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Molecular interactions of arbuscular mycorrhizal fungi with mycotoxin-producing fungi and their role in plant defense responses

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Cette thèse intitulée:

Tripartite interactions between plants, mycotoxin-producing fungi and arbuscular mycorrhizal fungi, using the approach of functional genomics

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a été évaluée par un jury composé des personnes suivantes :

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Résumé

Les trichothécènes de *Fusarium* appartiennent au groupe des sesquiterpènes qui sont des inhibiteurs la synthèse des protéines des eucaryotes. Les trichothécènes causent d'une part de sérieux problèmes de santé aux humains et aux animaux qui ont consommé des aliments infectés par le champignon et de l'autre part, elles sont des facteurs importants de la virulence chez plantes. Dans cette étude, nous avons isolé et caractérisé seize isolats de *Fusarium* de la pomme de terre infectée naturellement dans un champs. Les tests de pathogénicité ont été réalisés pour évaluer la virulence des isolats sur la pomme de terre ainsi que leur capacité à produire des trichothécènes. Nous avons choisi *F. sambucinum* souche T5 comme un modèle pour cette étude parce qu'il était le plus agressif sur la pomme de terre en serre en induisant un flétrissement rapide, un jaunissement suivi de la mort des plantes. Cette souche produit le 4,15-diacétoxyscirpénol (4,15-DAS) lorsqu'elle est cultivée en milieu liquide. Nous avons amplifié et caractérisé cinq gènes de biosynthèse trichothécènes (*TRI5*, *TRI4*, *TRI3*, *TRI11*, et *TRI101*) impliqués dans la production du 4,15-DAS. La comparaison des séquences avec les bases de données a montré 98% et 97% d'identité de séquence avec les gènes de la biosynthèse des trichothécènes chez *F. sporotrichioides* et *Gibberella zeae*, respectivement. Nous avons confrenté *F. sambucinum* avec le champignon mycorhizien à arbuscule *Glomus irregulare* en culture *in vitro*. Les racines de carotte et *F. sambucinum* seul, ont été utilisés comme témoins. Nous avons observé que la croissance de *F. sambucinum* a été significativement réduite avec la présence de *G. irregulare* par rapport aux témoins. Nous avons remarqué que l'inhibition de la croissance *F. sambucinum* a été associée avec des changements morphologiques, qui ont été observés lorsque les hyphes de *G. irregulare* ont atteint le mycélium de *F. sambucinum*. Ceci suggère que *G. irregulare* pourrait produire des composés qui inhibent la croissance de *F. sambucinum*. Nous avons étudié les patrons d'expression des gènes de biosynthèse de trichothécènes de *F. sambucinum* en présence ou non de *G. irregulare*, en utilisant le PCR en temps-réel. Nous avons observé que *TRI5* et *TRI6* étaient sur-exprimés, tandis que *TRI4*, *TRI13* et *TRI101* étaient en sous-exprimés en présence de *G. irregulare*. Des analyses par chromatographie en phase-gazeuse (GC-MS) montrent clairement que la présence de *G. irregulare* réduit significativement la production des trichothécènes par *F. sambucinum*. Le dosage du 4,15-DAS a été réduit à 39 µg/ml milieu GYEP par *G. irregulare*, comparativement à 144 µg/ml milieu GYEP quand *F. sambucinum* est cultivé sans *G. irregulare*.

Nous avons testé la capacité de *G. irregulare* à induire la défense des plants de pomme de terre contre l'infection de *F. sambucinum*. Des essais en chambre de croissance montrent que *G. irregulare* réduit significativement l'incidence de la maladie causée par *F. sambucinum.* Nous avons aussi observé que *G. irregulare* augmente la biomasse des racines, des feuilles et des tubercules. En utilisant le PCR en temps-réel, nous avons étudié les niveaux d'expression des gènes impliqué dans la défense des plants de pommes de terre tels que : chitinase class II (*ChtA3)*, 1,3-β-glucanase (*Glub)*, peroxidase (*CEVI16*), osmotin-like protéin (*OSM-8e*) et pathogenèses-related protein (*PR-1*). Nous avons observé que *G. irregulare* a induit une sur-expression de tous ces gènes dans les racines après 72 heures de l'infection avec *F. sambucinum*. Nous avons également trové que la baisse provoquée par *F. sambucinum* des gènes *Glub* et *CEVI16* dans les feuilles pourrait etre bloquée par le traitement AMF. Ceci montre que l'inoculation avec *G. irregulare* constitut un bio-inducteur systémique même dans les parties non infectées par *F. sambucinum.*

En conclusion, cette étude apporte de nouvelles connaissances importantes sur les interactions entre les plants et les microbes, d'une part sur les effets directs des champignons mycorhiziens sur l'inhibition de la croissance et la diminution de la production des mycotoxines chez *Fusarium* et d'autre part, l'atténuation de la sévérité de la maladie dans des plantes par stimulation leur défense. Les données présentées ouvrent de nouvelles perspectives de bio-contrôle contre les pathogènes mycotoxinogènes des plantes.

Mots-clés : Mycotoxines - *Fusarium sambucinum* - gènes du cluster trichothécènes - 4,15-diacetoxyscirpenol (4,15-DAS) - qRT-PCR - L'expression des gènes - champignons mycorhiziens à arbuscules - gènes de la défense

Abstract

Fusarium trichothecenes are a large group of sesquiterpenes that are inhibitors of eukaryotic protein synthesis. They cause health problems for humans and animals that consume fungus-infected agricultural products. In addition some of *Fusarium* trichothecenes are virulence factors of plant pathogenesis. In this study, sixteen *Fusarium* strains were isolated and characterized from naturally infected potato plants. Pathogenicity tests were carried out to evaluate the virulence of these isolates on potato plants and their trichothecene production capacity. We chose *F. sambucinum* strain T5 as a model for this study because it was the most aggressive strain when tested on potato plants. It induces a rapid wilting and yellowing resulting in plant death. This strain produced 4,15-diacetoxyscirpenol (4,15-DAS) when grown in liquid culture. We amplified and characterized five trichothecene genes (*TRI5*, *TRI4*, TRI3, *TRI11*, and *TRI101*) involved in the production of 4,15-DAS. Nucleotide BLAST search showed 98% and 97% sequence identity with trichothecene biosynthetic genes of *F. sporotrichioides* and *Gibberella zeae*, respectively. We used *F. sambucinum* to determine if trichothecene gene expression was affected by the symbiotic arbuscular mycorrhizal fungus (AMF) *Glomus irregulare*. We found that the growth of *F. sambucinum* was significantly reduced in the presence of *G. irregulare* isolate DAOM-197198 compared with controls that consisted of carrot roots without *G. irregulare* or *F. sambucinum* alone. Furthermore, inhibition of the growth *F. sambucinum* was associated with morphological changes, which were observed when *G. irregulare* hyphae reached *F. sambucinum* mycelium, suggesting that *G. irregulare* may produce compounds that interfere with the growth of *F. sambucinum*. Using real-time qRT-PCR assays, we assessed the relative expression of trichothecene genes of *F. sambucinum confronted or not with G. irregulare.* When *G. irregulare was confronted* with F. *sambucinum, TRI5* and *TRI6* genes were up-regulated, while *TRI4*, *TRI13* and *TRI101* were down-regulated. We therefore used GC-MS analysis to determine whether *G. irregulare* affects trichothecene production by *F. sambucinum*. We found that the production of 4,15-DAS trichothecene was significantly reduced in the presence of *G. irregulare* compared with controls that consisted of carrot roots without *G. irregulare* or *F. sambucinum* alone. Interestingly, 4,15-DAS pattern was reduced to 39 µg/ml GYEP medium by *G. irregulare* compared to 144 µg/ml GYEP with *F. sambucinum* grown with carrot roots or *F. sambucinum* alone respectively.

We tested the AMF capacity to induce defense responses of potato plants following infection with *F. sambucinum*. The response of AMF-colonized potatoes to *F. sambucinum* was investigated by tracking the expression of genes homologous with pathogenesis-related proteins chitinase class II (*ChtA3*), 1,3-β-glucanase (*gluB*), peroxidase (*CEVI16*), osmotin-like protein (*OSM-8e*) and pathogenesis-related protein (*PR-1*). We found that the AMF treatment up-regulated the expression of all defense genes in roots at 72 hours post-infection (hpi) with *F. sambucinum*. We also found that a decrease provoked by *F. sambucinum* in *gluB* and *CEVI16* expression in shoots could be blocked by AMF treatment. Overall, a differential regulation of PR homologues genes in shoots indicates that AMF are a systemic bio-inducer and their effects could extend into non-infected parts.

In conclusion, this study provides new insight into on the interactions between plants and microbes, in particular the effects of AMF on the growth and the reduction of mycotoxins in *Fusarium*. It also shows that AMF are able to reduce the disease severity in plants by stimulating their defense. The data presented provide new opportunities for bio-control against mycotoxin-producing pathogens in plants.

Keywords: Mycotoxins – *Fusarium sambucinum* – Trichothecenes cluster genes – 4,15-diactoxycscirpenol (4,15-DAS) – qRT-PCR – Gene expression – Arbuscular mycorrhizal fungi – Defense related genes.

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1. Introduction

1.1. Trichothecenes mycotoxins

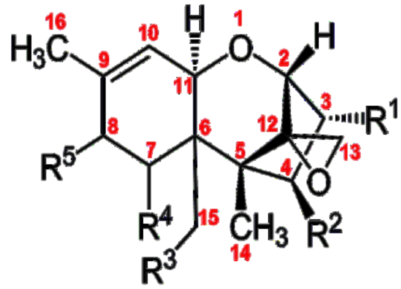
Mycotoxins are secondary metabolites produced by fungi and have been implicated as causative agents of health problems in human and animals that consume fungus-infected agricultural products. Mycotoxin is termed for toxic secondary metabolites produced by fungi that infect crops (Turner and Subrahmanyam, 2009). The reason for the production of mycotoxins is not yet known; they are neither necessary for growth nor the development of the producing fungi (Fox and Howlett, 2008). Because of their pharmacological activity, mycotoxins or their derivatives are usually used as antibiotics, plant growth regulators, and other drugs (Bennett and Klich, 2003). Among the myriad of mycotoxins that have been identified, trichothecenes are one of the most important group of mycotoxins.

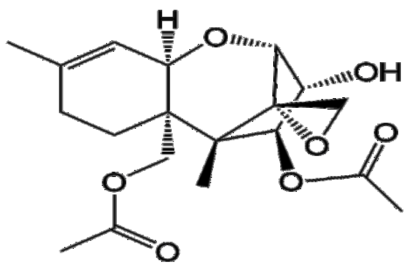
Trichothecenes are a family of terpene-derived mycotoxins produced by ascomycetes, mainly species of *Fusarium* and other fungal genera belonging to the order Hypocreales (Proctor et al., 2009), including *Myrothecium*, *Trichothecium*, *Cephalosporium*, and *Stachybotrys* (Desjardins et al., 1993). This group of structurally related mycotoxins has a strong impact on the health of animals and humans due to their potent inhibition effect of eukaryotic protein synthesis (Bennett and Klich, 2003), and cause moldy-grain toxicosis in animals (Yoshizawa, 2003). However, a high dose exposure in animals causes radiomimetic symptoms, including diarrhea, vomiting, leukocytosis and gastrointestinal hemorrhage, while extreme high doses trichothecenes cause a shock-like syndrome ultimately resulting in death (Pestka and Smolinski, 2005). Trichothecenes are also an agricultural concern due to their contribution to plant pathogenesis of *Fusarium* on some crops (Maier et al., 2006; Ismail et al., 2011).

The chemistry and toxicology of trichothecenes were reported in the sixties and the seventies (Bamburg et al., 1968; Tamm and Breitenstein., 1980.). The distinguishing chemical structure of trichothecenes is the presence of tricyclic nucleus named trichothecene ring (Figure 1.1) and they usually also contain an epoxide at C-12 and C-13 which is essential for toxicity (Desjardins et al., 1993). According to their chemical structure, trichothecenes are classified into four chemo-types (A, B, C and D) (Ueno, 1984; Ueno and Hsieh, 1985). Type A trichothecenes (T-2 toxin, HT-2 toxin, Diacetoxycsirpenol) are of special interest because they are even more toxic than the related type B trichothecenes (Deoxynivalenol, Nivalenol, 3- and 15-Acetyldeoxynivalenol). Type B trichothecenes differ from type A by the presence of a keto group at C-8 (Figure 1.1). However, type C and type D trichothecenes are a minor group of non-*Fusarium* trichothecenes containing 7, 8-epoxide (Kimura et al., 2007). Type D trichothecenes of non-*Fusarium* mycotoxins of a highly diverse group, include satratoxin, rordins, and verrucarins which are associated with *Stachybotrys* mycotoxins (Brasel et al., 2005). Molecular characterization of trichothecenes is highly needed as a model to investigate the secondary metabolism in fungi. This is not only needed for developing new approaches toward elimination trichothecene toxins, but should also help for future applications in the metabolic engineering of sesquiterpenes.

1.2. Trichothecene biosynthesis in *Fusarium* species

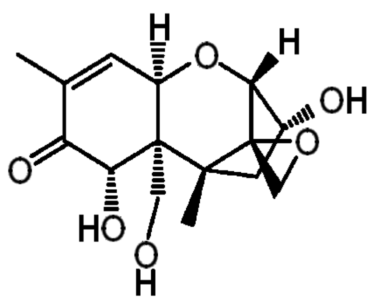
The most complete information for the trichothecene biosynthetic pathway has been obtained from studies on trichothecene production in *F. sporotrichioides* and *F. graminearum* (Brown et al., 2001; Alexander et al., 2009; Proctor et al., 2009). In *F. sporotrichioides*, the trichothecene biosynthesis pathway has been characterized using different approaches such as analysis of mutants, isotopic labeling of precursors and cross-feeding experiments (McCormick et al., 1999; Tag et al., 2001). In general, all trichothecenes have a skeleton derived from farnsyl pyrophosphate (FPP) that is converted by cyclization to trichodiene in the first step of biosynthesis pathway (Evans et al., 1973). The nontoxic compound trichodiene is then converted to the highly toxic compounds through a series of 14 additional steps involving oxygenation, isomerization, cyclization, esterification and deacetylation (Figure 1.2) (Desjardins et al., 1993; Kimura et al., 2007). In the trichothecene biosynthetic pathway, the first 9 steps appear to be shared across the Fusaria however; the trichothecene end product varies from species-to species and strain-to-strain.



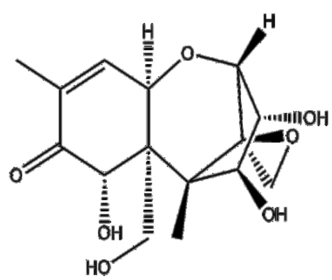


Diacetoxycsirpenol (DAS)

T-2 toxin



Deoxynivelanol (DON)



Nivalenol (NIV)

Figure 1.1: Chemical structure of Trichothecene skeleton (Source http://en.wikipedia.org/wiki/Trichothecene). Chemical structure of major trichothecene type A and type B. Type A trichothecene, Diacetoxycsirpenol (DAS) and T-2 toxin are often associated with *F. sambucinum* and *F. sporotrichioides* respectively. The type B trichothecenes Deoxynivalenol (DON) and Nivalenol (NIV) are mostly associated with *Fusarium* head blight (FHB), a destructive disease of wheat, barley, maize and other important cereal crops caused by *F. graminearum* and *F. culmorum*.

(Tag et al., 2001). For example, as shown in figure (1.3) the final end product in *F. sporotrichioides* is T-2 toxin (Desjardins et al., 1986), while in many *F. graminearum* strains is deoxynivalenol (DON) or nivalenol (NIV) (McCormick et al., 2004), they are mostly associated with *Fusarium* head blight (FHB), a devastating disease of wheat, maize and other important crops (Desjardins and Hohn, 1997). *F. sambucinum* predominantly produces diacetoxyscirpenol (DAS) and in some cases 4, 15-diacetoxyscirpenol (4-15-DAS, (Ismail et al., 2011), type A trichothecene that is often associated with *F. sambucinum*, but some isolates could produce T-2 toxin (Marasas et al., 1984). The trichothecene biosynthesis pathway has been investigated in *F. sporotrichioides* and *F. graminearum* by many authors (Alexander et al., 1998; Kimura et al., 1998; Alexander et al., 1999; Kimura et al., 2007; Alexander et al., 2009; Proctor et al., 2009) and in *F. sambucinum* (Hohn et al., 1993). The extensive research on trichothecene biosynthesis by *Fusarium* species begun in the mid-1980s (Kimura et al., 2007), using several approaches such as UV mutagenesis, gene disruption and feeding experiments. In *F. sporotrichioides*, UV mutagenesis and mutant screening has been performed using a monoclonal antibody to T-2 toxin to look for mutants that no longer produce T-2 toxin (Beremand, 1987). This approach identified the first four trichothecene biosynthetic genes (*TRI*), *TRI1*, *TRI2*, *TRI3* and *TRI4* (formerly *Tox1*, *Tox2*, *Tox3* and *Tox4*) (Beremand, 1987; McCormick et al., 1989, 1990). These UV-induced mutants provided the first evidence of the role of trichothecenes in plant pathogenesis (Desjardins et al., 1989), and permitted more studies that determined the identity and order of many of the intermediates in the T-2 toxin biosynthetic pathways in *F. sporotrichioides* (Beremand, 1987; McCormick et al., 1989, 1990), and interestingly in the cloning of several *TRI* genes (McCormick et al., 1996; McCormick et al., 2006). The early genetic studies conducted on *Gibberella pulicaris* (*F. sambucinum*) allowed mapping the genes by segregation analysis (Desjardins and Beremand, 1987; Beremand, 1989). Naturally occurring strains of *F. sambucinum* produce different kinds and levels of trichothecenes. These studies showed that progeny from crosses between strains which produce trichothecenes with an oxygen-containing group at (C8+) and those that do not (C8-) can segregate in a 1:1 ratio for this trait. These studies defined a genetic locus, which was designated as *Toxl* (*TRI1*) (Beremand and Desjardins, 1988). The segregation patterns observed for progeny obtained from crosses between high-toxin producers and low-toxin producers indicated that the quantity of trichothecene production is determined by several loci. One gene that controls quantitative aspects of toxin production segregates independently from both the *Tox*1 locus and another locus that controls toxin levels. Beremand and coworkers suggested that multiple loci are involved in the control of trichothecene biosynthesis in *F. sambucinum* (Beremand and Desjardins, 1988). *TRI* gene disruption is an approach that has been used to characterize the trichothecenes biosynthesis by *Fusarium* species. However, the first trichothecene gene, *TRI5* (formerly *Tox5*) was isolated from a λgt11 expression library containing genomic DNA from *F. sporotrichioides* using an antibody generated against purified trichodiene synthase protein (Hohn and Beremand, 1989). Sequence analysis of additional genomic fragments recovered from the library provided the sequence of the promoter and open reading frame of *TRI5*. In the study of (Hohn and Beremand, 1989), *TRI5* was disrupted by transforming fungal strain NRRL 3299 with a vector containing a doubly-truncated fragment of the *TRI5* open reading frame and a hygromycin resistance cassette. Transformants possessing a disrupted *TRI5* gene produced no trichothecenes. Furthermore, the feeding of intermediates beyond trichodiene indicated that the enzymes beyond trichodiene synthase were present and active in the *TRI5* transformants as shown by the conversion of these precursors to T-2 toxin (Hohn and Beremand, 1989). *TRI5* was subsequently cloned and disrupted in *G. pulicaris* and shown to be required for trichothecene production in this fungus (Hohn and Desjardins, 1992).

1.3. Trichothecene biosynthesis genes and gene cluster

The genetic functions of the cluster *TRI* genes were characterized using *F. graminearum* and *F. sporotrichioides* as model systems by Alexander and co-workers. They reported that trichothecene biosynthetic enzymes are encoded by genes at three loci: the single-gene *TRI101* locus, the two-gene *TRI1-TRI16*

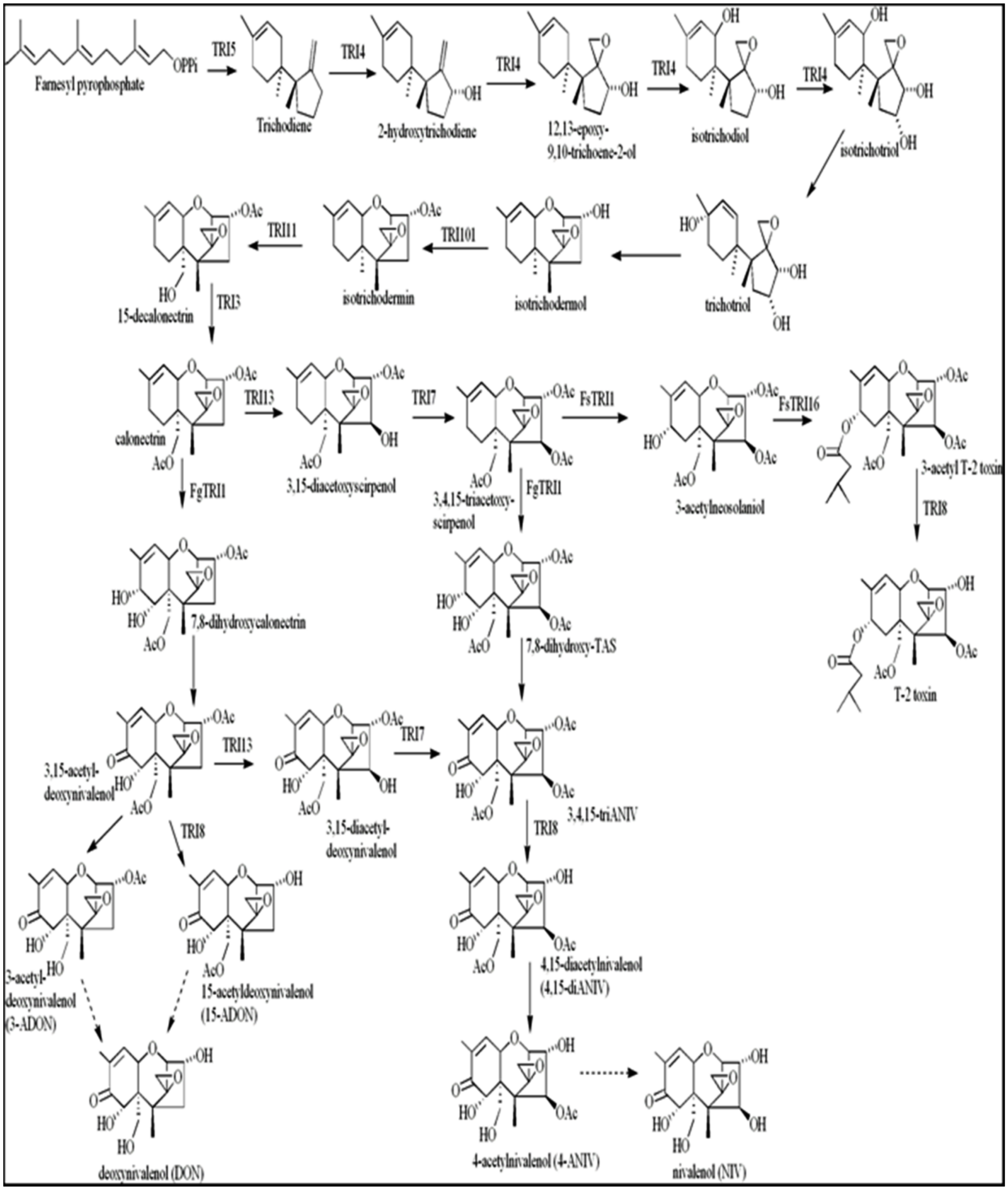
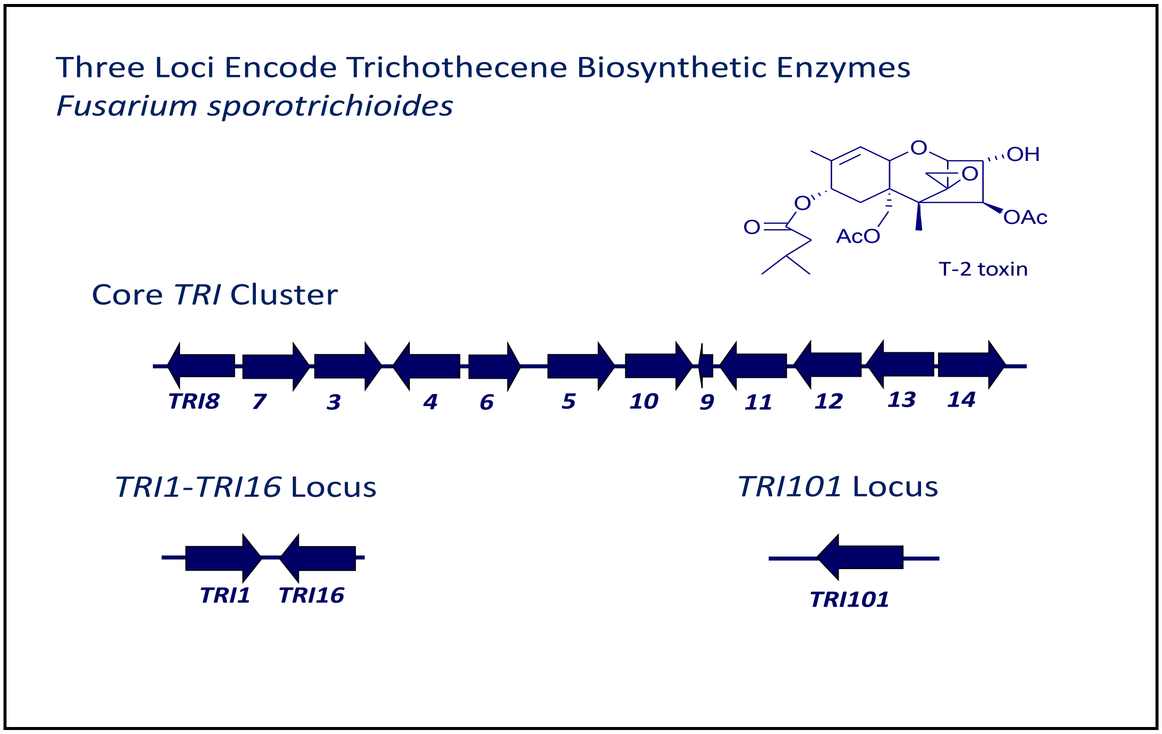


Figure 1.2: Proposed trichothecene biosynthetic pathway in *Fusarium* species (provided by Susan McCormick).

locus, and the 12-gene core *TRI* cluster (Alexander et al., 2009). The *F. sporotrichioides TRI1* enzyme catalyzes hydroxylation of trichothecenes at carbon atom 8 (C-8). This activity results in trichothecenes that have oxygen at C-8, but no oxygen atom at C-7. In contrast, the *F. graminearum TRI1* enzyme catalyzes hydroxylation of trichothecenes at C-8 and C-7, which results in trichothecenes that have oxygen atoms at both C-7 and C-8 (Figure 1.1) (Alexander et al., 2009). These differences in the activity of the *TRI1* enzyme from *F. sporotrichioides* and *F. graminearum* are part of the genetic and enzymatic basis for the difference in production of type A and B trichothecenes by these two species as well as other species of *Fusarium*. For example, *F. sporotrichioides* produces T-2 toxin, which has an oxygen atom at C-8 but not at C-7, whereas *F. graminearum* can produce DON and/or NIV, which have oxygen atoms at both C-7 and C-8 (McCormick et al., 2004). However *F. sambucinum* can produce trichothecenes that either have an oxygen atom at C-8 but not at C-7 (e.g. neosolaniol, 8-acetylneosolaniol) or that have no oxygen at C-7 or C-8 (e.g. diacetoxyscirpenol = DAS). Therefore, the greater similarity of the *F. sambucinum TRI1* to the *F. sporotrichioides TRI1* is consistent with the production of C-8 oxygenated (but not C-7 oxygenated) trichothecenes by *F. sambucinum* (Beremand and Desjardins, 1988; Alexander et al., 2009). The first trichothecene biosynthetic gene identified and characterized was the trichodiene synthase gene. It was identified by purifying the enzyme from *F. sporotrichioides*, raising antibodies to the purified enzyme, and using the antibody to screen a library of *F. sporotrichioides* genomic DNA that was expressed in *Escherichia coli* (Hohn and Vanmiddlesworth, 1986; Hohn and Beremand, 1989). This method identified the *TRI5* gene that encodes the first enzyme in trichothecene biosynthesis. To determine whether trichothecene biosynthetic genes were also clustered, individual cosmid clones derived from *F. sporotrichioides* genomic DNA were transformed into the *Tox1, Tox3*, and *Tox4* mutants of the fungus (Hohn et al., 1993). Two of the cosmid clones that included *TRI5* restored T-2 toxin production to the *Tox3* and *Tox4* mutants of *F. sporotrichioides*, but neither clone restored production to the *Tox1* mutant. These findings indicated that the genes (*TRI3* and *TRI4*) corresponding to *Tox3* and *Tox4* were located in the same cluster as *TRI5*. However, subsequent sequencing gene disruption, and expression analysis in *F. sporotrichioides* identified a trichothecene gene cluster (Figure 1.3) that contained *TRI3*, *TRI4* and *TRI5* and nine other genes including *TRI6*, *TRI7*, *TRI8*, *TRI9*, *TRI10*, *TRI11*, *TRI12*, *TRI13* and *TRI19* (Brown et al., 2002; Brown et al., 2004; Alexander et al., 2009). The cluster contains two regulatory genes and most of the biosynthetic enzymes necessary for the production of trichothecenes. This trichothecene gene cluster was subsequently identified and characterized in other *Fusarium* species such as *F. graminearum* and *F. sambucinum* and other species closely related to it (Brown et al., 2001; Brown et al., 2002; Ward et al., 2002; Ismail et al., 2011). *TRI7* and *TRI13* are non-functional in DON-producing strains of *F. graminearum* due to multiple insertions and deletions in their coding regions, whereas in NIV-producing strains, *TRI7* and *TRI13* are functional (Brown et al., 2002; Lee et al., 2002). This difference, combined with the finding that *TRI13* is responsible for trichothecene C-4 hydroxylation, identified the basis for NIV versus DON production in *F. graminearum* (Alexander et al., 2009). In addition to the core *TRI* cluster, two other loci encoding trichothecene biosynthetic enzymes have been identified in *Fusarium*. The first locus includes only *TRI101* gene that is involved in trichothecene biosynthesis (Kimura et al., 1998; McCormick et al., 1999; Ismail et al., 2011) which encodes an acetyltransferase that catalyzes esterification of acetate with the C-3 hydroxyl of trichothecenes. This acetylation reduces the toxicity of trichothecenes to *Fusarium*, and therefore likely functions as a self-protection mechanism in trichothecene-producing species (McCormick et al., 1999). The second locus encoding other trichothecene biosynthetic enzymes consists of two genes, *TRI1* and *TRI16* (Kimura et al., 2007; Alexander et al., 2009; Proctor et al., 2009). *TRI1* encodes a cytochrome P450 monooxygenase, but the deduced amino acid sequences of *FsTRI1*and *FgTRI1* are only 65% identical (Alexander et al., 2009). It has been reported that the two homologs vary in function between *F. sporotrichioides* and *F. graminearum*. The *FsTRI1* enzyme catalyzes hydroxylation of trichothecenes at C-8 only, whereas the *FgTRI1* enzyme catalyzes hydroxylation at C-7 and C-8 (McCormick and Alexander, 2006).



