THE CONTINUING BATTLE BETWEEN WHEAT AND *FUSARIUM GRAMINEARUM*: UNDERSTANDING THE MOLECULAR PHYLOGENETIC RELATIONSHIPS, CHEMOTYPE DIVERSITY AND TRICHOTHECENE BIOSYNTHESIS GENE EXPRESSION PATTERNS

By

CHAMI CHATHURANGI AMARASINGHE

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

In Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Department of Plant Science

University of Manitoba

Winnipeg, Manitoba

Canada

ABSTRACT

Fusarium head blight (FHB) continues to threaten the economic sustainability of wheat and barley production in Canada and worldwide. The overall goal of this thesis is to expand our current knowledge of the FHB pathogen, *Fusarium graminearum* and its trichothecene chemotype diversity. Continuous monitoring of trichothecene chemotypes may well inform on the potential risk and the type of *Fusarium* populations present in a given region. *Fusarium* populations in Winnipeg and Carman, Manitoba were examined using chemotype as a marker in the field. Rapid expansion of the 3-acetyldeoxynivalenol (3-ADON) chemotype was observed in Winnipeg and Carman. 3-ADON chemotype is consistently found at high frequencies over the previously common 15-acetyldeoxynivalenol (15-ADON) chemotype, suggesting that the shift in pathogen populations is continuing. This study provides the first evidence on the presence of nivalenol (NIV) producing *F. cerealis* strains in winter wheat in Manitoba, Canada. Therefore, discovery of NIV producing *F. cerealis* in wheat poses a serious concern for the wheat industry in Canada. Phylogenetic, chemotypic, phenotypic, and pathogenic abilities of 150 strains of *F. graminearum* species complex (FGSC) from eight countries were investigated. Type and amount of trichothecenes produced by a strain are key factors in determining the level of aggressiveness of that strain regardless of its species origin. The sequence variations of *TRI8* gene in different species in the FGSC were examined as *Fusarium* species may produce different types of trichothecenes depending on differences in the core trichothecene (*TRI*) cluster genes. The *TRI8* haplotypes did group according to chemotype rather than by species, indicating that 3-ADON, 15-ADON and NIV chemotypes have a single evolutionary origin. Comparison of *TRI* gene expression demonstrated that accumulation of *TRI* transcripts was higher in 3-ADON producing strains compared to 15-ADON and NIV strains. The presence of masked mycotoxins

deoxynivalenol-3-glucoside (D3G) in food and feed is an increasing concern. Canadian spring wheat cultivars inoculated with different chemotypes produce D3G upon *Fusarium* infection and moderately resistant/intermediate cultivars showed higher D3G/DON ratio compared to susceptible cultivars.

ACKNOWLEDGEMENTS

I gratefully acknowledge my supervisor Dr. Dilantha Fernando for his valuable guidance, mentorship, and advice. His strong support and continuous encouragement motivated me throughout my study leading to successful completion of my Ph. D. project. Also I would like to thank Dr. Fernando, for accepting me for the *Fusarium* project in his lab. His advice, own experience and guidance helped me to get through so many difficult situations. I was fortunate to have an advisor like you in my life.

I extend my sincere appreciation to Drs. Anita Brûlé-Babel, Barbara Sharanowski and Michele Piercey-Normore for serving as my Ph.D. committee members and for their invaluable guidance, support, and the critical review of the thesis. Also I would like to thank my external examiner Dr. Ravindra Chibbar for his valuable comments and revisions on my thesis.

I wish to thank Dr. Sheryl Tittlemier (Canadian Grain Commission, Winnipeg) and Dr. Senay Simsek (North Dakota State University, Fargo, USA) for providing facilities to carry out mycotoxin analysis in their labs.

I would like to acknowledge Drs. Thomas Miedaner (Universitaet hohenheim, Stuttgrat, Germany), Malgorzata Jedryczka (Polish Academy of Science, Pozan, Poland), Philip Jennings (The food and environment research agency, York, UK), Pawan Kumar Singh (CIMMYT, Mexico), Silvia Barcellos Rosa (Melhoramento de Sementes Ltd. , Brazil), Kemal Kazan (CISRO plant industry, Brisbane, Australia), Altus Viljoen (Stellenbosch University, Stellenbosch , South Africa), Rubella Goswami (North Dakota State University, Fargo, USA), Nancy J. Alexander (United States Department of Agriculture, Peoria, Illinois, USA), Sussane Vogelgsang (Federal Department of Economic Affairs FDEA, Zurich, Switzerland), Jianhua Wang (Huazhong agricultural University, Hubei, China), Tapani Yli-Mattila (University of

Turku, Turku, Finland), Friday Obanor (CISRO Plant Industry, Brisbane, Australia) and Julie Toussaint-Ferreyrolle (Arvalis Institut du Végétal, INRA, France) for generously providing *Fusarium* strains for my research.

Also I would like to thank technicians, Ms. Paula Parks, Ms. Mary Meleshko, Mr. Roger Larios, Mr. Lorne Adams, Mr. Doug Durninn, Ms. Eppie Austria and research associates Dr. Maria Stoimenova and Dr. Arvind Hirani (Dept. of Plant Science, University of Manitoba) for their assistance in both lab and field experiments.

I wish to thank all the members of support and administration staff of the Department of Plant Science, University of Manitoba for their kindness and support during my studies.

Special thanks to my past and present lab mates for their company, especially my dear friends, Ru Li and Xuehua. A friendly chat with you was priceless for me to share my happy and difficult times.

I would like to express my gratitude to the Canadian Wheat Board and the University of Manitoba Graduate Fellowship for funding my doctoral scholarships. Also I would like to acknowledge the funding provided by Agri-Food Research and Development Initiative (ARDI) Manitoba and Western Grains Research Foundation (WGRF).

Last but not least, I wish to thank my Amma, Thaththa, Sajeewa and my sister for their continuous support, patience and encouragement.

DEDICATION

Every challenging work needs encouragment, support & love from those who were very close to our hearts

My Ever Loving Parents, My Sister, My Husband Sajeewa & Our Cute Little Baby Girl Naylie

You were there for me every step of this challenging journey.

You are the pillars of my success !!!

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

FOREWORD

This thesis is written in manuscript style following the guidelines set by the University of Manitoba. A general introduction about the research project and literature review precedes the six manuscripts that comprise the main part of the thesis. Each manuscript has an abstract, introduction, materials and methods, results and discussion. The manuscripts are formatted according to the journal Plant Pathology, and they are followed by general discussion and conclusions, literature cited and appendices sections. The first manuscript (Chapter 3) has been published in Plant Pathology (2015, 64: 988-995). The second manuscript (Chapter 7) and third manuscript (Chapter 8) have been submitted to Frontiers in Microbiology and Food Additives & Contaminants: Part A, respectively.

CHAPTER 1

GENERAL INTRODUCTION

The infection and growth of plant pathogenic fungi in agriculturally important crops is a worldwide problem confining the food and feed supply for animal and human consumption. One quarter of the world's economically important food crops are potentially contaminated with mycotoxin producing fungi (Logrieco & Visconti, 2014). Mycotoxins are toxic fungal secondary metabolites that can contaminate a wide range of food and feed. Mycotoxins are invisible, odourless, and cannot be detected by smell or taste, but they can significantly reduce the food and farm animal production (Binder *et al*., 2007). Over the past two decades *Fusarium graminearum* species complex emerged as one of the most destructive fungal pathogens causing the important disease, fusarium head blight in various cereal species (Wang *et al*., 2011). The *Fusarium* infection compromises grain quality by contaminating grains with *Fusarium* mycotoxins and poses a significant threat to the cereal industry. Amongst the most noxious toxins produced by *Fusarium* spp. are trichothecenes, which are composed of approximately 200 different toxins that inhibit eukaryotic protein synthesis (Maresca, 2013). Trichothecenes are also highly phytotoxic and act as virulence factors in some host plants (Proctor *et al*., 2009). Based on the type of trichothecene produced, different chemotypes have been described for *F. graminearum* species. Chemotypes are specific spectra of secondary metabolites or toxins produced by related strains or species (Rep & Kistler, 2010). The chemotypes of *F. graminearum* include; Deoxynivalenol (DON) chemotype, which produces DON and/or its acetylated derivatives, and nivalenol (NIV) chemotype, which produces NIV and/or 4-acetylnivalenol (4-ANIV). The DON chemotype can be further classified in to 3-

acetyldeoxynivalenol (3-ADON) chemotype, producing DON and 3-ADON and 15-acetyldeoxynivalenol (15-ADON) chemotype producing, DON and 15-ADON (Miller *et al*., 1991).

During recent years, changes in agronomic practices, global climate change, human influence on global trade and exchange of agricultural commodities have increased the spread and severity of FHB disease worldwide (Wang *et al*., 2011; van der Lee *et al*., 2015). In addition, recently, new *F. graminearum* species have been reported in areas; where the pathogen did not exist before. These areas are highly reliant on agricultural imports (Starkey *et al*., 2007; Zhang *et al*., 2010; Gale *et al*., 2011). Since the introduction of the phylogenetic species recognition concept for *F. graminearum* by O'Donnell *et al*. (2000), 16 monophyletic species have been reported in the *F. graminearum* species complex (FGSC) (Starkey *et al*., 2007; Gale *et al*., 2011; Sarver *et al*., 2011). To date, the phylogenetic species recognition concept has become a valuable tool to study the geographical structure of the species. This concept also enables the identification of novel species in a specific geographic region. The recent discovery of novel species in the FGSC and different types of trichothecens produced by these species has initiated surveys of trichothecene diversity in many different geographic areas to identify toxigenic risks associated with these trichothecens in infected grains (Starkey *et al*., 2007; Zhang *et al*., 2010; Gale *et al*., 2011; Sarver *et al*., 2011; Pasquali & Migheli, 2014; Varga *et al*., 2015). Different mycotoxins have different toxicological properties; therefore, determining the chemotype of *Fusarium* strains in a specific region is important (Ward *et al*., 2008; Gilbert *et al*., 2010; Puri & Zhong, 2010; Schmale *et al*., 2011; Amarasinghe *et al*., 2015). The presence of a certain chemotype in a specific region, may inform the potential toxigenic risk associated with the food and feed produced in that region. Therefore, the knowledge of the type of chemotype present in a

specific region provides long term preparation for developing preventative tools and methods to reduce the toxigenic risk (Pasquali & Migheli, 2014). Different trichothecene chemotypes of *F. graminearum* have different fitness characteristics associated with the toxin chemotype (Ward *et al*., 2008; Gilbert *et al*., 2010; Puri & Zhong, 2010). Hence, these trichothecene chemotypes can be considered as different genetic populations (Mishra *et al*., 2009). Many studies have used variable number tandem repeats (VNTR), restriction fragment length polymorphism (RFLP) and simple sequence repeat (SSR) markers to determine the genetic diversity of these strains (Gale *et al*., 2007; Qu *et al*., 2008a; Ward *et al*., 2008). The gene flow among these populations is limited even if they co-exist in the same area. Therefore, monitoring chemotype diversity in a specific region is important to characterise the *F. graminearum* population in that region. Because trichothecenes act as virulence factors for FHB development, the discovery of a *Fusarium* population with higher mycotoxin production may have a stronger effect on FHB development than a population with lower mycotoxin production.

The production of different types of trichothecenes depends on the differences in the core trichothecene gene clusters in *Fusarium* strains. Based on the *Fusarium* spp. and the trichothecene chemotype, the number of functional genes in the core trichothecene cluster is different (Lee *et al*., 2001; Alexander *et al*., 2011). The trichothecene biosynthesis genes in *F. graminearum* arranged into a core cluster consists of 12 contiguous genes in chromosome 2, the two gene *TRI1-TRI16* locus (chromosome 1), the single gene loci *TRI101* (chromosome 4) and *TRI15* (chromosome 3) which are located outside the core cluster. The insertions/deletions and nucleotide polymorphisms within the trichothecene gene clusters have been used to develop tools to differentiate chemotypes of *Fusarium* spp. (Lee *et al*., 2001; Ward *et al*., 2002; Nicholson *et al*., 2003; Chandler *et al*., 2003; Wang *et al*., 2008; Ward *et al*., 2008; Amarasinghe *et al*., 2011;

Pasquali & Migheli, 2014). To date, genetic chemotyping methods which were developed based on gene diversity have been widely used to determine the chemotype of *Fusarium* spp. over the chemical analytical methods. Other than the gene diversity of trichothecene biosynthesis genes, the level of expression of trichothecene biosynthesis genes may contribute to the differential ability of chemotypes to produce different amounts of toxins (Hallen-Adams *et al*., 2011). Therefore, knowledge of gene expression profiles among different trichothecene chemotypes is helpful to discover the possible trichothecene chemotype specific gene expression levels and patterns among different chemotypes (Lee *et al*., 2014).

Mycotoxins such as trichothecenes are phytotoxic compounds. Plant detoxification mechanisms can modify these compounds to either less toxic or non-toxic compounds, than their parent mycotoxins. These converted mycotoxin derivatives are undetectable by conventional analytical techniques because their molecular structure has been changed in the plant. These mycotoxins are referred to as masked or hidden mycotoxins (Poppenberger *et al*., 2003; Lemmens *et al*., 2005; Berthiller *et al*., 2013; Kluger *et al*., 2015). Because these masked mycotoxins escape conventional analytical techniques, it may lead to underestimation of total mycotoxin content in a given sample. Several studies have shown that these masked mycotoxins can be converted back to their parent mycotoxins in the animal gut by gut microbes where they regain toxicity (Berthiller *et al*., 2011; Nagl *et al*., 2012; Gratz *et al*., 2013; Warth *et al*., 2013). One major pathway of detoxifying DON is the conjugation of DON to a glucose moiety giving rise to DON-3-Glucosides (D3G) (Berthiller *et al*., 2011). Therefore, to date, the risk of masked mycotoxins has become an emerging concern in food and feed safety in the cereal industry.

Considering all the above literature on fusarium head blight disease and the trichothecene mycotoxins, this study was designed to deepen and broaden our current knowledge on the wheat-

F. graminearum pathosystem. This thesis consists of nine chapters; Chapter 1 provides a general introduction to the thesis and chapter 2 provides an overview of current knowledge on the nature of FHB disease, the damage it causes, dynamics of the different chemotypes, trichothecene biosynthesis pathway, and recent understanding of how to best control the disease. Chapter 3 is focused on examining the distribution of *Fusarium* spp. and trichothecene chemotype diversity in winter wheat collected from Winnipeg and Carman, Manitoba. Chapter 4 evaluates the genetic variability of *F. graminearum* populations in Winnipeg and Carman, Manitoba using variable number tandem repeat markers. Chapter 5 presents a comparative analysis of worldwide *F. graminearum* populations focusing on the phylogenetic relationships, trichothecene chemotype diversity and variation in aggressiveness among *Fusarium graminearum* strains collected from different countries. Chapter 6 is focused on the nucleotide and amino acid sequence polymorphism in the *TRI8* gene among the *F. graminearum* strains obtained from different countries. Chapter 7 examines the trichothecene biosynthesis related gene expression patterns among the different trichothecene chemotypes during wheat infection and colonization. Chapter 8 presents an analysis of deoxynivalenol and deoxynivalenol-3-glucosides content in Canadian spring wheat cultivars and Chapter 9 provides a general discussion and thesis conclusions.

CHAPTER 2

LITERATURE REVIEW

2.1 The Disease- Fusarium head blight

Fusarium head blight (FHB), or scab, is one of the most important diseases affecting wheat, barley and other small grain crops throughout the cereal growing regions of the world. This disease has occurred sporadically in Canada and several recent epidemics have been reported. FHB is caused by several *Fusarium* species, which include *F. graminearum*, *F. culmorum*, *F. cerealis*, *F. poae*, *F. avenaceum*, *F. verticilliodes*, *F. pseudograminearum* and *F. sprotrichiodes* (Parry *et al*., 1995). Among these different *Fusarium* species, *F. graminearum* is the main causal agent of FHB worldwide. Until the year 2000, *F. graminearum* was considered as a single panmictic species that spanned all over the world (O'Donnell *et al*., 2000). However, recent research conducted on a world collection of *F. graminearum* strains have shown that *F. graminearum* is not a single species but consists of at least 16 phylogenetically distinct species (O'Donnell *et al*., 2000; O'Donnell *et al*., 2004; Starkey *et al*., 2007; Yli-Mattila *et al*., 2009; Sarver *et al*., 2011). Collectively, this group of species is now known as *F. graminearum* species complex (FGSC). In the FGSC, *F. graminearum sensu stricto* is cosmopolitan in distribution and has been reported in Asia, Africa, the Americas and Europe. Other species in the complex are reported to have limited geographical distribution (O'Donnell *et al*., 2004; Starkey *et al*., 2007; Yli-Mattila *et al*., 2009; Sarver *et al*., 2011).

FHB is a very important disease because it can affect both grain quality and quantity. *Fusarium* spp. can cause yield losses either by producing light weight, scabby seeds known as

fusarium damaged kernels (FDK), or causing sterility in the wheat kernels (Trail, 2009). One of the major concerns of FHB is the contamination of wheat kernels with *Fusarium* mycotoxins. This can greatly reduce the grain quality, thus making it unmarketable. These mycotoxins comprise a group termed trichothecenes, which include the compounds deoxynivalenol, nivalenol, T-2 toxins and HT-2 toxins (Foroud & Eudes, 2009). Because mycotoxins contaminated grains pose a serious threat to humans and farm animals, many countries have established maximum limits of trichothecenes in cereals and cereal products (Foroud & Eudes, 2009).

2.2 The Host - Wheat

Triticum aestivum L. or bread wheat is one of the earliest crops in the world that was domesticated over 10 000 years ago in south-western Asia (Faris, 2014). Being the third largest crop globally, wheat accounts for 29-30% of the world's total cereal production. There is an increased demand of wheat due to availability of various end products at lower prices over other cereal crops. Global wheat production on average in the year 2013 was ~700 metric tonnes. It is estimated that, growing world population would need around 840 million tonnes of wheat by 2050 (http://www.fao.org/worldfoodsituation/csdb/en/, accessed on 10 October, 2015). Therefore, there is a continuous effort to increase the global wheat production.

2.2.1 Wheat production in Canada

In terms of global wheat production, Canada ranks number sixth for producing this particular cereal crop. In fact, Canada contributes around 4% (25 Mt) of the global wheat production. The majority of Canadian wheat is grown in the Prairie Provinces of western Canada.

Saskatchewan contributes 46% of total production, followed by Alberta with 30%, and Manitoba with 14% (http://www.agr.gc.ca/, accessed on 10 October, 2015). Canada is famous for its high quality and consistency in wheat and accounts for ~20% of internationally traded wheat. Canadian wheat has been used for many end products such as bread, flour, semolina, breakfast cereals, noodles and other confectionary products. The recent FHB epidemics in western Canada have contributed to huge economic losses in the wheat industry (Gilbert & Haber, 2013). Therefore, extensive efforts are being taken by researchers to fight against the pathogen (*F. graminearum*) and to strengthen the host (wheat).

2.3 The Pathogen- *Fusarium graminearum*- A brief overview

The genus *Fusarium* includes many agriculturally important toxin-producing plant pathogenic fungi. The most economically important plant pathogens in the *Fusarium* genus includes; *F. graminearum* species complex, *F. solani* species complex, *F. oxysporum* species complex and *F. fujikurio* species complex (Aoki *et al*., 2014). Plant pathogenic fungi that belongs to the *Fusarium* genus are capable of causing many diseases such as blights, wilts, rots and cankers in many economically important field, horticultural, ornamental and forest crops (Ma *et al*., 2013). One of the major concerns of this genus is that they can produce a diverse array of toxic secondary metabolites or mycotoxins. These different types of mycotoxins include trichothecenes, fumonisins, zearalenone and fusarin c, which can contaminate cereals and other important agricultural products, making them unsuitable for food or feed (Desjardins & Proctor, 2007). The genus *Fusarium* is a complex group, and the taxonomy has always been controversial with species numbers ranging from over 1000 (in 1900) to over 100 (in 2008) (Summerell *et al*., 2010; Aoki *et al*., 2014).

2.4 Taxonomy of the genus *Fusarium*

The genus *Fusarium* belongs to the phylum Ascomycota, class Ascomycetes and order Hypocreales. The teleomorphs of *Fusarium* species are mostly classified in the genus *Gibberella* (Ma *et al*., 2013). The taxonomy of *Fusarium* provides a framework to identify existing species, identify the relationship between species within a genus, and identify new species. A species concept provides a discrete set of characters that are used to describe a species and differentiate species from each other. The main approach for the *Fusarium* classification is based on the morphology, which is known as morphological species concept. This concept uses the idea that the morphology of an individual represents the differences present within an entire species (Summerell *et al*., 2010). This concept has been used by fungal taxonomists for over 200 years and is supported by many studies (Nelson, 1991; Summerell *et al*., 2010; Cai *et al*., 2011; Aoki *et al*., 2014). The main traits for species to be placed in *Fusarium* genus are the shape and size of the macroconidia, microconidia and chlamydospores, and the type and the presence of conidiogenous cells (Leslie & Summerell, 2006). Other characters such as the nature of hyphae in the culture, pigments produced, growth rate, and secondary metabolite production are also used to differentiate species. This morphological species concept was first diagnosed by Link in 1809, and he described *Fusarium* as *Fusisporium* (Summerrell *et al*., 2010). All *Fusarium* taxonomic classification systems developed so far are based on the monumental work by Wollenweber and Reinking in 1935. They organized approximately 1000 named species of *Fusarium* into 16 sections, including 65 species, 55 varieties and 22 forms (Nelson, 1991; Summerrell *et al*., 2010). Wollenweber and Reinking separated the sections based on morphological characters such as: shape of microconidia or macroconidia, the presence or absence of chlamydospores, the shape of the basal foot cell on macroconidia, and the location of

chlamydospores terminal. The taxonomic system by Snyder and Hansen made nine species out of Wollenweber and Reinking's 65 species, and their system was primarily based on the morphology of the macroconidia (Nelson, 1991; Summerrell *et al*., 2010). Later, Nelson *et al.* (1983) proposed a simpler classification system that divided the genus in 12 sections. They ended up classifying 30 species plus an additional 16 species. One of the major limitations of the morphological species concept is that, in micro-fungi, the number of unique morphological characters in a species is far smaller than the number of species that need to be identified (Summerrell *et al*., 2010). Despite the limitations in morphological species recognition, it is widely used by many diagnosticians to determine species limits. Therefore, the morphological species standard will remain an important criterion in identifying species limits within *Fusarium* (Nelson, 1991; Aoki *et al*., 2014).

