

Screening and Evaluation of Potential Fungal
Antagonists for the Biological Control of Fusarium
Head Blight Incited by *Gibberella zeae*

By

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General Abstract

Fusarium head blight (FHB) is an important disease of wheat and other small grain cereals. The principal pathogen associated with FHB in Manitoba is *Gibberella zae* (Schwein.) Petch (anamorph = *Fusarium graminearum* Schwabe). Currently there are no biological control agents registered for the control of FHB. The overall objectives of the project were to identify isolates that may be used in the biological control of FHB and to investigate the interaction between the identified biocontrol agents and *G. zae*. In this study, 150 bacteria and 29 fungi were isolated from soil, wheat heads and crop debris from southern Manitoba. An additional 10 isolates of *Trichoderma harzianum* were obtained from the Canadian Collection of Fungal Cultures, CCFC (Ottawa, Ontario). The *T. harzianum* isolate, T-22 (RootShield) was included as a positive control. All were screened for inhibition of *Gibberella zae* using confrontation plate assays *in vitro* and seed, wheat head, and straw assays *in planta*. Only 6% of bacterial and 45% of fungal isolates tested in the confrontation plate assays. Of the 6% of the bacterial isolates none significantly reduced FHB disease on wheat heads or reduced perithecial production on wheat straw, and had a negative effect on seed germination, therefore were not further evaluated. *Chrysosporium* sp. and *Penicillium* spp. and *Trichoderma harzianum*, were the fungal species that inhibited the growth of *G. zae* by more than 50%. Of which *Trichoderma* isolates were the most effective and were able to over-grow *G. zae*. Fourteen of the 18 isolates tested, including six *Trichoderma* and two *Chrysosporium* isolates, significantly reduced perithecial production on wheat straw by 52-89% compared to the control. From this study, *Trichoderma harzianum* was identified as most potentially effective candidate for the biocontrol of *Gibberella zae*. Spore suspensions

and cell-free filtrates of *Trichoderma harzianum* isolates were evaluated for their effectiveness in reducing perithecial and ascospore production of *Gibberella zeae* on wheat straw. Five *T. harzianum* isolates, including T-22 (RootShield™), reduced perithecial formation by 70% or more. Perithecial reduction was highest (96-99%) when *T. harzianum* spore suspension or cell-free filtrate was applied to straw 24 hours prior to inoculation with *G. zeae*. Control was less effective when *T. harzianum* was applied at the same time (co-inoculated) or 24 hours after *G. zeae*. Field trials showed significant reduction of perithecia on residues treated with *T. harzianum* prior to placement on the soil surface. Identification of those compounds in the cell-free filtrate most likely to affect biocontrol was accomplished through the use of cluster analysis, ordination and regression methods. It was found that isolates that produce similar levels of biocontrol had similar chemical composition. Ultrastructural changes were observed primarily in the exterior cells of the outer cell wall. Cytoplasmic degradation, invagination of the plasma cell membrane and thin cell walls were observed in the treated samples. Immature perithecia were overgrown by *T. harzianum* 15 days after co-inoculation. Few perithecia were overgrown at later stages. The perithecia affected by *T. harzianum* collapsed 21 days after inoculation (dai), compared to the perithecia in the untreated samples which collapsed 28 dai.

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1.0 General introduction

Fusarium head blight (FHB) is an important disease of wheat (*Triticum aestivum* L.) and other small grain cereals such as barley (*Hordeum vulgare* L.), rice (*Oryza sativa* L.), and oats (*Avena sativa* L.). Several *Fusarium* species cause FHB, including *F. graminearum* Schwabe, *F. culmorum* (W.G. Smith), *F. avenaceum* (Corda; Fr.) Sacc., *F. poae* (Peck) Wollenw., *F. acuminatum* (Ellis & Everhart), *F. equiseti* (Corda) Sacc., and *F. sporotrichioides* (Sherb.) (Clear and Patrick 2000). The principal pathogen associated with FHB in Manitoba is *Gibberella zeae* (Schwein.) Petch (anamorph = *Fusarium graminearum* Schwabe), accounting for over 97% of the *Fusarium* species isolated (Gilbert et al. 2009). The disease results in yield and quality losses due to the presence of lighter-weight fusarium damaged kernels (FDK) and accumulation of the mycotoxin, deoxynivalenol (DON) which renders the grain unfit for consumption. Milling, baking, and pasta qualities are also altered (Charmley et al. 1994). In 1993, during Manitoba's most severe outbreak on record, losses were estimated at \$75 million dollars and annual losses have since been estimated at approximately \$50 million (Gilbert et al. 1994; Gilbert and Tekauz 2000).

Several practices have been suggested to control FHB. These include crop rotation of wheat and other susceptible small grain cereals with non-host crops such as canola or alfalfa (Dickson 1956; Sutton 1982; Parry et al. 1995), and plowing under of crop debris to help eliminate inoculum by accelerating decomposition and blocking perithecia and ascospore production (Teich and Hamilton 1985). Breeding FHB resistant wheat varieties offers a cost-effective solution for the control and management of the disease. However, at this time no registered resistant cultivars are available. Currently in

western Canada, only two moderately resistant Canada western red spring (CWRS) wheat varieties are available, 5602HR and Waskada. The CWRS wheat line, BW 365 also has partial resistance and may be registered in the near future (Seed Manitoba 2008).

Chemical control, using fungicides such as benomyl, tebuconazole, fludioxonil, and mancozeb, does result in higher yields, and lower DON concentration, lower disease incidence and severity (Jones 2000). The main factors that limit their success and use are potential chemical residues, the need for multiple applications, and high costs for these compounds. In addition, results have not always been consistent from year to year.

Biological control may be an effective method to reduce disease level when used in conjunction with other control methods; it also may prevent the possible emergence of fungicide-resistant strains that develop with over-use of chemicals. Several bacterial and fungal isolates have been shown to have antagonistic activity towards *G. zeae*. These include *Bacillus* spp. (Stockwell et al. 2001), *Cryptococcus nodaensis* (Khan et al. 2001), *Lysobacter enzymogenes* (Jochum et al. 2006), *Trichoderma harzianum* (Fernandez 1992), *Microsphaeropsis* sp. (Bujold et al. 2001), *Paenibacillus macerans* (Schisler et al. 2002), *Pseudomonas* sp. (Schisler et al. 2006), *Sporobolomyces roseus* (Milus 2001), and *Streptomyces* sp. (Fulgueira et al. 1996). The main mechanisms for control include competition, antibiosis and mycoparasitism. Several challenges must be overcome when developing potential biocontrol agents, including hostile environmental conditions on the host plant such as lack of nutrients and water, high UV radiation, high temperatures, and competition with other organisms. It is important to identify biological control organisms that are adapted to a wide range of environmental conditions in order to make the strains an economically viable option. Some problems associated with the development and

registration of BCAs include lengthy procedures by regulatory agencies to obtain permits, and the high costs of obtaining patents and field evaluations (Utkhede 1996).

Furthermore, fermentation and formulation must be cost-effective. Costs for development of new a BCA can exceed 8 million dollars (Harman 2009, Pers. Comm.). Therefore if investment companies are to see a profit, the product must be adapted for large-scale use.

The main source of inoculum for FHB is infected plant material left on the soil surface where *Gibberella zeae* overwinters as saprophytic mycelium. In the spring, warm, moist weather gives rise to perithecia and ascospores (Sutton 1982; Paulitz 1996; Inch et al. 2001). Maturation of perithecia usually corresponds to flowering/anthesis of spring wheat cultivars. The wind-borne ascospores are forcibly discharged and may potentially land on the host plant. These then germinate and colonize individual wheat spikelets and the pathogen may spread from spikelet to spikelet through the vascular bundles. Typical FHB disease symptoms develop including premature bleaching of the heads, brown discoloration of the rachis and peduncle, and pink/orange sporodochia (containing macroconidia). Fusarium damaged kernels (FDK) that develop after infection are lighter in weight than unaffected kernels, have a chalky white appearance, and accumulate trichothecene toxins such as deoxynivalenol (DON). A biological control method that targets perithecial development might lead to reduced inoculum and disease severity.

The overall objectives of this project were to identify potential fungal biocontrol agents of *Gibberella zeae* and to determine their mechanisms of control. The study is presented in four parts:

1. Screening and evaluation of potential biocontrol agents towards *G. zeae*
2. Examination of the effects of *T. harzianum* isolates and secondary

metabolites on perithecial production of *G. zeae*.

3. The development of a predictive model for biological control of FHB based on secondary metabolite production by *T. harzianum*
4. Investigation of the interactions between *T. harzianum* and *G. zeae* during perithecial development.

2.0 Literature Review

2.1 The host: wheat

Wheat (*Triticum* spp.) is thought to have been first cultivated approximately 10,000 - 12,000 years ago in the Fertile Crescent of the Near East. It was introduced to North America from Europe in the 16th century (Gooding and Davies 1997). Today it is one of the most widely grown cereal crops worldwide, ranking second to rice in total production (FAOSTAT 2006). Canada, producing approximately 27 million tonnes of grain per year on over 12 million hectares of land, is the second largest exporter of wheat and wheat products, with 20 million tonnes exported annually. China is the largest producer/importer of wheat, producing over 100 million tonnes per year and importing 10 million tonnes annually (Bailey et al. 2003).

Wheat belongs to the kingdom Plantae, division Magnoliophyta, class Liliopsida, order Poales, family Poaceae, tribe Triticeae, and genus *Triticum*. It is common for plants to exhibit polyploidy. In wheat there are up to three complete sets of genomes, A, B, and D that a species may contain and further classification of wheat can be based on ploidy level: diploid (14 chromosomes), tetraploid (28 chromosomes) and hexaploid (42 chromosomes). Major cultivated species of wheat include the diploid species, Einkorn (*Triticum monococcum* L.), which contains chromosomes AA, the tetraploid species Emmer (*Triticum dicoccoides* (Körn. ex Asch. & Graebner) Schweinf.) and Durum wheat (*Triticum turgidum* L. var. *durum* (Desf.) Mk.), which contains AAxBB. Bread wheat (*Triticum aestivum* L.), which is a hexaploid, contains all three sets of chromosomes, AAxBBxDD (Caligari and Brandham 2001). Wheat can be

further categorized based on growing season (Spring vs Winter), protein content (hard vs soft) and grain color (red, white or amber). Major commercial classes grown in western Canada include Canada western hard red spring (CWHRs) and Canada western hard white spring (CWHWS), Canada western amber durum (CWAD), Canada prairie spring red (CPSR), Canada prairie spring white (CPSW), Canada prairie red winter (CPRW), Canada western soft white spring (CWSWS) and Canada western extra strong (CWES). In Manitoba, 89% of the wheat grown is CWHRs, which tends to have superior milling and baking qualities. Other classes grown in Manitoba include CPRW (7.3%), CPSR (1.2%), CWES (0.9%) and CWSWS (0.2%) (Canadian Grain Commission).

2.1.1 Diseases of wheat

Wheat cultivars are susceptible to a variety of diseases (Bailey et al. 2003). Viral diseases include wheat streak mosaic and spot mosaic. Fungal pathogens attack various organs of the plant body. Leaf spot diseases such as *Bipolaris sorokiniana* (Sacc. Shoemaker), *Septoria tritici* Roberge in Desmaz., *Stagonospora nodorum*, and *Pyrenophora tritici-repentis* (Died.) Drechs produce lesions on the leaves. Stem rust *Puccinia graminis* Pers.:Pers. and leaf rust *Puccinia triticina* Eriks attack the stem and leaves respectively. These diseases may result in yield losses of up to 50% depending on the year and location (McMullen and Adhikari 2009). Fusarium head blight (FHB) affects the wheat inflorescence and is caused by several *Fusarium* species including *Gibberella zeae* (Schwein.) Petch (anamorph *Fusarium graminearum* Schwabe), *F. avenaceum* (Fr.:Fr.) Sacc., *F. culmorum* (Wm. G. Sm.) Sacc., and *F. poae* (Peck) Wollenw. (Clear and Patrick 2000). In Manitoba, the predominant species is *G. zeae*,

accounting for over 97% of the *Fusarium* species isolated. Other species found at low levels include *F. sporotrichioides* (2.2%), and *F. equiseti* (<1%) (Gilbert et al. 2009). *Fusarium* head blight results in yield losses but also produces trichothecene mycotoxins, including deoxynivalenol (DON) and nivalenol (NIV), which are a cause for concern regarding human and animal health and safety (McMullen et al. 1997).

2.2 The pathogen: *Gibberella zeae*

Gibberella zeae is the principal cause of FHB in North America, which is a destructive and economically important disease of small grain cereals. It was first reported in the late 1800's in England by Smith (1884), who attributed the disease to *Fusisporium culmorum*. In 1917, FHB caused by *Gibberella zeae*, formerly *G. saubinetii*, was reported on many of the small grain cereal crops in the eastern United States (Atanasoff 1920). From 1927 to 1980, periodic epidemics were reported in eastern Canada during periods of above average rainfall (Sutton 1982). In Manitoba, *F. graminearum* (an anamorph of *G. zeae*) was isolated from just 0.9% of cereal seed samples in the mid decades of the 1900's, and was not considered the predominant *Fusarium* species (Gordon 1944). The three common species of *Fusarium* that were isolated from seed samples at that time were *F. avenaceum* (20.1 %), *F. poae* (16.4%), and *F. equiseti* (7.8%) (Gordon 1944). It has been suggested that since the 1940's, *F. graminearum* has been spreading northwest from the U.S.A. into southern Manitoba and now reaches into parts of eastern Saskatchewan and Alberta (Clear and Patrick 2000). In 1984, two FHB-contaminated wheat samples from southern Manitoba were identified. High levels of deoxynivalenol (DON) were found in the hard red spring wheat cultivar,

Sinton, and in the amber durum wheat cv. Coulter, at levels of 12.6 and 9.6 ppm, respectively. Plentiful rainfall in the area of Letellier, near the U.S.A. border, during anthesis and corn/wheat rotations appeared to have favored the development of the disease (Clear and Abramson 1986). Studies have shown that continuous crops of wheat or wheat/corn rotations can potentially increase the amount of inoculum and lead to epidemics of FHB (Snyder and Nash 1968; Teich and Nelson 1984). Since 1984, the incidence of FHB caused by *F. graminearum* has steadily increased (Tekauz et al. 1986; Gilbert and Tekauz 2000). In 1992 in southern Manitoba, FHB was found in 36% of the wheat fields sampled and *F. graminearum* was the principal causal species (Gilbert et al. 1993). In 1993, during the most severe outbreak on record, infected heads were found in 96% of the fields examined and *F. graminearum* accounted for 88% of the *Fusarium* isolations. Losses from the epidemic were estimated at \$75 million dollars and annual losses since were estimated at approximately \$50 million (Gilbert et al. 1994; Gilbert and Tekauz 2000).

2.2.1 Host range

The anamorph of *G. zae*, *Fusarium graminearum* is pathogenic on corn and small grain cereal crops including wheat, barley, rice, and oats. The fungus may infect other plant species without causing disease symptoms. Connors (1942) indicated that *F. graminearum* was rare in Manitoba, although it was reported on several grass species in Minnesota, North Dakota, and South Dakota. These species included *Agropyron cristatum* (L.) Gaertn., *A. subsecundum* (Lk.) Hitchc., *Agrostis* sp., *Bromus tectorum* L., *Cenchrus echinatus* L., *Cortaderia selloana* (Schult.) Aschers. and Graebn., *Echinochloa*

crusgalli (L.) Beauv., *Hordeum distichon* L., *Lolium perenne* L., *Poa annua* L., *P. pratensis* L., and *Setaria lutescense* (Weigel) (Sprague 1950). More recently Inch and Gilbert (2003a) reported *Fusarium graminearum* was the predominant *Fusarium* species isolated from wild grasses and accounted for 59 to 62% of the isolations in Manitoba. Incidence of other *Fusarium* species ranged from 3 to 13 %. *Fusarium graminearum* was isolated from approximately 9% of the grasses examined, and occurred on 11 grass species including *Agropyron trachycaulum* (Link) Malte (slender wheat grass), *Agropyron repens* (L.) Beauv. (quack grass), *Agrostis stolonifera* L. (red top), *Bromus ciliatus* L. (fringed brome), *Bromus inermis* Leyss. (smooth brome), *Calamagrostis canadensis* (Michx.) Beauv. (canary reed grass), *Echinochloa crusgalli* (L.) Beauv. (barnyard grass), *Hierochloa odorata* (L.) Beauv. (sweet grass), *Phleum pratense* L. (timothy), *Poa pratensis* L. (Kentucky blue grass), and *Spartina pectinata* Link (prairie cord grass). The latter seven represented first reports of hosts for *F. graminearum*. Other *Fusarium* species isolated at low frequencies included, *F. sporotrichioides* (2.3%), *F. equiseti* (1.2%), *F. oxysporum* (1.1%), *F. avenaceum* (0.9%), *F. culmorum* (0.8%), and *F. poae* (0.7%). *Fusarium graminearum* also has been reported on broad-leaved host plant species including *Matricaria* spp. and *Viola arvensis* Murr. (Jenkinson and Parry 1994).

2.2.2 Taxonomy

The genus *Gibberella* belongs to the family Nectriaceae, order Hypocreales, subclass Hypocreomycetidae, class Sordariomycetidae, subphylum Pezizomycotina, phylum Ascomycota, kingdom Eumycota (Kendrick 2003). The genus *Fusarium* is divided into 16 taxonomic sections, four of which contain species that cause FHB. These

taxonomic sections include *Discolor*, *Roseum*, *Gibbosum*, and *Sporotrichella*. Each section has unique features that define the group. The section *Discolor*, contains *Fusarium graminearum*, *F. culmorum* and *F. crockwellense*. These three species are the predominant cause of FHB in temperate climates. Other important species from other sections include *F. equiseti*, *F. scirpi* and *F. acuminatum* (Section *Gibbosum*), *F. avenaceum* (Section *Roseum*), *F. poae*, *F. tricinctum*, and *F. sporotrichioides* (Section *Sporotrichella*).

Until recently *Fusarium graminearum* was considered to be a polyphyletic group consisting of both heterothallic and homothallic species, *Fusarium graminearum* group 1 and 2, respectively. Group 1 is primarily responsible for crown and foot rot of grains and Group 2 is the causal agent of FHB (Aoki and O'Donnell 1999). Based on current evidence, including morphological, molecular, and distribution data, Group 1 has been re-classified as *Fusarium pseudograminearum* (O'Donnell et al. 2000). Genetic analysis of 11 genes at six independent loci of Group 2 revealed that *F. graminearum* consists of eleven distinct lineages/species (O'Donnell et al. 2004; Starkey et al. 2007). Based on the molecular evidence, eight out of eleven lineages have been re-classified (Gale et al. 2002; O'Donnell et al. 2004). Lineage 7, *Fusarium graminearum sensu stricto*, is the predominant lineage in North America and Europe (O'Donnell et al. 2000; 2004). However, the re-classification is not accepted by all scientists (Leslie and Bowden 2008). The proposed new species all cause FHB and are morphologically identical. Even though flow is limited or absent it still may occur under certain circumstances (O'Donnell et al. 2000, 2004). Suggesting these new species are in fact one species.

Gibberella zeae is a homothallic fungus that is self-fertile but also is capable of out-

crossing with members of the same species. Sexual reproduction is regulated by the MAT loci (Lee et al. 2003). The fungus contains alternative forms of the MAT locus (idiomorphs). Lee et al. (2003) found that deletion of one of the idiomorphs resulted in the loss of ability for self-crossing. However, this did not affect the ability to outcross. The rate and importance of out-crossing under field conditions is unclear. Differentially-expressed proteins involved in cell wall biosynthesis were identified in a *mat1-2*-deleted strain of *G. zeae* during perithecial development. In total 13 genes were found to be ultimately leading to self-sterility (Lee et al. 2008).

Unlike most members of the family, *G. zeae* lack ascogonia and antheridia. The development of dikaryotic hyphae by somatic anastomosis induce perithecial development (Trail and Common 2000) and clusters of perithecia develop usually in association with the stomata and silica cells of wheat stem tissue (Guenther and Trail 2005). They are ovoid in shape with a rough tuberculate wall and range in size from 140-250 μm in diameter. The asci are clavate with a short stipe and contain eight, 4-celled ascospores in a biserial arrangement (Trail and Common 2000). The ascospores are hyaline and approximately 22 x 3.5 μm in size (Booth 1971). Macroconidia of the anamorph *F. graminearum* are asexual spores formed by mitotic division. The conidia are falcate to sickle shaped with a distinct foot cell at the base. They have 3-7 septa and are approximately 45 x 3.5 μm in size. Macroconidia can aggregate together to form pink/orange sporodochia (Booth 1971).

2.2.3 *Fusarium* mycotoxins

Mycotoxins are secondary metabolites produced by fungi that may cause mycotoxicoses in humans or animals if ingested. The severity of the reaction depends on the toxicity of the toxin, duration of exposure, age and size of the individual. The chemical structure of the toxins varies, but generally they are low molecular weight organic compounds. The most common food-borne toxins include, aflatoxins (which are acutely toxic), ochratoxins (produced by *Penicillium* and *Aspergillus* species), trichothecenes (produced mainly by *Fusarium* species), and fumonisins (common contaminants of corn produced by *Fusarium* species) (Peraica et al. 1999; Desjardins and Proctor 2007). Among these toxins, trichothecenes are associated with chronic and fatal toxicoses of humans and animals (Desjardins 2006). Trichothecenes are sesquiterpenoid toxins which have a tricyclic ring structure with a double bond at C-9 and C-10, and an epoxide at C-13. There are two major groups, Type A and Type B, which are defined based on the R-group at C-8. Type A, have either hydrogen (-H), hydroxyl (-OH) or an ester group and include T-2 and HT-2 toxins. Type B have a keto functional group and include deoxynivalenol (DON) and nivalenol (NIV). The major types of trichothecenes produced by *Fusarium* species varies depending on the species, location and nutrient source. Abramson et al. (1993) and Mirocha et al. (1989) found that *F. graminearum* produced mainly DON and its derivatives, 3-acetyl-DON (3ADON), 15-acetyl-DON (15ADON), NIV, T-2, HT-2 and zearalenone. *Fusarium graminearum*, *F. poae*, and *F. sporotrichioides* produce a greater amount of trichothecenes than *F. culmorum* and *F. equiseti* (Mirocha et al. 1989).

The mycotoxins, DON and NIV, may be important pathogenicity and virulence

factors in causing FHB. Atanassov et al. (1994) was able to demonstrate that strains that produced high levels of NIV showed the highest virulence on all wheat genotypes tested. Isolates which produced low to moderate amounts of NIV, showed low to moderate levels of virulence. However, there was no correlation between levels of DON and virulence. All isolates, regardless of DON levels were able to cause infection and symptom development. Very shortly after infection of the wheat floret by *G. zeae*, DON biosynthesis begins. The presence of DON has been found to be important in the necrosis of plant tissue and allows for movement of the fungus from the floret into the rachis (Jansen et al. 2005).

2.2.4 Life cycle

The main source of inoculum for FHB is *G. zeae* infested crop debris on the soil surface (Atanasoff 1920). The fungus overwinters on this tissue and in the spring perithecia and ascospores develop (Sutton 1982). Carbon sequestration from debris during vegetative growth is essential for the saprophytic survival and later perithecial initiation on straw. The role of antimicrobial compounds in preventing the colonization of the straw by other organisms is unknown (Trail 2009). Guenther and Trail (2002) found that perithecia developed on the internode tissues and was closely associated with the stomates and chlorenchyma tissue. On the nodes of wheat stems perithecia were associated with epidermal silica cells. The mature perithecia consist of three intergraded layers, the outer, middle, and inner layers. The outer layer is composed of thick-walled cells, 2-3 cells deep that are tubular and highly vacuolated. The middle layer consists of flattened, highly granulated cells. The inner wall consists of round cells with thin walls,

2-5 cells deep (Trail and Common 2000; Guenther and Trail 2005). Hallen et al. (2007) using an Affymetrix GeneChip probeset consisting of 17, 830 predicted genes, were able to identify over 2000 genes which were expressed only during sexual development. Of these, 175 were orphan genes (only found in association with *G. zeae*) and 162 were ion transporter genes including MirA-type siderophores, P-type, ATPases and potassium transporters.

The pathogen exhibits a haplontic life cycle; the chromosome complement of the mycelium is haploid during the majority of the life cycle (Trail 2009). *Gibberella zeae* is homothallic and therefore is self-fertile. A single mating type locus (MAT) with two idiomorphs, Mat1-1 and Mat 1-2 regulates the sexual development. If one of the genes are deleted the mutant is self-sterile but still capable of out-crossing (Lee et al. 2003). A brief diploid stage forms when two compatible nuclei fuse. Shortly after karyogamy, meiosis occurs and re-instates the haploid condition. During the initiation of perithecia, dikaryotic hyphae form in the xylem tissue and in the chlorenchyma cells and “pith” cavity of the host plant. The dikaryotic stage allows for somatic hybridization and recombination between nuclei without meiosis. It is unclear how and why the dikaryotic stage is induced (Trail et al. 2005). However, prior to perithecial formation, dense mats of hyphae containing abundant lipid bodies develop. This carbon sequestration may provide the fungus with the energy it needs for the next stages (Trail 2009). Uninucleate hyphae begin to coil and form perithecial initials. These cells enlarge and remain uninucleate except when under-going division. Once the ascogenous cells develop, apical paraphyses begin to elongate and become attached to the hymenium. Within the immature ascus, a diploid nucleus forms and undergoes meiotic and mitotic division, giving rise to the eight

haploid ascospores. As the perithecia mature the ascospores form a biseriate arrangement in the unitunicate asci. The intracellular siderophore (ferricrocin) has been found to be essential for intracellular iron sequestration, asci and ascospores development. *G. zeae* strains with deleted *nps2* gene, which codes ferricrocin, produced perithecia but the asci were abnormal in appearance and void of ascospores (Oide et al. 2007).

The mechanism of ascospore discharge is currently under investigation (Hallen et al. 2007, Hallen and Trail 2008; Trail 2009). Ascospores are forcibly discharged through a small tear or slit, the ostiole, at the apex of the perithecium (Trail and Common 2000). It has been postulated that dehydration of the perithecium and substrate triggers ascospore release and has been found to correspond to a decrease in relative humidity (RH) (Tschanz et al. 1975). This may result in the mechanical rupturing of the ascus wall. As the RH begins to increase, the osmotic pressure may also rise within the perithecium, due to an influx of mannitol, as well as the ions, potassium and chloride, into the asci, which leads to the forcible discharge of the ascospores (Trail et al. 2002; Trail et al. 2005). Launch speed of ascospores has been estimated at 34.5 m/s with an acceleration of 870,000g, which is the fastest recorded acceleration in biological organisms (Trail et al. 2005). Transporter proteins, such as Co/Zn/Cd cation transporters, ammonia permeases, chloride channels, potassium transport protein and mitochondria phosphate transport protein are some of the proteins involved in maintaining the intracellular environment and provide the osmotic pressure for ascospore discharge (Hallen et al. 2007). Ascospore discharge requires an estimated pressure of 1.54 MPa in the perithecium for high-speed release (Trail et al. 2005).

Air-borne ascospores are the primary inoculum source (Shaner 2003). They are dispersed primarily by wind and may then be deposited on the heads of wheat, barley, and oats (Sutton 1982). Wheat is most susceptible to infection during a narrow range of development, from anthesis to the soft dough stage of kernel development. Very little infection develops after this time (Schroeder and Christensen 1963).

Under favorable environmental conditions, the ascospores germinate on the exterior surface of the wheat spike and the hyphae begin to grow towards a stomatal opening or towards the exposed anthers during dehiscence (Bushnell et al. 2003). The presence of choline and betaine (fungal growth stimulants) in anthers, was found to stimulate hyphal extension but not ascospore germination (Strange and Smith 1978). Glume stomata appear to be an early entry point, as colonization and subcuticular growth of the hyphae can be observed on inoculated glumes. Glume epidermal cells are highly resistant to direct penetration by the hyphae due to the thick, crenulated cell walls and high levels of silica in the cell lumina (Hodson and Sangster 1988). Once inside the floret, the anthers, stigmas and lodicules are colonized. The fungus can spread systemically from floret to floret through the vascular bundles in the rachis (Ribichich et al. 2000). Using a green fluorescent protein, Guenther and Trail (2005) were able to follow the movement of the hyphae through the plant. Once inside, the vascular tissues become dysfunctional, which may lead to the premature death of the inflorescence and resulting in a typical bleached appearance (Bushnell et al. 2003). In the resistant wheat cultivar, Sumai 3, pectic gels form in the vascular tissue blocking the spread of the pathogen (Ribichich et al. 2000; Miller et al. 2004).

Under wet conditions, the surface of the florets become colonized by the mycelium and orange/pink sporodochia, containing macroconidia, may be produced. Macroconidia that arise from sporodochia may be an important source of inoculum for short-distance dispersal and infection on heads of secondary tillers later in the growing season (Shaner 2003). Both ascospores and macroconidia can infect at any time during head maturity (Hart et al. 1984) and are equally effective at causing FHB, despite the difference in size (Stack 1989). Macroconidia are generally splash-dispersed (Deacon 2006; Sutton 1982). Relatively few macroconidia are trapped from air relative to the number of ascospores (Inch et al. 2003a; Fernando et al. 2000; Markell and Francl 2003). Fusarium head blight is considered a monocyclic disease and the levels of disease directly correspond to the amount of primary inoculum (Wilhem and Jones 2005).

Insect damage may provide another point of entry and allow for the colonization of *F. graminearum*. The orange wheat blossom midge may be a vector responsible for the transmission and spread of FHB. Sporadic outbreaks of wheat midge have been recorded in Manitoba and Saskatchewan, corresponding to FHB epidemics (Lamb et al. 1999). Studies have shown that wheat spikes exposed to *F. graminearum* inoculated-wheat midge had a higher incidence (29%) of disease than plants exposed to the control midge (0%) (Mongrain et al. 1999). However, no direct correlation has been observed between the wheat midge and the spread of FHB under field conditions. In Saskatchewan wheat midge is a serious problem not only in areas where FHB occurs (Lamb et al. 1999).

2.2.5 Environmental effects on spore dispersal and deposition

Under field conditions in Manitoba, ascospore discharge is correlated with a drop in air temperature and a rise in relative humidity. Inch et al. (2005) reported spore deposition begins slowly at approximately 1500h, which corresponds to a drop in RH and rise in air temperature. Higher numbers of spores were trapped as RH increased, peaking at 2100 h and continuing until 0400h. Few ascospores were trapped between 0500 and 1400 h. Other studies have shown similar patterns of ascospore release and deposition. Ascospores are generally collected in the late afternoon and continue into the evening (Ayers et al. 1975; Paulitz 1996; Fernando et al. 2000). Schmale et al. (2003) collected 94% - 86% of the spores during the night and 6% - 14 % during the day. This suggests that deposition of spores in wheat fields occurs at night when conditions are favorable for germination and infection. High relative humidity and low temperatures are conducive for germination (Bayer and Verreet 2005; Gilbert et al. 2008).

Rainfall may be required prior to ascospore discharge. Many studies have demonstrated a distinct lag phase between rainfall and ascospore release. Generally, airborne ascospores are trapped 1-7 days after rainfall events of more than 5 mm (de Luna et al. 2002; Fernando et al. 2000; Paulitz 1996; Inch et al. 2005; Maldonado-Ramirez et al. 2005). Release has been found to be inhibited by periods of rain greater than 5 mm, continuous RH of more than 80%, or high RH with intermittent rainfall during the day (Paulitz 1996; Inch et al. 2005). A seasonal trend is also apparent, several studies have observed multiple spore release events over a 2-month period (Inch et al. 2005; Fernando et al. 2000; Paulitz 1996). This would suggest that a heterogeneous population of perithecia with varying levels of maturity exist at any given time. Ascospore discharge is

in part, related to the number of mature perithecia available at the time when optimal conditions for discharge occur (Tschanz et al. 1975). Dry weather appears to inhibit ascospore release (Inch et al. 2005). The relationship between rainfall and ascospore dispersal may be coincidental as after an initial stimulus such as light or moisture is removed or changed, ascospore discharge continues and occurs in a rhythmic pattern (Tschanz et al. 1975).

Wind plays an important role in the dispersal of ascospores. Fernando et al. (1997) found wind-driven gradients in plots inoculated with ascospores. The infection gradient upwind was much steeper than the gradient produced downwind with gradient slopes ranging from -10 to -43 m^{-1} . Seed infection declined up to 90 % within 5-22 m from the inoculum source. In plots inoculated with macroconidia a symmetrical pattern away from the focal centre was observed in which the gradient was much steeper than that produced by ascospores; slopes ranged from -0.48 to -0.79 m^{-1} . This would suggest that wind plays a lesser role in the dispersal of macroconidia. Macroconidia are primarily splash-dispersed and thus are transported only over short distances in rain droplets (Sutton 1982). Inch et al. (2005) and Fernando et al. (2000) detected relatively low concentrations of macroconidia from the air. Macroconidia are less likely to become airborne and trapped with cyclone or Burkard spore samplers, which primarily trap airborne spores and high amounts of kinetic energy would be required to release macroconidia into the air (Fernando et al. 2000). Markell and Francl (2003) trapped one macroconidium for every two ascospores. Both macroconidia and ascospores are equally effective in disease development, but the lower numbers of macroconidia present in the

air, suggest that ascospores are more important for infection of wheat heads (Stack 1989; Fernando et al. 2000; Inch et al. 2005).

2.2.6 Environmental effects on inoculum production

Under controlled conditions several hours of UV light in the range of 300-320 nm are required for initiation of the sexual state (Tschanz et al. 1976). However, Gilbert and Tekauz (2000) reported the formation of perithecia without exposure to UV light. Optimal temperatures for the production of perithecia range from 15 to 28.5°C and for ascospore production, from 25 to 28°C. Perithecia are not produced when temperatures exceed 30 °C (Tschanz et al. 1976; Sutton 1982). The optimal temperature range for macroconidia production is 28-32°C and decreases sharply below 16°C or above 36°C (Tschanz et al. 1976). Moisture levels are also an important factor in perithecial production. Optimum water potential of -0.45 to -1.5Mpa was found to favor perithecial production and was limiting at -2.36 to -4.0MPa (Sung and Cook 1981; Dufault et al. 2006). There appears to be a correlation between temperature and moisture levels. When temperatures are low, higher moisture levels are required to initiate perithecial production. Conversely, when temperatures are high, lower moisture levels are required (Dufault et al. 2006). Sung and Cook (1981) found under *in vitro* conditions perithecia must remain hydrated for ascospore development. However, under field conditions, which are subject to dry periods, perithecia were able survive extended dry periods and resume activity after re-wetting of the substrate occurred (Fernando et al. 2000). It is presumed that ascospore development becomes dormant when the conditions are not suitable for development and resumes maturation during favorable conditions. Under

natural field conditions in eastern Canada and Manitoba, perithecia form on over-wintered crop residues in late spring and early summer (Inch et al. 2005; Paulitz 1996; Sutton 1982).

Environmental conditions, such as temperature and relative humidity (RH) also affect ascospore germination (Gilbert et al 2008; Beyer and Verreet 2005). It appears temperature and duration of exposure are the main factors that affect survival and germination. Gilbert et al. (2008) found that germination rates *in vitro* decrease with an increase in temperature and duration of exposure. Ascospore germination was less than 50% at 30°C after 48 hours. Relative humidity also affects germination rates; high RH (90%) at 15, 20°C had higher germination rates than RH of 30 and 60%. Beyer and Verreet (2005) also found a significant correlation between RH, temperature, time and ascospore germination. However, in their study, ascospores did not germinate at low temperature combined with 30% RH. However, with increasing temperature and time, ascospore germination also increased; after 4 h of exposure at 20 and 30°C, ascospore germination reached 50%. It appears that ascospore discharge, which corresponds to an increase in RH (de Luna et al. 2002; Fernando et al. 2000; Paulitz 1996; Inch et al 2005; Maldonado-Ramirez et al. 2005) also corresponds to conditions that are favorable for ascospore germination under Canadian growing conditions.