TRI1 = C-8 hydroxylase
TRI3 = 3-acetyltransferase
TRI4 = P450 monooxygenase
TRI5 = trichodiene synthase
TRI6 = transcription factor
TRI7 = acetyltransferase
TRI8 = esterase

TRI9 = unknown
TRI10 = regulatory gene
TRI11 = P450 monooxygenase
TRI12 = transporter
TRI13 = P450 monooxygenase
TRI14 = unknown
TRI16 = acetyltransferase
TRI101 = 3-O-acetyltransferase

Figure 1.3: *Fusarium* trichothecene gene cluster. In *Fusarium*, trichothecene biosynthetic enzymes are encoded by *TRI* genes at three loci: the 12-gene core *TRI* cluster, the single-gene *TRI101* locus, and the two-gene *TRI1-TRI16* locus. (Provided by Susan McCormick; USDA-ARS)

The *TRI1 gene* is likely similar to the *Tox1* identified in *F. sambucinum* (Beremand and Desjardins, 1988; Beremand and McCormick, 1992). The *TRI16* was identified in *F. sporotrichioides* by its location next to *TRI1* and by its high level of expression under conditions that promote expression of core *TRI* cluster genes (Brown et al., 2003). This gene encodes an acyltransferase. Gene deletion analysis indicated that it is responsible for esterification of an isovalerate moiety by the C-8 hydroxyl during formation of T-2 toxin. The *F. graminearum TRI16* homologue is non-functional due to multiple insertions and deletions in its coding region. This is consistent with the absence of the isovalerate ester in trichothecenes produced by *F. graminearum* (Brown et al., 2003; McCormick et al., 2004).

1.4. Contribution of trichothecenes in plant pathogenesis

Trichothecene production appears to provide important benefits to the organisms producing them. All of the trichothecene-producing fungi are plant pathogens such as *F. acuminatum*, *F. crookwellense*, *F. culmorum*, *F.equiseti*, *F. graminearum* (*Gibberella zeae*), *F. lateritium*, *F. poae*, *F. sambucinum* (*Gibberella pulicaris*), and *F. sporotrichioides* (Marasas et al., 1984; Clark et al., 1995; Desjardins and Hohn, 1997; Ismail et al., 2011). The trichothecenes have been shown to be phytotoxic and play a role in virulence in several specific plant-pathogen interactions (Brian et al., 1961; Ismail et al., 2011). Evidence for contribution of the trichothecenes in plant pathogenesis was reported by using mutant strains of *F. sporotrichioides* and *F. sambucinum* that are unable to produce trichothecenes. These mutant strains exhibited reduction in their virulence on parsnip root (*Pastinaca sativa*) (Desjardins et al., 1989; Desjardins et al., 1992) and they play a role in the virulence of *F. graminearum* in *Fusarium* head blight (FHB) of wheat (Bernardo et al., 2007). However, the ability to produce trichothecenes was unnecessary to achieve wild-type levels of virulence on potato with *Gibberella pulicaris* (Desjardins et al., 1992) and trichothecene production by *F. graminearum* is not essential for infection of the maize host plant (Harris et al., 1999; Bai et al., 2002). Studies using trichothecene non-producing mutant strains on maize (Harris et al., 1999) and on wheat (Proctor et al., 1995a; Proctor et al., 1997; Desjardins et al., 2000; Bai et al., 2002) have clearly shown that trichothecenes act as virulence factors. The approach of using mutants and disruption of *TRI* genes revealed that trichothecenes are involved in plant pathogenesis. Desjardins and coworkers investigated the role of trichothecenes in a number of plant diseases by generating trichothecene-nonproducing mutants through the disruption of *TRI5* (Desjardins and Hohn, 1997). This approach was successful because *Fusarium* is haploid, and because *TRI5* occurs as a single copy. Diacetoxycsirpenol biosynthesis was blocked by disruption of *TRI5* in *Gibberella pulicaris* (*F. sambucinum*), which causes dry rot in a variety of plants. The virulence of trichothecene-nonproducing mutants was significantly reduced on parsnip roots, but was not changed on potato tubers. To determine whether the reduced virulence of the mutants was due specifically to *TRI5* disruption or to non-target effects of the transformation process, a *TRI5*$^{-}$ mutant was crossed to a *TRI5*$^{+}$ wild-type strain (*G. pulicaris* is heterothallic). Tetrad analysis resulted in either co-segregation of hygromycin resistance, trichothecene nonproduction, and reduced virulence on parsnip, or in the simultaneous loss of all three traits (Desjardins et al., 1992). These results were consistent with an earlier finding that production of trichothecenes is important for virulence of *F. sporotrichioides* on parsnip root (Desjardins and Plattner, 1989). This apparent effect of the host on the importance of trichothecenes in virulence is still unexplained, but suggests that the importance of trichothecenes in disease may differ from one plant species to another. It has been reported that deoxynivalenol (DON) biosynthesis also was blocked by disruption of *TRI5* in *Gibberella zeae* (*F. graminearum*) which causes seedling blights, root rots, and *Fusarium* head blight (FHB) of wheat, barley, rye, maize, rice, and other grains. Two trichothecene nonproducing mutants exhibited reduced wheat seedling blight and head scab virulence (Proctor et al., 1995b).

1.5. Trichothecene resistance

Understanding of the chemistry, biosynthesis, and gene regulation and function of the trichothecene toxins opens potential perspectives and new strategies to control trichothecene production and contamination of crops. It has been reported that trichothecenes could act as a virulence factor on particular host plants (Desjardins et al., 1992). In this respect, inhibition of trichothecene biosynthesis is not only needed for controlling mycotoxin production by an organism but it may decrease virulence and protect plants from infection. Strategies for controlling trichothecene production include the application of synthetic or naturally occurring trichothecene inhibitors in cultures of producing organism (Desjardins et al., 1987; Alexander et al., 2008), or directly to plants. Desjardins and coworkers reported that Ancymidol, a plant growth regulator, inhibited biosynthesis of diacetoxyscirpenol by *F. sambucinum in vitro* (Desjardins and Beremand, 1987). However, Ancymidol also inhibited biosynthesis of *T-2* toxin by a wild type strain of *F. sporotrichioides* and biosynthesis of diacetoxyscirpenol, deacetylated calonectrin, and deacetylated calonectrin by mutant strains of this species. The GC-MS analyses indicated that Ancymidol blocked trichothecene production in both strains after formation of trichodiene and before formation of trichothecenes containing four or more oxygen atoms. Alexander and coworkers showed that Xanthotoxin (8-methoxypsoralen) effectively blocked T-2 toxin production by *F. sporotrichioides* in liquid cultures (Alexander et al., 2008). The mechanism by which xantotoxin inhibited T-2 toxin production appeared similar to that of Ancymidol (Desjardins et al., 1987), as the addition of xanthotoxin to liquid culture of *F. sporotrichioides* caused a significant increase of the trichodiene accumulation. This suggested that xanthotoxin not only blocked trichothecene oxygenation reactions but, may also have induced the synthesis of trichodiene (Alexander et al., 2008). Using approaches of classical plant breeding or genetic engineering, transformation of genes encoding these inhibitors into plants might be decrease the virulence of trichothecene-producing fungi by altering genes encoding plant proteins that are target sites for trichothecenes (Desjardins et al., 1993). It has been reported that *TRI101* (encodes trichothecene 3-O-acetyltransferase) was originally identified as a cDNA that confers resistance to T-2 toxin when expressed in *Schizosaccharomyces pombe* (Kimura et al., 1998). This gene catalyses the C-3 acetylation of various *Fusarium* trichothecenes, including T-2 toxin, DON, and 4.15-DAS (Kimura et al., 1998b; Kimura et al., 1998; Kimura et al., 2007). Trichothecene 3-*O*-acetyltransferase (*TRI101*) catalyzes the conversion of toxic *Fusarium* trichothecenes to less toxic products and has, therefore, been proposed as a metabolic self-protection mechanism in *F. graminearum* (Kimura et al., 1998; McCormick et al., 1999). Trichothecene detoxification may also lead to improved plant resistance. Interestingly, glycosylation of the C-3 hydroxyl group has been correlated with moderate scab resistant lines derived from Sumai-3 wheat and a glucosyltransferase has been isolated from *Arabidopsis thaliana* that can detoxify deoxynivalenol (Lemmens et al., 2005). Other possible detoxifying enzymes are C-3 oxidase (Shima et al., 1997), epoxide reductase (Fuchs et al., 2002), and epoxide hydrolase (Weijers, 1997). An alternative strategy for reducing trichothecene production is to use non-producing strains as competitors for trichothecene-producing pathogens (Desjardins et al., 1993) or using biocontrol agents. One of the most effective control strategies for trichothecene production is to prevent fungal infection and toxin production in the host plant. In this regard, confrontation experiments have been conducted in dual cultures to assess the effect of symbiotic arbuscular mycorrhizal fungi (AMF) on DAS-producing *F. sambucinum* (Ismail et al., 2011). AMF form symbioses with plant roots, improving their mineral nutrient uptake and protecting them against soil-borne pathogens. In this system, *G. irregulare* significantly inhibited *F. sambucinum* growth and modulated expression of trichothecene biosynthetic genes. The AMF *G. irregulare* showed downregulation of the *TRI4* gene that encodes oxygenation reactions during trichothecene biosynthesis and upregulated of *TRI5* gene that encodes trichodiene synthase. This finding unveiled an important mechanism for modifying plant mycotoxin producing-pathogen interactions by introducing a third player, a symbiotic AMF. This opens new perspectives for controlling toxin production and plant pathogens. However, continued extensive research on understanding the mechanisms by which the AMF could prevent mycotoxin production, is highly required in applying this approach to improving food quality and safety.

1.6. Interactions of arbuscular mycorrhizal fungi (AMF) with pathogens

The role of AMF and their interactions with plants has gained increased attention in recent years. In particular, their interactions with pathogenic organisms have been characterized as being particularly relevant due to their important implications for plant fitness (Wehner et al., 2009). AMF form symbioses with a majority of all plant species (Smith and Read, 2008), in particular by improving their mineral nutrient uptake and protecting plants against soil-borne pathogens (Azcón-Aguilar and Barea, 1997; Lioussanne et al., 2009b; Wehner et al., 2009). Several mechanisms have been proposed to explain how this protection arises (Filion et al., 1999; St-Arnaud and Vujanovic, 2007; Pozo et al., 2009; Wehner et al., 2009). These mechanisms have been summarized by Wehner and colleagues as; *i*) Improved nutrient status of the host plant; *ii*) Competitive interactions with pathogenic fungi; *iii*) Anatomical or architectural changes in the root system; *iv*) Microbial community changes in the rhizosphere; and *v*) Induction of plant defense mechanisms

i) **Improved nutrient status of plants:** It has been reported that AMF improve their host plant's status through increasing supply nutrients (Smith, 2009). Several nutrients, particularly phosphorus (P), are fixed during the symbiotic association with plant in exchange for carbon (Pearson and Jakobsen, 1993). Many studies shown that when plants took up larger amount of nutrients through symbiosis with *Glomus intraradices* and *Glomus mosseae*, they became better able to tolerate to infection by pathogens (Karagiannidis et al., 2002; Yao et al., 2002). However, other studies have shown that the protection against pathogens is not correlated with the improving nutrient status of the host plant during the symbiotic association with AMF (Shaul et al., 1999; Fritz et al., 2006).

ii) **Competition with pathogenic fungi:** Interactions between AMF and pathogenic fungi could be direct through interference competition including chemical interactions and indirect via exploitation competition (Wehner et al., 2009). The competitive interactions have been proposed as mechanisms by which AMF can reduce the abundance of pathogenic fungi in roots (Filion et al., 2003). These have been observed through the relative abundance of AM fungi structures and pathogenic fungi in roots (Filion et al., 2003; Lioussanne et al., 2009b) or on growth medium (St-Arnaud et al., 1995; Ismail et al., 2011b). Probably, pathogenic and AMF compete for common resources within the root, including infection sites, space, and photosynthate (Whipps, 2004; Wehner et al., 2009). Interference competition may happen due to carbon availability in intercellular spaces and the rhizosphere (Graham, 2001) or may reduce the number of infection loci within the root system as a result of AMF colonization (Vigo et al., 2000). It has been recently reported that the competition interactions of AMF with pathogenic fungi is more for resources than for occupying the space within the roots (Wehner et al., 2009).

iii) **Anatomical or architectural changes in the root system:** Anatomical and arictectural changes occur through AMF colonization of plant roots. It has been reported that AM-colonized roots become more plentifully branched (Yano et al., 1996; Oláh et al., 2005; Gutjahr et al., 2009). The correlation between anatomical and morphological changes in the root system and protection against fungal pathogens has been demonstrated for several AMF species. Trifoliate orange seedlings colonized by four AMF species promoted formation of lateral roots (Yao et al., 2009). However, inoculation of tomato with *G. mosseae* did not significantly affect the branching of root system, but decreased the number of infection loci of *Phytophthora parasitica* (Vigo et al., 2000). It has been proposed that the abundance of lateral root tips and developing meristems make highly branched root systems more susceptible to pathogen attack, resulting in an increasing demand for AMF to protect them (Wehner et al., 2009). Norman and coworkers provided support for this hypothesis as they compared plants with inherently highly branched root systems and found that mycorrhizal plants had fewer necroses compared to non-mycorrhizal ones (Norman and Hooker, 2000). If mycorrhizal fungi frequently caused increased branching of the roots, but increased branching in itself leads to higher susceptibility to root pathogen attack, AMF must confer protection through additional mechanisms.

iv) **Changes of microbial community in the rhizosphere:** It has been reported that AMF influence microbial community structures in the rhizosphere. Several factors such as root exudation patterns, putative AMF effectors, and changes in root size and architecture may contribute quantitative and qualitative microbial community changes in the rhizosphere (Hodge, 2000; Artursson et al., 2006; Finlay, 2008; Lioussanne et al., 2009). The effect of AM fungi on microbial community structure in the rhizosphere may due to the impact of AM fungi on fungal pathogen populations (Filion et al., 1999; Larsen et al., 2003). It has been reported that the presence of *G. intraradices* and *Pseudomonas* in the rhizosphere produced significant amounts of the antibiotic 2,4-diacetylphloroglucinol (DAPG) that confers plant protection against *Gaeumannomyces graminis var. tritici* (Siasou et al., 2009). Other studies showed that bacterial strains putatively associated with AM fungi were equally effective at providing pathogen protection as their counterparts isolated from non-mycorrhizal soils (Li et al., 2007).

v) **Induction of plant defense mechanisms:** During arbuscular mycorrhizal (AM) colonization, recognition dialogue established between the host plant and AMF results in specific changes in host gene regulation (Genre et al., 2009; Oldroyd et al., 2009) leading to the production of specific multi-functional compounds. These compounds could be both involved in transduction pathways and capable of conferring disease resistance (Liu et al., 2007; Pozo and Azcón-Aguilar, 2007; Van Wees et al., 2008; Ismail et al., 2011). During mycorrhizal establishment, modulation of plant defense responses occurs upon recognition of the AMF in order to achieve a functional symbiosis (Pozo et al., 2009). As a consequence of this modulation, plant defense mechanisms become more activated. It has been reported that induced plant resistance against pathogens through this mechanism may either be systemic within the plant (Pozo et al., 2002a; Li et al., 2006; Liu et al., 2007) and/or through root exudation (Lioussanne et al., 2008; Lioussanne et al., 2009). Pozo and coworkers found that *G. mosseae* and *G. intraradices* induced local and systemic resistance in tomato against *Phytophthora parasitica* and found that the protection was effective for reducing disease symptoms through the induction of different hydrolytic enzymes (Pozo et al., 2002). Liu and coworkers compared the transcriptional response of *Medicago truncatula* to different AMF, including *Gigaspora gigantean* (Liu et al., 2007). The AMF were effective to induce a core set of genes, including some associated with defense mechanisms. Pozo and coworkers compared the response of non- mycorrhizal plants or plants colonized by either *G. mosseae* or *G. intraradices* to the application of different defense-related stimuli in the shoots and found stronger induction of defense- associated genes, particularly in *G. mosseae* colonized plants (Pozo et al., 2009).

1.7. Objectives

The general objective of my thesis is to understand the interactions of arbuscular mycorrhizal fungi with mycotoxin-producing fungi. We isolated and characterized 16 fungal strains belonging to the genus *Fusarium* from naturally infected potato plants and confirmed that nine isolates produced trichothecenes. One isolate, *F. sambucinum* strain T5 induced a rapid wilting and yellowing that resulted in plant death and was selected for further studies. Therefore my PhD project focuses on three main points:

1.7.1. Identification of the trichothecene compounds and genes involved in their biosynthesis in *F. sambucinum* and assessment of the effect of the AMF *G. irregulare* on growth and expression of *TRI* genes by *F. sambucinum*. I addressed three specific points:

i) Trichothecene production by *F. sambucinum* using molecular and chemical approaches.

ii) Effect of the AMF *G. irregulare* on the growth of trichothecene-producing *F. sambucinum in vitro.*

iii) *G. irregulare*-induced modulation of trichothecene gene expression in *F. sambucinum*.

This objective addresses the hypothesis that AMF *G. irregulare* affects expression of trichothecene biosynthetic genes of mycotoxin-producing fungi

1.7.2. Testing the AMF for controlling trichothecene production by the fungus *F. sambucinum*. I addressed two specific points that are:

i) Effect of the AMF *G. irregulare* on the survival of *F. sambucinum.*

ii) Does *G. irregulare* reduce trichothecene production by *F. sambucinum in vitro*?

This objective addressed the hypothesis that The AMF *G. irregulare* is not only affecting transcriptional regulation of mycotoxin genes, but also potentially rate-limiting to mycotoxin production

1.7.3 Test whether inoculation of potato with the AMF *Glomus irregulare* can activate defense-related genes following a subsequent infection with the mycotoxin-producing strain *F. sambucinum*.

i) Effect of *G. irregulare* on the disease severity of *F. sambucinum* on potato plants

ii) Effect of *G. irregulare* on the growth and yield of potato

iii) Effect of *G. irregulare* on transcriptional regulation of potato defense genes in response to infection by *F. sambucinum*.

This objective addresses the hypo this that AMF G. irregulare can effectively control mycotoxin-producing fungi *in vivo*.

2. A fungal symbiotic-plant modulates mycotoxin gene expression in the pathogen *Fusarium sambucinum*

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Author Contributions

Conceived and designed the experiments: MH. Performed the experiments: YI SM. Analyzed the data: YI SM. Contributed reagents/materials/analysis tools: MH SM. Wrote the paper: YI SM MH.

2.1. Abstract

Fusarium trichothecenes are fungal toxins that cause disease on infected plants and, more importantly, health problems for humans and animals that consume infected fruits or vegetables. Unfortunately, there are few methods for controlling mycotoxin production by fungal pathogens. In this study, we isolated and characterized sixteen *Fusarium* strains from naturally infected potato plants in the field. Pathogenicity tests were carried out in the greenhouse to evaluate the virulence of the strains on potato plants as well as their trichothecene production capacity, and the most aggressive strain was selected for further studies. This strain, identified as *F. sambucinum*, was used to determine if trichothecene gene expression was affected by the symbiotic arbuscular mycorrhizal fungus (AMF) *Glomus irregulare*. AMF form symbioses with plant roots, in particular by improving their mineral nutrient uptake and protecting plants against soil-borne pathogens. We found that that *G. irregulare* significantly inhibits *F. sambucinum* growth. We also found, using RT-PCR assays to assess the relative expression of trichothecene genes, that in the presence of the AMF *G. irregulare, F. sambucinum* genes *TRI5* and *TRI6* were up-regulated, while *TRI4, TRI13* and *TRI101* were down-regulated. We conclude that AMF can modulate mycotoxin gene expression by a plant fungal pathogen. This previously undescribed effect may be an important mechanism for biological control and has fascinating implications for advancing our knowledge of plant-microbe interactions and controlling plant pathogens.

2.2. Author Summary

Fungi are responsible for a large majority of plant diseases that can result in reduced growth or death of either some plant organs or the entire plant. Fungal plant pathogens can be controlled using chemicals or biological control agents. In recent years, the disease resistance induced in plants by arbuscular mycorrhizal fungi (AMF) has become increasingly useful in biocontrol of plant diseases. Many literature reports indicate that AMF are antagonists of soil-borne disease pathogens, and either suppresses the growth of the pathogen, or increase the resistance or tolerance of mycorrhizal plants to soil-borne diseases. Several hypotheses have been proposed to explain the mechanisms of the increased resistance in mycorrhizal plants: improvement of plant nutrition, competition, changed microbial flora in the rhizosphere, induced resistance, or systemic resistance in the plant. However, mechanisms involving a direct effect of AMF against fungal pathogens are not well studied. This study documenting the interaction of the AMF *Glomus irregulare* with the mycotoxin-producing *F. sambucinum* pathogen presents a breakthrough. We found that *G. irregulare* not only inhibits the growth of *F. sambucinum* but is also able to modulate mycotoxin gene expression by the pathogen.

2.3. Introduction

Secondary metabolites are compounds produced by many organisms including filamentous fungi. These compounds include pigments, toxins, plant growth regulators, antibiotics and numerous compounds used for pharmaceutical purposes (Proctor et al., 2009). Many fungal secondary metabolites increase the fitness of the organisms toward adverse environmental conditions (Calvo et al., 2002; Keller et al., 2005). The fungal genus *Fusarium* (teleomorph: *Gibberella*) consists of over 70 species, many of which are plant pathogens, and some species can produce secondary metabolites, known as mycotoxins, that are toxic to humans and animals (Leslie and Summerell, 2006; Proctor et al., 2009). Trichothecenes are a family of terpene-derived mycotoxins that impact humans, animals (Nielsen et al., 2009), and plants (Desjardins and Hohn, 1997). Trichothecene biosynthetic gene clusters have been characterized in *F. graminearum* and *F. sporotrichioides* (Brown et al., 2002; Proctor et al., 2009). In both species, there is a core cluster of 12 genes that are involved in the biosynthesis, regulation or transport of trichothecenes. These genes are: *TRI5* (encoding a terpene synthase); *TRI4*, *TRI11* and *TRI13* (encoding cytochrome P450 monooxygenase); *TRI3* and *TRI7* (encoding acetyl transferases), *TRI101* (encoding trichothecene 3-*O*-actyltransferase), *TRI8* (encoding an esterase), *TRI6* and *TRI10* (proposed to be regulatory genes) and *TRI12* (encoding a transporter). In addition, there are also two genes, *TRI9* and *TRI14* with unknown functions. The biosynthetic pathway for all trichothecenes begins with a cyclization of farnesyl pyrophosphate to produce the hydrocarbon trichodiene (Turner, 1975). Trichodiene is then converted to highly toxic molecules such as 4, 15-diacetoxyscirpenol (DAS), the predominant mycotoxin of *F. sambucinum*, through a series of oxygenation, isomerization, cyclization, esterification, and deacetylation steps (Figure 2.1).

Control strategies against fungal pathogens that produce mycotoxins are mainly based on the use of fungicides (Edwards et al., 2001), although biological-control agents (Cooney et al., 2000) and plant-resistant varieties have been reported. However, the use of AMF to control mycotoxin-producing microorganisms has not been previously reported.

AMF inhabit plant roots and not only form a symbiotic association with most plant species but also interact with a wide range of other soil organisms such as soil bacteria (Lecomte et al., 2010). AMF are well known to promote plant growth and are largely used as commercial inoculants and bio-fertilizers worldwide. Many literature reports showed that AMF can reduce the incidence and severity of root diseases and protect plants against soil-borne pathogens (St-Arnaud and Vujanovic, 2007). However, the mechanisms by which AMF may act as biological control agents are not known. Three mechanisms have been hypothesized: soil microbial community changes, antagonisms and stimulation of plant defenses. AMF can inhibit or promote soil microorganisms (Lecomte et al., 2010). It has been reported that soluble substances released by the extra radical mycelium of *Glomus intraradices* grown *in vitro*, stimulated both the growth of *Pseudomonas chlororaphis* and the germination of *Trichoderma harizianum* conidia. In contrast, the germination of *Fusarium oxysporum* f. sp. *chrysanthemi* conidia was reduced in the presence of the AMF extract (Filion et al., 1999).

In this study, we identified 16 isolates belonging to the genus *Fusarium* from naturally infected potato plants and confirmed that nine produced trichothecenes. One isolate, *F. sambucinum* strain T5 induced a rapid wilting and yellowing that resulted in plant death and was selected for further studies. The objective of this work was to assess the effect of the AMF *G. irregulare* on growth and expression of *TRI* genes by *F. sambucinum*. We addressed three specific points: i) trichothecene production by *F. sambucinum* using molecular and chemical approaches; ii) effect of the AMF *G. irregulare* on the growth of trichothecene-producing *F. sambucinum in vitro*; and iii) *G. irregulare*-induced modulation of trichothecene gene expression in *F. sambucinum*.

2.4. Results and Discussion

2.4.1. Characterization of *TRI* genes of *F. sambucinum*

We isolated sixteen strains of *Fusarium* from roots and tubers of naturally infected potato plants, and found that nine of these produced trichothecenes. We chose *F. sambucinum* strain T5 as a model for this study because it was the most aggressive strain when tested on potato plants, inducing a rapid wilting and yellowing that resulted in plant death (Figure 2.2). This strain produced 4, 15-diacetoxyscirpenol (DAS) when grown in liquid culture (Figure 2.3). We used ITS regions of ribosomal rRNA genes and morphology to confirm its identity. Nucleotide BLAST search showed 100% sequence identity with *Gibberella pulicaris* strain NBAIM: 455 (anamorph: *F. sambucinum*). This strain is a causal agent of dry rot of tuber crops (Ayers and Robinson, 1956). We used degenerate primers (Table 2.1) and DNA from *F. sambucinum* to amplify fragments corresponding to five trichothecene genes (*TRI5*, *TRI4*, *TRI101*, *TRI3*, and *TRI11*) involved in production of DAS (Figure 2.1). PCR gave bands of the expected sizes ranging from 1.1 to 1.5 Kb. These PCR products were sequenced and, when compared to the NCBI database using nucleotide BLAST searches, showed 98% and 97% sequence identity with trichothecene biosynthetic genes of *F. sporotrichioides* and *Gibberella zeae,* respectively.

To study the impact of *G. irregulare* on the growth of *F. sambucinum,* we used confrontation cultures using an *in vitro* system. We found that the growth of *F. sambucinum* was significantly reduced in the presence of *G. irregulare* isolate DAOM-197198 compared with controls that consisted of carrot roots without *G. irregulare* or *F. sambucinum* alone (Table 2.2). *G. irregulare* significantly reduced the growth of *F. sambucinum* after 3, 5, 7 and 15 days (Figure 2.5).

2.4.2. Expression of *TRI4, TRI5, TRI6, TRI13* and *TRI101* genes.

To test whether *G. irregulare* modulates the expression of *TRI* genes, we carried out real-time qRT-PCR assays on *TRI4* and *TRI5* on total RNA extracted from *F. sambucinum* grown alone or confronted with *G. irregulare* isolates DAOM-197198 and DAOM-234328 during 3 and 5 days. *TRI4* and *TRI5* genes encode P450 oxygenases and trichodiene, respectively (Ward et al., 2002). We used □-tubulin and the translation factor EF1α as reference genes for RT-PCR assays. Figure 2.6 shows relative expression patterns of *TRI4* and *TRI5* genes when *F. sambucinum* was confronted with the two isolates of *G. irregulare*. Interestingly, the relative expression of *TRI5* in *F. sambucinum* was up-regulated ($p<0.001$) by a factor of 17 and 8 after 3 days of confrontation with *G. irregulare* isolates DAOM-197198 and DAOM-234328*,* respectively. *TRI5* encodes a trichodiene synthase and is the first enzyme involved in the trichothecene biosynthesis pathway (Proctor et al., 2009). In contrast, the relative expression of *TRI4* (encoding a multifunctional P450 oxygenase) was down-regulated ($p<0.003$) by a factor of 0.46 and 0.43 when confronted with isolates DAOM-197198 and DAOM-234328*,* respectively. Relative expression of *TRI5* and *TRI4* genes was not affected ($p>0.22$) when *F. sambucinum* was grown alone or confronted with carrot root without *G. irregulare* (Figure 2.4 A and C) and (Table 2.3). We used carrot root as a control because *G. irregulare* is an obligate biotroph that requires plant roots for its culture. These controls clearly show that over-expression of *TRI5* and down-regulation of *TRI4* was due to *G. irregulare.*

Interestingly, the two isolates of *G. irregulare* showed different modulation levels on the expression of *TRI5* and *TRI4* genes in *F. sambucinum* (Table 2.3). This difference in the response of *G. irregulare* isolates could be explained by their genetic composition. It is well documented in the literature that AMF have a high intra- and inter-isolate genetic diversity (Hijri and Sanders, 2005; Boon et al., 2010).

To test the effect of *G. irregulare* on other trichothecene biosynthetic genes, we carried out additional qRT-PCR assays on *TRI6, TRI13,* and *TRI101,* using an experimental setup similar to that used for *TRI4* and *TRI5* (Fig. 2.6 B and D) and (Table 2.3). Relative expression of the transcription factor *TRI6* gene was only up-regulated ($p<0.03$) by a factor of 2.8 and 3.6, after 3 and 5 days of confrontation with *G. irregulare* DOAM-*234328*, respectively (Fig. 2.6B and D) and (Table 2.3). Surprisingly, isolate DOAM-197198 produced no significant effect ($p>0.08$) on relative expression of *TRI6* from *F.*

sambucinum. This finding strongly supports genetic heterogeneity and phenotypic differences among isolates of *G. irregulare*. Boon and coworkers investigated and analyzed the intra-isolate genomic and cDNA sequence variation of two genes, large subunit ribosomal RNA (LSU rDNA) of *Glomus* sp. DAOM-197198 and the *POL1*-like sequence (PLS) of *Glomus etunicatum*. For both genes, they showed high sequence variation at the genome and transcriptome level, furthermore, reconstruction of LSU rDNA secondary structure showed that all variants are functional (Boon et al., 2010). This result supports strongly the hypothesis that AMF may differ in their interactions with other microorganisms.

TRI6 encodes a zinc finger protein involved in regulation of trichothecene biosynthesis (Proctor et al., 1995b) with expression of *TRI5* and *TRI4* genes dramatically reduced or silenced in *TRI6* disruption mutants (Pirgozliev et al., 2003). Relative expression patterns of *TRI13* and *TRI101* were greatly down-regulated ($p<0.001$) after 3 and 5 days of confrontation with *G. irregulare* isolates, respectively (Fig. 2.6B and D). *TRI101* encodes a trichothecene 3-O-acetyltransferase that acetylates the C-3 of various *Fusarium* trichothecenes, converting them to less toxic products (McCormick et al., 1999a; Garvey et al., 2008). In biosynthesis of DAS, the *TRI101* acetyltransferase catalyzes the conversion of isotrichodermol to isotrichodermin (McCormick et al., 1999; Garvey et al., 2008).