Another theory used to define species limits is the biological species concept. This concept was introduced by Mayr (1940, 1963) and describes species as groups of populations that actually, or potentially, interbreed with each other. One of the major limitations of applying the biological species concept on the *Fusarium* spp. is that many strains of *Fusarium* are asexual or they rarely produce a sexual stage (Nelson, 1991; Summerell *et al*., 2010). The biological species criterion has been successfully used in the *Gibberella fujikuroi* complex (Leslie, 1995), where standard female fertile tester strains were used to identify the species limits of an unidentified strain. This method, in combination with the development of PCR- based tests for mating type, has been used widely for species identification (Kerenyi *et al*., 2004; Steenkamp *et al*., 2000).

The phylogenetic species concept is a more recent development in fungal taxonomy and was first defined as the small monophyletic clade of organisms that share a derived character set

(Cracraft, 1983). This concept uses the difference in DNA sequences of selected genes and yields quantitative measures of genetic relatedness (Summerrell *et al*., 2010). In 2000, Taylor *et al*. (2000) introduced a more developed criterion, genealogical concordance phylogenetic species recognition (GCPSR), to identify the species limits of sexual species. To date, the GCPSR criterion has been widely used in fungal classification (O'Donnell *et al*., 2000; Pringle *et al*., 2005; Summerrell *et al*., 2010; Cai *et al*., 2011; Glienke *et al*., 2011; Geiser *et al*., 2013; Aoki *et al*., 2014). This criterion can discriminate species more accurately and it is more convenient than other criteria. GCPSR is better at revealing species difference in fungi than any other organisms, because fungi have simple morphological characteristics and it is complicated to do *in vitro* crosses for many fungi (Taylor *et al*., 2000).

Phylogenetic species criterion has been very helpful in defining species limits in *Fusarium* species, for which sexual reproduction is difficult to induce under *in vitro* conditions. Approximately 300 phylogenetic species have been discovered in the *Fusarium* genus using molecular phylogenetics; however, most of the species have yet to be described formally (Aoki *et al*., 2014). O' Donnell *et al*. (2000) revealed the cryptic species within *F. graminearum* for the first time using the phylogenetic species recognition criterion. In their study, they illustrated seven phylogenetically distinct species in *F. graminearum* using six single copy gene phylogenies. Later, other researchers also exhibited the presence of new cryptic species of *F. graminearum* from different parts of the world (Cai *et al*., 2011). To date, 16 monophyletic species of *F. graminearum* have been discovered using multilocus phylogenies, which resulted in the renaming of *F. graminearum* as *F. graminaerum* species complex (FGSC) (O Donnell *et al*., 2004; Starkey *et al*., 2007; Sarver *et al*., 2011). The translation elongation factor 1 α gene (*EF-1 α*) is the most frequently used gene in species recognition in *Fusarium* (Cai *et al*., 2011). Other

genes that used to delimit species boundaries in *Fusarium* include the gene reductase (*RED*), mating type locus genes (*MAT)*, phosphate permase (*PHO*), trichothecene-3-*O*- acetyltransferase (*TRI101*), β-tubulin and internal transcribed regions (ITS) of the rRNA gene repeats (Summerrell *et al*., 2010; Park *et al*., 2011; Aoki *et al*., 2014). Interestingly, use of morphological species recognition criteria such as conidial morphology and colony characters was only able to distinguish six species among the 16 monophyletic species that were initially detected using molecular phylogenetic approaches. However, it appears that the incongruence between morphological and phylogenetic species recognition is consistent with the recent evolutionary origin of FGSC (O'Donnell *et al*., 2013; Aoki *et al*., 2014).

2.5 Life cycle and symptoms of FHB

Fusarium head blight is a monocyclic disease, indicating that *F. graminearum* has only one infection cycle per season (Bai & Shaner, 1994; Shaner, 2003). Wheat is susceptible to *F. graminearum* at the anthesis stage, where disease development is dependent upon the interaction of three major factors: 1) the pathogen 2) the host and 3) the contributing environmental conditions (Shaner, 2003). All three components (pathogen, host and the environment) form the disease triangle, a well-known conceptual model in plant pathology that helps determine plant disease outcome. Disease development in all plants, including those infected with FHB, occurs if the interaction between these three factors is conducive to instigate an infection. The disease cycle of FHB is initiated from the *Fusarium*-infected crop residues on the soil surface from the previous cropping season (Shaner, 2003; Parry *et al*., 1995; Bai & Shaner, 2004). FHB is a monocyclic disease; therefore, the amount of primary inoculum that is available during one cropping season will have a huge effect on FHB epidemic development (Sutton, 1982; Bai &

Shaner, 1994). In the spring, during warm, moist, and windy environmental conditions, the pathogen is able to produce sporodochia which contain macroconidia (asexual spores) and later produce perithecia, sexual fruiting structures which give rise to ascospores (sexual spores). These ascospores disperse by wind, land on susceptible host plants and subsequently initiate infection (Fernando *et al*., 1997; Shaner, 2003). In addition to ascospores and macroconidia, chlamydospores and hyphal fragments also act as a source of inoculum for FHB development. However, ascospores and macroconidia were identified as the major sources of inoculum in FHB epidemics (Sutton, 1982; McMullen *et al*., 1997; Fernando *et al*., 2000; Inch & Gilbert, 2003; Liddell, 2003; Osborne & Stein, 2007). It has been reported that the presence of sticky substances on ascospore wall make them better attach to the wheat spike than macroconidia (Markell & Francl, 2003). The amounts of crop residue in the soil and the degree of infection of the crop determine the amount of FHB inoculum available for the next cropping season (Dill-Macky & Jones, 2000). The optimal temperature for *F. graminearum* perithecial and ascospore production is 29ºC and 25-28ºC respectively. The discharge of ascospores is triggered by the combined effect of a drop in air temperature and a rise in relative humidity (Paulitz *et al*., 1996). Interestingly, rainfall events have also been shown to have a direct effect on the perithecial formation and ascospore development. A positive correlation has been observed between the release of ascospores with rain fall events and the time of the day (Fernando *et al*., 2000). It has also been reported that the release of ascospores usually follow a 1 to 3-day gap after a rain fall event (Fernando *et al*., 2000). The temperature in which ascospore release has been shown to occur is between 10-30ºC, with the optimum temperature for discharge at 16ºC. No ascospore discharge was observed at high temperatures i.e. at or above 26ºC (Sutton, 1982). The dispersal of *F. graminearum* inoculum occurs downwind from inoculated wheat spikes (Fernando *et al*.,

1997). Environmental factors such as temperature, pH, relative humidity, aeration and light determines the successful germination of ascospores on the host tissue. Ascospores initiate germination within 4 hrs at 20ºC with relative humidity of 100% (Beyer & Verreet, 2005). The highest rate of ascospore germination was observed at 90% relative humidity, the lowest rate at 60% relative humidity and there was no germination below 50% relative humidity (Beyer & Verreet, 2005; Gilbert *et al*., 2008). It has been reported that ascospores are viable up to 21 days following discharge and remain viable under most environmental conditions (Beyer & Verreet, 2005). High relative humidity of 92-94%, in combination with warm temperatures during the flowering stage of host plant, makes the best conditions for *F. graminearum* spores to germinate and establish infection on susceptible plants (Sutton, 1982).

The most distinctive symptom of FHB is premature bleaching of spikelets (Fig 2.1a). These symptoms first appear on the first florets to flower, generally near the middle of the spike. The moisture content is higher in the middle of the spike than in the antipetals or basipetals. Then symptoms spread both apically and basally from the point of infection (Shaner, 2003; Bushnell *et al*., 2003; Osborne & Stein, 2007). Wet and humid weather conditions at the flowering stage induce production of salmon orange to pink spore masses on the infected spikelets. These asexual spore masses are called sporodochia. Later in the growing season, black perithecia, which contain ascospores (sexual spores), may appear on spikelets (Osborne & Stein, 2007). The infected spikelets contain shriveled, light weight, chalky white kernels usually with pink mycelia. These kernels are commonly termed, fusarium damaged kernels (FDK) or tombstone kernels (Fig 2.1b) (Bushnell *et al*., 2003; Goswami & Kistler, 2004). In addition, *Fusarium* strains are capable of producing mycotoxins in infected plants and kernels.

(a) (b)

Figure 2.1 Distinctive symptoms of fusarium head blight (a) wheat spikes showing premature bleaching and (b) fusarium damaged kernels.

2.5.1 Pathways of *Fusarium* infection in wheat

Upon landing on a susceptible host plant, macroconidia germinate within 5-6 hrs on wheat glumes. Emerging germ tubes rapidly elongate over the glume and floret surfaces and form a complex mycelial network across the exterior surfaces of the host tissue (Pritsch *et al*., 2000). *Fusarium* hyphae use several routes to enter the floret and grow over the adaxial surfaces of palea and lemma to infect plant tissue. One of the routes is direct, via the penetration of the epidermal cell wall using a penetration peg, while another route is through stomata. Wounds also offer another potential route for direct penetration into, or through the epidermis. Following primary penetration, *Fusarium* hyphae begin subcuticular growth, which is the initial stage of pathogenesis. This serves as a mechanism for fungal growth and leads to direct penetration of epidermal cells. A study conducted using the immunogold labelling technique illustrated that hyphal penetration is initiated through enzymatic degradation (Kang & Buchenauer, 2000). It has been reported that fungal hyphae take approximately 76 hrs to reach the developing caryopsis across the outer surfaces of the lemma and glume. Once the fungus is established in one floret, it

can then spread from one floret to another through a systemic and necrotic infection of the rachis and rachis node (Schroeder & Christensen, 1963; Ribichich *et al*., 2000). The main pathway of fungal spread in the wheat spike is through the vascular bundles in the rachis and rachilla (Kang & Buchenauer, 2000; Ribichich *et al*., 2000). When the hyphae spread vertically and horizontally (Fig 2.2) within the rachis, the host cell tissue and organelles become fragmented. Also, it interrupts the water and nutrients transport to the developing tissue which results in premature death of infected spikelets. Within the wheat rachis, the hyphae spread both apically and basally and finally extend into the peduncle below the spike (Kang & Buchenauer, 2000).

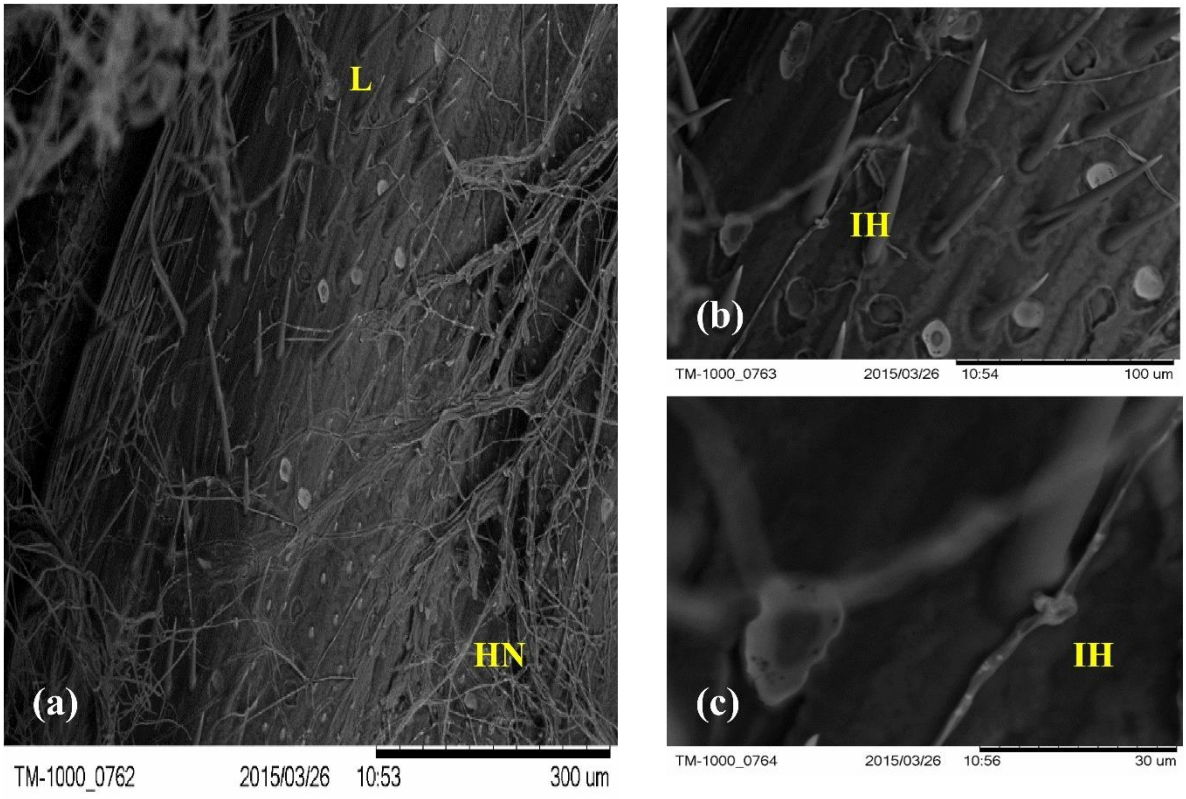


Figure 2.2 Scanning electron micrographs showing infection of wheat spikes (cultivar Roblin) by *Fusarium graminearum*. (a) Hyphal network (HN) on wheat lemma (L) 72 hours post-inoculation. (b) and (c) infection hyphae (IH) penetrating the wheat tissue.

2.6 *Fusarium* mycotoxins- Trichothecenes

Trichothecenes are toxic sesquiterpenoid compounds composed of a common tricyclic 12, 13-epoxytrichothec-9-ene (EPT) core structure (Cole *et al*., 2003; Grove, 2007; McCormick *et al*., 2011). A cyclopentyl moiety is also fused to the tetrahydropyran ring through C-2 and C-5, while C-12 forms part of an epoxide functionality, which is known to be important for toxicity. Trichothecenes have been classified in to four groups (Types A, B, C and D) based on the substitution pattern in the trichothecene core (Fig 2.3).

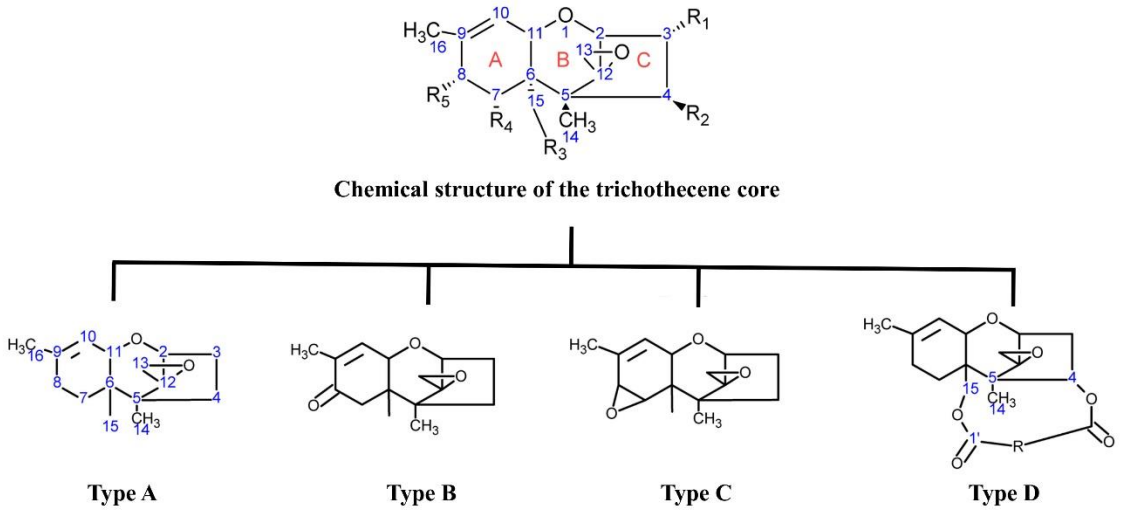


Figure 2.3 General chemical structure, numbering system of the tetracyclic trichothecene nucleus, Type A, Type B, Type C and Type D trichothecenes (modified from Foroud & Eudes, 2009; Shank *et al*., 2011).

The classification of Types A, B and C trichothecenes is based on the substitution groups at the C-8 position. The major type A trichothecenes in *Fusarium* species include T-2 toxin (T-2) and HT-2 toxin (HT-2) which possess an isovalerate moiety at the C-8 position (Table 2.1). Type A trichothecene production is more common among the *F. sprotrichiodies* and *F. poae* species

and is reported to be more toxic in mammals than Type B trichothecenes. Type B trichothecenes generally contain a keto group at the C-8 position; however, unlike other genera, *Fusarium* Type B trichothecenes typically have a hydroxyl group at the C-7 position. A common Type B trichothecenes associated with FHB is deoxynivalenol (DON). Other Type B trichothecenes include nivalenol (NIV), acetylated derivatives of nivalenol (4-ANIV), 3- acetyldeoxynivalenol (3-ADON) and 15-acetyl deoxynivalenol (15-ADON) (Fig 2.4). *Fusarium* species such as *F. graminearum* and *F. culmorum* are the major producers of DON and NIV that are associated with FHB disease (McCormick *et al*., 2011). Type C and D trichothecenes are not associated with FHB and have a C-7/C-8 epoxide and an additional ring linking the C-14 and C-15 respectively.

Table 2.1 Different trichothecene types, their molecular weight, oxygenation and esterification at different carbon positions

Trichothecene	**MW[1]**	**Oxygenation and esterification at different carbon positions[2]**				
		R1	**R2**	**R3**	**R4**	**R5**
DON	296	-OH	-H	-OH	-OH	=O
3-ADON	338	-OAc	-H	-OH	-OH	=O
15-ADON	338	-OH	-H	-OAc	-OH	=O
NIV	312	-OH	-OH	-OH	-OH	=O
T-2	466	-OH	-OAc	-OAc	-H	-OIsoval
HT-2	424	-OH	-OH	-OAc	-H	-OIsoval

[1] MW= Molecular Weight (Daltons).
[2]OAc= Acetyl Ester= -O-CO-CH_3, OIsoval= Isovalerate Ester= -$OCOCH_2CH((CH_3)_2)$.

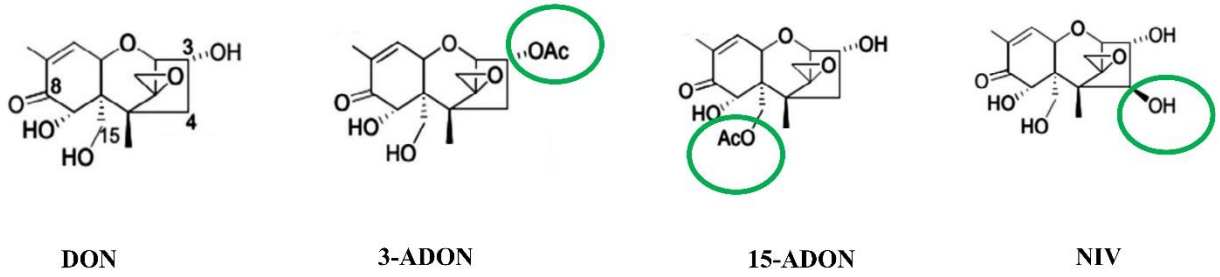


Figure 2.4 Chemical structures of deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON) and nivalenol (NIV) (modified from Varga *et al*., 2015)**.**

2.6.1 Trichothecene biosynthesis

The expression profile of trichothecene mycotoxins is highly complex in *Fusarium* species. To date, 16 genes have been characterized in the trichothecene biosynthesis pathway (McCormick *et al*., 2011). In *Fusarium*, trichothecene biosynthetic enzymes are encoded by genes at four loci located on different chromosomes in the genome; the single-gene *TRI101* locus (chromosome 4), *TRI15* locus (chromosome 3), the two-gene *TRI1-TRI16* locus (chromosome 1), and the 12-gene core *TRI* cluster (chromosome 2) (Desjardins, 2006; Alexander *et al*., 2009; Hallen-Adams *et al*., 2011; McCormick *et al*., 2011). The arrangement of trichothecene biosynthesis related genes in *F. graminearum* is shown in Figure 2.5. The enzymes encoded by each gene are listed in Table 2.2.

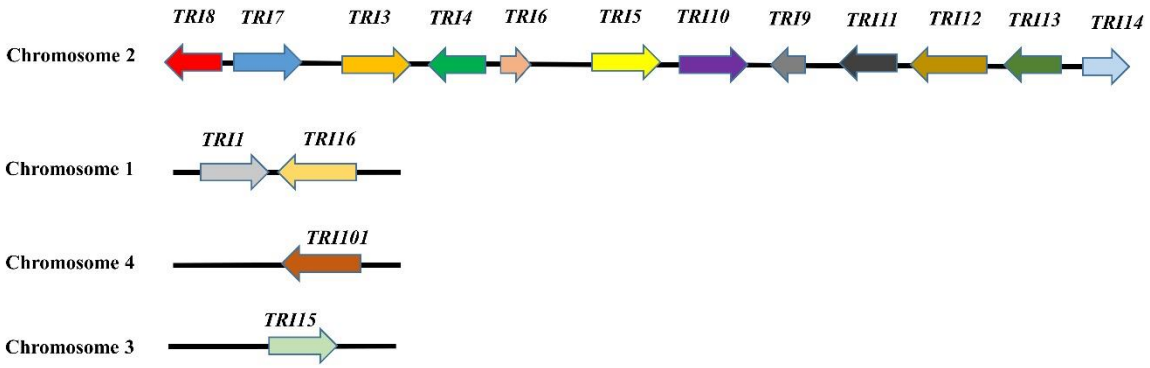


Figure 2.5 The arrangement of trichothecene biosynthesis related genes in *Fusarium graminearum*.

