Figure 5.10 Comparison of the relative density of residual mycotoxins (SMCD 2220-01 with mycotoxins, ■) on 3 weeks incubation based on XIC. Contaminated but not inoculated culture (only mycotoxins, □) was used as a control. Data are means and standard deviations of three replicates.

Table 5.1 Chemical structures, molecular formula, and weight of tested mycotoxins for this study.

Mycotoxins	Structure	Molecular formula	Molecular weight (Exact Mass)
Deoxynivalenol		$C_{15}H_{20}O_6$	296.3157 (296.1260)
3-Acetyl-deoxynivalenol		$C_{17}H_{22}O_7$	338.3524 (338.1366)
15-Acetyl-deoxynivalenol		$C_{17}H_{22}O_7$	338.3524 (338.1366)
Zearalenone		$C_{18}H_{22}O_5$	318.3643 (318.1467)

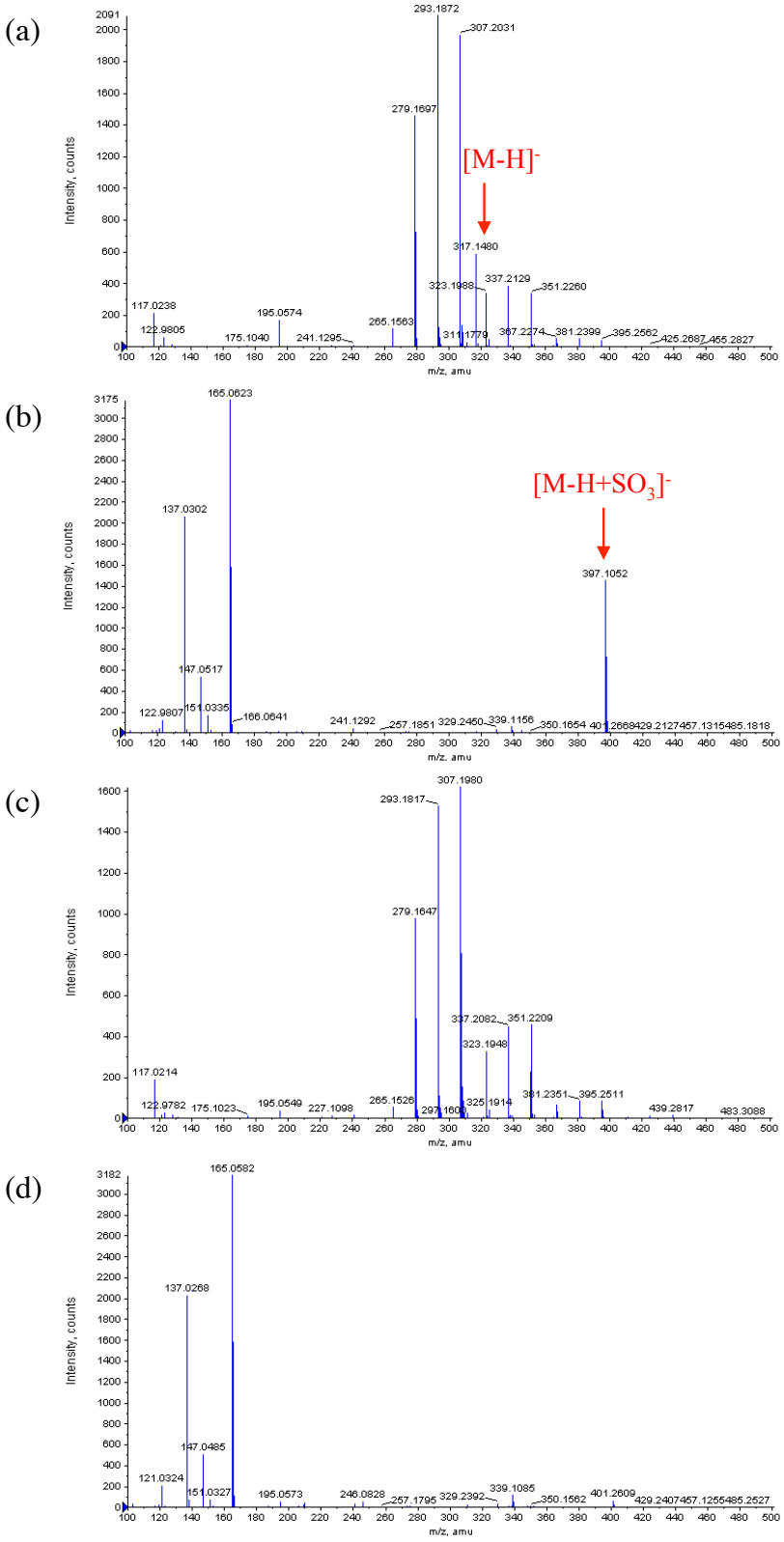


Figure 5.11 Representative mass spectra of extracts analyzed by ESI-TOF-MS in negative-ion mode for ZEN. Zearalenone (a), SMCD 2220-01 with zearalenone (b), potato dextrose medium (c), and SMCD 2220-01 (d).