2.2.7 Survival of *Gibberella zae* on crop residues and soil

Under field conditions in Manitoba, seed-borne *G. zae* survived for at least 24 months on fusarium damaged kernels (FDK) left on the soil surface or buried up to 10 cm (Inch and Gilbert 2003b). In eastern Canada, survival time was somewhat shorter; *G.*

zeae was isolated from all types of corn and wheat residues located above the soil and from corn kernels, wheat stems, and wheat spikelets from the soil surface, but the duration of survival of *G. zeae* on buried residues was only 1 year and 2 years on corn stems and wheat kernels located on the soil surface (Konga and Sutton 1988). Perithecia and ascospores matured on kernels left on the soil surface providing a potential source of inoculum to infect the spikes of small grain crops. Fusarium damaged kernels may act as a source of inoculum leading to disease development when susceptible crops are planted in contaminated fields. Chongo et al. (2001) found that root and crown rot severity increased, and seedling emergence decreased in cereal and non-cereal crops planted in soils inoculated with *F. graminearum*-infected seed. However seed-borne inoculum is not thought to be an important factor in FHB development (Gilbert et al. 2003; Wong et al. 1992).

Soil type also influences survival of *G. zeae*. Under field conditions in Australia, recovery of the fungus was significantly greater from straw residues collected from soils located at Blue Nobby (104 weeks) than from those buried at Tremain (42 weeks) or at the North-West Wheat Research Institute (96 weeks) location (Burgess and Griffin 1967). The rate at which host tissues decompose also influences the survival of *G. zeae*. The FDK that were buried decomposed at a faster rate than those left at the soil surface. Kernels which are buried have a greater surface area in contact with soil micro-organisms which may influence the decomposition rate. *Gibberella zeae* is a poor competitor with other soil microflora and requires a suitable substrate within the soil to survive (Nyvall 1970). Once a residue decomposes, the fungus needs to colonize a new substrate. Recovery of *G. zeae* on wheat residues corresponded to the rate of decomposition (Dill-

Macky et al. 1998). Furthermore, survival of *G. zeae* was longer on tissues that break down slowly and consequently recovery of *G. zeae* after 52 weeks was confined to nodal rather than internodal tissues, 94% and 0%, respectively (Burgess and Griffin 1968). Other environmental parameters such as temperature, rainfall, soil temperature, and soil moisture levels may also influence rate of residue decomposition and fungal survival. Increased soil moisture (more frequent wetting) has been found to reduce the recovery of *G. zeae*. After 28 weeks, the recovery of *G. zeae* decreased when moisture was adjusted every three weeks to pF 2.5. compared to 72 weeks when moisture was adjusted every 6 weeks (Burgess and Griffin 1968).

Macroconidia of *F. graminearum* will form chlamydospores when placed in soil (Nyvall 1970). However, *F. graminearum* is not found in high frequencies in the soil and is generally associated only with plant residues (Sutton 1982). The predominant species of *Fusarium* found in soil samples from cereal plots in Manitoba in 1950 were *Fusarium oxysporum* Schlecht. Emend. Snyder & Hans (51.91 %) and *F. equiseti* (24.95 %) (Gordon 1954). No isolates of *F. graminearum* were obtained. In eastern Australia, *F. graminearum* was isolated from small pieces of organic debris in 4 out of 5 soils. However, it was only isolated from 2 of 5 soils using the soil dilution technique (Wearing and Burgess 1978).

2.3 The disease

2.3.1 Symptoms of fusarium head blight

In general, only *F. graminearum*, *F. culmorum*, and *F. avenaceum* produce significant head blight symptoms on wheat (Miller 1994). At the infection site, water-

soaked brown lesions and discoloration can be seen. Eventually the pathogen enters into the vascular system where it may spread from spikelet to spikelet resulting in premature bleaching of one or more spikelets of the head. The infected spikelets generally become sterile and are the site of accumulation of the toxin DON. The fungus is capable of colonizing multiple components of the inflorescence including the flowering structures, glumes, rachis and kernels. Pink/orange sporodochia may be seen around the edges of glumes on infected florets, and the rachis and peduncle can darken, turning a chocolate-brown color (Parry et al. 1995). Fusarium damaged kernels are shriveled, chalky-white in appearance, and are lighter in weight. The degree to which the kernels are affected depends on the stage in the wheat life cycle they were infected. Early season infections result in kernels that are small and almost 100% mycelium. When kernels are infected late in the season, they may resemble healthy kernels (Bushnell et al. 2003). The time of infection also influences DON accumulation in the kernels. Del Ponte et al. (2007) found that the highest levels of DON occurred when plants were inoculated at the kernel watery ripe stage (GS 73) and early milk stage (GS 77), with 98.0 and 89.7 mg/kg of DON, respectively.

2.3.2 Disease control

Several practices have been suggested to control FHB and to reduce inoculum levels. These include crop rotation of wheat and other susceptible small grain cereals with non-host crops such as canola or alfalfa (Dickson 1956; Sutton 1982; Parry et al. 1995) and plowing under of crop residues to eliminate inoculum by accelerating debris decomposition and blocking ascospore dispersal (Teich and Hamilton 1985). Buried crop

residues can support *G. zeae* as a saprophyte (Sutton 1982) and can persist in the soil as chlamydospores (Nyvall 1970). The fungus will survive for at least 24 months on buried infected kernels (Inch and Gilbert 2001b). The role of infected seeds is not clear. Gilbert et al. (2003), found no difference in disease severity when wheat was planted with *Fusarium*-infested seed compared to the control plots. However, these FDK can provide an inoculum reservoir that may be able to infest crop debris on the soil surface. With the increase in zero tillage practice, large quantities of crop residues remain on the surface of the soil, which can support *G. zeae* over winter (Warren and Kommedahl 1973; Windels and Kommedahl 1984; Dill-Macky 1998). Fungicides are able to reduce disease incidence and DON accumulation in small grain cereals, but results have not always been consistent from year to year. Seed treatments however, have proved to be effective against seedling blight and in increasing stand establishment (Gilbert and Tekauz 1995).

Recommendations for the control of fusarium head blight include deep tillage; burying crop residues, to accelerate decomposition and potentially eliminate this source of inoculum (Sutton 1982). Gilbert and Tekauz (1994) showed there was no apparent effect of tillage practice on disease development in Manitoba. However, the effects of various tillage practices were difficult to demonstrate due to high background levels of inoculum. Rotations with a non-host crop after a susceptible host such as corn or barley may reduce disease incidence (Sutton 1982; Teich and Hamilton 1985; Parry et al. 1995). Crop densities may affect the incidence of FHB. High stand densities which increase the humidity in the canopy may lead to a higher incidence of disease (Hatmanu 1972). The amount and type of nitrogen fertilizer used also may influence the development of disease. Teich (1987) found that the use of urea fertilizer on wheat resulted in lower

incidence of disease compared to wheat fertilized with ammonium nitrate. Weed control may also reduce disease incidence. Fields with an excess of weeds present were shown to have higher incidence of FHB than fields free of weeds (Teich and Nelson 1984). Weeds may potentially increase the amount of inoculum available and may increase the humidity and shading of crop plants, which can lead to higher incidence of disease (Hanson et al. 1950).

Fungicides can be divided up into four broad groups depending on their mode of action. The first type, inhibit or interfere with the electron transport chain in the mitochondria resulting in the disruption of cellular respiration. This is the mode of action of many fungicides such as the strobilurins (Azoxystrobin, Pyraclostrobin and Trifloxystrobin). The second type, which includes the dithiocarbamates and benzonitrile (Dithane, Manzate, and Chlorothalonil), inhibits enzyme function. The third type inhibits protein and nucleic acid synthesis and includes fungicides such as dicarboximides (Iprodione, Vinclozolin), benzimidazoles (Thiophanate Methyl) and acetanilide (Metalaxyl and Mefanoxam). The last group of chemicals interferes with membrane function and synthesis. They are demethylation inhibitors (Propiconazole, Tebuconazole and Triadimefon, which interfere with the demethylation of ergosterol, which is an important component in the cell membranes of fungi (Crop protection 2009).

Chemical control can help minimize disease incidence, but the efficacy of many of the currently registered fungicides has not been consistent. Many factors affect the level of control including effectiveness of the active ingredients, application timing, and efficient application of the product. Fungicides are effective in suppression only (Wale 1994; Wilcoxson 1996; McMullen et al. 1997) and plant diseases are difficult to treat

once symptoms are observed (McMullen et al. 1997). Foliar fungicides, such as tebuconazole (Folicur) and propiconazole (Tilt) have been found to be effective in reducing FHB severity and DON accumulation in moderately resistant wheat cultivars (Wilcoxson et al. 1989; Wilcoxson 1996), but are not effective on highly susceptible varieties (McMullen et al. 1997; Mesterhazy and Bartok 1997). Seed treatment fungicides have been found to be effective in preventing seedling blight and result in increased seed germination and stand establishment (Gilbert and Tekauz 1995).

As early as the 1920's plant pathologists and plant breeders noticed differences in FHB resistance levels in the various wheat varieties grown (Hanson et al. 1950). Several types of resistance to FHB have been identified and include both passive and active resistance mechanisms (Mesterhazy 1995). Passive mechanisms include plant height, presence or absence of awns, spikelet density, and time of flowering. Active forms of resistance are divided into 5 main types. Type I resistance: varieties are resistant to initial infection, Type II resistance: the varieties are susceptible to infection, however the infection is restricted to a few florets and does not spread to the entire inflorescence (Schroeder and Christensen 1963). Mesterhazy (1995) later expanded the definition of physiological resistance types to include Type III, kernel resistance, Type IV, yield tolerance and Type V, low DON accumulation in FDK (Mesterhazy 1999; Miller et al. 1985).

Each of these resistance types involves multiple genes. Current sources of resistant genes are primarily derived from the Chinese spring wheat cultivars Sumai-3 and Ning (Wilcoxson et al. 1992). Another source of resistance genes comes from The Brazilian spring wheat cv. Frontana (Singh et al. 1995) Wild species, may be a further

source of FHB resistance genes. Studies by Gilchrist et al. (1999) and Wan et al. (1999) have identified a number of wild species with resistance. However, there are significant problems in transferring these genes into wheat breeding lines, including incompatible pairing of chromosomes and poor agronomic traits (Chen et al. 1997). Quantitative trait loci (QTL) mapping studies have found resistances loci on all wheat chromosomes with the exception of 7D (Buerstmayer et al. 2009).

According to Seed Manitoba 2008, varieties with good resistance to FHB include Canada western red spring wheat 5602HR, Waskada and soon to be registered BW 365. Under high levels of disease all varieties are susceptible to FHB. The use of susceptible cultivars has been blamed in part for the severe FHB outbreaks in North America (McMullen et al. 1997).

Recent advancements in our understanding of genomics and the release of the complete genome sequence of *G. zeae* by the Broad Institute in 2003 (www.broad.mit.edu/annotation/genome/fusarium_group/Multihome.html) have changed the way in which we approach control strategies. Genes can be targeted which may disrupt various stages of the pathogen's life cycle. Over 30% of the genes identified are unique to fungi and therefore could be a target for control with little or no affect on the host plant and animals. Current work is underway investigating and determining the function of various genes. From this, novel control methods could be developed including more effective fungicides and genetically engineered organisms (Trail 2009).

2.4 Biological control

Biological control involves the suppression and reduction of disease causing organisms by the use of other organisms (Cook and Baker 1983). Biological control dates back to the ancient Greeks, who used moulds and other plants to treat infections. Other early work, noted that cultures contaminated with mould prevented the growth of bacteria. In 1874, William Roberts observed that *Penicillium glaucum* prevented the establishment of bacteria in cultures. Louis Pasteur (1877) also observed similar phenomena when cultures of anthrax bacilli were contaminated with *Penicillium notatum* (Wainwright and Swan 1986). By the 1920s there was an increase in the reports of plant diseases controlled with antagonistic bacteria and fungi found in soils. Millard and Taylor (1927) were able to demonstrate that microbes in suppressive soils were able to reduce potato scab caused by *Streptomyces scabies*. They suggested that the bacteria produced acidic compounds that were responsible for the control. Through a series of simple experiments, Henry (1931) was able to demonstrate that various concentrations of suppressive soils were able to reduce foot rot caused by *Helminthosporium sativum* and *F. graminearum*. The first commercially available biocontrol agent (BCA) was introduced in 1963, for the control of *Fomes annosus* on tree stumps by *Peniophora gigantean* (Baker 1987). Cook and Baker (1974) published the first book on biological control. Currently, approximately 40 biocontrol agents have been registered and are commercially available for the control of plant diseases (Fravel and Larkin 1996).

The first commercial chemicals available for the control of plant diseases were lime sulfur in 1802 and the Bordeaux mixture in 1882 to control grape vine powdery mildew caused by *Uncinula necator* (Agrios 2005). However, over-use of both chemicals

lead to ecological degradation and effects on the microbial composition of the soil. The use of biological control allows for a natural alternative, which may have minimal negative effects on the environment.

When selecting BCAs, only organisms native to a particular area should be used. The potential agent also should attack only the target organism and have no detrimental effects on other non-target species. The ability of a micro-organism to adapt and interact in the environment makes it more suitable for long-term control than using synthetic pesticides (Weller et al. 2002). Limitations associated with biological control involve potential unpredictable interactions between the BCAs and the environment and possible difficulties and expense to produce enough inoculum for large-scale use (Cook 1993). However, with a better understanding of the interactions of the antagonist with the pathogen and the environment, effective application and disease control may be achieved. A variety of methods for control have been identified, which include antibiosis, competition, parasitism, and induction of host resistance. Biological control can be a safe and effective method to reduce disease levels and may be used as part of an integrated pest management system. When developing a biocontrol method several factors will influence efficacy including the type of biocontrol agent, the method of application and the longevity and ability of the BCA to survive in the environment.

2.4.1 Mechanisms of biological control

2.4.1.1 Competition

All organisms occupy an ecological niche, which is the role an organism plays in the environment and its required resources. Competition occurs when the niches of two or

more organisms overlap and resources are limited. The competitive exclusion principal (Gause 1934) states that “When ecological niches overlap, one of the organisms will have little or no access to the resources, this will result in displacement due to competition.” Factors which micro-organisms compete for include space and nutrients such as carbon, nitrogen and iron. Soil and plant surfaces generally have limited resources therefore only the organisms that are the strongest competitors will be able to effectively colonize the area (Singh and Faull 1988). Competition is a common mechanism of biocontrol of many soil-borne plant pathogens when non-pathogenic species compete for the same limited resource. Rhizosphere-colonizing bacteria and fungi may prevent access to the root systems of the plant host. Several studies have demonstrated *Fusarium* suppression by *Trichoderma* and non-pathogenic *Fusarium* species (Sivan and Chet 1989; Mandeel and Baker 1991; Couteaudier 1992). Mandeel and Baker (1991) demonstrated that addition of non-pathogenic strains of *Fusarium oxysporum* to the soil reduced infection by *F. oxysporum* f. sp. *cucumerinum*. Competition for infection sites and acquired systemic resistance were suggested as possible mechanisms to explain the observed reduced infection levels. Competition for nutrients between germinating oospores of *Pythium aphanidermatum* and bacteria was correlated with disease suppression in the rhizosphere of wheat, tomato, cucumber, melon, bean and cotton plants under greenhouse conditions (Elad and Chet 1987). The same authors also found a 94 % reduction in damping-off caused by *Pythium ultimum* in cucumbers and 100-fold increase in the population numbers of *Pseudomonas putida* and *P. cepacia* on the roots of cucumbers when applied as a seed treatment.

Iron is an important nutrient required for various metabolic processes. Iron is generally abundant in soils but usually present in a form that is not readily available to aerobic micro-organisms. Under these conditions many bacterial and fungal species will synthesize iron-chelating siderophores, which are extra-cellular, low molecular weight, water soluble molecules with a high affinity for ferric iron. These molecules bind with iron and allow the cell to take up the iron where it is released and used for metabolic processes including the biosynthesis of ATP, reduction of ribotide precursors of DNA, and formation of heme (Leong 1986; Neilands 1995). Most micro-organisms have the ability to produce siderophores, however their affinity for iron can vary and is generally species-specific. Therefore the species that produces the siderophore with the greater affinity for iron can successfully out-compete another species for iron. Iron competition by siderophore-producing biocontrol agents appears to be involved in the control of soil-borne plant pathogens.

2.4.1.2 Antibiosis

Antibiosis is the production of compounds or antibiotics that inhibit the growth of other micro-organisms. These compounds include low-molecular weight molecules, proteins, and enzymes (Raaijmakers et al. 2002). Most fungi and bacteria produce these secondary metabolites during regular growth or they may be induced by the presence of other organisms. The type of antimicrobial compounds produced varies depending on the substrate on which the organism is growing, therefore compounds produced *in vitro* may differ from those produced *in situ* (Park et al. 1991). *Trichoderma* and *Gliocladium* species produce a variety of compounds; these include gliotoxin, viridin, and gliovirin

(Howell and Stipanovic 1983). Gliotoxin is a sulfur-containing antibiotic, an inhibitor of farnesyl transferase that blocks thiol groups in the cell membranes (Jones and Hancock 1988). When *T. virens* was added to soil it reduced the incidence of *Pythium ultimum*-induced seedling disease. Gliotoxin was detected in the surrounding soil and control was directly attributed to its presence (Lumsden et al. 1992). Biocontrol activity decreased when gliotoxin-deficient mutants were applied to the soil indicating that gliotoxin was responsible for disease suppression (Wilhite et al. 1994).

Production of volatile compounds by various bacteria has also been found to be effective in controlling plant pathogens. Volatile ammonia produced by *Enterobacter cloacae* was shown to minimize pre-emergence damping-off caused by *Pythium* spp. (Howell et al. 1993). Fernando et al. (2005) identified several volatile compounds produced by *Pseudomonas* spp. which were effective in the reduction of *in vitro* growth of mycelia and sclerotia of *Sclerotinia sclerotiorum*. These compounds included nonanal, benzothiazole, dimethyl trisulfide, n-decanal, cyclohexanol, 2-ethyl, and 1-hexanol.

2.4.1.3 Mycoparasitism

Mycoparasitism is the antagonistic interaction between two fungal species where one species infests or exploits the other. This association may be a useful method for biological control of fungal plant pathogens. Mycoparasites can be classified into two types: biotrophs or necrotrophs, depending on their mechanism. Biotrophic parasites require a living host for nutrients and other essential compounds. Initial stages of the interaction do not result in the destruction of the host. However, over time the parasite will destroy and kill the host. This relationship generally is very specific and requires

recognition between the two species (Jefferies and Young 1994). Obligate biotrophs have limited use as biocontrol agents due both to their specific host requirements and the fact that they are incapable of surviving without a living host. Examples of obligate biotrophs includes *Tetragoniomyces uliginosus* and its host *Rhizoctonia solani* (Bauer and Oberwinkler 1990) and *Sporodesmium sclerotivorum* and its host *Sclerotinia minor* (Bullock et al. 1986). The majority of mycoparasitic biocontrol agents are necrotrophic fungi that are facultative parasites obtaining both nutrients from dead organic material and from living hosts. These include *Trichoderma* spp., *Gliocladium* spp., *Talaromyces flavus*, and *Coniothyrium minitans*. These species tend to attack a broad range of plant pathogens using specialized structures and the production of various enzymes and antibiotics.

Production of cell wall degrading enzymes (CWDE) plays an important role in the mycoparasitic interaction. Lytic enzymes are able to hydrolyze a wide variety of polymers including proteins, cellulose, chitin, and DNA. Generally these enzymes are produced by the organism to decompose organic material and obtain nutrients such as carbon and nitrogen. However, they may be useful for biocontrol of pathogenic organisms, as chitin and cellulose are key components in the cell walls of many fungi. For example, *Trichoderma* and *Gliocladium* species produce cellulases, proteases and chitinases that are effective in the biocontrol of many fungal plant pathogens (Howell 2003; Kubicek and Harman 1998; Lorito et al. 1996).

2.4.1.4 Induced resistance

Induced systemic resistance (ISR) and systemic acquired resistance (SAR) are two types of induced resistance and are cellular responses in plants that are triggered by the presence of non-pathogenic bacteria or fungi. ISR and SAR confer resistance and long-term immunity against plant pathogens (Agrios 2005). Three different pathways have been identified in plant systems. Two of these pathways involve the production and accumulation of pathogenesis-related (PR) proteins such as chitinases, glucanases, thaumatins, oxidative enzymes (peroxidases, polyphenol oxidases, lipoxygenase), and low molecular weight compounds (phytoalexins) (Pieterse and van Loon 1999; Pieterse et al. 2001; Ryals et al. 1996; Schneider and Ullrich 1994). The pathway for SAR involves the accumulation of salicylic acid and is also referred to as salicylic acid-dependent. SAR generally is induced by avirulent infections and results in the immunity of the plant to future infection by plant pathogens. The second pathway, jasmonic-acid dependent is a type of ISR and involves the accumulation of jasmonic acid and ethylene. This pathway is generally induced by herbivory; the grazing of the plant by insects and nematodes. The third type of induced resistance is rhizobacteria-induced systemic resistance (RISR). This type of resistance is induced in plants by colonization of the roots by rhizosphere bacteria. Cornrath et al. (2002) referred to this phenomenon as “Priming” and is different from ISR and SAR because in the RISR pathway, the PR proteins and phytoalexins do not accumulate in the absence of attack by the plant pathogen. Once the plant is challenged by a pathogen, this system will induce a similar cascade of proteins as in the jasmonic-acid- and salicylic acid-dependent induced systems (Harman et al. 2004; Pozo et al. 2002). Priming has been shown to lower disease incidence in tobacco when

plants were preconditioned with methyl jasmonate; this resulted in a quicker and stronger lipid peroxidase and protein phosphorylation response (Dubery et al. 2000).

Fungi belonging to the genera *Trichoderma* and plant growth promoting rhizobacteria including *Pseudomonas*, *Enterobacter*, *Bacillus* and *Streptomyces* provide protection in plants against various viral, bacterial and fungal plant pathogens (Belimov et al. 2001; Harman et al. 2004; Pieterse et al. 2000; Pieterse et al. 2001). Seed treatment by several strains of *T. virens* (G-6, G-6.5 and G-11) reduced disease incidence of *R. solani* by up to 78% and was found to induce terpenoid phytoalexins (Howell et al. 2000). *Trichoderma harzianum* (T-1) and *T. virens* (T-3) were also found to be effective in the control of green-mottle mosaic virus of cucumber when added to the soil (Lo et al. 2000). Hoffland et al. (1996) found that pretreatment of radish seeds with *Pseudomonas fluorescens* strain WCS417 protected against various plant pathogens and increased yields.

2.5 Biocontrol of fusarium head blight

Several isolates of bacteria, fungi and yeast have been screened and identified as potential BCAs of FHB, the majority of which are bacteria and include *Pseudomonas* (Schisler et al. 2006; Perondi et al. 1996), *Bacillus subtilis*, *Paenibacillus* sp. (Stockwell et al. 1997), *Lysobacter enzymogenes* (Jochum et al. 2006), and *Brevibacillus* sp. (Palazzini et al. 2007). The actinomycete, *Streptomyces* sp. (Fulgueira et al. 1996) and several yeasts, including *Cryptococcus nodaensis* (Khan et al. 2001), *Sporobolomyces roseus* (Perondi et al. 1996; Luz 2000; Schisler et al. 2001) are also reported to show effective control. The number of fungal BCAs are fewer and include *Trichoderma*

harzianum (Inch and Gilbert 2007; Fernandez 1992), *Clonostachys rosea*, non-pathogenic *Fusarium* spp. ((Luongo et al. 2005; Kucuk and Kivanc 2008) and *Microsphaeropsis* sp. (Bujold et al. 2001). Various stages in the life cycle of *G. zae* can be targeted for biological control including prevention of spikelet infection and systemic movement within the rachis, survival of the pathogen in crop debris, and prevention of perithecia and ascospore development.

2.5.1 Spikelet infection

Wheat is most susceptible to infection from early flowering to soft dough stage (Zadoks scale 60-85), which usually lasts approximately two weeks (Anderson 1948; Fernando et al. 1997). Therefore a bioprotectant can be applied during this short window of opportunity. Stockwell et al. (1997) identified a bacterial species, *Paenibacillus macerans*, and a yeast species, *Sporobolomyces roseus*, which when sprayed on heads during anthesis reduced yield loss and increased 100-kernel weight. Khan et al. (2001) screened over 700 bacterial isolates obtained from wheat anthers. Approximately 5% of those isolates prevented development of FHB on heads in plant head bioassays and three strains were effective in controlling FHB. *Bacillus* strains (AS 43.3 and 43.4) and *Cryptococcus* strain OH 182.9 reduced disease severity by 77, 93 and 56% respectively. All 3 isolates resulted in an increase in 100-kernel weight (Khan et al. 2001). Jochum et al. (2006) found that *Lysobacter enzymogenes* strain C3 reduced FHB severity to less than 10% infected spikelets; control plants showed greater than 80% infection. Induced resistance and the production of chitinases were thought to be the main modes of action. Palazzini et al. (2007) identified two species *Brevibacillus* sp. (BRC263) and

Streptomyces sp. (BRC87B), which resulted in reduction in DON accumulation to undetectable levels in kernels.

2.5.2 Saprophytic survival and ascospore production on crop debris

Stages within the life cycle of *G. zae* that may be a target for control include the period of saprophytic survival in crop debris and perithecia and ascospore development. Infested crop debris is the main source of inoculum for disease development (Sutton 1982) and the amount of inoculum correlates with the density of crop residues (Dill-Macky and Jones 2000). Fernandez (1992) found a significant reduction in the saprophytic survival, colonization and re-isolation of various fungal pathogens, including *Fusarium graminearum* and *Cochliobolus sativus* on wheat straw treated with *Trichoderma harzianum* under field conditions. *Microsphaeropsis* sp. (isolate P130A) was found to be effective at reducing perithecial production of *G. zae* under both controlled and field conditions. Best control was achieved when the BCA was applied two weeks prior to application of a spore suspension of *G. zae*. Moderate control was seen if *G. zae* was co-inoculated or applied after *G. zae* (Bujold et al. 2001). Luongo et al. (2005) found that *Clonostachys rosea* and *Epicoccum nigrum* were able to reduce conidial production of *F. culmorum* and *F. graminearum* on wheat straw by 83 to 100% under field conditions. *Fusarium equiseti* and *F. oxysporum* also significantly reduced sporulation by more than 55%.

2.6 *Trichoderma harzianum*

2.6.1 Taxonomy

The genus *Trichoderma* contains 33 species, for many of which the sexual state has not been identified. This has made the classification of *Trichoderma* species difficult (Gams and Bissett 1998). Traditional taxonomy of the group was based upon morphological differences among the asexual sporulation structures. However in recent years, isozyme profiles, molecular data, sequencing of the ITS region, ribosomal DNA, and DNA fingerprinting has helped classify and identify species (Fujimori and Okuda 1994; Meyer et al. 1992; Ziman et al. 1994). *Trichoderma harzianum* was first classified in 1930 as *Sporotrichum narcissi* Tochinai & Shimada, later as *Trichoderma narcissi* (Tochinai & Shimada) Tochinai & Shimada in 1931, and then as *Trichoderma lignorum* var. *narcissi* (Tochinai & Shimada) Pidopl. in 1953. It was finally classified as *Trichoderma harzianum* Rifai, in 1969. *Trichoderma harzianum* Rifai belongs to the kingdom Eumycota, division Ascomycota, subdivision Pezizomycotina, order Hypocreales, family Hypocreaceae (Gams and Bissett 1998).

2.6.2 Biology

Trichoderma harzianum grows rapidly and sporulates abundantly in culture making it ideal for use in biological control. No known sexual state exists; the fungus reproduces entirely by the production of asexual spores (conidia) by mitotic division. Conidiation appears effuse, granular and powdery. The general appearance of the colony is yellowish-green to dark green. Conidiophores are regularly verticillate resulting in a pyramidal structure. The phialides are 10µm in length, flask-shaped, generally 3 to 4

verticillate and sometimes paired. The conidia (5.0 x 2.5µm) are smooth, subhyaline to pale green, and are subglobulose to obovoid. The reverse side of the colonies is generally a brownish yellow color due to the presence of yellowish crystals. Optimal temperature for mycelial growth on malt agar is 30°C with a maximum growth temperature of 36°C (Gams and Bissett 1998).

2.6.3. Biological control of plant pathogens

Trichoderma harzianum is one of the best known biocontrol agents and is effective against many plant pathogens including *Pythium* (Benhamou and Chet 1997), *Rhizoctonia* (Elad et al. 1984), *Botrytis* (Harman et al. 1996), *Fusarium* (Thangavelu et al. 2004), *Sclerotinia* (Barak et al. 1985) and *Phytophthora* (Sharifi et al. 2004).

Trichoderma harzianum, strain T-22, is commercially available and sold under the trade name RootShield™ (Bioworks, Geneva, NY). This product contains a hybrid species made by the protoplast fusion of two strains: *T. harzianum*, T-95, a rhizosphere-competent strain isolated from Columbian soil and T-12 from New York soil (Stasz et al. 1988). This strain is able to colonize plant root systems and provides long-term protection against plant pathogens. RootShield is sold as a planter box or in-furrow treatment and provides protection against various soil borne pathogens such as *Pythium* spp., *Fusarium* spp., and *Rhizoctonia* spp. (Harman et al. 1996). Control is comparable to and equally effective as a fungicide treatment when applied before infection. The antagonistic activity of T-22 is due to a combination of mycoparasitism, production of various CWDEs, competition, induced resistance, inactivation of the pathogen's enzymes, solubilization of inorganic plant nutrients, and antibiosis (Harman et al. 2004;

Howell 2003; Harman 2006). A second *T. harzianum* strain, T-39 (TRICHODEX, 20P[®]), is effective in the control of powdery mildew of grapes and *Botrytis cinerea*. The mode of action of this strain was directly attributed to competition for nutrients and disruption of lytic enzyme production by the pathogen (Elad and Kapat 1999).

2.6.4. Mechanisms of control

2.6.4.1 Induced systemic resistance

Trichoderma isolates have been shown to form beneficial associations with plants that result in improved seed germination, seedling establishment, increased disease resistance, and increased plant vigor and growth. Induced systemic resistance (IRS) occurs when *Trichoderma* enters into the root system of a plant, and causes a series of biochemical changes resulting in the confinement of *Trichoderma* to a limited area within the roots (Harman 2006; Shoresh and Harman 2008). Some of the elicitors of this plant defense response may include various proteins including enzymes, avirulence (avr)-proteins, oligosaccharides and cell wall fragments released by the plant caused by the production of CWDE (Hanson and Howell 2004). T-22 is a rhizosphere competent strain that can grow continuously within and alongside the plant and therefore may confer long-term resistance. Application of T-22 to the soil reduced foliage disease severity by *Alternaria solani* in tomato plants and conferred long-term resistance to the disease. Leaf infection was reduced by more than 90% for at least 100 days after treatment (Seaman 2003). Meyer et al. (1998) found that amending the soil with T-39 reduced infection and colonization of *B. cinerea* in the leaves of tomato, pepper, bean and tobacco by 25 – 100%. Cucumber roots inoculated with *T. asperellum* (formerly *T. harzianum*) (T-203)

resulted in a decrease in disease development by *Pseudomonas syringae* pv. *lachryman*. Increased peroxidase and chitinase activity, the deposition of callose on the inner surface of the infected cell walls and the production of several PR proteins and hydrolytic enzymes in roots and leaves during the early stages of root colonization (Yedidia et al. 1999; Yedidia et al. 2000; Yedidia et al. 2003). Root colonization of corn seedlings by T-22 has been shown to induce changes in the proteome of the shoot system (Shoresh and Harman 2008a). Shoresh and Harman (2008b) identified 91 of 114 up-regulated and 20 of 30 down-regulated proteins in the shoots. The majority of the up-regulated proteins were found to be involved in carbohydrate metabolism, photosynthesis, genetic information processing, amino acid metabolism, stress response, and defense and resistance. Several proteins were unknown or unidentified. Cotton roots inoculated with *T. virens* induced a proteinaceous elicitor Sm1, which is responsible for ISR against *Colletotrichum graminicola* (Djonovic et al. 2007). Similar levels of control of were achieved when just the purified Sm1 elicitor protein produced by *T. virens* was applied to cotton roots. Sm1 was found to trigger the production of reactive oxygen species and expression of defense-related genes (Djonovic et al. 2006).

2.6.4.2 Competition

Trichoderma harzianum competes aggressively for space in the rhizosphere and for nutrients exuded from the plant roots (Howell 2003). Competition is also thought to be one of the mechanisms involved in the control of *B. cinerea* in the phyllosphere of the host plant. Zimand et al. (1996) found a reduction in the germination and germtube biomass of *B. cinerea* on the leaf surface during the early interaction with *T. harzianum*.

The nutrient status of the soil affects efficacy of *T. harzianum* as a BCA. Chlamyospore germination of *F. oxysporum* was reduced when *T. harzianum* was added to soils with low concentrations of glucose and asparagine. However, when the soil was amended with glucose and asparagine at concentrations of 0.3 and 0.06 mg/g soil, respectively, chlamyospore germination was not significantly reduced (Sivan and Chet 1989).

Trichoderma harzianum also effectively colonized wound sites, thereby preventing the establishment of plant pathogens. *Pythium* infection was prevented on cucumber roots when *T. harzianum* was applied to soil containing pathogenic *Pythium* species (Thrane et al. 1997).

2.6.4.3 Mycoparasitism

Trichoderma harzianum is also an effective mycoparasite that can cause death of the host (Barnett and Binder 1973). The mycoparasitic interaction between *T. harzianum* and the host fungi, *B. cinerea*, *R. solani*, *Pythium*, and *Sclerotium rolfsii* is well documented (Chet et al. 1981; Kullnig et al. 2000; Inbar and Chet 1995; Lorito et al. 1996). There are four distinct stages that occur between *T. harzianum* and the host (Chet et al. 1990). The first is the attraction of *Trichoderma* hyphae by chemotrophism. Chet et al. (1981) observed atypical branch formation of *T. harzianum* when grown in close proximity to the host fungus. Amino acids and sugars are thought to trigger this response (Chet et al. 1990). The second is specific recognition of the host, either physically or chemically, involving lectins on the surface of the host and chitin oligomers. Lectins are glycoproteins which are involved in the interaction of the cell surface with its extra-cellular environment (Barondes 1981). The role of lectins in the early recognition of *R.*

solani by *T. harzianum* was demonstrated by Elad et al. (1983); they found that lectins on the mycelial surface of *R. solani* were agglutinated by pre-incubation of fucose or galactose. The surface of *T. harzianum* has galactose residues on its cell surface, which may be involved in recognition. Nylon fibers coated with purified lectins from *Sclerotium rolfsii* induced coil formation of *Trichoderma*; untreated fibers did not produce the response (Inbar and Chet 1995) suggesting that lectins are responsible for recognition of the host. The third stage of interaction between *Trichoderma* and an appropriate host involves the attachment and coil formation which is induced during contact between the two species (Barak et al. 1985; Omero et al. 1999). The hyphae of *T. harzianum* attach to the host via specialized hook and appressorium-like structures (Elad et al. 1984; Harman et al. 1981). Cell-to-cell contact appears to trigger *T. harzianum* to coil around the hyphae of the other organism (Elad et al. 1984). The last stage of mycoparasitism is the secretion of lytic enzymes which are essential for penetration and colonization and ultimately result in the cell death of the other organism (Elad et al. 1996).

Trichoderma harzianum produces various CWDEs including proteases (Flores et al. 1997), β -1,3-glucanases (Cruz et al. 1995) and chitinases (Baek et al. 1999; Woo et al. 1998; Limon et al. 1999). Biocontrol is usually not attributed to a single enzyme; rather enzymes act synergistically (Lorito et al. 1994). Chitin and β -1,3-glucan are major components of many fungal cell walls (Bartnicki-Garcia 1968). Chitinases are crucial for apical growth, cell division, and nutrient acquisition for saprophytic and mycoparasitic fungi (Papavizas 1985; Kuranda and Robbins 1991). Strains of *T. harzianum* which excrete higher levels of CWDE have higher levels of biocontrol activity. Kucuk and Kivanc (2008) identified four strains of *T. harzianum* which produced high levels of

glucanase and chitinase and significantly reduced the radial growth of *G. zea* by more than 50% in dual culture plate assays. Transformant strains that overexpress 33-kDa chitinase have increased antifungal activity towards *R. solani*. In dual cultures, transformants produced a large zone of lysis and were able to overgrow *R. solani* colonies. Inhibition of *R. solani* was also possible when grown on plates that were previously inoculated with the transformants; growth was reduced by 37 and 67% when compared to the control (Limon et al. 1999). Disruption of CWDE genes also demonstrates the relative importance of their role in biocontrol. Woo et al. (1999) created endochitinase mutants of *T. harzianum* by disrupting the ech42 gene encoding for 42-kDa endochitinase (CHIT42). The biocontrol activity of the mutants was reduced. In confrontation plate assays with the mutant *T. harzianum* strain, control against *B. cinerea* and *R. solani* was reduced by 40% compared with the wild type strain. Antifungal activity was restored if additional CHIT42 was applied. Control of *B. cinerea* on bean leaves was also reduced by 30% compared with the wild type. However, control of *Pythium ultimum* was not significantly different for the mutant versus the wild type strain. The relative importance of these enzymes in biological control is dependent on specific pathogen-host interactions (Lora et al. 1995).