Table 2.2 Genes involved in trichothecene production and the enzymes encoded by each gene

Gene	Function	Reference
TRI1	C-7 monooxygenase	Brown *et al*., 2003
TRI3	Trichothecene-15-*O*-acetyltransferase	McCormick *et al*., 1999; Kimura *et al*., 2007
TRI4	Cyctochrome P450 monooxygenase	Kimura *et al*., 2007; Tokai *et al*., 2007
TRI5	Trichodiene synthase	Hohn & Beremand, 1989; Desjardins, 2006
TRI6	Transcriptional regulator	Proctor *et al*., 1995a; Kimura *et al*., 2007
TRI7	C-4 acetyltransferase	Brown *et al*., 2001
TRI8	Trichothecene-3-*O*-esterase	Brown *et al*., 2001; Kimura *et al*., 2007
TRI9	Unknown	Brown *et al*., 2001; Kimura *et al*., 2007
TRI10	Global regulator	Tag *et al*., 2001
TRI11	C-15 oxygenase	McCormick *et al*., 1999
TRI12	Trichothecene efflux pump	Alexander *et al*., 1999; Kimura *et al*., 2007
TRI13	C-4 oxygenase	Lee *et al*., 2002
TRI14	Unknown	Foroud & Eudes, 2009
TRI15	Cys_2-His_2 zinc finger protein	Alexander *et al*., 2004
TRI16	C-8 acyltransferase	Peplow *et al*., 2003
TRI101	Trichothecene-3-*O*-acetyltransferase	Kimura *et al*., 1998; 2007

The first step in the trichothecene biosynthesis pathway initiates with the cyclization of farnesyl pyrophosphate, which is the main metabolic intermediate that in turn convert into trichodiene (Hohn & VanMiddlesworth, 1986; Hohn & Bermand, 1989) (Fig 2.6). This reaction is catalyzed by the enzyme terpene cyclase trichodiene synthase (Tri5) which is encoded by

TRI5. Trichodiene, then undergoes a series of four oxygenation reactions mediated by a cyctochrome P450 monooxygenase encoded by *TRI4* (Hohn *et al*., 1995). These four oxygenation reactions include hydroxylation at C-2, epoxidation at C-12/C-13, hydroxylation at C-11, and hydroxylation at C-3 positions (McCormick *et al*., 2006; Alexander *et al*., 2009). The next two steps in the pathway occur non-enzymatically, where the initial step is the formation of trichotriol by an isomerization that replaces the C-9 hydroxyl with a C-11 hydroxyl. The second step is the formation of isotrichodermol, which takes place through a series of hydroxylation and acetylation or acylation steps (McCormick *et al*., 1990; Alexander *et al*., 2009; McCormick *et al*., 2011). Isotrichodermol is then converted to isotrichodermin by *TRI101* gene which encodes an acetyltransferase enzyme. The formation of isotrichodermin reduces the toxicity of the tricothecene intermediate, by providing a self-protection mechanism for trichothecene-producing fungi (Kimura *et al*., 1998). The next step in trichothecene biosynthetic pathway is hydroxylation of the C-15 position in isotricodermin mediated by cytochrome P450 monooxygenase encoded by *TRI11* (Alexander *et al*., 1998). Following C-15 hydroxylation, the acetyltransferase that is encoded by *TRI3* catalyzes the transfer of an acetyl moiety to the C-15 oxygen, generating calonectrin (McCormick *et al*., 1996). The trichothecene biosynthesis pathway leading to the formation of calonectrin is similar in all the T-2, HT-2, DON and NIV producers. However, upon the production of calonectrin, the pathways diverge according to the type of trichothecenes that are produced within the strains. In T-2 and NIV producing species, trichothecene biosynthesis proceeds with C-4 hydroxylation of calonectrin which is mediated by the *TRI13*- encoded cytochrome P450 monooxygenase. Due to the absence of a functional *TRI13* gene, DON producing strains are unable to hydroxylate the trichothecenes at position C-4. In T-2 and NIV producing strains, oxygen at the C-4 position is converted into an acetyl moiety by the

enzyme acetyltransferase encoded by *TRI7* (Alexander *et al*., 2009). In contrast, DON producing strains do not have a functional *TRI7* gene and they lack the ability to produce C-4 acetylated trichothecenes (Lee *et al*., 2002). In *F. sporotrichioides*, a Type A producer, trichothecene biosynthesis proceeds with C-8 hydroxylation of calonectrin, which is controlled by the *TRI1* gene. Then, follows an addition of an isovaleryl moiety at the C-8 position encoded by *TRI16*. The final T-2 toxin is produced by removing an acetyl group from the C-3 position, which is mediated by the C-3 deacetylase enzyme encoded by the *TRI8* gene (McCormick *et al*., 2006; McCormick *et al*., 2011). In trichothecene Type B strains, *TRI1* gene mediates the addition of hydroxyl groups at both the C-7 and C-8 positions (McCormick *et al*., 2004; McCormick *et al*., 2011). Later, the hydroxyl group at C-8 position is converted to a keto group; however, this step is not fully understood yet. The final step in Type B trichothecene biosynthesis is similar to that of Type A producers, where the removal of the C-3 acetyl group, or the C-15 acetyl group, is carried out by an esterase encoded by *TRI8* (McCormick *et al*., 2002; McCormick *et al*., 2011). Differential activity of Tri8 determines the synthesis of either 3-ADON or 15-ADON in *F. graminearum* (Alexander *et al*., 2011). The core *TRI* cluster also contains other genes such as *TRI9, TRI12* and *TRI14*. The role of *TRI9* gene product in trichothecene production has not yet been characterized, but it has been reported that *TRI9* gene encodes a unique 43- amino acid protein that does not resemble any known protein to date (Brown *et al*., 2001). The *TRI12* gene encodes a major facilitator super family (MFS) transporter involved in trichothecene efflux (Alexander *et al*., 1999). Also the role of the *TRI14* gene has not yet been fully characterized, but it has been reported that, *TRI14* mutants do not produce trichothecenes when inoculated into wheat spike florets. However, *TRI14* deletion mutants synthesize DON on cracked maize kernel

medium and exhibit wild-type colony morphology and growth rate on complex and minimal agar media (Dyer *et al*., 2005).

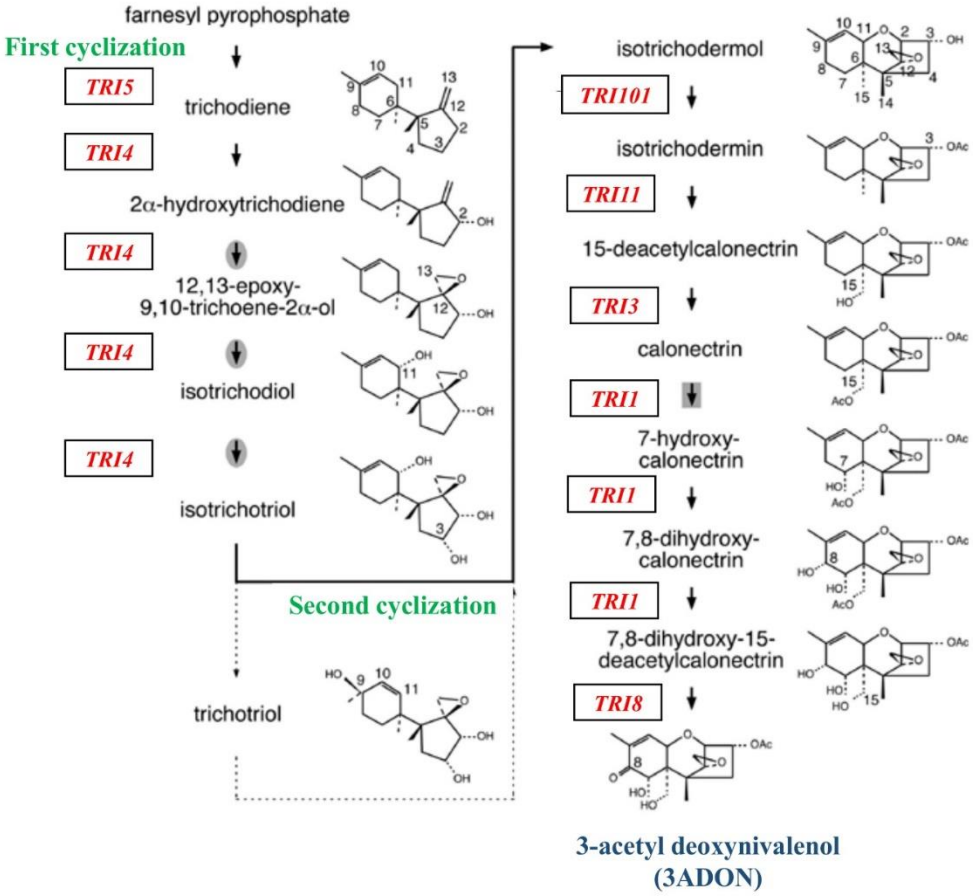


Figure 2.6 Trichothecene biosynthesis pathway in 3-acetyldeoxynivalenol producing *Fusarium graminearum* strains (modified from Tokai *et al*., 2005).

2.6.2 Biological activity of trichothecenes in animals

The trichothecene mycotoxins are toxic to humans, other mammals, birds, fish, invertebrates, plants, and eukaryotic cells in general. The acute toxicity of the trichothecene mycotoxins varies somewhat with the particular toxin and the animal species studied. The research on the biological effects of trichothecenes in animal systems began in 1960 -70's with the discovery of T-2 toxin,

nivalenol, deoxynivalenol, and other trichothecenes. Toxicity tests performed with different types of trichothecenes have shown that Type A-toxins, such as DAS and T-2 toxin, are more toxic than Type B-toxins such as DON and NIV (Leeson *et al*., 1995). Among different animal species, trichothecenes are especially toxic to monogastric animals such as cats, dogs, humans, rodents and swine. All animal species evaluated to date are susceptible to DON according to the order of swine > mice > rats > poultry ≈ ruminants (Prelusky *et al*., 1994). Differences in metabolism, absorption, distribution, and elimination of DON among different animal species might account for this differential sensitivity. Swine are most vulnerable to trichothecene contamination, where deoxynivalenol contamination of 2 μg/g or less can cause feed refusal and vomiting. In contrast, the deoxynivalenol level of tolerance is 10-fold higher in other animals like chickens, turkeys and cows. The rumen microorganisms also play a key role in the metabolism and detoxification of trichothecenes in animals. The major pathways of trichothecene metabolism in animals include glycosylation, reduction of the epoxide ring, and ester hydrolysis (Berthiller *et al*., 2011).

The potential impact of DON on human and animal health may occur after ingestion of DON contaminated foods from wheat, barley, corn and other grains. DON was also detected in flour, bread, noodles, beer and malt (Pestka & Zhou, 2000; Pestka, 2007). Compared to T-2 toxins, DON does not constitute an acute threat to public health. In a few cases, short-term nausea and vomiting have been recorded (Perkowski *et al*., 1990). Other effects include diarrhea, abdominal pain, headache, dizziness and fever. Although DON is less toxic than other trichothecenes, extremely high doses of DON can cause shock-like death (Yoshizawa *et al*., 1983).

2.6.3 Biological activity of trichothecenes in plants

Trichothecenes can cause phytotoxicity in a wide variety of whole plants and plant tissue. The toxicity of trichothecenes to plant cells was first studied by Brian *et al*. (1961). Since then, trichothecenes are considered as phytotoxic compounds and they can produce disease symptoms such as wilting, chlorosis, necrosis and various other non-specific symptoms within plant tissue (Desjardins, 2006; Nishiuch *et al*., 2006). Trichothecenes can affect protein synthesis, electron transport, membrane integrity, leaf chlorophyll content and other functions of plant tissue (Desjardins, 2006). Also, trichothecenes have been reported to act as virulence factors during fungal infection (Proctor *et al*., 1995b). The relationship between *Fusarium* pathogenicity and trichothecene production in plants was examined by Proctor *et al*. (1995b) using *Fusarium* knock-out mutants. They examined the effect of trichothecene production on seedling emergence and height of *Fusarium* inoculated wheat, maize, oat and rye using both a non-trichothecene producing *Fusarium* strain (*Tri5*$^{-}$) and a wild type strain. Their findings illustrated that mutant strains had reduced FHB incidence and severity compared to the wild type. A similar study, conducted by Harris *et al*. (1999), also reported that non-trichothecene producing strains showed less virulence compared to wild type on inoculated maize under field conditions. Interestingly, it has also been shown that even though the trichothecene mutants were less able to spread from the point of infection, they still retained their ability to initiate infection (Bai *et al*., 2001). Taken together, the findings in these studies suggest that trichothecenes act to enhance the ability of *Fusarium* to cause disease on susceptible host plants (Desjardins, 2006).

2.6.4 Role of deoxynivalenol in *Fusarium* pathogenicity

Deoxynivalenol plays a major role in the entire life cycle of *F. graminearum*. As a hemibiotrophic pathogen, *F. graminearum* proceeds through a biotrophic and a necrotrophic phase during the colonization of their host. The *F. graminearum* life cycle initiates with the landing of a rain-splashed conidium or wind-dispersed ascospore on the exposed vulnerable parts of a host plant. After this point, the fungus can germinate and penetrate the plant tissue (Audenaert *et al*., 2013). In the initial phase, *F. graminearum* grows biotrophically into the intercellular spaces. Studies by Desjardins *et al*. (1996) and Cowger & Arellano (2013) have shown expression of the *TRI5* gene at the hyphal tip during the biotrophic phase, indicating active trichothecene biosynthesis takes place at this stage in life cycle. Recently, it has also been shown that very low DON concentrations can inhibit apoptosis like programmed cell death (PCD) and can disrupt the biotroph type defense in *Arabidopsis* cells (Diamond *et al*., 2013). Therefore, it is believed that DON is recruited by the pathogen to interfere with PCD and the associated cascade of defence responses. It has been shown that mutants with an inactive *TRI5* gene are less virulent and are reduced in their ability to spread in the rachis. These studies clearly indicate that DON not only moves into host plant tissue in advance of the colonising fungus, but is a requirement for the spread of the fungus (Desjardins *et al*., 1996; Bai *et al*., 2002; Jansen *et al*., 2005). The second phase, or the necrotrophic phase, follows the biotrophic phase where the fungus exhibits a more aggressive and invasive intracellular growth. At this particular phase, high concentrations of DON were shown to trigger hydrogen peroxide (H_2O_2) synthesis and initiate cell death. It has been reported that the presence of H_2O_2 is a prerequisite for DON production. Hydrogen peroxide is one of the first defense molecules reacting with the invading *Fusarium* hyphae and it has been reported that H_2O_2 establishes a positive feedback loop leading

not only to an increase in DON production, but also elevate levels of H_2O_2 (Ponts *et al*., 2006; Audenaert *et al*., 2010). The involvement of H_2O_2 as an inducer of DON production has been shown in studies using ferulic acid, an anti-oxidative phenolic acid, which can inhibit the trichothecene biosynthesis *in vitro* by repressing *TRI* gene expression (Boutigny *et al*., 2010). Production of conidia and ascospores is important for pathogen's survival and a major component in the pathogen life cycle. Many reports provide evidence of a link between DON production and formation of reproductive structures in *Fusarium* (Lysoe *et al*., 2011; Jonkers *et al*., 2012). Functional analysis of the *FgStuA* gene (a transcription factor with homology to APSES transcription factors in fungi) have shown that a deletion of this gene greatly reduces the pathogenicity on wheat spikes and production of secondary metabolites (Lysoe *et al*., 2011). There are, however, limited studies on the role of DON in saprophytic phase in *F. graminearum.* One particular study carried out by Tunali *et al*. (2012) elucidated that saprophytic growth on media and DON production from saprophytic colonization of straw and grain were linked to high FHB aggressiveness.

2.6.5 Detoxification of DON in plants

Trichothecenes act as virulence factors that enhance fungal infection. Therefore, a reduction of mycotoxin contamination in grain could also limit fungal infection. The resistance mechanisms in plants have the potential to reduce the trichothecene contamination and inhibit the spread of the disease. The resistance mechanism attributed to the low toxin accumulation trait, is referred to as Type V resistance to *Fusarium* (Mesterházy *et al*., 1995; Mesterházy *et al*., 1999). Type V resistance has been classified in to two classes. Class 1, includes the mechanisms by which the plants chemically transform trichothecenes to less toxic compounds, or detoxification. Class 2

comprises mechanisms that reduce the accumulation of trichothecenes by inhibiting their biosynthesis through the action of plant endogenous compounds. One of the main mechanisms of class 1 resistance is glycosylation (Berthiller *et al*., 2005). In this process, trichothecenes are converted to stable, non-reactive storage forms with increased water solubility. These compounds are known as masked mycotoxins. In the converted products, the reactive site of the toxin is masked by a sugar residue, changing the toxin to a less toxic compound (Jones & Vogt, 2001; Berthiller *et al*., 2005). A paper published by Savard (1991) illustrated the synthesis of the first glycoside derivative of deoxynivalenol. Poppenberger *et al*. (2003) identified a UDP glucosyltransferase in *Arabidopsis thaliana* which had the ability to catalyse the glycosylation of DON. The presence of glycoside derivatives of deoxynivalenol in cereals has been identified in several studies (Berthiller *et al*., 2005; Lemmens *et al*., 2005). In the study conducted by Lemmens *et al*. (2005), a QTL, termed *Qfhs.ndsu-3BS* was reported to be linked with DON resistance, which was hypothesized to encode the enzyme DON-glucosyltransferase or regulate the expression of such an enzyme. Also, they established a strong positive correlation between the ratio of DON 3-*O*-glucoside/DON and DON resistance in DON-treated wheat ears. However, there is a possibility that these less toxic glycosylated products can be re-converted into toxic forms in the digestive tract of humans and animals by gut microbes (Gareis *et al*., 1990).

Another strategy to reduce the toxicity of deoxynivalenol is through acetylation. Acetylation has been used by *Fusarium* species as a self- protection mechanism from their own toxins (Berthiller *et al*., 2005; Boutigny *et al*., 2008). The *TRI101* gene, which encodes a trichothecene-3-*O*-acetyltransfearse, is responsible for catalysing 3-*O*-acetylation of trichothecenes (Kimura *et al*., 1998). It has been shown that 3-*O*- acetylation of trichothecenes led to a reduced toxicity on *in vitro* protein synthesis in the rabbit reticulocyte lysate translation

system (Kimura *et al*., 1998). Studies done in transgenic wheat, barley and rice plants expressing the *TRI101* gene have shown to reduce DON accumulation or FHB symptoms in greenhouse plants; however, there are no reports on the natural occurrence of trichothecene acetylation as a detoxification process in plants (Okubara *et al*., 2002; Kimura *et al*., 2006; Ohsato *et al*., 2007). Several research groups have reported conflicting observations regarding the phytotoxicity of 3-ADON (Wang & Miller 1988; Wakulinski 1989; Bruins *et al*., 1993; Eudes *et al*., 2000). The higher phytotoxicity of 3-ADON observed with non-transgenic plants may be attributed to C-3 deacetylation inside the cells. It has been reported that C-3 acetyl is not very stable in the biosynthesis of *Fusarium* trichothecenes; it is mostly lost (i.e., deacetylated) but subsequently restored (i.e., re-acetyalted) in the 3-ADON producer *F. culmorum* (Zamir *et al*., 1996). Oshato *et al.* (2007) explained that the stable transgenic expression of *TRI101* in model cereal rice plants constantly eliminates C-3 deacetylated trichothecenes within plant cells. In turn, this could protect cereals from the phytotoxic effect of trichothecenes.

Class 2 resistance strategy is via the limitation of mycotoxin biosynthesis during the growth of the plant. The biosynthesis of trichothecene involves a number of oxygenation steps that require molecular oxygen. Therefore, the changes in the oxidative parameters of the nutrient source and the kernels can regulate the trichothecene production in plants (Ponts *et al*., 2006). Antioxidant compounds such as phenolic compounds, peptides and hydrogen peroxide can interfere with the trichothecene biosynthesis (Huang *et al*., 1997; Ponts *et al*., 2006).

2.7 Chemotypes of *F. graminearum* species complex

Until the year 2000, *F. gramineraum* was considered as a single panmictic species. However, employing the GCPSR approach, 16 phylogenetically distinct species were identified within the

FGSC (O'Donnell *et al*., 2000; O'Donnell *et al*., 2004; Starkey *et al*., 2007; O'Donnell *et al*., 2008; Gale *et al*., 2011; Sarver *et al*., 2011). The species within the FGSC have evolved in Africa, South America, North America, Asia, Europe and Australia. These 16 species include: *F. austroamericanum*, *F. meridionale*, *F. boothii*, *F. mesoamericanum*, *F. acacia-mearnsii*, *F. asiaticum*, *F. graminearum sensu stricto*, *F. cortaderiae*, *F. brasilicum*, *F. gerlachii*, *F. vorosii*, *F. aethiopicum*, *F. ussurianum*, *F. nepalenses*, *F. louisianense* and genetically distinct U.S. Gulf Coast population of *F. graminearum s.s.* All 16 species within the FGSC are believed to be self-fertile as they possess a homothallic mating type locus organization (O'Donnell *et al*., 2014).