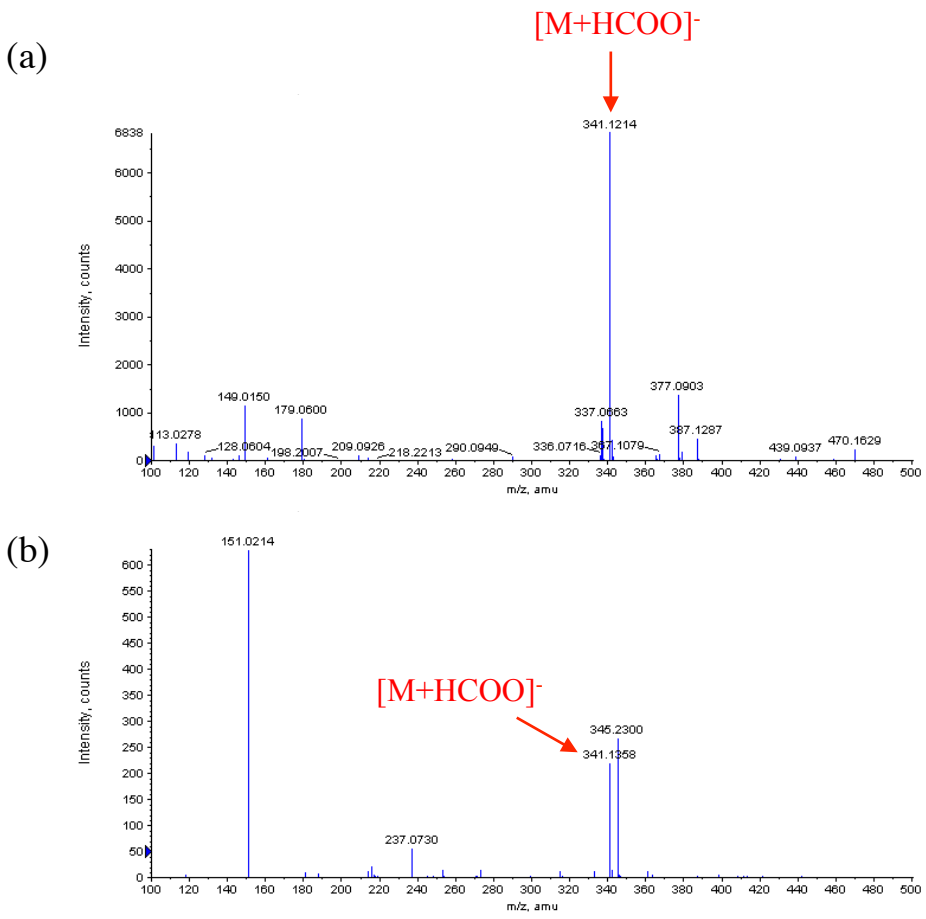


Figure 5.12 Representative mass spectra of extracts analyzed by ESI-TOF-MS in negative-ion mode for DON. Deoxynivalenol (a) and SMCD 2220-01 with deoxynivalenol (b).

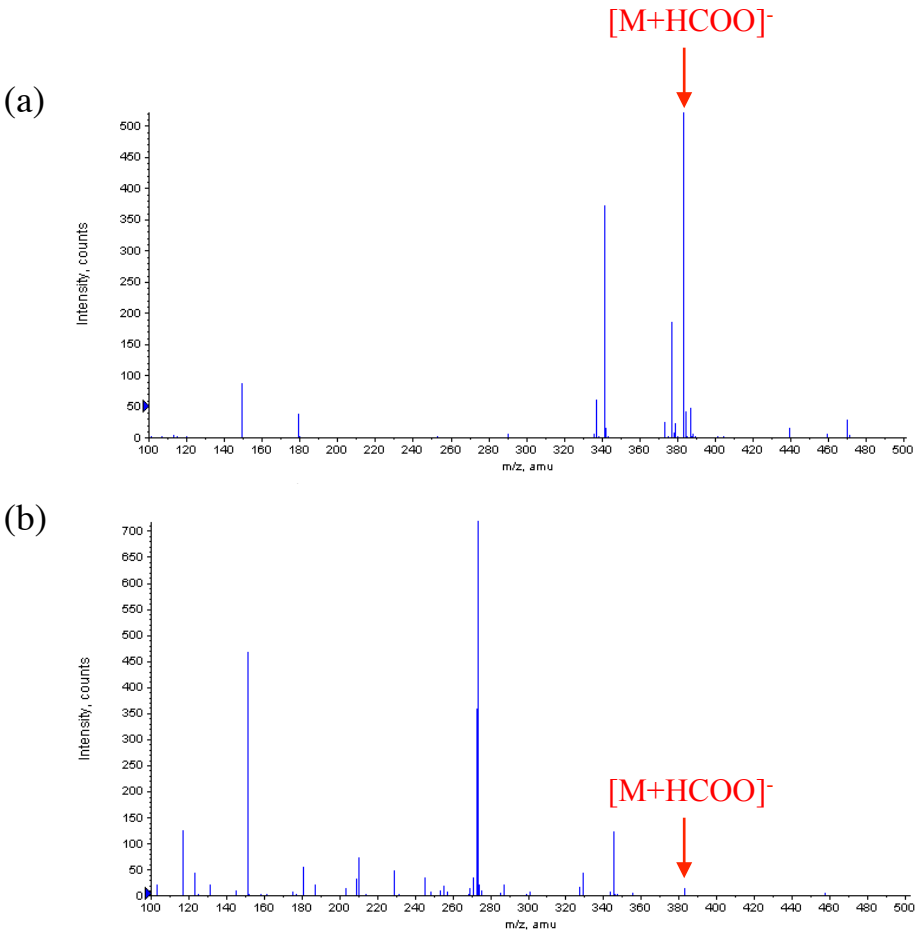


Figure 5.13 Representative mass spectra of extracts analyzed by ESI-TOF-MS in negative-ion mode for 3-ADON. 3-Acetyl-deoxynivalenol (a) and SMCD 2220-01 with 3-acetyl-deoxynivalenol (b).