Control of mycotoxin-producing fungal pathogens has largely relied on the use of chemicals (Edwards et al., 2001). Modulation of trichothecene biosynthetic gene expression by AMF may be a safer way of limiting mycotoxin production. The impact of AMF on plant pathogenic fungi has been studied under ecological conditions and in a large number of host-pathogen interactions (St-Arnaud and Vujanovic, 2007; Lioussanne et al., 2009). These interactions can be direct, such as competition with the pathogen, or indirect, including (1) alleviation of abiotic stress such as enhanced nutrition of the host plant, (2) biochemical induced changes, and (3) interactions with microbiota in the rhizosphere. Most of the direct effects have been a result of AMF interacting with pathogens in the rhizosphere in which complex associations exist among plant roots, soil, and microorganism (Wehner et al., 2009). However, changes in plant root physiology due to AMF association are certain to have significant impacts on the rhizosphere microflora through alteration of root exudates and other nutrient-related mechanisms (St-Arnaud and Vujanovic, 2007; Lioussanne et al., 2009). A direct interaction between *G. irregulare* and *F. oxysporum* has been studied with axenic system designed by St-Arnaud et al., (1995) (St-Arnaud et al., 1995) in which *G. irregulare* altered the growth of *F. oxysporum*. In this study, we also showed that *G. irregulare* had a significant inhibitory effect on growth of a virulent and mycotoxin-producing isolate of *F. sambucinum*. In addition, *G. irregulare* significantly induced down-regulation of three trichothecene biosynthetic genes, *TRI4, TRI13, and TRI101*. Confrontation with *G. irregulare* increased the expression of the trichodiene synthase gene *TRI5,* which may be a response to the down-regulation of the P450 oxygenase gene *TRI4.* Up-regulation of *TRI6*, a *G. irregulare* regulatory gene in trichothecene biosynthesis, may also contribute to the increase in *TRI5* expression (Alexander et al., 2008). Our study confirms and demonstrates AMF influence on the growth of *F. sambucinum* and furthermore have an effect on mycotoxin biosynthetic gene expression. The effect of *G. irregulare* on gene expression in *F. Sambucinum* could result from a direct effect of the AMF, but also from an indirect effect. *G. irregulare* could induce carrot roots to produce volatiles with activity against trichothecene production in *F. Sambucinum*.

2.4.3. Conclusion

We conclude that AMF can modulate mycotoxin gene expression of a plant fungal pathogen. This effect may be an important mechanism involved in biological control of plant pathogens.

Table 2.1: Primers sets used for PCR and qRT-PCR assays.

Primer	Nucleotide sequences (5′-3′)	Target gene	Reaction	Reference
ITS1 ITS4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	*ITS*	PCR	(Proctor et al., 2009)
1912 1914	TGTGTMGGYGCWGAGGCVATYGTTGG ACRGCAGCRGTCTGRCACATGGCGTA	*TRI3*	PCR	,,
1450 1455	ACCTTGAGTTCTACCATGAAGTCATC GCACTGTCTAGARCCCTGAGAGAAGT	*TRI4*	PCR	,,
1558 1559	GGCATGGTCGTGTACTCTTGGGTCAAGGT GCCTGMYCAWAGAAYTTGCRGAACTT	*TRI5*	PCR	,,
1482 1483	CACACYCTCCTSATGCTYTGTGGACT TCCCAMACTGTYCTYGCCAGCATCAT	*TRI11*	PCR	,,
109 178	CCATGGGTCGCRGGCCARGTSAA AACTCSCCRTCIGGYTTYTTNGGCAT	*TRI101*	PCR	,,
β-tubulin-F β-tubulin-R	GCCATGAAAGGAGGTTGAGGA AAGCCTTGCGTCGGAACATA	*Ref. gene*	qPCR	(Alexander et al., 2008)
EF 1α-F EF 1α-R	GTACGCCTGGGTTCTTGACA GAGCGTCTGGTAGGCATGTTAG	*Ref. gene*	qPCR	,,
TRI4-F TRI4-R	GCCACTGCTGCTACTGTTG GGTCGTTGTCCAGATGTTCTTG	*TRI4*	qPCR	,,
TRI5-F TRI5-R	TGGAGAACTGGATGGTCTGG GACATAGCCGTGCATGAAGC	*TRI5*	qPCR	,,
TRI6-F TRI6-R	AGTGCCAAGTCAGCTCATCG GAGCACGATCCTTGCGAGTT	*TRI6*	qPCR	,,
TRI13-F TRI13-R	CTGCGGTGGAACCGCTGGTA ACACTGGCGTTGTCCGTAAG	*TRI13*	qPCR	,,
TRI101-F TRI101-R	ATCGCCAACGAACCACTTG TGATGCTGCTTGACGGATTC	*TRI101*	qPCR	,,

Table 2.2: Effect of *G. irregulare* isolate (DOAM-197198) on the growth of *F. sambucinum*.

Treatment	Fungal growth (cm^2)							
	3-days		5-days		7-days		15-days	
	Growth*	SD**	Growth	SD	Growth	SD	Growth	SD
Fs + M medium	**14.50[a]**	**1.42**	**25.62[a]**	**3.26**	**29.71[a]**	**3.73**	**42.69[a]**	**4.18**
Fs + Carrot roots	**15.79[a]**	**2.02**	**25.30[a]**	**3.87**	**28.74[a]**	**2.92**	**46.04[a]**	**4.33**
Fs + DAOM-197198	**7.55[b]**	**2.15**	**9.31[b]**	**1.67**	**11.40[b]**	**1.42**	**12.63[b]**	**1.54**

*Values are means of 20 replicates. Within each column values followed by the
Same letters are not significantly different using one-way ANOVA analysis.
**Standard deviation of the mean.

Table 2.3: Changes in expression levels of *TRI4, TRI5, TRI6, TRI101* and *TRI13* genes from *F. sambucinum*

Gene	Treatment†	Time post-inoculation (3-days)			Time post-inoculation (5-days)		
		Expression	*p* value	Regulation††	Expression	*p* value	Regulation††
TRI4	Fs +C. roots	0.98	0.944	Not-affected	0.773	0.450	Not-affected
	Fs +Gi197198	0.45	0.001	Down	0.394	0.001	Down
	Fs +Gi234328	0.430	0.001	Down	0.475	0.001	Down
TRI5	Fs +C. roots	0.96	0.938	Not-affected	1.055	0.929	Not-affected
	Fs +Gi197198	17.124	0.001	Up	20.784	0.004	Up
	Fs +Gi234328	8.035	0.004	Up	6.423	0.001	Up
TRI6	Fs +C. roots	1.11	0.800	Not-affected	2.719	0.198	Not-affected
	Fs +Gi197198	0.606	0.286	Not-affected	2.139	0.083	Not-affected
	Fs +Gi234328	2.811	0.001	Up	3.638	0.001	Up
TRI101	Fs +C. roots	2.24	0.264	Not-affected	0.635	0.564	Not-affected
	Fs +Gi197198	0.056	0.001	Down	0.033	0.001	Down
	Fs +Gi234328	0.054	0.001	Down	0.001	0.001	Down
TRI13	Fs +C. roots	4.41	0.001	Up	0.566	0.604	Not-affected
	Fs +Gi197198	0.030	0.004	Down	0.347	0.004	Down
	Fs +Gi234328	0.756	0.390	Not-affected	0.113	0.001	Down

Fs + c. root, *F. sambucinum* inoculated with carrot roots without AMF

Fs +Gi197198, *F. sambucinum* inoculated with *G. irregular* isolate DOAM-197198

Fs +Gi234328, *F. sambucinum* inoculated with *G. irregular* isolate DOAM-234328

†† Regulation: (up & down) gene expression in sample group is significant and different in comparison to control group $p < 0.05$, and (not-affected) gene expression in sample group is not different in comparison to control group $p > 0.05$

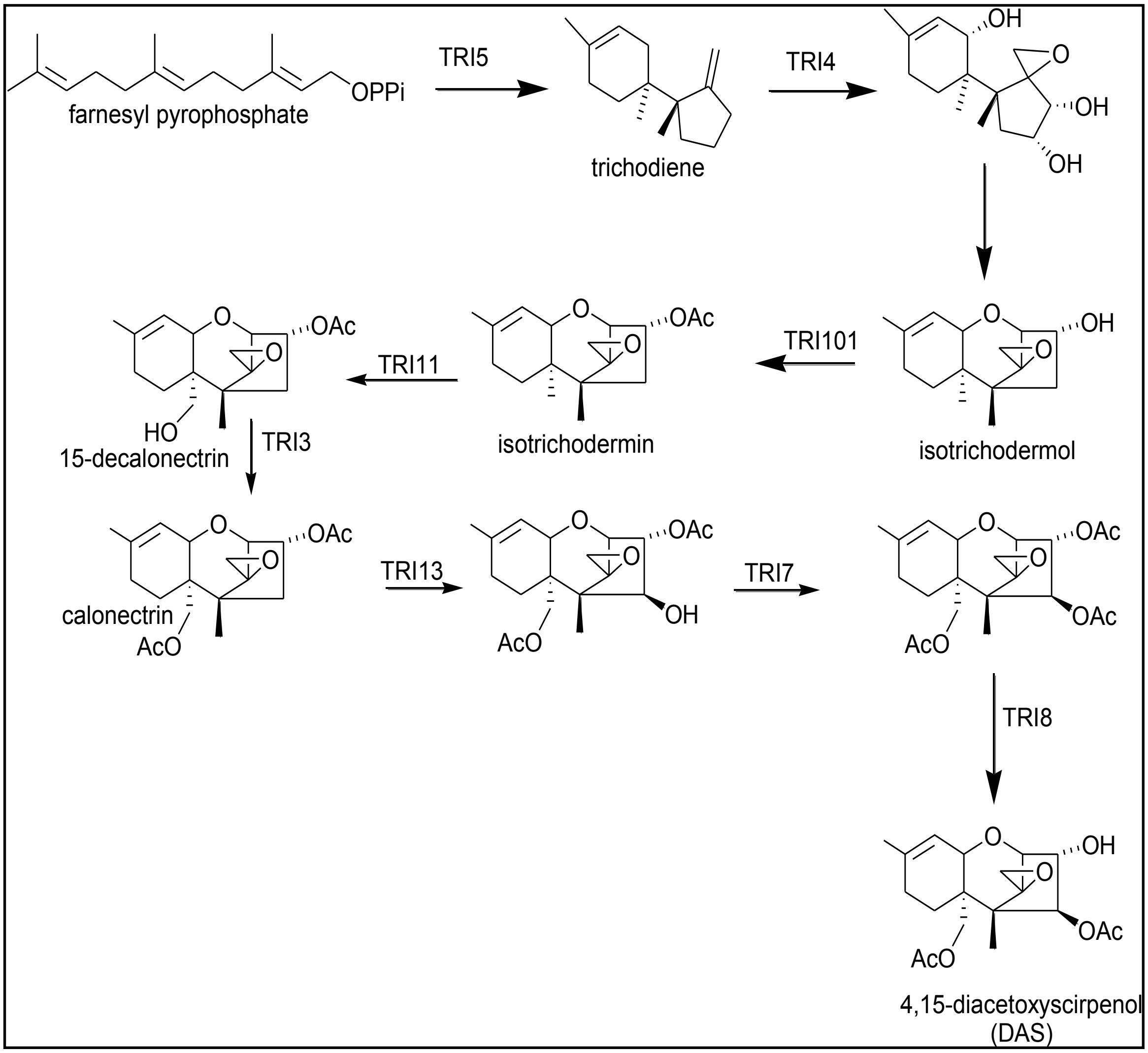

Figure 2.1: Proposed biosynthetic pathway for 4, 15- diacetoxyscirpenol (4, 15-DAS) adapted from Susan McCormick

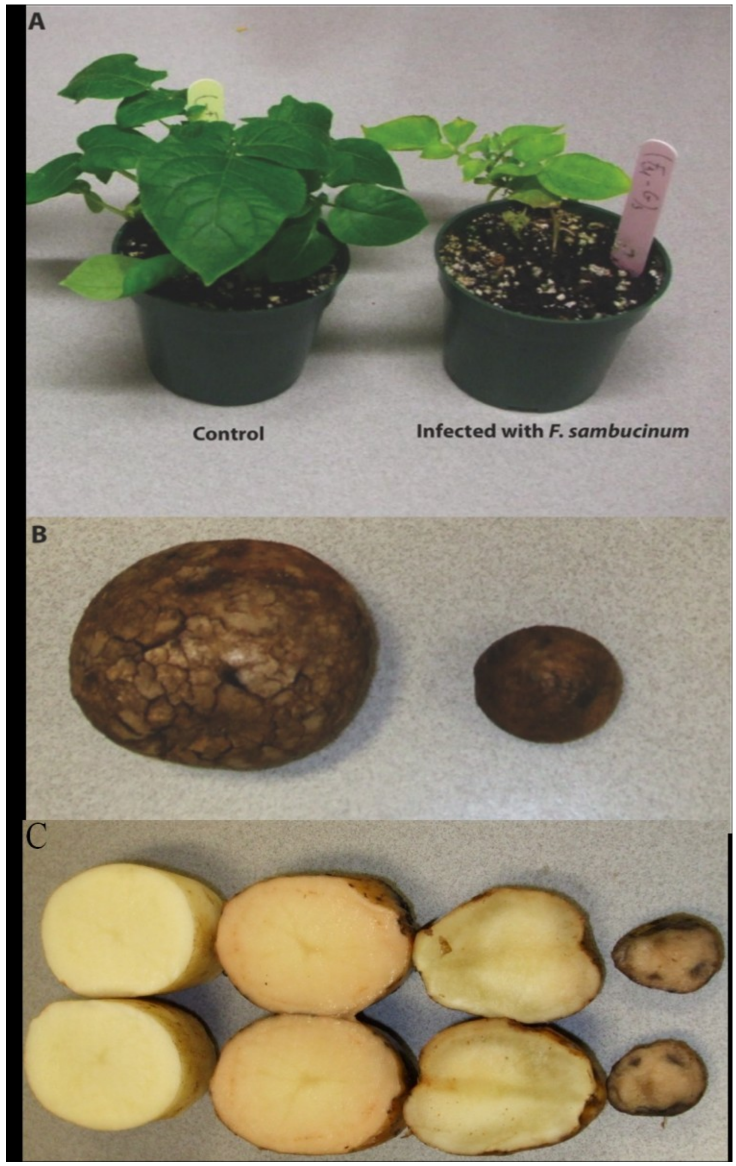

Figure 2.2: Artificial inoculation of potato plants with *F. sambucinum* strain T5. (A) Potato plant infected with *F. sambucinum* (right) and non-infected plants (left). (B) Potato tubers harvested from pots infested with *F. sambucinum*. (C) Infected tubers that show rotting consisted of a brown decay of tuber tissues, however tubers harvested from healthy plant do not show any symptoms of rot.

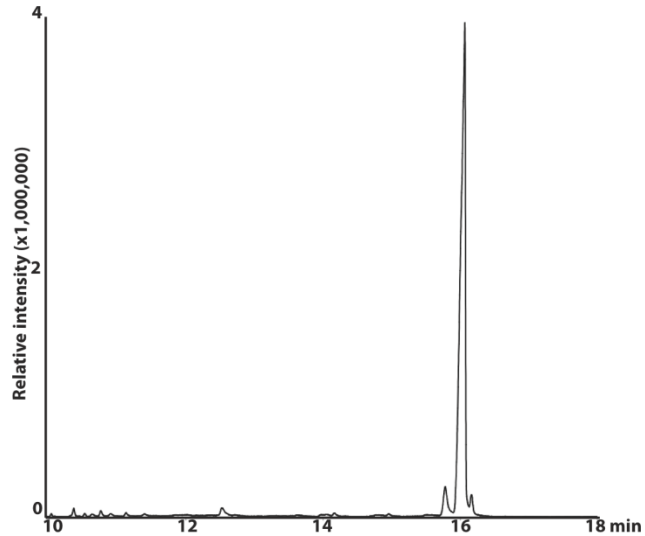

Figure 2.3: GC-MS traces of Diacetoxycsirpenol (DAS). Reconstructed ion chromatogram of an extract of 7 day-old liquid stage 2 cultures of *F. sambucinum* (T5) shows DAS elutes at 16.1 minutes.

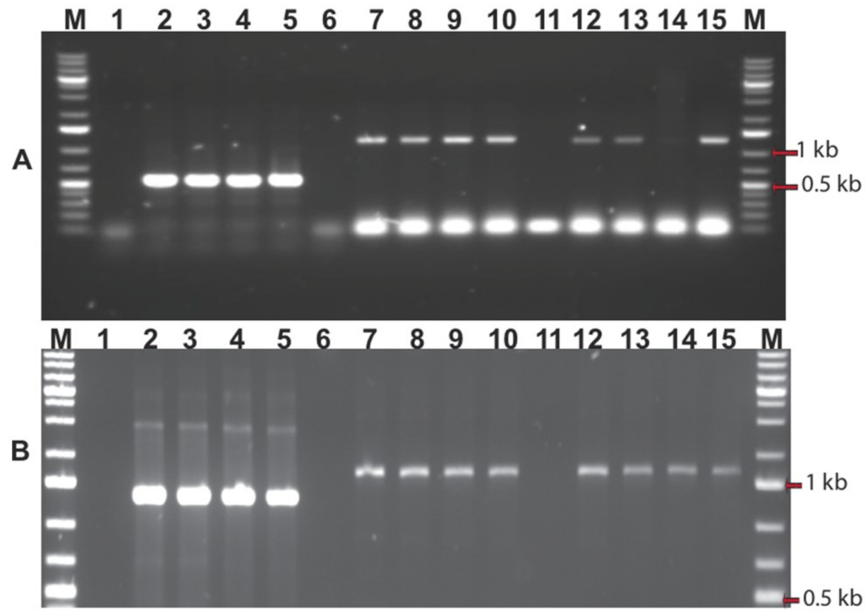

Figure 2.4: PCR amplification of ITS and *TRI* genes. Panel A: Agarose gel electrophoresis showing PCR products of ITS and *TRI* from four *F. sambucinum* strains T3, T5, T6 and T8 isolated from naturally infected potato. Lanes (2–5) show ITS PCR products from strains T3, T5, T6 and T8 respectively. Lanes (7–10) and (12–15) show *TRI5* and *TRI101* PCR products from T3, T5, T6 and T8 respectively. Lanes, 1, 6 and 11 are negative controls. Lane M shows 1 Kb ladder. Panel B: shows PCR patterns of *TRI3* (lanes 2–5), *TRI4* (lanes 7–10 and *TRI11* (lanes 12–15) from strains T3, T5, T6 and T8, respectively. Lanes, 1, 6 and 11 are negative controls. Lane M shows 1 kb ladder.

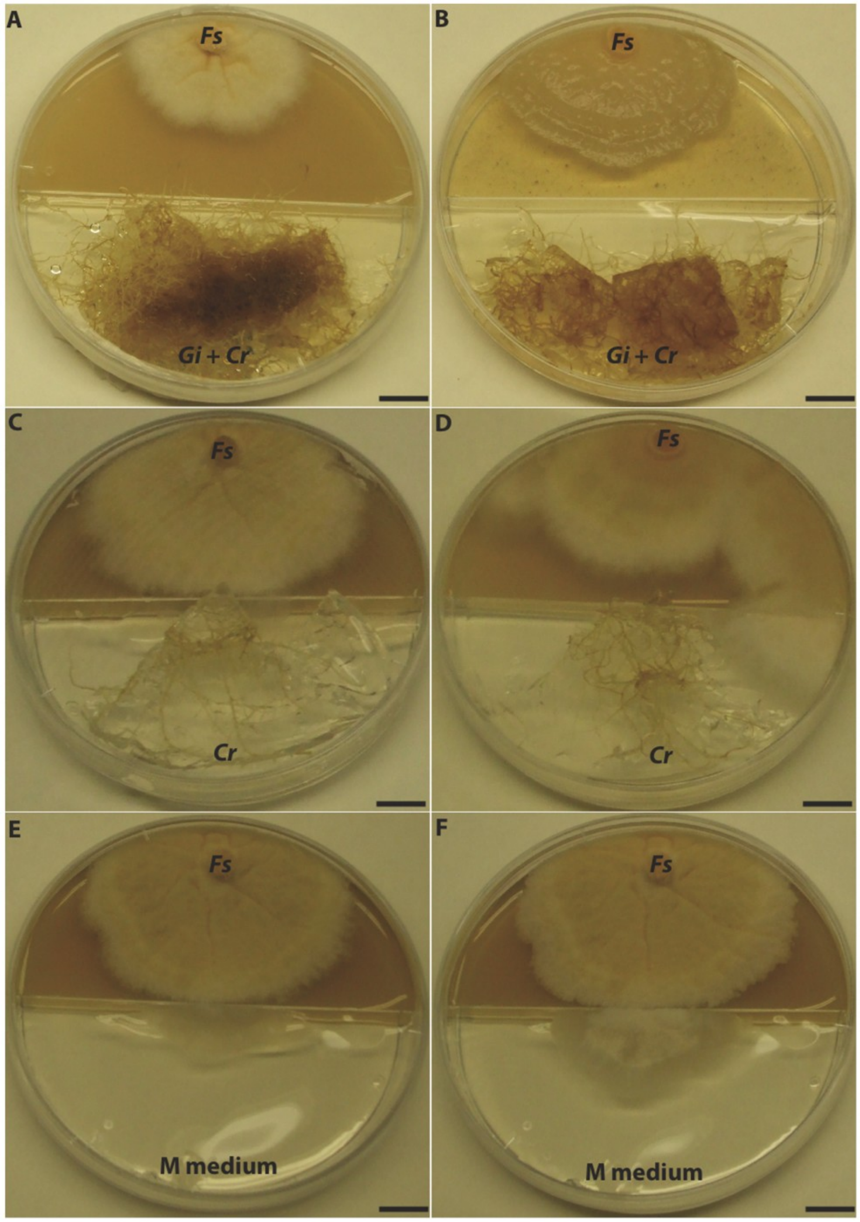

Figure 2.5: Confrontation cultures between *G. irregulare* and *F. sambucinum.* Experiments of confrontation between *F. sambucinum* and 2 isolates of *G. irregulare* DOAM-197198 (A) and DOAM-234328 (B) were performed in two-compartment Petri plates. Controls consisted of carrot roots without AMF (C & D) and M medium (E & F) without carrot roots or AMF.

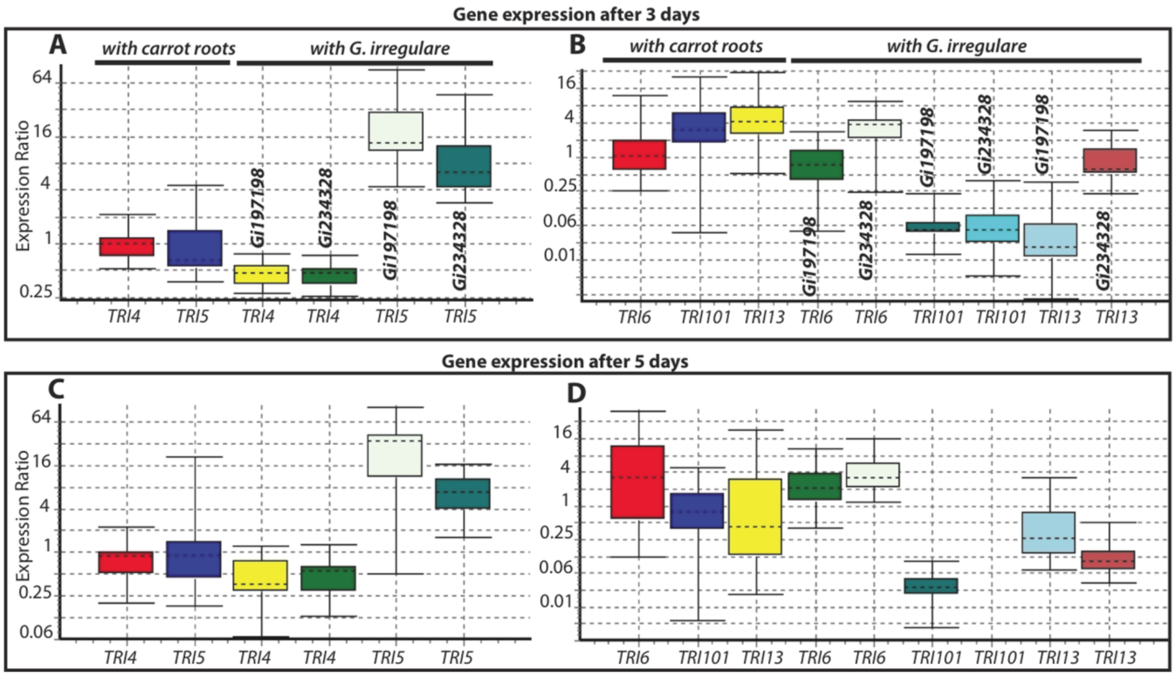

Figure 2.6: Relative expression patterns of *TRI4, TRI5, TRI6, TRI101* and *TRI13* genes of *F. sambucinum* inoculated with *G. irregulare* and or with carrot roots compared to *TRI4, TRI5, TRI6, TRI101* and *TRI13* genes of *F. sambucinum* growing alone as control. Changes in relative expression of *TRI* genes were calculated from *F. sambucinum* after 3 days (A and B) and 5 days (C and D) of confrontation either with carrot roots lacking AMF, or with *G. irregulare* colonized carrot roots. Panels A and C show relative expression patterns of *TRI4* and *TRI5* for *F. sambucinum* confronted with carrot roots and against *G. irregulare* isolates DAOM-197198 (Gi197198) and DAOM-234328 (Gi234328), respectively. Panels B and D show relative expression patterns of *TRI6*, *TRI101* and *TRI13* of *F. sambucinum* against carrot roots and *G. irregulare* isolates DAOM-197198 (Gi197198) and DAOM-234328 (Gi234328), respectively. The bars represent standard error at 95% confidence interval. The significance is reported according to the hypothesis test ($p < 0.05$) as shown in Table (2.3).

2.5. Materials and Methods

2.5.1. Fungal strains and growth conditions

Fusarium sambucinum strain T5 was isolated and characterized from naturally infected potato plants (cultivar Riba) from a field located in 2420 Rue Principale, Saint-michel, Québec (45° 11'46''N-73°36'20.52''W). The strain was grown and maintained on V-8 juice agar medium and in GYEP medium (2% glucose, 0.1% yeast extract, 0.1% peptone) (Seo et al., 2001). Two isolates of the AMF *G. irregulare* DAOM-197198 and DAOM-234328 were grown *in vitro* in co-culture with Ri T-DNA-transformed carrot roots (*Daucus carota* L.) on a minimal (M) medium. Spores for both isolates of *G. irregulare* were collected from plates by dissolving the Gellan gum as described (Hijri and Sanders, 2004).

2.5.2 Pathogenicity of *F. sambucinum* (T5) on potato plants.

F. sambucinum (strain T5) was used to inoculate oat (*Avena sativa*) seeds as described by (Yao et al., 2002). Potato seedlings (cultivar Riba) were germinated and maintained *in-vitro* using a technique adapted from (Suttle, 1998)**.** Four week-old potato seedlings grown in the green-house were infected with *F. sambucinum* by gently pushing the soil at the base of plantlets to expose portions of the root system and five non-infected (mock) or fungus-infected seeds were then placed directly in contact with uncovered roots at five points equidistant from the stem. Disease symptoms (wilting of rotting) were observed and recored on plants and potato tubers.

2.5.3. Dual culture assays.

The confrontation cultures between *G. irregulare* and *F. sambucinum* were performed *in vitro* using two-compartment Petri dishes (100 x 15 mm). One compartment was filled with 25 ml GYEP agar medium (2% glucose, 0.1% yeast extract, 0.1 % peptone and 2% agar) for *F. sambucinum*. The other compartment of the plates was gently filled with 25 ml M medium. GYEP and M media were connected with a bridge by adding droplets of M medium over the separation of the two compartments that allowed fungal hyphae to cross from one compartment to the other. Approximately 2 cm^2 of *G. irregulare* (isolates DAOM-197198 and DAOM-234328) and transformed carrot root co-cultures were individually transferred into each compartment containing M medium. Because AMF grow slowly, plates were incubated at 25 ◦C for 4 weeks until the AMF hyphae grew to reach the bridge. The cultures were examined weekly and carrot roots were trimmed aseptically to prevent their growth into the distal compartment. Controls consisted of Ri T-DNA transformed carrot without AMF and *F. sambucinum* alone (neither roots nor AMF). An agar disk of 0.5 cm diameter containing *F. sambucinum* strain T5 was used to inoculate the distal compartment containing GYEP agar medium on the side opposite *G. irregulare*. Additional controls were performed used a disk of the *F. sambucinum* adjacent to M medium alone (Fs + M) or with carrot-roots not inoculated with *G. irregulare* (Fs + Cr). Each combination of *F. sambucinum*/*G. irregulare* and controls was replicated 20 times and plates were randomly placed in the dark and incubated at 25□C. The growth rate of *F. sambucinum* was checked every 2 days and pictures were recorded to measure the growth area using Image J software available at (http://rsbweb.nih.gov/ij/). Results are reported as means of *F. sambucinum* growth alone on M medium (Fs + M), in the presence of *G. irregulare* (*Gi197198*), or with non-inoculated carrot roots (Fs + Cr).