The members within the FGSC are capable of producing trichothecene mycotoxins that contaminate cereals, and cause significant health risks to humans and farm animals (Proctor *et al*., 1995b). The species in the FGSC produce B-type trichothecenes such as deoxynivalenol (DON), nivalenol (NIV) and their acetylated derivatives, i.e., 3-acetyl-deoxynivalenol (3-ADON) and 15-acetyl-deoxynivalenol (15-ADON) (Ward *et al*., 2002). There are three trichothecene chemotypes within the B-trichothecene producing *Fusarium* species. These trichothecene chemotypes are categorized based on the type of trichothecenes produced by each strain. These three chemotypes include 1) 3-ADON chemotype (produce DON+3-ADON), 2) 15-ADON chemotype (DON+15-ADON) and 3) NIV chemotype (NIV+ 4-ANIV) (Fig 2.5). To date, 16 genes have been found in the trichothecene biosynthesis pathway and these genes are evolving under strong balancing selection (Ward *et al*., 2002; O'Donnell *et al*., 2004; Aoki *et al*., 2014). Balancing selection refers to a number of selective processes by which multiple alleles are actively maintained in the gene pool of a population at frequencies above that of gene mutation. It has been reported that approximately half of the species within the B-type trichothecene

producing clade still segregate for trichothecene toxin chemotype (Sarver *et al*., 2011; Aoki *et al*., 2014).

2.7.1 Distribution of FGSC species and chemotypes in North America

Field surveys on FHB and mycotxin chemotypes have been performed in Canada and the USA over the last decades. Both in Canada and the USA *F. graminearum s.s* is the most prevalent species causing FHB in wheat. Before year 2000, 15-ADON producing *F. graminearum s.s* strains were dominant in Canada; however, after the year 2000, a chemotype shift was reported, where 15-ADON producing strains were rapidly replaced by the 3-ADON producing strains in western Canada (Ward *et al*., 2008; Guo *et al*., 2008). Ward *et al*. (2008) analysed the trichothecene chemotype distributions across Canada and revealed a distinct longitudinal cline in which 3-ADON producing strains were significantly higher in eastern Canada than in western Canada. They also reported that the 3-ADON chemotype frequency in western Canada increased more than 14-fold from 1998 to 2004. Based on analyses of genetic variation at variable number tandem repeat (VNTR) loci, the 3-ADON populations in North America appeared to be more closely resemble the Italian population than the native 15-ADON population. Taken together, these observations indicated that the 3-ADON strains were first introduced to eastern Canada and have spread west across Canada, then moved down towards the upper Midwest of the United States. Strains producing 3-ADON represent genetically different populations and demonstrate different phenotypic characteristics than the native 15-ADON chemotypes: 3-ADON populations show higher growth rates, produce more conidia, and have the capacity to accumulate more trichothecenes than 15-ADON populations. Therefore, 3-ADON populations are considered more toxigenic and potentially more vigorous than the native 15-ADON populations. It is

believed that these characteristics provided a direct fitness advantage to the 3-ADON populations which allowed them to invade the local 15-ADON populations and spread rapidly in the region (Ward *et al*., 2008; Puri & Zhong, 2010, Kelly *et al*., 2015).

A similar situation was also observed in the upper Midwest of the United States, where Gale *et al.* (2007) reported a high frequency of 3-ADON strains among the more prevalent 15-ADON strains. A genetic analysis using restriction fragment length (RFLP) fingerprinting showed that 3-ADON strains were genetically more similar to the Italian strains. It was hypothesised that the 3-ADON strains might have migrated from Europe into Canada and the USA. In the upper Midwest USA, Gale *et al*. (2011) reported a presence of the "Northland population", which has the 3-ADON genotype, but surprisingly these strains do not produce any trichothecenes on inoculated spikes. Apart from *F. graminearum s.s*, other species within the FGSC have been discovered in the United States. Starkey *et al*. (2007) reported the presence of *F. gerlachii*, a new species within the FGSC, which is present at very low frequency in Minnesota, North Dakota and Wisconsin. The species and chemotype distribution is more complex in southern parts of the United States. New *Fusarium* populations have been discovered in small grain growing regions of Louisiana, named Gulf coast population that consists of 3-ADON producers and *F. louisianense*, which consist of NIV producers (Gale *et al*., 2011; Starkey *et al*., 2011). Also Gale *et al*. (2011) reported the presence of NIV producing *F. asiaticum* strains for the first time in the United States. The discovery of NIV producing *F. louisianense* and *F. asiaticum* species seems to overlap with the rice growing areas in the southern United States.

Very recently Varga *et al*. (2015) have reported the presence of a novel chemotype named as NX-2 in strains collected from the Midwestern US. These strains produce a novel Type A trichothecene known as, 3α-acetoxy-7α, 15-dihydroxy-12, 13-epoxytrichothec-9-ene, which is structurally similar to 3-ADON but lacks a keto group at C-8 position in the core trichothecene structure. Genetic analysis revealed that the allelic variations in the *TRI1* gene are responsible for the NX2 chemotype (Liang *et al*., 2014; Varga *et al*., 2015).

2.7.2 Distribution of FGSC in South America

Surveys done in both North and South America have shown that *F. graminearum s.s*, appears to be the dominant species associated with FHB in wheat and barley. In South America, six other species in the FGSC were discovered in wheat and other cereal crops in the Southern Cone (geographic region composed of the southernmost areas of South America), Argentina, Brazil and Uruguay. These species include *F. meridionale*, *F. austroamericanum*, *F. boothii*, *F. brasilicum*, *F. cortaderiae* and *F. asiaticum*. Among these species, *F. meridionale*, *F. brasilicum*, and *F. boothii* are supposed to be endemic to South America. Other species in the FGSC are either geographically limited, or appeared to exhibit a possible host preference (Ward *et al*., 2008; Astolfi *et al*., 2012). Studies on FHB in South America have been mainly done in the Southern Cone, Argentina, Brazil and Uruguay. In Argentina, the fourth largest exporter of wheat in the world, the main causal agent of FHB in wheat appears to be *F. graminearum s.s* 15-ADON producers (Reynoso *et al*., 2011; Malbrán *et al*., 2014). Although *F. graminearum s.s* is more prominent in wheat, other members in the FGSC such as *F. meridionale* and *F. boothii* were more prevalent in infected maize fields of northwest Argentina. All strains of *F. meridionale* were found to be NIV chemotype and *F. boothii* strains were either DON or 15-

ADON type (Sampietro *et al*., 2010). In Brazil, a multiyear survey conducted using 671 FGSC strains discovered five species and three trichothecene genotypes. *F. graminearum* 15-ADON genotype was more prominent followed by *F. meridionale* (NIV), *F. asiaticum* (NIV), *F. cortaderiae* (NIV/3-ADON) and *F. austroamericanum* (NIV/3-ADON) (Del Ponte *et al*., 2013).

A similar species and chemotype distribution was observed in Uruguay. Umpiérrez-Failache *et al*. (2013) observed significant regional differences in the composition of FGSC species and trichothecene types within Uruguay *Fusarium* strains. *F. graminearum s.s* with the 15-ADON type were the most prevalent in western provinces while *F. asiaticum* and the NIV type predominated in the new wheat production zone along Uruguay's eastern border with Brazil. These areas overlap with the rice growing areas in Uruguay.

2.7.3 Distribution of FGSC in Europe

Before the year 2000, *F. culmorum* was the major causal agent of FHB in Europe (Waalwijk *et al*., 2003); however, a rapid population shift from *F. culmorum* to *F. graminearum*, which has led to *F. graminearum* being the principle species of FHB in Europe (Nielsen *et al*., 2011). Studies on species and chemotype distribution of FGSC were reported in many countries in Europe including: UK, Germany, Hungry, Netherlands, Norway, Italy, Finland, Austria, Poland and France (Jennings *et al*., 2004; Szécsi *et al*., 2005; Tóth *et al*., 2005; Stepien *et al*., 2008; Waalwijk *et al*., 2008; Talas *et al*., 2011; Prodi *et al*., 2009; 2011; Somma *et al*., 2014; Boutigny *et al*., 2014; Przemieniecki *et al*., 2014; Aamot *et al*., 2015). In all countries, *F. graminearum s.s* was identified as the dominant FGSC species causing FHB in wheat, barley, and maize. The populations were predominately 15-ADON, but some 3-ADON and NIV chemotypes were also observed. In Hungry, a novel species within the FGSC was discovered using the multilocus

genotyping (MLGT) assay, and named as *F. vorosii*. *F. vorosii* formed a sister group with *F. asiaticum* in the combined multilocus phylogeny and found to be a 15-ADON producer (Starkey *et al*., 2007).

2.7.4 Distribution of FGSC in Asia

During the last decade, FHB outbreaks have been more frequent and severe in China, making wheat a less attractive crop for farmers. The recent epidemics took place in wheat growing areas in the middle and lower valleys of the Yangtze River in southwest China. A large scale survey was done in China covering 15 provinces, where 457 *Fusarium* strains were isolated from infected wheat spikes. Among the discovered *Fusarium* species, *F. asiaticum* appeared to be the most prominent species followed by *F. graminearum s.s* and *F. meridionale* (Zhang *et al*., 2012). Most of the *F. asiaticum* strains identified were either 3-ADON or NIV producers, whereas all the *F. graminearum s.s* strains were of the 15-ADON type. A similar study conducted on barley also revealed that *F. asiaticum* is the primary source of FHB in southern China and only a limited percentage of other species in the FGSC were present (Zhang *et al*., 2010). In contrast, *F. graminearum s.s* was found to be the major source of FHB in the North/Northeast China (Zhang *et al*., 2012).

In Japan, the distribution of *Fusarium* species in wheat and barley showed a distinct geographic distribution. *F. graminearum s.s* was prominent in the north parts of Japan and *F. asiaticum* was more prevalent in the southern region (Suga *et al*., 2008). The distribution of species was strongly correlated with the mean temperatures in the regions. *F. graminearum s.s* favored the cooler climates, whereas *F. asiaticum* preferred warmer climates. Trichothecene chemotype distribution was also different between the two species. All *F. graminearum s.s*

strains were either 3-ADON or 15-ADON type, but the majority (70%) of the *F. asiaticum* strains were of the NIV type. In the central part of Japan, both *F. graminearum s. s.* and *F. asiaticum* were isolated at similar frequency, but no hybrids were detected in the area. This evidence suggested a limited gene flow between these species under natural conditions. In addition to the above species, a novel FGSC species, *F. vorosii* was also isolated from infected wheat heads in Hokkaido, Japan (Starkey *et al*., 2007).

In regions of Asian Russia and European Russia, *F. graminearm s.s* is the major causal agent of FHB in wheat, barley and other cereals. The trichothecene chemotypes were discovered in equal frequencies, where 15-ADON producing strains represented a slightly higher frequency than the 3-ADON type; however, NIV producing strains were not identified in the survey (Yli-Mattila *et al*., 2009). A novel Asian clade within the FGSC was also discovered by Yli-Mattila *et al*. (2009) from the Russian Far East. The authors in this publication collected 223 FGSC strains from Finland, European Russia, Asian Russia and China and subjected them to MLGT where a novel species in the FGSC, termed *F. ussurianum*, was identified. This species illustrated a 3-ADON chemotype based on the limited sampling. In addition to the newly described species, a 15-ADON producer, *F. vorosii* was also found in Far East Russia.

A study done by Sarver *et al*. (2011) illustrated the discovery of a new species in the FGSC, termed *F. nepalenses*, which was isolated from samples collected from infected rice in Nepal. *F. nepalenses* formed a sister group with *F. ussurianum* and *F. asiaticum* in a phylogenetic tree constructed using a combined set of 12 gene sequences.

2.7.5 Distribution of FGSC in Africa

There have been limited studies conducted on species distribution and chemotype diversity of FGSC in Africa. In a study carried out by O'Donnell *et al*. (2008), 31 strains were collected from wheat seeds in Ethiopia where they discovered a new species in FGSC, termed *F. aethiopicum*. *F. aethiopicum* was reported to be a 15-ADON producer. Also, another new species in FGSC was discovered from South Africa, *F. acacia-mearnsii*, and found to be a NIV producer. Both *F. aethiopicum* and *F. acacia-meransii* are sister species and may be endemic to the Southern Hemisphere. A detailed study done by Boutigny *et al*. (2011) reported that *F. graminearum s.s* was the predominant FHB causing agent in South America and most of the strains were of 15-ADON type. Apart from *F. graminearum s.s*, five other species in the FGSC were also found in infected wheat, barley and above-ground parts of corn, which included *F. boothii*, *F. meridionale*, *F. cortaderiae*, *F. acacia-mearnsii* and *F. brasilicum*.

2.7.6 Distribution of FGSC in Australia and New Zealand

In Australia, both *F. graminearum* and *F. pseudograminearum* act as the major cause of FHB (Obanor *et al*., 2013). The presence of other species in the FGSC has not yet been reported. Both DON and 3-ADON chemotypes have been detected in the infected wheat samples in Western Australia (Tan *et al*., 2012). In New Zealand *F. graminearum s.s* is the most predominant species. *F. graminearum s.s* strains showed both DON and NIV chemotypes in grain samples collected from six regions in New Zealand (Lauren *et al*., 1992; Di menna *et al*., 1997). NIV producing *F. cortaderiae* species have also been reported in infected maize kernels in New Zealand (Monds *et al*., 2005).

2.8 Management of FHB

2.8.1 Genetic resistance

Cultivation of *Fusarium* resistant varieties is one of the major management strategies to control FHB and reduce the risk of mycotoxin contamination. Therefore, breeding for improved FHB resistance has become an important goal for wheat breeders. Resistance to FHB in wheat is a quantitative trait governed by many genes (Buerstmayr *et al*., 2009). There are five types of resistance to FHB: Type I (resistance to initial infection), Type II (resistance to spread of symptoms within the head), Type III (resistance to kernel infection), Type IV (tolerance), and Type V (resistance to DON accumulation (Schroeder & Christensen, 1963; Miller *et al*., 1985; Wang & Miller, 1988; Mesterházy *et al*., 1995; Mesterházy *et al*., 1999; Bushnell *et al*., 2003). So far, no specific genes have been identified to confer resistance to FHB. Nonetheless, many quantitative trait loci associated with FHB resistance have been detected. A review on FHB resistance QTL lists 52 QTLs associated with FHB (Buerstmayr *et al*., 2009). QTLs linked to FHB resistance have been identified in all chromosomes in the wheat genome except for 7D (Buerstmayr *et al*., 2009). Even chromosome 7D has now been found to be involved in FHB resistance (Cativelli *et al*., 2011). FHB resistance QTLs can be divided in to two main categories, 3BS (*Fhb1*) for Type II resistance and 5AS for Type I resistance. Most of the research has focused on Type II resistance and studies on other types of resistance are limited. Lemmens *et al*. (2005) reported a QTL, *Qfhs.ndsu-3BS* that was linked with toxin accumulation. They hypothesised that *Qfhs.ndsu-3BS* either encodes for a DON-glucosyltransferase or regulates the expression of such an enzyme. A large number of resistance sources showing variable resistance to FHB are available from different geographical regions. These sources of resistance can be divided into three main groups; the first group is composed of exotic, highly resistant lines such

as the Chinese spring wheat lines, Sumai3, Ning 7840, Ning 8343, Nubai, Wangshubai and Nobeokabouzu. These are the most widely used and best characterized FHB resistance sources in the world. Most of these lines show Type II resistance and have the 3BS QTL (*Fhb1*) (Chen *et al*., 2005; Cuthbert *et al*., 2006; Mardi *et al*., 2006; Yu *et al*., 2008). Other resistance sources, Frontana and Encruzilhada from Brazil, show Type I resistance (Mesterházy, 1997). The second group is composed of alien species that exhibit more FHB resistance than Sumai3. These species are *T. aestivum-Leymus racemosus*, *T. aestivum –Roegneria komoji* and *T. aestivum-R. ciliarisc* (Chen *et al*., 2005). The third group includes local sources showing FHB resistance, the level of FHB resistance in these lines are lower compared to group 1 and group 2 lines. Although these lines, showed less resistance, it is important to incorporate these local sources into breeding programs as they show the best agronomic adaptation in a given region (Chen *et al*., 2005).

2.8.2 Chemical control

Use of chemicals is one of the major components of integrated FHB management. It has been shown that no single strategy is effective against managing FHB. Several research trials provided evidence that a combination of strategies such as crop rotation, cultivar selection, and fungicide use reduced FHB severity and DON levels in an additive manner (McMullen *et al*., 2008; Amarasinghe *et al*., 2012; Gilbert & Haber, 2012; Wegulo *et al*., 2012). Fungicides having the triazole chemistry, such as prothioconazole, tebuconazole and metconazole, are considered to be the most effective fungicides (Edwards *et al*., 2001; Simpson *et al*., 2001; Pirgozliev *et al*., 2002; Mesterházy *et al*., 2003). Triazole based fungicides inhibit the 14α demethylase, an enzyme that is essential for ergosterol biosynthesis (Klix *et al*., 2007). Use of fungicides to control FHB under field conditions has generally been inconsistent. Therefore, before 1997, plant pathologists

were doubtful about the use of fungicides on FHB control. During that time propiconazole or Tilt from Syngenta Crop Protection was used extensively, but the product showed reduced efficacy in controlling FHB. However, in 1997, with the introduction of tubeconazole fungicides (Folicur, Bayer Crop Science) and the positive results from research, the attitudes regarding the use of fungicides in controlling FHB began to change. The amount of disease on crops, cultivar resistance, climatic factors, crop sensitivity and yield potential are the main factors that should be considered when selecting appropriate fungicides and doses (Mesterházy, 2003). A study conducted by Paul *et al*. (2008) used a multivariate meta-analysis to determine the effective combinations and doses of propiconazole, prothioconazole, tebuconazole, metconazole fungicides. Their results suggested that although all of the tested triazoles provided significant reduction in FHB and DON accumulation, metconazole and prothioconazole were more effective than tebuconazole, and propiconazole was the least effective (Paul *et al*., 2008). In addition, they observed that the fungicides were more effective for spring wheat than in winter wheat, suggesting that the timing of fungicide application also plays a key role in FHB control. A similar result in terms of fungicide application was reported by Hollingsworth *et al*. (2006), where prothioconazole alone and in combination with tebuconazole, and metconazole were more effective in controlling FHB than either tebuconazole or propioconazole alone (Hollingsworth *et al*., 2006). These findings together clearly illustrate a trend in the effectiveness of these three chemicals. The timing of fungicide application also plays a key role in FHB control. It has been reported that multiple fungicide applications might give an additional protection against FHB development and DON accumulation. A study done by Edward & Godeley (2010) reported that the application of prothioconazole at Zadoks growth stages (GS), 31, 39 and 65 controlled FHB severity by 50, 58 and 83%, respectively. The reduction of FHB severity achieved by all three

timings was 97%. The reduction of DON content at the above growth stages was 27%, 49% and 57%, respectively (Edward & Godely, 2010). A study conducted in Japan illustrated that the application of fungicides 20 days after anthesis (late milk stage) reduced FDK and mycotoxin contamination without reducing FHB severity (Yoshida *et al*., 2012). Interestingly, some evidence has suggested that the application of fungicides several weeks before wheat anthesis may increase the FHB disease severity as fungicides may inhibit the growth of non-toxigenic microorganisms and subsequently promote the spread of toxigenic *Fusarium* species in the field. (Henriksen & Elen, 2005). Taken together, these findings suggest that there may be a narrow window in which the application of these fungicides determines the overall effectiveness in terms of disease control.

2.8.3 Biological control

The use of fungicides has adverse effects on humans, animals, microorganisms, and the environment. Therefore, use of biocontrol agents may help to minimize the risks posed by fungicide application. Many microorganisms have been identified to show antagonistic reaction towards *Fusarium* spp. and include bacteria, fungi and yeast. Bacterial genera like *Bacillus*, *Pseudomonas* and *Streptomyces* have been widely studied as antagonistic agents against *Fusarium* spp. Studies done by Wang *et al*. (2007) and Chan *et al*. (2009) have reported that compounds produced by *Bacillus* spp. can supress growth and DON production in infected host plants. Some antagonistic bacteria can degrade DON by using DON as a source of carbon. Sato *et al*. (2012) identified 13 strains of DON degrading bacteria (DDB) in which, nine belonged to the gram-positive genus *Nocardioides* and the other four belonged to the gram-negative genus *Devosia*. All bacteria identified were able to reduce the DON concentrations from 100

ppm to less than 0.5 ppm. The gram positive genus *Nocardiodies* utilized DON as a carbon source unlike the gram negative genus *Devosia* (Sato *et al*., 2012). Another study conducted by Palazzini *et al*. (2007), identified 22 strains of bacteria from a total of 365 that were capable of reducing the FHB severity and DON accumulation. These bacteria appeared to belong to *Brevibacillus* spp. and *Streptomyces* spp. This suggests that these types of bacterial strains could be utilized to reduce DON contamination by 60-100% in irradiated wheat grains.