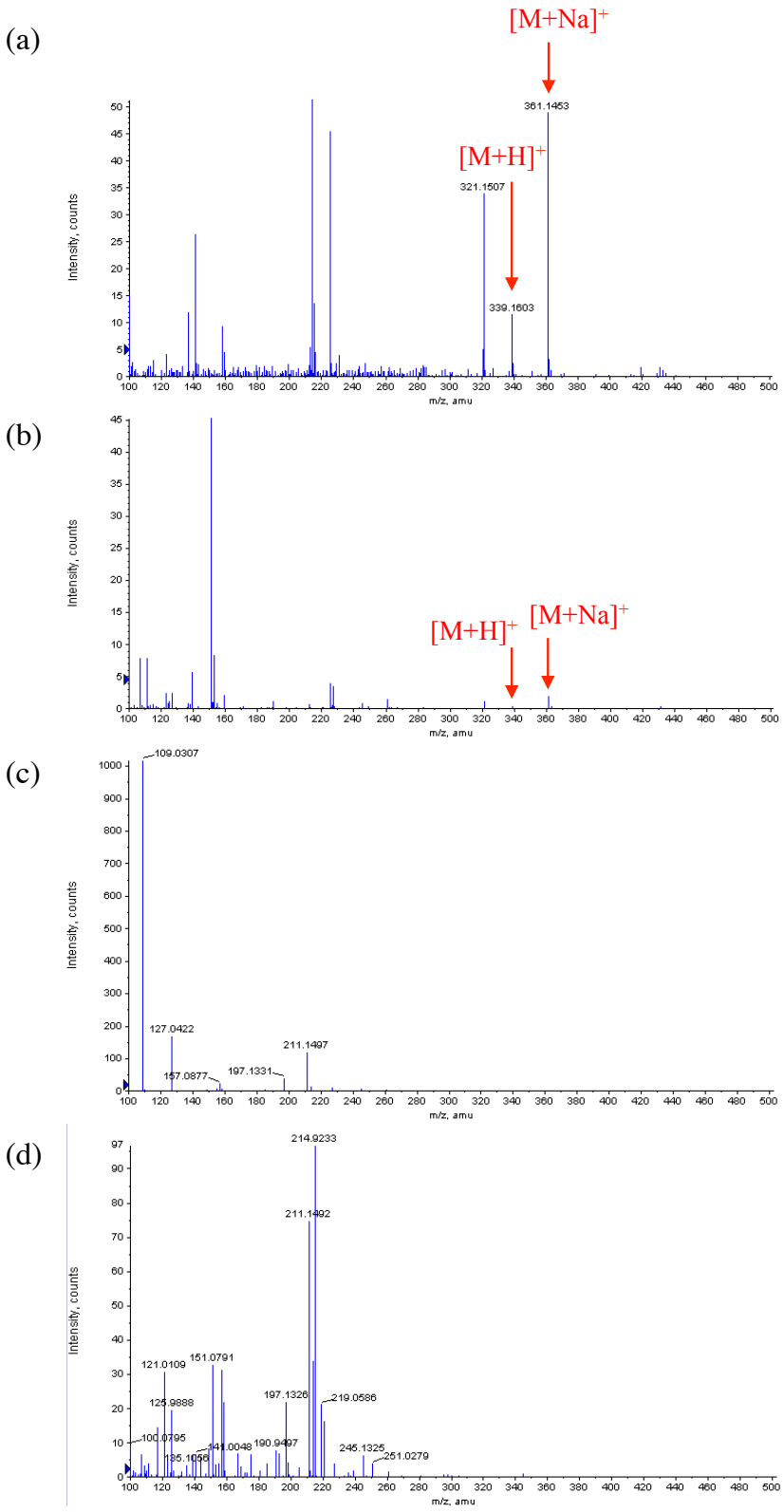


Figure 5.14 Representative mass spectra of extracts analyzed by ESI-TOF-MS in positive-ion mode for 15-ADON. 15-Acetyl-deoxynivalenol (a), SMCD 2220-01 with 15-acetyl-deoxynivalenol (b), potato dextrose medium (c), and SMCD 2220-01 (d).

5.6 Discussion

Mycotoxin-degrading microbes have been isolated mainly from agricultural environments. Microbial detoxification or biotransformation of mycotoxins includes different types of reaction, such as acetylation, glucosylation, ring cleavage, hydrolysis, deamination, and decarboxylation (McCormick, 2013). Biotransformation of zearalenone has been reported by following several fungi producing different metabolites: *Rhizopus* spp. producing *α*–zearalenol and *β*-zearalenol (Brodehl *et al.*, 2014); *Aspergillus ochraceous* and *Aspergillus niger* producing *α*–zearalanol and *β*-zearalanol (El-Sharkawy & Abul-Hajj, 1988b); *Cunninghamella bainieri* producing 2, 4-dimethoxyzearalenone and 2-methoxyzearalenone (El-Sharkawy & Abul-Hajj, 1988b); *Rhizopus arrhizus* producing zearalenone 4-sulfate (El-Sharkaway *et al.*, 1991); *Thamidium elegans* and *Mucor bainieri* producing zearalenone-4-*β*-D-glucoside (El-Sharkawy & Abul-Hajj, 1987). Interestingly, a mycoparasite *Clonostachys rosea* showed the ability to detoxify zearalenone to a ring cleavage product, 1-(3,5-dihydroxy-phenyl)-10'-hydroxy-1'*E*-undecene-6'-one (Kakeya *et al.*, 2002). Moreover, it was shown that the detoxification of zearalenone by zearalenone hydrolase of *Clonostachys rosea* is crucial for the successful mycoparasitism of *Clonostachys rosea* against *F. graminearum* (Kosawang *et al.*, 2014). In this study, as a first report, SMCD 2220-01 showed clearly the decrease in the residual zearalenone by 97%, and zearalenone sulfate was detected as a metabolite of zearalenone by SMCD 2220-01. The biodegradability of this mycoparasite might be closely related to its mycoparasitic ability. Additionally, SMCD 2220-01 indicated the decrease in residual 15-ADON, 3-ADON, and DON by 72%, 60%, and 89%. It has been known that trichothecene C-3 acetyltransferase plays an important role in microbial biotransformation of trichothecenes such as deoxynivalenol (Khatibi *et al.*, 2010; Kimura *et al.*, 1998). Complete mineralization of trichothecenes has not been reported so far. *Aspergillus tubingensis* isolated from soil showed 94.4% biotransformation rate after two weeks of cultivation supplemented with DON; biotransformants of DON was not identified (He *et al.*, 2008).