2.5.4. DNA extraction, PCR amplification and sequencing.

DNA was extracted from freshly harvested fungal mycelium grown in liquid GYEP medium for 2-4 days. Fungal mycelium was lypholized in liquid nitrogen and ground using mortar and pestle and DNA was extracted with DNeasy Plant Mini Kit (Qiagen, Canada) following the manufacturer's instructions. PCR amplifications of trichothecene genes: *TRI1*, *TRI3*, *TRI4*, *TRI4*, *TRI6*, and *TRI101* were performed on using the primer sets listed (Table 2.1). Primers were synthesized by Alpha DNA Oligonucleotide Synthesis. All primer sequences were adapted from Robert proctor and co-workers at the US department of agriculture, agricultural research services (USDA-ARS). The GenomeWalker protocol (Clontech) was used to amplify regions of DNA flanking *TRI* gene fragments that had been amplified with primers (Table 2.1) that were designed based on *F. graminearum* and *F.*

sporotrichioides sequences (Proctor et al., 2009). With this strategy, we obtained *TRI3*, *TRI4*, *TRI5*, *TRI11*, *TRI101* sequences for the *TRI* cluster region in *F. sambucinum*. The resulting sequence data were used to design *Fusarium*-specific primers for qPCR assays. However, primers for *TRI6* and *TRI13* were obtained from (Alexander et al., 2008) PCR amplifications in 50 µL all contained: 1x *Taq* buffer, 0.25 mM of each dNTP, 0.5 µM of each primer, 1U of *Taq* DNA polymerase (Fermentas), and 50 ng of DNA template. Reactions were carried out using a thermal cycler EP Master cycler S (Eppendorf) under the following parameters: pre-denatured at 94°C for 1 min, followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 51 °C for 30 sec and elongation at 72 °C for 90 sec, and a final, elongation at 72 °C for 5 min. For each trichothecene gene, a negative control without DNA template was performed. PCR amplificons were visualized on a 1% agarose gel stained with ethidium bromide and visualized under UV light. In our PCR assays with degenerate *TRI* primers, it happens, but not often that we get amplifications of multiple bands with a set of primers. The typical band that corresponds to the target *TRI* gene fragment was purified by extraction with an equal volume of a 1:1 (v/v) mixture of TRIS-equilibrated phenol and chloroform: isoamyl alcohol (24:1). The resulting aqueous phase was mixed with 2 vols of NaI solution and 5 ml of UltraBind solution, and then further purified by the Ultra-Clean DNA Purification kit (Mo Bio Laboratories) as specified by the manufacturer. Reactions that showed clear amplification bands were sequenced at the Genome Quebec Innovation Center (Montreal, Qc), using the specific primers (Table 2.1). Two sequencing reactions were performed for each PCR amplicon. Recovered sequences were assembled and analyzed using Vector NTI software (Invitrogen) and compared to the NCBI database using Nucleotide BLAST search. Nucleotide sequences were deposited in the EMBL nucleotide sequence database under the accession numbers: HQ445900 for ITS and HQ445905 to HQ445907 for *TRI* genes.

2.5.5. Chemical analysis of the trichothecenes.

To induce mycotoxin production in liquid culture, a two-stage medium protocol modified from the method of Miller and Blackwell (1986) was employed (Miller and

Blackwell, 1986). The cultures of the anamorph *F. sambucinum* (T5) were grown at 25 □C on a rotary shaker at 200 rpm in the dark. After 7 days of incubation in the second stage medium, a 5 ml aliquot containing fungal material was extracted with 2 ml ethyl acetate. Extracts of 10 replicates were analyzed with gas chromatography and low resolution mass spectrometry (GCMS) using a Hewlett Packard 6890 gas chromatograph fitted with a HP-5MS column (30 m × 0.25 mm film thickness) and a 5973 mass detector. Trichothecenes were identified by comparison of retention time and mass spectra with standard compounds.

2.5.6. RNA isolation and real-time qRT-PCR assays.

Total RNA of *F. sambucinum* was extracted from each treatment combination with three biological replicates. Fungal material was prepared as described in the DNA extraction section. Total RNA was isolated using TRIZOL® Reagent according to the manufacturer's instructions (Qiagen, Canada). cDNA libraries were constructed by RevertAid™ 270 H Minus M-MuLV kit (Fermentas) according to the manufacturer's instructions. Real-time (RT) PCR reactions were performed in a volume of 10 µl containing 2 µl water, 1 µl of each primer, 1 µl cDNA and 5 µl CYBR green Maxima® SYBR Green/ROX qPCR Master Mix. A liquid handling Workstation ep*Motion* 5070 (Eppendorf) was used to optimize RT-PCR assays with small reaction volumes. All genes were run in triplicate on each plate and 3 biological replicates of each treatment were performed. A negative control using Mili-Q water was prepared for each sample. RT-PCR was run on EP RealPlex MasterCycler (Eppendorf) using the following conditions: an initial denaturation step at 95°C for 10 min followed by 39 cycles of 95 °C for 15 sec, 60 °C for 45 sec (annealing and extension). A final extension was carried out by 95°C for 15 sec followed by 60 °C for 1 min. A melting curve was performed from 55 to 95°C with a 0.2°C reading interval. We used two house-keeping genes, □-tubulin and elongation factor EF1□, in our qRT-PCR assays. All samples had only a single peak, indicating a pure qRT-PCR product and no contamination or primer dimer formation. The relative expression levels ΔC_t of gene of interest *TRI4*, *TTRI5*, *TRI6*, *TRI13* and *TRI101* genes were calculated in relation to □-

tubulin elongation factor EF1□□□To determine the change in expression level of each *TRI* gene, we used C_t of untreated *TRI* genes and untreated reference genes from cDNA of *F. sambucinum* grown alone as calibrator (control). The treated *TRI* genes and treated reference genes from treatment combinations (Fs + Gi) and (Fs + C. roots) were normalized in relations to control (Fs alone). Data analysis was performed on REST 2009 Software available at http://www.gene-quantification.de/rest-2009.htm as described below in statistical analysis section.

2.5.7. Experimental design and statistical analysis.

Experiments were performed using a factorial arrangement (1 pathogen) × (2 AMF fungi + control) in a randomized complete design with 20 replicates. Analysis of variance (one-way ANOVA) was used to examine the significant effect of the AMF *G. irregulare* isolate 197198 on growth of *F. sambucinum*. Post-hoc comparison between the treatments were done using Tukey`s HSD test using SPSS software v. 17 (SPSS Inc., Chicago, Illinois). We used Relative Expression Software Tool (REST 2009) for group-wise comparison and statistical analysis of relative expression results as described in (Pfaffl et al., 2002). The relative expression ratio of a target gene is computed, based on its real-time PCR efficiencies (*E*) and the crossing point (CP) difference (Δ) of treatment (*F. sambucinum* + *G. irregulare*) and (*F. sambucinum* + carrot roots) versus a control (*F. sambucinum*). For calculating efficiency (*E*) of qRT-PCR reactions: $E = 10^{-1/slope} - 1$. For calculating relative expression ratio of a target gene:

$$Relative\ expression\ ratio = (E_{target})^{\Delta CP_{target(control-sample)}} / (E_{ref})^{\Delta CP_{ref(control-sample)}}$$

The target (*TRI*) gene expression is normalized by non-regulated two reference genes ß-tubulin and elongation factor EF1α. We used the hypothesis test *P* (H_1) that represents the probability of the alternate hypothesis that the difference between sample and control groups is due only to chance. The hypothesis test performs at least 2000 times of random reallocations of samples and controls between the groups. Statistical difference are significant when $p<0.05$.

2.6. Acknowledgments

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3. Control of *Fusarium* trichothecene mycotoxin production by an arbuscular mycorrhizal fungus.

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3.1. Abstract

Many plant pathogenic fungi belonging to the genus *Fusarium* produce mycotoxins commonly known as trichothecenes. This family of mycotoxins exerts its destructive effects by inhibiting protein synthesis in eukaryotes. We previously characterized trichothecene-producing *Fusarium* strains isolated from naturally infected potato plants. *Fusarium sambucinum* showed its capacity to produce trichothecene 4, 15-Diacetoxyscirpenol (4, 5-DAS). Using dual cultures, the symbiotic arbuscular mycorrhizal fungus (AMF) inhibited the growth of *F. sambucinum* and modulated expression of trichothecene biosynthetic and regulatory genes. However, the effect of AMF in mycotoxin production was not known. Here we show that the AMF *Glomus irregulare* significantly reduced the production of 4, 15-DAS by *F. sambucinum* when confronted in dual cultures. We hypothesized that trichodiene accumulates due to a down-regulation of the *TRI4* gene in the biosynthesis pathway in *F. sambucinum.* When we tested trichodiene on *F. sambucinum,* we observed no effects on the growth or any changes in morphology of the mycelium.

Keywords: Trichothecene resistance – *Fusarium sambucinum* – mycotoxin – Arbuscular mycorrhizal fungi.

3.2. Introduction

Trichothecenes are a large family of sesquiterpenoid secondary metabolites produced by a number of *Fusarium* species and other molds (Desjardins et al., 1993a; Kimura et al., 2007). They are important inhibitors of protein synthesis in eukaryotes (Bennett and Klich, 2003), and cause destructive effects, particularly in animals that consume contaminated grains (Yoshizawa, 2003). Trichothecenes are also an agricultural concern because they contribute to plant pathogenesis of *Fusarium* on some crops (Desjardins and Hohn, 1997; Maier et al., 2006). The trichothecene known as diacetoxyscirpenol (DAS) is one of the most economically important trichothecenes reported to be produced by *Fusarium sambucinum* (Desjardins et al., 1992; Ismail et al., 2011). It is the most prevalent and is commonly found in barley, corn, rye, safflower seeds, wheat, and mixed feeds (Miller, 1994). When DAS is ingested in high doses by agricultural animals, it causes nausea, vomiting, and diarrhea (Bennett and Klich, 2003). The biosynthetic pathway of DAS in *F. sambucinum* involves a series of oxygenation and esterification reactions controlled by several trichothecene genes (Desjardins et al.,1992 ;Hohn et al., 1993 Ismail et al., 2001; Achilladelis et al, 1968). The pathway then proceeds through a sequence of oxygenation, esterification, and deacetylation steps to produce more complex trichothecene 4, 15-diacetoxyscirpenol (4, 15-DAS). The toxicity of mycotoxins, including Trichothecenes, is mostly studied in animals or animal cell lines. However, the characterization of Trichothecenes as virulence factors has recently become an interest in the phytotoxicity (McCormick, 2009). In addition to their effect as inhibitors of protein synthesis, trichothecene also affect mitochondrial function, electron transport, changes in seed germination, root and shoot growth, leaf chlorosis and necrosis, bleaching and degradation of chlorophyll (Katouli and Marchant, 1981; McLean, 1996; McCormick, 2009). Strategies of trichothecene resistance that have been proposed include the application of synthetic or naturally occurring trichothecene inhibitors in cultures of *Fusarium* (Desjardins et al., 1987; Alexander et al., 2008), or directly onto plants. Xanthotoxin (8-methoxypsoralen) could effectively block T-2 toxin production by *F. sporotrichioides* in liquid cultures (Alexander et al., 2008). However, the mechanism by which xantotoxin inhibited T-2 toxin production was similar to Ancymidol (Desjardins et al., 1987), where the addition of xanthotoxin to liquid culture of *F. sporotrichioides* caused a significant increase of the trichodiene accumulation. This suggested that xanthotoxin not only blocks trichothecene oxygenation reactions but, may in some way also induce the synthesis of trichodiene (Alexander et al., 2008). It has been reported that the *TRI101* gene encoding an acetyltransferase gene that controls the addition of a C-3 acetyl group (Kimura et al., 1998; Kimura et al., 1998) can protect *Fusarium* from Trichothecenes. This acetyl group protects the fungus from its own toxin during biosynthesis. Therefore since trichothecenes have been identified as virulence factors in *Fusarium* head blight (FHB) in wheat (Proctor et al., 1995b), a strategy for improving plant resistance to trichothecene toxicity is to express *TRI101* in plants (McCormick, 2009). In order to improve plant resistance to Trichothecenes, the *TRI101* has been transferred into several plants species including rice (Okubara et al., 2002; Ohsato et al., 2007), and barley (Manoharan et al., 2006) in order to introduce resistance to the trichothecene toxins and thereby increase resistance to *Fusarium*.

The use of non-producing strains to compete with trichothecene-producing strains has been reported to be an alternative strategy for trichothecene resistance (Desjardins et al., 1993) as has use of other biocontrol agents. However, the most effective strategy for trichothecene resistance is to prevent the fungal infection and toxin production by the producing organism. The arbuscular mycorrhizal fungi (AMF) have been shown to reduce the populations of pathogenic fungi in root rhizosphere (St-Arnaud and Vujanovic, 2007), and to inhibit the growth of the mycotoxin-producing fungus *Fusarium sambucinum* (Ismail et al., 2011). AMF interact with soil microbes to promote inhibitory or stimulatory reactions of which some are clearly competitive, while others may be mutualistic (Filion et al., 1999). It has been reported that the inhibition of *F. sambucinum* growth was associated with morphological changes when the fungus was confronted with the AMF *Glomus irregulare*. The growth of *Pseudomonas chlororaphis* and the germination of *Trichoderma harizianum* conidia were stimulated by substances released by the extra radical mycelium of *Glomus intraradices* grown *in vitro* (Filion et al., 1999). Beside the direct competitive interactions with other pathogenic fungi, the AMF symbiosis affects the community and diversity of other organisms in the soil. This can be achieved by changes in the plant species and plant exudates type and amount (Marschner and Timonen, 2005). However, the recent research has shown that AMF release an unidentified diffusion factor, known as the myc-factor, which stimulates the activation of plant nodulation by the nitrogen fixing and rhizobial bacteria (Kosuta et al., 2003). An unidentified diffusion compound may be similar to the myc-factors released by AMF and are proposed to affect the normal growth of *F. sambucinum*.

Using quantitative RT-PCR assays, we showed that *F. sambucinum* trichothecene biosynthetic genes have been modulated by the AMF *G. irregulare.* In particular the expression of *TRI5* and *TRI6* were up-regulated, while *TRI4*, *TRI13* and *TRI101* were down-regulated (Ismail et al., 2011). However, we do not know yet if these changes in genes affect the production of mycotoxin in *F. sambucinum.* The objective of this study was therefore to test the effect of the AMF *G. irregulare* on mycotoxin production of *F. sambucinum.* We aimed to test whether *G. irregulare* controls 4, 15-diactoxyscirpenol production by *F. sambucinum*.

3.3. Materials and Methods

3.3.1. Fungal strain and growth conditions

Fusarium sambucinum strain T5 was grown and maintained on V-8 juice agar and in GYEP agar media (2% glucose, 0.1% yeast extract, 0.1% peptone and 2 % agar) (Ismail et al., 2011). Two isolates of AMF *Glomus irregulare* (DAOM-197198 & DAOM-23438) were grown *in vitro* in co-culture with RiT-DNA-transformed carrot roots (*Daucus carota* L.) on minimal (M) medium. *F. sambucinum* and *G. irregulare* cultures were incubated at 25°C in the dark.

3.3.2. Dual culture assays and quantitative analysis of the Trichothecenes

The confrontation cultures between *G. irregulare* and *F. sambucinum* were performed as described by (Ismail et al., 2011). Treatment combinations were consisted of *F. sambucinum* inoculated with *G. irregulare* isolate (DOAM-197198) and (DOAM-234328) (Gi +Fs), *F. sambucinum* inoculated with carrot roots without AMF (Fs + C. roots) and *F. sambucinum* alone (Fs). Ten replicates were used for each treatment combination. The plates were incubated in a complete randomized design at 25°C for 7 days. To measure 4, 15-diacetoxyscirpenol, GYEP agar medium with *F. sambucinum* biomass was quantitatively transferred *to a 250* ml *beaker* containing 100 ml ethyl acetate. The beakers were covered with aluminum foil and shacked on a rotary shaker at 200 rpm for 1 hour. Ethyl acetate extracts were transferred in new 250 ml beakers and left overnight to evaporate. The trichothecene extracts were re-suspended in 2 ml ethyl acetate and mixed well with a glass pipette and then transferred to 2 ml vial tubes. Extracts of ten replicates for each treatment combination were analyzed with gas chromatography and low resolution mass spectrometry (GCMS) using a Hewlett Packard 6890 gas chromatograph fitted with a HP-5MS column (30 m×0.25 mm film thickness) and a 5973 mass detector.

3.3.3. Effect of AMF *G. irregulare* on *F. sambucinum* survival

To investigate the impact of *G. irregulare* on *F. sambucinum*, we set up two independent and complementary experiments as follows: (i) for the effect of G. irregulare on the morphology of the mycelium and growth of *F. sambucinum*, plates (9 cm in diameter) containing 40 ml of M medium were inoculated with a 2 cm^2 piece of agar containing *G. irregulare* mycelium (*DAOM*-197198 or DAOM-234328) and transformed carrot root co-cultures. Because AMF grow slowly, plates were incubated at 25°C for 4 weeks until the AMF extra-radical hyphae grew. Controls consisted of plates inoculated with Ri T-DNA transformed carrot without AMF and M medium without any inoculation. After the hyphae of *G. irregulare* had successfully grown, plates containing GYEP agar medium were inoculated with an agar disk of 0.5 cm diameter containing *F. sambucinum* strain T5. (ii) For volatile substances affecting the growth of *F. sambucinum*, a plate containing *G. irregulare* on M medium with transformed carrot roots was sealed together with a plate of *F. sambucinum* on GYEP agar medium after removing covers. Plate pairs were sealed twice with paradigm. Control plates were prepared using a plate of the *F. sambucinum* with a plate of M medium (Fs+M) or with carrot roots not inoculated with *G. irregulare* (Fs+Cr). Each combination of *F. sambucinum*/*G. irregulare* and controls was replicated 20 times and plates were randomly placed in the dark and incubated at 25°C. The growth of *F. sambucinum* was checked and the plate positions were changed regularly every 2 days. To test the effect of AMF *G. irregulare* on *F. sambucinum* survival, *an* agar disk of 0.5 cm from each culture combination was individually re-cultured on plates containing GYEP agar medium. The growth rate of *F. sambucinum* was checked every 2 days and pictures were taken to measure the growth area using Image J software available at (http://rsbweb.nih.gov/ij/).

3.3.4. Effect of trichodiene on growth of *F. sambucinum*

Trichodiene was kindly obtained from Dr. Susan McCormick's laboratory at the National Center for Agricultural Utilization Research, Peoria Illinois, USA. 2 mg of *t*richodiene were dissolved in 400 µl of acetone and added to 40 ml of GYEP agar medium. An agar disk of 0.5 cm diameter containing *F. sambucinum* strain T5 was used to inoculate the plates containing GYEP agar medium. Controls consisted of *F. sambucinum* grown either on GYEP agar with 400 µl of acetone or GYEP agar only. Each treatment was replicated 12 times and plates were randomly placed in the dark and incubated at 25°C. The growth rate of *F. sambucinum* was checked every 2 days and pictures were recorded to measure the growth area using Image J software.

3.3.5. Experimental design and statistical analysis

Experiments were performed using a factorial arrangement (1 pathogen) × (2 AMF fungi + control) in a randomized complete design with 10 replicates. Analysis of variance (one-way ANOVA) was used to examine the significant effect of the AMF *G. irregulare* on DAS production *F. sambucinum*. The same analysis of variance (one-way ANOVA) was used to examine the significant effect of trichodiene on the growth of *F. sambucinum*. Post-hoc comparison between the treatments were done using Tukey`s HSD test using SPSS software v. 17 (SPSS Inc., Chicago, Illinois)

3.4. Results and discussion

To study the impact of *G. irregulare* on 4, 15-DAS production by *F. sambucinum*, we used confrontation cultures using an *in vitro* system as described in (Ismail et al., 2011b). We used GC-MS to quantify 4, 15-DAS in ethyl-acetate extracts of *F. sambucinum* grown on GYEP agar medium. The relative intensities (10 reads for each treatment combination) of 4,15-DAS were greatly decreased in the presence of *G. irregulare* isolate DAOM-197198 and isolate DAOM-23438 compared with controls that consisted of carrot roots without *G. irregulare* or *F. sambucinum* alone (Figure 3.2). Interestingly, the DAS concentrations were significantly reduced to 39 and 42 µg/ 1ml of GYEP medium by both AMF strains *G. irregulare* DAOM-197198 and DOAM-234328 respectively (Figure 3.3). We assessed DAS production by either *F. sambucinum* grown with carrot roots or *F. sambucinum* grown alone. In the presence of carrot roots, *F. sambucinum* produced 144 µg/ ml, while when the fungus was grown alone, 4, 15-DAS production was 126 µg/ ml (Figure 3.3). The quantitative differences observed in this study in DAS production by *F. sambucinum* growing under different treatments have been reported in other *F. sambucinum* treated with a plant growth regulator Ancymidol (Desjardins et al., 1987). In other *Fusarium* species, xanthotoxin has been shown to reduce T2-toxin production in growth culture of *F. sporotrichioides* (Alexander et al., 2008). Analysis of culture extracts by GC-MS showed that DAS concentrations decreased in cultures of *F. sambucinum* inoculated with two isolates of *G. irregulare* (DOAM-197198 and DOAM 234328) compared to those in cultures of *F. sambucinum* grown alone or inoculated with carrot roots without AMF (Table 3.1). This result indicates our previous work that AMF modulate the expression of *TRI* genes involved in DAS biosynthesis (Ismail et al., 2011). We reported that *G. irregulare* inhibited *F. sambucinum* growth in vitro and furthermore, AMF modulated expression of a number of trichothecene biosynthetic genes including *TRI5*, *TRI4*, *TRI6*, *TRI13* and *TRI101* (Ismail et al., 2011). However, we did not see significant difference between both AMF isolate on DAS production by *F. sambucinum*.

AMF have been shown to affect pathogenic fungi by several mechanisms (St-Arnaud and Vujanovic, 2007; Wehner et al., 2009). In this regard, direct competition via interference competition including chemical interactions has been proposed as a mechanism by which AMF can reduce the abundance of pathogenic fungi in plant roots (Azcón-Aguilar and Barea, 1997; St-Arnaud and Vujanovic, 2007).

To test whether trichodiene affects the fungal growth, we grew *F. sambucinum* on GYEP agar containing trichodiene 2 µg/40ml medium. The results of this study (Figure 3.4) show that trichodiene neither affects the growth nor induced any morphological changes associated with *F. sambucinum*. These results confirm that despite the fact that *F. sambucinum* produces volatile sesquiterpenes including trichodiene, trichodiene has no effect on the fungus itself. It has been reported that *F. sambucinum* is a source of many volatile sesquiterpenes, which are associated with strain toxicity (Jelen et al., 1995). The biosynthetic gene *TRI4,* was shown to control the conversion of trichodiene into a toxic product through several oxygenation steps as shown in (Figure 3.1) (McCormick et al., 2006). This suggests that the inhibitors that block *TRI4* enzymes would effectively block the production of trichothecene and could decrease the virulence of the fungus. AMF *G. irregulare* has been shown to down-regulate the expression of *TRI4* of *F. sambucinum* (Ismail et al., 2011), in a way similar to a number of compounds such as Ancymidol and xanthotoxin that have been used to block T-2 toxin biosynthesis in *F. sporotrichioides* (Desjardins et al., 1987; Alexander et al., 2008).

The effect of AMF on DAS production suggests new strategy for prevention of trichothecene from entering human and animal food chains, as well as their role in plant pathogen protection. AMF are commonly used as biofertilizers in agriculture to enhance mineral uptake, in particular phosphorus (Roy-Bolduc and Hijri, 2011). The fact that AMF control mycotoxin production and induce plant defense genes underscore the importance of integrating AMF in modern production systems and encouraging their use for improving plant resistance and producing safe food.

Table (3.1) Diacetoxycsirpenol concentrations of *F. sambucinum* cultures

Reps.	DAS concentrations µg/ml			
	Fs alone	Fs + C. roots	Fs + Gi-197198	Fs + Gi-234328
1	111	109	28	16
2	104	159	22	43
3	129	109	45	40
4	149	133	52	50
5	159	222	34	38
6	128	95	35	49
7	128	126	44	65
8	133	185	35	70
9	84	175	53	24
10	132	127	38	26
Average	126	144	39	42

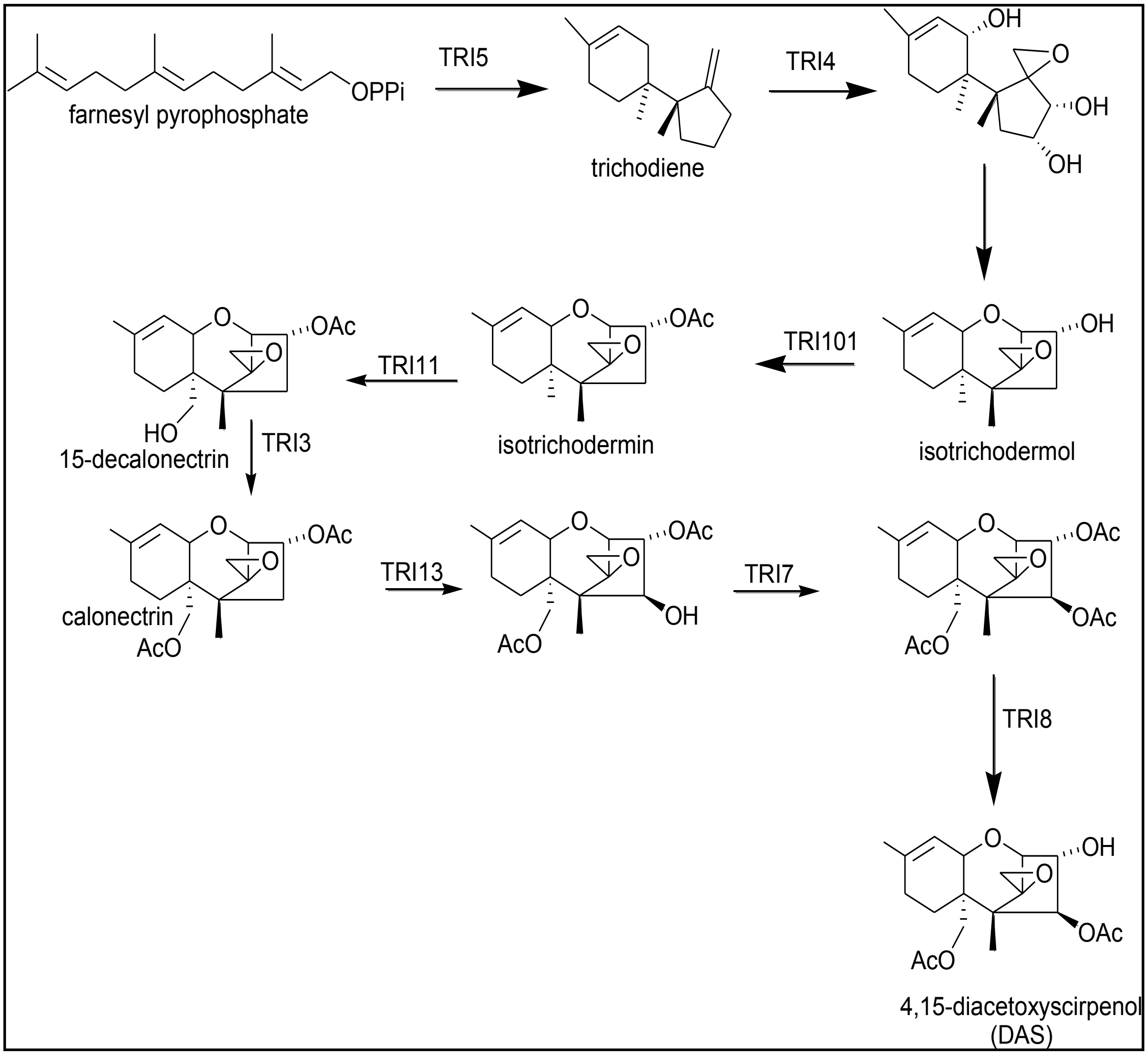

Figure 3.1: Proposed biosynthetic pathway of 4, 15-diactoxyscirpenol (4, 15-DAS) adapted by Susan McCormick.

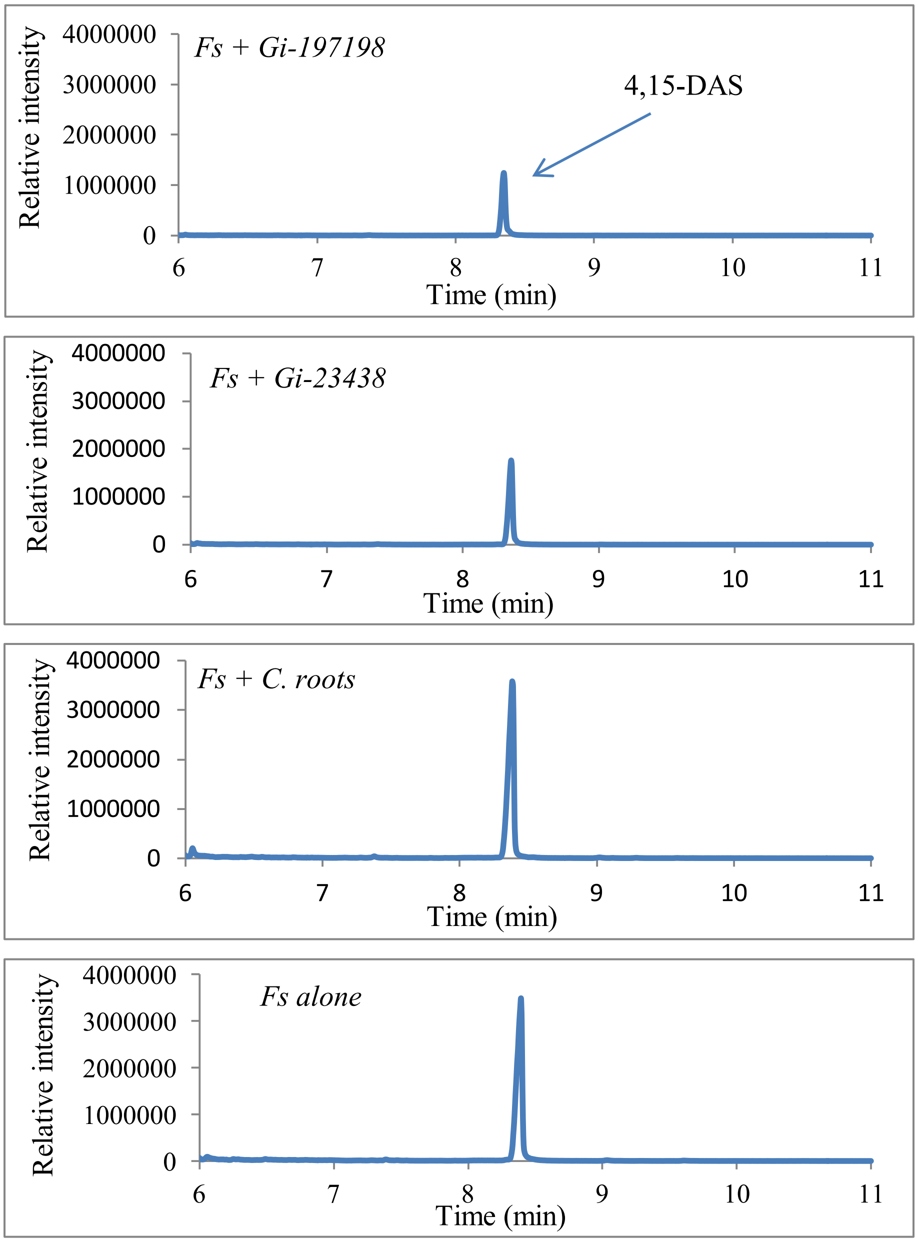

Figure 3.2: GC-MS Reconstructed ion chromatogram of ethyl-acetate extracts showing 4.15-diacetoxyscirpenol (4.15-DAS) patterns by *F. sambucinum* with different treatments; Fs = *F. sambucinum*; Gi = *G. irregulare*; and Cr = Carrot roots. 4, 15-DAS elutes at 9 minutes.