Antagonistic fungi may also reduce the survival and growth of nectrotrophic pathogens in crop residues and help increase the rate of decomposition (Kohl & Fokkema, 1998). For example, *Trichoderma* spp. have been used as fungal biocontrol agents and have proved to be the most effective, where more than 50 products are registered worldwide. These fungal species utilize various products as their carbon and nitrogen source; therefore, they are well adapted to live on other fungi. Also, they are resistant to many pesticides and other toxic substances, making them ideal for use in an integrated disease management system. *Trichoderma harzianum* is among the most effective biocontrol agents in reducing perithecial production, both under controlled and field environments (Inch & Gilbert, 2007). A study done using scanning electron microscopy illustrated that *T. harzianum* produced secondary metabolites that interacted with perithecial development and resulted in abnormal perithecia, and a reduction in pathogen colonization in host tissue (Inch & Gilbert, 2011). Other *Trichoderma* strains namely, *T. gamsii* 6085, 6317 and *T. volutinum* 4837 reduced both *F. graminearum* growth and DON production on a rice substrate. These *Trichoderma* strains were able to reduce *F. graminearum* DON production from 60-92% at 14 days post-inoculation (Matarese *et al*., 2012). A study done by Schoneberg *et al*. (2015) demonstrated that all 10 *Trichoderma* strains used in their study significantly reduced the colony area of *F. graminearum* in co-culture. Also, the fungi were able

to reduce the number of perithecia and ascospores on wheat straw by 88-100% when inoculated before the pathogen. Luongo *et al*. (2005) studied the potential of saprophytic fungi collected from other cereal crops to supress the sporulation of *Fusarium* spp. in wheat straw. They discovered that strains of *Clonostachy rosea* consistently reduced the sporulation of *F. graminearum* and *F. culmorum* on wheat straw. However, when these strains were tested under field conditions the results were not conclusive. Another strain of *C. rosea*, ACM941 has been extensively studied by Xue *et al*. (2009). This particular group examined the antibiosis ability of *C. rosea* ACM941 towards *Gibberellazeae* under *in vitro*, greenhouse, and field conditions compared to the registered fungicide Folicur (tebuconazole). They found that ACM941 reduced the mycelial growth of *G. zeae* by 53% in dual culture and completely suppressed the macroconidium germination of *G. zeae* in co-culture. Under the simulated natural epidemic conditions *C. rosea* ACM941 reacted similar to the fungicide Folicur, and it reduced infected spikelets by 44-51%, FDK by 33-68%, and deoxynivalenol (DON) in grains by 10-28% (Xue *et al*., 2009). Even though research has identified effective biocontrol agents against *F. graminearum*, there is only limited success in field applications. Therefore, it appears that much research is still needed to develop more effective, stable, and less expensive biocontrol agents.

2.8.4 Cultural control

Cropping history, crop rotation, and the type of tillage play a main role in FHB disease development and DON production. The primary inoculum survives saprophytically on host crop debris, therefore, to avoid subsequent infections; a minimum of two years of rotation is required. Wheat-corn rotations are still practised in some regions. A study conducted by Dill-Macky & Jones (2000) reported that FHB disease severity was higher when wheat was grown after corn

than after wheat. Similar results have been observed by Pirgozliev *et al*. (2003), where FHB infection increased by 15% when wheat was grown after corn; however, when wheat was grown after alfalfa and oats, the increment was only 4%. These findings suggest that the rate of decomposition of corn residues is likely slower than other residues and the amount of inoculum on corn may be higher than other crops (Schaafsma *et al*., 2001). Unlike rotation, it appears that the results reporting the effect of tillage in FHB development is not consistent. Some studies have shown that tillage does not have a significant effect on FHB severity and kernel infection (Miller *et al*., 1998; Dill-Macky & Jones, 2000). In contrast, a study done by Schaafsma *et al*. (2005) reported that previous crop, size of the field, and tillage affect the FHB index, FDK, and DON production in infected fields. Another cultural practice that can be applied to reduce the inoculum levels in the field (Pirgozliev *et al*., 2003) is weed control, as weeds have a tendency to act as a source of inoculum for FHB development. A study done by Teich & Nelson (1984) showed that fields with higher weed densities had higher numbers of infected heads than in fields free of weeds.

CHAPTER 3

NIVALENOL PRODUCING *FUSARIUM CEREALIS* ASSOCIATED WITH FUSARIUM HEAD BLIGHT IN WINTER WHEAT IN MANITOBA, CANADA.

C. C. AMARASINGHE[1], S.A. TITTLEMIER[2] AND W. G. D. FERNANDO[1]

[1]Department of Plant Science, University of Manitoba, Winnipeg, Manitoba Canada. R3T2N2

[2]Grain Research Laboratory, Canadian Grain Commission, Winnipeg, Manitoba, Canada.

The author C. C. Amarasinghe carried out the research, analyses, interpretation of data and wrote the manuscript as a part of her Ph. D thesis. The major supervisor Dr. W.G.D. Fernando supervised the work and reviewed the manuscript. Dr. S.A. Tittlemier conducted the GC-MS/MS for mycotoxin analysis and reviewed the manuscript. The authors read and approved the final manuscript.

The manuscript was published in Plant Pathology 2015, 64: 988-95.

3.1 Abstract

Fusarium head blight (FHB) in small grain cereals is primarily caused by the members of the *Fusarium graminearum* species complex. These produce mycotoxins in infected grains, primarily deoxynivalenol (DON); acetylated derivatives of DON, 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON); and nivalenol (NIV). This study reports the isolation of *F. cerealis* in infected winter wheat spikes for the first time in Canada. A phylogenetic analysis based on *TRI101* gene and *F. graminearum* species-specific primers revealed two species of *Fusarium*: *F. graminearum sensu stricto* (127 strains) and *F. cerealis* (5 strains). Chemotype determination based on *TRI3* gene revealed that 65% of the strains were 3-ADON, 31% were 15-ADON and 4% NIV producers. All the *F. cerealis* strains were of NIV chemotype. *F. cerealis* strains can often be misidentified as *F. graminearum* as the morphological characteristics are similar. Although the cultural and macroconidial characteristics of *F. graminearum* and *F. cerealis* strains were similar, the aggressiveness of these strains on susceptible wheat cultivar Roblin and moderately resistant cultivar Carberry differed significantly. The *F. graminearum* 3-ADON strains were most aggressive followed by *F. graminearum* 15-ADON and *F. cerealis* NIV strains. This study confirms the continuous shift of chemotypes from 15-ADON to 3-ADON in North America. In Canada, the presence of NIV is limited to barley samples and the discovery of NIV producing *F. cerealis* species in Canadian wheat fields may pose a serious concern to the Canadian wheat industry in the future.

3.2 Introduction

Fusarium head blight (FHB), also called scab, is an economically important fungal disease in many crops including wheat, barley and oat (Parry *et al*., 1995). Although many species of *Fusarium* contribute to this disease, *Fusarium graminearum* (teleomorph *Gibberella zeae*) is considered to be the major pathogen of FHB in many countries (Parry *et al*., 1995; McMullen *et al*., 1997). *Fusarium culmorum*, *Fusarium avenaceum*, *Microdochium nivale*, *Fusarium verticilloides, Fusarium oxysporum* and *Fusarium poae* are the other related species that play a minor role in FHB development (Liddell, 2003; Parry *et al*., 1995). These fungi, in association with FHB in cereals, produce mycotoxins that lead to contamination of grains posing a concern to the cereal industry. For example, contamination of wheat grains with potent mycotoxins alters the milling, baking and pasta making properties of the grains.

Fusarium cerealis (syn. *Fusarium crookwellense*) is an important pathogen that causes root rot and seedling blight of cereals. *F. cerealis* has been reported in North America, South Africa, Australia, New Zealand, China, Japan and European countries (De Nijs *et al*., 1996; Tan *et al*., 2004; Srobarova *et al*., 2008). In Europe, *F. cerealis* was often found within the FHB pathogen complex causing red rot in corn (Logrieco *et al*., 2003). *F. cerealis* is reported to be the second most widespread pathogen on corn heads and leaves in New Zealand (Lauren *et al*., 1999). Sugiura *et al*. (1994) reported the presence of *F. cerealis* in the complex of wheat blight pathogens in Japan. Recently, Schmale *et al*. (2011) have reported on one strain of *F. cerealis* found in wheat fields in eastern USA.

The type B trichothecene mycotoxins deoxynivalenol (DON) and nivalenol (NIV) are the main mycotoxins found in *Fusarium*-infected wheat kernels. *F. graminearum*, *F. culmorum* and *F. cerealis* strains produce type B trichothecenes. Although *F. graminearum* strains produce

DON and its acetylated derivatives 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON), and NIV, no single *F. graminearum* strain is known to produce both DON and NIV (Goswami & Kistler, 2005). Unlike *F. graminearum*, *F. cerealis* strains do not produce DON, but they produce NIV (Ichinoe *et al*., 1983). It has been reported that *F. cerealis* may also produce fusaric acid, zearalenone, fusarin C and diacetoxyscirpenol (Bacon *et al*., 1996; Bottalico *et al*., 2002).

In Canada, DON is the primary mycotoxin in *Fusarium*-infected grain (McMullen *et al*., 1997), but in Asia and Europe both DON and NIV are common contaminants of grain (Ichinoe *et al*., 1983). The acetylated derivatives of DON, 3-ADON and 15-ADON, increase the total DON contamination in wheat. Although NIV is less pathogenic to plants, it has been reported that NIV is more toxic to animals than DON (Ueno, 1977; Visconti *et al*., 1991). Both DON and NIV pose a significant threat to the human and animal systems by inhibiting the protein synthesis process.

Molecular techniques have allowed the differentiation of *F. graminearum* from other species (Demeke *et al*., 2005) and provided genetic information on the species populations (Cumagun *et al*., 2004; Fernando *et al*., 2006). PCR assays have also been developed to characterize the different chemotypes (3-ADON, 15-ADON, NIV) of *Fusarium* spp. (Ward *et al*., 2008). These molecular techniques help to identify the type of mycotoxins produced by different *Fusarium* spp. and their genetic diversity. Lee *et al*. (2002) reported the importance of *TRI13* gene in the *TRI5* cluster. The *TRI13* gene is responsible for regulating the DON-NIV switch in *Fusarium* strains. High throughput multilocus genotyping (MLGT) has been extensively used in *Fusarium* species identification. One of the major genes used in species identification is *TRI101* (O'Donnell *et al*., 2004; Starkey *et al*., 2007). This gene encodes a trichothecene 3-*O*-acetyltransferase that transfers an acetyl group from acetyl-CoA to the C-3

hydroxyl moiety of the trichothecene molecule (Garvey *et al*., 2008). Disruption of *TRI101* in *Fusarium sporotrichioides* blocks the production of T-2 toxin, a trichothecene-derived metabolite, and leads to the accumulation of an intermediate, isotrichodermol, suggesting that the *TRI101* gene product is necessary for the production of trichothecenes (McCormick *et al*., 1999).

To the best of the author's knowledge, NIV producing *F. graminearum* strains have not yet been reported in *Fusarium*-infected wheat fields in Canada. Gale *et al*. (2011) reported that populations of *F. graminearum* collected from infected wheat spikes in Louisiana were mainly composed of NIV producing strains. However, in Canada testing for NIV is not routinely conducted at mills or elevators. Therefore, the discovery of *Fusarium* spp. that is capable of producing NIV may cause increasing concern to the wheat industry in Canada. This study reports the presence of NIV producing *F. cerealis* in infected winter wheat plots in Manitoba, Canada, for the first time. The objectives of this study were 1) to identify *Fusarium* spp. in winter wheat plots grown in Carman, Manitoba, Canada; 2) to determine the chemotype diversity of *Fusarium* spp.; and 3) to characterize the ability of *Fusarium* spp. to induce FHB on wheat in greenhouse pathogenicity experiments.

3.3 Materials and methods

3.3.1 Sample collection and isolation

Wheat spikes with symptoms were collected from seven naturally infected winter wheat lines, CDC Buteo, Accipiter, CDC Falcon, McClintock, Moats, 39M*11 and AC Readymade, grown at the Ian Morrison Research Farm, Carman, Manitoba, Canada in 2013. Twenty to twenty-five spikes were collected from each cultivar plot. Wheat spikes with top, middle and bottom infection as well as full spike and peduncle infection were collected separately, placed in paper

bags and transported to Winnipeg, Manitoba. Samples were stored at -20°C until processing. Isolations were done from each spike. Infected seeds were surface disinfected in 1% sodium hypochlorite for 1 min and air dried on sterile filter paper. Seeds were plated individually on potato dextrose agar (PDA) (Difco Laboratories, ON, Canada) and incubated at 25°C for 4-7 days under fluorescent light. The plates were then periodically examined for *Fusarium*-like mycelia. As *Fusarium* spp. grew out of the seeds, colonies were chosen and sub-cultured to obtain pure cultures. All strains were then plated onto synthetic nutrient agar (SNA) (0.2 g glucose, 0.2 g sucrose, 1 g potassium dihydrogen phosphate, 1 g potassium nitrate, 0.25 g magnesium sulfate anhydrous, 0.5 g potassium chloride and 14 g technical agar in 1 L of distilled water) plates in order for sporulation to occur. Sporodochia in the SNA medium were washed using a drop of 50 µL sterile distilled water, and the macroconidia suspension was spread over water agar (WA) plates. These plates were incubated under the same conditions for 4-7 days and a single germinating macroconidium was transferred onto PDA plates and incubated at 25°C for 4-7 days under fluorescent light. These single spore strains were preserved on filter paper discs and stored at -20°C until further use.

3.3.2 DNA extraction

Single spore strains were placed on PDA and incubated as described above. Once plates were covered with fungal growth, mycelia were harvested, lyophilised and stored at -20°C until further use. DNA extraction was carried out according to Fernando *et al*. (2006). Briefly, the lyophilised mycelia (~100 mg) were broken into smaller pieces using sterile toothpicks followed by grinding in 600 µL TES buffer (100 mM Tris, 10 mM EDTA, and 2% sodium dodecyl sulfate) in a 1.5 mL microcentrifuge tube using a pellet pestle. After this, 140 µL of 5 M NaCl

and 70 µL of 10% cetyltrimethylammonium bromide (CTAB) were added to the tube and vortexed. The mixture was incubated at 65°C for 20 min. Following incubation, 600 µL of phenol: chloroform: isoamyl alcohol (25:24:1 vol/vol) was added and then centrifuged at 10,000 rpm for 15 min. The supernatant was transferred into a new tube and the latter step was repeated. DNA was precipitated by adding 80 µL of 5 M NaCl and 1 mL of 100% ethanol, followed by centrifugation at 13,000 rpm for 5 min. The DNA pellet was washed with 200 µL of ice-cold 80% ethanol. After air-drying, the pellet was suspended in 400 µL of warm sterile water (65°C). Following full resuspension, DNA was treated with RNase (0.75% vol/vol) and stored at -20°C until further use.

3.3.3 Identification of strains to species using PCR and sequencing

Strains were first amplified using *F. graminearum* species-specific PCR primers described by Demeke *et al*. (2005). Two primers were used in the PCR, Fg16F (5′- CTCCGGATATGTTGCGTCAA-3′) and Fg16R (5′-GGTAGGTATCCGACATGG CAA-3′), which amplify a fragment of 450 bp. PCR was performed in a 25 µL reaction volume containing 20 ng template DNA, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris HCl (pH 8.0), 0.2 mM of each dNTP (Invitrogen Life Technologies, CA, USA), 0.4 µM of each primer, and 0.75 units Taq DNA polymerase (Invitrogen Life Technologies, CA, USA). PCR amplification protocol consisted of an initial denaturation at 95°C for 3 min, followed by 35 cycles of 30 s at 95°C, 1 min at 56.7°C, 1 min at 72°C, and a final extension of 72°C for 5 min. PCR products were separated on 1% agarose gel.

To identify strains that did not amplify with *F. graminearum* species-specific primers, a portion of the *TRI101* gene was amplified and bidirectionally sequenced. The primers used for

amplification were TRI101F (5′- CCATGGGTCGCRGGCCARGTSAA-3′) and TRI101R (5′-AACTCSCCRTCIGGYTTYTTNGGCAT-3′) (Proctor *et al*., 2009). PCR was performed in a 25 µL reaction volume as described above. The PCR amplification protocol consisted of an initial denaturation at 95°C for 2 min, followed by 30 cycles of 40 s at 95°C, 30 s at 54°C, 90 s at 72°C, and a final extension of 72°C for 7 min. For sequence analysis, PCR-amplified DNA fragments were purified with Exosap-IT PCR product clean up kit (Affymetrix Inc., Santa Clara, CA, USA), according to the manufacturer's instructions. Sequencing reactions were carried out using the BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA), with reaction products analyzed on an Applied Biosystems 3730xl DNA Analyzer at Eurofins MWG Operon (Huntsville, AL, USA). Sequences were assembled, trimmed and edited using GENEIOUS v. 5.4.5 (Drummond *et al*., 2011). The sequences of the PCR products were aligned manually using BIOEDIT v. 7.1.3 sequence alignment editor (Hall, 1999). The final data set had an aligned length of 1208 bp. For estimating the appropriate model of sequence evolution, a hierarchical likelihood ratio test (hLRT) was carried out using MODELTEST v. 3.7 (Posada & Crandall, 1998). The hLRT criterion indicates that the HYM +G (Tamura & Nei, 1993) represent the optimal model for the data set. The Bayesian inference of phylogeny was performed using MR. BAYES v. 3.2.1 on the CIPRES SCIENCE GATEWAY v. 3.1. Posterior probability (PP) distributions of trees were created using the Markov Chain Monte Carlo (MCMC) method and following search strategies suggested by Huelsenbeck *et al*. (2002). Four chains were run simultaneously (10 000 000 generations), starting from random trees. Chains were collected every 1000 generations and the respective trees were written to a tree file. All runs reached a plateau in log likelihood score, which was indicated by the standard deviation of split frequencies (0.02) and the potential scale reduction factor, was close to one, indicating that our

four MCMC chains converged. The initial 2500 trees were discarded as burn-in before the stationary phase was reached. The 50% majority rule consensus tree was developed from the remaining 7500 trees. Calculation of the consensus tree and of the posterior probability of clades was done based upon the trees sampled after the burn-in, and trees were compiled and drawn using FIGTREE v. 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree). Sequences from Agricultural Research Services- ARS (NRRL) culture collection, reference strains were downloaded from GenBank and used in the alignment for species identification. *Fusarium pseudograminearum* was used as the out-group.

3.3.4 Determination of chemotypes using PCR assay

Chemotype identity of each strain was determined using multiplex PCR primers 3CON, 3NA, 3D3A, 3D15A (Ward *et al*., 2002). The multiplex PCR primers generated an 840 bp fragment from NIV producing strains, a 610 bp fragment from 15-ADON producers and a 243 bp fragment from 3-ADON producers, respectively. PCR was performed in a 15 µL volume containing 20 ng template DNA, 2.0 mM $MgCl_2$, 50 mM KCl, 10 mM Tris HCl (pH 8.0), 0.2 mM each dNTP (Invitrogen Life Technologies, CA, USA), 0.4 µM each primer, and 0.75 units Taq DNA polymerase (Invitrogen Life Technologies, CA, USA). PCR cycling conditions consisted of an initial denaturation at 94°C for 4 min, followed by 35 cycles of 1 min at 94°C, 40 s at 58°C, 40 s at 72°C, and a final extension of 72°C for 6 min. PCR amplicons were separated on a 2% agarose gel.

3.3.5 Analysis of colony morphology and macroconidia of *F. graminearum* and *F. cerealis*

To determine the cultural characteristics of *F. cerealis* and *F. graminearum*, strains were grown on PDA for 1 week in the dark at 22-23ºC. The macroconidia were produced in SNA, nutrient-deficient medium and observed under a microscope (Leica Microsystems Inc., ON, Canada) using LEICA APPLICATION SUITE v. 2.8.1.

3.3.6 Aggressiveness experiments and statistical analysis

F. cerealis and *F. graminearum* strains were individually inoculated on the susceptible wheat cultivar Roblin and moderately resistant wheat cultivar Carberry. Three strains each of *F. graminearum* 15-ADON, *F. graminearum* 3-ADON, and *F. cerealis* NIV strains were used in this study. Wheat plants were grown in plastic pots containing Sunshine Mix™ (Sun Gro Horticulture Ltd, MA, USA). Plants were fertilized once every two weeks with N-P-K (20:20:20) from two weekold stage till flowering. One tablespoon (~20 g) of the fertilizer was added to a gallon of water, which was used for watering the plants. The plants were arranged in a completely randomized design with ten replicates. *Fusarium* inoculations were done by point inoculations as described by Cuthbert *et al*. (2006). Three to four spikes were inoculated per plant; once individual spikes were close to 50% anthesis. Two florets in a spikelet were inoculated by injecting 10 µL of a macroconidial suspension adjusted to $5x10^4$ spores/mL between the lemma and palea of a floret. Following inoculation, a glassine bag was placed over the spike to increase humidity. The bag was removed 48 h post-inoculation. Disease severity was rated 7, 10, 14 and 21 days post-inoculation (dpi). Disease severity was rated using the FHB disease scale (Stack & McMullen, 1995). Disease severity measured the average percentage of spikelets that were infected, on a scale of 0% (indicating no infection) to 100% (indicating

completely infected spikes). Analysis of variance (ANOVA) for disease severity was performed using 'PROC MIXED' procedure of the SAS software (SAS version 9.3, SAS Institute Inc., Cary, NC). The wheat cultivar, strain and their interaction were considered as fixed effects.

3.3.7 Analysis of nivalenol in *F. cerealis* infected wheat samples

In order to confirm the production of NIV by *F. cerealis* strains, the infected wheat kernels were subjected to GC-MS analysis. Wheat kernels from ten replicated heads of each strain were pooled, ground and analysed by GC-MS according to the protocol described by Tittlemier *et al*. (2013). Eight *Fusarium* trichothecenes, DON, 3-ADON, 15-ADON, NIV, T-2 toxin, and its conversion product HT-2 toxin, diacetoxyscirpenol (DAS) and fusareon-X (FUS-X) were included in the GC-MS analysis with a quantitation limit of 0.05 mg kg^{-1}.

3.4 Results

Fusarium species identification was done by PCR using *F. graminearum* species-specific primers Fg16F/R and also by sequencing a portion of *TRI101* gene. Based on the Fg16F/R species-specific PCR assay, a 450 bp fragment characteristic of *F. graminearum* was obtained in 127 strains. Thus, these 127 strains were identified as *F. graminearum* (Fig 3.1). However, five strains did not show the expected 450 bp fragment. Therefore, these 5 strains along with 22 strains, randomly selected from the 127 PCR-positive strains for Fg-16F/R species-specific primers, were sequenced based on the *TRI101* gene. Partial sequences of *TRI101* gene were used to identify the five unknown strains to species level.