5.7 Conclusions

SMCD 2220-01 showed the ability to degrade mycotoxins such as ZEN by 97%, 15-ADON by 72%, 3-ADON by 60%, and DON by 89% through TLC and HPLC-HR-ESI-MS. The transformant of ZEN by SMCD 2220-01 was identified as zearalenone sulfate as a result of

HPLC-HR-ESI-MS. These findings clearly indicate that the mycoparasite not only parasizes on the host, but also degrades *Fusarium* mycotoxins.

6. GENERAL DISCUSSION

Biological control has been considered as a promising alternative to synthetic chemical pesticides. Biological control agents, such as *Trichoderma* spp. and *Clonostachys rosea* as a generalists have been studied well; it is likely that the BCA specialists remain behind compared with the BCA generalists. The BCA specialits can be more useful if they are more understood. *Sphaerodes mycoparasitica* Vujan. SMCD 2220-01 as a promising candidate of the BCA specialist was isolated from wheat and asparagus field associated with *Fusarium avenaceum* and *F. oxysporum*. Previous research showed the mycoparasitism of the mycoparasite with several *Fusarium* strains and the potential of the mycoparasite to degrade *Fusarium* mycotoxins. However, the interrelationship between the mycoparasite and the host *Fusarium* spp. were not clear. Thus, the overall aim of this thesis was to better understand mycoparasite interactions with plant pathogenic *Fusarium* spp. and their mycotoxins.

In chapter 3, the results indicated that the mycoparasite possesses the broad host compatibility with twelve *Fusarium* strains and the different level of host compatibility. The broad host compatibility, confering polyphagous lifestyle of this mycoparasite is an unique characteristic of this mycoparasite and has not observed on another species *Sphaerodes* mycoparasite such as *Sphaerodes retispora* var. *retispora* which is known as monophagous mycoparasite on *Fusarium oxysporum* (Harveson *et al.*, 2002). Furthermore, SMCD 2220-01 showed the adaptability to particular *Fusarium* filtrates (SMCD 2242, 2243, 2134, and 2423). These results indicate that specificity of this mycoparasite can be based on not only a genetic basis, but also a phenotypic plasticity, which was described by Little et al. (2006). These findings also imply that this mycoparasite are closely related with secondary metabolites produced by *Fusarium* strains. Moreover, adaptation procedure may increase the host compatibility of the mycoparasite, which has a great potential in agriculturally applications to provide a benefit for managing this mycoparasite to control plant pathogen *Fusarium*, even at species level.

To better understand the mycoparasitism of this mycoparasite, in chapter 4, the mycoparasitism with fungal surface hydrophobicity were investigated based on the fact that fungal-fungal interaction including mycoparasitism occurs the fungal interfaces by communicating and/ or contacting fungal surfaces or cell walls. The results demonstrated that the mycoparasite has diphasic interactions (biotrophic-attraction and antagonistic-inhibition) with different *Fusarium* hosts; this phenomenon may occur because of the different types of host cell wall and secondary metabolites produced by *Fusarium* strains (Ojha & Chatterjee, 2011). Furthermore, it was shown that the mycoparasite changes the host fungal surface hydrophobicity during the mycoparasitism with diphasic lifestyles. There is a possibility that the mycoparasite produces and/or accumulate particular substances (e.g. VOCs) on the contact zone and/or on the host cell wall after intra-penetration as well as secretes lytic enzymes for decomposition process; VOCs are known to modify the morphology of *Fusarium* strain (Vergara-Fernández *et al.*, 2011). A lysis is a key step found in mycoparasitism of *Trichoderma* spp. (Gajera & Vakharia, 2012).

Fusarium spp. are not only plant pathogenic, but also mycotoxigenic fungi. Thus, in chapter 5, *Fusarium* mycotoxin-degrading ability of the mycoparasite was examined. SMCD 2220-01 showed clearly the decrease in the residual zearalenone by 97%, and zearalenone sulfate was detected as a transformant of zearalenone by SMCD 2220-01. Biotransformation of ZEN has been reported by several fungi producing different metabolites. Specially, SMCD 2220-01 showed the same metabolite as produced by *Rhizopus arrhizus* (El-Sharkaway *et al.*, 1991). Interestingly, it seems that mycotoxin-degrability and mycoparasitic ability are closely related. For example, the detoxification of zearalenone by zearalenone hydrolase of *Clonostachys rosea* plays a key role in the successful mycoparasitism of *Clonostachys rosea* against *F. graminearum* reported by Kosawang et al. (Kosawang *et al.*, 2014). The overall results in this thesis explained partly physiological, ecological, and biological aspects of *Sphaerodes mycoparasitica*'s specific lifestyles including diphasic mycoparasitisms and biodegradability.