2.6.4.4 Antibiosis

The ability to produce compounds varies among species and among isolates within the same species. Environmental conditions, such as the substrate, pH, and temperature, also will influence antimicrobial compound production (Brain and Hemming 1945; Brain and McGowan 1945). In addition, the genus *Trichoderma*

produces a wide variety of volatile and non-volatile compounds that have been found to be effective at suppressing many plant-pathogens (Dennis and Webster 1971). However, control is not attributed to a single compound, but rather to being part of a more complex system involving a number of different constituents. Numerous compounds have been identified and demonstrated to have effective biocontrol activity on various plant pathogens. Alkyl pyrones have been isolated from several *Trichoderma* species including *T. harzianum*, *T. viride*, *T. koningii* and *T. hamatum* (Ghisalberti and Savasithamparam 1991; Simon et al. 1998). Claydon et al. (1987) identified two strains of *T. harzianum* that produced the volatile pentyl analogues, 6-n-pentyl-2H-pyran-2-one and 6-n-pentenyl-2H-pyran-2-one. These secondary metabolites were effective at reducing the incidence of damping off in lettuce seedlings by *Rhizoctonia solani* during *in vitro* testing. Isonitriles, such as isonitrin A-D and isonitrinic acids E and F have been isolated from *Trichoderma* species and found to be effective against various fungi, and gram negative and gram positive bacteria (Fujiwara et al. 1982; Okuda et al. 1982). The polyketide harzianolide, was effective against *Gaeumannomyces graminis* under greenhouse conditions (Ordentlich et al. 1992). This antibiotic was also produced by a strain of *T. harzianum* which reduced the disease incidence of *F. oxysporum* f. sp. *melonis* and *F. oxysporum* f. spp. *vasinfectum* (Rebuffat et al. 1989). The secondary metabolites, T22azaphilone, 1-hydroxy-3-methyl-anthraquinone, 1,8-dihydroxy-3-methyl-anthraquinone, T39butenolide, harzianolide, and harzianpyridone, isolated from cell-free filtrates of isolates T-22 and T-39, were found to inhibit the *in vitro* growth of many plant pathogenic fungi, including *P. cinnamomi*, *B. cinerea*, *L. maculans*, *G.*

graminis var. *tritici*, *R. solani*, and *P. ultimum* (Vinale et al. 2006; Vinale et al. 2009).

The mode of action for many of these compounds is not known.

Trichoderma species and related genera also produce various types of antibiotic peptides, referred to as peptaibols. Peptaibols are short chain peptides (10-20 amino acids in length) and characterized by an acetylated N-terminus and an amino alcohol at the C-terminus with high amounts of α -aminoisobutyric acids (Iida et al. 1994). Peptaibols form voltage-gated ions in the membranes, which results in membrane disruption and hemolysis (Fox and Richards 1982). Over 200 peptaibols have been identified, sequenced, and compiled in a database at www.cryst.bbk.ac.uk/peptaibol (Whitmore and Wallace 2004). The peptaibols, trichozianines A and B were induced when *T. harzianum* was grown in culture media containing cell walls of *B. cinerea*, and were found to completely inhibit the mycelial growth and spore germination of *B. cinerea* (Schirmbrock et al. 1994).

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3.0 Screening and evaluation of potential antagonists for the biological control of fusarium head blight (FHB) of wheat incited by *Gibberella zeae*.

3.1 Abstract:

To identify potential biological control agents of fusarium head blight, 150 bacteria and 29 fungi were isolated from soil, wheat heads and crop debris from southern Manitoba. An additional 10 isolates of *Trichoderma harzianum* were obtained from the Canadian Collection of Fungal Cultures, CCFC (Ottawa, Ontario). The *T. harzianum* isolate, T-22 (RootShield) was included as a positive control. All strains were screened for inhibition of *Gibberella zeae* using confrontation plate assays *in vitro*, and wheat head, seed and straw assays *in planta*. In the plate assays, 6% of the bacterial, and 45% of the fungal isolates tested, including *Trichoderma harzianum*, *Chrysosporium* sp. and *Penicillium* spp., inhibited the growth of *G. zeae* by more than 50%. *Trichoderma* isolates showed the highest levels of inhibition and were able to over-grow *G. zeae*. None of the 6% bacterial isolates that reduced mycelial growth of *G. zeae* significantly improved seed germination, reduced FHB on wheat heads or perithecial production on wheat straw. The bacterial treatments decreased seed germination. None of the fungal isolates tested improved seed germination, and *Chrysosporium* sp. and *Penicillium* spp. significantly reduced germination rates by 30 – 52% compared to the control. Treatments with the fungal biocontrol agents applied to the wheat heads were not effective at suppressing FHB development. However, 14 of the 18 isolates tested, including six *Trichoderma* and two *Chrysosporium* isolates, significantly reduced perithecial production on wheat straw by 52-89% compared to the control. From this study, *T. harzianum* isolates were identified as the best potential candidates for the biocontrol of

FHB in field trials as they had no negative affects on seed germination and reduced reducing perithecial development on wheat straw.

3.2 Introduction:

Fusarium head blight (FHB) is an economically important disease of wheat and other small grain cereals. In Manitoba, *Gibberella zeae* (anamorph = *Fusarium graminearum*) is the main causal agent (Gilbert and Tekauz 2000). Several other species of *Fusarium* cause FHB including *F. acuminatum* Ellis and Everhart, *F. avenaceum* (Fr.) Sacc., *F. crookwellense* Burgess, Nelson, Toussoun, *F. culmorum* (W.G. Smith) Sacc., *F. equiseti* (Corda) Sacc., *F. poae* (Peck) Wollenw., and *F. sporotrichioides* (Sherb.). Of these, *F. graminearum*, *F. culmorum*, and *F. avenaceum* develop significant visual head blight symptoms (Miller 1994). Symptoms include premature bleaching, poorly filled kernels (also known as fusarium damaged kernels), and production of mycotoxins such as zearalenone, and deoxynivalenol (DON). These toxins cause significant gastrointestinal problems if consumed and may result in feed refusal in swine. Besides yield and quality losses, seedling germination and seedling vigor is reduced (Polley et al. 1991).

Several practices have been suggested to control FHB. Resistant varieties are not available in Canada, although varieties with moderate resistance to FHB including Canada western red spring 5602HR, Waskada and BW 365 (Seed Manitoba 2008) have recently been registered. However, under high disease pressure all varieties will develop disease symptoms of FHB to a greater or lesser degree (McMullen et al. 1997). Chemical control can help minimize disease incidence, but the efficacy of many of the currently registered fungicides has varied. Factors affecting the level of control include effectiveness, application timing, and efficiency of application. Foliar fungicides, such as tebuconazole (Folicur) and propiconazole (Tilt) have been found to be effective in reducing FHB severity and DON accumulation. Results have not consistent from year to

year (Mesterhazy and Bartok 1996; Wilcoxson et al. 1992). Plant diseases are difficult to treat once symptoms are observed and therefore fungicides should be applied prior to flowering. This might result in unnecessary applications in years when little disease develops. Predictive models based on climate data and location may help prevent over-use of chemicals (De Wolf et al. 2003). Burying crop debris may reduce inoculum levels (Khonga and Sutton 1988). However, recent studies have shown that conventional tillage does not substantially reduce disease incidence when compared to other tillage systems. Zero tillage resulted in only slightly higher levels of disease (71%) compared to conventional tillage (moldboard plow) (64%) with DON levels 11.1 ppm and 8.1 ppm, respectively (Dill-Macky and Jones 2000).

Biological control may be useful as part of an integrated pest management system when used in conjunction with planting resistant cultivars, and chemical and cultural control methods. Various stages within the life cycle of the pathogen may be targeted for control, including application of biocontrol agents (BCAs) to the wheat head at anthesis to prevent spore germination and fungal colonization during the susceptible period. Schisler et al. (2006) screened 738 potential BCAs isolated from anthers. Only 48 of the 738 were able to reduce FHB symptoms by 25-50% in greenhouse trials. Under field conditions, in two locations, *Pseudomonas* sp. AS 64.4 reduced disease severity by 63% and 46% compared to the controls. Other effective BCAs that have been identified include *Cryptococcus* strain OH 182.9, and *Bacillus* strains AS 43.3 and AS 43.4. These three isolates were found to increase 100-kernel weight and reduce disease severity by 56%, 77% and 93%, respectively (Khan et al. 2001). One way of increasing the effectiveness of BCAs may be to combine them with fungicide treatments. The bacterium, *Lysobacter enzymogenes* strain C3 was found to provide more effective

control and consistent results when the treatment was combined with tebuconazole treatments. Application of both the BCA and fungicide reduced disease severity to 40% compared to the treatment with BCA (47%) or fungicide (54%) alone (Jochum et al. 2006).

Other target sites in the life cycle include crop debris. Infected plant material left on the soil surface is the main source of inoculum (Sutton 1982). Application of BCAs to this material has been found to reduce the saprophytic colonization of *Fusarium* species. Isolates of *Clonostachys rosea* were found to suppress sporulation of *Fusarium culmorum* by 51- 100% and *F. graminearum* from 17-100% (Luongo et al. 2005). *Trichoderma harzianum* also was able to reduce colonization by *F. graminearum*; the pathogen was isolated from 19 to 68 % of the straw sampled over a 6-month period (Fernandez 1992). Bujold et al. (2001) were able to demonstrate a reduction in perithecial development on straw under both greenhouse and field conditions using *Microsphaeropsis* sp. The BCA significantly reduced ascospore production when applied 2 weeks before, at the same time and 4 weeks after inoculation with *G. zeae*. Treatment of seeds with a BCA may help improve seedling germination and stand development. Seed treatments with *Pseudomonas fluorescens* strains MKB 100 and MKB 249 reduced seedling blight caused by *F. culmorum* by 53-93% (Khan et al. 2005). To date no BCAs have been implemented in field-based biological control of FHB.

The objectives of the study were to: 1) evaluate potential antagonists of *G. zeae* in confrontation assays and to determine their efficacy for control of FHB and 2) investigate the effect of application of those antagonists shown to be effective in the confrontation plate assays to *G. zeae*-infected plant material including seeds, straw, and wheat heads.

3.3 Materials and methods

3.3.1 Isolation of potential antagonistic organisms

Fungi and bacteria were isolated from soils, wheat heads and crop debris from three Manitoba locations, Glenlea, Portage, and Tolstoi Tall-Grass Prairie Preserve in 2001. Plant materials were placed in sterile distilled water, sonicated for 1 minute, and 100 µl of the suspension spread onto potato dextrose agar (Difco Laboratories, Detroit) amended with the antibiotic streptomycin (PDA+S). Soil samples were plated using the soil dilution method (Rodriguez-Kabana 1967). For each location, 10 g of soil was sampled from 3 locations and pooled. One gram of soil was placed into 10 ml of distilled water and mixed. Further dilutions were created by transferring 1 ml from the stock solution to 9 ml of distilled water (10^{-2}), and from this another 1 ml was transferred to 9 ml of distilled water (10^{-3}).

For the isolation of fungal isolates, 100 µl from the 10^{-3} dilution, was transferred to Petri dishes containing PDA+S. Pure cultures of the isolates were obtained by transferring a small amount of mycelium from a single colony to a new Petri plate containing PDA+S. Isolates were then transferred to PDA slants and stored under mineral oil at 4°C until needed. The *Trichoderma harzianum* isolates used in this study were obtained from the CCFC and included DAOM 167088 (T-188), 175926 (T-926), 190830 (T-030), 199083 (T-083), 216472 (T-472), 222130 (T-130), 222136 (T-136), 222137 (T-137), 222151 (T-151), 222183 (T-183). Isolate T-22 is the commercial product RootShield™ a known biocontrol agent of many greenhouse crops and was selected as a positive control. The isolates used in this study and their sources are listed in Table 3.1.

For the isolation of bacterial isolates, 100 µl from the 10⁻³ dilution, was transferred to Petri dishes containing tryptic soy broth agar (TSBA) (Difco Laboratory) and incubated at 25°C for 24h. Pure cultures of the isolates were obtained by transferring cells of the strains of interest to a new Petri plate containing TSBA. Isolates were then transferred to 10% glycerol and stored at -80°C until needed.

3.3.2 Confrontation assays

Dual cultures were set up by placing 200 µl of a bacterial suspension (5 x 10⁸ CFU/ml) or mycelial plugs (5 mm) of fungal antagonists with *G. zeae*, 40 mm apart on a Petri dish containing TSBA (for the bacteria) or PDA (for the fungi). The plates were incubated under continuous fluorescent light at 20°C for 7 days. Growth and overgrowth of isolates were measured and percent mycelial inhibition was determined using the following formula:

$$\% \text{ mycelial inhibition} = \frac{(R1 - R2)}{R1} * 100$$

Where: R1 = average radius of mycelial growth on control plates
R2 = average radius of mycelial growth on assay plates

3.3.3 Head blight assay

To determine the effect of fungal and bacterial antagonists on the development of head blight symptoms a plant head bioassay was conducted. Three isolates of *G. zeae* used in this study included DAOM 192132, 192131, 170785 and were obtained from the Canadian Collection of Fungal Cultures (Ottawa, Ontario). These isolates were chosen

based on field trials and their ability to cause FHB under field conditions (R. Kaethler Pers. Comm. 2000). DAOM 192132 and 192131 were isolated from wheat kernels in Manitoba in 1988 and DAOM 170785 was isolated from *Zea mays* kernels in Ontario in 1979. Conidial suspensions were prepared by inoculating liquid carboxymethyl cellulose (CMC) (KH₂PO₄ 1.0g, MgSO₄.7H₂O 0.5g, NaCl 0.5g, FeSO₄.7H₂O 0.01g, MnSO₄.H₂O 0.01g, NH₄NO₃ 0.3g, CMC 10.0g) with cultures of *G. zea* grown on potato dextrose agar and incubated under aeration to promote conidial production. After 5 days at room temperature the spore concentration was adjusted by adding distilled water for a final concentration of 5×10^4 spores ml⁻¹. Inoculum preparation of the fungal antagonists were prepared by inoculating flasks containing autoclaved millet with mycelial disks of the isolates and incubating them for 8 days at 28°C under cool white fluorescent lights. Conidial suspensions were prepared by adding distilled water to the flasks and hand shaking for 2-3 minutes. The suspensions were then filtered through double layer cheesecloth and the spore concentration adjusted to 5×10^5 conidia ml⁻¹. One ml of Tween 20 (Polyoxyethylene 20-sorbitan monolaurate, Fisher Bioreagent[®] New Jersey) was added to the spore suspensions prior to inoculation. Inoculum preparations of the bacterial antagonists were prepared by inoculating 500 ml flasks containing tryptic soy broth. The flasks were incubated at 25°C at 250 rpm for 24 h.

The FHB-susceptible wheat cultivar, CDC Teal, was grown in 6 in. plastic pots with a 24 h regime of 22°C for 16 hours under sodium halide lights and at 17°C for 8 hours in the dark. Isolates that showed greater than 50% inhibition in the confrontation plate assays were tested on four heads per pot. At anthesis (Zadok's growth scale 69; Zadok et al. 1974), spikes were spray-inoculated with 3-5 ml of a spore suspension ($5 \times$

10^5 spores/ml) of the fungal antagonist or 5×10^8 spores/ml of the bacterial antagonist then 24 hours later with *G. zeae* at a concentration of 5×10^4 spores/ml. Inoculated plants were placed in a humidity chamber (100% RH) for 24 hours. Heads inoculated with *G. zeae* alone or with distilled water alone were included as controls. Ten heads per replication were rated for disease severity and incidence 21 days after inoculation. FHB index was calculated based on the calculation:

$$\text{FHB Index} = (\text{disease severity} * \text{disease incidence})/100$$

Each treatment was replicated four times. Treatment means were compared using Tukey's method ($P \leq 0.05$) (SAS Institute, Cary NC). Disease severity and incidence data were transformed using a square root transformation to normalize the distribution of the data.

3.3.4 Seed treatment

To determine the effect of BCAs on seed germination, isolates that showed greater than 50% inhibition in the confrontation plate assays were tested on naturally infested seed samples of the spring wheat cultivar, Roblin. Ten seeds per replicate were plated on PDA to determine initial levels of infection. There were a total of four replicates. Forty seeds per treatment were soaked in a fungal suspension (5×10^5 spores/ml), bacterial suspension (5×10^8 spores/ml) or water (control) for 24 h in a dessicator under vacuum to ensure the fungi/ bacteria were closely adhering to the seeds. These were then placed in a glass (Petri dish) humidity chamber and allowed to germinate. After 3-4 days seed germination was determined. The experiment was

conducted twice. Treatment means were compared using Tukey's method ($P \leq 0.05$) (SAS Institute, Cary NC).

3.3.5 Straw residue treatment

To examine the effect of BCAs on colonization and perithecial production of *G. zeae*, sterilized wheat stem tissues (with one node per 3 cm piece) were co-inoculated with 100 μ l spore suspension of a fungal antagonist (5×10^5 conidia ml^{-1}) or bacterial suspension (5×10^8 spores/ml) followed by a 100 μ l macroconidial suspension of *G. zeae*. The inoculated tissues were placed in Petri dishes containing 3 g sterile vermiculite. The treatments were replicated four times. Plates containing residues were placed at room temperature under UV light to induce perithecia development for 2-3 weeks (Tschanz et al. 1975). The vermiculite and residue pieces were watered every four days with 3 ml of sterile water and examined for perithecial production. Treatment means were compared using Tukey's method ($P \leq 0.05$) (SAS Institute, Cary NC). Perithecia data were transformed using a square root transformation to normalize the distribution of the data.

3.4 Results

Bacterial Antagonists

Of the 150 bacterial isolates, 6% were able to reduce mycelial growth of *G. zeae* in the confrontation plate assays. The bacterial isolates did not significantly increase seed germination and several strains reduced seed germination. FHB symptoms were not significantly reduced when wheat heads were co-inoculated with the bacterial isolates. Of

the nine bacterial isolates screen, none of them significantly reduced perithecial production on co-inoculated wheat straw (Appendix 1).

3.4.1. Confrontation assays

A total of 40 fungal isolates were screened for antagonistic activity against *G. zea* (DAOM 192132) confrontation plate assays. Of these, 18 (45%) of the isolates inhibited the growth of *G. zea* by more than 50% (Table 3.1). Species that showed a 50% reduction in mycelial growth of *G. zea* included all 12 of the *Trichoderma harzianum* isolates, 3 isolates of *Chrysosporium* sp., and the 3 *Penicillium* species. *Penicillium* strains were the most effective at reducing mycelial growth of *G. zea* with an average mycelial inhibition of 77%. *Chrysosporium* and *Trichoderma* strains were also reduced average radial mycelial growth of *G. zea* by 51 and 64%, respectively (Figure 3.1). All *T. harzianum* isolates produced a yellow pigment in the agar medium. The ability for potential BCAs to over-grow *G. zea* in culture varied. The majority of fungal isolates did not over-grow *G. zea*. However, 10 of the *T. harzianum* isolates, all 3 strains *Chrysosporium* sp., two *Aspergillus* spp. and one *Mucor* sp. were able to over-grow *G. zea* by 1 to 32 mm (Table 3.1).

3.4.3 Seed treatment

Artificially infested seed samples of spring wheat cultivar, Roblin, were inoculated with the 18 fungi which inhibited growth of *G. zea* by more than 50% in confrontation plate assays. The germination rate of the untreated control sample was 73%. No isolate of *T. harzianum* significantly improved seedling germination, but nor did they significantly reduce germination. The other isolates, *Chrysosporium* sp. (F-23, F-24,

and F-35) and the *Penicillium* spp. (F-4, F-5, and F-19) significantly decreased germination rates; only 30-52% of the seeds germinated (Figure 3.2).

3.4.4 Head blight assay

The 18 antagonistic isolates identified in the confrontation plates assays were evaluated to determine their efficacy in controlling FHB symptoms of wheat incited by 3 strains of *G. zeae*. There was no significant decrease in disease symptoms on heads treated with the fungal antagonists as indicated by the FHB index. Two of the *G. zeae* isolates (DAOM 192132 and 192131) had significantly higher levels of disease than isolate DAOM 170785 (Table 3.2). The *Penicillium* and *Chrysosporium* isolates resulted in discoloration of the wheat heads 3 to 5 days after inoculation with the fungal isolates.

3.4.5 Straw residue treatment

Strains of *G. zeae* differed significantly in their ability to produce perithecia under laboratory conditions. Isolates 192132 and 170785 produced more perithecia than isolate 192131, with an average of 135, 123 and 44 perithecia per 3 cm piece of wheat stem tissue, respectively. Fourteen of the 18 isolates evaluated, including seven *T. harzianum* and 2 *Chrysosporium* isolates (F-24, and F-35) significantly reduced perithecial development by more than 50% (Table 3.3).

Table 3.1: List of fungal isolates, their sources, mycelial growth inhibition (%), and their overgrowth (mm) of *Gibberella zeae* (DAOM 192132) in confrontation plate assays after 7 days.

Isolate #	Identification	Source	Mycelial Inhibition (%)	Over-growth of <i>G. zeae</i> (mm)
F-4	<i>Penicillium</i> sp.	Soil (Tolstoi)	82.4	0
F-9	<i>Penicillium</i> sp.	Soil (Tolstoi)	70.6	0
F-19	<i>Penicillium</i> sp.	Soil (Tolstoi)	76.5	0
F-23	<i>Chrysosporium</i> sp.	Soil (Tolstoi)	49.4	32
F-24	<i>Chrysosporium</i> sp.	Soil (Tolstoi)	50.6	10
F-35	<i>Chrysosporium</i> sp.	Soil (Tolstoi)	52.9	30
F-28	Unknown1	Soil (Tolstoi)	23.5	0
F-33	Unknown2	Soil (Tolstoi)	16.5	0
F-37	Unknown3	Soil (Tolstoi)	5.9	0
F-39	Unknown4	Soil (Tolstoi)	8.2	0
F-22	<i>Aspergillus</i> sp.	Soil (Tolstoi)	32.9	1
F-27	<i>Aspergillus</i> sp.	Soil (Tolstoi)	40.0	2
F-38	<i>Mucor</i> sp.	Soil (Tolstoi)	32.9	15
F-31	* <i>Trichoderma harzianum</i>	Soil (Tolstoi)	58.8	0
T-188	* <i>Trichoderma harzianum</i>	Alpine eutric soil (Alberta)	69.4	1
T-926	* <i>Trichoderma harzianum</i>	Log on the ground (Ontario)	64.7	0
T-030	* <i>Trichoderma harzianum</i>	Gas Reactor (Ontario)	65.9	9
T-083	* <i>Trichoderma harzianum</i>	Soil sample (Ontario)	64.7	25
T-472	* <i>Trichoderma harzianum</i>	Unknown (CCFC)	65.9	9
T-130	* <i>Trichoderma harzianum</i>	Cucumber plant (BC)	58.8	20
T-151	* <i>Trichoderma harzianum</i>	Mushroom farm (Pennsylvania)	62.4	23
T-183	* <i>Trichoderma harzianum</i>	Mushroom farm (unknown)	60.0	20
T-136	* <i>Trichoderma harzianum</i>	Mushroom farm (Ontario)	60.0	5
T-137	* <i>Trichoderma harzianum</i>	Mushroom farm (unknown)	60.0	8
T-22	<i>Trichoderma harzianum</i>	RootShield™	74.1	12
F-2	<i>Fusarium equiseti</i>	Soil (Portage)	38.8	0
F-8	<i>Fusarium equiseti</i>	Soil (Portage)	48.2	0
F-25	<i>Fusarium culmorum</i>	Soil (Portage)	45.9	0
F-1	<i>Fusarium poae</i>	Soil (Portage)	35.3	0
F-40	<i>Fusarium sporotrichioides</i>	Soil (Portage)	42.4	0
F-3	<i>Alternaria</i> sp.	Soil (Portage)	23.5	0
F-6	<i>Alternaria</i> sp.	Soil (Portage)	11.8	0
F-21	<i>Alternaria</i> sp.	Soil (Portage)	25.9	0
F-5	Unknown5	Soil (Portage)	8.2	0
F-16	Unknown6	Soil (Portage)	23.5	0
F-17	Unknown7	Soil (Portage)	14.1	0
F-10	<i>Fusarium pseudograminearum</i>	Soil (Portage)	43.5	0
F-15	<i>Fusarium culmorum</i>	Wheat Head (Portage)	30.6	0
F-16	<i>Fusarium equiseti</i>	Wheat Head (Portage)	28.2	0
F-26	<i>Alternaria</i> sp.	Wheat Head (Portage)	18.8	0

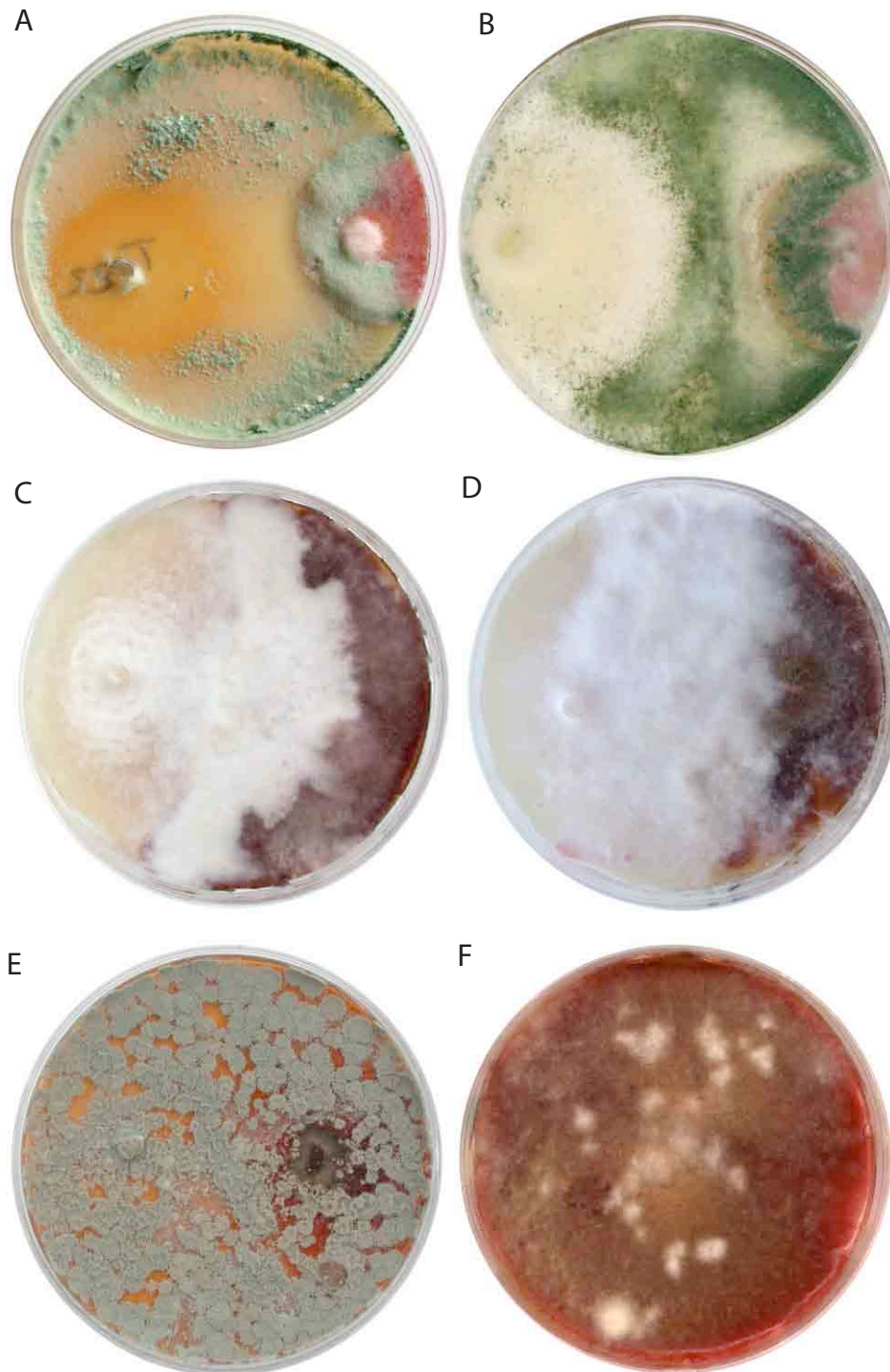


Figure 3.1: Confrontation plate assays on potato dextrose agar 7 days after inoculation of A) *Trichoderma harzianum* T-51, B) *T. harzianum* T-22, C) *Chrysosporium* sp. F-23, D) *Chrysosporium* sp. F-35, E) *Penicillium* sp. F-4, and F) *Gibberella zeae* (control).

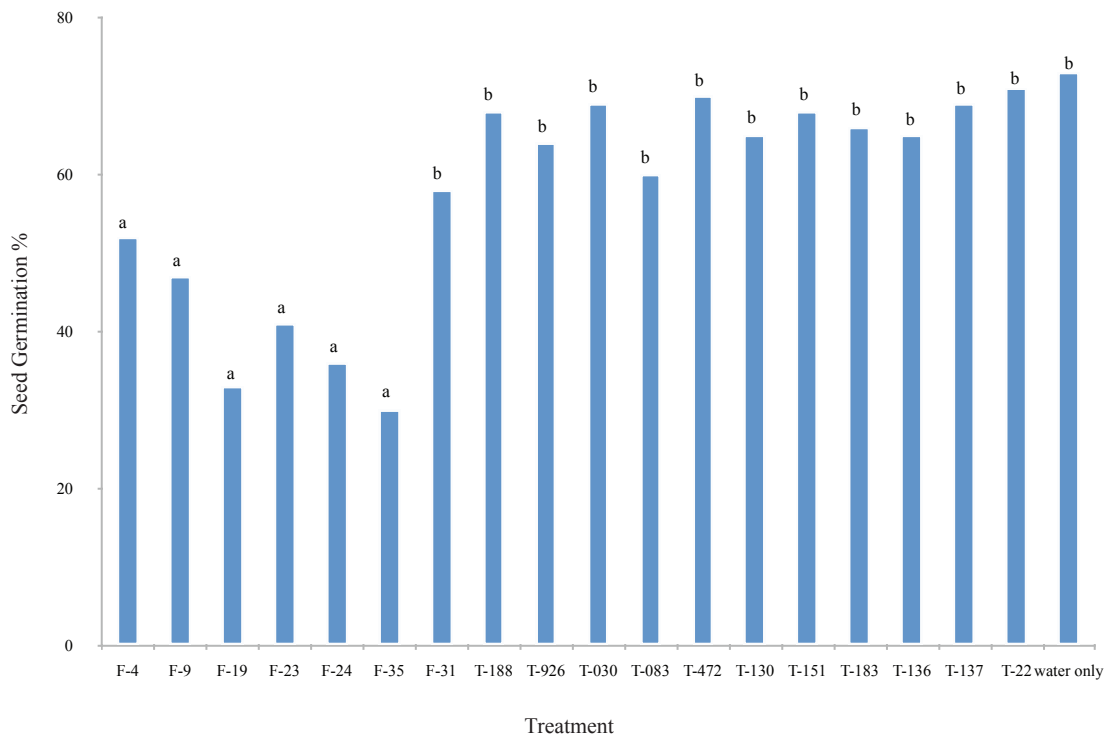


Figure 3.2: Average percent seed germination of naturally infected seeds of the spring wheat Roblin treated with a spore suspension of fungal antagonists or water only. Average value for two experiments, n=80 seeds/treatment. Values of the bars with the same letters are not significantly different at $P \leq 0.05$ according to Tukey's test.

Table 3.2: Effect of treatment with a spore suspension of fungal antagonists on disease as measured by the FHB index of fusarium head blight caused by three *Gibberella zeae* isolates (DAOM 19132, 192131, and 170785) on wheat cultivar CDC Teal.

Treatment	<i>Gibberella zeae</i> isolate		
	192132	192131	170785
	^x FHB Index	^x FHB Index	^x FHB Index
F-4	72	58	28
F-9	77	71	32
F-19	83	65	26
F-23	83	68	28
F-24	72	72	31
F-35	78	63	32
F-31	80	62	38
T-188	86	67	41
T-926	85	69	34
T-030	73	63	38
T-083	78	77	32
T-472	78	66	37
T-130	77	69	26
T-151	79	70	30
T-183	75	62	32
T-136	85	69	31
T-137	86	72	36
T-22	77	69	30
<i>G. zeae</i> only	86	71	32
Water only	0	0	0

Ten heads per replication were rated for disease severity and incidence

Average value for two experiments

Within rows values with no letter, or followed by the same letter, are not significantly different at ($P \leq 0.05$).

^xFHB Index = (disease severity * disease incidence)/100

Table 3.3: Effects of fungal antagonists on perithecial development of *Gibberella zeae* (isolates DAOM 192132, 192131, and 170785) under controlled conditions on wheat straw.

Treatment	<i>Gibberella zeae</i> Isolates					
	192132		192131		170785	
	Average Number of Perithecia ^z	Perithecia Reduction (%) ^y	Average Number of Perithecia ^z	Perithecia Reduction (%) ^y	Average Number of Perithecia ^z	Perithecia Reduction (%) ^y
F-4	128	5	41	7	101	18
F-9	112	17	38	14	99	20
F-19	102	24	30	32	118	4
F-23	110	19	23	48	132	0
F-24	38*	72	10*	77	42*	66
F-35	29*	79	9*	80	37*	70
F-31	99	27	22*	50	87	29
T-188	95*	30	41	7	84	32
T-926	127	6	46	0	113	8
T-030	65*	52	28	36	78	37
T-083	94	30	37	16	83	33
T-472	17*	87	6*	86	22*	82
T-130	29*	79	24	45	27*	78
T-151	42*	69	26	41	56*	54
T-183	30*	78	19*	57	37*	70
T-136	126	7	69	0	116	6
T-137	105	22	61	0	119	3
T-22	15*	89	3*	93	16*	87
<i>G.zeae</i> only	135 ^a	0	44 ^b	0	123 ^a	0
Water only	0	-	0	-	0	-

^z Average is based on 4 replicates and two experiments (n=48)

Values are back-transformed from least squares means of arc-sine square root transformed data.

Within rows values followed by a different letter are significantly different at ($P \leq 0.05$).

*Within columns, values are significantly different from the controls according to Tukey's least significant difference ($P \leq 0.05$)

^y Perithecial reduction as a percent of control.

3.5 Discussion

Pre-selection of potential candidates is generally done *in vitro* on agar plates. This allows for the relatively rapid selection and identification of potential BCAs. However, a number of factors may influence the biocontrol activity of isolates, such as growth medium, temperature and light (Schisler and Slininger 1997). Correlations between *in vitro* and *in planta* results are not always constant and may differ dramatically (Lindow 1988). Only 6% of the bacterial isolates evaluated were able to reduce mycelial growth of *G. zea* in confrontation plates assays by more than 50%. However, none of the bacteria significantly reduced *G. zea* *in planta*. It is possible that the bacterial isolates were unable to colonize and survive on the wheat straw or heads. Almost half fungal isolates (18/40) evaluated in this study were able to reduce mycelial growth of *G. zea* in confrontation plates. Half (9/18) of those isolates were able to significantly reduce perithecial development of *G. zea*. This is the first report of *T. harzianum* and *Chrysosporium* sp. reducing perithecial development on wheat straw. Bujold et al. (2001) were able to reduce perithecia and ascospore production of *G. zea* on both wheat and corn stubble when they applied the saprophytic fungus, *Microsphaeropsis* sp. 2 weeks prior, same time or 2 weeks after inoculation with *G. zea*. *Trichoderma harzianum* has been shown to reduce saprophytic colonization of wheat straw under field conditions (Luongo et al. 2005; Fernandez 1992).