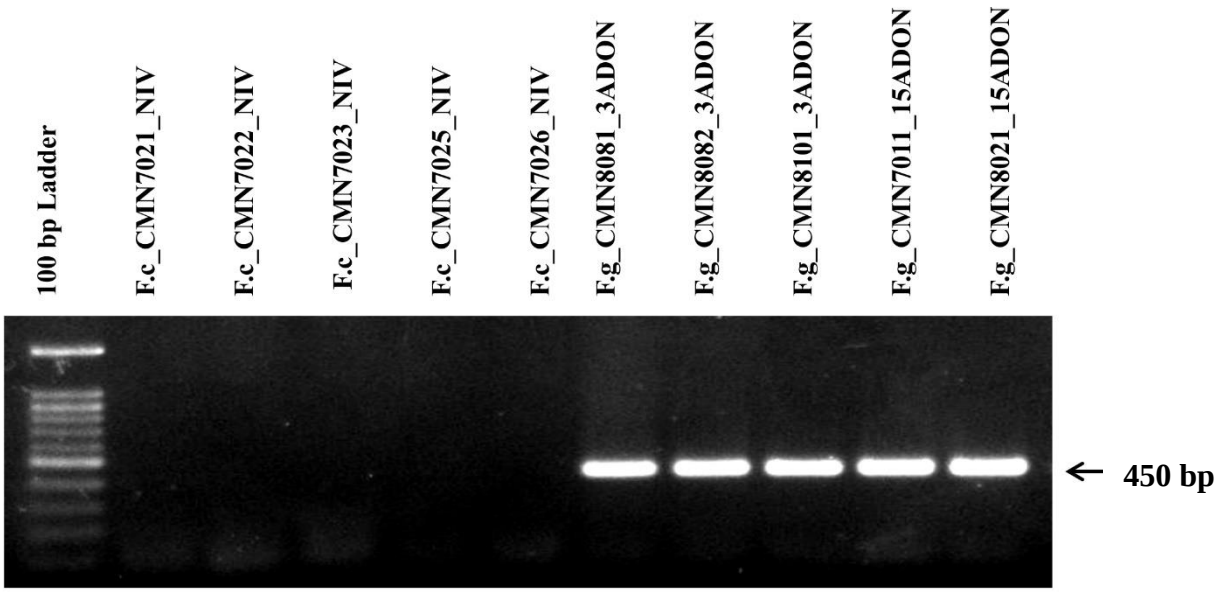


Figure 3.1 Representative PCR amplicons for Fg16 F/R *Fusarium graminearum* species-specific primers**.** PCR products of 450 bp are produced by *Fusarium graminearum* strains. Representative strains of *Fusarium cerealis* NIV (lane2-6), did not give any amplification for Fg16 F/R *Fusarium graminearum* species specific primers. *Fusarium graminearum* 3-ADON (lane7-9) and *Fusarium graminearum* 15-ADON (lane 10-11) gave the amplified product of 450 bp.

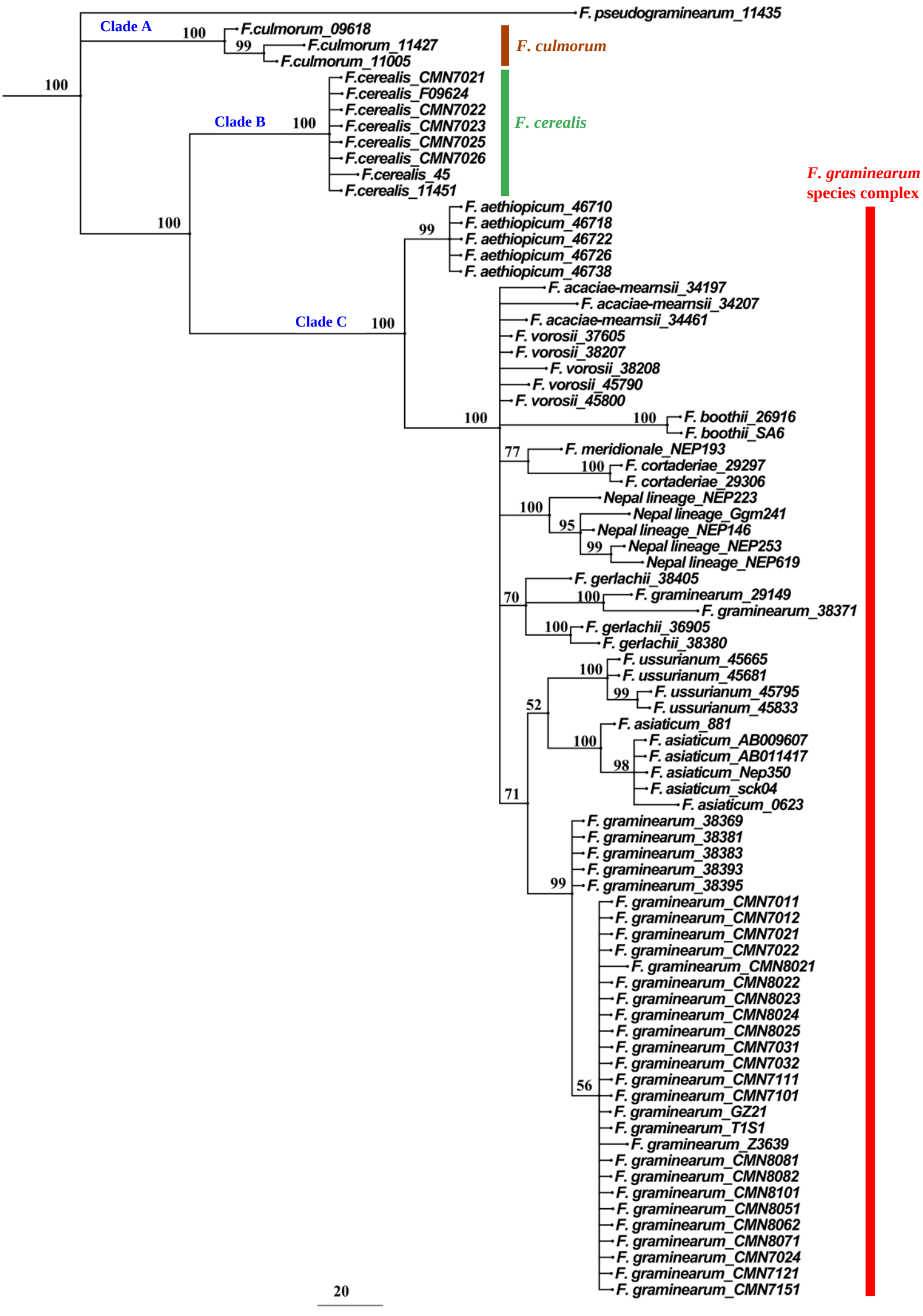

F. pseudograminearum_11435
Clade A
100
F.culmorum_09618
99
F.culmorum_11427
F.culmorum_11005
F. culmorum
100
F.cerealis_CMN7021
F.cerealis_F09624
F.cerealis_CMN7022
Clade B
100
F.cerealis_CMN7023
F.cerealis_CMN7025
F.cerealis_CMN7026
F.cerealis_45
F.cerealis_11451
F. cerealis
F. graminearum species complex
100
F. aethiopicum_46710
99
F. aethiopicum_46718
F. aethiopicum_46722
F. aethiopicum_46726
F. aethiopicum_46738
F. acaciae-mearnsii_34197
F. acaciae-mearnsii_34207
Clade C
100
F. acaciae-mearnsii_34461
F. vorosii_37605
F. vorosii_38207
F. vorosii_38208
F. vorosii_45790
F. vorosii_45800
100
100
F. boothii_26916
F. boothii_SA6
77
F. meridionale_NEP193
100
F. cortaderiae_29297
F. cortaderiae_29306
100
Nepal lineage_NEP223
Nepal lineage_Ggm241
95
Nepal lineage_NEP146
99
Nepal lineage_NEP253
Nepal lineage_NEP619
F. gerlachii_38405
70
100
F. graminearum_29149
F. graminearum_38371
100
F. gerlachii_36905
F. gerlachii_38380
100
F. ussurianum_45665
F. ussurianum_45681
99
F. ussurianum_45795
52
F. ussurianum_45833
F. asiaticum_881
100
F. asiaticum_AB009607
98
F. asiaticum_AB011417
F. asiaticum_Nep350
F. asiaticum_sck04
71
F. asiaticum_0623
F. graminearum_38369
F. graminearum_38381
F. graminearum_38383
F. graminearum_38393
99
F. graminearum_38395
F. graminearum_CMN7011
F. graminearum_CMN7012
F. graminearum_CMN7021
F. graminearum_CMN7022
F. graminearum_CMN8021
F. graminearum_CMN8022
F. graminearum_CMN8023
F. graminearum_CMN8024
F. graminearum_CMN8025
F. graminearum_CMN7031
F. graminearum_CMN7032
56
F. graminearum_CMN7111
F. graminearum_CMN7101
F. graminearum_GZ21
F. graminearum_T1S1
F. graminearum_Z3639
F. graminearum_CMN8081
F. graminearum_CMN8082
F. graminearum_CMN8101
F. graminearum_CMN8051
F. graminearum_CMN8062
F. graminearum_CMN8071
F. graminearum_CMN7024
F. graminearum_CMN7121
F. graminearum_CMN7151
20

Figure 3.2 Phylogenetic tree based on a total of 1208 bp of *TRI101* gene sequences derived from Bayesian analysis. Representative strains of *Fusarium graminearum* (22) and *Fusarium cerealis* (5) are shown in the figure by _CMN strain code name. Other representative strains from different *Fusarium* spp. were downloaded from GenBank. NRRL strain designations and strain codes follow the underscore after the species name. Numbers at the nodes represent the posterior probability values derived from the Bayesian analysis.

A phylogenetic tree based on 1208 bp of *TRI101* gene sequence revealed three distinct clades (Fig 3.2). These three clades represent *F. culmorum* (clade A), *F. cerealis* (clade B) and *F. graminearum* species complex (clade C). The five non-*Fusarium graminearum* strains (herein called unknown) in the collection (CMN7021, CMN7022, CMN7023, CMN7025, CMN7026) clustered with the known *F. cerealis* strains downloaded from GenBank (*F. cerealis* strain 45, *F. cerealis* NRRL 11451 and *F. cerealis* strain F09624). The unknown strains formed a distinct monophyletic clade with the known *F. cerealis* strains with 100% posterior probability. This analysis confirms that the unknown *Fusarium* strains belong to the *F. cerealis* clade. These *F. cerealis* strains were recovered from the winter wheat cultivar Accipiter. The other 22 *F. graminearum* strains that are already identified from Fg16F/R PCR assay formed a monophyletic clade with other *F. graminearum sensu stricto* strains downloaded from GenBank (GZ21, T1S1, and Z3639) (Fig 3.2). These strains did not cluster with other species in the *F. graminearum* species complex. This shows that they represent *F. graminearum s.s* or lineage 7, that are commonly distributed in Canada and North America.

The trichothecene chemotypes of all 127 *F. graminearum* and 5 *F. cerealis* strains were evaluated by using a multiplex PCR assay based on the *TRI3* gene (Fig 3.3). According to the multiplex PCR assay, 65% of the strains were 3-ADON and only 31% were 15-ADON. Five strains showed the amplified band for NIV, which represented 4% of the total strains. All the NIV producing strains were found to be *F. cerealis*.

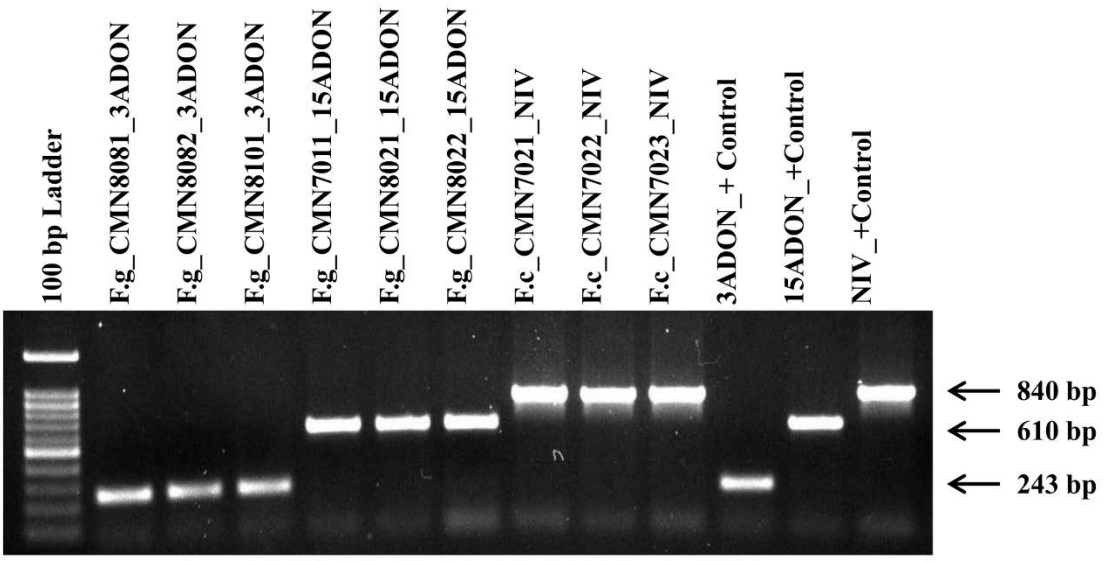


Figure 3.3 Representative PCR amplicons for multiplex PCR assay**.** PCR products of 243, 610 and 840 bp are produced for 3-ADON, 15-ADON and NIV strains, respectively. Representative strains of *Fusarium graminearum* 3-ADON (lane 2-4), and *Fusarium graminearum* 15-ADON (lane 5-7) and *Fusarium cerealis* NIV (lane 8-10), are shown. Positive controls of 3-ADON, 15-ADON and NIV strains are shown in lane 11-13, respectively.

The morphological and cultural characteristics of *F. graminearum* and *F. cerealis* were also compared. Colonies of both *F. graminearum* and *F. cerealis* grew rapidly, with flocculent aerial mycelia. Although *F. graminearum* mycelia were fuzzy with white-pink or pink coloration, mycelia of *F. cerealis* were fuzzy with a red-pink color. The reverse side of *F. graminearum* culture plate was more pinkish than the *F. cerealis* strains. Macroconidia of *F. cerealis* were sickle-shaped, thick-walled with the dorsoventral side significantly curved compared to the ventral side. Although most of *F. cerealis* macroconidia had five septa, few had four septa (Fig 3.4). Similar to *F. cerealis* macroconidia, *F. graminearum* macroconidia were also sickle-shaped but the curve of *F. graminearum* macroconidia on the dorsoventral side was

less than on the ventral side. In addition, most *F. graminearum* macroconidia had five septa and some had three or four septa.

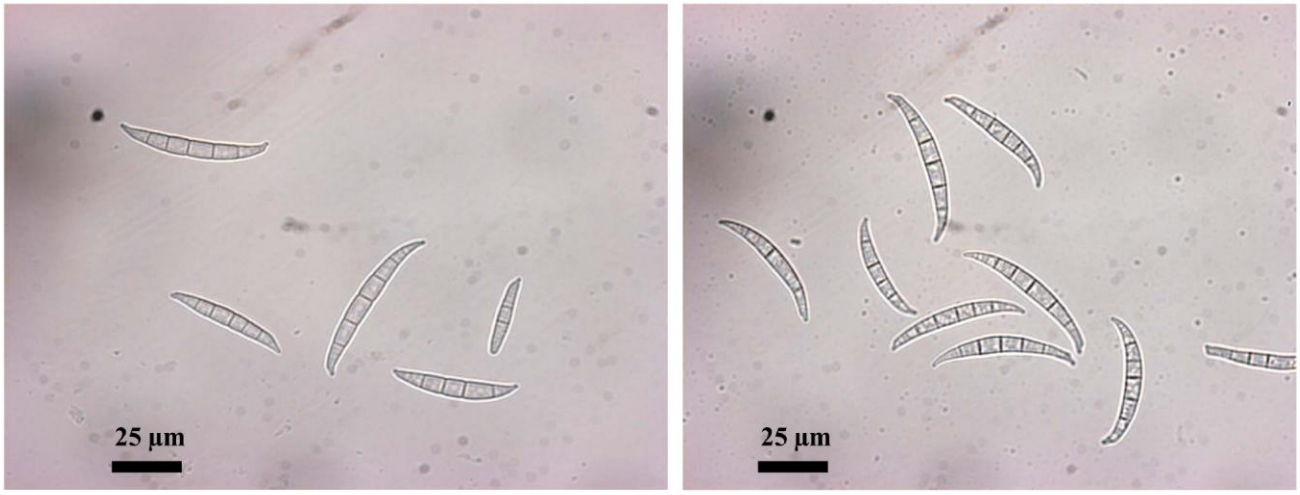


Figure 3.4 Macroconidia of *Fusarium graminearum* (a) and *Fusarium cerealis* (b) on synthetic nutrient agar medium**.**

Finally, pathogenicity tests of *F. cerealis* and *F. graminearum* were conducted on susceptible (S) and moderately resistant (MR) wheat cultivars. The results showed that the aggressiveness of *F. cerealis* was lower than that of *F. graminearum* on both S and MR wheat cultivars. *F. graminearum* strains showed disease symptoms five dpi whereas *F. cerealis* took seven days to show symptoms in S cultivar Roblin and 10-12 days in MR cultivar Carberry. The highest disease severity on both cultivars, Roblin and Carberry was shown by 3-ADON strains followed by 15-ADON and NIV strains. Significant differences for disease severity were also observed among the *Fusarium* strains both at 14 dpi (Fig 3.5) and 21 dpi (Fig 3.6). Data from GC-MS also confirmed the production of NIV by *F. cerealis* strains. The examined *F. cerealis* strains produced NIV in infected grains of S and MR wheat cultivars at levels of 1.39-6.15 and

0.66-0.99 mg kg^{-1}, respectively. The other examined trichothecenes (DON, 3-ADON, 15-ADON, T-2 toxin, HT-2 toxin, DAS and FUS-X) were not detected in the analysed grain samples.

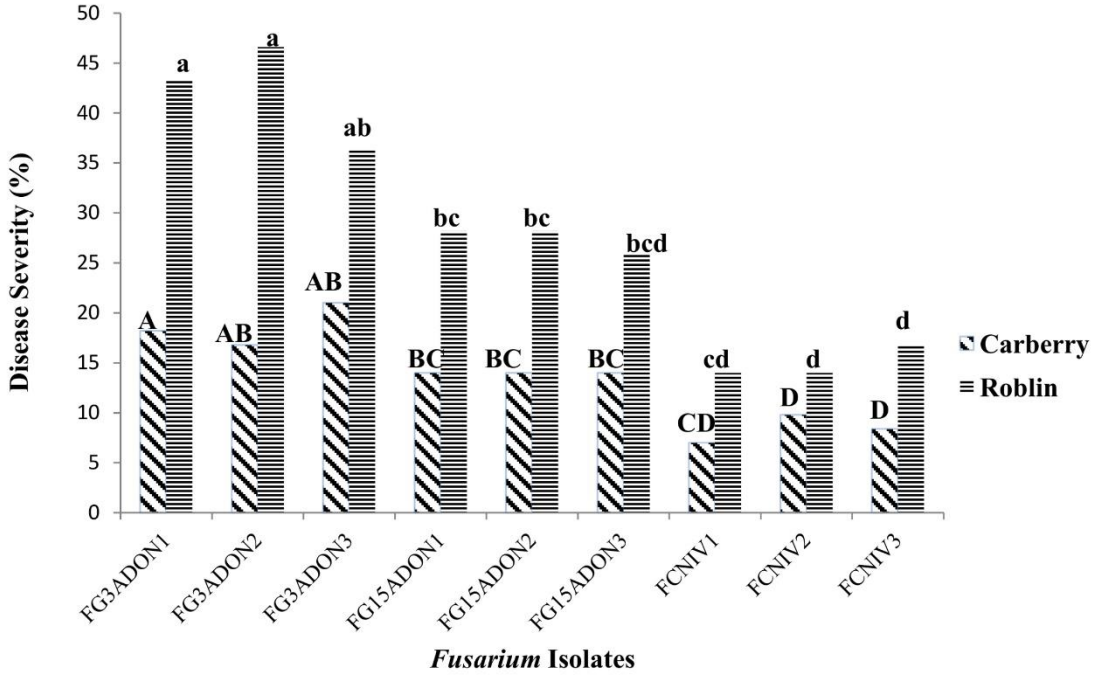


Figure 3.5 Mean fusarium head blight disease severity of Carberry and Roblin cultivars at 14 days post-inoculation with *Fusarium graminearum* 3-ADON strains, 15-ADON strains and *Fusarium cerealis* NIV producing strains**.** Means with the same letter for disease severity are not significantly different.