7. GENERAL CONCLUSIONS

Mycotoxin contamination of grains by plant pathogenic and mycotoxigenic *Fusaria* is a chronic threat to crop, human, and animal health. Global food security research is seeking environmentally friendly solutions in applying BCA products to prevent economic losses due to reduced quantity and quality of crops. This reality is even more aggravated by fluctuating environmental conditions under global climate change (Jurado *et al.*, 2006). Each infected crop or harvested grain lot may contain a different spectrum of *Fusarium* spp., while the amount of associated mycotoxins varies greatly. This complication implies difficulties with certain BCAs, particularly the group of non-specific mycoparasitic generalists that typically have a necrotrophic lifestyle, to reduce the risk of multiple grain contamination by the *Fusarium* species complex. The counter attack of mycotoxigenic *Fusarium* and its mycotoxins can repress the chitinase gene expression involved in biocontrol and reduce BCA efficacy (Harveson & Kimbrough, 2001). Interestingly, these necrotrophic BCAs are also known producers of phytotoxic molecules, in addition to triggering higher levels of DON produced by *Fusarium*'s active defense in environmental samples. In contrast, host-specific mycoparasites with typical biotrophic lifestyles that can also be polyphagous, such as *S. mycoparasitica,* control more than one *Fusarium* species (e.g., *F. graminearum*, *F. culmorum, F. avenaceum*, and *F. equiseti*) (Rodríguez *et al.*, 2011) and multiple mycotoxins (e.g., aurofusarin, DON, 3-ADON, 15-ADON, and ZEN) (Vujanovic & Goh, 2011b) in a single grain lot. This group of mycoparasites seems well suited for use in an optimized BCA product to reduce *Fusarium*-associated risks under a changing environment and to protect human and animal health, as well as the global economy.

In this thesis, *Sphaerodes mycoparasitica* as a promising BCA candidate was tested to demonstrate the mycoparasite's efficacy in the future application through investigation of the mycoparasite interaction with plant pathogenic *Fusarium* spp. and their mycotoxins. Major findings are as follows: (1) the mycoparasite possesses the broad and different level of host compatibility with twelve *Fusarium* strains, as well as adaptability; and (2) the mycoparasite

parasitizes different hosts using diphasic interactions such as biotrophic-attraction and antagonistic-inhibition; and (3) the mycoparasite has *Fusarium* mycotoxin-degrading ability. The findings of this thesis successfully showed the promising possibility of this mycoparasite to be developed as a specific BCA to control *Fusarium* pathogens and mycotoxins.

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9. APPENDIX

9.1 The standard curve of the tested mycotoxins analyzed by TLC

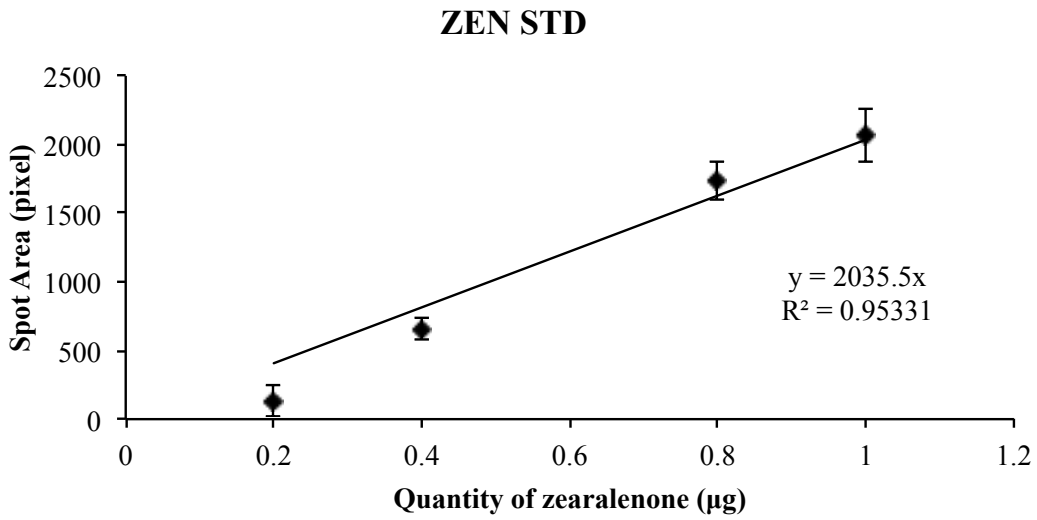

Figure 9.1 The standard curve of ZEN obtained with TLC analysis.