Penicillium species colonized the Petri dishes within 4 days, which resulted in reduction of growth of *G. zea*, suggesting that the mode of action was primarily competition. Many fungi can produce fungicidal metabolites that may diffuse through the medium causing a reduction in growth. *Penicillium* species are known to produce a wide

variety of antimicrobial compounds (Ciegler et al. 1971). *Trichoderma harzianum* and *Chrysosporium* sp. were able to overgrow *G. zeae* in culture suggesting that mycoparasitism may be involved. Both species are known to produce antimicrobial compounds, such as alkyl pyrones (Ghisalberti and Sivasithamparam 1991), 6-n-pentyl-2H-pyran-2-one and 6-n-pentenyl-2H-pyran-2-one (Claydon et al. 1987), isonitriles (Fujiwara et al. 1982), polyketides, harzianolide (Ordentlich et al. 1992), and extracellular enzymes, including proteases (Flores et al. 1997), β -1,3-glucanases (Cruz et al. 1995) and chitinases (Baek et al. 1999) which may be involved in mycoparasitism (Alfonso et al. 1991, El-Katatny et al. 1999). *Trichoderma* species and related genera are also known to produce various types of antibiotic peptides, referred to as peptaibols (Figure 2.5). Peptaibols are short chain peptides (10-20 amino acids in length) and characterized by an acetylated N-terminus and an amino alcohol at the C-terminus with high amounts of α -aminoisobutyric acids (Iida et al. 1994).

Non-pathogenic isolates of *Fusarium* may be strong competitors against pathogenic species. In this study only potentially pathogenic *Fusarium* species were isolated. *Fusarium equiseti* was able to reduce the mycelial growth of *G. zeae*, but only by 48%. Other *Fusarium* species evaluated included *Fusarium sporotrichioides*, *F. culmorum*, and *F. pseudograminearum*, but owing to the potential for these species to cause FHB and seedling blight and their limited success in the confrontation plate assays they were not further evaluated for an effect on seed germination or to reduce FHB or perithecial production.

Trichoderma harzianum neither negatively or positively affected seed germination indicating that the strains would not interfere with stand establishment.

Further evaluation is required to determine the long-term effects on seed germination and seedling growth. *Trichoderma harzianum* has been shown to be an effective rhizosphere colonizer and to increase seedling establishment. When added as a seed treatment, *T. harzianum* grows with the developing root system and provides protection from soil-borne pathogens (Harman 2000). Three *Penicillium* spp. and three isolates of *Chrysosporium* sp. decreased seed germination suggesting that these species would not be effective BCAs and might be detrimental to seedling development and stand establishment. Even though three *Chrysosporium* isolates were able to reduce perithecial development such a species would not be an option for biocontrol because of its effect on seed germination.

In this study, isolates of *T. harzianum* were identified as potential BCAs in controlling perithecial development of *G. zeae* on wheat straw. Also these isolates showed no negative effects on seed germination which is an important factor to consider if applying *T. harzianum* strains to crop residues on the soil.

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4.0 The Effect of Application time of *Trichoderma harzianum* on perithecial production of *Gibberella zeae* on wheat straw

4.1 Abstract

To determine the effects of application time a spore suspensions or cell-free filtrate of *Trichoderma harzianum* isolates were evaluated for their effectiveness in reducing perithecial and ascospore production of *Gibberella zeae* on wheat straw. Isolate T-22, which is registered in the US as a biological control agent (RootShield™), was included in the study as a positive control. When spore suspensions of *T. harzianum* strains were co-inoculated with *G. zeae* all eleven isolates of *T. harzianum* significantly reduced perithecial development on wheat straw. Five *T. harzianum* isolates, including T-22, reduced perithecial formation by 70% or more. Isolates of *G. zeae* tested in this study varied in their ability to produce perithecia. Isolate 192132 produced the greatest number of perithecia and was used to further evaluate the effect of application time of the biological control agents. Perithecial reduction was highest (96-99%) when *T. harzianum* spore suspension or cell-free filtrate was applied to straw 24 hours prior to inoculation with *G. zeae*. Control was less effective when *T. harzianum* was applied at the same time (co-inoculated) or 24 hours after *G. zeae*. Field trials showed significant reduction in perithecia numbers on residues treated with *T. harzianum* prior to placement on the soil surface. Both *T. harzianum* and *G. zeae* were re-isolated from residues sampled after 30 and 60 days of exposure to the environment.

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4.2 Introduction

Fusarium head blight (FHB) is an important disease of wheat and other small grain cereals. The principal pathogen associated with FHB in Manitoba is *Gibberella zae* (Schwein.) Petch (anamorph = *Fusarium graminearum* Schwabe) (Gilbert et al. 2006). Economic losses may arise from the accumulation of the mycotoxin, deoxynivalenol (DON), which makes the grain unfit for human and animal consumption. Milling, baking, and pasta-making qualities are altered when grain is contaminated with DON (Charmley et al. 1994). In 1993, during Manitoba's most severe outbreak on record, losses were conservatively estimated at \$75 million dollars and annual losses have since been estimated at approximately \$50 million (Gilbert et al. 1994; Gilbert et al. 2001).

Gibberella zae over-winters on infested crop residues and gives rise to perithecia in the spring (Sutton 1982; Paulitz 1996; Inch et al. 2005). Adequate rainfall in spring favors the development of perithecia which subsequently produce ascospores later in the growing season. Wind-borne ascospores are regarded as the primary source of inoculum. They are forcibly discharged from perithecia 2-5 days after a rainfall event and then can be deposited on heads of susceptible crops at or around the time of anthesis (Paulitz 1996; Inch et al. 2005). Macroconidia are produced on infected heads and crop residues. These are splash-dispersed by rain and may be important for the secondary infection of tillers later in the growing season (Fernando et al. 1997).

Several practices may control FHB and reduce inoculum levels. These include crop rotation of wheat and other susceptible small grain cereals with non-host crops such as canola or alfalfa (Sutton 1982; Parry et al. 1995), and plowing under of crop residues to eliminate inoculum by accelerating decomposition and blocking ascospore dispersal

(Teich and Hamilton 1985). With the increase in zero tillage practice, large quantities of crop residues remain on the soil surface that can support *G. zeae* over winter and give rise to primary inoculum in the spring (Dill-Macky 1998; Dill-Macky and Jones 2000; Inch and Gilbert 2003).

In the several studies that report success for biological control of FHB two main approaches have been investigated. The first involves the application of antagonist organisms such as *Lysobacter enzymogenes*, *Bacillus subtilis*, and *Cryptococcus* species to wheat heads (Yuen et al. 2003; Schisler et al. 2002; Khan et al. 2001; McMullen and Bergstrom 1999). The second approach examines the efficacy of application of antagonistic fungi, such as *Microsphaeropsis* species (Bujold et al. 2001), *Trichoderma harzianum* (Fernandez 1992), non-pathogenic *Fusarium* species, *Clonostachys rosea* and *C. cladosporioides* (Luongo et al. 2005) to crop residues. *Trichoderma* is one of the most studied biological control agents. It has been implicated in the control of many plant pathogens including *Pyrenophora tritici-repentis* and *Mycosphaerella graminicola* (Perello et al. 2006), *Rhizoctonia solani* (Baek et al. 1999; Howell et al. 2000), *Botrytis cinerea* (Elad and Kapat 1999; Kapat et al. 1998) and *Sclerotium rolfsii* (El-Katatny et al. 2001; Wells et al. 1972).

Several mechanisms of control using *T. harzianum* have been identified including mycoparasitism, production of antibiotics, competition, and production of various enzymes including chitinases and glucanases (Bélanger et al. 1995; El-Katatny et al. 2001; Inbar and Chet 1995; Lorito et al. 1996; Schirmbock et al. 1994; Sivan and Chet 1989). *Trichoderma harzianum* has been shown to reduce colonization of straw residues by *Fusarium* species (Fernandez 1992) and to cause a reduction in mycelial growth in

culture (Chapter 3.0), but has not been studied for the effect on the production of perithecia. Application of antagonistic organisms to *G. zeae*-infested crop residues targeting saprophytic colonization, perithecia and ascospore development may reduce primary inoculum and potentially accelerate decomposition of residues. This would be an important consideration for residue management and might be used as part of an integrated pest management system. The objectives of the study were to: 1) evaluate the efficacy of *T. harzianum* spore suspensions and cell-free filtrates as biocontrol agents to reduce perithecia, ascospore and macroconidia production of *G. zeae* on wheat straw residue and; 2) determine the effects of time of application of spore suspensions and cell-free filtrate *T. harzianum* on perithecial production of *G. zeae*.

4.3 Materials and methods

4.3.1 Microorganisms and cultivation

Ten *Trichoderma harzianum* isolates (treatments) used in this study were obtained from the Canadian Collection of Fungal Cultures, CCFC (Ottawa, Ontario) and included T-030 (DAOM 198030), T-472 (DAOM 216472), T-130 (DAOM 222130), and T-151 (DAOM 222151) and T-183 (DAOM 222183), T-083 (DAOM 199083), T-088 (DAOM 167088), T-926 (DAOM 175926), T-136 (DAOM 222136), T-137 (DAOM 222137). An additional isolate T-22 (strain KRL-AG2), which is registered in the US as a biological control agent, RootShield™ (Bioworks, New York), was also included as a positive control. T-22 is a hybrid species made by the protoplast fusion of two strains, *T. harzianum*, T-95 and T-12 (Stasz et al. 1988). The *G. zeae* isolates 1H (DAOM 192132), 1L (DAOM 192131) and N1 (DAOM 170785) were obtained from the Canadian

Collection of Fungal Cultures (Ottawa, Ontario) and were chosen based on pathogenicity and ability to produce perithecia on residue under laboratory and field conditions. DAOM 192132 and 192131 were isolated from wheat kernels in Manitoba in 1988 and DAOM 170785 was isolated from *Zea mays* kernels in Ontario in 1979. All cultures were grown on potato dextrose agar (PDA) (39 g Difco PDA, 1L distilled water) for 5-7 days under fluorescent white light at 20°C. Cultures were then stored at 5°C until needed.

4.3.2. Inoculum preparation

Petri plates of potato dextrose agar (PDA) containing each *T. harzianum* culture were flooded with sterile distilled water and the spores gently scraped off from surface. The suspension was filtered through a double layer of cheesecloth and number of spores were determined using a hemacytometer. The suspension was adjusted to a final concentration of 5.5×10^5 spores/ml by adding sterile distilled water. A surfactant (Tween 20TM) was added just prior to inoculation (0.2 ml per 100ml inoculum). Cell-free filtrate was also prepared by inoculating 500 ml of liquid organic medium (glucose 10 g, peptone 1g, yeast extract 0.1g, KH₂PO₄ 1 g, MgSO₄·7H₂O 0.3g, distilled water 1L) with a 200 µl spore suspension (5.5×10^5 spores/ml) of *T. harzianum*. The flasks were placed on a rotary shaker set at 100 rpm under cool white fluorescent light (Sylvania cool white 30W) at room temperature for 10 days. The cultures were filtered through Whatman #1 filter paper (Maidstone, England) and then through a 0.22µm Polyethersulfone, sterilizing, low-protein binding membrane (Corning, NY). Non-inoculated organic medium was also filtered and served as a control.

A macroconidial suspension of *G. zea* was prepared using fresh fungal cultures grown on PDA. The cultures were cut into 1 cm sections and added to streptomycin-amended carboxymethyl cellulose (CMC) liquid medium (carboxymethyl cellulose 15 g, NH_4NO_3 1g, KH_2PO_4 1g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5g, yeast extract 1 g, distilled water 1L, streptomycin sulfate 0.2g added to the cooled medium). The flask was attached to a vacuum line and incubated under aeration to promote macroconidial production. After 7 days at room temperature, macroconidia were counted using a hemacytometer and the suspension adjusted to a final concentration of 5×10^4 macroconidia/ml by adding sterile distilled water. A surfactant (Tween 20TM) was added just prior to inoculation (0.2 ml per 100ml inoculum).

4.3.3 Evaluation of the efficacy of *Trichoderma harzianum* spore suspension for the biological control of perithecial production of *Gibberella zea* on wheat stem tissue

To determine the effects of *T. harzianum* spore suspension on the production of perithecia of three isolates of *G. zea* on wheat straw, six pieces of sterilized straw, 3 cm long and including one node, were inoculated by pipette with 200 μl of a macroconidial suspension of a single isolate of *G. zea* (5×10^4 macroconidia/ml) followed 15 minutes later with 200 μl of a spore suspension (5.5×10^5 spores/ml) of one of the eleven *T. harzianum* isolates (including T-22). These were then placed in Petri dishes containing 3g of sterile moistened vermiculite. Each treatment had 4 replicates. Plates containing the treated straw were placed in a randomized complete block design under fluorescent cool white and UV light to promote perithecial development (Tschanz et al. 1976). Moisture

levels of the vermiculite were monitored daily and sterile distilled water was added when necessary. The experiment was conducted twice.

4.3.4 Effects of application time of spore suspension or cell-free filtrate of *Trichoderma harzianum* on perithecial production of *Gibberella zeae* in vitro

To determine if the time of application of *T. harzianum* spore suspension or cell-free filtrate influences perithecial production three application times were chosen; application of *T. harzianum* 24 hours before *G. zeae* (-24 h), at the same time (0 h), or applied 24 hours after *G. zeae* (+24 h). The *T. harzianum* isolates that reduced *G. zeae* perithecia numbers by more than 70% in the previous experiment were applied to pieces of sterilized straw (3cm long plus one node). Six pieces of straw per isolate (treatment) were inoculated by pipette with 200 µl of a spore suspension or cell-free filtrate of *T. harzianum* for each application time. Straw inoculated with either *G. zeae* alone or cell-free filtrate of *T. harzianum* were included as controls. Petri dishes containing 3g of sterile moistened vermiculite and treated straw were placed under fluorescent cool white and UV light in a randomized complete block design. Each treatment had 4 replicates. After 3 weeks, all perithecia on the straw were counted and average number of perithecia per piece was calculated. One week later, ascospores were trapped and counted. Ascospores, discharged from mature perithecia adhered to the lid of the Petri plate and were suspended in 5 ml sterile distilled water and counted. The average number of ascospores/ml was determined using a hemacytometer. The average number of macroconidia was estimated by placing the residue into 10 ml of distilled water and

shaken vigorously for 30 seconds and numbers were determined using a hemacytometer. The experiment was conducted twice.

4.3.5 Effect of *Trichoderma harzianum* isolates on perithecial production of *Gibberella zeae* on straw residue under field conditions

Isolates of *T. harzianum* that showed reduction of perithecia development *in vitro* were also evaluated in the field. Pieces of water-soaked autoclaved wheat straw residue (3 cm long and including one node) were immersed in a macroconidial suspension (5.0×10^4 spores/ml) of *G. zeae* isolate 192132 for 5 minutes. The inoculated residues were transferred to filter paper and air-dried. After 15 minutes the residues were sprayed with a spore suspension (5.5×10^5 spores/ml) of a single *T. harzianum* isolate. Residue inoculated only with *G. zeae* served as the control. Each treatment consisted of six pieces of straw (3 cm long and including one node). There were 4 replications for each sample time. In the first week of June in 2003 and 2004 each experimental unit was placed in a separate nylon mesh bag and all bags were placed on the soil surface and secured using metal pegs, in a randomized complete block design at a research farm in Portage la Prairie, MB.

Four bags per treatment at each sample time were collected after 30 and 60 days of exposure to the environment in 2003 and 2004. Straw residues were surface-sterilized in a 1% NaOCl₃ solution for 1 minute and rinsed in distilled water. The sterilized residues were examined for perithecia and numbers were counted on each piece of straw. They were then placed on PDA amended with 0.2 g streptomycin sulfate and incubated under fluorescent white light for 7 days at 20°C. Colonies of *T. harzianum* and *G. zeae* were

identified based on colony and spore morphology. Presence or absence of *T. harzianum* and *G. zea*e from straw was recorded and percentage of recovery was calculated.

4.3.6 Statistical analysis

The main effects of isolate differences of *G. zea*e, *Trichoderma* treatments, application time on perithecia, ascospore and macroconidia formation, and re-isolation of *G. zea*e and *T. harzianum* were determined using ANOVA (SAS procedure PROC GLM). Treatment means were compared using Tukey's method ($P \leq 0.05$) (SAS Institute, Cary NC).

Perithecia data were transformed using a square root transformation, in order to normalize the distribution.

4.4 Results

4.4.1 Evaluation of the efficacy of *Trichoderma harzianum* spore suspension in the biological control perithecial production of *Gibberella zea*e

The ability of three *G. zea*e isolates to produce perithecia on straw varied (Table 4.1). Significantly higher numbers of perithecia were produced with isolate 192132 (143) compared to isolates 170705 (103) and 192131 (36). All eleven *T. harzianum* isolates significantly reduced perithecia numbers for isolate 192132. However, the efficacy of *T. harzianum* to reduce number of perithecia varied among different *G. zea*e isolates. Five *T. harzianum* isolates (T-22, T-130, T-151, T-183, T-472), significantly reduced the number of perithecia by more than 70% when a spore suspension was co-inoculated on wheat straw with all isolates of *G. zea*e. The most effective *T. harzianum* isolates were T-

472 and T-22, which consistently reduced number of perithecial produced by 83-89% across the three *G. zeae* isolates (Figure 4.1).

4.4.2 Effect of application time of spore suspension or cell-free filtrate of *Trichoderma harzianum* on perithecial production of *Gibberella zeae* on straw

The ANOVA indicated that there was a significant interaction between application time and treatment with *T. harzianum* ($P \leq 0.05$) (Appendix 2). Perithecial production was reduced on straw treated with either a spore suspension of *T. harzianum* or cell-free filtrate, 24 hours before inoculation with *G. zeae*. Control was less effective, but still significant, when *T. harzianum* or cell free-filtrate were co-inoculated or applied 24 hours after *G. zeae* inoculation (Tables 4.2 and 4.3). Straw treated with *T. harzianum* in which low numbers of perithecia developed also showed significant reduction in ascospore production (Tables 4.2 and 4.3). However, there was no significant difference in the average number of ascospores produced per perithecium. Application of, *T. harzianum* 24 hours before *G. zeae*, at the same time, or 24 hours after *G. zeae*, did not prevent or reduce macroconidial production (Table 4.4).

4.4.3 Field evaluation of *Trichoderma harzianum* isolates on perithecial production of *Gibberella zeae* on straw residue

Under field conditions, all five *T. harzianum* isolates significantly reduced numbers of perithecial of *G. zeae* on straw residues after 30 and 60 days of exposure. Similar trends were observed in both 2003 and 2004, although in 2004 fewer perithecia developed on both the control and treated residues (Table 4.5). There was no significant

difference in the percent recovery/re-isolation of *G. zeae* isolates from residues treated with *T. harzianum* spore suspensions. *Trichoderma harzianum* was recovered from 83.3 to 95.8% of residues sampled in July and August. Isolation of *G. zeae* was slightly lower, ranging from 79.2-87.5% (Table 4.6).

Table 4.1: Effects of *Trichoderma harzianum* spore suspension on perithecial production of *Gibberella zeae* (isolates DAOM 192132, 192131, and 170785) on wheat straw.

Treatment	192132		170785		192131	
	Average Number of Perithecia ^z	Perithecial Reduction (%) ^y	Average Number of Perithecia ^z	Perithecial Reduction (%) ^y	Average Number of Perithecia ^z	Perithecial Reduction (%) ^y
T-083	86*	40	75*	26	36	0
T-130	21*	85	22*	78	10*	72
T-183	26*	82	16*	84	15*	58
T-151	38*	73	14*	85	8*	78
T-22	21*	85	15*	85	4*	89
T-030	51*	64	48*	53	26	28
T-472	18*	87	17*	83	5*	86
T-137	120*	16	99	3	28	22
T-136	103*	28	86	16	23	33
T-188	86*	39	54*	47	28	22
T-926	99*	31	76*	25	33	8
<i>G. zeae</i>	143 ^a	-	103 ^b	-	36 ^c	-

^z Average is based on 4 replicates for two experiments (n=48)

Values are back-transformed from least squares means of arc-sine square root transformed data.

Within rows the values followed by a different letter are significantly different at ($P \leq 0.05$).

Within a column, means followed by an asterisk are significantly different from the controls, according to Tukey's least significant difference ($P \leq 0.05$).

^y Perithecial reduction as a percent of control.

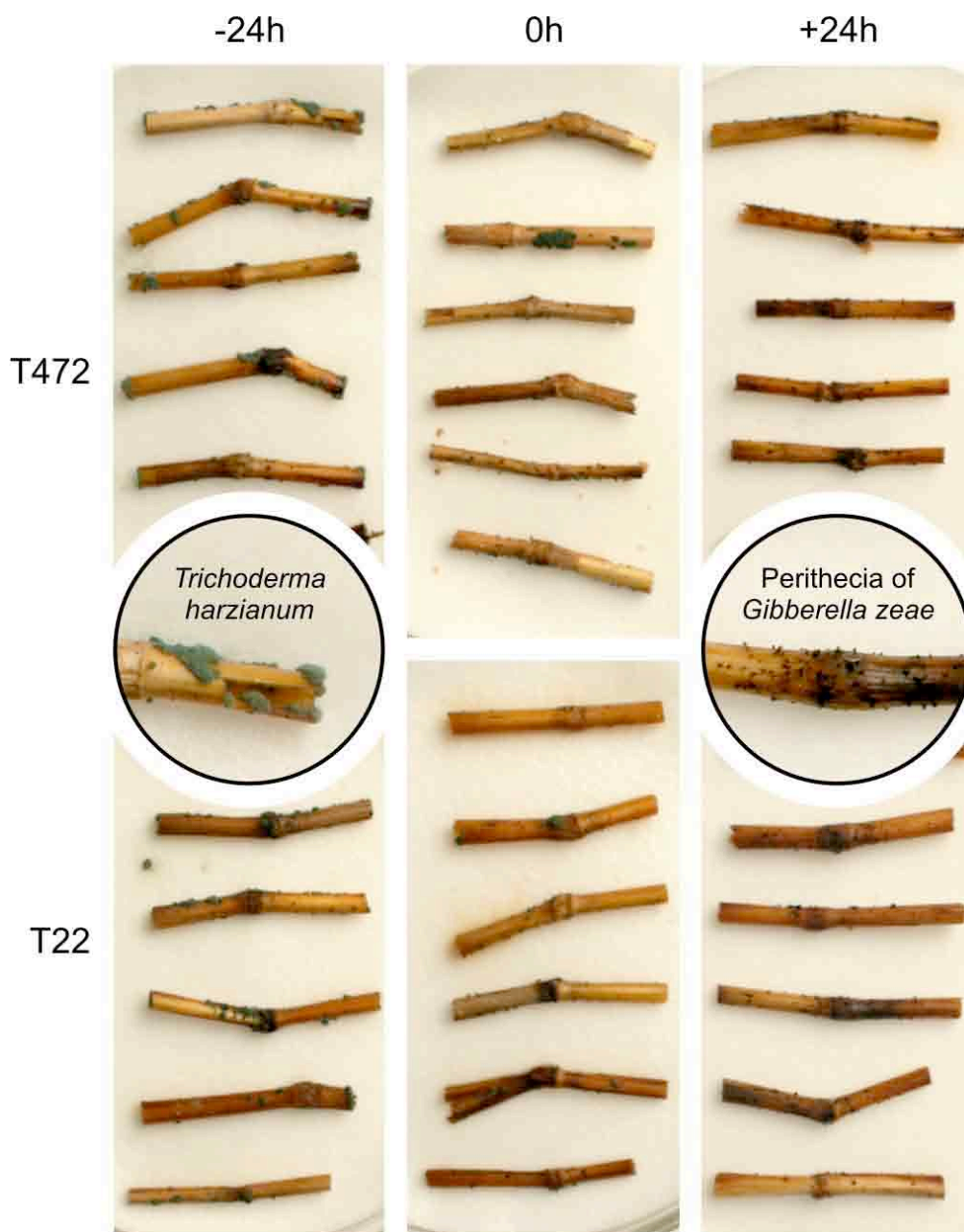


Figure 4.1: Perithecial production of *Gibberella zeae* on wheat stem tissue inoculated 24 before (-24 h), 24 after (+24 h) or co-inoculated (0 h) with *Trichoderma harzianum* strains T-22 (RootShield™), and T-472.

Table 4.2: Perithecial production of *Gibberella zeae* (DAOM 192132) on wheat straw when inoculated with spore suspensions or cell-free filtrates of *Trichoderma harzianum* 24 h before *Gibberella zeae* (-24h), co-inoculated (0h), or 24 h after *G. zeae* (+24h).

Treatment	Application time					
	-24h		0h		+24h	
	Average number of perithecia ^z	Perithecial Reduction (%) ^y	Average number of perithecia ^z	Perithecial Reduction (%) ^y	Average number of perithecia ^z	Perithecial Reduction (%) ^y
Spore Suspension						
T-151	2* ^a	98	42* ^b	60	94 ^c	5
T-130	4* ^a	96	35* ^b	67	55* ^c	44
T-183	3* ^a	97	30* ^b	72	43* ^b	57
T-472	1* ^a	99	23* ^b	78	56* ^c	43
T-22	1* ^a	99	20* ^b	81	39* ^c	61
Cell-Free Filtrate						
T-151cf	3* ^a	97	35* ^b	67	44* ^b	56
T-130cf	4* ^a	96	28* ^b	74	46* ^b	54
T-183cf	2* ^a	98	27* ^b	75	51* ^c	48
T-472cf	3* ^a	97	21* ^b	80	42* ^c	58
T-22cf	2* ^a	98	20* ^b	81	39* ^c	61
Control (<i>G.zeae</i>)	89 ^d	-	106 ^d	-	99 ^d	-

^z Average is based on 4 replicates for two experiments (n=48)

Within rows the values followed by a different letter are significantly different at ($P \leq 0.05$).

Within a column, means followed by an asterisk are significantly different from the controls, according to Tukey's least significant difference ($P \leq 0.05$).

^y Perithecial reduction as a percent of control.

Table 4.3: Ascospore production on wheat straw when inoculated with *Trichoderma harzianum* spore suspensions or cell-free filtrates 24 h before *G. zeae* (-24h), co-inoculated (0h), or 24 h after *G. zeae* (+24h).

Treatment	Average ^z number of ascospores (x 10 ⁵) ml ⁻¹		
	Application time		
	-24h	0h	+24h
Spore Suspension			
T-151	0.14* ^a	4.52* ^b	8.50* ^c
T-130	0.21* ^a	3.41* ^b	4.90* ^c
T-183	0.16* ^a	2.97* ^b	5.12* ^c
T-472	0.10* ^a	2.10* ^b	6.25* ^c
T-22	0.12* ^a	1.98* ^b	3.89* ^c
Cell-Free Filtrate			
T-151cf	0.28* ^a	1.81* ^b	2.81* ^c
T-130cf	0.19* ^a	1.42* ^b	3.19* ^c
T-183cf	0.15* ^a	1.44* ^b	4.22* ^c
T-472cf	0.18* ^a	1.49* ^b	4.01* ^c
T-22cf	0.13* ^a	1.51* ^b	3.89* ^c
Control (<i>G.zeae</i>)	11.07 ^d	12.23 ^d	10.59 ^d

^z Average value for two experiments

Within rows the values followed by a different letter are significantly different at ($P \leq 0.05$).

Within a column, means followed by an asterisk are significantly different from the controls, according to Tukey's least significant difference ($P \leq 0.05$).

Table 4.4: Macroconidial production on wheat straw when inoculated with *Trichoderma harzianum* spore suspensions or cell-free filtrates 24 h before *G. zeae* (-24h), co-inoculated (0h), or 24 h after *G. zeae* (+24h).

Treatment	Average ^z number of macroconidia (x 10 ⁶) ml ⁻¹		
	Application time		
	-24h	0h	+24h
Spore Suspension			
T-151	1.30*	1.55*	1.83*
T-130	1.45*	1.53*	1.90*
T-183	1.57*	1.48*	2.03
T-472	1.33*	1.45*	1.90*
T-22	1.38*	1.55*	1.98*
Cell-Free Filtrate			
T-151cf	1.40*	1.53*	1.98*
T-130cf	1.43*	1.68*	2.00
T-183cf	1.58*	1.60*	2.08*
T-472cf	1.48*	1.60*	1.93*
T-22cf	1.38*	1.50*	1.93*
Control (<i>G.zeae</i>)	2.23	2.13	2.28

^z Average value for two experiments

Within a column, means followed by an asterisk are significantly different from the controls, according to Tukey's least significant difference ($P \leq 0.05$).

Table 4.5: Perithecial production of *Gibberella zeae* on wheat straw treated with *Trichoderma harzianum* spore suspension after 30 and 60 days exposure to the environment at Portage la Prairie, Manitoba in 2003 and 2004.

Treatment	2003			2004		
	30d	60d	60d	30d	60d	60d
	Average number of perithecia ^z	Perithecial Reduction (%) ^y	Average number of perithecia ^z	Perithecial Reduction (%) ^y	Average number of perithecia ^z	Perithecial Reduction (%) ^y
T-151	12*	91	12*	90	6*	85
T-130	35*	74	24*	80	14*	65
T-188	22*	84	15*	88	9*	78
T-472	8*	94	9*	93	4*	90
T-22	6*	96	10*	92	5*	88
Control (<i>G. zeae</i>)	135 ^a	-	123 ^a	-	40 ^b	-
					52 ^b	-

^z Average is based on 4 replicates for each sampling time (n=24)

Values are back-transformed from least squares means of arc-sine square root transformed data.

Within rows values followed by a different letter are significantly different at ($P \leq 0.05$).

Within a column, means followed by an asterisk are significantly different from the controls, according to Tukey's least significant difference ($P \leq 0.05$).

^y Perithecial reduction as a percent of control.

Table 4.6: Incidence of recovery (%) of *Gibberella zeae* and *Trichoderma harzianum* from wheat straw, co-inoculated with spore suspensions, after 30 and 60 days exposure to the environment at Portage la Prairie in 2003 and 2004.

Treatment	2003						2004					
	July			August			July			August		
	<i>T. harzianum</i>	<i>G. zeae</i>	<i>T. harzianum</i>	<i>G. zeae</i>	<i>T. harzianum</i>	<i>G. zeae</i>	<i>T. harzianum</i>	<i>G. zeae</i>	<i>T. harzianum</i>	<i>G. zeae</i>	<i>T. harzianum</i>	<i>G. zeae</i>
T-151	91.7	79.2	91.7	75.0	87.5	79.2	91.7	79.2	91.7	87.5	91.7	87.5
T-130	95.8	83.3	91.7	79.2	91.7	83.3	91.7	83.3	95.8	79.2	95.8	79.2
T-183	83.3	83.3	87.5	79.2	87.5	83.3	87.5	83.3	83.3	79.2	83.3	79.2
T-472	95.8	79.2	91.7	79.2	95.8	79.2	95.8	79.2	95.8	83.3	95.8	83.3
T-22	87.5	83.3	95.8	75.0	91.7	75.0	91.7	75.0	87.5	83.3	87.5	83.3
<i>G. zeae</i>	0.0	91.7	0.0	95.8	0.0	91.7	0.0	91.7	0.0	95.8	0.0	95.8

4.6 Discussion:

The results of the study indicate that spore suspensions and cell-free filtrate of *T. harzianum* effectively reduced perithecial development of *G. zeae* on wheat straw residue under both laboratory and field conditions. A number of factors may influence results when evaluating potential biological control agents. Wheat straw was chosen as the substrate to simulate natural conditions, as it is important to evaluate the potential biological control agent on the substrate on which the pathogen develops. Both wheat and corn straw have been found to be suitable substrates producing perithecia under laboratory and field conditions (Bujold et al. 2001). In a previous experiment, all eleven *T. harzianum* isolates were effective at reducing the growth of *G. zeae* in culture (Inch and Gilbert 2002; Chapter 3.0). The correlation between results from agar bio-assays and field bio-assays are not always consistent (Lindow 1988). Media selection has been shown to influence antagonistic activity (Schisler et al. 2000) and production of antimicrobial compounds (Schisler and Slininger 1997).

Pathogen variation can also influence the efficacy of biocontrol agents in the control and management of the disease. High levels of genetic variation were demonstrated among and between populations of *G. zeae* (Zeller et al. 2004; Zeller et al. 2003; Ghisalberti et al. 1990). The isolates of *G. zeae* used in this study differed in their ability to produce perithecia. Isolate 192131 produced fewer perithecia than isolates 192132 and 170785. *Trichoderma harzianum* was effective at reducing numbers of perithecia, but only one isolate T-472, plus the positive control T-22 consistently reduced perithecia production by 83-89%. *Trichoderma harzianum* isolates used in this studied showed considerable variation in their ability to reduce perithecial numbers of *G. zeae*.

Analysis of randomly amplified polymorphic DNAs demonstrated that there is substantial genetic variation among *T. harzianum* isolates and this may influence the biocontrol activity (Gomez et al. 1997).

Under laboratory conditions application of *T. harzianum* spore suspension or cell free-filtrate, before *G. zae* was established on straw, provided the best control, which is in agreement with other studies. Bujold et al. (2001), found that application of *Microsphaeropsis* species two weeks before or at the same time as *G. zae* significantly reduced numbers of perithecia. Control of *Pyrenophora tritici-repentis* on residues was more effective with the early application of *Limonomyces roseipellis* (Pfender 1988, Pfender et al. 1993). It was also demonstrated that later application of antagonists was less effective in control (Bujold et al. 2001, Pfender 1988, Pfender et al. 1993). Mechanisms of control were not identified, but competition and production of antimicrobial compounds may be responsible.

Cell-free filtrate of *T. harzianum* was as effective as spore suspensions of *T. harzianum* at reducing perithecial numbers when applied 24 hours prior or co-inoculated with *G. zae*. This suggests that anti-microbial compounds are interfering with perithecial development and may be using specific resources such as space and nutrients or altering the environment, e.g. raising or lowering the pH, which may be required for perithecial development of *G. zae*. *Trichoderma harzianum* is known to produce various antimicrobial compounds, peptaibols, secondary metabolites, and cell wall degrading enzymes which may be responsible for the antagonistic activity (Schirmbock et al. 1994; Inbar and Chet 1995, Bélanger et al. 1995, Elad and Kapat 1999; El-Katatny et al. 2001). The antagonistic activity and production of antibiotics vary depending on the *T.*

harzianum isolate used as reported for the inhibition of growth of *Agaricus bisporus* and *Gaeumannomyces graminis* var. *tritici* in culture (Mumpuni et al. 1998; Ghisalberti et al. 1990).

Under field conditions, reduction of *G. zae* perithecia numbers were observed on residues treated with *T. harzianum* prior to placement in the field. Isolates which reduced perithecia numbers on residue under laboratory conditions were also effective under field conditions. Perithecia numbers were greater in 2003 than in 2004, this may be attributed to the difference in weather conditions. Precipitation levels were greater in June 2003 (98.6 mm) than in June 2004 (35.5 mm). However, July and August 2003 were drier than in 2004. Rainfall is important in the development of perithecia and may account for this difference in perithecial production (Inch et al. 2005; Paulitz 1996). However, the general trend in 2003 and 2004 was the same, all five isolates significantly reduced perithecia under field conditions. Recovery of *T. harzianum* was slightly greater than *G. zae* from treated residues. However, these differences were not significant among isolates tested. The growth rate in culture of *T. harzianum* is greater than *G. zae*; this may partly account for the difference in re-isolation. Fernandez (1992) found a significant reduction in recovery of *F. graminearum* on residues treated with *T. harzianum*. Luongo et al. (2005) demonstrated that sporulation and colonization of *G. zae* was suppressed on treated corn and wheat residues.

Application of *T. harzianum* spore suspension or cell-free filtrate to wheat residues significantly reduced perithecia numbers, both under laboratory and field conditions. When *T. harzianum* was applied to residues 24 hours before *G. zae* or at the same time, the isolates were more effective at reducing perithecia and ascospore

numbers. The effects of spring versus fall treatment were not investigated in this study. However, application in the fall to residues may provide better control. Newly harvested material may be less colonized by *G. zeae*. For effective fall treatment, *T. harzianum* would have to be able to survive and over-winter on residues. This would have to be assessed on a regional basis, due to differences in climatic conditions. Investigation on the mechanisms involved in the control of perithecial production of *G. zeae* by *T. harzianum* and the role of antimicrobial compounds is underway.