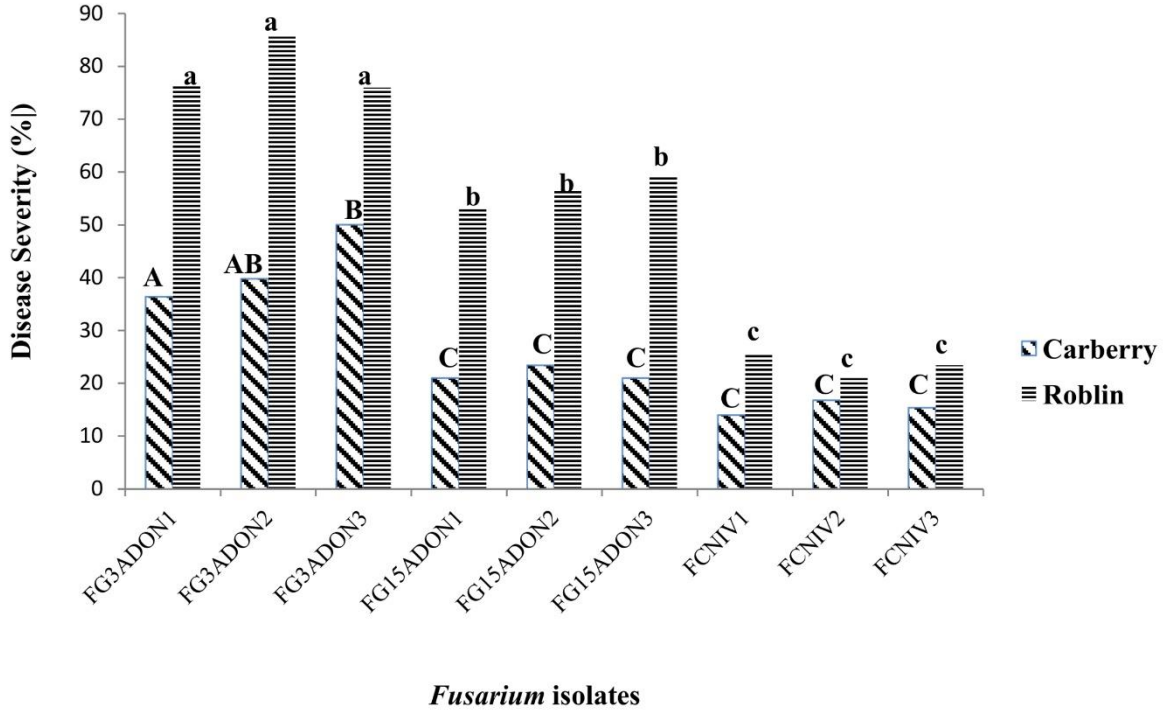


Figure 3.6 Mean fusarium head blight disease severity of Carberry and Roblin cultivars at 21 days post-inoculation with *Fusarium graminearum* 3-ADON strains, 15-ADON strains and *Fusarium cerealis* NIV producing strains**.** Means with the same letter for disease severity are not significantly different.

3.5 Discussion

To the best of the author's knowledge, this study provides the first evidence of the presence of NIV producing *F. cerealis* strains in winter wheat fields in Carman, Manitoba, Canada. Miller *et al*. (1991) have reported the presence of two *F. crookwellense* strains in Ontario, Canada. Since then, there have been no other reports of *F. cerealis* strains from Canada. Recently, a single NIV producing *F. cerealis* strain was identified in New York (Schmale *et al*., 2011). Zhang *et al*. (2011) and Castanares *et al*. (2013) also reported the presence of *F. cerealis* in barley seeds collected from China and Argentina. Such recent reports on the occurrence of *F. cerealis* raises concern on the emergence of *F. cerealis* as one of the major pathogens causing FHB. *F. cerealis*

and *F. graminearum* are morphologically very similar and difficult to distinguish from each other in commonly used media, with their macroconida having a similar shape (Yli-Mattila & Gagkaeva, 2010). Several studies reported that *F. cerealis* macroconida are stout, thick-walled, with curved apical and basal cells and usually 5-septate. Similarly, *F. graminearum* macroconida were sickle-shaped, elliptically curved and have five septa (Sugiura *et al*., 1994; Yli-Mattila & Gagkaeva, 2010). Therefore, molecular techniques have been more effective in distinguishing these two species.

This study, used *TRI101* sequences of *F. graminearum* species complex, *F. culmorum*, *F. cerealis* and *F. pseudograminearum* (as the out-group) from GenBank for phylogenetic analysis. *F. graminearum* species complex included *F. acacia-mearnsii*, *F. aetheiopicum*, *F. asiaticum*, *F. boothii*, *F. cortaderiae*, *F. gerlachii*, *F. graminearum sensu stricto*, *F. meridionale*, Nepal lineage (*F. nepalense*), *F. ussurianum*, *F. vorosii*, and novel *F. graminearum* strains from the Louisiana Gulf-coast population. *Fusarium graminearum s.s* strains collected from winter wheat plots in Carman, Manitoba, Canada, formed a distinct cluster with other *F. graminearum s.s* strains obtained from GenBank. The *F. cerealis* strains identified in this study formed a distinct cluster with known *F. cerealis* strains from GenBank. The analyses of *TRI101* gene sequences confirmed the presence of *F. cerealis* in infected wheat spikes collected from winter wheat plots in Carman, Manitoba, Canada.

All of the *F. cerealis* strains in this study were of NIV chemotype. This finding was similar to the reports from Germany, Poland, Japan and Russia (Sugiura *et al*., 1994; Chandler *et al*., 2003). Other reports indicate that *F. cerealis* is also capable of producing the mycotoxin zearalenone in infected barley seeds (Di Menna *et al*., 1991; Miller *et al*., 1991). Until 2012, the presence of NIV chemotypes in Canadian grain samples was limited to barley samples

(Tittlemier *et al*., 2013); no NIV chemotypes have been found in infected wheat samples. Therefore, the finding of NIV producing *F. cerealis* strains in wheat fields in Carman, Manitoba, Canada, causes serious concern for the wheat industry in Canada. A chemotypic shift from 15-ADON strains to 3-ADON strains was also observed in this study, as reported by Ward *et al.* (2008) and Guo *et al*. (2008) in western Canada. Specifically, of the 132 *Fusarium* strains studied, 65% were 3-ADON and 31% were 15-ADON strains.

The pathogenicity tests revealed that *F. cerealis* strains were able to cause FHB in wheat, but were less aggressive than *F. graminearum* strains. For the *F. cerealis* strains, the first symptoms on the MR cultivar appeared 10-12 days after single floret inoculation, whereas in *F. graminearum* strains the symptoms appeared on day 5. Similar lower virulence of *F. cerealis* strains in wheat has been reported in other studies (Sugiura *et al*., 1994; Desjardins *et al*., 2004). Miller (1994) reported that the pathogenicity of FHB complex species varied from *F. graminearum* (highest aggressiveness) >*F. culmorum* >*F. avenaceum*> *F. cerealis* (lowest aggressiveness). The regional and annual distribution of FHB complex species is affected by temperature, where *F. culmorum* is more prevalent in cooler climates, followed by *F. cerealis* and *F. graminearum* in warmer climates (Miller, 1994).

Although NIV is not very toxic to plants or wheat, it is more toxic to animals than DON. DON and NIV differ only at the C4 position in the chemical structure: NIV has a hydroxyl group at the C4 position whereas DON has a hydrogen molecule. The presence of a hydroxyl group in nivalenol increases the toxicity in animals tenfold as compared to the hydrogen in DON (Visconti *et al*., 1991). NIV contamination is rare in Canada and North America compared to that in Europe and Asia (Desjardins *et al*., 2004, Schmale *et al*., 2011). Therefore, discovery of NIV producing *Fusarium* species in Canada indicates the need for extensive sampling of wheat fields

across the country. NIV production is a more ancestral trait and DON-producing strains have a selective advantage over NIV-producing strains. However, recent reports from USA on NIV-producing *F. graminearum* and *F. cerealis* strains and other *Fusarium* strains with novel traits show the risk of changing profiles of mycotoxins and species in the FHB complex (Starkey *et al*., 2007; Gale *et al*., 2011). This study shows the importance of testing for NIV in naturally infected grain samples and the need for extensive research for other *Fusarium* species in the FHB complex that could be a potential threat to the wheat industry in Canada.

CHAPTER 4

THE DOMINANCE OF 3-ACETYLDEOXYNIVALENOL CHEMOTYPE OF *FUSARIUM GRAMINEARUM* IN WINTER WHEAT IN WINNIPEG AND CARMAN, MANITOBA

4.1 Abstract

Trichothecene contamination in agriculturally staple crops such as wheat, barley and maize during *Fusarium graminearum* colonization, is an increasing concern in major cereal producing countries. Deoxynivalenol (DON), also known as vomitoxins and its acetylated derivatives, 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON) are the trichothecenes most commonly detected, often at the ppm levels. Recently, a shift from 15-ADON to the more aggressive 3-ADON has been observed in Manitoba. The aim of this research was to determine the trichothecene diversity in Manitoba, Canada and to analyse the extent to which the trichothecene chemotype can be used as a marker to represent *F. graminearum* population identity. Natural infection in spikes of winter wheat occurring at the top, middle and bottom as well as the full spike and peduncle were collected separately from winter wheat cultivars; CDC Buteo, CDC Falcon, CDC Kestrel, McClintock, Accipiter, 39M*11, Pergrine and AC Readymade from University of Manitoba research field stations in Winnipeg and Carman in 2010 and 2013. Characterization of single-spore strains and identification of *Fusarium* chemotypes from each spike was done with PCR using *F. graminearum*-specific primers and multiplex PCR respectively. The overall percentage of chemotypes in Winnipeg, 2010 was 86% and 14 % for 3-ADON and 15-ADON, respectively. There was a 4% increase of 3-ADON

strains in Winnipeg in 2013 compared to 2010. The frequency of 3-ADON strains in Carman, 2010 was 57% and 15-ADON strains were 43%. In 2013, there was an 8% increase in the frequency of 3-ADON strains in Carman compared to 2010. Population genetic analyses using variable number tandem repeat (VNTR) markers recovered a significant genetic differentiation between 3-ADON and 15-ADON populations. Results from the current study clearly indicate the dominance of the 3-ADON chemotype in winter wheat in Winnipeg and Carman, Manitoba.

4.2 Introduction

One of the major challenges for the wheat industry worldwide is Fusarium head blight (FHB), caused mainly by the members in the *Fusarium graminearum* species complex. Wheat yield loss of up to 50% due to FHB has been reported (McMullen *et al*., 1997; Goswami & Kistler, 2005). *Fusarium graminearum* produces trichothecene mycotoxins that reduce wheat quality, pose a significant food safety risk, and cause illness in humans and animals, resulting in difficulties for wheat marketing, and processing (Rocha *et al*., 2005; Guo *et al*., 2008; Foroud & Eudes, 2009). Deoxynivalenol (DON) is one of the most important toxins produced by *F. graminearum*, which inhibits protein biosynthesis in eukaryotic organisms (Rocha *et al*., 2005) and results in health risks. These health risks include feed refusal, vomiting, diarrhea, emesis and anorexia (D'Mello *et al*., 1999). The members of the *Fusarium graminearum* species complex are composed of different strains that produce one of the three strain-specific profiles of trichothecene metabolites (Miller *et al*., 1991; Ward *et al*., 2002; O'Donnell *et al*., 2004). These are nivalenol (NIV), a C-4 oxygenated derivative of DON; 3-ADON, an acetyl ester derivative of DON at the 3-position oxygen; and 15-ADON, an acetyl ester derivative of DON at the 15-position oxygen (Miller *et al*., 1991).

In general, most of the Canadian wheat cultivars are susceptible to FHB, and wheat breeders are continuously working on to improve the FHB resistance in each wheat class (Gilbert & Haber, 2013). Each wheat cultivar responds differently to *F. graminearum* infection and level of resistance in wheat cultivars range from moderately resistant to highly susceptible. Among different winter wheat cultivars, Canada red winter wheat cultivar, Emerson is reported to be resistance to FHB (Seed Manitoba, 2015). Winter wheat protects soil from wind and erosion in the fall, winter and spring, helps spread field operations and yields at least 20% higher than the spring wheat. It is believed that winter wheat has the ability to escape excessive heat/drought and FHB as compared to the spring wheat due to early flowering (Gilbert & Haber, 2013).

Historically, FHB was more common in eastern Canada. Since 1984, FHB has been most prevalent in the Red River Valley region, particularly south of Winnipeg. The current interest in FHB research in Canada initiated with the FHB epidemic in 1993, which was the worst epidemic reported to date in the wheat growing regions of the upper American Midwest and southern Manitoba, Canada (Gilbert & Tekauz, 2000). The 1993 FHB epidemic in southern Manitoba was estimated to have caused losses totaling $75 million (Gilbert & Tekauz, 2000; Gilbert & Haber, 2013). After 1993 epidemic, a higher severity of FHB was again reported in 1997 especially in western Manitoba and southeastern Saskatchewan. The movement of FHB infection from eastern Canada to western Canada would threaten Canada's major wheat producing regions, which are largely free of FHB infection. Also, previous studies had indicated that 15-ADON chemotype strains were dominant in North America (Abramson *et al*., 1993, 2001; Miller *et al*., 1991). Before 1998, the 15-ADON chemotype was the most prevalent chemotype in North America, but between 1998 and 2004, the 3-ADON chemotype became predominant over the 15-ADON chemotype in Manitoba (Miller *et al*., 1991; Guo *et al*., 2008; Ward *et al*., 2008; Gilbert *et al*.,

2014; Kelly *et al*., 2015). There is growing evidence of a *F. graminearum* population shift in North America. Ward *et al*. (2008) showed a chemotype shift from 15-ADON to the more aggressive 3-ADON from eastern to western Canada. Similarly, Guo *et al*. (2008) indicated a potential chemotype shift from 3-ADON to 15-ADON in wheat fields in Manitoba. These studies have also revealed that 3-ADON chemotype strains produce more DON than 15-ADON chemotype strains (Ward *et al*., 2008) and chemotype differences can have a significant impact on pathogen fitness (Ward *et al*., 2002). These situations cause increasing concern to the cereal industry. The reason for the rapid increase in 3-ADON population in Canada is not yet understood. It has been reported that the 3-ADON populations in North America are genetically more similar to Italian populations than the native 15-ADON populations, indicating that transcontinental introduction may have had an effect on the rapid shift (Ward *et al*., 2008; Kelly *et al*., 2015). Despite the initial reports that suggested panmictic local populations of the pathogen in wheat growing areas of North America (Dusabenyagasani *et al*., 1999; Zeller *et al*., 2003), recent studies indicated localised heterogeneity among these populations (Starkey *et al*., 2007; Gale *et al*., 2007; Ward *et al*., 2008).

In this study we investigated the distribution of *F. graminearum* trichothecene chemotypes in winter wheat fields in Winnipeg and Carman, Manitoba over a two-year period. In addition, using variable number tandem repeat (VNTR) markers, the population structure of *F. graminearum s.s* was characterized from Winnipeg and Carman, Manitoba.

4.3 Materials and Methods

4.3.1 Sample collection and isolation

Winter wheat spikes showing FHB disease symptoms were collected from six naturally infected winter wheat cultivars CDC Buteo, CDC Falcon, CDC Kestrel, McClintock, Peregrine and AC Readymade grown at The Point, University of Manitoba Research Station, Winnipeg, Manitoba and Ian Morrison Research Station, Carman, Manitoba in 2010. In 2013, wheat heads with FHB symptoms were collected from seven naturally infected wheat cultivars, CDC Buteo, CDC Falcon, Accipiter, McClintock, Moats, 39M*11 and AC Readymade from above locations. Winter wheat spikes were collected showing FHB symptoms at the top, middle and bottom of the spikes as well as the full spike and peduncle. This sampling method was utilized to minimize any effect that could arise from an uneven distribution of *Fusarium* spp. on wheat spikes. Twenty to twenty-five spikes were collected from each cultivar plot. The collected spikes were stored at -20°C until single spore isolations were made. Single spore isolations were done from each spike as reported in Amarasinghe *et al.* (2015).

4.3.2 DNA extraction

DNA extraction was carried out according to the protocol described by Fernando *et al*. (2006) using a cetyltrimethylammonium bromide (CTAB). The extracted DNA was treated with RNase (0.75% vol/vol) and stored at -20°C until further use.

4.3.3 Identification of strains to species level

Identity of strains was confirmed using *F. graminearum* species-specific PCR primers described by Demeke *et al*. (2005). The primers, Fg16F (5′- CTCCGGATATGTTGCGTCAA-3′) and

Fg16R (5′-GGTAGGTATCCGACATGG CAA-3′), were used in the PCR, which amplify a fragment of 450 bp. The PCR reaction was performed in a 25 µL reaction containing 20 ng of template DNA, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris HCl (pH 8.0), 0.2 mM each dNTP (Invitrogen Life Technologies, CA, USA), 0.4 µM each primer, and 0.75 units of Taq DNA polymerase (Invitrogen Life Technologies, CA, USA). The PCR products were run on 1% agarose gel. The strains that could not be identified using the Fg16F/R species-specific primers, a portion of the *TRI101* gene was amplified and sequenced bi-directionally according to Amarasinghe *et al*. (2015). The primers used for amplification were TRI101F (5'-CCATGGGTCGCRGGCCARGTSAA-3') and TRI101R (5'-AACTCSCCRTCIGGYTTYTTNGGCAT-3') (Proctor *et al*., 2009).

4.3.4 Determination of trichothecene chemotypes of strains

Chemotype identity of each *F. graminearum* strain was determined using the multiplex PCR primers developed by Ward *et al*. (2008). The multiplex primers used in the PCR were 3CON (5′-TGGCAAAGACTGGTTCAC-3′), 3D15A (5′-ACTGACCCAAGCTGCCATC-3′), 3D3A (5′-CGCATTGGCTAACACATG-3′), and 3NA (5′-GTGCACAGAATATACGAGC-3′). These primers amplify a 840 bp fragment for the NIV chemotype, a 610 bp fragment for the 15-ADON chemotype and a 243 bp fragment for the 3-ADON chemotype. The PCR reaction was performed in a 15 µL volume containing 20 ng of template DNA, 2.0 mM $MgCl_2$, 50 mM KCl, 10 mM Tris HCl (pH 8.0), 0.2 mM each dNTP (Invitrogen Life Technologies, CA, USA), 0.4 µM each primer, and 0.75 units of Taq DNA polymerase (Invitrogen Life Technologies, CA, USA). The PCR amplicons were separated on a 2% agarose gel.

4.3.5 VNTR analysis and statistical analysis

Analysis of population structure was conducted on 302 strains (from 2010) and 289 strains (from 2013) from Winnipeg and Carman, using the variable number tandem repeat (VNTR) markers reported by Suga *et al*. (2004), Gale *et al*. (2005) and Vogelgsang *et al*. (2009). A total of 12 VNTR markers (*HK630, HK913, HK917, HK957, HK965, HK967, HK977, HK1059, HK1073, HK1043, HK1003* and *Fuss20*) were used to analyze the population structure of the strains. All forward primers were produced by adding the M13 primer sequence (5'-CACGACGTTGTAAAACGAC-3') at the 5' end of each VNTR marker used in this study. Polymerase chain reaction assays for each marker, contained a total volume of 10 µL consisting of 1X buffer, 200 µM of dNTP (Invitrogen Life Technologies, CA, USA), 1.5 mM $MgCl_2$, 1.0 µM of each forward, reverse and M13 primer, 1 unit of Taq polymerase (Invitrogen Life Technologies, CA, USA), and DNA template at 20 ng/µL. PCR amplification was performed according to the protocol described by Puri & Zhong (2010). PCR reaction cycles were consisted with initial denaturation at 94 ºC for 3 min; followed by 3 cycles of 94 ºC for 30 s, 56 ºC for 30 s, and 72 ºC for 1 min, an additional 25 cycles of 94 ºC for 30 s, 52 ºC for 30 s and 72 ºC for 45 s, and final extension at 72 ºC for 5 min. Then the reaction products were scored relative to a GS500 ROX (Applied Biosystems, Carlsbad, CA, USA) internal size standard using an ABI 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA). The data were processed using the genotyping software GENOGRAPHER v. 2.1. VNTR allele data were analysed by the program STRUCTURE v. 2.2 (Pritchard *et al*., 2000) which analyses multi-locus genotype data to investigate population structure using a Bayesian model based clustering method. The Bayesian model is based on maximizing linkage equilibrium within clusters and disequilibrium between them (Liang *et al*., 2014). The Monte Carlo Markov Chain approach was run for 10,000

iterations with a 1000 burn in period. The admixture model was selected for the analysis and the *K* value was set ranging from *K* =1 to *K* =10 with 10 replications to calculate the convergence of likelihood value for each value of *K* (Liang *et al*., 2014). Analysis of molecular variance among and within populations were estimated using GENALEX v. 6.5 (Peakall & Smouse, 2012). The genetic distances between populations were measured based on F_{ST}. Significance of F_{ST} values were calculated from 1000 random permutations of the data set. The gene flow (*Nm*) between populations was also estimated.

4.4 Results

4.4.1 Identification of *Fusarium* species and trichothecene chemotypes

A total of 982 *Fusarium* strains were recovered from winter wheat in Winnipeg and Carman in years 2010 and 2013 (Winnipeg 2010 (n=360), Winnipeg 2013 (n=162), Carman 2010 (n=328) and Carman 2013 (n=132)). The primers Fg16F/R generated a *F. graminearum s.s* specific 450 bp amplified product, which was observed in all 977 strains except in five strains analysed. Phylogenetic analysis of the *TRI101* gene sequences indicated that the five strains belong to *F. cerealis* and all the other strains belong to *F. graminearum s.s* (Amarasinghe *et al*., 2015). Among the 360 strains collected in 2010 from Winnipeg, 82% were of the 3-ADON chemotype and only 18% of the strains were of 15-ADON chemotype (Fig 4.1a). In 2013, 86% were 3-ADON chemotypes and only 14% were found to be 15-ADON (Fig 4.1b).

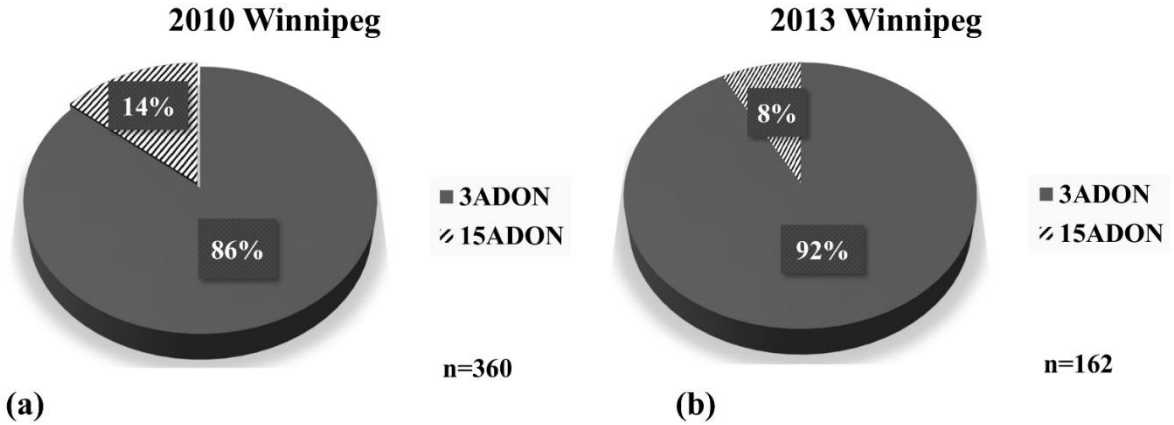


Figure 4.1 Trichothecene chemotypes frequency among the *Fusarium graminearum* strains collected during (a) 2010 and (b) 2013 in Winnipeg, Manitoba, Canada.