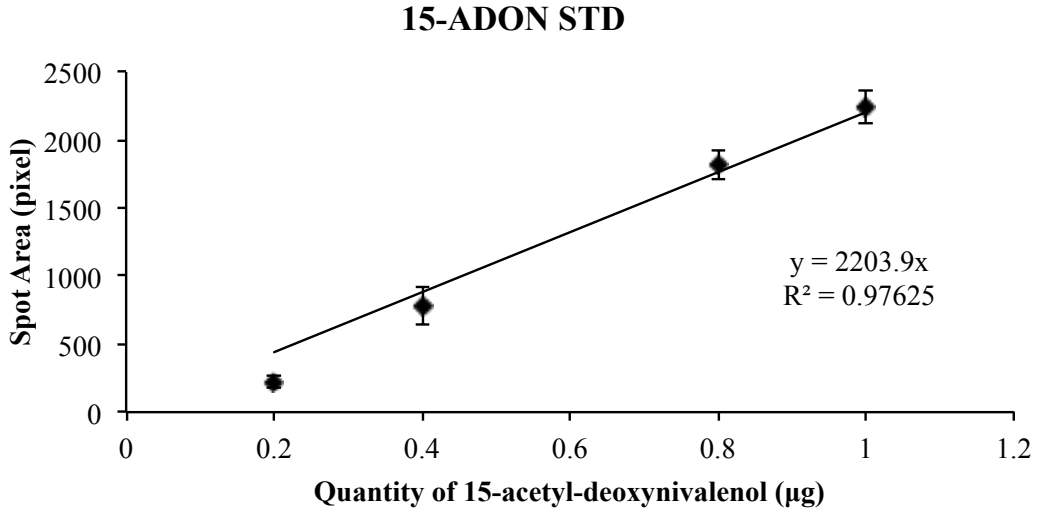

Figure 9.2 The standard curve of 15-ADON obtained with TLC analysis.

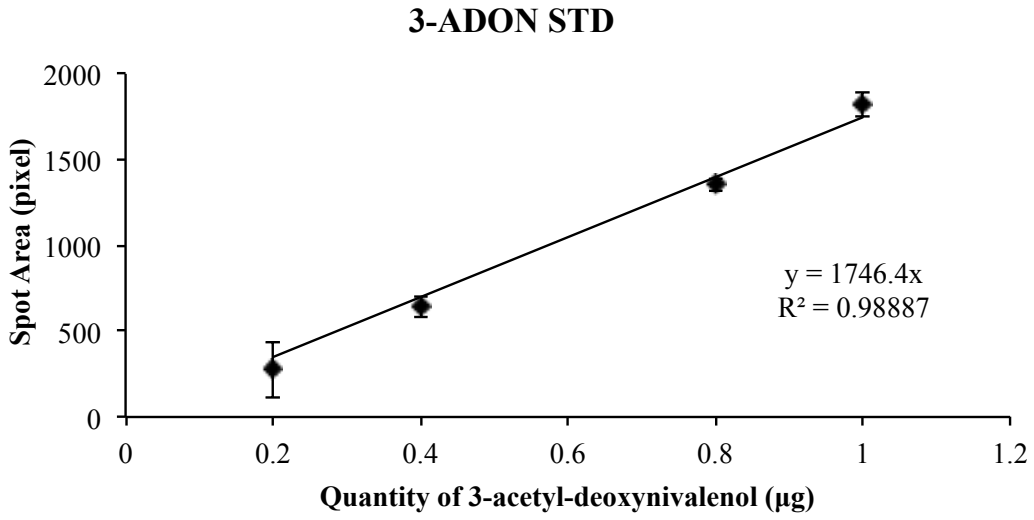

Figure 9.3 The standard curve of 3-ADON obtained with TLC analysis.

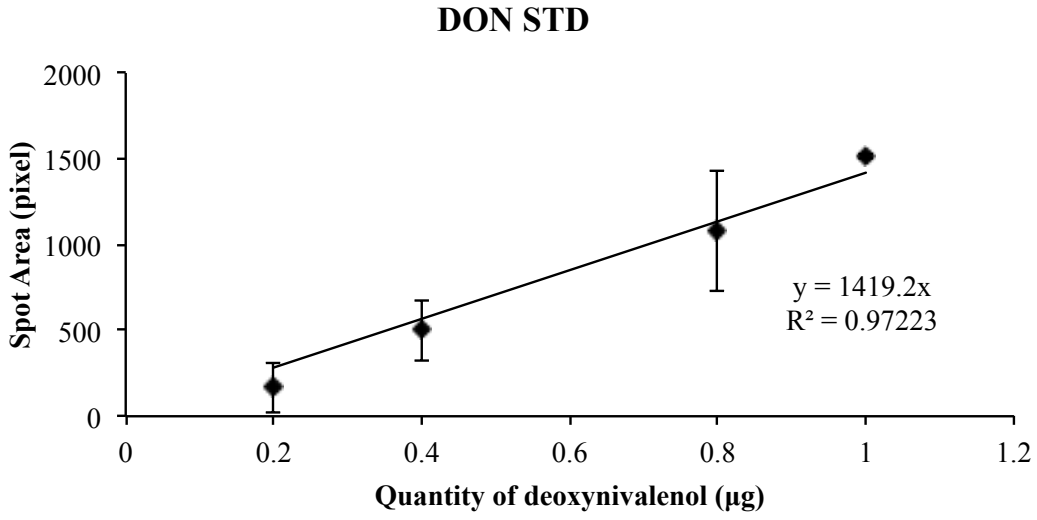

Figure 9.4 The standard curve of DON obtained with TLC analysis.