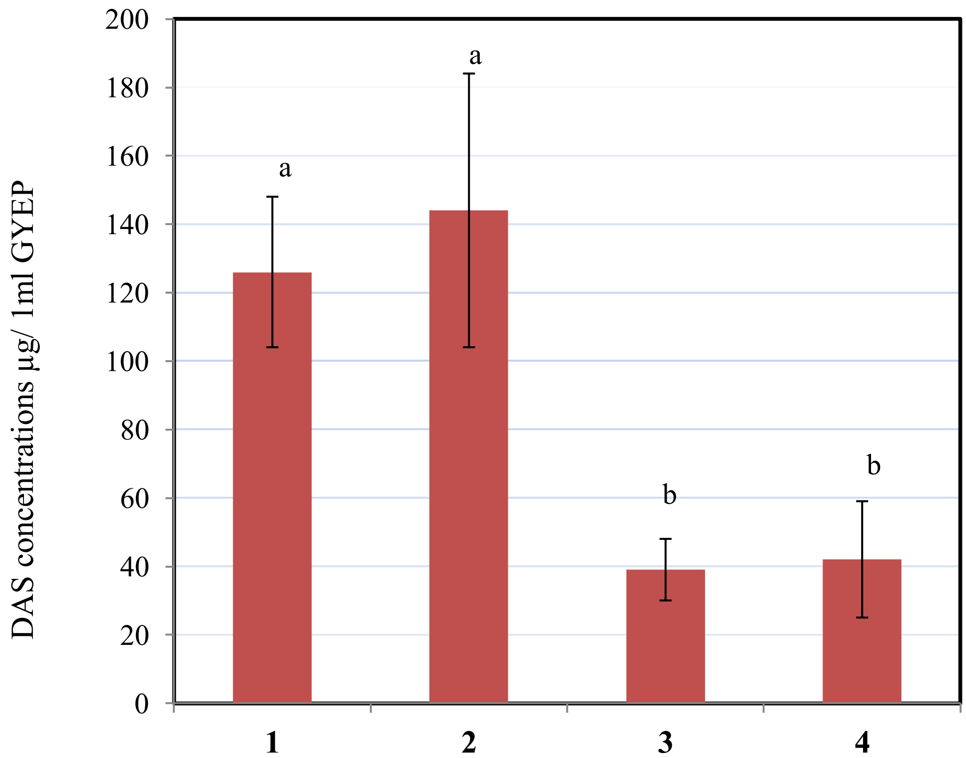

Figure 3.3: GC-MS quantitative profiles of 4, 15-diactoxyscirpenol (DAS) in 1 ml of GYEP medium. Confrontation cultures using an in vitro system showing effect of *G. irregulare* on DAS production by *F. sambucinum*. Treatment combinations consisted of *F. sambucinum* growing alone (1); *F. sambucinum* growing with carrot roots without any *G. irregulare* (2); *F. sambucinum* growing with *G. irregulare* DOAM-917198 (3) and *F. sambucinum* growing with G. irregulare DOAM-234328 (4). DAS of 10 replicates of each treatment combination was extracted in ethyl-acetate. DAS concentrations were detected by GC-MS analysis as showing in (Table 3.1). Treatments with the same letter are not significant ($p < 0.05$).

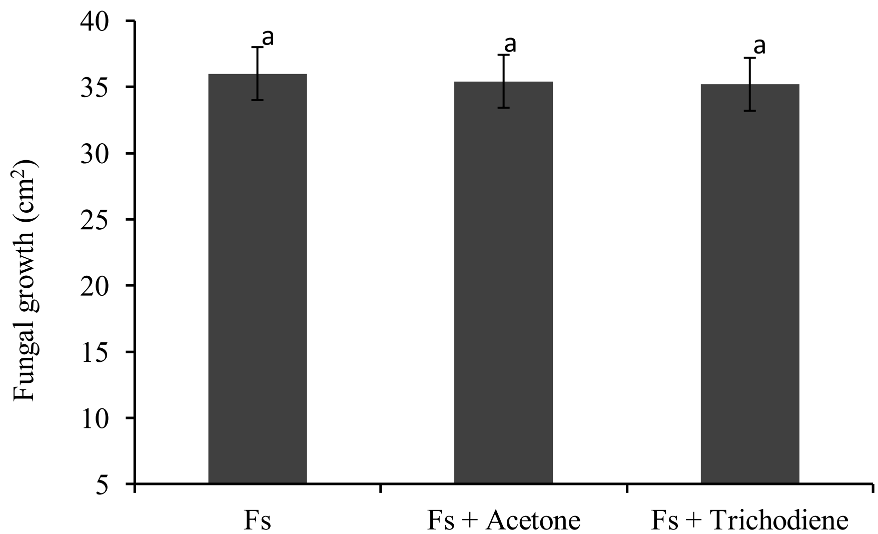

Figure 3.4: Effect of trichodiene on growth of *Fusarium sambucinum*: *F. sambucinum* grown on GYEP agar medium containing trichodiene 2 µg/40ml medium. The fungal growth area was measured after 7 days of inoculation using imageJ software. Two controls consisted of *F. sambucinum* grown either on GYEP agar medium with 4 ml acetone or GYEP alone. Treatments with the same letter are not significant ($p< 0.05$)

4. Arbuscular Mycorrhization with *Glomus irregulare* induces expression of potato PR homologues genes in response to infection by *Fusarium sambucinum*

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4.1. Abstract

Arbuscular Mycorrhizal Fungi (AMF) are symbiotic, root-inhabiting fungi colonizing a wide range of vascular plant species. We previously showed that AMF modulate the expression of mycotoxin genes in *Fusarium sambucinum*. Here, we tested the hypothesis that AMF may induce defense responses in potato to protect against infection with *F. sambucinum*. We analyzed the response of AMF-colonized potato plants to the pathogenic fungus *F. sambucinum* by monitoring the expression of defense-related genes *ChtA3*, *gluB, CEVI16*, *OSM-8e* and *PR-1.* As response to *F. sambucinum* infection, we found that the AMF treatment up-regulated the expression of all defense genes except *OSM-8e* in potato roots at 72 and 120 hours post infection (hpi). However, we found variable transcriptional regulation with *gluB* and *CEVI16* in shoots at both times 72 and 120 hpi in AMF-colonization and infected plants. Overall, differential regulation of defense-related genes in leaf tissues indicates that AMF are a systemic bio-inducer and their effect could extend into non-infected parts. Thus, AMF significantly suppressed disease severity of *F. sambucinum* on potato plants compared with those infected and non-mycorrhizal plants. Furthermore, the AMF treatment decreased the negative effects of *F. sambucinum* on biomass and potato tuber production.

Keywords: Keywords: Potatoes; PR proteins; Gene expression; arbuscular mycorrhizal fungi; *Glomus irregulare*; *Fusarium sambucinum*; mycotoxins; induced resistance; qRT-PCR.

4.2. Introduction

Fusarium sambucinum Fuckel (teleomorph: *Gibberella pulicaris*) is an aggressive fungal pathogen that causes wilting and yellowing resulting in plant death (Ismail et al., 2011) and is the causal agent of tuber sprout rotting and dry rot in potato (Wharton et al., 2006). In the course of the disease, the fungus produces the trichothecene mycotoxin diacetoxyscirpenol (DAS) that plays a role in pathogenesis and is toxic to humans and animals (Desjardins and Hohn, 1997; Ismail et al., 2011). Thus, in addition to the negative economic impacts on the potato production, *F. sambucinum* constitutes a significant health hazard to humans and livestock. Current approaches to control mycotoxin producing *Fusaria* are limited and mainly depend on the use of fungicides to suppress the fungal growth and development. The heavy use of fungicides often leads to contamination of the agro-ecosystems and fungicide-resistant strains, in addition to its hazards to the health of humans and animals. Therefore, alternative control approaches are required to fulfill grower's needs to sustain production systems. During the last years, a great effort has been undertaken to develop alternative and safe biological control methods for plant diseases (Lahlali and Hijri, 2010). Among them, the use of induced resistance to plant pathogens has become an important area of research (Walters et al., 2005). Indeed, various biotic and abiotic stresses can contribute to increased resistance to pathogens, which can be expressed locally or systemically in the plant (Loon, 1997; Walters et al., 2005).

Plant defense responses can be induced by exogenous application of chemicals such as salicylic acid (SA), ethylene and methyl jasmonate (Gaffney et al., 1993; Clarke et al., 2000), as well as by non-pathogenic micro-organisms such as fluorescent *Pseudomonas* (Bakker et al., 2007) and Arbuscular Mycorrhizal Fungi (AMF) (Stein et al., 2008). AMF form symbiosis with roots of a wide range of vascular plant species. They interact with a diverse array of soil bacteria and fungi (Lecomte et al., 2011), providing several benefits to plants such as drought tolerance, access to phosphorous and other essential nutrients. AMF are well-known to promote plant growth and are largely used as commercial inoculants and as bio-fertilizers worldwide. In addition, AMF have been more recently shown to help plants withstand attack by various pathogens and grazers, a concept called Mycorrhiza-

Induced Resistance MIR, reviewed in (Pozo et al., 2009). Although AMF have been repeatedly shown to reduce the incidence and severity of root diseases and protect plants against soil-borne pathogens (Yao et al., 2002; St-Arnaud and Vujanovic, 2007), the exact mechanisms of this beneficial effect, are elusive. This is due in large part to the complex interactions that AMF maintain with the entire soil microbial community. Nonetheless, it is believed that ultimately, AMF are able to impact plant resistance to pathogens by enhancing plant nutrition, competing with root pathogens for colonization, modifying the rhizosphere microbial community and by modulating the expression of genes associated with defense pathways in the host (St-Arnaud and Vujanovic, 2007; Lioussanne et al., 2009). The effect of AMF on expression of defense-related genes is more often apparent at the site of pathogen challenge, where the accumulation of reactive oxygen species (ROS), phytoalexins and hydrolytic enzymes (e.g. chitinases and glucanases) are correlated with enhanced penetration resistance (Pozo et al., 2009).

In addition, the accumulation of pathogenesis-related proteins (PRs) that extend into non-infected plant parts can have a significant effect on defense capabilities throughout the plant (van Loon et al., 2006b; Stein et al., 2008). Indeed, defense-related genes that are activated by AMF are key players in the defense against several root pathogens (Liu et al., 2007). Previous studies have identified a large number of defense-related genes in both compatible and incompatible plant-pathogen interactions (Liu et al., 2007; Lehtonen et al., 2008). However, in potato plants challenged with *Rhizoctonia solani,* 24 induced genes related to cell defense were identified by microarray analysis (Lehtonen et al., 2008). These genes encode chitin-hydrolyzing enzymes such as acidic chitinases of classes II, III, and IV, members of the pathogenesis-related (PR) protein groups (including 1,3-β-glucanase and lignin-catalyzing peroxidases), osmotin-like proteins, defense-associated signaling kinases, host protein protecting substances, and enzymes leading to phytoalexin accumulation (van Loon et al., 2006b; Lehtonen et al., 2008). It has been reported that several genes could be induced after AMF colonization in host plant and are involved in plant defense against pathogens. These genes encode pathogenesis-related proteins such as PR-1a, β-1, 3 glucanase, and PR-10 in tomato, pea and parsley (Haneef Khan, 2010). In tobacco roots, AMF-induced defense genes encode isozymes catalyzing to peroxidation and production of phytoalexins and phenolic compounds such as phenylalanine ammonia lyase (PAL) and peroxidase (Blilou et al., 2000).

The aim of this study was to test whether inoculation of potato with the AMF *Glomus irregulare* isolate DAOM-197198 can activate homologous genes of pathogenesis-related (PR) proteins and affect disease severity following a subsequent infection with the mycotoxin-producing strain *F. sambucinum*. In addition, we explored the hypothesis that AMF root colonization of potato plants affects homologous PR genes throughout the plant, and not just at the site of infection. We performed an experimental trial in growth chambers where mycorrhizal and non-mycorrhizal potato plants were monitored after infection with *F. sambucinum*. Changes in relative expression of defense-related genes were assessed by real-time PCR on tissues isolated from roots and leaves. We chose five genes *ChtA3*, *gluB CEVI16*, *OSM-8e* and *PR-1* because their expressions have been previously characterized as pathogenesis-related proteins (Lehtonen et al., 2008).

4.3. Materials and methods

4.3.1. Fungal strains and growth conditions

Fusarium sambucinum strain T5 was previously isolated naturally infected potato cv. "Riba" collected from farm located in 2420 Rue Principale, Saint-michel, Québec (45° 11'46''N-73°36'20.52''W) (Ismail et al., 2011). The strain was grown and maintained on V-8 juice agar medium and in GYEP medium (2% glucose, 0.1% yeast extract, and 0.1% peptone) (Ismail et al., 2011a). The AMF *Glomus irregulare* suggested new name for *Rhizophagus irregularis* (Schüßler and Walker, 2010) isolate DAOM-197198 was grown *in vitro* in co-culture with RiT-DNA transformed carrot roots (*Daucus carota* L.) on minimal (M) medium. *F. sambucinum* and *G. irregulare* cultures were incubated at 25°C and in the dark.

4.3.2. *In vitro* propagation of potato seedlings and AMF inoculation

Potato seedlings (cultivar Riba) were germinated and maintained *in-vitro* using a technique adapted from (Suttle, 1998). Four weeks-old seedlings were individually transplanted under sterile conditions into 18 cm diameter pots containing a mixture of loamy soil (Montreal Botanical garden), peat-based growing substrate and turface (3/1/1; v/v/v). The soil mixture was sterilized twice by autoclaving at 120°C for 45 min. To avoid contamination, each individual pot was placed inside a plastic bag (Sun-Bag, Fisher Scientific) that allows gas exchange through a 0.22 µm opening filter-membrane. AMF inoculation was achieved using sterile spores of *G. irregulare* produced *in vitro* as follows. Spores were freshly collected from plates by dissolving the Gellan gum as described previously by (Hijri and Sanders, 2004; Hijri et al., 2007), and then washed twice for 1 min by shaking in 0.05% (w/v) aqueous Tween 20. A final spore suspension of approximately 200 spores per ml was made in autoclaved water. Inoculation of seedlings with the AMF *G. irregulare* was achieved by adding 5 ml of spore suspension to soil mixture. Non-mycorrhizal plant controls were made by adding 5 ml autoclaved water to soil mixture. Four weeks after inoculation with AMF, randomized samples were collected from plant roots to check for mycorrhizal colonization using an ink and vinegar root staining method (Vierheilig et al., 1998).

4.3.3. Preparation and inoculation of potato seedlings with *F. sambucinum*

F. sambucinum (strain T5), virulent on potato (Ismail et al., 2011) was used to inoculate oat (*Avena sativa*) seeds as described by (Yao et al., 2002). Four week-old seedlings were infected with *F. sambucinum* by gently pushing the soil at the base of plantlets to expose portions of the root system and five non-infected (mock) or fungus-infected seeds were then placed directly in contact with uncovered roots at five points equidistant from the stem. Each combination of *F. sambucinum* and or *G. irregulare* and control plants was replicated 12 times. Macroscopic disease severity of *F. sambucinum* (wilting or rotting) was recorded weekly. Disease assessment was performed 1, 2, 3 and 4 weeks post-inoculation with *F. sambucinum*. An arbitrary scale (0–5) was designed to assess disease severity where 0 stands for no symptom and 5 stands for plant death.

4.3.4. Selection of homologous genes of potato PR proteins.

Five defense-related genes were selected from *S. tuberosum* EST sequences available in public databases and reported by (Lehtonen et al., 2008). The selected genes are homologous to potato pathogenesis-related (PR) proteins including classII chitinase (*ChtA3*), 1, 3-beta-glucanase (*gluB*), osmotin-like protein (*OSM-8e*), putative peroxidase (*CEVI16*), and pathogenesis-related (*PR-1*) protein precursor. We used β-tubulin, elongation translation factor 1α (*EF1α*), and actin as reference genes in all qRT-PCR assays.

4.3.5. RNA isolation and RT-PCR assays.

For total RNA, 72 and 120 hours post-inoculation (hpi) with *F. sambucinum*, the apical portions from roots and shoots from all seedlings were sampled. Each sample was ground rapidly in liquid nitrogen, and stored at –80°C until use. The frozen material was crushed and ground in liquid nitrogen and total RNA was isolated using TRIZOL® Reagent according to the manufacturer's instructions (Qiagen, Canada). cDNAs were constructed by RevertAid™ 270 H Minus M-MuLV kit (Fermentas) according to the manufacturer's instructions and then the cDNAs were amplified with gene-specific primers designed by

(Lehtonen et al., 2008) and listed in (Table 4.1). The reaction efficiency of each sample was determined according to (Pfaffl et al., 2002) prior to running the qRT-PCR. QPCR reactions were performed in a volume of 10 µl containing 2 µl water, 1 µl of each primer, 1 µl cDNA and 5 µl CYBR green Maxima SYBR Green/ROX qPCR Master Mix. A liquid handling Workstation ep*Motion* 5070 (Eppendorf) was used to optimize qRT-PCR assays with small reaction volumes. All genes were run in triplicate on each plate and 3 biological replicates of each treatment were performed. A negative control using Mili-Q water was prepared for each sample. qRT-PCR was run on EP RealPlex MasterCycler (Eppendorf) using the following conditions: an initial denaturation step at 95°C for 10 min followed by 39 cycles of 95°C for 15 sec, 58°C for 45 sec (annealing and extension). A final extension was carried out by 95°C for 15 sec followed by 58°C for 1 min. A melting curve was performed from 55 to 95°C with a 0.2°C reading interval. We used three house-keeping genes, ß-tubulin elongation factor EF1α and actin, in our RT-PCR assays. All samples had only a single peak, indicating a pure RT-PCR product and no contamination or primer dimer formation. The relative expression levels ΔC_t of gene of interest *ChtA3*, *gluB*, *CEVI16*, *OSM-8e* and *PR1* genes were calculated in relation to □-tubulin, elongation factor EF1□□actin based on. To determine the change in expression level of each gene, we used C_t of untreated (control) defense genes and untreated reference genes from cDNA of AMF-noncolnization healthy plants (no *G. irregulare*, no *F. sambucinum*) as calibrator (control). The treated defense genes and treated reference genes from treatment combinations (-Gi +Fs), (+Gi -Fs) and (+Gi +Fs) were normalized in relations to control (-Gi -Fs). Data analysis was performed on REST 2009 Software available at http://www.gene-quantification.de/rest-2009.htm.

4.3.6. Experimental design and Statistical analysis

Experiments were performed in the growth chamber using a factorial arrangement (1 cultivar) × (1 AMF fungus + control) × (*F. sambucinum*-infected + non-infected). The treatments were arranged in a randomized complete block design with 12 replicates. Each experimental unit consisted of eight plants randomly distributed in each block. Analysis of variance was used to examine the significant difference in disease severity of *F.*

sambucinum and potato growth parameters and yield. The data sets were analyzed by MANOVA using JMP 6 (SAS Institute, Cary, USA). A P value of 0.05 was used as a threshold to accept the significance of effects. Treatment means were compared based on least significant differences (LSD), where significant treatment effects were found. The data was tested for normality using Shapiro-Wilk's test. Analysis of relative expression of the target genes compared to reference genes was performed using the Relative Expression Software Tool version 2009 (REST 2009) for group-wise comparison and statistical analysis as described by Pfaffl et al 2002 (Pfaffl et al., 2002). The relative expression ratio of a target gene is computed, based on its real-time PCR efficiencies (E) and the crossing point (CP) difference (Δ) of an unknown sample versus a control. The efficiencies (E) of qRT-PCR reactions were calculated according to the equation; $E = 10^{-1/slope} - 1$. The relative expression ratio of each target gene was calculated according to the equation;

$$Relative\ expression\ ratio = (E_{target})^{\Delta CP_{target(control-sample)}} / (E_{ref})^{\Delta CP_{ref(control-sample)}}$$

The target (defense–related) gene expression is normalized by non-regulated three reference genes ß-tubulin elongation factor EF1α and actin. We used the hypothesis test *P(H*$_1$*)* that represents the probability of the alternate hypothesis that the difference between sample and control groups is due only to chance. The hypothesis test performs at least 2000 times of random reallocations of samples and controls between the groups. Statistical difference are significant when p<0.05.

4.4. Results:

For all experiments, three-week old cultured potato seedlings were pre-inoculated with *G. irregulare* isolate DAOM-197198. Four weeks later, plants were inoculated with *F. sambucinum*. AMF colonization as well as disease progression was monitored weekly for 4 weeks after *F. sambucinum* inoculation, then plants were harvested and biomass and molecular analyses were performed.

4.4.1. Effect of *G. irregulare* colonization on disease severity and potato biomass production.

We first assessed the impact of the AMF *G. irregulare* on disease severity caused by *F. sambucinum* and plant growth in a growth chamber trial (Fig. 4.1). Disease severity (wilting and yellowing) on potato plants was assessed weekly for a period of 4 weeks post-inoculation with *F. sambucinum,* and was found to be significantly reduced in mycorrhizal plants compared with controls (Fig. 4.2). To explore the role of *G. irregulare* on growth of potato plants, we measured shoot fresh weight, root dry weight, and yield of tubers (Fig. 4.3A, B and C). *G. irregulare* had a marked effect on potato biomass production (root and shoot) compared to control plants. Remarkably, inoculation with *G. irregulare* significantly increased potato biomass and tuber yield compared with infected nonmycorrhizal plants. In contrast, infection with *F. sambucinum* caused a significant decrease in tuber yield, root and shoot weight compared to control plants. Co-inoculation of *G. irregulare* and *F. sambucinum* completely abolished the negative effects of the *F. sambucinum* pathogen on potato biomass production.

4.4.2. Effect of AMF-colonization on the gene expression of *ChtA3*, *gluB*, *CEVI16*, *OSM-8e* and *PR1*.

To test whether *G. irregulare* affects the expression of gene homologous to potato pathogenesis-related (PR) proteins, we carried out real-time PCR assays on genes *ChtA3* (class II chitinase), *gluB* (1, 3-β glucanase), *CEVI16* (peroxidase precursor), *OSM-8e* (osmotin-like protein) and *PR1* (pathogenesis-related protein), after successful root colonization by *G. irregulare*. In root tissues, relative expression of *ChtA3, and CEVI16* were up-regulated ($p < 0.05$) by factors of 7.44 and 4.90 at 72 hpi and 6.92 and 3.23 at 120 hpi respectively (Fig. 4.4 and Table 4.2). At 72 hpi, the expression pattern of *gluB* was down-regulated ($p < 0.05$) by a factor of 0.40 meanwhile, at 120 hpi, expression of *gluB* was not affected ($p > 0.05$) by AMF colonization. Relative expression of OSM-8e was not affected ($p > 0.05$) at neither at 72 nor at 120 hpi; was meanwhile PR1 gene expression only up-regulated by factor 2.08 at 120 hpi (Fig. 4.4A and B). In shoot tissues, relative expression of *ChtA3*, *CEVI16*, OSM-8e and PR1 were up-regulated due AMF colonization at both times 72 and 120 hpi. However, relative expression pattern of gluB was down-regulated at both times by factors of 0.60 and 0.20 respectively (Fig. 4.4C and D). Thus, AMF colonization of potato roots affects the gene expression of several defense-related genes, both at the site of colonization as well as in remote organs such as leaves.

4.4.3. Expression of *ChtA3*, *gluB*, *CEVI16*, *OSM-8e* and *PR1* in roots.

To test whether *G. irregulare* affects the expression of genes homologous to potato PR proteins in the context of *F. sambucinum* infection, we repeated our real-time PCR assays on genes *ChtA3, gluB, CEVI16, OSM-8e* and *PR1*. Interestingly, the first sampling of plant roots, at 72 hpi by *F. sambucinum*, did not show any detectable infection structure or symptoms, yet relative expression of *ChtA3, gluB, CEVI16 and PR1 in roots* was up-regulated ($p<0.05$) by factors of 3.86; 3.11; 3.70 and 4.29 respectively, at this time (Fig. 4.5A and Table 4.2). *T*he relative expression of *OSM-8e* was not significantly affected ($p > 0.05$). In contrast, infection of non-mycorrhizal plants with *F. sambucinum* did not affect relative expression of *ChtA3*, *CEVI16*, *OMS-8e* and *PR1* ($p > 0.05$), and the relative expression of *gluB* was down-regulated ($p<0.029$) by a factor of 0.48. During the second sampling of roots, at 120 hpi, the plants exhibited disease symptoms such as wilting and rotting. Figure 4.5B shows relative expression of defense-related genes at 120 hpi with *F. sambucinum*. In the mycorrhizal plants, relative expression of *ChtA3*, *gluB*, *CEVI16*, *OSM-8e* and *PR1* were all substantially greater ($p < 0.05$) in roots of infected plants, by factors 11.84, 5.17, 4.77, 6.24 and 8.13 fold, respectively. In contrast, in non-mycorrhizal plants infected by *F. sambucinum,* we only observed significant ($p < 0.05$) up-regulation of *ChtA3*, *OSM-8e* and *PR1I,* by factors 7.68, 3.79 and 2.53, respectively, while relative expression levels of *gluB* and *CEVI16* remained unaffected (p> 0.05).

4.4.4. Expression of *ChtA3, gluB, CEVI16, OSM-8e* and *PR1* genes in shoots

To test whether *G. irregulare* has a systemic effect on the expression of defense-associated genes, we assessed gene expression of *ChtA3*, *gluB*, *CEVI16* and *OSM-8e* in potato shoots, which are not directly affected by *F. sambucinum*. Fig. 4.6A and Table 4.2 show that overall, relative levels of *ChtA3*, *gluB*, *CEVI16* and *OSM-8e* are enhanced in shoot of mycorrhizal plants at 72 hpi with *F. sambucinum,* by factors 4.11, 1.79, 1.73 and 2.17, respectively, whereas relative expression of *PR1* was not significantly affected. In shoot of non-mycorrhizal and infected plants, we observed an up-regulation of *ChtA3* (p<0.05) by a factor 2.66, while *gluB* and *CEVI16* genes were down-regulated (p<0.001) by factors 0.23 and 0.35, respectively, and no significant effect was observed on the relative expression of *PR1* (p>0.75). Similarly, we assessed the relative expression of defense-related genes in shoot at 120 hpi. Fig. 4.6B and Table 4.2 show that in mycorrhizal plants, the relative expression of *ChtA3*, *CEVI16*, *OSM-8e* and *PR1* was up-regulated (p<0.05) by factors 8.10; 3.13; 5.38 and 6.22, respectively, whereas the relative expression of *gluB* was down-regulated (p<0.001) by a factor 0.24. On the other hand, in the non-mycorrhizal plants, we observed an up-regulation of *ChtA3* and *OSM-8e* (p<0.05) by factors 3.24 and 4.75 respectively, whereas relative expression of *gluB* was greatly down-regulated by a factor 0.06 (Fig. 4.6B).

Table 4.1: Potato defense-related genes and primers sets used in this study (Lehtonen et al., 2008)

Accession number	Target gene	Forward primer (5'-3')	Reverse primer (5'-3')
BQ118564	1,3-β-glucanase	CACATTGCTTCTGGGATGGA	TTTAACATCAGGCCAGAAATCTTTAA
BQ517484	Class II chitinase	GCAGCTAACTCGTTTCCAGCTT	AAAGGCAGCCATTTCCTTCTT
BQ121967	Peroxidase precursor	TGCCCCTGACCCTTCAATAG	CATCCCCGTTTTGTGGACAT
BQ121995	Basic PR-1 protein	AACCTAGCTGCCGCTTTCC	TCTCATCGACCCACATCTTCAC
BQ515720	Osmotin-like protein	TTGCCAGACCGGTGATTGT	GCTAGGGTGTTTGGCGATTTAC
X55746.1	Actin	GTACGTCGCTATTCAGGCAGTCTT	CAGAATCCAGCACAATACCTGTTG
GO514912	β –tubulin	AAATGTGGGATGCCAAGAAC	TATCGCACACGCTTGACTTC
DQ294264	Elongation factor 1-α	GCCTGGTATGGTCGTCACTT	GGGTCATCTTTGGAGTTGGA

Table 4.2: Relative expression patterns of homologues PR genes *ChtA3, gluB, CEVI16, OSM-8e and PR1* genes from *potato* compared with three reference genes *ß-tubulin*, *actin* and *EF-1α*.

Gene	Treatment†	Time post-inoculation (72 hpi)			Time post-inoculation (120 hpi)		
		Expression	*p* value	Regulation††	Expression	*P* value	Regulation††
Roots							
ChtA3	Gi	7.44	0.001	Up	6.92	0.001	Up
	Fs	1.47	0.282	Not-affected	7.68	0.001	Up
	Gi + Fs	3.86	0.001	Up	11.84	0.001	Up
gluB	Gi	0.40	0.015	Down	0.53	0.062	Not-affected
	Fs	0.48	0.029	Down	0.67	0.169	Not-affected
	Gi + Fs	3.11	0.004	Up	5.17	0.001	Up
CEVI16	Gi	4.90	0.001	Up	3.23	0.001	Up
	Fs	0.57	0.116	Not-affected	1.07	0.806	Not-affected
	Gi + Fs	3.70	0.001	Up	4.77	0.001	Up
OSM-8e	Gi	1.07	0.821	Not-affected	1.21	0.532	Not-affected
	Fs	0.71	0.326	Not-affected	3.79	0.001	Up
	Gi + Fs	1.41	0.370	Not-affected	6.24	0.001	Up
PR1	Gi	0.83	0.539	Not-affected	2.08	0.001	Up
	Fs	0.67	0.169	Not-affected	2.53	0.004	Up
	Gi + Fs	4.29	0.001	Up	8.13	0.001	Up
Leaves							
ChtA3	Gi	8.53	0.001	Up	3.59	0.001	Up
	Fs	3.24	0.006	Up	2.66	0.002	Up
	Gi + Fs	8.10	0.001	Up	4.11	0.002	Up
gluB	Gi	0.60	0.010	Down	0.20	0.004	Down
	Fs	0.24	0.002	Down	0.23	0.001	Down
	Gi + Fs	0.06	0.001	Down	1.79	0.001	Up
CEVI16	Gi	5.32	0.001	Up	1.76	0.018	Up
	Fs	0.99	0.990	Not-affected	1.73	0.031	Up
	Gi + Fs	3.13	0.006	Up	0.35	0.001	Down
OSM-8e	Gi	5.44	0.001	Up	3.87	0.001	Up
	Fs	4.75	0.001	Up	2.16	0.001	Up
	Gi + Fs	5.38	0.001	Up	1.31	0.076	Not-affected
PR1	Gi	2.09	0.002	Up	3.28	0.001	Up
	Fs	0.75	0.370	Not-affected	1.22	0.140	Not-affected
	Gi + Fs	6.22	0.001	Up	0.92	0.757	Not-affected

† Treatment consists of (Fs) *F. sambucinum* alone, and (Gi + Fs) *G. irregulare* DAOM-197198 and *F. sambucinum.*

†† Regulation: (up & down) gene expression in sample group is significant and different in comparison to control group $p < 0.05$, and (not-affected) gene expression in sample group is not different in comparison to control group $p > 0.05$

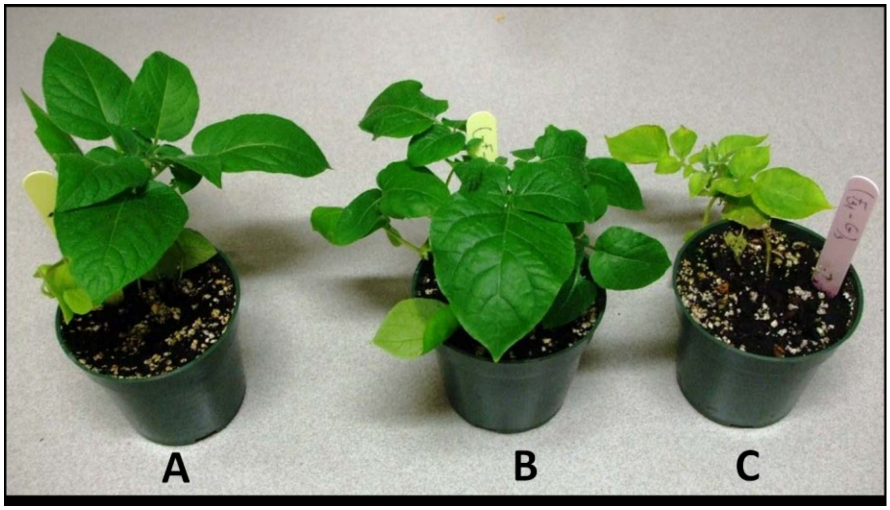

Figure 4.1: Artificial inoculation of potato plants with AMF *G. irregulare* isolates DAOM-197198 and/ or *F. sambucinum* strain T5. (A) Control plants, (B) potato-inoculated with *G. irregulare* DOAM-197198, and (C) potato infected with *F. sambucinum*.