4.7 Literature cited

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5.0 The development of a predictive model for biological control of fusarium head blight based on secondary metabolite production by *Trichoderma harzianum*

5.1 Abstract

Fusarium head blight (FHB) is an important disease of wheat and other small grain cereals and causes significant economic and yield losses. The principal pathogen associated with FHB in Manitoba is *Gibberella zeae* (anamorph = *Fusarium graminearum*). *Gibberella zeae* over-winters on infested crop residues and gives rise to perithecia and ascospores. Wind-borne ascospores are the primary source of inoculum. Members of the genus *Trichoderma* include well-known biocontrol agents which have been used in the control of many plant pathogens. Application of a cell-free filtrate of *T. harzianum* to *G. zeae*-infested straw was shown to reduce perithecial production. Secondary metabolites were extracted from eleven *T. harzianum* isolates using diethyl ether. The extracts and cell free filtrates were screened for antifungal activity against perithecial production. Isolates T-472 and T-22 (RootShield™ positive control) significantly reduced perithecia numbers by more than 70%. Reverse phase high performance liquid chromatography was performed on extracts of *T. harzianum* and a series of alkylphenones. From this the bracketed alkylphenone retention indices were calculated. Identification of compounds most likely to affect biocontrol was accomplished through the use of cluster analysis, ordination and regression methods. It was found that isolates that cause similar levels of biocontrol had similar chemical compositions. Unique compounds in several isolates of *T. harzianum*, show promise as

potential biocontrol agents. A multivariate weighted average regression approach is presented as a means to predict bioactivity of compounds suspected to be involved in this control.

*** This Chapter was prepared in collaboration with Dr. David Walker, Department of Environment and Geography.**

5.2 Introduction

Members of the genus *Trichoderma* are well-known biocontrol agents and have been used in the control of many plant pathogens including *Rhizoctonia*, *Botrytis*, *Sclerotinia*, (Elad et al. 1984; Harman et al. 1993; Lorito et al. 1996;), *Pythium* (Benhamou and Chet 1997), *Fusarium graminearum* (Inch and Gilbert 2007, Fernandez 1992), and *Cochliobolus sativus* (Fernandez 1992). Commercial formulations of *T. harzianum* are available including isolate T-39 (TRIDODEX 20SP™, Makhteshim Chemical Works) which is used for the biological control of *Botrytis cinerea* and powdery mildew on grapes (Elad 2000), and T-22 (RootShield™), registered for the biocontrol of greenhouse crops (Harman et al. 2004). *Trichoderma* species have a number of characteristics that promote their usefulness for biological control. Members of the genus are easily isolated from soil, plants, and decaying wood, have a rapid growth rate, and colonize substrates very quickly (Howell 2003). Modes of action depend on the species and their interactions with target hosts. Rhizosphere competition by *T. harzianum* and induced localized or systemic resistance have been demonstrated to provide the plant with resistance to abiotic stresses, improved nutrient uptake efficiency, and protection from various plant pathogens (Harman et al. 2004). Other mechanisms include a combination of mycoparasitism, competition, and antibiosis (Howell 2003; Harman 2006). *Trichoderma* produces various cell wall degrading enzymes (CWDEs) including proteases (Elad and Kapat 1999), β -1,3-glucanases (Cruz et al. 1995), and chitinases (Baek et al. 1999). These enzymes have been found to be essential for penetration and colonization of the host and ultimately result in cell death of the other organism (Elad 1996). They appear to be synergistic in their activity as greater control is achieved when a

mixture of the compounds is used (Lorito et al. 1996). Members of the genus *Trichoderma* also produce a wide variety of volatile and non-volatile compounds that are effective in suppressing plant-pathogens (Dennis and Webster 1971). These compounds include alkyl pyrones (Claydon et al. 1987), isonitriles (Fujiwara et al. 1982), polyketides (Ordentlich et al. 1992), trichorzianines (Rebuffat et al. 1989), trichokindins (Iida et al. 1994), T22azaphilone, 1-hydroxy-3-methyl-anthraquinone, 1,8-dihydroxy-3-methyl-anthraquinone, T39butenolide, harzianolide, and harzianpyridone (Vinale et al. 2009). The exact mode of action for most of these compounds is not known. *Trichoderma* species and related genera are also known to produce various types of antibiotic peptides, referred to as peptaibols. Over 200 peptaibols have been identified, sequenced, and compiled in a database at www.cryst.bbk.ac.uk/peptaibol (Whitmore and Wallace 2004).

The complex multivariate inter-relationships of compounds involved in biocontrol are not well understood and there are few published examples that attempt to predict possible bioactivity from chemical signatures. Cell-free filtrates of *T. harzianum* were able to reduce perithecial numbers when applied before or co-inoculated with *G. zeae* (Inch and Gilbert 2007; Chapter 4.0). This suggests that the cell-free filtrates have antimicrobial compounds present that are effective in reducing perithecial development and are potential biopesticides. It has been demonstrated that metabolite production by *T. harzianum* is species- and strain- specific (Vinale et al. 2006).

High throughput screening is commonly used in the pharmaceutical industry when screening for new bioactive compounds. Isolation and characterization of new potential strains can be expensive and time-consuming. Using reverse phase high performance liquid chromatography (RP-HPLC) and regression analysis may provide a

method for rapid screening and identification of useful isolates. In RP-HPLC analysis bracketed alkylphenone retention indices (RIs) may be used to indicate presence of specific compounds (secondary metabolites) or groups of compounds which share similar polarity and structural properties. Slight differences in retention times of a peak and RIs may result when compounds are closely related. The chromatogram UV spectrum is used to determine whether compounds with similar RIs are the same or related compounds (Thrane et al. 2001). Peaks on the chromatograms that have similar shape and retention time are considered the same compound. When comparing several chromatograms from different RP-HPLC runs, the magnitude and shape of the chromatogram are sensitive to experimental conditions and sample concentration. In addition the concentration and types of secondary metabolites produced by an isolate can vary depending on growth medium, temperature, light, and extraction method (Vinale et al. 2009). Therefore the protocols must be standardized to ensure consistency of the peaks in the chromatogram when comparing multiple runs. The use of RIs to determine presence or absence of specific peaks avoids problems associated either with magnitude or absolute concentration. The pattern and complexity of peaks in a chromatogram is a reflection of the presence of different secondary metabolites and these chemical signatures can be used for identification and classification through multivariate analysis (Cserhati 2008; Nielsen et al. 1999). Thrane et al. (2001) classified 44 *Trichoderma* strains using image analysis of complete chromatographic matrices obtained from HPLC. However, recent studies have focused on integration of descriptive methods with models that can predict the relationships between spectral patterns observed and sample composition (Nord et al. 2004). Many regression and regression-like modeling approaches have been suggested,

one of which is the application of multivariate calibration such as partial least squares (PLS) of the absorbance signals in chemometrics (El-Gindy et al. 2004; Hassel et al. 2002; Parisotto et al. 2007). Although the data for PLS must meet the same standard assumption of linear regression, it has the advantage that predictions are more robust even when the number of variables exceeds the number of observations (ter Braak and Schaffers 2004). The use of PLS in combination with spectrophotometric approaches has been shown to be effective in quantifying compounds in a multivitamin mixture. This method was cheaper and had fewer time-consuming steps than using HPLC alone (El Gindy et al. 2004). PLS regression analysis was also used to quantify organic and amino acids in beer using ¹H NMR spectroscopy data (Nord et al. 2004). However, PLS is a linear model and many systems do not exhibit strict linearity (Hassel et al. 2002). When dealing with non-linear data, weighted averaging partial least squares (WA-PLS) (ter Braak and Juggins 1993) or weighted averaging calibration can be used (ter Braak and van Dam 1989). These methods have been frequently employed in paleolimnology and paleoecology to predict past environmental conditions using current diatom composition (Bigler and Hall 2002; Hausmann and Pienitz 2007), and in ecology to reconstruct past hurricane damage (Ross et al. 2001). The advantage of these approaches is that they can be used to develop prediction models in which non-linear monotone descriptors can predict one or more response variables. WA and WA-PLS have only recently been applied in chemometrics and in bioassay approaches. Zhang et al. (2004) compared NIR spectral datasets from green tea to look at overall composition of peaks similar to HPLC spectra. This method could be implemented in biological control to predict the potential performance of a biocontrol agent based on spectral data, such as HPLC, prior to field

trials. There has been little use of such approaches to develop predictive models of biological control from HPLC data.

The objectives of the study were to 1) investigate the effect of secondary metabolites, produced in cell-free filtrates of *T. harzianum*, on the perithecial production of *G. zeae* and 2) identify unique biochemical signatures among isolates of *Trichoderma* that describe and predict their bioactivity in controlling FHB. This was addressed by: 1) identifying compounds or related compounds in isolates of *Trichoderma* that have a known level of biocontrol using flash column chromatography and reverse phase high performance liquid chromatography (HPLC), 2) determining the multivariate associations among isolates of *Trichoderma* and similarities among their chemical composition based on bracketed alkylphenone RIs, 3) determining the relationship between chemical composition based on RIs and known levels of biocontrol activity, and 4) by developing a model to predict biocontrol from RIs.

5.3 Materials and Methods

5.3.1 Isolates Used

Ten *Trichoderma harzianum* isolates used in this study varied in their ability to reduce perithecial numbers (Chapter 4.0). Isolates from a variety of substrates (none with known biocontrol activity) were requested from the Canadian Collection of Fungal, CCFC (Ottawa, Ontario) and included T-030 (DAOM 198030), T-472 (DAOM 216472), T-130 (DAOM 222130), and T-151 (DAOM 222151) and T-183 (DAOM 222183), T-083 (DAOM 199083), T-088 (DAOM 167088), T-926 (DAOM 175926), T-136 (DAOM

222136), T-137 (DAOM 222137). An additional isolate T-22 (strain KRL-AG2), which is registered in the US as a biological control agent, RootShield™ (Bioworks, New York), was included as a positive control. T-22 is a hybrid species made by the protoplast fusion of two strains of *T. harzianum*, T-95 and T-12 (Stasz et al. 1998). The *G. zeae* isolate used was obtained from the CCFC (DAOM 192132) and was chosen based on aggressiveness and ability to produce perithecia on wheat straw under laboratory and field conditions (Chapter 3.0). All cultures were grown on potato dextrose agar (PDA) (39 g Difco PDA, 1L distilled water) for 5-7 days under fluorescent white light at 20°C. Cultures were then stored at 5°C until needed.

5.3.2 Inoculum production

A macroconidial suspension of *G. zeae* was prepared using 5-8 day old fungal cultures grown on potato dextrose agar (PDA). The cultures were cut into 1 cm sections and added to streptomycin-amended carboxymethyl cellulose (CMC) liquid medium (carboxymethyl cellulose 15 g, NH₄NO₃ 1g, KH₂PO₄ 1g, MgSO₄·7H₂O 0.5g, yeast extract 1 g, distilled water 1L, streptomycin sulfate 0.2g added to the cooled medium). The flask was attached to a vacuum line and incubated under aeration to promote macroconidial production. After 7 days at room temperature, macroconidia were counted using a hemacytometer and the suspension adjusted to a final concentration of 5.0 x10⁴ macroconidia/ml by adding sterile distilled water. A surfactant (Tween 20™) was added just prior to inoculation (0.2 ml per 100ml inoculum).

5.3.3 Cell-free filtrate

Cell-free filtrate was prepared by inoculating 500 ml of liquid organic medium (glucose 10 g, peptone 1g, yeast extract 0.1g, KH_2PO_4 1 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.3g, distilled water 1L) with a 200 μl macroconidial suspension (5.0×10^5 spores/ml) of *T. harzianum*. The flasks were placed on a rotary shaker set at 100 rpm under cool white fluorescent light (Sylvania cool white 30W) at room temperature for 10 days. The cultures were filtered through Whatman #1 filter paper (Maidstone, England) and then through a 0.22 μm polyethersulfone, sterilizing, low-protein binding membrane (Corning, NY). Non-inoculated organic medium was also filtered and served as a control.

5.3.4 Extraction of secondary metabolites from cell-free filtrate

Organic compounds were extracted from the cell-free filtrate using diethyl ether and hexane. The cell-free filtrate was placed into a 1L separatory funnel and ether was added (1/10 volume, i.e. 50 ml ether to 500 ml filtrate), and inverted several times to extract compounds. The aqueous phase was drained off and the organic phase containing the compounds was collected. This step was repeated three times to ensure all organic compounds were extracted from the filtrate. Following this, 10 ml of hexane was added to the aqueous phase and inverted several times. A small amount of magnesium sulfate (MgSO_4) was added to remove any remaining water. This was then filtered to remove the MgSO_4 . The ether was evaporated using a rotovap, which left an oily residue on the bottom of the flask. This residue was re-suspended in 4 ml ether and transferred to 4 ml vials with Teflon-lined caps. Nitrogen gas was used to further concentrate the samples to 1 ml and the samples were then stored at -5°C until needed. The initial resolution of

extracted compounds was done by thin layer chromatography (TLC). A solvent system consisting of 50:50 ether: hexane was used to separate the compounds on a silica gel TLC plate: 5, 10, 20, and 40 µl was spotted on the plates which were placed in the solvent. Once the solvent front reached 1 cm from the top edge of the plate, it was removed and the solvent front was marked. The retention factor (R_f) values were calculated using the following formula:

$$R_f = \frac{\text{distance traveled by the compound}}{\text{distance traveled by the solvent front}}$$

5.3.5 Bioassay

The extracts were screened for antifungal activity by immersing pieces (1 cm) of wheat stem tissue in 1 ml of concentrated extract (0.3 mg/ml) from which the ether was allowed to evaporate, and applying 500µl of a macroconidial spore suspension (5.0×10^4 spores/ml) of *G. zea*. Treated pieces of straw were placed on moistened vermiculite, incubated at 20°C under fluorescent white light, and monitored for perithecial development. Treated straw inoculated with *G. zea* only, and ether followed by *G. zea*, were included as controls. Each treatment was replicated four times and the entire experiment was repeated once.

5.3.6 Column chromatography

To prepare the flash column (40 x 3 cm), 500 ml of silica gel (230-400 mesh, Sigma) was mixed with 400 ml of 3:1 hexane: ethyl acetate. A small piece of cotton was placed at the base of the column followed by a thin layer of sand (white quartz -50+70 mesh, Sigma). The silica slurry was gradually added to the column and packed tight with

forced air. This was then topped with a thin layer of sand to prevent damage to the silica gel when adding the solvents.

The concentrated ether extracts of T-472 and T-22 were eluted through a silica-gel flash column with 200 ml of each of the following solvent systems, 3:1 hexane: ethyl acetate, 1:1 hexane: ethyl acetate, 1:3 hexane: ethyl acetate, 100% ethyl acetate, 100% methanol (gradient non-polar to polar). The eluted fractions (~9 ml) were monitored by TLC using a 1:1 hexane: ethyl acetate solvent system. For isolate T-472, a total of 71 fractions were collected, and fractions with similar R_f values from TLC assays were pooled together to yield 12 sub-fractions (A-L). For isolate T-22 a total of 14 sub-fractions (A-M) were collected. The solvent was evaporated off and the sub-fractions were re-suspended in ether and stored at -5°C until needed. Each sub-fraction was screened for active compounds against perithecial development.

5.3.7 Reverse phase high performance liquid chromatography

Reverse phase high performance liquid chromatography (RP-HPLC) was performed on extracts of *T. harzianum* on a Waters 2690 separation module with autosampler and Waters 996 photodiode array detector. This was fitted with a 5µm LiChrospher® 100 RP-18 guard column (LiChroCART® 4-4) and a reverse-phase 5 µm 250-4 LiChrospher® 100 RP column ((LiChroCART® 4-4). Results were analyzed using Empower Software version 2.0. The column was eluted at a flow of 1 ml/min with a gradient using a solvent system composed of A: 0.1% H₃PO₄⁻ acidified water and B: HPLC grade acetonitrile. The gradient used for the analysis was: (time [min]/A[%]/B[%]) = 0/100/0, 5/95/5, 10/95/5, 14/90/10, 20/80/20, 23/80/20, 30/65/35, 35/65/35, 43/50/50,

48/25/75, 55/0/100, 60/0/100. Injection volumes were 50 µl per sample and each sample was replicated three times.

5.3.8 Calculation of retention index

Chromatographic peaks were monitored at 225 nm and characterized by their UV-VIS spectrum and alkylphenone RI. A series of internal alkylphenone standards (Frisvad and Thrane 1987) were included at a concentration of 20 µmol/ml: acetophenone, butyrophenone, decanophenone, hexaphenone, octophenone, propiophenone, valerophenone. The bracketed alkylphenone RIs were calculated from the following equation:

$$RI = \frac{(T_{sm} - T_{p1}) \Delta z}{T_{p2} - T_{p1}} + z \cdot 100$$

where RI = bracketed retention index, T_{sm} = retention time of the secondary metabolite, T_{p1} = the retention time of the alkylphenone that elutes before the secondary metabolite, T_{p2} = the retention time of the alkylphenone that elutes after the secondary metabolite, z = the number of carbon atoms in the alkylphenone that elutes before the secondary metabolite and Δz = the difference between the number of carbon atoms in the alkylphenones that elute before and after the secondary metabolite.

5.3.9 Determination of compounds from RIs

The compounds were labeled for analysis based on Thrane et al. (2001). Each peak in the HPLC was identified and numbered. After all peak signatures were labeled, the compounds were aggregated based on similar RIs. The choice to merge or maintain a particular label was based on a visual assessment of the chromatogram and by

preliminary statistical and multivariate analyses. Different RIs were assumed to represent unique compounds.

5.3.10 Data file structure and datasets

Two approaches were used to encode the data. The first was a binary method in which an RI-based chemical was identified as being ‘present’ (coded as 1) or ‘not present’ (coded as 0) (Thrane et al. 2001). Association measures and methods of analysis were selected for binary data examination according to Legendre and Legendre (1998), where the influence of concentration of a particular compound is ignored and trace presence or abundance of a compound are both counted as “1” or present. To account for this, a second matrix was developed using a rank-order criterion for chromatogram peak concentrations.

The second method used rank order values to indicate ‘abundance’ or magnitude and has been used extensively in social psychology and marketing in combination with correspondence analysis (Teil 1975). The relative height of each peak on the chromatogram was used to rank the concentration of the compounds. The ranks were recorded in reverse order such that the largest value was associated with the highest peak for an isolate. The number of compounds identified within an isolate thus influenced the ‘size’ of largest rank value. Using a reverse rank order for concentration tries to reflect the true relationship in magnitude without using absolute values.

5.3.11 Multivariate data analysis

5.3.11.1 Presence-absence dataset

Cluster analysis of the binary HPLC data was performed using the Jaccard coefficient association measure (to form a distance matrix) followed by the unweighted pair group method with arithmetic mean (UPGMA). The Jaccard coefficient ignores shared zeros when calculating distances in a binary matrix which is preferred when abundant mutual absences are present in the dataset (Legendre and Legendre 1998). Choice of UPGMA has the advantage of reducing the chaining effects in data where group structure may be weak (Grondona et al. 1997); Thrane et al. 2001; Nord et al. 2004). Nearest neighbor (single-linked) cluster analysis was also performed to test the robustness of the data analysis. Ordination of these data was performed using principal coordinates analysis (PCoA, metric multidimensional scaling) using the Jaccard measure of association. PCoA is similar to principal components analysis (PCA) except that it can account for a variety of distance-based association measures (Legendre and Legendre 1998) and differs from PCA in the fact that it recovers coordinates for the objects without retaining information about correlations among the variables.

5.3.11.2 Rank-order dataset

Cluster analysis of the rank-order dataset was performed using a chord distance matrix and UPGMA as the clustering algorithm (Grondona et al. 1997; Thrane et al. 2001; Nord et al. 2004). The chord distance is a standardized form of the Euclidean distance that scales object vectors to unit length (Legendre and Legendre 1998). This method preserves relative magnitude but not absolute magnitude. Therefore it is

appropriate for data containing shared zeros and semi-metric data. Correspondence analysis (CA) was performed to ordinate the ranked data. CA is frequently used with rank-order data in social psychology and marketing and is commonly known as perceptual mapping or correspondence factor analysis (Teil 1975; Torres and Greenacre 2002). Recently this method has been applied to fungal HPLC data (Sonjak et al. 2005) and can work with a variety of matrices consisting of positive values (Legendre and Legendre 1998) or rank-order data (Teil 1975; Faust and Wasserman 1993).

To determine the relationship between biocontrol activity and chemical composition of the isolates, a weighted averaging calibration (WA) was performed. This method can be used to develop predictive models between monotonic non-linear predictor variables and a linear response variable (ter Braak and Schaffers 2004). The WA predicts which chemical or combination of chemicals (weighted by the rank of the concentration) are effective in the biocontrol and inhibition of perithecial development. During the calculation of the weighted averaging, averages are taken twice. This leads to a reduction of the range of the predicted response values or "shrinking" of the gradient. Various methods are available to "de-shrink" the predicted response such as "inverse deshrinking" regression or "classical de-shrinking" (Marchetto 1994). Inverse de-shrinking minimizes the mean squared error in the data set, and tends to reduce estimates at the ends of the gradient. This can potentially result in underestimation for models when the potential values predicted are unbounded. Inverse de-shrinking was used in this study because perithecial inhibition was measured in percentage and was therefore bounded. To determine which compounds may be responsible for the bioactivity, tolerance down-

weighting was used to eliminate the influence of compounds shared by all isolates, since these are unlikely to contribute to biocontrol.

5.3.11.3 Software

All statistical analyses were performed using CRAN-R (R Development Core Team 2009). Contributed packages analogue and paltran were used to perform WA, CA was used to perform correspondence analysis, the package Vegan was used for computing associations for chord and Jaccard metrics, and Ade4 was used for PCoA.

5.4 Results

The control treatments (wheat straw inoculated with *G. zeae* alone and ether followed by *G. zeae*) had an average value of 151 and 148 perithecia per 1 cm section. Reduction of perithecial numbers was most effective when either the cell-free filtrate or the entire extract was applied to the wheat straw; the numbers of perithecia were reduced to an average of 41 and 52 perithecia per section, respectively, indicating that the active compound(s) were extracted by the ether: hexane solvent. Three of the 12 sub-fractions (H, I, and L) of isolate T-472 extract significantly reduced perithecia numbers to 96, 89, and 97 perithecia per straw piece, respectively (Figure 5.1). When sub-fractions H, I, and L were combined and applied, perithecial numbers were reduced to 96 per piece of straw. Isolate T-22 showed a similar trend, sub-fractions, A, B, G, H, and I significantly reduced perithecia numbers to 91, 102, 94, 87, and 92 respectively. When these sub-fractions were combined, perithecia numbers were reduced to 87 (Figure 5.1). None of the sub-fractions was able to reduce perithecia numbers to the levels of the total extract or the cell-

free filtrate, indicating that are two or more active compounds were responsible for the reduction of perithecia numbers.

The RP-HPLC chromatographs revealed quantitative differences among isolates and secondary metabolites produced (Appendix 3). The initial data matrix consisted of 11 isolates by 107 RI compounds, of which 66 unique secondary metabolites were identified after aggregation of similar RIs. All analyses proceeded on 11 (isolates) x 66 (RI compounds).

5.4.1 Presence-absence data

The unweighted paired-group dendrogram of isolates using a Jaccard association matrix of binary relationships is presented in Figure 5.2. Branch lengths indicated distance (differences) in the chemical composition of isolates. In this dendrogram isolates with the highest biocontrol (T-472 and T-22) formed a tight cluster on the basis of having similar presence absence patterns in peak concentrations as identified by HPLC. There was some evidence present of a lack of structure or chaining. The first two axes of the principal coordinates analysis of the 11 isolates based on the Jaccard association matrix is presented (Figure 5.3). The first axis recovered 25% of the dispersion in the distance matrix and clearly separated isolates T-472 and T-22 from the other isolates. Isolates associated with low-medium biocontrol (Table 5.1) showed no definite pattern on the ordination plot, similar to the results from the cluster analysis. Nearest neighbor clustering analysis was also performed, however, a large amount of chaining and weak group structure resulted. Isolates T-22 and T-472 form a tight cluster as seen in the UPGMA dendrogram. However all other isolates were clustered into one large group (Appendix 4).

5.4.2 Rank-order data

Unweighted paired-group dendrogram of isolates using a chord distance association matrix of concentration relationships (reverse rank-order) showed a three group structure (Figure 5.4). Unlike the binary analysis, when the mean percentage of perithecial inhibition (biocontrol) for each group was calculated, a clear trend was evident. Typically the level of biocontrol within each group was consistently either high, medium or low. Two of the three isolates, T-472 and T-22 in the leftmost class had the highest control; the exception was T-188. This isolate had the fewest number of compounds (8 major compounds) of all isolates tested, but of those, several were shared with T-472 and T-22, which produced 15 and 13 major compounds, respectively.

The correspondence between compound rank-order and the isolates tested in this study is provided in Figure 5.5. Like the PCoA analysis, the first axis of the correspondence analysis extracted 25% of the total information in the dataset and separated isolates T-472 and T-22. Unlike the previous ordination, CA also provided an ordinate for each of the RI defined compounds in the study. Compounds C18, C22, C27 and C42 showed highest affinities to these two isolates, and occurred in both isolates. However, they were not present in T-188. As the RI labels were applied in order of polarity and size, these compounds eluted early indicating greater polarity and smaller size.

Results of the weighted averaging calibration model are presented in Figure 5.6. The R^2 for the model relationship was relatively strong at 0.745 with a root mean square error (RMSE) of 14% and a mean residual of zero. Isolates with the highest predicted biocontrol were T-472 and T-22 (Table 5.1, predicted values). The WA plot of observed

and fitted perithecial inhibition (Figure 5.6A) demonstrated the relatively strong relationship in the model. Observations were evenly distributed on either side of the dashed line representing the 1:1 relationship as evident in the residual plot (Figure 5.6B). Some curvilinearity was evident in the model and residuals indicating a slight deviation from a perfectly linear or 1:1 relationship.

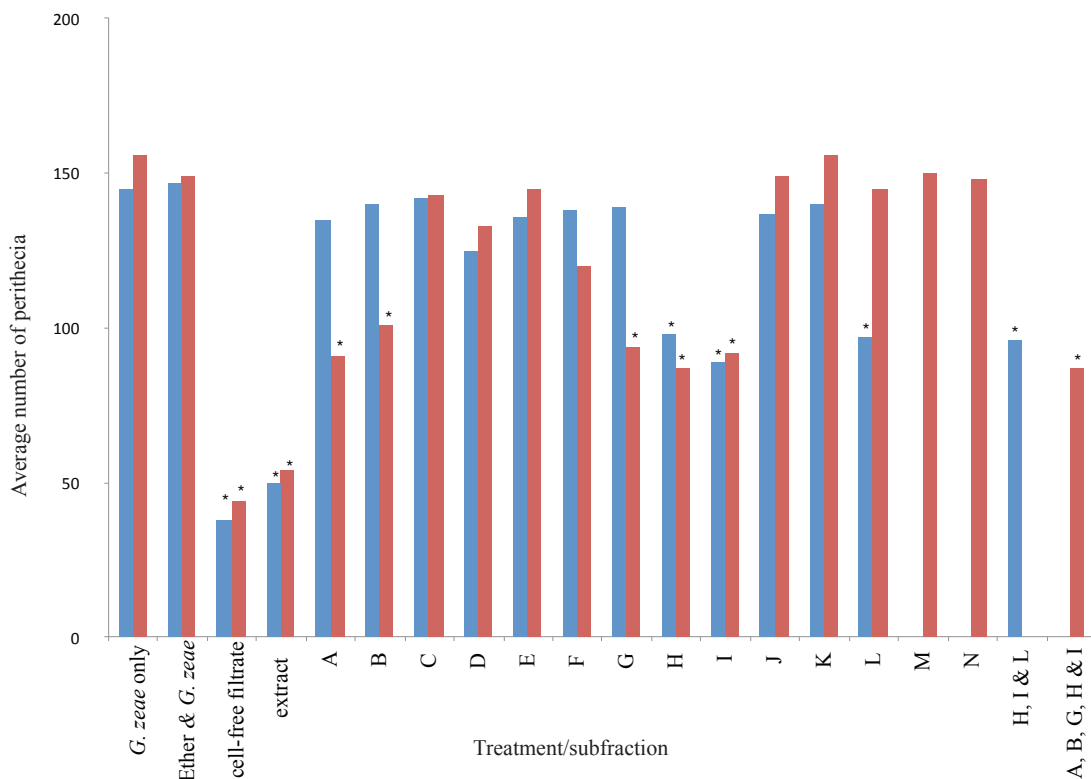


Figure 5.1: Average number of perithecia on wheat straw co-inoculated with a macroconidial suspension of *Gibberella zeae* and extracts of sub-fractions of *Trichoderma harzianum* isolates T-472 (blue bars) and T-22 (red bars) collected from column chromatography. Experiment was conducted twice. Average based on 6 pieces of straw per treatment per replicate. Bars with an asterisk (*) are significantly different from the controls, *Gibberella zeae* alone and ether + *Gibberella zeae*.

Note:

H, I & L are the combined sub-fractions of isolate T-472

A, B, G, H & I are the combined sub-fractions of isolate T-22

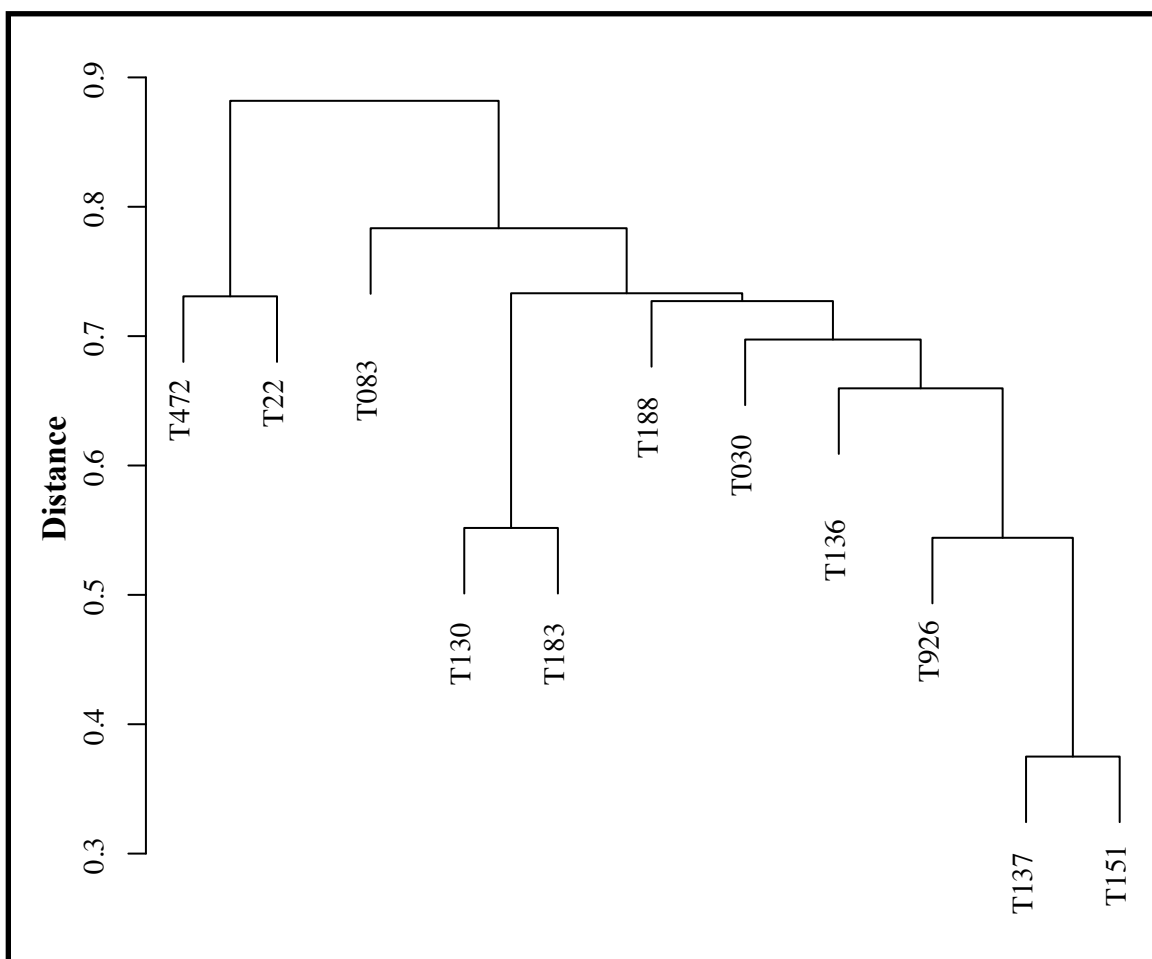


Figure 5.2: Unweighted paired-group dendrogram of isolates using a Jaccard association matrix of binary relationships (presence-absence) of compounds detected in HPLC. Branch lengths indicate distance (differences) in the chemical composition of isolates.

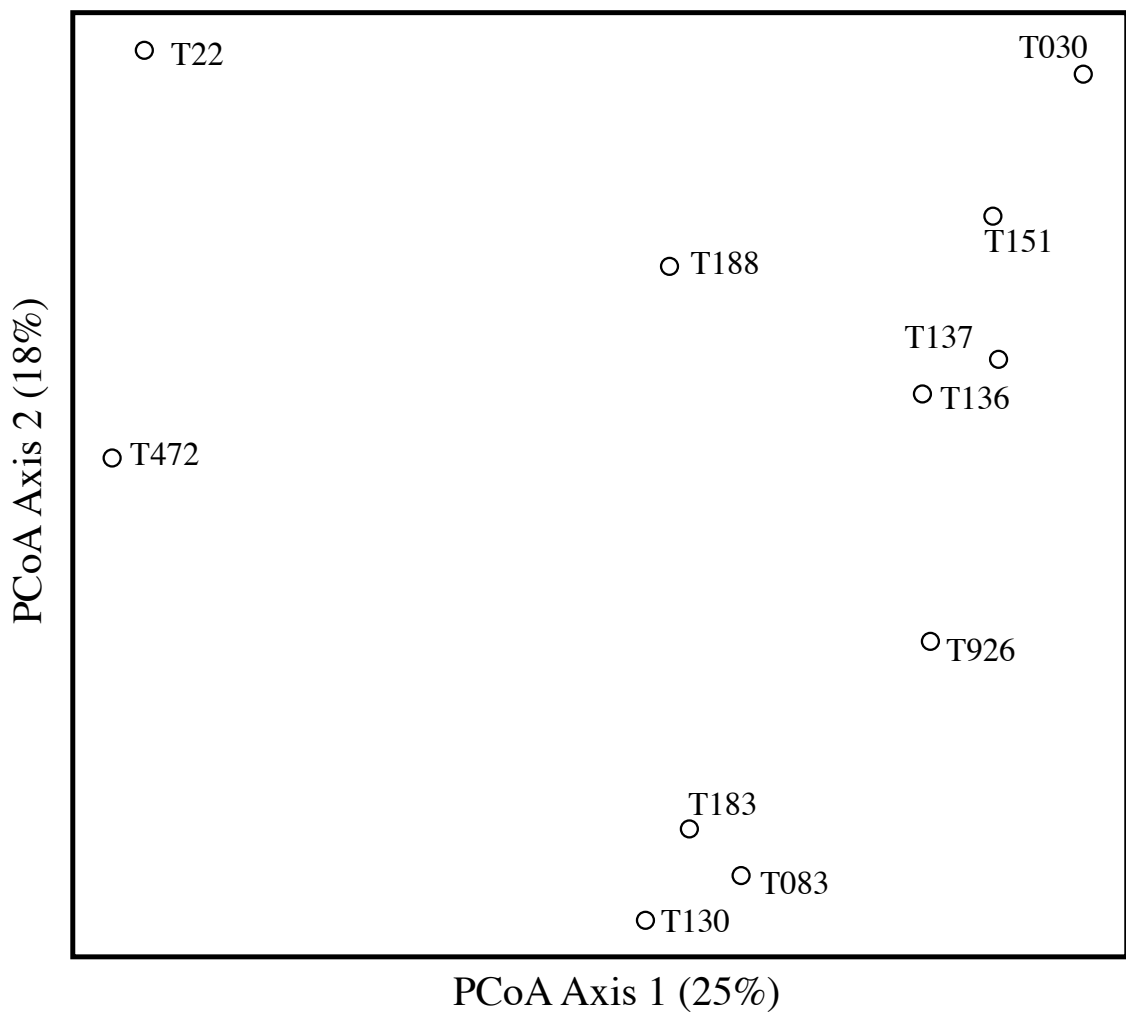


Figure 5.3: The first two axes of the principal coordinates analysis of the 11 isolates based on a Jaccard association matrix calculated from the binary data. Percentage of the overall dispersion in the data matrix accounted for by each component is provided.