9.2 The standard curve of the tested mycotoxins analyzed by HPLC-HR-ESI-MS

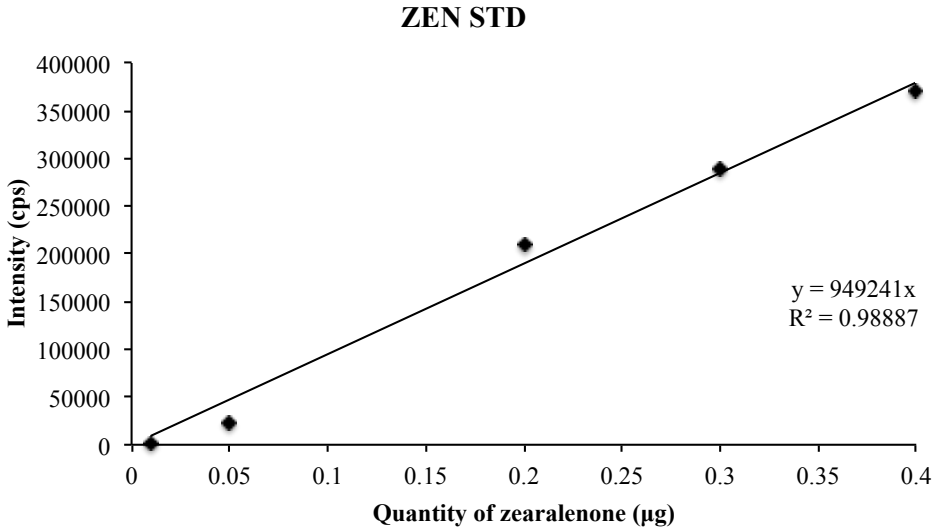

Figure 9.5 The standard curve of ZENobtained with XIC of HPLC-HR-ESI-MS analysis. The used quantities were 0.01 µg, 0.05 µg, 0.2 µg, 0.3 µg, and 0.4 µg.

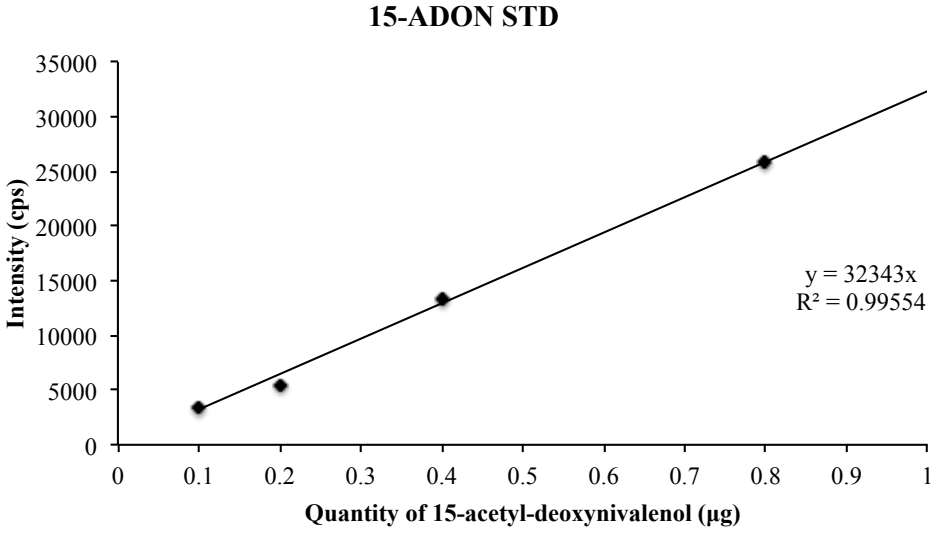

Figure 9.6 The standard curve of 15-ADON obtained with XIC of HPLC-HR-ESI-MS analysis. The used quantities were 0.1 µg, 0.2 µg, 0.4 µg, and 0.8 µg.

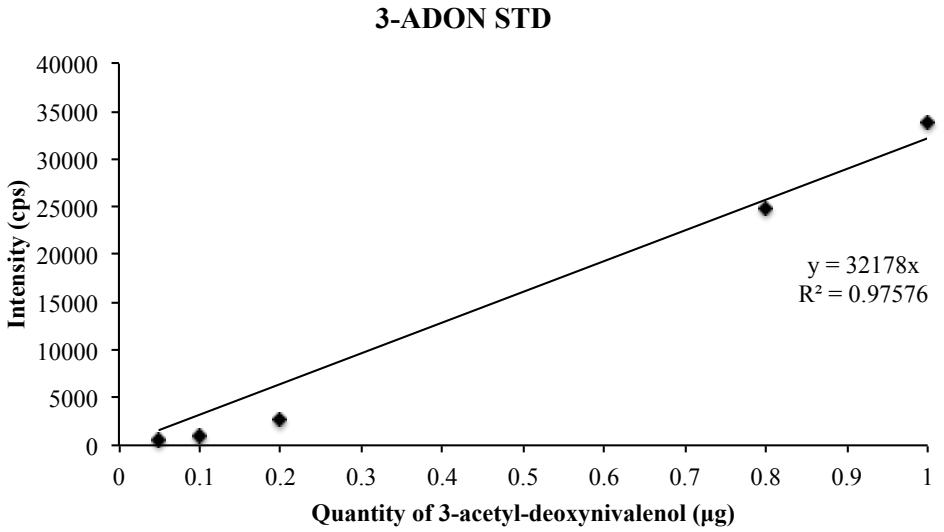

Figure 9.7 The standard curve of 3-ADON obtained with XIC of HPLC-HR-ESI-MS analysis. The used quantities were 0.05 µg, 0.1 µg, 0.2 µg, 0.8 µg, and 1.0 µg.