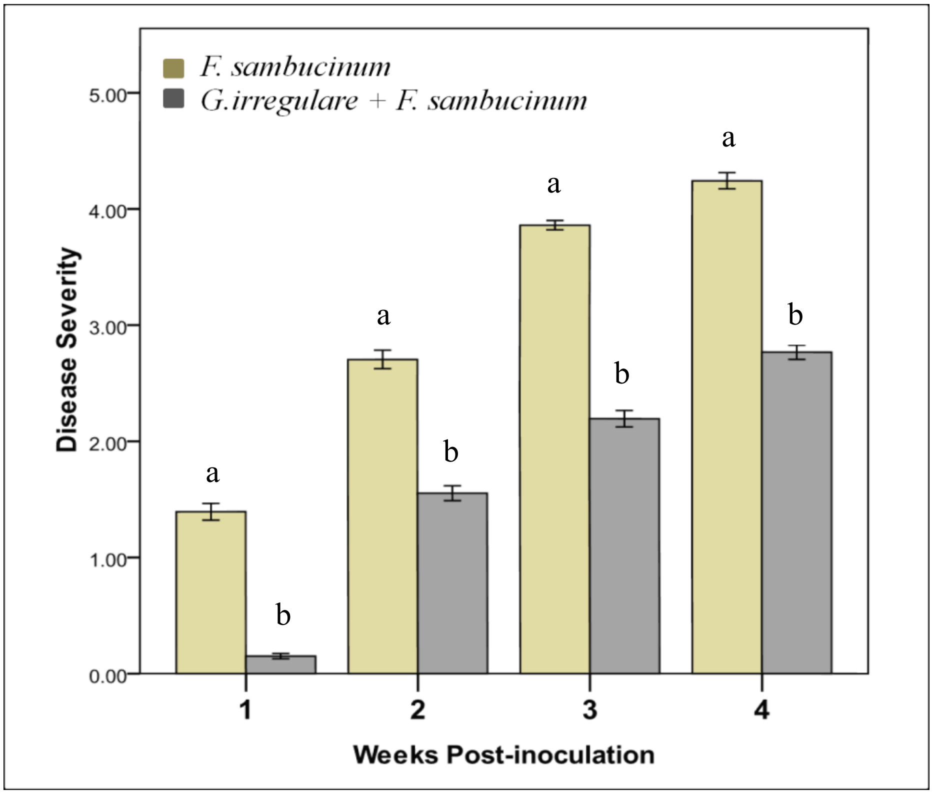

Figure 4.2: Effect of the AMF *G. irregulare* on disease severity caused by *F. sambucinum and* potato growth parameters. Disease severity estimated on plant shoots during 4 weeks following infection with *F. sambucinum.* An arbitrary scale (0–5) was performed to assess disease severity where 0 stands for no symptoms, and 5 stands for plant death. Treatments with different letters are significant ($p < 0.05$).

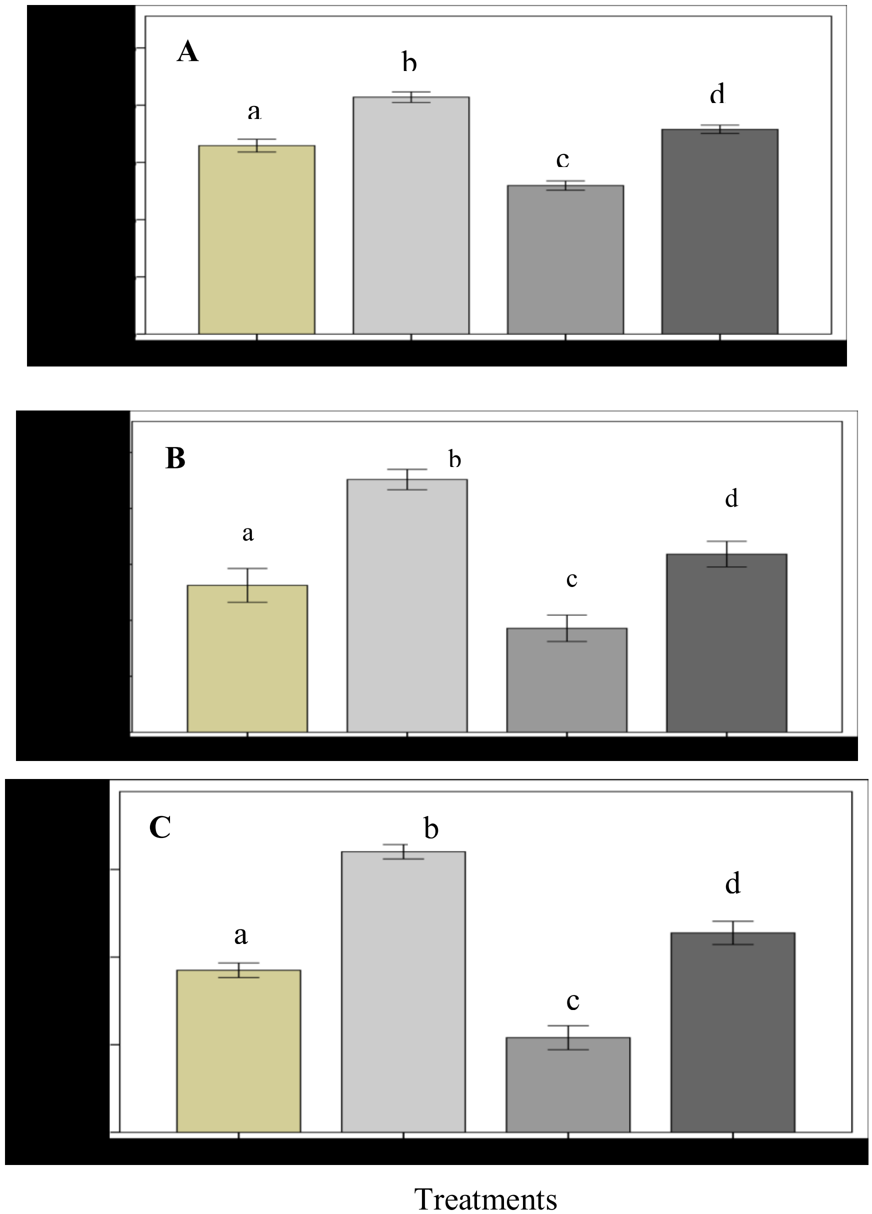

Figure 4.3: Effect of the AMF *G. irregulare* on potato growth parameters. Shoot fresh weight (A), roots dry weight (B) and yield of tubers (C) for AMF-infected (Gi + Fs), AMF-healthy (Gi), non-mycorrhizal infected (Fs) and control plants (Co). Treatments with different letters are significant ($p < 0.05$).

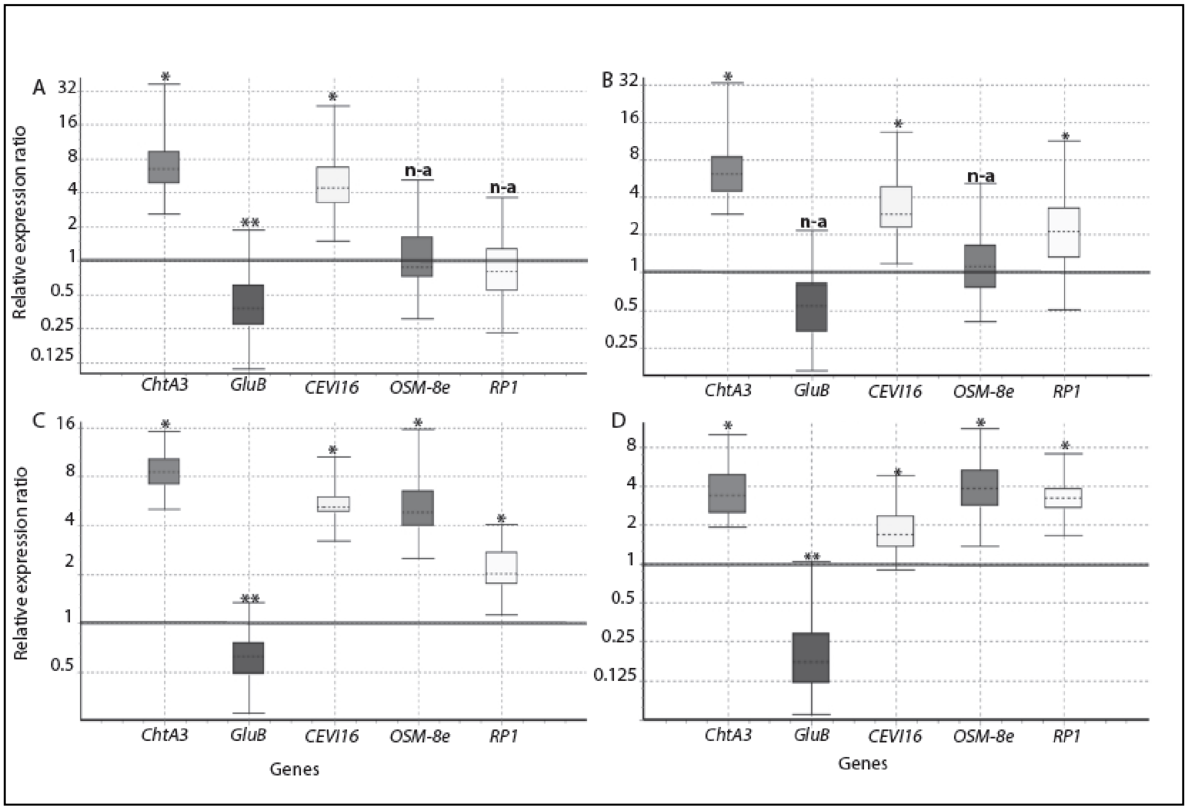

Figure 4.4: Relative expression levels of *ChtA3*, *gluB*, *CEVI16*, *OSM-8e* and *PR1* genes from AMF-inoculated potato roots and shoots compared to control plants AMF-noncolnization. Relative expression patterns of *defense-related* genes of roots of *AMF-inoculated healthy potato* after 72 hpi (A) and 120 hpi (B), and leaves after 72 hpi (C) and 120 hpi (D). Expression level of such defense gene was calculated in relation to reference genes. Changes in expression level of such defense gene was normalized with the same gene in control nonmycorrhizal plants (-Gi). *Up-regulated; **Down-regulated; n-aNot-affected. The bars represent standard error at 95% confidence interval. The significance is reported according to the hypothesis test ($p < 0.05$) as shown in Table (4.2).

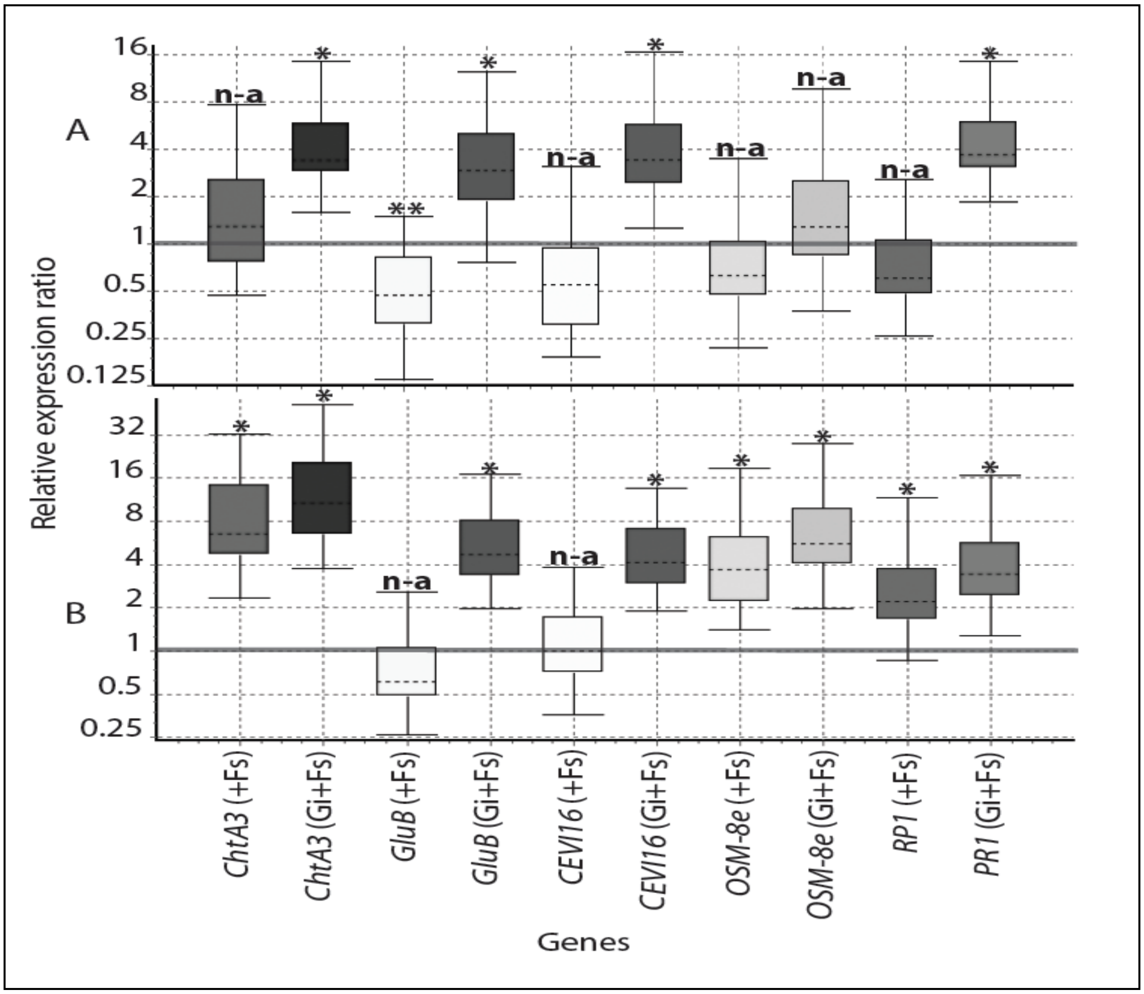

Figure 4.5: Relative expression patterns of *ChtA3*, *gluB*, *CEVI16*, *OSM-8e* and *PR1* genes from potato roots compared to control AMF-non-colonized healthy plants (-Gi –Fs). The expression patterns of *defense-related* genes of non-mycorrhizal and mycorrhizal plants after 72 hpi (A) and 120 hpi (B) with *F. sambucinum* were calculated relative to levels in control plants (non-mycorrhizal and not infected with *F. sambucinum*). RT-PCR was performed using cDNA constructed from roots of control plants, mycorrhizal-plants infected with *F. sambucinum* and non-mycorrhizal-plants infected with *F. sambucinum.* Panel A shows relative expression patterns of *ChtA3, gluB, CEVI16, OSM-8e* and *PR1* 72 hpi with *F. sambucinum*. Panel B shows relative expression patterns of the same genes 120 hpi with *F. sambucinum*. *Gi*, treatment with *G. irregulare*; *Fs*, treatment with *F. sambucinum.* [*]Up-regulated; [**]Down-regulated; [n-a]Not-affected. The bars represent standard error at 95% confidence interval. The significance is reported according to the hypothesis test ($p < 0.05$) as shown in Table (4.2).

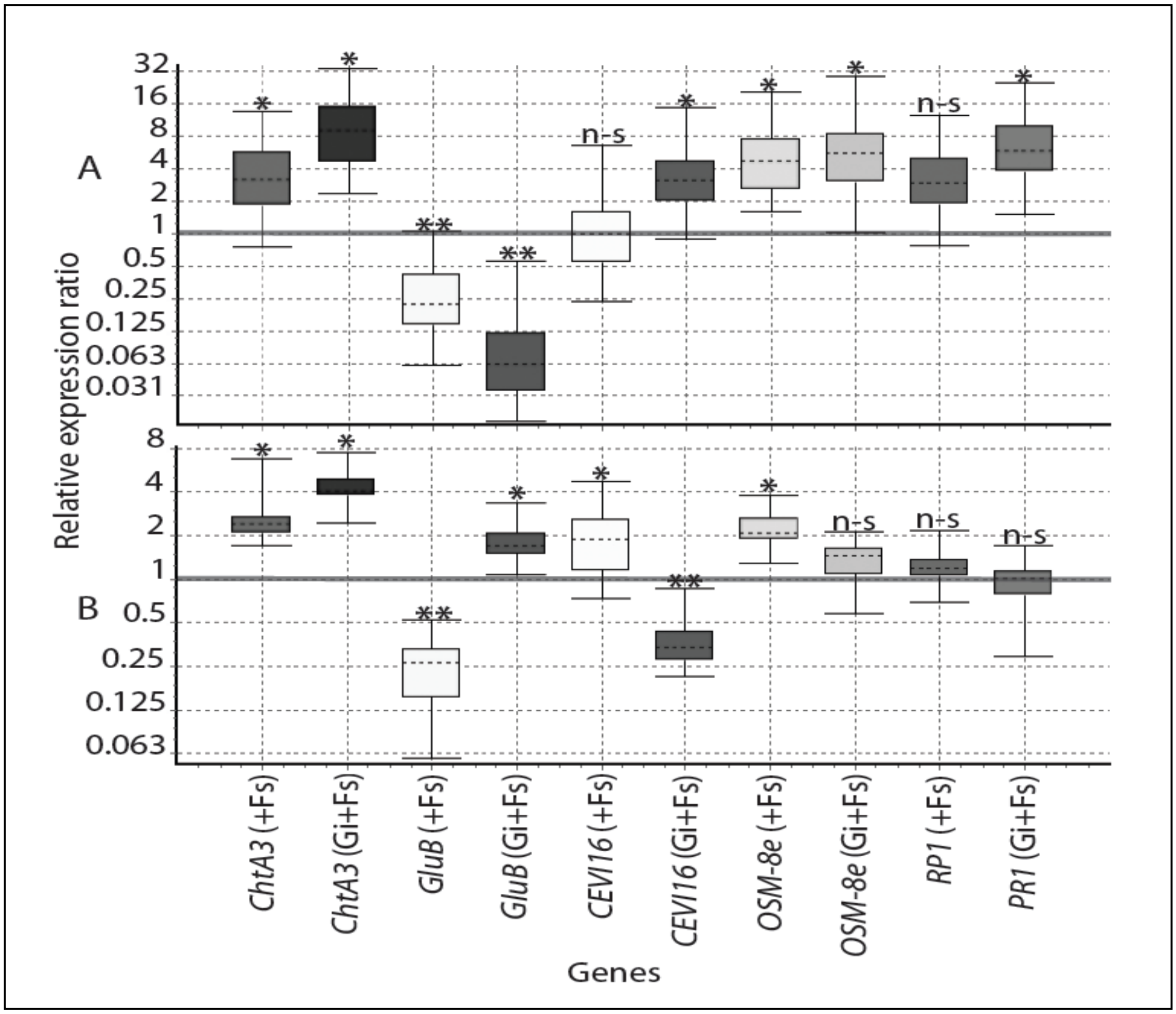

Figure 4.6: Relative expression patterns of *ChtA3*, *gluB*, *CEVI16*, *OSM-8e* and *PR1* genes from potato shoot compared to control AMF-non-colonized healthy plants. The expression patterns of *defense-related* genes of non-mycorrhizal and mycorrhizal plants after 72 hpi (A) and 120 hpi (B) with *F. sambucinum* were calculated relative to levels in control plants (non-mycorrhizal-plants and not infected with *F. sambucinum*). RT PCR was performed using cDNA constructed from shoot of mycorrhizal-plants infected with *F. sambucinum*; non-mycorrhizal-plants infected with *F. sambucinum* and control plants (non-mycorrhizal and not infected with *F. sambucinum*). Panel A shows relative expression patterns of *ChtA3, gluB, CEVI16, OSM-8e* and *PR1* after 72 hpi with *F. sambucinum*. Panel B shows relative expression patterns of *ChtA3, gluB, CEVI16, OSM-8e* and *PR1* after 120 hpi with *F. sambucinum*. *Gi*, treatment with *G. irregulare*; *Fs*, treatment with *F. sambucinum.* [*]Up-regulated; [**]Down-regulated; [n-s]Not-affected. The bars represent standard error at 95% confidence interval. The significance is reported according to the hypothesis test ($p < 0.05$) as shown in Table (4.2).

4.5. Discussion

The results of this study show the effects of the AMF *G. irregulare* isolate DAOM-197198 on potato disease severity caused by *F. sambucinum*, as well as the affects of the expression patterns of homologous genes of PR proteins in potato. It has been reported that *F. sambucinum* produces trichothecene 4, 15-diacetoxyscirpenol (4, 15-DAS) (Ismail et al., 2011), which is toxic to plants and can contribute to pathogenesis of *Fusarium* on some tuber crops (Desjardins and Plattner, 1989; Desjardins et al., 1992). *F. sambucinum* T5 induces wilting and yellowing on leaves of infected potato plant as shown in (Figure 4.1). However, the effect of the AMF *G. irregulare* is observed as a significant reduction in disease severity of the pathogen and a significant promotion of plant growth and yield. Many authors have reported that AMF not only reduce disease severity of several fungal pathogens on potatoes but can also have beneficial effects on potato growth and yield (Yao et al., 2002; Smith et al., 2003). It has been reported that AMF reduced infection of other pathogens such as *P. infestans* on strawberry (Norman et al., 1996), and *R. solani* on mung bean (Kasiamdari et al., 2002). Our results also show that potato shoot fresh weight and root dry weight have been significantly increased in mycorrhizal plants supporting the idea that AMF enhances plant growth (James, 1998). It is well known in the literature that AMF promotes plant growth by increasing mineral uptake in particular phosphorus uptake (Gavito et al., 2003). The promoting effect of AMF on potato tubers was reported by (Niemira et al., 1996), and it was suggested that AMF could affect hormone balance in potatoes leading to increased production of tubers (Yao et al., 2002).

We chose five representative genes homologues to potato pathogenesis-related (PR) proteins, whose role in defense reactions in potato had been previously reported (Lehtonen et al., 2008). These genes encode members of potato PR proteins such as class II chitinase and 1,3-β glucanase, that catalyze the hydrolysis of chitin and 1,3- β -D-glucoside linkages in 1,3- β -D-glucanase of fungal cell wall (Beerhues, 1994). Putative peroxidase (*CEVI16*), displays diverse expression profiles in the plant host and participates in several physiological processes such as lignification, auxin catabolism, wound healing (Hiraga et al., 2001; Kawano, 2003), and the generation of reactive oxygen species (ROS) that play an important role in plant defense mechanisms (Kawano, 2003). *OSM8e* and *PR-1* precursor encode osmotin-like protein and pathogenesis-related (PR-1) proteins, respectively (Ruiz et al., 2005; Van Loon et al., 2006; Lehtonen et al., 2008).

4.5.1. Expression of defense related genes in AMF-colonized healthy potatoes

In this study, real-time PCR assays indicate that inoculation with the AMF *G. irregulare* potentiates systemic induction of chitinase class II and peroxidase encoded by *ChtA3* and *CEVI16* meanwhile, expression of genes encoding osmotin-like protein (OMS-8e) and *PR-1* precursor was not affected after the successful colonization by AMF. Interestingly, AMF induces down-regulation of *gluB* which encodes 1, 3-β glucanase. It has been reported that the pattern of PR proteins accumulation and the expression of defense-related genes varies during roots colonization by AMF (Pozo et al., 2002b; Gao et al., 2004). However, the induction of root chitinase and glucanase isoforms during the AMF symbiosis appears to be a specific response, since differential induction of chitinase and glucanase isoforms after symbiotic or pathogenic fungal interactions has been reported in various plants (Dumas-Gaudot et al., 1994; Pozo et al., 1998b; Pozo et al., 1999). The accumulation of reactive oxygen species (ROS) catalyzed by peroxidase isoforms and the activation of phenylpropanoid metabolism has been reported in AMF root colonization (Garcia-Garrido and Ocampo, 2002; Pozo et al., 2002). Despite the localization of AMF in plant roots, our RT-PCR assays indicate that *G. irregulare* has a systemic effect on defense-related genes, as monitored in potato shoot tissues. *G. irregulare* induced the up-regulation of *ChtA3*, *CEVI16*, *OSM-8e* and *PR-1* genes, and down-regulation of *gluB*. This finding is in agreement with previous studies reporting systemic regulation of defense-related genes in roots and shoots of mycorrhizal plants (Liu et al., 2007).

4.5.2. Expression of defense related genes in AMF-colonized and/or infected potatoes

We performed RT-PCR to assess the relative expression of *ChtA3*, *gluB*, *CEVI16*, *OSM-8e* and *PR-1* in roots and shoot of potatoes at 72 and 120 hpi of infection with *F. sambucinum*. In this study, RT-PCR assays indicate that inoculation with *G. irregulare* changes transcriptional regulation of defense-related genes in roots and shoot of potato plants. At 72 hpi, the roots of potato plants were colonized by *F. sambucinum* but no apparent infection structures or symptoms were observed. Despite the lack of obvious infection symptoms, defense-related genes were induced at 72 hpi, suggesting that there is a delay between pathogen penetration and lesion appearance. During this delay, the timely induction of defense genes is likely critical for the control of later disease development. In general, the regulation of expression of homologous PR genes begins early, prior to development of any visible infection structure on potato plants and the relative expression values of defense-related genes in shoot tissues are higher than those in root tissues after 72 and 120 hpi. Our data support the hypothesis that the early regulation of defense-related genes might be correlated with AMF-root colonization and induction of plant defense against pathogens. This is in agreement with previous findings where inoculation of pea roots with the AMF *G. mosseae* induced over-expression of seven defense-related genes including chitinase-encoding genes (Ruiz-Lozano et al., 1999).

We have found up-regulation of gene expression of *ChtA3* and *gluB* in root tissues of mycorrhizal plants at 72 hpi and 120 hpi with *F. sambucinum*. This finding may explain the reduced disease severity in mycorrhizal plants compared to non-mycorrhizal plants where only *ChtA3* expression was up-regulated at 120 hpi. This shows that the AMF contributes to the induction of homologous genes of PR proteins (Ruiz-Lozano et al., 1999). To test whether AMF induce a systemic effect in mycorrhizal plants, we assessed the relative expression of *ChitA3* and *gluB* genes in shoots. In general, the inoculation with AMF up-regulated *ChitA3* in plant shoots at 72 and 120 hpi with *F. sambucinum*. We found that the expression of *gluB* was down-regulated in shoot tissues of *F. sambucinum* infected-plants at 72 and 120 hpi and it becomes slightly up-regulated in shoots of mycorrhizal plants at 72 hpi with *F. sambucinum*. Chitinases and glucanases are generally induced in plants during invasion by fungal pathogens and by fungal elicitors and their activities are considered a part of a non-specific defense response occurring in plants after pathogen attack or environmental stress (Pozo et al., 1998; Pozo et al., 1999). Chitinases and glucanases could be also secreted by other mycoparasitic fungi such as *Trichoderma* that attack cell walls in other fungal pathogens and were therefore used in the development of biocontrol agents (Chet and Inbar, 1994).

The results of this study also show that AMF could regulate expression of other defense-related genes in potato, such as *CEVI16* that encodes a putative peroxidase, *OSM-8e* that encodes an osmotin-like protein and *PR1* that encodes the pathogenesis-related (*PR-1*) protein and thus promote resistance against *F. sambucinum* infection. By analysis of gene expression with RT-PCR, previous studies revealed similar expression patterns for putative peroxidase, osmotin-like protein and *PR-1* during plant responses to a variety of fungal infection and environmental stresses (Zhu et al., 1995; Lehtonen et al., 2008). Also, plants respond to adverse environmental stress and pathogen attack by osmotin and osmotin-like proteins encoded by *OMS-8e* that have been classified as plant pathogenesis-related (PR) type-5 proteins (Zhu et al., 1995; van Loon et al., 2006), and reported to delay symptom development following infection of potato plants with *P. infestans* (Liu et al., 1994). During this event, most of the host plants show cytological and molecular reactions near the fungal appressoria or around the colonizing hyphae involving phenylpropanoid biosynthesis and pathogenesis-related (PR) proteins including hydrolytic enzymes (Gianinazzi-Pearson et al., 1996). It has been reported that the beneficial microorganisms enhance resistance in plants through priming of the defense mechanisms and no through a direct activation of defense (Van Wees et al., 2008), by preconditioning of plant tissues for quick and more effective defense responses against pathogen invaders (Pozo et al., 2004b; Van Wees et al., 2008).

The impact of AMF on plant pathogenic fungi has been studied under field conditions and in a large number of host-pathogen interactions (St-Arnaud and Vujanovic, 2007a; Lioussanne et al., 2009a; Ismail et al., 2011a). These interactions can be direct, such as a competition with the pathogen, or indirect, such as an alleviation of abiotic stress through enhanced nutrition of the host plant, biochemical changes, and interactions with microorganisms in the rhizosphere. Furthermore, direct effects of AMF on fungal pathogens have been reported *in vitro* where *G. irregulare* suppressed the growth of a virulent and mycotoxin-producing isolate of *F. sambucinum* (Ismail et al., 2011). Most of the direct effects have been a result of AMF interacting with pathogens in the rhizosphere in which complex associations exist among plant roots, soil, and microorganisms (Lecomte et al., 2011), and changes in plant root physiology due to AMF association are certain to have significant impacts on the rhizosphere microflora through alteration of root exudates and other nutrient-related mechanisms (St-Arnaud and Vujanovic, 2007; Lioussanne et al., 2009).

4.6. Conclusion

In this study we conclude that mycorrhization of potato plants with AMF decreased diseases severity of *F. sambucinum* compared with nonmycorrhizal infected plants. AMF *G. irregulare* can improve growth and yield whether potato plants are healthy or infected with *F. sambucinum*. Treatment of *G. irregulare* alters transcription regulation of potato defense gene cluster in response of infection with *F. sambucinum*.