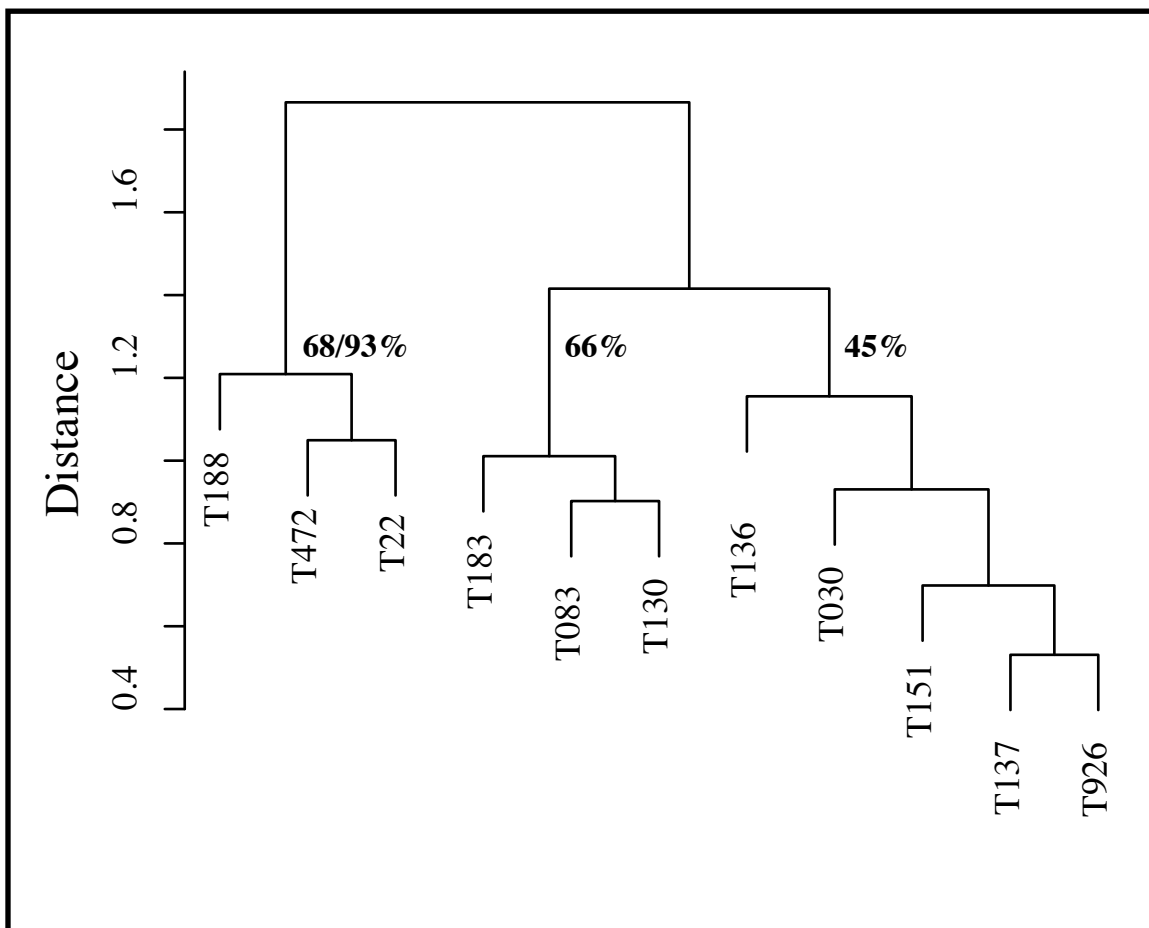


Figure 5.4: Unweighted paired-group dendrogram of isolates using a chord distance association matrix of concentration relationships (reverse rank-order) of compounds detected in HPLC. Branch lengths indicate distance (differences) in the chemical composition of isolates. The mean percentage of perithecial inhibition (biocontrol) is provided at the three group level and annotated on the dendrogram. Group 1 is reported with and without T188.

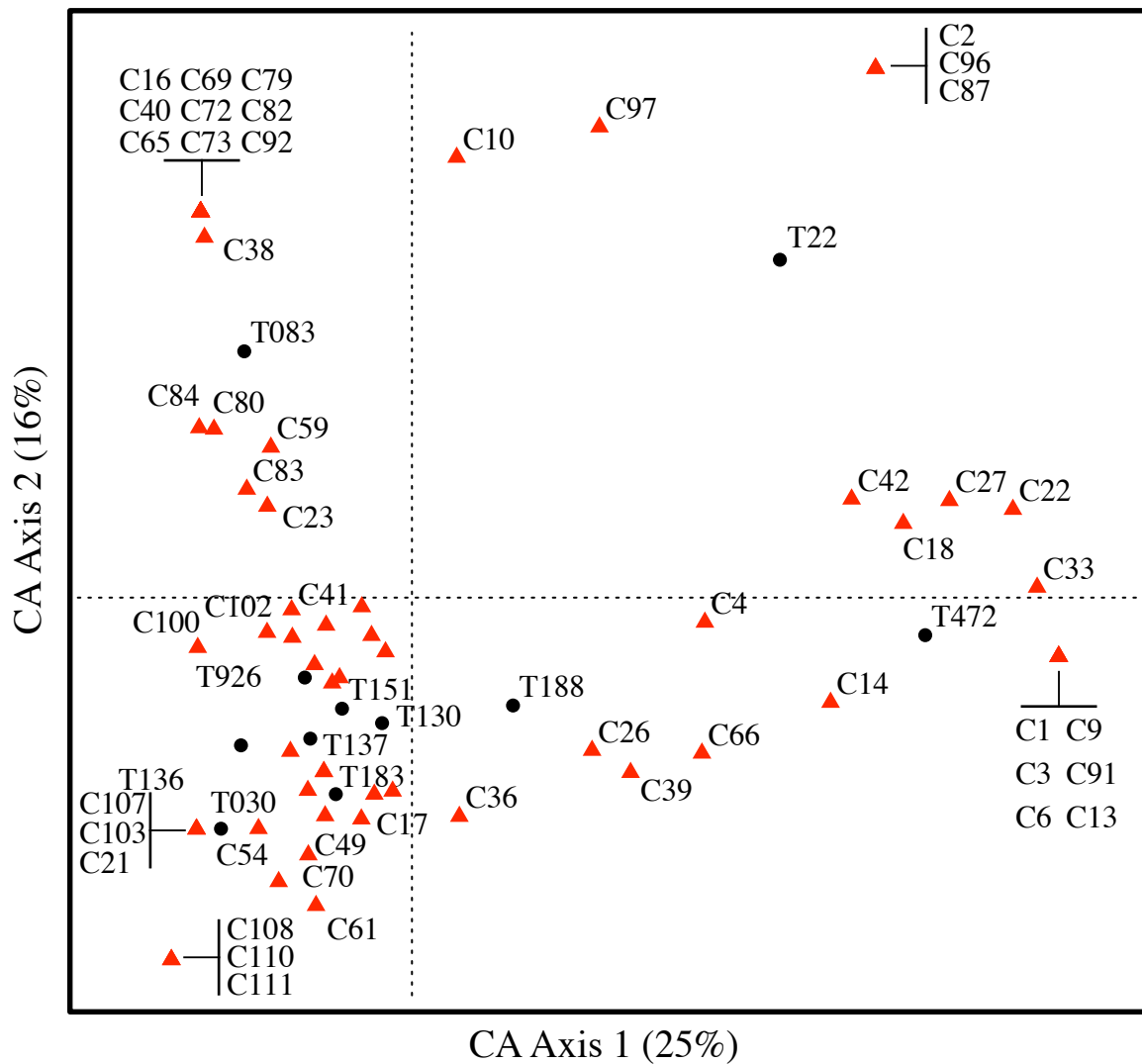


Figure 5.5: Correspondence analysis of the rank-order concentration data. Isolate scores are indicated by a black dot and labeled appropriately. Compound scores are red triangles, most compounds with large absolute scores are labeled for the purposes of discussion. Percentage of total inertia accounted for by each axis is provided.

Table 5.1: Statistics for the weighted averaging calibration (WA) analysis of perithecial inhibition (%) and the observed and predicted values for perithecial reduction (%) of *Gibberella zeae* on straw treated with cell-free filtrate extracts of isolates of *Trichoderma harzianum*.

<i>T. harzianum</i> isolate	Predicted perithecial inhibition (%)	Observed perithecial inhibition (%)
T-030	46	64
T-083	38	43
T-130	63	75
T-136	25	25
T-137	45	23
T-151	54	84
T-183	65	82
T-188	58	18
T-22	88	92
T-472	102	94
T-926	46	31

WA Performance:

RMSE	R²	Avg. Bias	Max. Bias
14.363	0.745	0.000	-13.413

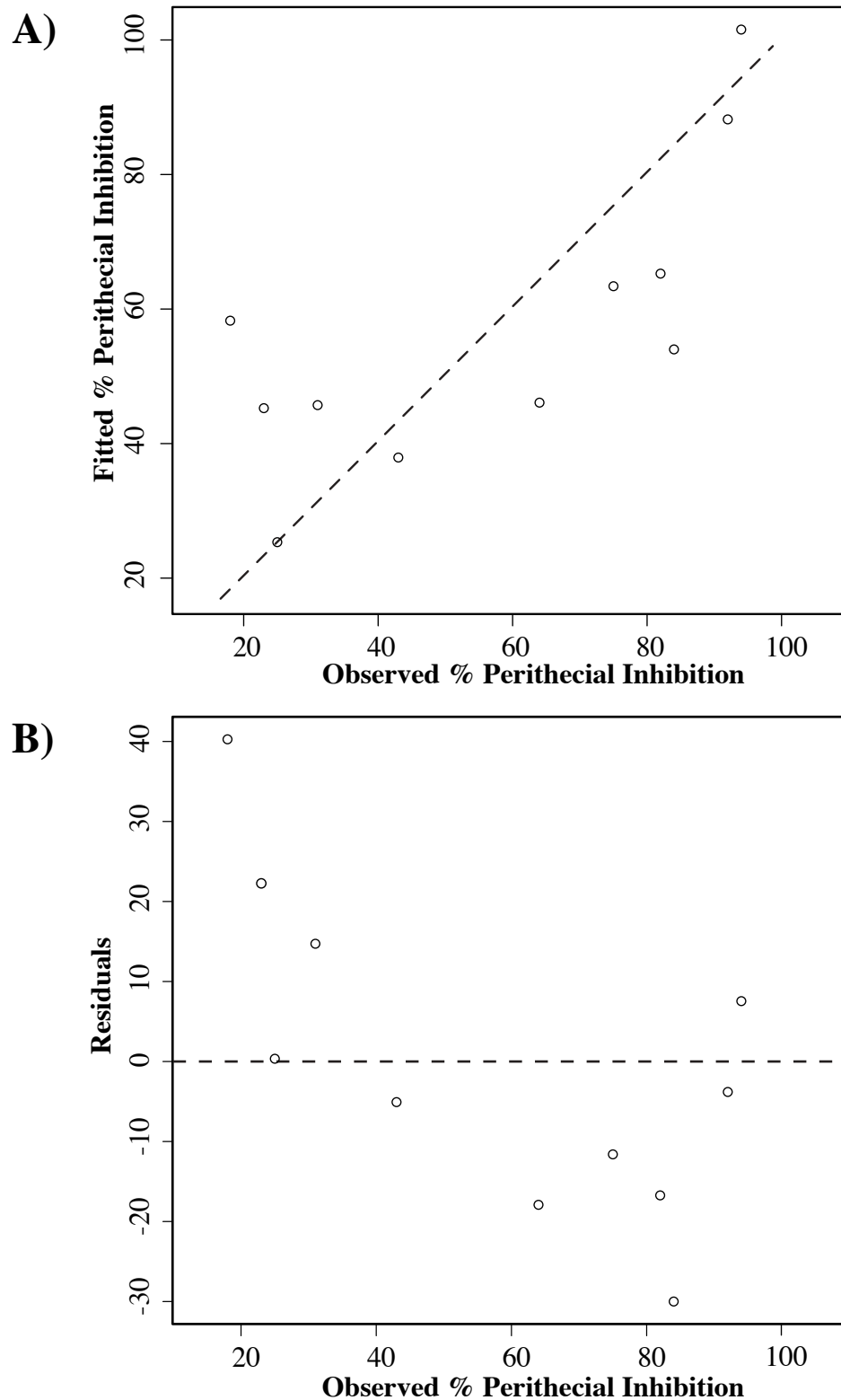


Figure 5.6: Plots derived from the weighted averaging calibration model: a) inferred and observed percentage perithecial inhibition, the dashed line represents the 1:1 relationship; b) model residuals against observed percentage perithecial inhibition, the dashed line represents the mean bias (mean of the residuals).

5.5 Discussion

The ten *Trichoderma* isolates evaluated in this study showed a wide range of different secondary metabolite production and concentrations. The positive control, T-22 (RootShield™) and T-188 produced several similar compounds, however, the isolates differed greatly in their ability to reduce perithecial development. This may have been due to the fact that either the bioactive compound was not present in T-188 or that the relative concentration of compounds was only approximately half that of compounds present in T-22 and too low for control. Generally, isolates with similar chemical composition and biocontrol activity clustered together indicating that certain chemical combinations may be responsible for control. However, caution must be used when screening potential biocontrol agents based on the metabolites produced. Elad (2000) demonstrated that *Trichoderma* isolates which produced various CWDE were not always effective in biocontrol. In his study, all five isolates tested were shown to produce endochitinase, chitobiosidase, and N-acetyl-B-D-glucosaminidase. However, only one isolate (a known biocontrol agent, T-39) produced high concentrations of these enzymes. The other four isolates produced the enzymes but in lower quantities.

When compounds were separated, the biocontrol activity was lost. Control was more effective when the entire extract or cell free-filtrate was applied to the wheat straw pieces, suggesting that there may be two or more compounds working synergistically that are responsible for the reduction of perithecia. Many studies have demonstrated that mixtures of compounds are more effective at control than the application of single compounds. A mixture of hydrolytic CWDE and peptaibols produced a greater inhibitory effect on plant pathogens such as *Botrytis cinerea*, *Rhizoctonia solani*, *Pythium ultimum*,

Sclerotium rolfsii (Elad et al. 1984; Harman et al. 1993; Lorito et al. 1996; Lorito et al. 1994; Schirmböck et al. 1994; Benhamou and Chet 1997).

None of the compounds produced by the eleven isolates was able to be accurately identified using GC-MS chromatography. However, some generalizations can be made regarding the polarity and general composition of the chemicals. Based on the column and solvent used, small polar molecules would have eluted first from the column. These compounds were probably small polar proteins, such as peptaibols (Whitmore and Wallace 2004). The compounds that eluted in the middle were most likely larger aromatic hydrocarbons, similar to many polyketide-derived antibiotics including harzianopyridone (Vinale et al. 2009), trichorzianines (Rebuffat et al. 1989), and 6-pentyl- α -pyrone (Claydon et al. 1987). These are commonly produced by *T. harzianum* and have antimicrobial properties. The compounds with the highest retention times were most likely long chain, non-polar fatty acids, which may include ergosterol, a common component of fungal plasma membrane.

Image analysis of HPLC chromatograms has been shown to be effective at identifying *Trichoderma* strains. Thrane et al. (2001) concluded that secondary metabolite profiles are different enough to elucidate strain differences. Compounds with similar retention times, RI values and UV spectrum can be considered the same compound. Nielsen and Smedsgaard (2002) screened 474 mycotoxins and calculated the RI values for these compounds. However, the problem with using their RI values to classify unidentified compounds is the difficulty in reproducing exactly the HPLC methods they used. Temperature, solvent and column differences can all result in different retention times. Even multiple runs on the same column can result in differences

due to drifting of the retention times. To avoid errors in the binary matrix, the RI values in this study were calculated based on the average retention time for three replicates. If a compound was identified in only one or two of the replicates it was excluded from the analysis. Also to prevent cross contamination between sample injections, a wash cycle was run each time between samples. The WA calibration model was able to predict biocontrol efficacy based on secondary metabolite composition spectrum. It was found that *T. harzianum* isolates that reduced perithecial development to the same degree had secondary metabolites of similar chemical composition and concentration. Compounds unique to several isolates of *T. harzianum* show promise for biocontrol. However, in many cases there is generally little correlation between laboratory screening and field testing of biocontrol activity, and compounds produced in a Petri dish may differ from those produced under field conditions. Enzymes and antimicrobial compounds are influenced by substrate, temperature, and other organisms (Howell 2003).

The study demonstrated that using chemical signatures in combination with multivariate analysis can limit errors due to subjective visual analysis of the chromatographic data (Thrane et al. 2001; Nielsen and Smedsgaard 2002; Grondona et al. 1997). The WA calibration prediction model may be used for the rapid screening of *T. harzianum* isolates. The method used a regression model to predict the biocontrol activity from species responses to the environment, suggesting that some bioactivity is a direct function of secondary metabolites. The practical value of WA or WA-PLS approaches is that models could predict the performance of isolates prior to field studies. Furthermore, as suggested by El Gindy et al. (2004), spectrophotometric analysis using

WA-PLS performed well, compared with HPLC, at lower cost and fewer time-consuming steps.

In conclusion several compounds may be responsible for the reduction in perithecial production and act synergistically. *Trichoderma* isolates, T-22 and T-472, which had the highest biocontrol activity had unique chemical signatures which suggests that the compounds produced by these two strains in sufficiently high concentration may be responsible for reducing perithecial production. These unique signatures can be used in a weighted average prediction model to rapidly screen other *Trichoderma* isolates to identify other potentially useful strains. Further evaluation and creation of knock-out strains are required to accurately identify which compounds are responsible for control.

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6.0 Structural and ultrastructural study of the interaction between *Trichoderma harzianum* and perithecial development of *Gibberella zeae*

6.1 Abstract

Chronological events associated with the interaction between a strain of *Trichoderma harzianum*, T-472, with known biological control activity against perithecial production of *G. zeae* was studied using light, transmission and scanning electron microscopy, in order to determine the mechanisms of control. *Trichoderma harzianum* colonized the substrate very quickly and was able to outcompete the pathogen, *G. zeae*. The autoclaved mulched wheat straw inoculated with *G. zeae* only, had an average of 157 perithecia per plate and larger clusters of perithecia formed, consisting of 5 -15 perithecia. There was a general reduction in the number of perithecia that formed on straw which was treated with *T. harzianum*, with an average of 15 perithecia per plate and smaller clusters consisting of 3 to 6 perithecia. These mature perithecia from treated straw produced less pigment and were lighter in color. The thickness of the outer wall was also affected in the treated samples. In the untreated samples the outer wall consisted of 3- 4 cell layers, whereas in the treated samples, it was composed of just 1 to 3 cell layers. Generally immature perithecia were over-grown by *T. harzianum* approximately 15 days after co-inoculation with the biocontrol agent and pathogen. Few perithecia were colonized at later stages. The affected perithecia collapsed 21 days after inoculation (dai), compared to the perithecia in the untreated samples which collapsed 28 dai.

Ultrastructural changes were observed primarily in the exterior cells of the outer perithecial wall. Cytoplasmic degradation, invagination of the plasma cell membrane and thin cell walls occurred in the treated samples by 21 dai. Appressorium-like structures

and abundant mycelium were seen on the perithecia of the treated samples. However, direct penetration was not observed. It is possible that the perithecial structures are resistant to penetration by mycelium of *T. harzianum*.

6.2 Introduction:

Gibberella zeae (Schwein.) Petch (anamorph *Fusarium graminearum* Schwabe) is the main cause of fusarium head blight (FHB) in Manitoba (Gilbert et al. 2009). The disease attacks many small grain cereals and has resulted in millions of dollars in losses (Gilbert et al. 2000). *Gibberella zeae* is a facultative parasite that attacks living host tissue. They also are weak saprophytes and colonize senescent tissue and crop debris. Unlike most members of the family Nectriaceae, *G. zeae* lack ascogonia and antheridia. Instead, the development of dikaryotic hyphae in the xylem tissue and in the chlorenchyma cells of the host plant has been shown to induce perithecial development (Guenther and Trail 2005). The production of the dikaryotic stage allows for hybridization and recombination between nuclei without meiosis. Prior to perithecial formation, dense mats of hyphae containing abundant lipid bodies develop. This carbon sequestration may provide the fungus with the energy it needs for the next stages. Uninucleate hyphae begin to coil and form perithecial initials. These cells enlarge and remain uninucleate except when under going division. Once the ascogenous cells develop, apical paraphyses begin to elongate and become attached to the hymenium at the base of the developing perithecium. Within the immature ascus, a diploid nucleus forms and undergoes two meiotic and one mitotic division, giving rise to eight haploid ascospores (Trail and Common 2000). Asci are unitunicate and clavate with a short stipe and contain the eight 4-celled ascospores in a biseriate arrangement. The ascospores are hyaline and approximately 22 x 3.5 µm in size (Booth 1970). Intracellular iron sequestration by the siderophore, ferricrocin, is required for normal asci and ascospores

development. Oide et al. (2007) was able to demonstrate that ferricrocin-deficient strains were able to produce perithecia, but asci were abnormal in appearance and lacked ascospores.

The mature perithecia consist of three intergraded layers, the outer, middle, and inner layers. The outer wall is composed of thick-walled cells, 2-3 cells deep that are described as spherical and highly vacuolated (Trail and Common 2000). In the mature perithecia, this is the site of pigment deposition. The middle layer consists of flattened, highly granulated cells. These cells become progressively flattened as the perithecium develops. The inner wall consists of round multinucleate cells with thin walls, 3-5 cells thick. The middle and inner wall are completely compressed when the perithecium is fully matured (Booth 1971; Trail and Common 2000; Guenther and Trail 2005). The perithecia are ovoid/flask shaped with a rough tuberculate wall. Size ranges from 140-250 μm in diameter (Booth 1971). Environmental conditions such as temperature and moisture are important factors in the development and maturation of perithecia under field conditions. In the spring during favorable environmental conditions, purplish-black perithecia develop from which ascospores are subsequently released through the ostiole (Fernando *et al.* 2000; Inch et al. 2005; Sutton 1982; Paulitz 1996). Perithecia are formed in clusters representing various stages of maturity and are usually associated with the stomata and silica cells of wheat. Generally perithecia form denser clusters on nodes than internodes where silica cells are more abundant (Guenther and Trail 2005; Paulitz 1996).

Trichoderma harzianum (Inch and Gilbert 2007; Chapter 3.0; Chapter 4.0). and *Microsphaeropsis* sp. (Isolate P130A) (Bujold et al. 2001) were found to be effective at reducing perithecial production of *G. zae* under both controlled and field conditions.

However the direct effect on perithecial development of *G. zae* was not studied and the mechanisms involved in control of perithecial development using *T. harzianum* are not clear. It may result from competition, antibiosis, mycoparasitism or production of cell wall degrading enzymes (CWDE). *Microsphaeropsis* sp. has been found to effectively reduce the development of apple scab, *Venturia inaequalis* for which the mechanism of control was attributed to mycoparasitism and the production of CWDE, leading to the breakdown and degradation of the host hyphae (Carisse et al. 2000; Benyagoub et al. 1998). Benyagoub et al. (1998) were able to demonstrate that *Microsphaeropsis* sp., causes the breakdown of the cell wall by localizing cell wall components (glucans, cellulose and chitin) of *Venturia inaequalis* using gold-complexed β -1,4-exoglucanase and wheat germ agglutinin/ovomucoid-gold-complexed to cellulosic β -1,4-glucans and chitin monomers, respectively. The production of CWDEs by *Microsphaeropsis* sp. adversely affected the growth and development of *Venturia inaequalis* hyphae.

When developing an effective biocontrol method an understanding of the interactions between the antagonist and fungal pathogen in order to target application and optimize effectiveness of the biocontrol agent. This study is the first report in the literature on the direct interaction of *Trichoderma harzianum* on perithecial development. Other studies have primarily focused on the mycelial interaction and quantifying CWDE and antibiotic production.

The objective of this study was to investigate the interaction between *T. harzianum* and perithecial development of *G. zae* using light, transmission electron, and scanning electron microscopy in order to gain a better understanding of the mechanisms involved in the biological control.

6.3 Materials and methods

6.3.1 Inoculum preparation

To investigate the interactions between *T. harzianum* (Isolate T-472) and *G. zeae* (DAOM 192132), sterilized wheat stem tissue were mulched using a blender and a thin layer was distributed over the surface of potato dextrose agar (PDA) in Petri dishes. A macroconidial suspension of *F. graminearum* was prepared by inoculating liquid carboxymethyl cellulose (CMC) (carboxymethyl cellulose 15 g, NH_4NO_3 1g, KH_2PO_4 1g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5g, yeast extract 1 g, distilled water 1L, streptomycin sulfate 0.2g added to the cooled medium) and incubated under aeration to promote conidial production. After 5 days at room temperature the spore concentration was adjusted by adding distilled water for a final concentration of 5×10^4 spores/ml. *Trichoderma harzianum* cultures were grown on PDA for 10 days under fluorescent cool white light at 20°C. The spores were gently scraped from the surface and stored in 1.5 ml centrifuge tubes at 5°C until needed. The plates containing sterilized straw mulch +PDA were sprayed with a macroconidial suspension of *G. zeae* (5×10^4 conidia/ml) and with a spore suspension of *T. harzianum* isolates (5.5×10^5 spores/ml). Plates were placed at 20°C under UV light to promote perithecial development (Tschanz 1976).

6.3.2 Fixation

Residues were sampled at 8, 10, 15, 21, and 28 days after inoculation (dai). Samples were fixed in 3% (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 20 hours under a light vacuum at 4°C, then either post fixed for 2 hours at

4°C in 2% osmium tetroxide (OsO₄) in the same buffer or processed further without OsO₄ fixation. Tissues were dehydrated in a graded series of ethanol/water 30%, 50%, 70%, 85%, 95%, 2x100% for 20 minutes each followed by a graded series of propylene oxide/ethanol washes 50%, 75%, 2x100% for 30 minutes each. Osmicated samples were placed on a rotating rack, to ensure mixing of resin, and infiltrated with a mixture of propylene oxide and Spurr's resin (Spurr 1969) without activator in proportions of 3:1, 1:1, 1:3 for 24 hours followed by 3 days of 100% Spurr's with activator. Resin was changed daily. The samples were then transferred to fresh resin in aluminum weighing boats and placed in an oven at 70°C for polymerization of resin. After 24 hours samples were cut out of the block and mounted on blank epoxy resin stubs. Non-osmicated samples were embedded in JB-4 plus resin (Polysciences Inc.). Samples were placed in the infiltration solution for 3 days, resin was changed daily and placed on a slow rotator to allow for complete saturation. Samples were then transferred to embedding solution and placed in aluminum weighing dishes. Polymerization was carried out under anaerobic conditions by placing the dishes on ice in a desiccator flooded with nitrogen gas.

6.3.3 Light microscopy

Material embedded in JB4 was cut 2µm thin using a glass knife on a microtome (Sorvell Porter Blum JB4). The sections were placed in a drop of distilled water on coated glass microscope slides which were put on a slide warmer set at 80°C until all the water had evaporated. They were then covered with 1% aq. acid fuchsin for 1 minute on a slide warmer set at 80°C. Excess stain was removed by immersing the slides under running water for 2 minutes. Slides were then immersed in 0.05% toluidine blue O in

benzoate buffer (pH 7.4) for 1 minute and rinsed under running water for 2 minutes. The slides were blow-dried. Several drops of glycerol were placed on the slide and overlaid with a cover-slip. The slides were viewed using a Nikon optiphot light microscope and photographed using an Olympus Q-color 3 digital camera. Fresh samples were also examined using both a dissecting and compound microscope. Perithecia were removed from the straw and placed on a drop of distilled water. A cover-slip was placed over the perithecia which were examined using a light microscope.

6.3.4 Transmission electron microscopy (TEM)

Ultra-thin sections (100 nm) were cut with a diamond knife (Microstar) on an Ultracut Reichert-Jung Ultramicrotome and mounted on 200 nm nickel mesh grids and stained with uranyl acetate and lead citrate using a triple staining method. Multiple grids were stained by placing them into a grid support holder in a Petri dish containing KOH pellets. The grids were flooded with Reynold's lead citrate for 4 minutes, after which they were rinsed for 1 minute each in 3 changes of distilled water. The grids were placed back into the Petri dish and flooded with 1% uranyl acetate in 50% methanol for 10 minutes. They were rinsed for 1 minute in each of 3 changes of distilled water. The grids were then flooded one more time with Reynold's lead citrate for 2 minutes and rinsed three times in distilled water. Sections were examined and photographed with a Hitachi H-7000 electron microscope, operating at 75 kV. Negatives were developed using Kodak D-19 developer and then scanned.

6.3.5 Scanning electron microscopy (SEM)

Hexamethyldisilazane (HMDS) was used to prepare samples for SEM (Nation 1983). Treated residues were collected at 8, 10, 14, 21, and 28 days after inoculation and immersed in 3% (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 20 hours at 4°C under a low vacuum and rinsed in 3 washes of the buffer. Samples were dehydrated using a series of ethanol washes: 70%, 85%, 95% and 100% each for 15 minutes and then immersed in 2 changes of HMDS for 15 minutes each, after which samples were air-dried at room temperature. The prepared samples were mounted on SEM stubs with double sided sticky tape and stored in a desiccator under vacuum. The mounted samples were gold coated with an Edward Sputter Coater S150B and viewed with a Cambridge Stereoscan 120 scanning-electron microscope.

6.4 Results

Complete colonization of the mulched wheat straw and abundant spore production by *G. zeae* (Fig 6.1 A) or *T. harzianum* was observed when plates were inoculated with only one species (Fig 6.1 B). However, when the two species were co-inoculated, *T. harzianum* colonized the substrate faster and dominated colonization. *Gibberella zeae* colonized the straw more slowly and produced fewer macroconidia compared to the control (Fig 6.1 C). Perithecial production after 28 days was higher on the control plates with an average of 157 (n=8, Std dev 36) perithecia per plate. On treated wheat straw, numbers were reduced to an average of 15 (n=8, Std dev 7) perithecia per plate. Mature perithecia (21 dai) from wheat straw inoculated with *G. zeae* were purplish-black and the cells of the outer wall were uniform in appearance (Fig. 6.2 A and C). The cells of the

outer wall of the perithecia from straw treated with *T. harzianum* were lighter in color, clustered and non-uniform in appearance (Fig. 6.2 B and D).

The outermost walls of perithecia from untreated straw were 3-4 cell layers deep and the cells were thick-walled, large, tuberculate, and highly vacuolated. The cells of the middle wall appeared flattened and granular in appearance while the cells of the inner walls were flattened with numerous densely stained bodies (Fig. 6.2 E). The outermost wall of the perithecia from straw treated with *T. harzianum* were generally 1-2 cell layers deep and highly vacuolated. The inner layer was thinner than that of the untreated perithecia and composed of 2-3 compressed cell layers (Fig. 6.2 E). Collapsed paraphyses and ascospores in the 8-celled stage within the asci were observed in both the treated and untreated samples (Fig. 6.2 E and F). The cells of the outer walls of the perithecia of *G. zae* from control samples were tuberculate to slightly flattened in appearance and evenly distributed across the surface (Fig. 6.3 A). The perithecia from straw treated with *T. harzianum* were abnormal in appearance. The cells of the outer wall appeared highly degraded, giving the perithecia a smooth appearance (Fig. 6.3 B and C).

In the transmission electron micrographs, the cytoplasmic content in the control cells appeared granular and evenly distributed throughout the cell (Fig 6.4 A, C, and E). In the treated samples, there was a high degree of cellular degradation/disorganization, formation of globular masses, and the plasma membrane appeared to be retracted from the cell wall at several points (Fig. 6.4 F). The secondary cell wall of the treated samples lacks the striations seen in the untreated samples. The primary cell wall is starting to degrade (Fig. 6.4 B, D).

The perithecia from treated samples were colonized by *T. harzianum* at various stages of maturity. Early colonization of both immature and mature perithecia can be observed. Spores of *T. harzianum* and mycelia were found closely associated with the perithecial surface (Fig. 6.5 A – D). An appressorium-like structure can be seen on a collapsed perithecium of *G. zeae* (Fig. 6.5 E and F). Perithecia of *G. zeae* on untreated samples tended to grow in large clusters of 5- 15 perithecia on the mulched wheat straw and containing perithecia at various stages of maturity; immature and mature perithecia can be seen growing adjacent to each other (Fig. 6.6 A and C). The clusters that formed on treated straw tended to be small in comparison to the control, usually consisting of 3 to 6 perithecia (Fig. 6.6 B and D).

In the untreated samples, a few immature perithecia were aborted during early stages of development, 24% (36 out of 150) of the immature perithecia observed were prematurely aborted. The majority of the immature perithecia are well formed and round in appearance (Fig. 6.6 A and C) Not every immature perithecium is affected by *T. harzianum*. Some immature perithecia on treated straw appeared to escape colonization and appeared normal in appearance (Fig. 6.6 B). When straw was co-inoculated, many of the immature perithecia prematurely collapsed due to the presence of *T. harzianum*. Spores and mycelia of *T. harzianum* were closely associated with the collapsed immature perithecia (Fig. 6.7 D and F). Of the 100 immature perithecia examined on the treated samples 78% of them were found to be prematurely collapsed. Some of the perithecia from the treated sample reached maturity and looked normal in appearance. After ascospore discharge, approximately 28 days, the perithecia collapsed. This can be seen in both the untreated and treated samples (Fig 6.8 A-F).