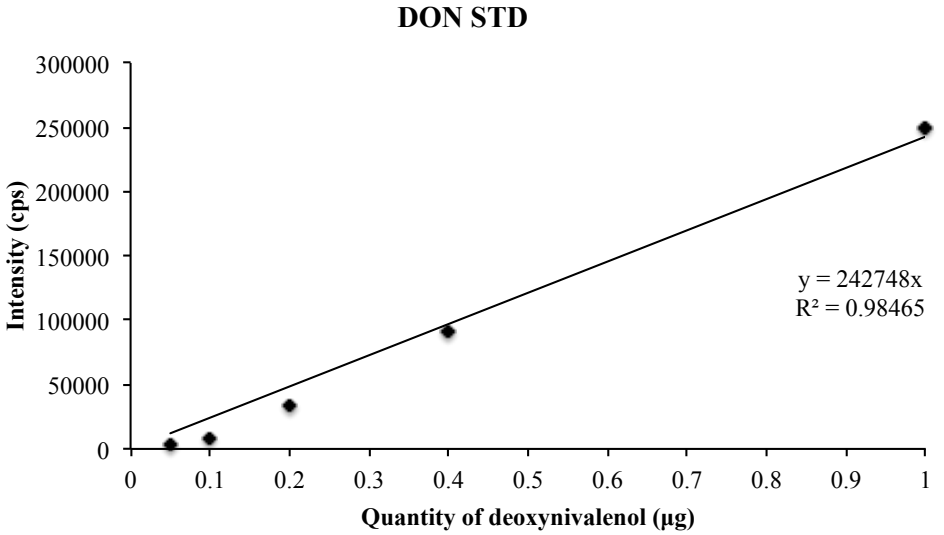

Figure 9.8 The standard curve of DON obtained with XIC of HPLC-HR-ESI-MS analysis. The used quantities were 0.05 µg, 0.1 µg, 0.2 µg, 0.4 µg, and 1.0 µg.

9.3 Representative mass spectra of the tested mycotoxins analyzed by ESI-TOF-MS

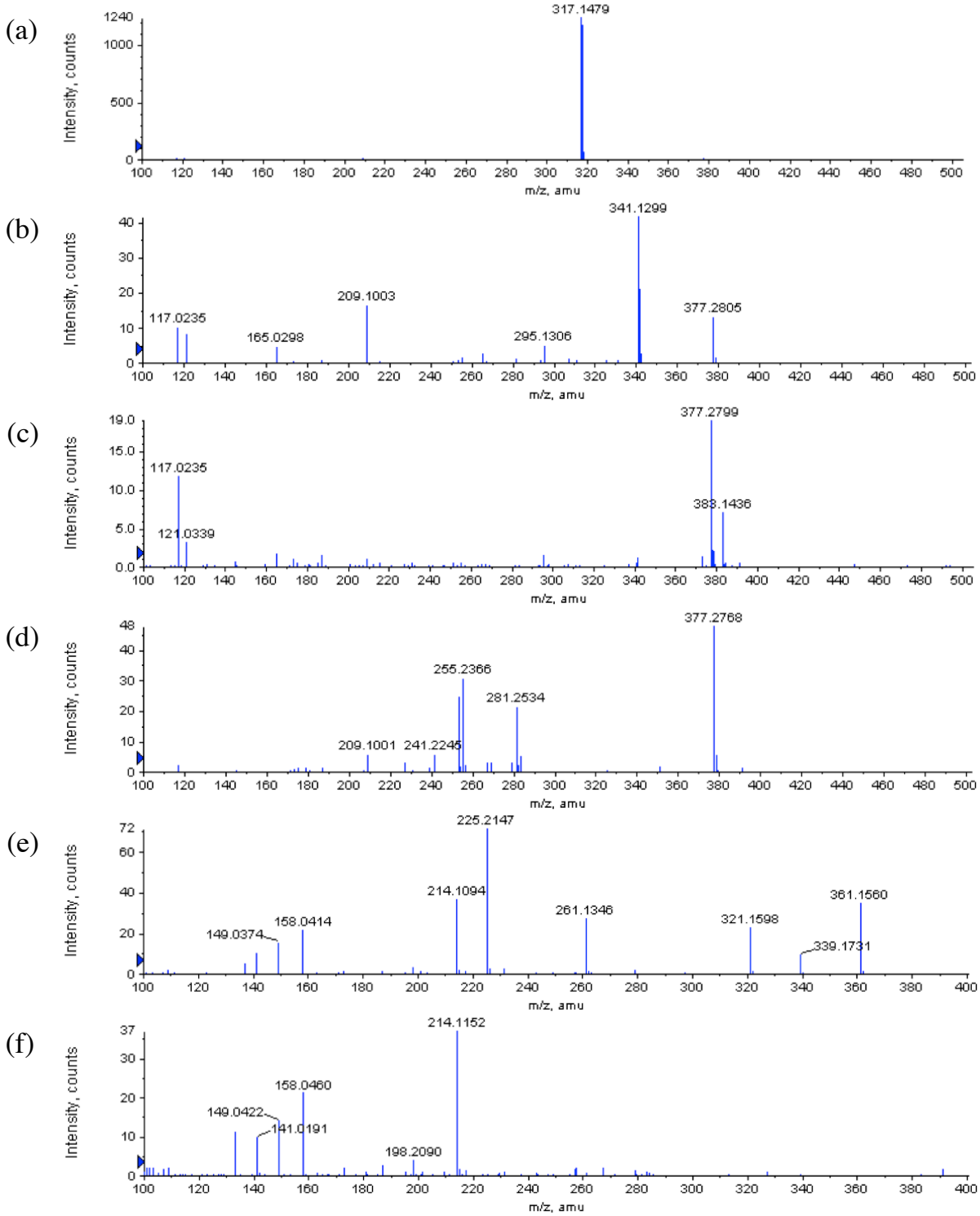

Figure 9.9 Representative mass spectra of standard mycotoxins analyzed by ESI-TOF-MS in negative-ion mode and positive-ion mode. One ppm of zearalenone (a), deoxynivalenol (b), 3-acetyl-deoxynivalenol (c), and MeOH (d) in negative-ion mode, and 1 ppm of 15-acetyl-deoxynivalenol (e) and MeOH (f) in positive-ion mode.