4.7. Acknowledgments

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4.8. Supplementary information

Relative expression of defense-related genes in qRT-PCR assays taking into account a comparison between mycorrhizal plant and mycorrhizal and infected plant

Results

Expression of *ChtA3*, *gluB*, *CEVI16*, *OSM-8e* and *PR1* in potato roots.

In order to test whether *G. irregulare* affects the impact of pathogen infection on the defense-related genes, we performed an additional analysis of qRT-PCR data. In analysis, we compared mycorrhizal plants and/ or infected plants directly with mycorrhizal non-infected plants. We found at 72 hpi in roots, the expression levels of all *ChtA3*, *gluB* and *CEVI16* were down-regulated in treatment combinations of nonmycorrhizal healthy plants (Ctrl), infected nonmycorrhizal (Fs) and mycorrhizal-infected (GiFs) plants, except *ChtA3* was not affected ($p > 0.05$) in infected mycorrhizal plants as shown in (S. Figure 1A) and (Table S.1). By 72, OSM-8e showed up regulation in plant root tissues with all treatment combinations. In the healthy nonmycorrhizal plants (Ctrl) *OSM-8e* showed up regulation by factor of 9.98; and 10.02 in nonmycorrhizal infected plants (Fs) in comparison to mycorrhizal healthy (Gi) plants. The expression level of *OSM-8e* was higher in mycorrhizal-infected plants by factor of 21.49 compared with mycorrhizal healthy (Gi) plants as control (Figure S.1A and Table S.1). However, the relative expression of *PR-1* was not significantly affected ($p > 0.05$) in both treatment combinations Ctrl and Fs. In contrast, the treatment combination of GiFs showed up-regulation of *PR-1* expression by factor of 4.28 ($p < 0.001$). During the second sampling of roots at 120 hpi, the transcriptional regulation of defense-related genes was different in some genes compared to those were quantified at time point 72hpi. In comparison to mycorrhizal healthy (Gi) plants, we found that the treatment combination GiFs significantly upregulated expression of *ChtA3*, *OSM-8e* and *PR-1* by factors of 1.71; 6.97 and 2.03 ($p < 0.05$) respectively (Figure S 1B and Table S1). However, the same treatment combination did not affect expression of *gluB* and *CEVI16* at the same time pint 120 hpi. The infection with *F. sambucinum* led to down-regulation of expression levels of *gluB* and *PR1* by factors of 0.26 and 0.29 ($p<0.001$) respectively, and it only upregulated expression of *OSM-8e* by factor of 1.79 ($p< 0.004$).

Expression of *ChtA3, gluB, CEVI16, OSM-8e* and *PR1* genes in shoots

We calculated expression levels of defense-associated genes in potato shoot taking into account a direct comparison between mycorrhizal (Gi) plants as control (baseline). Figure (S 2A) and Table (S1) show that overall in mycorrhizal infected (GiFs) plants, expression of defense-related genes *ChtA3*, *CEVI16* and *OSM-8e* were up-regulated at 72 hpi by factors of 2.29; 12.05; 8.36 and 4.61 respectively, except the expression of gluB was downregulated by factor of 0.12 ($p< 0,001$). At the same time 72 hpi, the infection by *F. sambucinum* (Fs) only up-regulated expression of *CEVI16* by factor of 3.82 ($P< 0.001$), whereas the same treatment down-regulated expression of *gluB* and *PR-1* by factors of 0.15 and 0.55 ($p< 0.001$) respectively. The expression levels of *ChtA3* and *OSM-8e* were not affected ($p> 0.63$ and $p> 0.12$) in shoots of plant infected with *F. sambucinum* (Figure S. 1 A and Table S1). Similarly, we assessed the relative expression of defense-related genes in shoot at 120 hpi. We found similar transcriptional regulation of defense related genes with GiFs treatment combination in comparison to those at 72hpi in shoots. Figure (S2 B) Table (S1) show that in mycorrhizal infected (GiFs) plants, the relative expression of *ChtA3*, *CEVI16*, *OSM-8e* and *PR1* was up-regulated ($p<0.05$) by factors 3.61; 2.20; 3.00 and 1.96, respectively, whereas the relative expression of *gluB* was down-regulated ($p<0.015$) by a factor 0.51. On the other hand, in the infected (Fs) plants, we only found an up-regulation of *ChtA3* by factor 2.14 ($p< 0,002$) and down-regulation of gluB and *OSM-8e* ($p<0.006$ and $p< 0.001$) by factors 0.52 and 0.16 respectively, whereas relative expression levels of *CEVI16* and *PR-1* were not affected ($p> 0.65$ and $p> 0.12$).

Discussion

In this study, we investigated the hypothesis that AMF can modulate transcriptional regulation of a number of potato defense-related genes in response to infection by a mycotoxin-producing fungus *F. sambucinum*. We also investigated that the transcriptional regulation of defense-related genes occurs, not only locally but also systemically by assessing gene expression levels in both root and shoot tissues through time points of 72 and 120 hours post-inoculation (hpi) with *F. sambucinum*. We studied the kinetic of potato genes involved in plant defense including class II chitinase (*ChtA3*), 1,3-β glucanase (*gluB*), peroxidase (*CEVI16*), osmotin-like protein (*OSM-8e*) (Ruiz et al., 2005; Van Loon et al., 2006; Lehtonen et al., 2008). Many plant genes, including those related to antimicrobial enzymes have been identified as defense-related due to their responses to pathogen infection (Van Loon et al., 2006). Class II chitinase and 1,3-β glucanase have been shown to catalyze the hydrolysis of chitin and 1,3- β -D-glucoside linkages in 1,3- β -D-glucanase of fungal cell wall (Beerhues, 1994). Putative peroxidase displays diverse expression profiles in the plant host and participates in several physiological processes such as lignification, auxin catabolism, wound healing (Hiraga et al., 2001; Kawano, 2003), and the generation of reactive oxygen species (ROS) that play an important role in plant defense mechanisms (Kawano, 2003). In this study, qRT-PCR assays showed varied modulation levels of the defense-related genes upon time post-inoculation with the fungal pathogen and plant tissues. The relative differences in expression of plant defense genes upon infection process have been reported (Ma et al., 2010). In root tissues, we found downregulation of genes encoded enzymes of class II chitinase, 1, 3-β glucanase and peroxidase at 72 hpi in both infected non-mycorrhizal plant and infected mycorrhizal plant compared to mycorrhizal healthy plant. However, the expression of class II chitinase, 1, 3-β glucanase and peroxidase was slightly increased in roots at 120 hpi with the same treatment combinations. On the other hand, the expression level of gene encodes osmotin-like protein showed up-regulation at both 72 and 120 hpi. The down-regulation of *ChtA3*, *gluB* and *CEVI16* levels is due to the direct comparison with mycorrhizal healthy plant wherever the defense genes are upregulated. An additional factor, by which the modulation level of defense-related genes is downregulated in roots, is the effect of infection by *F. sambucinum* on plant defense. The AMF-colonization of plant roots has been shown to induce accumulation of plant defense compounds related to mycorrhization (Gianinazzi-Pearson et al., 1996). Accumulation of reactive oxygen species, activation of phenylpropanoid metabolism and accumulation of specific isoforms of hydrolytic enzymes such as chitinases and glucanases has been reported in mycorrhizal roots (Pozo et al., 1996; Pozo et al., 2009). Although, osmotin-like protein encoded by *OSM-8e* was upregulated in roots of infected and non-mycorrhizal plants, but the expression level of the *OSM-8e* was higher in mycorrhizal-infected plants by 21 folds in root tissues at the early stage of infection with *F. sambucinum*. This finding can be supported by the hypothesis that plants respond to adverse environmental stress and pathogen attack by osmotin and osmotin-like proteins encoded by *OMS-8e* that have been classified as plant pathogenesis-related (PR) type-5 proteins (Zhu et al., 1995; van Loon et al., 2006), and reported to delay symptom development following infection of potato plants with *P. infestans* (Liu et al., 1994).

In the present study, we found that AMF induced transcriptional regulation of *ChtA3*, *CEVI16*, *OSM-8e* and *PR-1* genes in shoot of mycorrhizal-infected plants at 72 and 120 hpi compared to mycorrhizal healthy plants. These results confirm the broad spectrum effect of AMF not only at infection site in roots but also it can extend into arial parts. Liu and co-workers (2007) described a complex pattern of changes in gene expression in roots and shoots associated with mycorrhizal colonization in *Medicago truncatula*. Defense-related genes were among those with altered expression levels, and the authors correlated that finding with increased gene expression in shoots (Liu et al., 2007). In the present study, we found that the treatment with *G. irregulare* decreased the impact of *F. sambucinum* on modulation levels of defense genes. In potato shoot, the infection with *F. sambucinum* to AMF-non-colonized plants only upregulated expression of chitinase classic (*ChtA3*) at 120hpi and peroxidase (*CEVI16*) at 72 hpi, compared to the double inoculation *G. irregulare*/*F. sambucinum* (GiFs).The beneficial organisms including AMF have been shown to protect plants against pathogens by preconditioning of plant tissues for a quick and more effective activation (Conrath et al., 2006; Pozo et al., 2009). Therefore, the beneficial micro-organisms develop the ability of enhancing resistance not through a direct activation of defense, which would be too expensive for the plant in the absence of challenging attackers, but through priming of the defense mechanisms (Pozo et al., 2004; Van Wees et al., 2008). This mechanism known as priming and it seems to be successfully triggered by certain beneficial microorganisms including AMF (Pozo and Azcon-Aguilar, 2007; Pozo et al., 2009). AMF-colonization of plant roots lead to priming of plant defense by accumulating of more PR-1 and basic b -1,3 glucanases mycorrhizal plant rather than non-mycorrhizal plants upon *Phytophthora* infection (Pozo et al., 2002) and chitinase in response to nematode *Meloidogyne incognita* (Li et al., 2006).

Conclusion

AMF symbioses have an important impact on plant interactions with *F. sambucinum*. The association leads to reduction of damaged caused by fungal pathogen by modulation the expression of defense genes including those encode pathogenesis-related (PR) proteins. QRT-PCR assays revealed relative downregulation of class II chitinase (*ChtA3*), 1, 3-β glucanase (*gluB*), peroxidase (*CEVI16*) in roots of infected and AMF/infected plants. However, the AMF treatment was able to upregulate expression of PR-1 in roots of infected plants. In plant shoot, the AMF treatment induced expression of *ChtA3*, *CEVI16*, *OSM-8e* and *PR-1* in response to infection with *F. sambucinum*.

Table (S.1) Expression factors of potato defense-related genes under treatment combination of AMF and/or infection with *F. sambucinum* in comparison with AMF-colonized healthy plants.

Gene	Treatment†	Time post-inoculation (72 hpi)			Time post-inoculation (120 hpi)		
		Expression	*p* value	Regulation††	Expression	*P* value	Regulation††
Roots							
ChtA3	Ctrl	0.15	0.001	DOWN	0.16	0.002	DOWN
	Fs	0.16	0.001	DOWN	0.90	0.601	Not-affected
	GiFs	0.84	0.424	Not-affected	1.71	0.031	UP
gluB	Ctrl	0.13	0.000	DOWN	2.09	0.007	UP
	Fs	0.04	0.000	DOWN	1.00	0.970	Not-affected
	GiFs	0.29	0.000	DOWN	1.40	0.104	Not-affected
CEVI16	Ctrl	0.23	0.001	DOWN	0.34	0.002	DOWN
	Fs	0.02	0.001	DOWN	0.26	0.001	DOWN
	GiFs	0.14	0.000	DOWN	1.23	0.221	Not-affected
OSM-8e	Ctrl	9.98	0.001	UP	2.92	0.001	UP
	Fs	10.02	0.000	UP	1.73	0.001	UP
	GiFs	21.49	0.001	UP	6.97	0.001	UP
PR1	Ctrl	1.40	0.249	Not-affected	0.15	0.001	DOWN
	Fs	0.67	0.068	Not-affected	0.29	0.001	DOWN
	GiFs	4.28	0.001	UP	2.03	0.002	UP
Shoots							
ChtA3	Ctrl	0.42	0.001	DOWN	0.44	0.003	DOWN
	Fs	0.91	0.631	Not-affected	2.41	0.002	UP
	GiFs	2.29	0.001	UP	3.61	0.002	UP
gluB	Ctrl	0.29	0.000	DOWN	0.07	0.002	DOWN
	Fs	0.15	0.001	DOWN	0.52	0.006	DOWN
	GiFs	0.12	0.001	DOWN	0.51	0.015	DOWN
CEVI16	Ctrl	0.68	0.031	DOWN	0.84	0.531	Not-affected
	Fs	3.82	0.000	UP	0.91	0.652	Not-affected
	GiFs	12.05	0.002	UP	2.20	0.003	UP
OSM-8e	Ctrl	0.66	0.094	Not-affected	0.38	0.004	DOWN
	Fs	1.46	0.122	Not-affected	0.16	0.001	DOWN
	GiFs	8.36	0.000	UP	3.00	0.001	UP
PR1	Ctrl	1.73	0.003	UP	0.75	0.193	Not-affected
	Fs	0.55	0.036	DOWN	1.51	0.112	Not-affected
	GiFs	4.61	0.000	UP	1.96	0.007	UP

† Ctrl, AMF-non-colonized/non-infected plant; Fs, *F. sambucinum* infected plants; GiFs, AMF-colonized and F. sambucinum infected plants.

†† Expression ratio was calculated in Ctrl, Fs and GiFs versus AMF-colonized *F. sambucinum* non-infected plants (Gi)

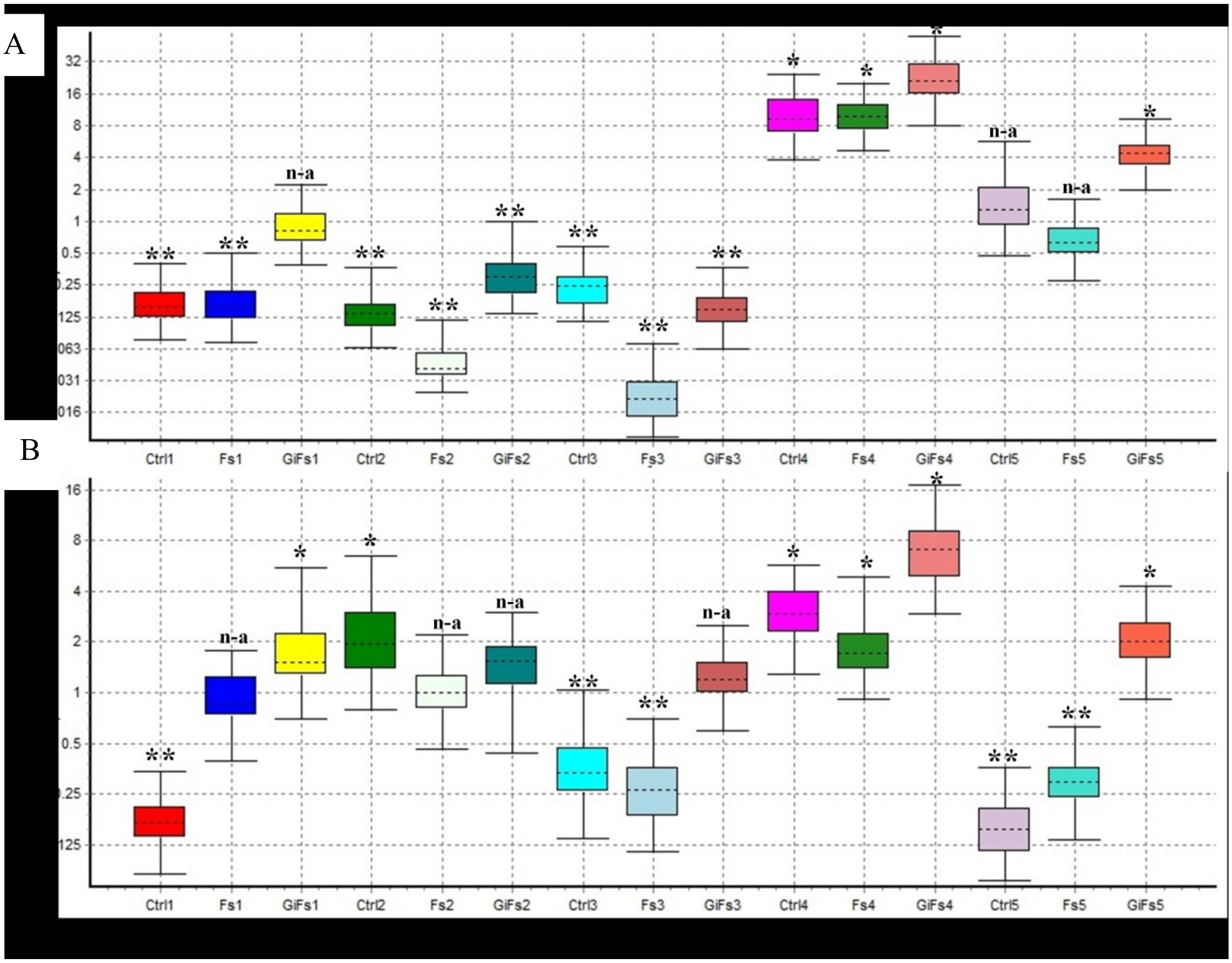

Figure S1: Relative expression patterns of *ChtA3*, *gluB*, *CEVI16*, *OSM-8e* and *PR1* genes from *potato roots* compared to AMF-colonized healthy plants (Gi). The expression patterns of *defense-related* genes of non-mycrrhizal healthy (Ctrl); *F. sambucinum*-infected (Fs) and G. irregulare colonized/infected plants (GiFs) after 72 hpi (A) and 120 hpi (B). Panel A shows relative expression patterns of *ChtA3, gluB, CEVI16, OSM-8e* and *PR1* at 72 hpi with *F. sambucinum*. Panel B shows relative expression patterns of *ChtA3, gluB, CEVI16, OSM-8e* and *PR1* after 120 hpi with *F. sambucinum*. Gi, *G. irregulare*; Ctrl, treatment with (no *G. irregulare*; no *F. sambucinum*) *Fs*, treatment with *F. sambucinum* and GiFs, *G. irregulare*/*F. sambucinum*. The bars represent standard error at 95% confidence interval. The significance is reported according to the hypothesis test ($p < 0.05$) as shown in Table (S.1).

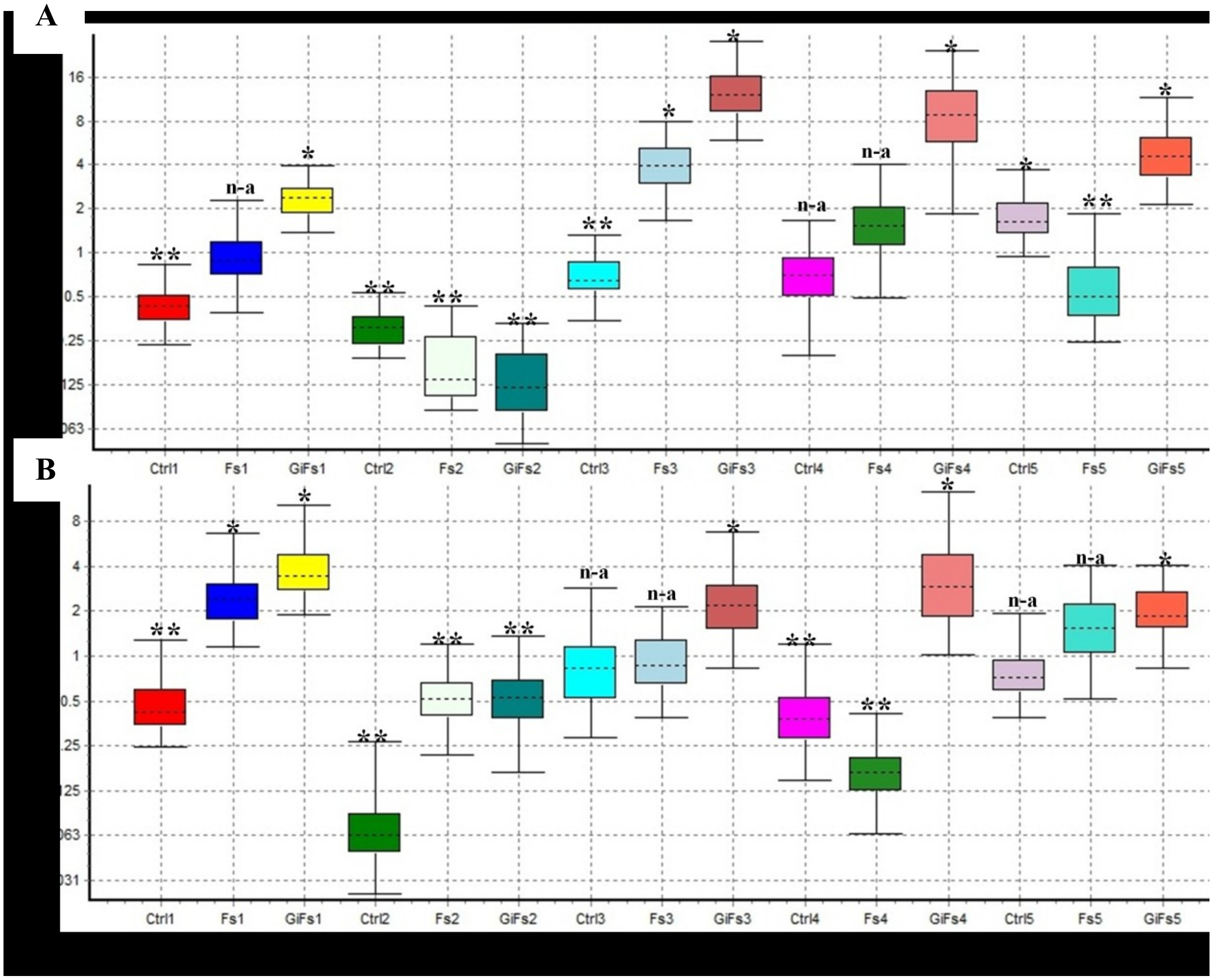

Figure S2: Relative expression patterns of *ChtA3, gluB, CEVI16, OSM-8e* and *PR1* genes from *potato shoots* compared to AMF-colonized healthy plants (Gi). The expression patterns of defense-related genes of non-mycrrhizal healthy (Ctrl); *F. sambucinum*-infected (Fs) and G. irregulare colonized/infected plants (GiFs) after 72 hpi (A) and 120 hpi (B). Panel A shows relative expression patterns of *ChtA3, gluB, CEVI16, OSM-8e* and *PR1* at 72 hpi with *F. sambucinum*. Panel B shows relative expression patterns of *ChtA3, gluB, CEVI16, OSM-8e* and *PR1* after 120 hpi with *F. sambucinum*. Gi, *G, irregulare*; Ctrl, treatment with (no *G. irregulare*; no *F. sambucinum*) *Fs*, treatment with *F. sambucinum* and GiFs, *G. irregulare*/*F. sambucinum*. *Up-regulated; **Down-regulated; n-aNot-affected. The bars represent standard error at 95% confidence interval. The significance is reported according to the hypothesis test ($p < 0.05$) as shown in Table (S.1).

5. Discussion and conclusion

5.1. Characterization of trichothecenes in *F. sambucinum*

This study contributed to the identification of trichothecene toxins and the genes of their biosynthesis pathway in *F. sambucinum*. The fungal strain was isolated from naturally infected potato in Montreal region (Quebec). The pathogen is considered one of the most important diseases of potato, affecting tubers in storage and whole seed or seed pieces after planting (Wharton et al., 2006). As well as having rotted tubers, all diseased tubers had rot. When potato plants were artificially inoculated, the fungus induced a rapid wilting and yellowing that resulted in plant death (Ismail et al., 2011b). This finding supported the idea that *F. sambucinum* is not associated only with causing dry rot of potato during storage, but also is able to infect plants in the field and to induce symptoms on plant roots and shoots (Wharton et al., 2006; Ismail et al., 2011). We showed that *F. sambucinum* strain T5 produces 4, 15-diacetoxyscirpenol (DAS) that is belonging to trichothecenes mycotoxins. Trichothecenes are one of the most important mycotoxins that have an immunosuppressive effect on the health of humans and animals due to its multiple inhibitory effect on eukaryotic cells, including inhibition of protein, DNA and RNA and inhibition of mitochondrial function and cell division (Bennett and Klich, 2003; He et al., 2010). In addition, trichothecenes play an important role in plant pathogenesis in some specific plant-pathogen interactions (McCormick, 2009). In this study, we identified five sequences from genomic DNA of *F. sambucinum* that exhibited high identities to *TRI4, TRI5, TRI3, TRI11* and *TRI101*. These genes encode enzymes that are involved in the trichothecene biosynthesis. It has been reported that the biosynthesis pathway of trichothecenes in *Fusarium* species such as *F. sporotrichioides* and *F. graminearum* involves a series of oxygenation and esterification reactions controlled by up 15 genes, most of which are located in a 25 Kb cluster (Desjardins et al., 1993a; Kimura et al., 2007; Alexander et al., 2009; McCormick, 2009). The GC-MS approach identified 4, 15-Diacetoxyscirpenol (DAS) in culture extracts of *F. sambucinum* strain (T5), that is belong to trichothecene type A and highly toxic to human and animals (Bennett and Klich, 2003), and involved in the pathogenesis in some tuber crops (Ellner, 2002; Ismail et al., 2011b). DAS causes inhibition of protein, DNA and RNA synthesis in eukaryotes (Miller and Ewen, 1997; Rocha et al., 2005), and contributes to pathogenesis and virulence of *F. sambucinum* causing dry rots in a variety of plants such as potato and parsnip (Desjardins et al., 1992; Desjardins et al., 1993a; Desjardins and Hohn, 1997).

5.2. Interactions of the AMF *G. irregulare* with *F. sambucinum* and their impact on expression of trichothecene genes.

We used the confrontation cultures using an *in vitro* system to determine whether AMF affect fungal growth and modulate expression of trichothecene genes in *F. sambucinum*. In this study, the AMF *G. irregulare* significantly suppressed growth of *F. sambucinum* after 3, 7 and 15 days compared with controls. These results indicate the antagonistic effect, particularly competition interactions of AMF with pathogens that have been described by many authors (St-Arnaud et al., 1995; Filion et al., 1999; Wehner et al., 2009). Here, we report direct interactions as a mechanism by which AMF can reduce growth of *F. sambucinum* on growth media. These direct interactions have been proposed as interference competition, including chemical interactions (Wehner et al., 2009). In addition to direct interaction, AMF involve indirect interactions to reduce abundance of pathogenic fungi in roots (St-Arnaud and Vujanovic, 2007b; Wehner et al., 2009). However, both direct and indirect interactions have generally been proposed in response to the effect of AMF on pathogenic microorganisms in roots and soil (Filion et al., 2003; Lioussanne et al., 2009) or on growth medium (St-Arnaud et al., 1995; Ismail et al., 2011). For example, it has been shown that fungal pathogens and AMF accomplish common resources within plant root, including infection sites, space, photosynthate within the root (Whipps, 2004; Wehner et al., 2009), and interference competition may happen due to carbon availability in intercellular spaces and the rhizosphere (Graham, 2001).

Our study demonstrates that AMF change the modulation levels of a number of trichothecene biosynthetic and regulatory genes including *TRI4*, *TRI5*, *TRI6*, *TRI13* and *TRI101* in *F. sambucinum*. QRT-PCR assays showed that expression levels of *TRI5* and *TRI6* were up-regulated in *F. sambucinum* due to confrontation with *G. irregulare*, while

AMF down-regulated TRI4, *TRI13* and *TRI01*. AMF inhibit the activity of two of P450 enzymes required of for DAS biosynthesis in *F. sambucinum*. The results of this study show that both the C-3 and C-4 enzymes encoded by *TRI4* and *TRI13* were sensitive and down-regulated by *G. irregulare*. *TRI4* encodes the C-3 oxygenase (McCormick et al., 2006), controls the conversion of trichodiene to form this toxic product. This suggests that inhibitors that block the *TRI4* enzyme would effectively block the production of a mycotoxin. Alexander and coworkers reported that the expression of *TRI4* was downregulated in *F. sporotrichioides* cultures treated with xanthotoxin (8-methoxypsoralen) (Alexander et al., 2008). The results of this study show that the AMF also down-regulated *TRI101* gene that encodes a trichothecene 3-O-acetyltransferase. The *TRI101* controls the early addition of an acetyl group et C-3 of various *Fusarium* trichothecenes, converting them to less toxic products (Kimura et al., 1998a; McCormick et al., 1999a). This acetyl group remains in place through the remaining oxygenations and esterification (McCormick et al., 1999).

5.3. AMF control trichothecene production *in vitro*.

This study shows that AMF significantly reduced 4, 15-DAS production by *F. sambucinum*. This finding confirms that AMF not only modulate expression of *TRI* genes but also effectively reduce toxin production *in vitro*. This effect of AMF is of high importance for biological control of mycotoxin-producing *Fusarium* in order to alleviate the use of chemicals such as fungicides. Several alternative strategies for improving trichothecene resistance have been reported. Most of these strategies are based on the use of chemicals, plant metabolites, herbicides, trichothecene biosynthesis (McCormick, 2009), or by transforming *TRI101* gene in plants such as tobacco (Muhitch et al., 2000), wheat (Okubara et al., 2002), barely (Manoharan et al., 2006), and rice (Ohsato et al., 2007). However, the mechanisms by which AMF control trichothecene production remains unknown.

5.4. AMF control *F. sambucinum* in potato plants.

We report that the AMF *G. irregulare* reduced the severity of *F. sambucinum* infection by a systemic induction of pathogenesis-related (PR) protein homologue genes in potato plants. This shows that AMF have an indirect effect to control *F. sambucinum* by inducing plant defense mechanisms. We found that AMF impact positively the plant growth and yield and reduce the disease severity of the pathogen. Beneficial effects of AMF on plant growth and health are well known (Yao et al., 2002; Smith, 2009). This study also shows that AMF could systemically regulate expression of potato PR homologues genes including classII chitinase (*ChtA3*) and 1, 3-β glucanase (*gluB*), putative peroxidase (*CEVI16*), OSM8e and PR-1 precursor that encode an osmotin-like protein and pathogenesis-related (*PR-1*) proteins respectively. It has been reported that induction of defense responses and pathogenesis-related (PR) proteins varies during root colonization by AMF (Pozo et al., 2002a; Pozo* et al., 2009), in particular genes encoding chitinase and glucanase isoforms and appears to be a specific response during the AMF symbiosis and pathogenic fungi in various plants (Dumas-Gaudot et al., 1992; Pozo et al., 1996; Pozo et al., 1998). We found that expression patterns of the chitinase class II gene (*ChtA3*) and 1, 3-β-glucanase (*gluB*) were similar during the interactions between *G. irregulare* and *F. sambucinum* in potato roots and shoot. This supports the hypothesis of a co-regulation of chitinase and glucanase genes in plant defense mechanisms. This hypothesis has been also supported by previous studies with systemic activation of 1, 3-β-glucanase that was observed in potato sprout associated with the induction of acidic chitinases belonging to classes 2, 3 and 4 (Lehtonen et al., 2008), and other studies reported that potato plants become more resistant to *P. infestans* with the elevation and co-expression of the enzymatic activity levels of chitinase and 1, 3-β-glucanase (kombrink et al., 1988).