Figure 6.1: Petri plates of potato dextrose agar with sterilized wheat straw mulch, 7 days after inoculation with spore suspensions of A) *Gibberella zeae*; B) *Trichoderma harzianum*, isolate T-472; C) co-inoculated with *G. zeae* and T-472

A



B



C



Figure 6.2: Photographs and light micrographs of perithecia of *Gibberella zeae* on A) straw inoculated with *G. zeae* only; B) *Trichoderma harzianum* (T-472) treated straw; C) straw treated with *G. zeae* only, with dark prominent outer wall (ow); D) T-472 treated straw, with small lighter-colored perithecia with uneven pigment deposition in the cells of the outer wall (ow); E) mature perithecia, stained with acid fuchsin and toluidine blue, at 21 days after inoculation (dai) from straw treated with *G.zeae* only, with mature ascospores (as), and paraphyses (pa), thick outer wall (ow) 3-4 cells deep, middle wall (mw) and compressed inner wall (iw); F) mature perithecia at 10X, stained with acid fuchsin and toluidine blue, at 21 dai from straw treated with T-472, with mature ascospores (as), and paraphyses (pa), thin outer wall (ow) 1-3 cells deep, middle wall (mw) and compressed inner wall (iw).Scale bars for E and F = 30 μ m

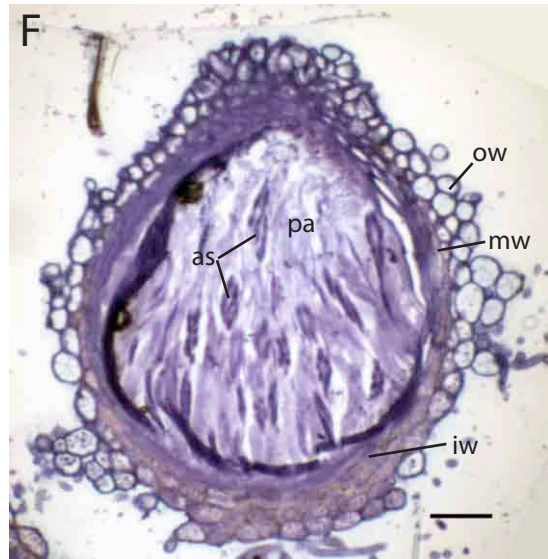
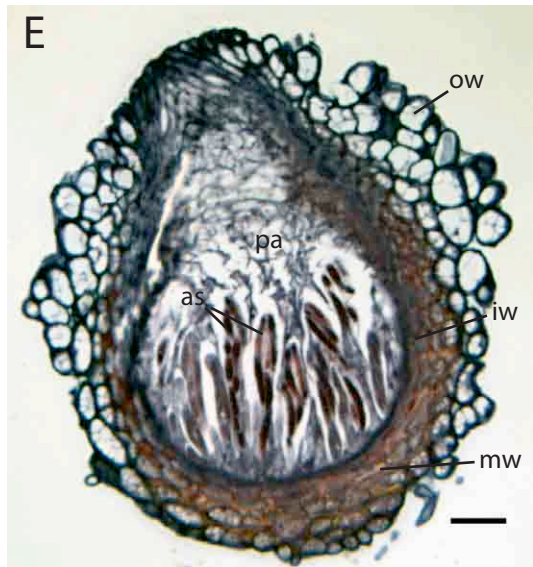
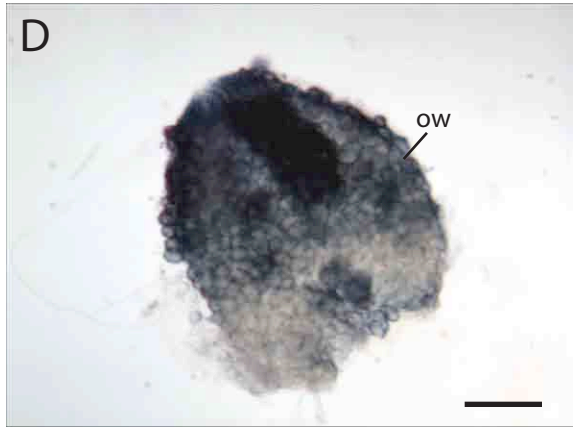
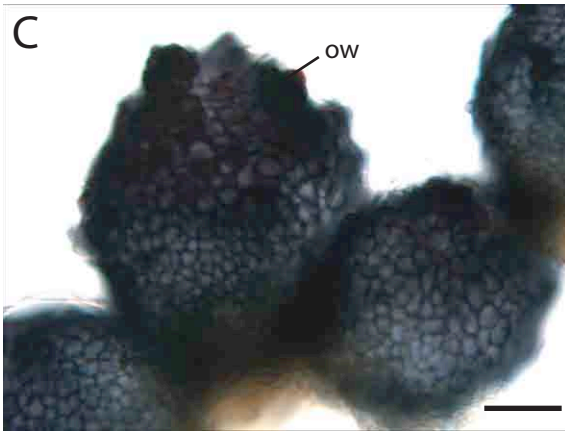
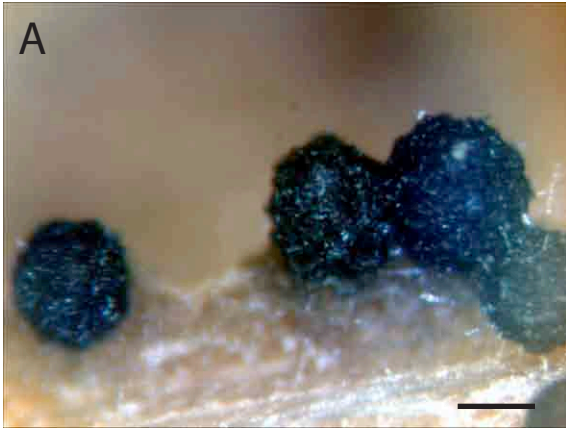


Figure 6.3: Scanning electron micrographs of mature perithecia of *Gibberella zeae* 28 days after inoculation (dai) on A) straw inoculated with *G. zeae* only. Outer wall (ow) consists of tuberculate cells covering the entire surface; B) and C) perithecium from straw treated with *Trichoderma harzianum* (T-472). The outer wall (ow) at the apex of the perithecium lacks tuberculate cells and may be covered in some material resulting in a smooth surface. The perithecium is closely associated with hyphae (hy) and *T. harzianum* spores (Ts). Scale bars = 20 μm

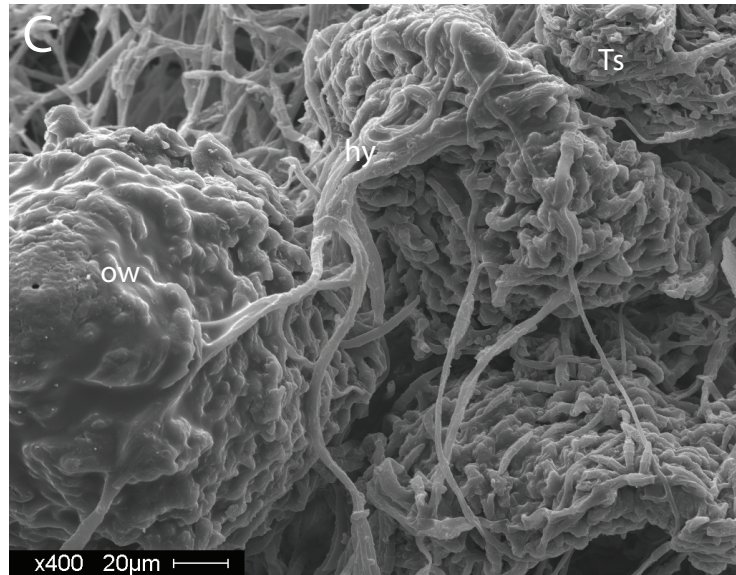
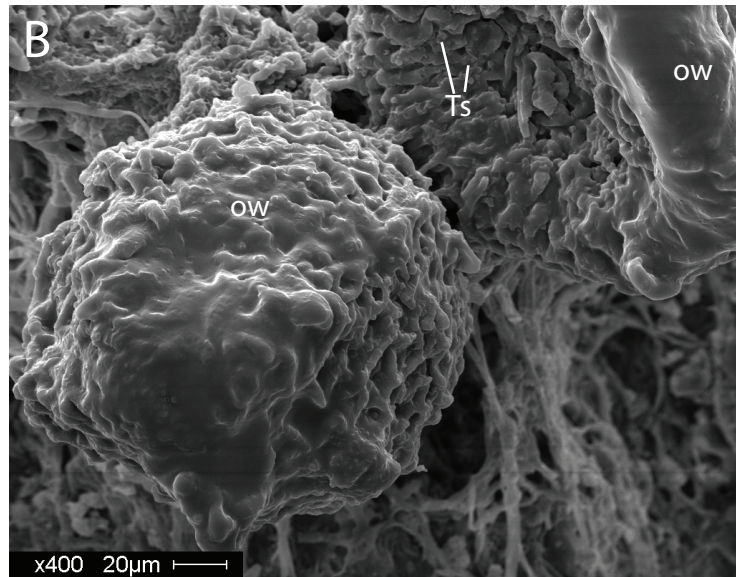
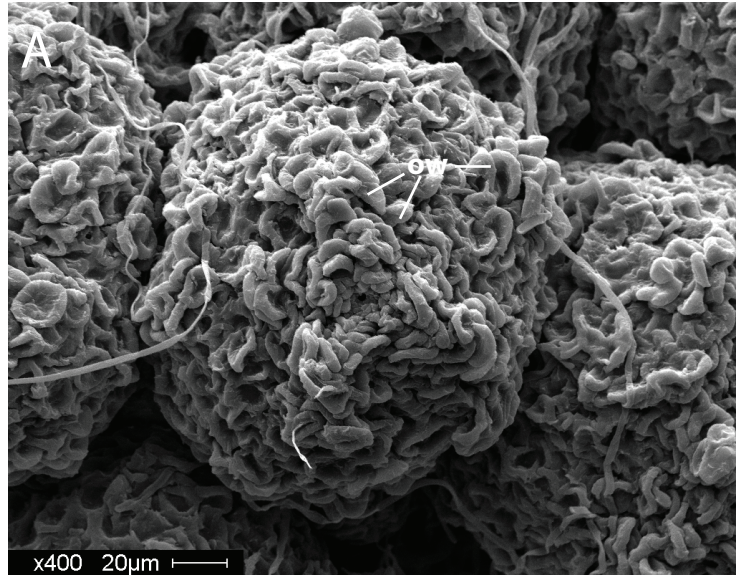


Figure 6.4: Transmission electron micrographs of the cells of the outer wall (ow) of mature perithecia of *Gibberella zeae* from straw treated with A) *Gibberella zeae* only, with thick secondary cell wall (2cw) and a thinner primary cell wall (1cw), and organized granular cytoplasm (cy) with organelles, B) outer wall (ow) of perithecium treated with T-472, with thin secondary cell wall (2cw). Primary cell wall (1cw) appears to be degraded; large globular masses (g) are present within the cytoplasm. C) cell of the outer wall of a perithecium from straw treated with *G. zeae* only, with granular cytoplasm (cy), thick secondary cell wall (2cw) and primary cell wall (1cw), and intact plasma membrane (pm); D) cells of the outer wall of a perithecium from straw treated with T-472. Appearance of large globular masses (g) within the cytoplasm (cy), thin secondary cell wall (2cw), degraded primary cell wall (1cw), invaginated plasma membrane (pm); E) *G. zeae* only, with thick secondary cell wall (2cw) and a thinner primary cell wall (1cw), organized granular cytoplasm (cy) with organelles, F) outer wall (ow) of perithecium treated with T-472, thinner secondary cell wall (2cw), cell wall (1cw) appears to be degraded, and the cytoplasm (cy) is disorganized.

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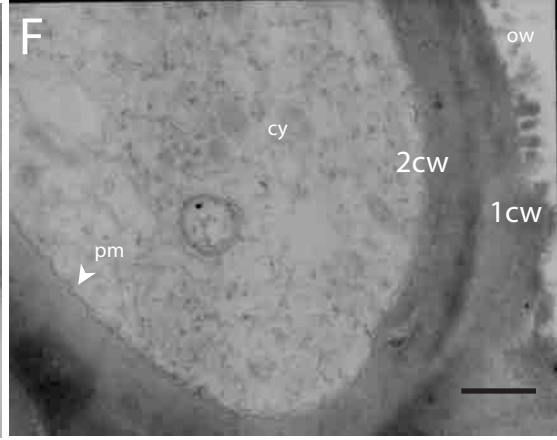
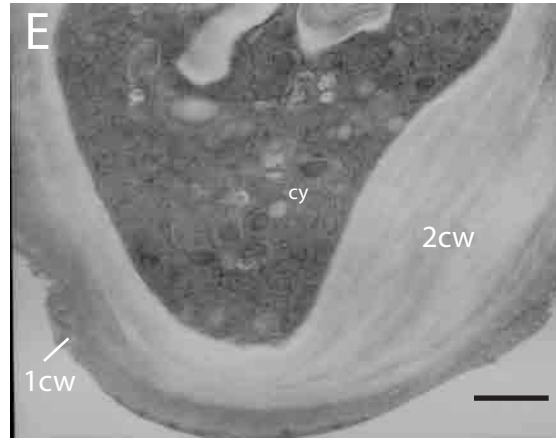
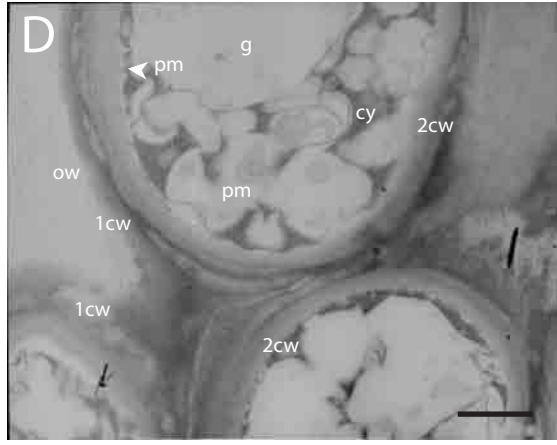
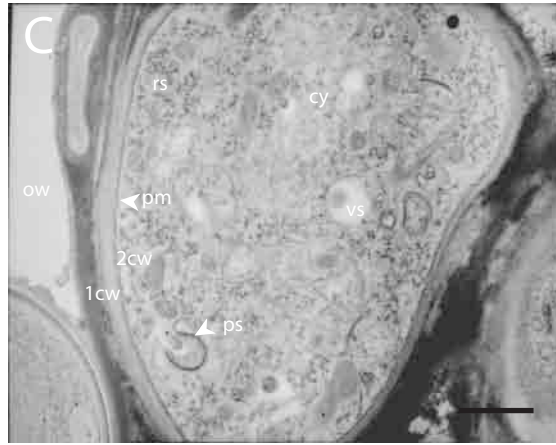
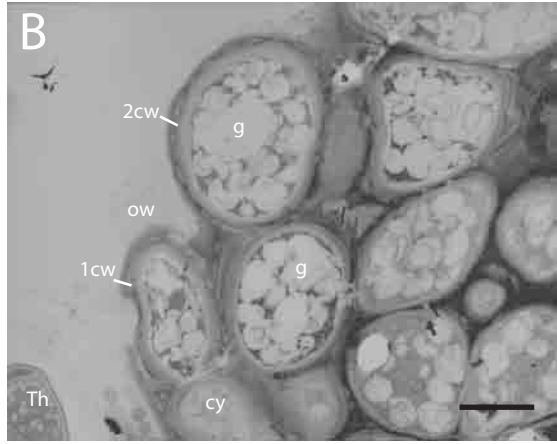
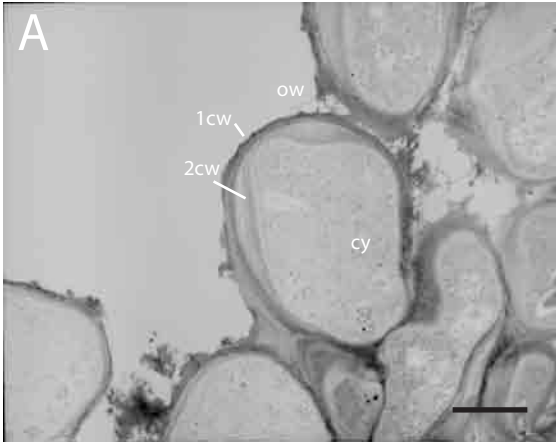


Figure 6.5: Scanning electron micrographs of perithecia from straw treated with *Trichoderma harzianum*, T-472. A) immature perithecia (Pi), 15 days after inoculation (dai) heavily colonized by *T. harzianum* spores (Ts) and hyphae (hy) B) immature perithecium, 15 dai heavily colonized by hyphae (hy); C) immature perithecium with *T. harzianum* spores and hyphae (hy) closely associated with the surface; D) higher magnification of *T. harzianum* spores (Ts) and hyphae growing along the surface; E) mature collapsed perithecium, 21 dai, colonized by *T. harzianum* spores and hyphae; F) higher magnification of the outer wall of mature perithecium with a hypha terminating with an apressorium-like structure (*). Scale bars for A and B = 10 μm ; scale bars for B and E = 20 μm ; scale bars for D and F = 5 μm

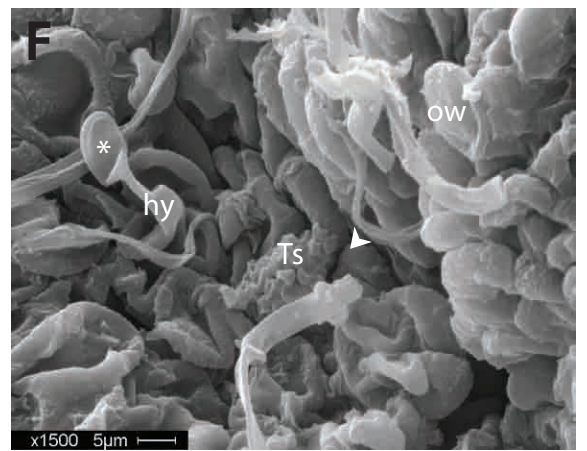
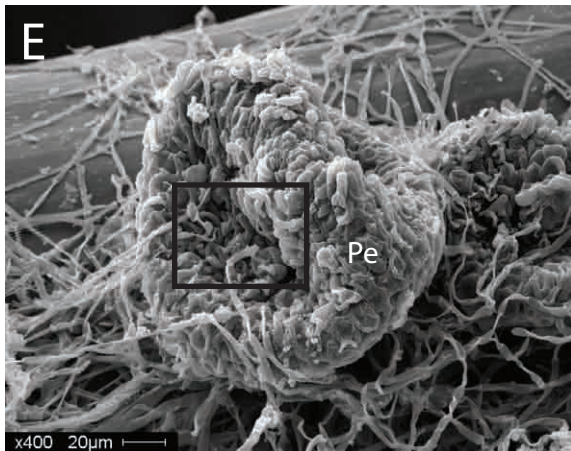
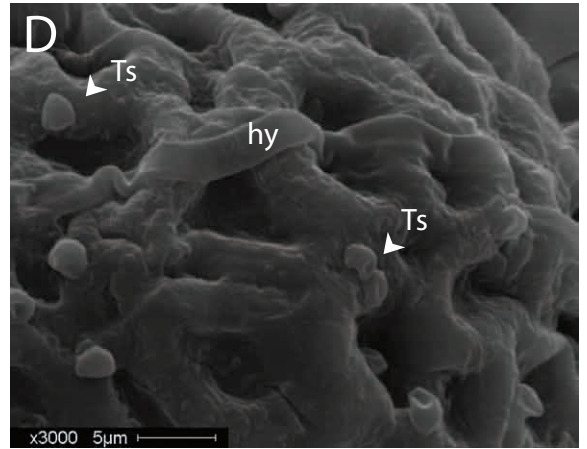
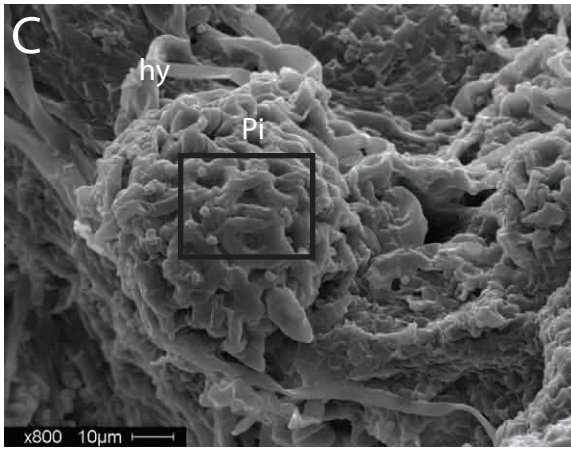
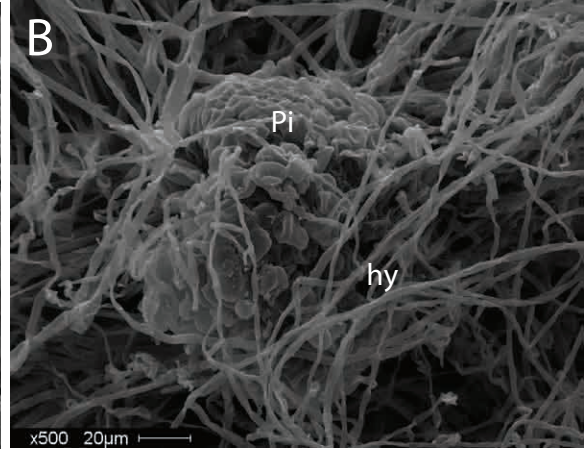
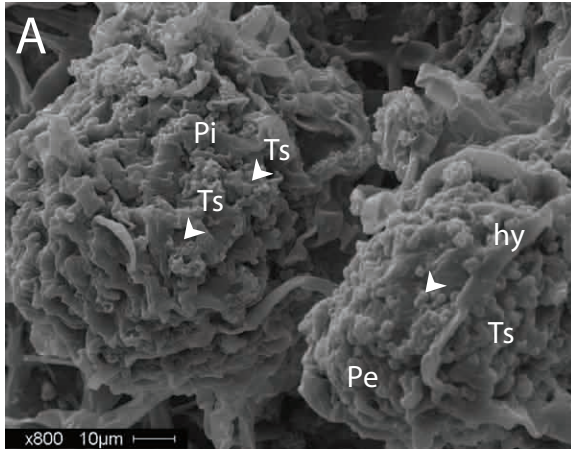


Figure 6.6: Scanning and light micrographs of clusters of perithecia from A) straw inoculated with *Gibberella zeae* only, 21 days after inoculation (dai), cluster of perithecia of different stages of maturity, mature perithecia (Pe) and immature perithecia (Pi); B) small cluster of perithecia at 21 dai from straw treated with *Trichoderma harzianum*, isolate T-472. Perithecia are at various stages of maturity, with mature collapsed perithecia (Pe) which have released ascospores and an immature perithecium (Pi); C) straw inoculated with *G. zeae* only, 28 dai, with cluster of perithecia which have collapsed, numerous ascospores and open ostiole (os) can be seen D) small cluster of perithecia at 28 dai from straw treated with *Trichoderma harzianum*, isolate T-472, perithecia are at various stages of maturity, with mature perithecia (Pe) and immature perithecia (Pi) Scale bars for A, B, C, D = 50 μ m

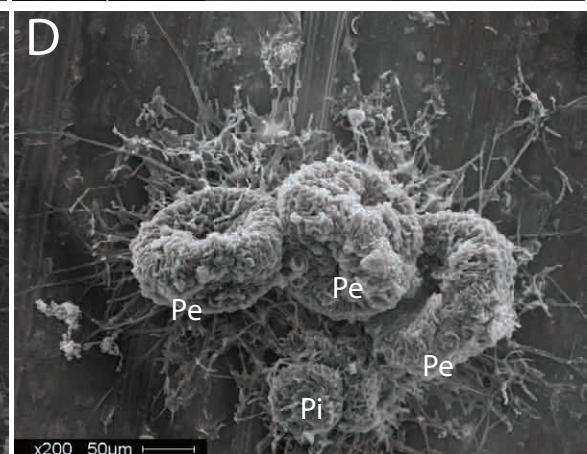
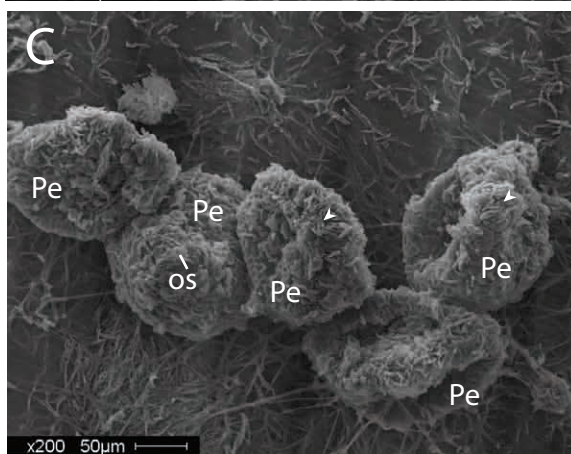
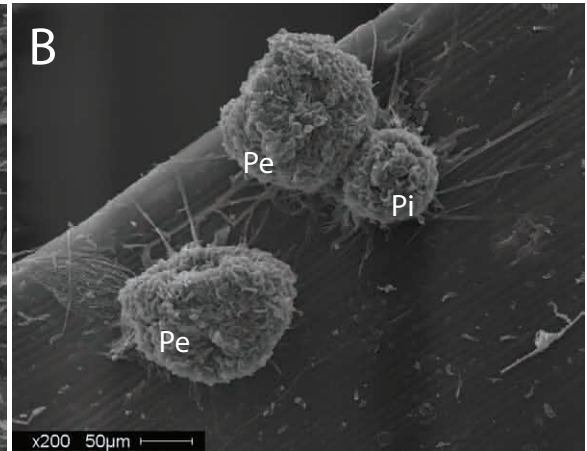
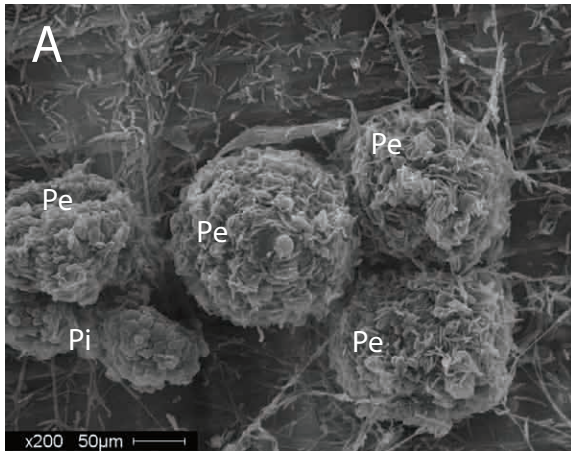


Figure 6.7: Scanning electron micrograph of immature perithecia 15 days after inoculation (dai) A) immature perithecium (Pi) from straw inoculated with *Gibberella zeae* only; B) immature perithecium (Pi) from straw treated with *Trichoderma harzianum*; C) immature perithecium (Pi) from straw inoculated with *G. zeae* only; D) collapsed immature perithecia (Pi) from straw treated with *Trichoderma harzianum*; E) immature perithecia (Pi) from straw inoculated with *G. zeae* only; F) higher magnification of immature perithecia (Pi) from straw treated with *T. harzianum* with hyphae and spores of *T. harzianum* closely adhering to the surface. Scale bars A, B, C, D, E = 20 μm ; scale bar F = 10 μm

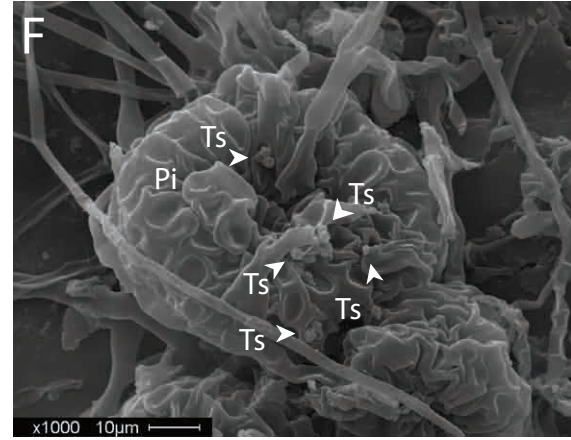
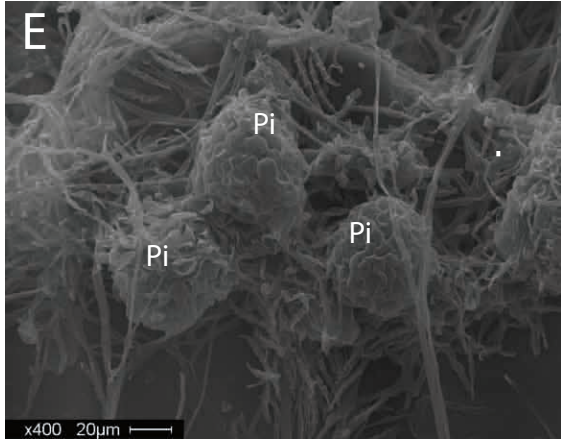
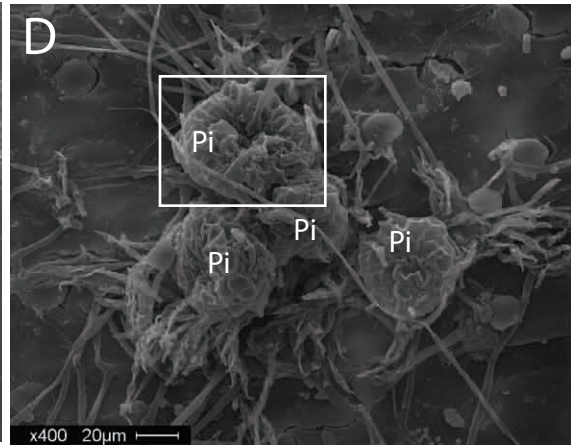
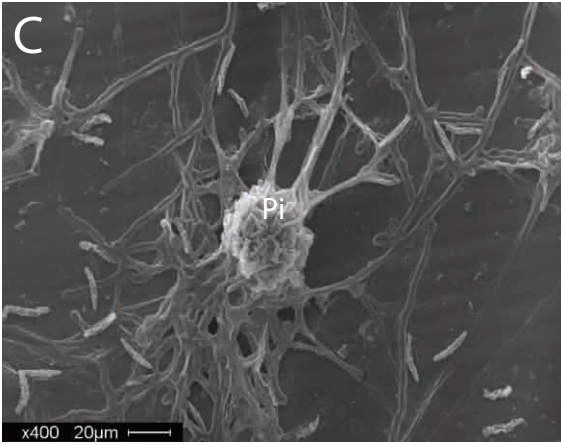
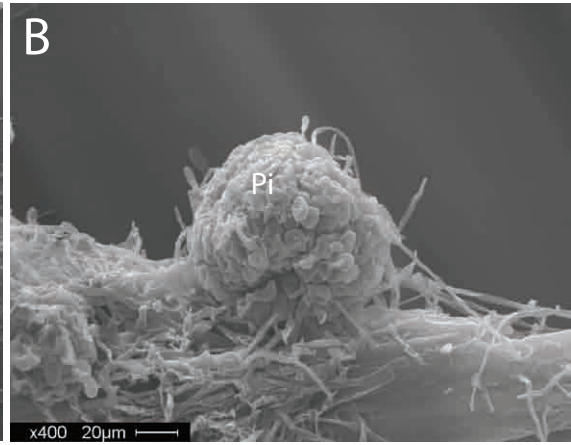
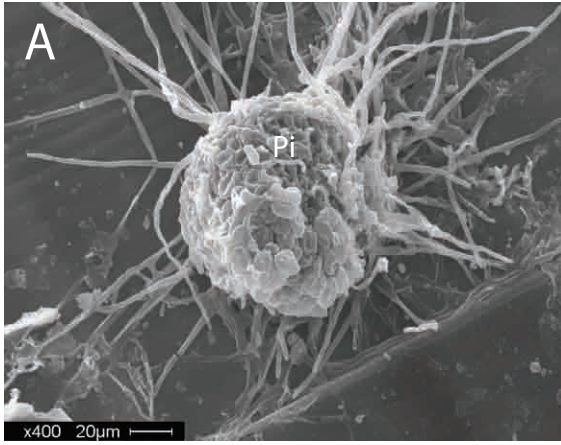
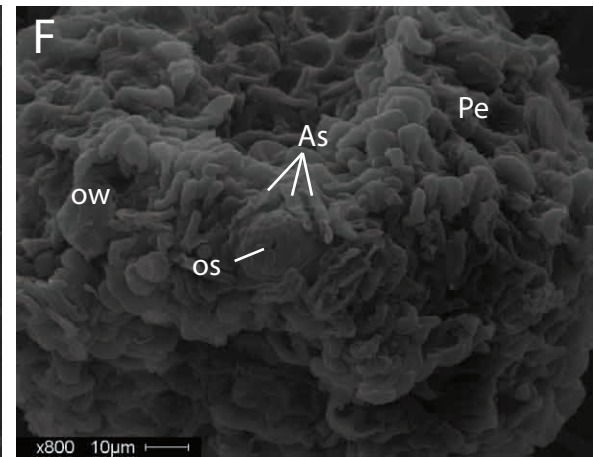
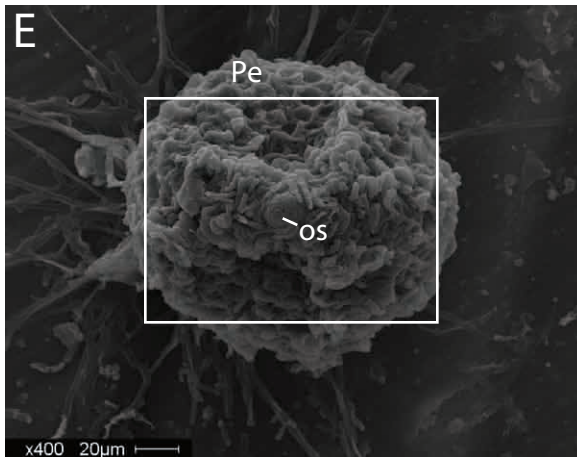
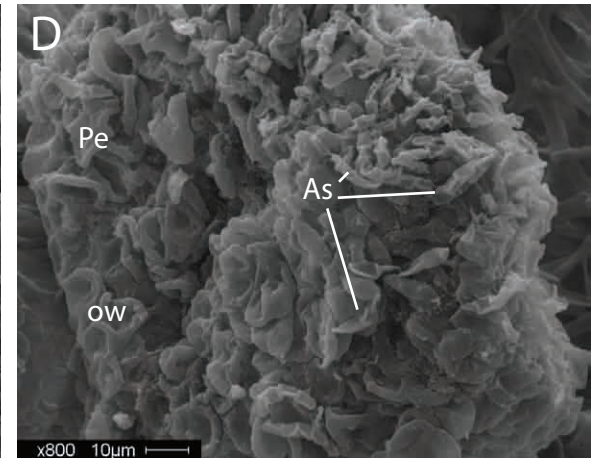
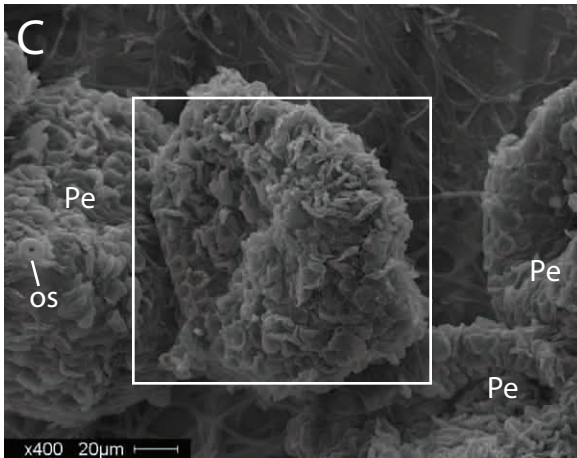
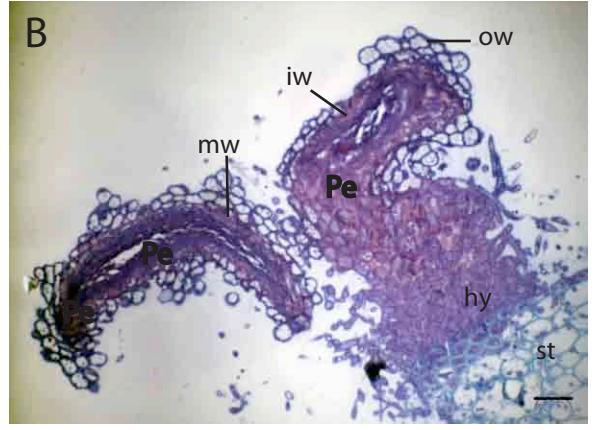
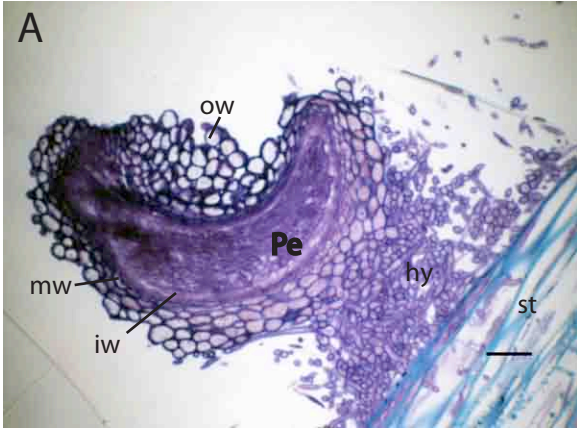


Figure 6.8: Collapsed perithecia of *Gibberella zeae* at 28 days after inoculation (dai) A) light micrograph of a perithecium from straw inoculated with *G. zeae* only, with thick outer wall (ow) 3-5 cells deep, thin middle (mw) and inner wall (iw) and hyphal mat (hy) at the base of the perithecium on straw tissue (st) B) light micrograph of a perithecium from straw treated with *Trichoderma harzianum* showing thin outer wall (ow), 1-3 cells deep, thin middle (mw) and inner wall (iw) and hyphal mat (hy) at the base of the perithecium on straw tissue (st), C) scanning electron micrograph of a mature perithecium with open ostiole (os) which has collapsed and released ascospores D) higher magnification of a mature perithecium (Pe) which has collapsed and released ascospores (as); E) scanning electron micrograph of perithecium (Pe) with open ostiole (os) from straw treated with *Trichoderma harzianum*; F) higher magnification of a mature perithecium (Pe) which has collapsed and released ascospores (as) through the open ostiole (os). bars A and B = 30 μm ; scale bar C and E = 20 μm ; scale bars D and F = 10 μm



6.5 Discussion

Trichoderma harzianum is a well-known biocontrol agent and strains T-22 (RootShield™) and T-39 (TRICHODEX, 20P™) are currently registered for the biocontrol of greenhouse fungal plant pathogens (Elad 1996; Kubicek and Harman 1998; Harman 2000). Multiple modes of action have been identified such as competition (Howell 2003), induced resistance (Harman 2006), solubilization of inorganic plant nutrients (Altomare et al. 1999), inactivation of the pathogen's enzymes involved in the infection process (De Meyer et al. 1998), and mycoparasitism (Barnett and Binder 1973). The fungus is also known to produce CWDE including chitinases (Baek et al. 1999), β 1-3 glucanases, endo β 1-6 glucanases (Cruz et al. 1995) proteases (Flores et al. 1997; Elad and Kapat 1999), antibiotics (Dennis and Webster 1971; Claydon et al. 1987; Fujiwara et al. 1982; Vinale et al. 2004; Vinale et al. 2009), and antibiotic peptides, such as peptaibols (Iida et al. 1994),

The mature perithecia consist of three intergraded wall layers, the outer, middle, and inner wall (Trail and Common 2000; Guenther and Trail 2005). These three layers were observed in the perithecia from both the control and treated samples. Differences in external morphology were seen in the perithecia from the straw treated with *T. harzianum*. Cells in the outer wall layer were only 1-3 cells deep and showed signs of cellular degradation in the light and TEM micrographs. Small invaginations of the plasma membrane were observed suggesting membrane perturbation. Belanger et al. (1995) observed similar changes to the plasma membrane of *Botrytis cinerea* when co-inoculated with *T. harzianum*. The invagination of the membrane occurred 12 hours before physical contact between the two organisms. *Trichoderma harzianum* is known to

produce various types of peptaibols. Peptaibols form voltage-gated ions in the membranes, which results in membrane disruption and hemolysis (Fox and Richards 1982). Disorganization of the cytoplasm was also observed in the exterior cells 21 dai. Production of antimicrobial compounds may deregulate the host metabolism leading to the breakdown and degradation of cellular material (Benhamou and Chet 1997). The outer wall of the exterior cells of the perithecia also appeared to be affected by the presence of *T. harzianum*. This may be attributed to the production of various CWDEs by *T. harzianum*. Benhamou and Chet (1997) were able to demonstrate through cytochemical labeling that CWDEs produced by *T. harzianum* affected cellulose and β -glucans in the cell wall of *Pythium ultimum*. This breakdown/weakening of the cell wall allows for local penetration of host by *T. harzianum*. Several other studies have reported that cell wall synthesis is a major target site for mycoparasitism (Lora et al 1995; Lorito et al. 1996; Kucuk and Kivanc 2008).