Our finding supports that PR homologues genes are involved in plant defense against *F. sambucinum*. Many beneficial microorganisms have been showed to enhance resistance in plants through priming of the defense mechanisms (Van Wees et al., 2008) or by preconditioning of plant tissues for quick and more effective defense responses against pathogen invaders (Van Wees et al., 2008; Pozo et al., 2009)

5.5. Conclusion

This study introduces an alternative strategy for controlling mycotoxins and for improved resistance against *Fusarium* using arbuscular mycorrhizal fungi (AMF). Many species of the genus *Fusarium* produce trichothecenes, a large group of sesquiterpenes that are found in agricultural products and threaten food safety. They are potent inhibitors of protein synthesis in eukaryotes and contribute to virulence of *Fusarium* on plant crops. This thesis brings new knowledge on molecular plant-microbe interactions and advances our understanding of the role of AMF in controlling soil-borne pathogens. This study is particularly important because it shows that AMF are able to manipulate expression of gene in the mycotoxin biosynthesis pathway in *F. sambucinum.* This funding will greatly contribute in developing biocontrol agents against mycotoxin producing strains. Furthermore, we show clear evidence that AMF play a key role in stimulating plant defense genes and thus reducing the disease severity in potato plants attacked by an aggressive fungal strain such as *F. sambucinum* T5. However, AMF did not prevent the virulence of *F. sambucinum* T5 in potato plants but reduced significantly the disease severity and increased potato yield. This will be greatly translated into securing food stocks worldwide as potato in one of the major crops and its production is expected to overcome grain production in the near future. The innovative finding of this research is the discovery that AMF control the production of mycotoxin in *F. sambucinum* strain T5. These results are novel and outstanding because AMF reduced dramatically the mycotoxin production when *F. sambucinum* is confronted with *G. irregulare in vitro.*

Overall, we can conclude that AMF modulate the expression of mycotoxin genes in particular they down-regulate key genes in the mycotoxin biosynthesis pathway and this is translated as a reduction in the mycotoxin production.

6. Perspective

The toxicity of trichothecenes is determined by the pattern of oxygenation, acetylation and esterification through a complex pathway that consists of 15 genes. In *F. sambucinum*, this pathway produces 4, 15-diactoxyscirpenol (4, 15-DAS) that is very toxic. Research to determine how *F. sambucinum* and other trichothecene-producing *Fusarium* species protect themselves from trichothecene toxicity, identified an acetyl transferase gene (*TRI101*) that controls the addition of a C-3 acetyl group, as a way to detoxify trichothecenes. The acetyl group protects the fungus from its own toxin during trichothecene biosynthesis and can be thought of an off/on switch for toxicity. However, this acetyl group is removed by the *TRI8* eaterase as a final step by which the fungus can release toxin in a host or a medium (e.g. plant – animal – growth media). It has been shown that gene disruption of *TRI101* resulted in the accumulation of isotrichodermol (Figure 1.3), and indicated that the gene also controlled a key step in trichothecene biosynthesis (McCormick et al., 1999b). However, loss of *TRI101* expression by the AMF may induce fungus self-toxicity by blocking acetylation of the C-3 of *F. sambucinum* trichothecene (figure 2.1). This finding shows that AMF may play a potential role to inhibit the function of *TRI101*. It is therefore important in future to understand the genetic and molecular basis and mechanisms by which AMF manipulate the trichothecene-producing fungus to kill itself by disrupting the *TRI101*. In this regard, the *TRI101*$^{-}$ mutants could be used in the confrontation systems with AMF. It is also important to identify and characterize the C-3 deacetylation gene *TRI8* (the gene encoding C-3 esterase) in cDNAs of *F. sambucinum* cultures confronted with AMF. Quantification of *TRI* genes and DAS production by *F. sambucinum* in AMF-colonized plants deserve further study.

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ANNEX

Supplementary information

1. Biodiversity of fungal populations associated with naturally infected potatoes

Youssef Ismail and Mohamed Hijri

1. Introduction

The potato (*Solanum tuberosum*) is so rich in starch that it ranks as the world's fourth most important food crop, after maize, wheat and rice. Potato plants are susceptible to a wide variety of diseases that can severely reduce yield, including the mold *Phytophthora infestans*, the causal agent of late blight of potato, which is remaining one of the most destructive factors of potato production. Potatoes are also subjected to infection by *Rhizoctonia solani* Kühn (teleomorph *Thanatephorus cucumeris* [Frank] Donk) (Anderson, 1982; Gvozdeva et al., 2006; Lehtonen et al., 2008). In *Rhizoctonia solani*, at least 14 different, genetically defined populations of anastomosis groups (AG) determined by anastomosis between hyphae of strains belonging to the same AG (Ogoshi, 1987; Carling et al., 2002; Sharon et al., 2006; Sharon et al., 2008). It has been reported that potatoes are mostly infected with isolates of AG-3 (Anderson, 1982; Lees et al., 2002; Balali et al., 2007). Several species of Fusaria, particularly *Fusarium sambucinum,* Fuckle (teleomorph: *Gibberella pulicaris* (Fries) Sacc.) is known to be important on potato in particular, in North America (Shattock, 2002). It has been reported that several *Fusarium* species produce mycotoxic sesquiterpenoid known as trichothecenes, including diacetoxyscirpenol (DAS), Deoxynivalenol (DON), nivalenol (NIV) and T-2 toxin (Desjardins *et al.*, 1993). These toxins are potent inhibitors of protein synthesis and are a significant agricultural problem due to their adverse affect on human, animal health (Bennett and Klich, 2003)

2. Research objectives:

The main objective of this study is to identify and characterize the diversity of fungal population isolated from naturally infected potato plants. However, this preliminary study provided my PhD project with mycotoxic *Fusarium* strains. In this part we focused on the following:

1) Collection and sampling of naturally infected potatoes from Montreal region.
2) Isolation of fungal genera associated with infected potato
3) Identification of Fungi Using Ribosomal Internal Transcribed Spacer (ITS) DNA Sequences.
4) Evaluation of fungal isolates based on their virulence using artificial inoculation on potato

3. Materials and Methods

3.1.Sampling and isolation.

Samples of Infected potato cv. "Riba" were collected from potato farm located in 2420 Rue Principale, Saint-michel, Québec (45° 11'46''N-73°36'20.52''W). Parts of potato plants shown different symptoms were divided into three groups, roots, leaves and tubers. Each group was taken and gently washed in tap water to remove soil granules. Small parts like 0.5-cm pieces from each group were surface-sterilized with consecutive washes of 70% ethanol (1 min). Potato dextrose agar (PDA) medium with 50 ppm each streptomycin, tetracycline, and penicillin was used for isolating fungi**.** The plates were incubated at 25 °C until development and then all plates purified and single cultures were transferred and maintained on V-8 medium.

3.2.DNA extraction and PCR amplification

DNAs from were isolated freshly harvested spores and hyphae with the Qiagen Plant DNA extraction kit (Qiagen, Canada) following the manufacturer's instructions. Primer sets of ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-CCTCCGCTTATTGATATGC-3′) were used to amplify ITS region in the isolated fungi. PCR was achieved in volume of 50 µL containing: 1 X Taq buffer , 0.25 mM of dNTP, 0.5 µM of each primer, 1U of *Taq* DNA polymerase (Fermentas), and approximately 40 ng of DNA template. PCRs were carried out with a programmable thermal controller (Eppendorf) and were pre-denatured at 94°C for 2 min, followed by 34 cycles of denaturation at 94 °C for 20 sec. annealing at 58 °C for 20 sec, and extension at 72 °C for 1 min, finally, re-extension at 72 °C for 10 min. Negative controls, without DNA template were prepared in each series of amplifications in order to detect possible contaminants in reagents or reaction buffers. PCR amplification products were visualized on an 1% agarose gel run at 100 V for 30 min before being stained with ethidium bromide (EB, 0.5 mg/L) for 20 min. The gel was rinsed in distilled water for 10 min, and was visualized under UV light. PCR product of each fungal isolate was sequenced at Genome Quebec Innovation Center at McGill University, using two sequencing reactions for each sample with ITS1 and ITS4 primers.

Sequences were analyzed using Vector NTI software (Invitrogen) and compared to database using Nucleotide Blast search at (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.3.Rapid assay of virulence using potato slices:

Rapid virulence assays of each of fungi were performed using potato slices technique for each group of fungi consisted of *R. solani* group, *Fusarium* group and other minor fungal groups. However, healthy potato tubers were washed carefully in distilled water and blotted dray on a sterile filter paper. Slices of (7x7x2 cm) were taken from individual tubers. The slices were sterilized in 0.6 % sodium hypochlorite for 10 min., then rinsed twice in sterile distilled water and blotted dry on a sterile filter paper. Three potato slices were placed in 15 cm diameter Petri dish (each plate contains a witted filter paper). A 0.5 cm disc of each fungus was placed on potato slices (in center) with 3 replicates for each fungus. The control slices were received discs of V8 media. The Petri dishes were incubated in the dark at 25 ^{o} C. The progress of each fungus was measured as spreading (cm) and browning (100%) at 0, 24 and 48 hours post inoculation.

3.4.Amplification of trichothecene genes of *Fusarium* species

The degenerated primer sets as shown in (Table 1) were used to amplify *TRI* genes from genomic DNA of *Fusarium* isolates. The PCR reactions were achieved using in volume of 50 µL containing: 1 X Taq buffer , 0.25 mM of dNTP, 0.5 µM of each primer, 1U of *Taq* DNA polymerase (Fermentas), and approximately 50 ng of DNA template. PCRs were carried out with a programmable thermal controller (Eppendorf) and were pre-denatured at 94°C for 1 min, followed by 30 cycles of denaturation at 94 °C for 30 sec. annealing at 51 °C for 30 sec, and extension at 72 °C for 1.5 min, finally, re-extension at 72 °C for 5 min. Negative controls, without DNA template were prepared in each series of amplifications in order to detect possible contaminants in reagents or reaction buffers. PCR amplification products were visualized on an 1% agarose gel run at 100 V for 30 min before being stained with ethidium bromide (EB, 0.5 mg/L) for 20 min. The gel was rinsed in distilled water for 10 min, and was visualized under UV light. PCR product of each fungal isolate was sequenced at Genome Quebec Innovation Center at McGill University.

3.5. Chemical analysis of the trichothecenes:

Mycelia were washed from V8 plates with 3.5 ml water and used to inoculate 50 ml of 1st stage media (GYEP: 3 g NH_4CL, 2 g $MgSO_4.7H2O$, 0.2 g $FeSO_4.7H_2O$, 2 g KH_2PO_4, 2 g peptone, 2 g yeast extract, 2 g malt extract, 20 g glucose in 1 L distilled water) in 250 ml Erlenmeyer flasks. The cultures were grown at 25 °C on a rotary shaker at 200 rpm in the dark. After three days, 1st stage cultures were transferred to a 250 ml Nalgene beaker and dispersed with a stick blender. The macerated culture was transferred to a 50 ml conical tube and centrifuged 5 min at 1600 rpm. Half of the medium was removed and the remaining of fungal mass and medium was mixed well. 1.5 ml of the concentrated 1st stage culture was transferred into 20 ml of 2nd stage media contain (1g $(NH_4)_2HPO_4$, 3g KH_2PO_4, 0.2g $MgSO_4$-$7H_2O$, 5g NaCl, 40g sucrose, 10g glycerol in 1L of distilled water in a 50 ml flask). The second stage cultures were put back on the shaker at 200 rpm at 25 °C in the dark. After 7 days, a 5 ml aliquot (fungal biomass and medium) were extracted with 2 ml ethyl acetate. The extract was dried under a nitrogen stream, re-suspended in ethyl acetate and analyzed with GCMS by Susan McCormick in the US Department of Agriculture.

3.6. Inoculum preparation of fungal strains

All strains were grown on V-8 juice agar plates for 1 week. For preparing the inocula, 50 g of sterile oat kernels in 250-ml Erlenmeyer flasks were received five plugs from each fungus and incubated at 25 °C for 3 weeks according to the method of (Cardoso and Echandi, 1987). The colonized oat kernels were stored at 4 °C until further use. Sterile oat kernels will be used as mock Inoculum for the control treatment.

3.7. Plant materials and AMF inoculation

The medium used in this technique was adapted from, (Suttle, 1998)**.** The procedures of *in vitro* tissue culture of potato plantlets were kindly conducted by Denis Lauzer at the IRBV. Three-week-old potato plantlets were individually rooted and grown in sowing trays (72 cells/tray). Each cell (diameter: 3 cm, volume: 50 ml) was half-filled with low phosphorus peat-based growing substrate. For inoculating the plantlets, 10 ml of spore suspension of *Glomus intraradices* were amended to root systems. Plants were kept in growth chamber under 16 h/day. Three weeks later, the rooted plantlets were individually transplanted with their cell content into 9 - cm-diameter pots containing a mixture of soil, peat-based growing substrate (3/1/1; v/v/v) previously sterilized at 121 °C for 45 min. for 2 times. To visualize the AMF colonization, fresh roots were cleared by boiling 4 min in 10% KOH, rinsed three times with tap water and stained by boiling for 4 min in a 5% ink (Shaeffer; jet-black)/household vinegar (=5% acetic acid) solution (Vierheilig et al., 1998; Vierheilig et al., 2005) After staining, the percentage of root colonization was determined according to the method of (Newman, 1966). Eight-weeks-old potato plants were inoculated with *Fusarium sambucinum*. Soil at the base of potato plantlets was gently pushed aside to expose portions of the roots system. Non-infected (mock) or *Fusarium*-infected oats (5 seeds per pot) were then placed directly in contact with uncovered roots at five points equidistant from the stem. Roots were covered with soil immediately. The symptoms of disease appearing were observed daily on both infected and non-infected plants.

4. Results

4.1. *Rhizoctonia solani* population

Fourteen strains of *Rhizoctonia solani* have been isolated from different parts of naturally infected potato such as roots (isolate codes R), tubers (isolate codes T) and leaves (isolate codes L). All strains were associated with the anastomosis group (AG-3) which is mainly infecting potatoes (Table 1). In this study we have successfully amplified the internal transcribed spacers (ITS) from the DNA of *Rhizoctonia* isolates using ITS1 and ITS4 primers. The PCR fragment sizes ranged between 550 – 700 bp. NCBI blast and database search of given sequences showed genetic variation among *Rhizoctonia* strains. Nine isolates have been identified as *Rhizoctonia solani* L1, L2, R1, R3, R4, R6, R8, R10 and T2 with accession Numbers (Table 1). However, six sequences have been identified for the teleomorph *Thanatephorus cucumeris* isolates R2, R5, R7, R9 and T1 (Table 1).

4.2. *Fusarium* population

Eleven strains of *Fusarium* were identified from roots and tubers of potatoes. Five strains (isolates T3 & T4 from tubers and R11, R12 and R16 from roots) were identified as *Fusarium oxysporum*. However, six sequences have been identified of trichothecenes-producing *Fusarium* include *Gibberella pulicaris* isolate T5 (anamorph: *Fusarium sambucinum*), *Gibberella zeae* (*F. graminearum*) isolate R16 and T6; *Fusarium culmorum* strain R3 and *Fusarium cerealis* isolate R30.

4.3. Virulence of selected fungi

The virulence of 10 isolates was determined *in vitro* on potato slices (6 isolates for *Rhizoctonia solani* & 4 isolates of trichothecene-producing *Fusarium*). The strains from *Rhizoctonia* have been selected from different intraspecific isolates and with high similarity (Table 1). Data mentioned in (Table 3) demonstrate the ability of fungi to infect of potato tissues with using the speeding zone (cm) and browning percentage as parameters for assessing virulence of fungi. *Rhizoctonia solani* isolate R8 was the most virulent strain on potato slices which had the highest spreading area (1.2 & 2.5), and browning percentage (15 and 35 %), at 24 and 48 hour post-inoculation respectively. However, the spreading and browning were well characterized in *Fusarium* strains. The strain T5 (*Fusarium sambucinum*) was the most aggressive on potato slices however, the spreading and browning were (1.8 & 3 cm and 45% & 75 %) at 24 and 48 hpi respectively (Table 3).

4.4. Molecular and chemical characterization of *Fusarium* Trichothecenes

The trichothecene gene cluster has been amplified by degenerate primers for amplification of *TRI1*, *TRI3*, *TRI4*, *TRI5*, *TRI11*, *TRI101 Fusarium* strains include; *Fusarium sambucinum* (T5), *F. graminearum* (R16), *F culmorum* (R3) and *F.cerealis* (R30). The amplified *TRI* genes gave PCR products of expected size 850 -1350 bp (Figure 1). However, the detection of the trichothecene genes by PCR isn't associated with mycotoxin productions for a given strain. Some strains have the biosynthetic genes necessary for mycotoxin production but they may not be activated for mycotoxin production. Thus, chemical analyses are the accurate approach to investigate trichothecene production. GC-MS analysis of culture extracts revealed different trichothecene chemo types produced by *Fusarium* strains. Figure (2) shows different trichothecene chemo types produced by several *Fusarium* isolates. 4, 15-deoxyscirpenol (4.15-DAS) chemo type was associated only with the extracts in *Fusarium sambucinum* isolate T5 (retention time = 16 min) and no traces of diacetoxyscirpenol was found in the other strains (Fig. 2A). Deoxynivalenol (DON) and 15-deoxynivalenol (15ADON) were formed by *F graminearum* isolate R16 and *F. cerealis* isolate R30 (Fig 2 B&D). However, 3 trichothecene chemo types were detected of *Fusarium culmorum* isolate R3 culmorine (retention time = 8 min), deoxynivalenol (DON) and 3-acetyldoxynivalenol (3-ADON) (retention time = 15 and 16 min respectively)

Table 1: NCBI search and sequence analyses of PCR products of *Rhizoctonia solani* isolates.

No	Isolate Code	Description	Anastomosis group	Accession No. in GenBank	Max identity
1	L1	*Rhizoctonia solani* isolate RT	AG-3	FJ746964	99%
2	L2	*Rhizoctonia solani* isolate RT1	AG-3	FJ746966	99%
3	R1	*Rhizoctonia solani* isolate RT1	AG-3	FJ746966	99 %
4	R2	*Thanatephorus cucumeris*	AG-3	AY387559	99 %
5	R3	*Rhizoctonia solani* isolate RT	AG-3	FJ746964	99 %
6	R4	*Rhizoctonia solani*, isolate: ST3-1.	AG-3	AB000041	99 %
7	R5	*Thanatephorus cucumeris* isolate Scl-24	AG-3	AB019019	100 %
8	R6	*Rhizoctonia solani* isolate RT 23-2	AG-3	FJ746965	100 %
9	R7	*Thanatephorus cucumeris* isolate T31	AG-3	AY387528	98 %
10	R8	*Rhizoctonia solani* isolate RT	AG-3	FJ746964	97 %
11	R9	*Thanatephorus cucumeris* isolate T102	AG-3	AY387569	98 %
12	R10	*Rhizoctonia solani isolate* RT	AG3	FJ746964	100 %
13	T1	*Thanatephorus cucumeris* isolate PWK-4	AG-3	FJ515892	96 %
14	T2	*Rhizoctonia solani* isolate RT	AG-3	FJ746964	99 %

L; strains isolated from leaves; (R) roots; (T) tubers

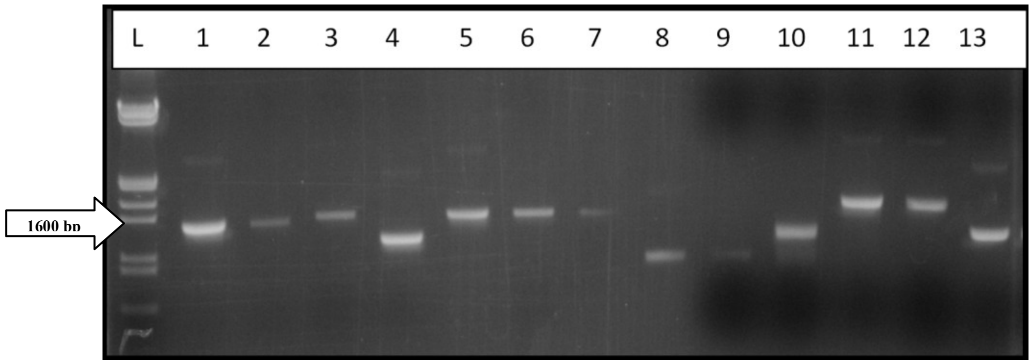
Table 2. NCBI search and sequence analyses of PCR products of *Fusarium* isolates.

No	Isolate code	Description	Accession No.	Identity
1	T3	*Fusarium oxysporum*	FN397202	100 %
2	T4	*Fusarium oxysporum*	GQ365156	100 %
3	T5	*Gibberella pulicaris* (anamorph: *Fusarium sambucinum*)	EU214565	99 %
4	T6	*Gibberella zeae*	GQ221859	99 %
5	R11	*Fusarium oxysporum f. sp. niveum*	EU588396	100 %
6	R12	*Fusarium oxysporum f. sp. lycopersici*	DQ452454	99 %
7	R3	*Fusarium culmorum*	DQ459870	99 %
8	R14	*Fusarium lunulosporum*	DQ459868	97 %
9	R30	*Fusarium cerealis*	EU214569	99 %
10	RR16	*Fusarium oxysporum*	FJ654694	100 %
11	R16	*Gibberella* (anamorph: *Fusarium graminearum*)	GQ221859	100 %
12	R18	*Pythium ultimum var. ultimum*	FJ415980	98 %
13	R19	*Sclerotinia sclerotiorum*	AF455526	99 %
14	R20	*Diaporthe* sp.	EU311609	99 %
15	R21	*Phomopsis* sp.	EF589868	98 %

L; strains isolated from leaves; (R) roots; (T) tubers

Table 3. Virulence assay of fungal strains on potato slices

Isolate code	Description	Lesion spreading (cm)			Lesion Browning (%)		
		0 H	24 H	48H	0H	24 H	48 H
R3	*Rhizoctonia solani*	0.5	0.8	1.2	0.0	10	15.0
R5	*Rhizoctonia solani*	0.5	0.8	0.9	0.0	8.0	12.0
R7	*Thanatephorus cucumeris*	0.5	0.6	0.9	0.0	10.0	20.0
R8	*Rhizoctonia solani*	0.5	1.2	2.5	0.0	15.0	35.0
R9	*Thanatephorus cucumeris*	0.5	0.7	0.9	0.0	10.0	15.0
T1	*Thanatephorus cucumeris*	0.5	1.0	2.0	0.0	15.0	25.0
R30	*Fusarium cerealis*	0.5	0.8	1.2	0.0	20.0	30.0
R3	*Fusarium culmorum* strain	0.5	1.0	2.2	0.0	30.0	45.0
R16	*Gibberella zeae* strain (anamorph: *Fusarium graminearum*)	0.5	1.0	1.5	0.0	15.0	25.0
T5	*Gibberella pulicaris* (anamorph: *Fusarium sambucinum*)	0.5	1.8	3.0	0.0	45.0	75.0



B

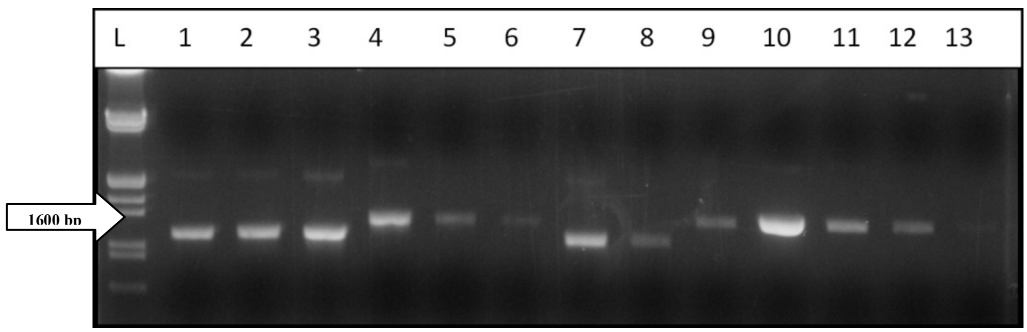


Figure (1) PCR amplification of *TRI* genes of 4 *Fusarium* species. Agarose gel electrophoresis showing PCR products of *TRI* from four *Fusarium* strains. *F. sambucinum* (T5), *F. culmorum* (R3), *F. graminearum* (R16), and *F. cerealis* (R30) isolated from naturally infected potato. Panel A: Ladder λ (L); *TRI1*-R3, *TRI1*-R16 and *TRI1*-T5 (lanes 1–3); *TRI3*-T5 (Lane 4); *TRI4*-R3, *TRI4*-R16 and *TRI4*-T5 (lanes 5-7); *TRI5*-R3 and *TRI5*-R16 (lanes 8 &9); *TRI11*-R3 and *TRI11*-T5 (lanes 10 &11); *TRI101*-R3 (lanes 12). Panel B: Ladder λ (L); *TRI101*-R16, *TRI101*-R30 and *TRI101*-T5 (lanes 1-3); *TRI3*-R3, *TRI3*-R16 and *TRI3*-R30 (lanes 4-6); *TRI5*-T5 and *TRI5*-R16 (lanes 7&8) and *TRI101*-T5 (lanes 11-13)

2. Quantitative analysis of 4,15-Diactoxuscirpenol (DAS) in ethyl-acetate extracts of *Fusarium sambucinum*

A. Growing *F. sambucinum* on 12 plates of GYEP agar

1. Prepare 500 ml of GYEP (25 g sucrose; 0.5 g Yeast extract; 0.5 Peptone; 10 agar) and autoclave for 20 min.
2. Fill 40 ml of GYEP agar into each plate with pipette under hotter conditions
3. Transferred 0.5 disc from pure culture of *F. sambucinum* into each half
4. Incubate plates in the dark on 26 C for 7 days. The fungus grows in each half

B. DAS Extraction

1. Prepare small cuts of F. sambucinum and GYEP agar of each half using knife and transfer them into a glass baker 250 ml
2. Add 100 Ethyl Acetate into each baker and seal with aluminum paper
3. Shake bakers on 150 rpm for 30 min.
4. Gently transfer liquid (Ethyl-acetate DAS)into a new 250 ml baker
5. Dry Ethyl-acetate under air dryer or leave baker over-night
6. Rinse DAS with 1 ml of Ethyl-Acetate and transfer liquid into big vials with glass pipette and repeat it 3 times and Vortex vials for 10 sec.
7. Dry Vial under Nitrogen Stream
8. Re-suspend DAS with 1 m of Ethyl-acetate by 1 ml syringe and vortex for 10 seconds
9. Gently transfer liquid into small vials and lid them with caps

10. Determine DAS and note both peak height and area correlation on GC-MS in each vial.

C. Creation standard curve of DAS

Prepare serial concentrations of DAS in 100; 200; 300; 400; 600; 800; 1000 µl in s vials 2ml and then determine both Peak height and Area correlation for each concentration on GC-MS as follow.

1. Calculating factors

Equation on excel =correl(DAS serials100:1000, DAS Pk height100:1000)enter

Equation on excel =correl(DAS serial100:1000, DAS area corr.100:1000)enter

2. Calculation of DAS (µg) in a vial:

Equation on excel =Forecast(DAS area corr, DAS serials 100:1000, DAS area corr100:1000)entre

3. Calculation of DAS (µg) in 1m a plate:

Equation on excel =DAS(µg) in vial/20enter

Table (1) GCMS of 4, 15-DAS readings (Peak height and Area counts) of ethyl-acetate extracts of *F. sambucinum* grown on GYEP agar medium

GCMS traces of 4,15-DAS

DAS standard curve	Pk height	DAS conc. (ug/ml)	Area Count	DAS conc. (ug/ml)	Pk height	Area Count	ug/ml in vial	ug/ml in plate
100	351858	100	8165470	Fs Plate 1A.D	4051534	114501930	1256.51	94.26
200	769272	200	17835731	Fs Plate 1B.D	2978357	80491330	885.44	66.42
400	1536411	400	36641508	Fs Plate 2A.D	4726716	152032579	1665.98	124.98
600	2212314	600	54056819	Fs Plate 2B.D	3520762	96936308	1064.86	79.88
800	2724819	800	72145383	Fs Plate 3A.D	3660945	108599279	1192.11	89.43
1000	3234170	1000	91295616	Fs Plate 3B.D	2880865	77699742	854.99	64.14
				Fs Plate 4A	3037387	83089992	913.79	68.55
				Fs Plate 4B	3532244	93433764	1026.65	77.01
				Fs Plate 5A	3231205	91756012	1008.34	75.64
				Fs Plate 5B	2979624	81299069	894.26	67.08
				Fs Plate 6A	3028578	80784651	888.64	66.66
				Fs Plate 6B	3126149	83974487	923.44	69.27

Das standard curve was made up in 1mL ethyl-acetate
Fs = *F. sambucinum* grown on GYEP agar medium

Table (2) GCMS of 4, 15-DAS readings of ethyl-acetate extracts of *F. sambucinum* confronted with *Glomus irregulare*.

DAS standard curve	Area counts	sample	ug/plate	ug/ml	sample	ug/plate	ug/ml
100	4163845	Fs + Gi1	568	28	Fs + M	2183	109
200	8583673	Fs + Gi1	442	22	Fs +M	3183	159
400	17971363	Fs + Gi1	893	45	Fs + M	2170	109
600	28102627	Fs + Gi1	1042	52	Fs + M	2661	133
800	36862159	Fs + Gi1	689	34	Fs + M	4434	222
900	40502991	Fs + Gi1	696	35	Fs + M	1896	95
1000	46193745	Fs + Gi1	878	44	Fs + M	2512	126
		Fs + Gi1	701	35	Fs + M	3708	125
		Fs + Gi1	1062	53	Fs + M	3509	175
		Fs + Gi1	754	38	Fs + M	2536	127
		Fs + C root	2215	111	Fs + Gi2	315	16
		Fs + C root	2080	104	Fs + Gi2	853	43
		Fs + C root	2575	129	Fs + Gi2	804	40
		Fs + C root	2987	149	Fs + Gi2	1005	50
		Fs + C root	3186	159	Fs + Gi2	753	38
		Fs + C root	2552	128	Fs + Gi2	975	49
		Fs + C root	2551	128	Fs + Gi2	1293	65
		Fs + C root	2662	133	Fs + Gi2	1404	70
		Fs + C root	1682	84	Fs + Gi2	478	24
		Fs + C root	2633	132	Fs + Gi2	517	26

Das standard curve was made up in 1.5 ml ethyl-acetate
Fs + Gi1 = *F. sambucinum* confronted with *G. irregulare* isolate DOAM-197189
Fs + Gi2 = *F. sambucinum* confronted with *G. irregulare* isolate DOAM-234328
Fs + C root = *F. sambucinum* confronted with carrot roots without *G. irregulare* (control)
Fs + M = *F. sambucinum* alone

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