In general fewer perithecia formed on straw treated with *T. harzianum*. This may be due to *T. harzianum* preventing the formation of the dikaryotic stage, which appears essential for the initiation of perithecia (Trail and Common 2000). The release of inhibitory compounds or cell wall degrading enzymes from *T. harzianum*, or competition for nutrients may be preventing the formation of the dikaryotic stage of perithecial development. Carbon sequestration and accumulation of lipids have been suggested to be required for perithecial formation (Trail and Common 2000; Trail 2009). Production of compounds may be inhibiting or altering gene expression during initiation perithecia. Hallen et al. (2007) identified over 2000 gene sequences specific to perithecial

development. It is unclear what the effects are on perithecial production if these genes are silenced at any stage of development.

Over-growth of the perithecia by *T. harzianum* was evident in the SEM micrographs. It appears the young immature perithecia are more susceptible to attack by *T. harzianum*. At 15 dai, mycelia and numerous spores of *T. harzianum* could be observed covering the surface of the young perithecia. Trail and Common (2000) found that many perithecia were aborted at the early stages of development, rather than at the later stages. However it was unclear why this occurred. Later colonization of mature perithecia could be seen 21 dai. The perithecia in the *T. harzianum* treated samples collapsed earlier than the controls, where collapse generally followed ascospore discharge after about 28 days. *Trichoderma harzianum* may be producing compounds that are disrupting ion transport of potassium and chloride ions into the perithecium. These ions have been demonstrated to increase the turgor pressure inside the perithecium and are responsible for the forcible discharge of ascospores (Trail et al. 2005). No direct penetration was observed in the micrographs, an appressorium like structure was seen on a collapsed perithecium 21 dai. Only a few appressorium-like structures were found. However, as direct penetration was not observed, perithecia may be resistant to penetration and the cells of the exterior wall may act as the first line of defense against mycoparasitism.

The number and size of the clusters of perithecia that formed on the treated and untreated straw also differed. In the untreated samples numerous clusters of perithecia formed which consisted of 5 to 15 perithecia compared to 3 to 6 perithecia in the treated sample. The reason for fewer perithecia forming in the treated sample may be due to a

combination of competition for space and resources. Also, the production of secondary metabolites has been found to reduce perithecial numbers. Inch et al. (2009) correlated secondary metabolite production with biocontrol activity. Isolates of *T. harzianum* that reduced perithecial numbers had unique chemical fingerprints (Chapter 5.0).

Pigment biosynthesis in perithecial wall also appeared to be affected by the presence of *T. harzianum*. In the untreated samples the mature perithecia developed the characteristic dark purplish black pigment, the function and chemical structure of which is unknown. In the treated samples the perithecia which developed to maturity were much lighter in colour than seen in untreated samples indicating a reduction in pigment production. This suggests that *T. harzianum* may produce compounds that interfere with pigment biosynthesis. Frandsen and Giese (2008) identified cluster of six genes, responsible for the biosynthesis of the pigment. The cluster consists of *PGL1*, *pglM* (monooxygenase), *pglJ* (O-methyltransferase), *pglV* (short chain dehydrogenase), *pglX* (zinc dependent oxidoreductase) and *pglR* (binuclear zinc cluster transcription factor). The genes are co-expressed during perithecium development. The cluster of encoded enzymes are not homologous to the enzymes for 1,8-dihydroxynaphthalene (DHN) melanin biosynthesis as seen in other fungi, which suggests that *Gibberella* pigment is not DHN melanin. However, the pigment produced probably functions in a similar fashion to melanin and may confer protection from UV radiation and lytic enzyme attack (Prota et al. 1998).

In conclusion morphological changes in perithecial development were observed when straw was treated with *Trichoderma harzianum*. In general fewer perithecia formed and the perithecia that did form frequently collapsed prematurely. Possible mechanisms

may be the production of secondary metabolites that may have inhibited the formation of the dikaryotic stage. These compounds may have interfered with potassium uptake, which is essential for the increase in osmotic pressure inside the perithecium. Competition for space and nutrients may have prevented perithecial formation. Intracellular changes, such as degradation of the protoplasm, were also observed in the cells of the outer perithecial wall. Cell wall breakdown appears to be occurring in mature perithecia. This may have been due to the production of CWDEs. Further investigation of the role of CWDEs on the outer perithecial wall is required.

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7.0 General discussion and conclusions

This dissertation has contributed new information and ideas towards the biological control of *G. zea* using fungal antagonists as follows:

- 1) Identified *Trichoderma harzianum* as an effective biological control agent against perithecial production on wheat straw production under both laboratory and field conditions. *Penicillium* and *Chrysosporium* species from the soil were also found to be effective at reducing mycelial growth. However, these species significantly reduced seed germination.
- 2) Application of *Trichoderma harzianum* spore suspension and cell-free filtrate were found to be most effective in reducing number of perithecia when applied to wheat straw 24 hours prior to a macroconidial suspension of *Gibberella zea*.
- 3) Perithecial production of *Gibberella zea* was reduced when cell-free extract/filtrate was applied to wheat straw. This suggests that antimicrobial compounds may be partial responsible for control and these compounds act synergistically.
- 4) Developed a predictive model to identify and predict biological control activity of *Trichoderma harzianum* strains based on their chemical signatures/ retention indices using a multivariate weighted average regression model.
- 5) Determined that the potential mechanisms of biological control of perithecial production of *G. zea* by *T. harzianum* may be partly due to a combination of competition and antibiosis/production of secondary metabolites.

One of the foremost challenges when developing biological control is rapid and efficient identification of BCAs. When screening potential bacterial and fungal antagonists, it is estimated that only 1 to 10% of the isolates screened *in vitro* will show some capacity to inhibit the growth of the pathogen (McSpadden Gardner and Fravel 2002). In this study, 45% of the fungi, including T-22 (RootShield) and others which were obtained from either CCFC or isolated from soil, wheat heads, and crop debris inhibited the growth of *G. zeae* by more than 50%. However, a screen that uses confrontation assays on Petri plates containing agar provides only limited information and may not be applicable to *in planta* situations (Lindow 1988). For example, inhibition zones provide only information regarding antimicrobial production, but even there a number of factors will influence the type and amount of compounds produced including selection of the growth media, temperature, light, and pH (Schisler and Slininger 1997). None of the isolates that were screened showed clear inhibition zones. However, many of the isolates reduced the growth of *G. zeae* and in some instances were able to over-grow *G. zeae* suggesting that a number of factors may be responsible for control including, growth rate, mycoparasitism, the direct colonization of the pathogen, and competition for space and nutrients which may, or may not involve production of secondary metabolites to prevent the growth and establishment of the pathogen.

The next step in evaluating potential BCAs are *in planta* tests where antagonists are applied to a target site and evaluated for their efficacy of control. Fusarium head blight is a monocyclic disease. The primary inoculum arises from *G. zeae* infested plant debris left on the soil surface. In the spring, when conditions are favorable, perithecia develop and subsequently give rise to wind-dispersed ascospores. Perithecial development is an ideal

stage to target with BCAs, as eliminating this stage would disrupt the life cycle of the pathogen and reduce inoculum development. Several fungi have been identified which reduce the saprophytic colonization of crop debris by *G. zeae* including *Trichoderma harzianum*, *Clonostachys rosea*, and non-pathogenic *Fusarium* species (Luongo et al. 2005; Bujold et al 2001; Kucuk and Kivanc 2008; Fernandez 1992). However, these studies did not examine the effects of perithecial development on treated debris. One of the new findings of this study was the effect of *T. harzianum* strains and *Chrysosporium* isolates on perithecial production. Isolates of both genera were able to significantly reduce perithecial development on wheat straw. Only one other fungal antagonist, *Microsphaeropsis* sp. (isolate P130A) has been found to significantly reduce perithecial development on wheat and corn debris (Bujold et al. 2001).

To control perithecial development the BCA would be applied to the soil where it would colonize crop debris and survive in the soil. Therefore, the presence of the BCA should not disrupt any aspect of the life cycle of the plant. The effects on wheat seed germination of isolates of both *T. harzianum* and *Chrysosporium* sp. were checked; the latter inhibited seed germination and just *T. harzianum* was further evaluated to determine the effects of application time on perithecial production. The appropriate time of application of a BCA is critical to ensure optimal efficacy and control. Perithecial reduction was greatest when *T. harzianum* was applied 24 hours before the pathogen. Early application allowed the BCA to establish and colonize the residue before *G. zeae*. The cell-free filtrate of *T. harzianum* was also effective at reducing perithecial development suggesting that secondary metabolites are at least partly responsible for the biological control activity. *Trichoderma harzianum* is known to produce a variety of

antimicrobial compounds and cell wall degrading enzymes. The secondary metabolites were extracted using diethyl ether and fractionated using column chromatography. The whole extract retained the biocontrol activity. However, when the compounds were separated they lost their ability to significantly reduce perithecia numbers. Therefore, control may be the result of a synergistic effect of two or more compounds produced by *T. harzianum*.

A predictive model was also developed. Reverse phase-high performance liquid chromatography revealed that each *T. harzianum* isolate had a unique chemical signature. The retention times were converted to a retention index and arranged in a reverse rank order based on relative concentration. The combination of compounds most likely to affect biological control were identified through cluster analysis, ordination and a weighted average calibration model. This model was able to predict biocontrol efficacy based on the spectrum of secondary metabolite composition. Isolates that reduced perithecial production generally produced similar metabolites. This method could be used for the rapid screening of potential BCAs at a lower cost and with fewer time-consuming steps such as confrontation plate assays.

The interaction between *T. harzianum* and *G. zae* has not been fully established in the literature. Competition and production of secondary metabolites were established as control mechanisms. However, it was initially unclear whether or not mycoparasitism is a factor in the bioactivity. *T. harzianum* is a well known mycoparasite of many plant pathogens. Structural and ultrastructural changes in the perithecial outer wall were observed using light, scanning and transmission electron microscopy. The thickness of the outer wall in the treated samples was 3-4 cell layers deep, whereas the outer wall from

treated samples was only 1-3 cell layers deep. Ultrastructural changes were also observed primarily in the exterior cells of the outer wall. Cytoplasmic degradation, invagination of the plasma cell membrane and thin cell walls occurred in the treated samples. Immature perithecia, observed 10 days after inoculation, appeared to be closely associated with *T. harzianum* which may have caused them to collapse prematurely. This may be responsible for the reduction in the number of mature perithecia observed on treated straw. *Trichoderma harzianum* also was observed overgrowing mature perithecia. Appressoria-like structures were seen in 6% of the SEM samples. In the mature perithecia of both untreated and treated samples, ascospores developed and were discharged. The ascospores from treated samples were found to be viable. However, the reduction in ascospore numbers is attributed to the fact that fewer perithecia developed rather than the average number of ascospores per perithecium.

In conclusion, *T. harzianum*, isolate T-472 (obtained from the CCFC, unknown substrate) was identified as an effective biocontrol agent which reduced perithecial development of *G. zae* on treated wheat straw under laboratory and field conditions. Large-scale field trials are required to determine the efficacy of this strain in reducing disease incidence and severity. Intuitively, if the numbers of perithecia are reduced, disease levels will also be lower. Due to the long-range transport of ascospores, optimal implementation of this biocontrol agent would be on a regional scale to provide on overall reduction of disease inoculum. The main mechanisms of control were attributed to competition and secondary metabolite production. However, the identification of the compounds responsible for the control were not identified in this study. Further evaluation and identification of these compounds is required.

7.1 Literature cited

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Appendix 1: Effect of bacterial suspensions on percent mycelial inhibition, percent seed germination, fusarium head blight (FHB Index), and perithecial production (% perithecia reduced) of the *Gibberella zeae* strain (DAOM 19132) on the wheat cultivar CDC Teal.

Bacterial Isolate	Mycelial inhibition (%)	Seed germination (%)	^w FHB index	^y Perithecia reduction (%)
B-1	63	66	76	22
B-5	75	78	87	10
B-31	68	69	81	5
B-35	61	64	89	31
B-59	63	48	84	2
B-68	50	55	87	10
B-70	66	71	92	22
B-73	72	62	96	20
<i>G. zeae</i>	100	-	88	0
water only	-	78	0	-

Ten heads per replication were rated for disease severity and incidence

Average value for two experiments

^wFHB Index = (disease severity * disease incidence)/100

^y Perithecial reduction as a percent of control.

Appendix 2) ANOVA of the interaction between application time of *Trichoderma harzianum* spore suspensions on A) perithecial, B) ascospore and C) macroconidial production of *Gibberella zeae* on wheat straw

Appendix 2A) Interaction between application time of *Trichoderma harzianum* spore suspensions on perithecial production of *Gibberella zeae* on wheat straw

Source	DF	Type III SS	Mean Squares	F Value	Pr >F
rep	1	2.634	2.634	15.03	0.0003
isolate	10	512.357	51.236	292.49	<0.0001
time	4	394.286	98.572	562.71	<0.0001
time*isolate	40	91.756	2.294	13.19	<0.0001

R Squared	Coeff Var	Root MSE	speri Mean
0.988	7.235	0.419	5.785

Appendix 2B) Interaction between application time of *Trichoderma harzianum* spore suspensions on ascospore production of *Gibberella zeae* on wheat straw

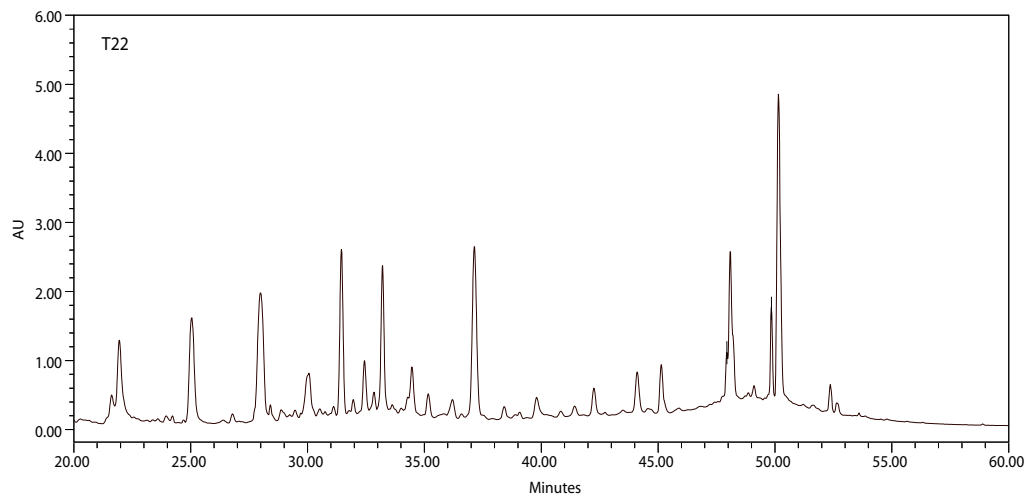
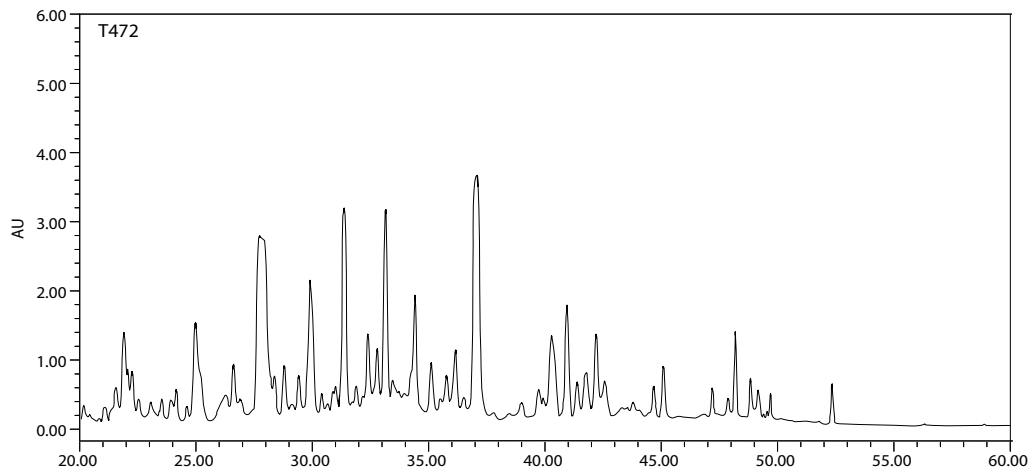
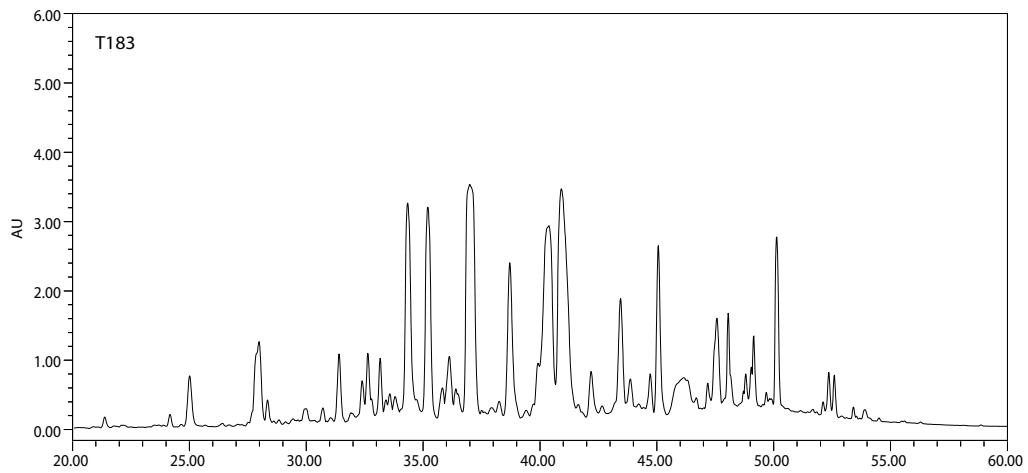
Source	DF	Type III SS	Mean Squares	F Value	Pr >F
rep	1	3769.701	3769.701	1.5	0.2259
isolate	10	2049409.713	204940.971	81.3	<0.0001
time	4	5214880.155	1303720.039	517.18	<0.0001
time*isolate	40	904019.031	22600.476	8.97	<0.0001

R Squared	Coeff Var	Root MSE	asco Mean
0.979	4.962	50.208	1011.646

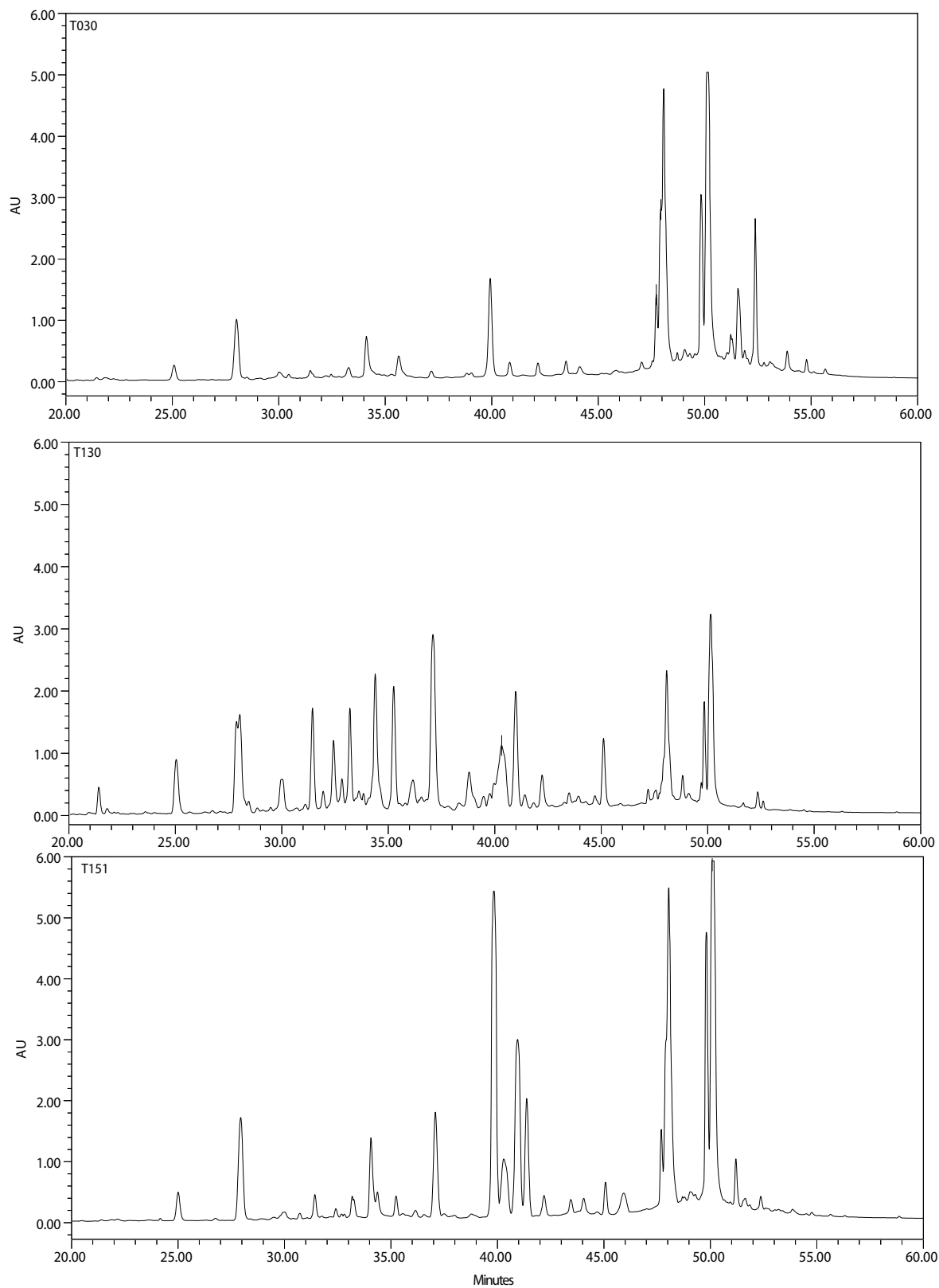
Appendix 2C) Interaction between application time of *Trichoderma harzianum* spore suspensions on macroconidial production of *Gibberella zeae* on wheat straw

Source	DF	Type III SS	Mean Squares	F Value	Pr >F
rep	1	12063.486	12063.486	1.69	0.1977
isolate	10	131435.034	13143.593	1.85	0.0702
time	4	145426.727	36356.682	5.11	0.0012
time*isolate	40	134622.485	3365.562	0.47	0.9937

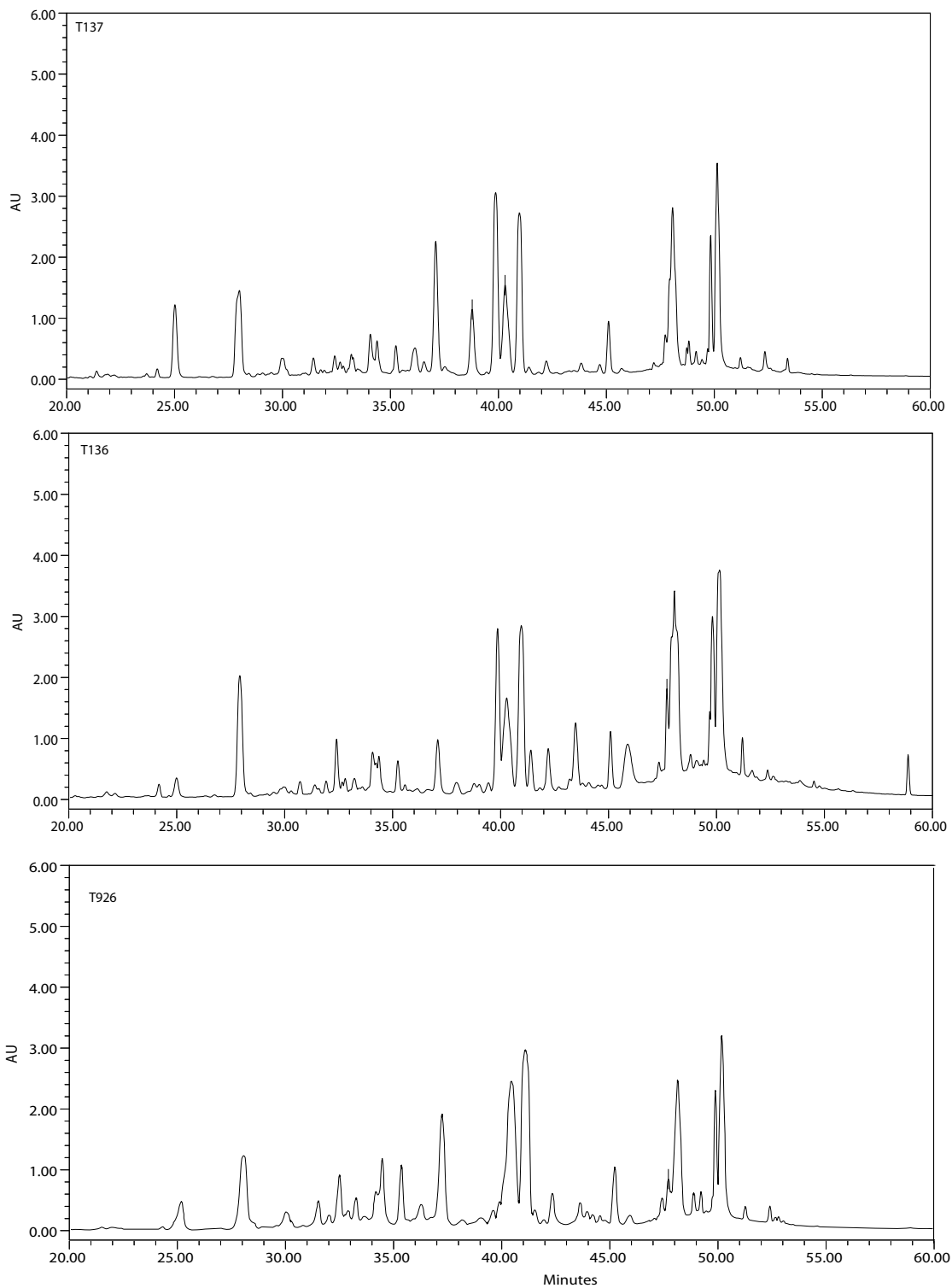
R Squared	Coeff Var	Root MSE	macro Mean
482	5.391	84.385	1565.036



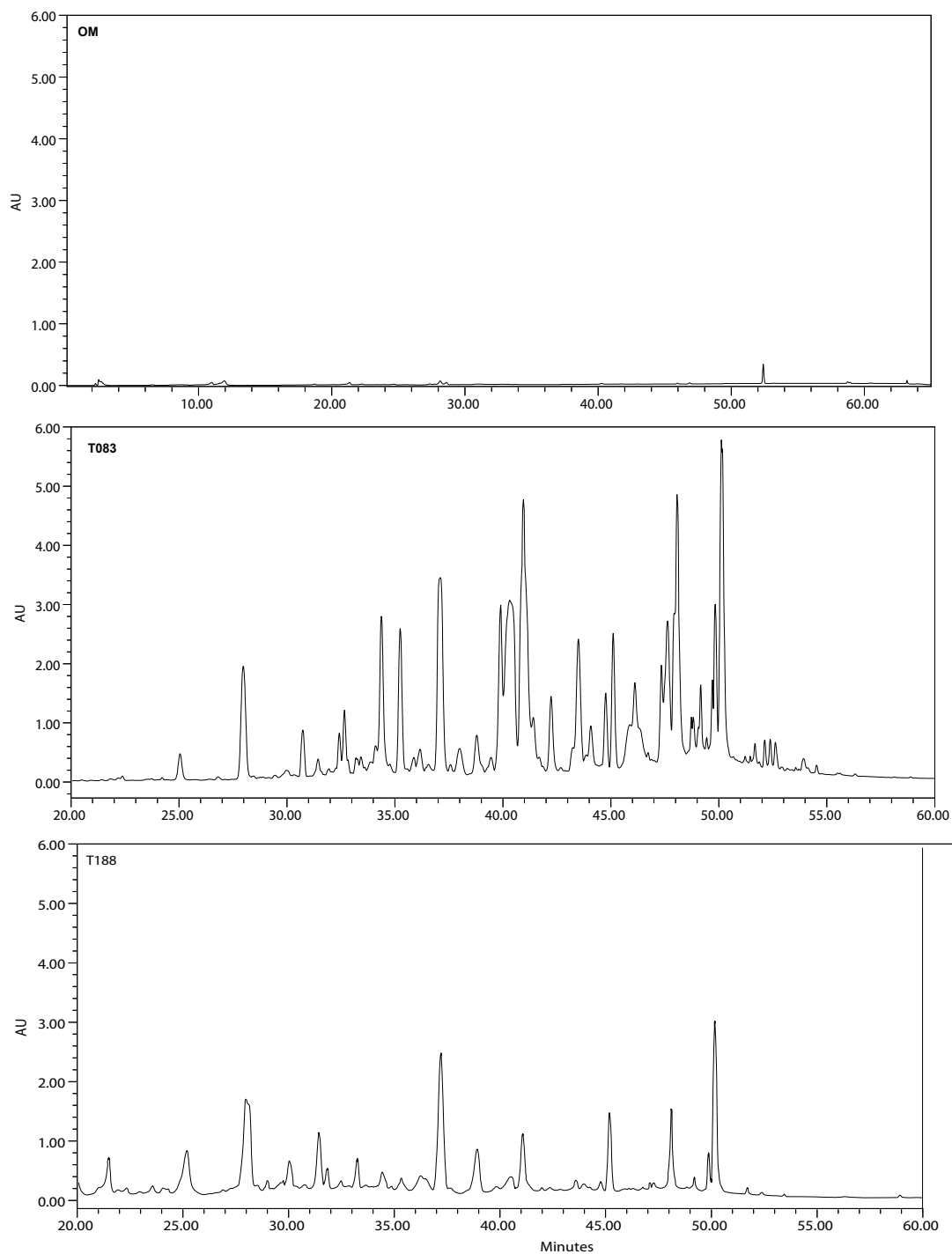
Appendix 3a: Reverse phase- high performance liquid chromatograph of cell-free filtrate extract of *Trichoderma harzianum* isolates T-183, T472, and T-22



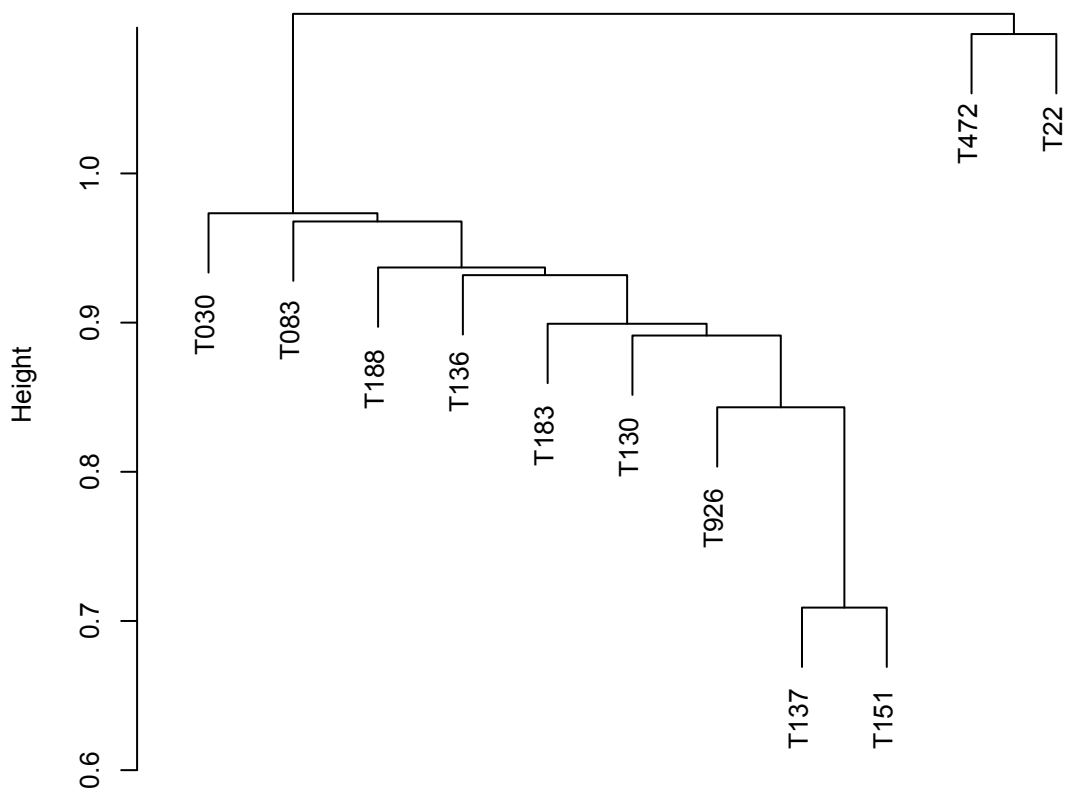
Appendix 3b: Reverse phase- high performance liquid chromatograph of cell-free filtrate extract of *Trichoderma harzianum* isolates T-030, T-130, and T-151



Appendix 3c: Reverse phase- high performance liquid chromatograph of cell-free filtrate extract of *Trichoderma harzianum* isolates T-136, T-137, and T-926



Appendix 3d: Reverse phase- high performance liquid chromatograph of cell-free filtrate extract of *Trichoderma harzianum* isolates T083, T-188 and organic



Appendix 4: Nearest Neighbor (single-linkage) dendrogram of isolates using a Jaccard association matrix of binary relationships (presence-absence) of compounds detected in HPLC. Branch lengths indicate distance (differences) in the chemical composition of isolates.