In 2013, a 4% increase of 3-ADON strains was observed compared to 2010 in Winnipeg. In 2010, Carman, 57% of the strains were 3-ADON and 43% of the strains were 15-ADON chemotype (Fig 4.2a). In 2013, there was an 8% increase in 3-ADON strains (65% of the total strains) than 2010 in Carman (Fig 4.2b). The percentage of 15-ADON producing strains was 31%. Interestingly 5% of the strains found were NIV producers and all of them belonged to *F. cerealis* (Fig 4.2b).

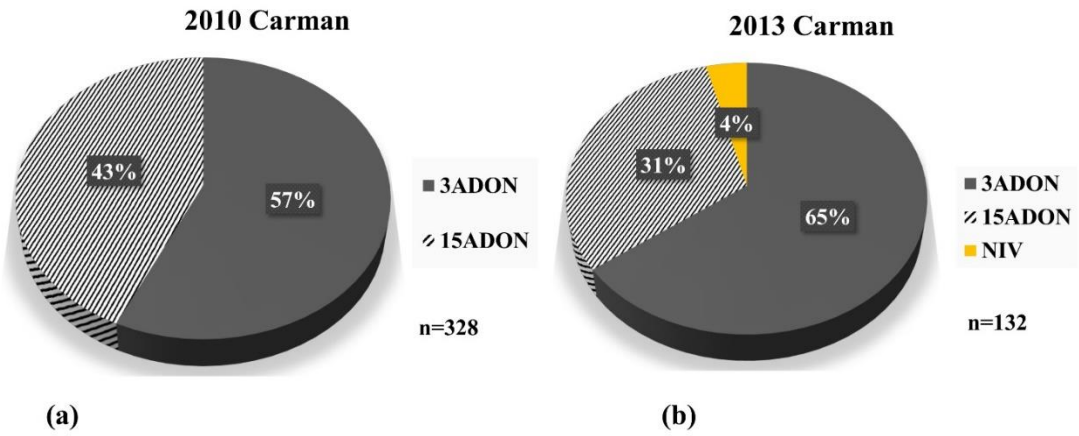


Figure 4.2 Trichothecene chemotypes frequency among the *Fusarium graminearum* and *Fusarium cerealis* strains collected during (a) 2010 and (b) 2013 in Carman, Manitoba, Canada.

Winter wheat cultivars differed in their reaction towards the two chemotypes. In 2010 Winnipeg, the highest percentage of 3-ADON chemotype strains were observed in the cultivar CDC Falcon (90%) and the lowest percentage of the same chemotype strains were recorded in the cultivar McClintock (82%) (Fig 4.3a). In 2013, the highest percentage of 3-ADON chemotypes was observed in the cultivar Moats in which all the isolated strains belonged to the 3-ADON producers (100%) (Fig 4.3b). In Carman, the highest percentage of 3-ADON chemotypes was isolated from the cultivar CDC Buteo, 62% in 2010 (Fig 4.4a) and 80% in 2013 (Fig 4.4b).

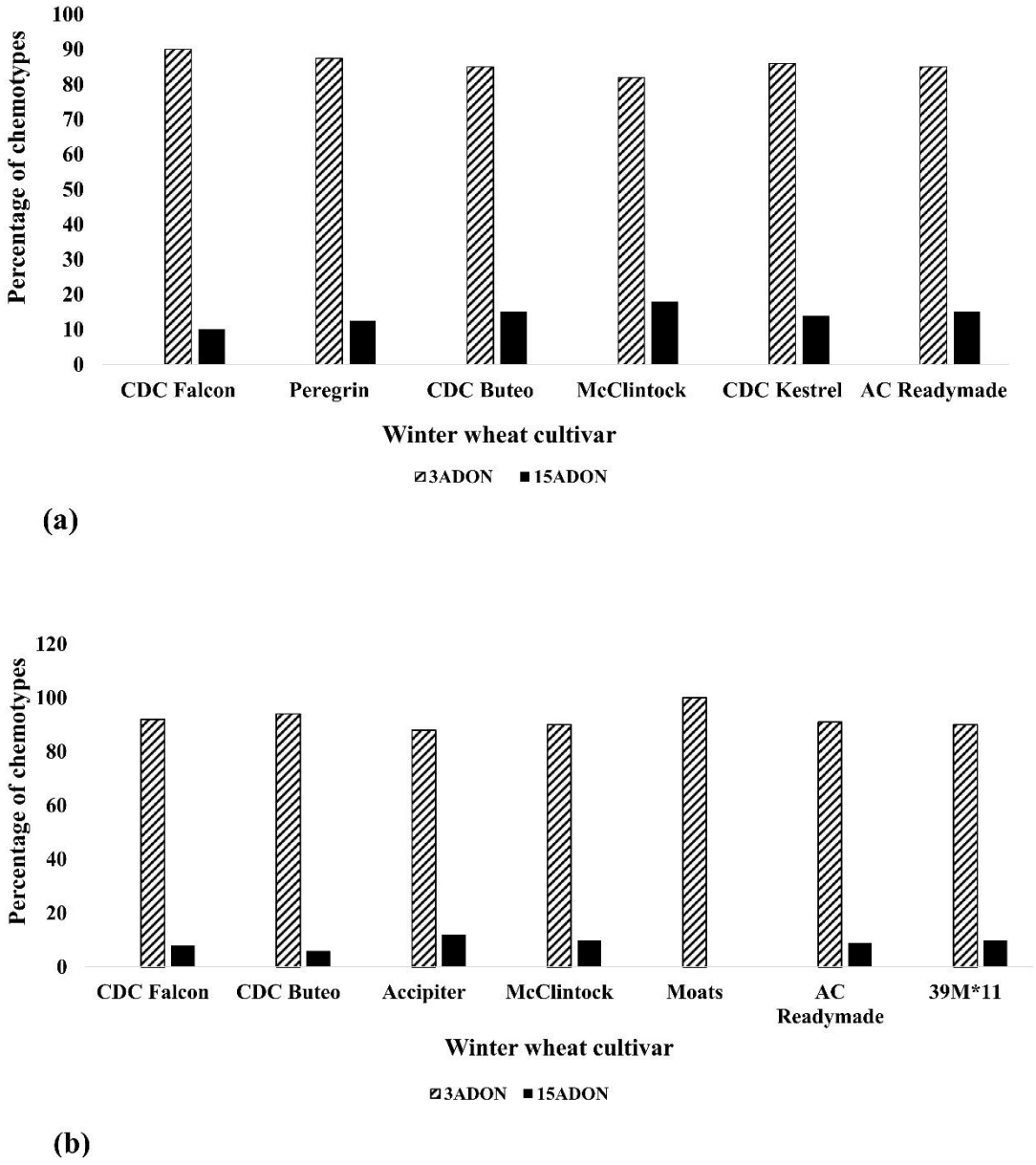


Figure 4.3 Frequency of *Fusarium graminearum* chemotypes isolated from each winter wheat cultivar from Winnipeg in (a) 2010 and (b) 2013.

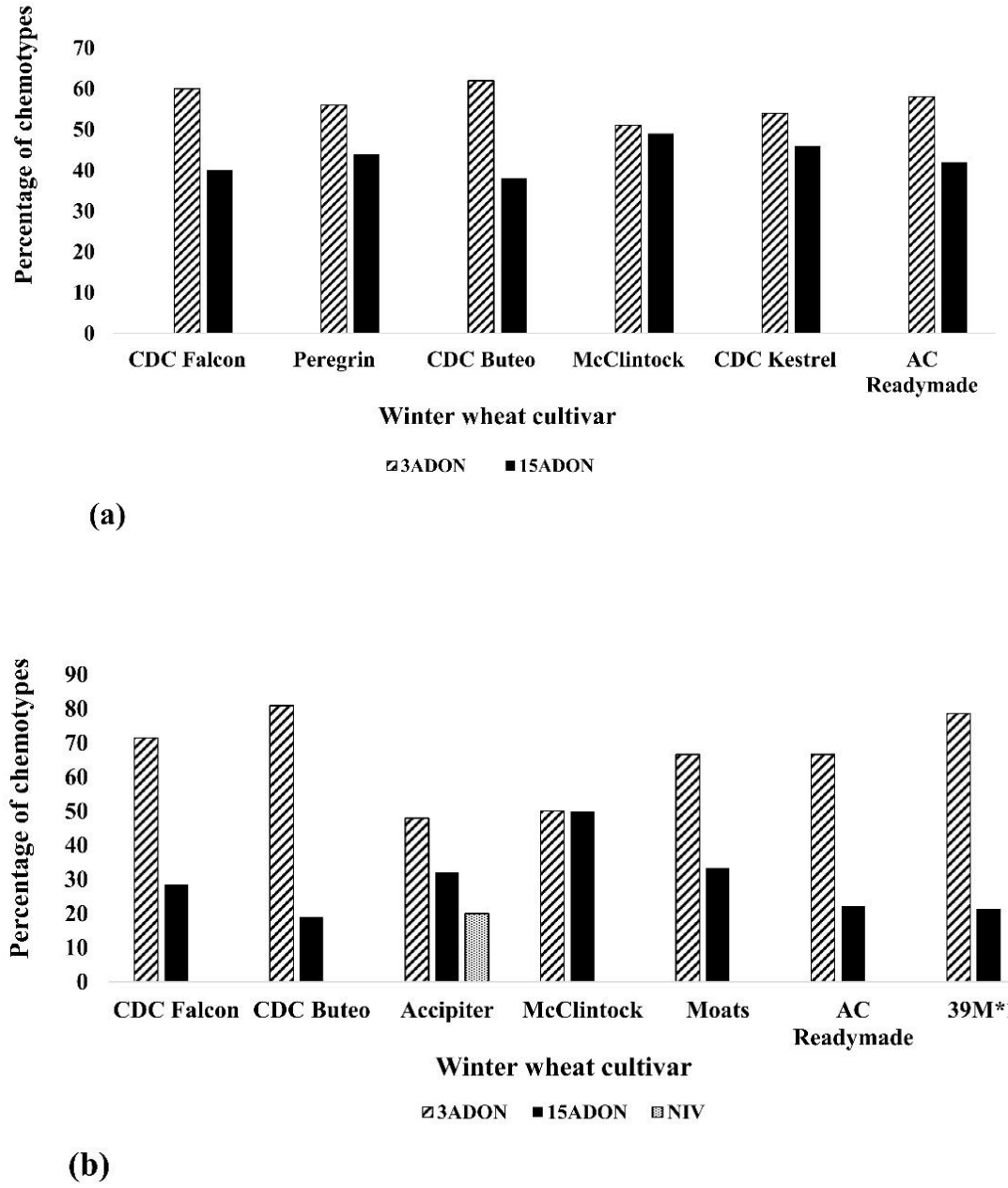


Figure 4.4 Frequency of *Fusarium graminearum* chemotypes isolated from each winter wheat cultivar from Carman in (a) 2010 and (b) 2013.

4.4.2 Population analyses

Twelve VNTR markers were used to genotype the *F. graminearum s.s* strains collected from Winnipeg and Carman in 2010 and 2013 (n=591). Data were analysed using the software STRUCTURE v. 2.2. At first, we assumed a genetic cluster number between one and five (K=1 to 5). The results from the initial simulation were analysed using STRUCTURE HARVESTER (Earl & vonHoldt, 2012) to determine the optimum population structure. According to the output from STRUCTURE HARVESTER, the optimum value for K was K=2, which resulted in two distinct clusters with nearly 90% of the strains being assigned to one of the clusters (Figure 4.5) . These two distinct clusters can be identified as 3-ADON chemotype and 15-ADON chemotype (These clusters or populations will be designated as 3APOP and 15APOP respectively) (Fig 4.6). Most of the 15-ADON chemotype strains were assigned to 15APOP with very high membership value of *Q* higher than 0.8 (83% of the strains). The *Q* memebership value represents the probability that each individual belongs to the respective cluster/population and *Q* value ranges between 0 to 1. Additionally, 17% of the 15-ADON producing strains were also placed into 15APOP, but at a lower *Q* value between 0.5 and 0.8. Also, seven strains of 15-ADON chemotype were assigned to 3APOP with *Q* ranging from 0.80 to 0.98. Considering the 3-ADON population, 87% of the 3-ADON chemotype strains were placed in to 3APOP with *Q* values higher, or equal to, 0.8. Also, 13% of the 3-ADON producing strains were placed into 3APOP at lower *Q* values ranging from 0.5 to 0.7. Two 3-ADON producing strains were placed into 15APOP with higher *Q* values ranging from 0.90-0.99.

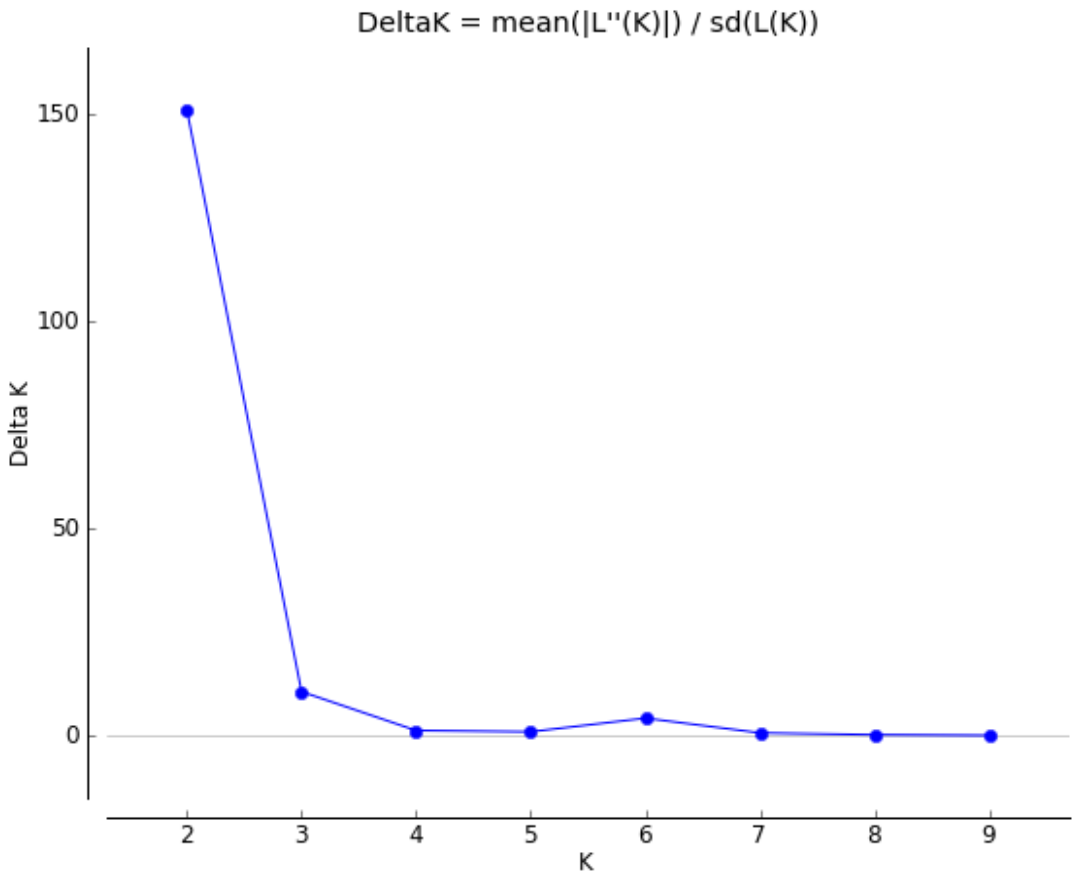


Figure 4.5 Structure harvester output showing the delta K values with the respective number of clusters.

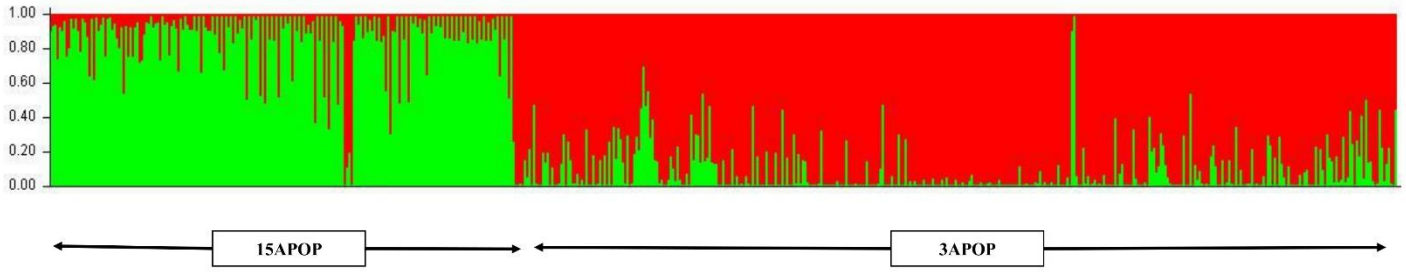


Figure 4.6 Visual representations of strain distribution and admixture estimates based on VNTR data for 591 *Fusarium graminearum sensu stricto* strains collected from Winnipeg and Carman in 2010 and 2013. Green= *Fusarium graminearum s.s* with 15-ADON chemotype, Red= *Fusarium graminearum s.s* with 3-ADON chemotype. Individual strains are represented by a distinct vertical line colored to represent the estimated portion of the strains genome derived from each population. The horizontal axis consists of a single vertical bar for each 591 strains.

The population differentiation within *F. graminearum s.s* strains was further examined using the analysis of molecular variance (AMOVA) on *F. graminearum s.s* strains collected from Winnipeg and Carman in 2010 and 2013. In the AMOVA analysis, strains were grouped according to their chemotypic origin for each location (Table 4.1). In Winnipeg high genetic variation was observed between the two populations (44%) (Table 4.1). The genetic differentiation between 3APOP and 15APOP was 0.442 (F_{ST} = 0.442). F_{ST} is a measure of the extent of genetic differentiation between populations. F_{ST} value can range from 0.0 (no differentiation) to 1.0 (complete differentiation, populations fixed for different alleles). The gene flow between populations was low (*Nm*= 0.631). Similarly, a high genetic variation was observed between the two populations at the Carman location (34%). In Carman the genetic differentiation between 3APOP and 15APOP was 0.339 (F_{ST} = 0.339) and gene flow was 0.988 (*Nm*= 0.988). In comparison to the Carman location, much higher population genetic differentiation and lower gene flow was observed between the 3APOP and 15APOP in Winnipeg.

Table 4.1 Analysis of molecular variance (AMOVA) of *Fusarium graminearum* 3-ADON and 15-ADON population collected from Winnipeg and Carman during 2010 and 2013

Location	Source	df	SS	MS	Est.	%	F_{ST} [1]	P [2]
Winnipeg	Among Pops	1	222.550	222.50	1.744	44%	0.442	0.001
	Within Pops	310	682.572	2.202	2.022	56%		
	Total	311	905.112		3.946	100%		
Carman	Among Pops	1	221.282	221.282	1.609	34%	0.339	0.001
	Within Pops	277	869.298	3.138	3.138	66%		
	Total	278	1090.581			100%		

[1] F_{ST} is a measure of the extent of genetic differentiation between populations.
[2]P- probability.

4.5 Discussion

The chemotype of a *F. graminearum* strain provides important epidemiological information on the *Fusarium* population colonizing cereal crops in a given region/country. Our study revealed that the frequency of *F. graminearum* strains with 3-ADON chemotype is increasing rapidly over the native 15-ADON chemotype in Winnipeg and Carman, Manitoba from 2010-2012. The 3-ADON chemotype is consistently found at high frequencies in Winnipeg and Carman, Manitoba, suggesting that the chemotype shift in *Fusarium* populations is still ongoing in Manitoba. Similar results have been reported by Ward *et al*. (2008), Guo *et al*. (2008) and Kelly *et al*. (2015) which demonstrated that *F. graminearum* population with a 3-ADON chemotype is continuously increasing in Canada since 2000. A study done by Ward *et al*. (2008) have shown that frequency of 3-ADON producing strains in western Canada increased more than 14 times from 1994 to 2004. populations of *F. graminearum* in Canada and found that the 3-ADON chemotype frequency in western Canada increased more than 14 fold between 1998 and 2004. In their study, the 3-ADON population was more aggressive and produced more toxin compared to

the 15-ADON population. Therefore, continuous monitoring of the distribution of 3-ADON population in Canada is very important. Similarly, a study done by Guo *et al*. (2008) examined FHB infected wheat samples collected from 15 wheat fields in Manitoba from 2004 to 2005 and found that percentage of 3-ADON chemotype changed from 0 to 95.7% from 2004 to 2005. To date, no clear reasons have been reported to explain the rapid increment in 3-ADON producing strains in North America. One possible explanation to chemotype shift could be the changing wheat cultivars, changes in agricultural practices such as crop rotation or climatic conditions. The emergence of FHB is driven primarily by the weather conditions. In Canada, it is believed that the changing precipitation patterns play a key role in disease development. High precipitation in July, that align with the anthesis of spring wheat is a major contribution factor for FHB development in the prairies (Gilbert & Haber, 2013). The lower frequency of 3-ADON strains in the eastern provinces of Canada can be explained by the climate differences among the regions. It is hypothesised that the environmental conditions in western Canada provide a selective advantage to 3-ADON strains over the 15-ADON strains. Environmental conditions such as, temperature also affects the distribution of *F. graminearum* species in the FGSC (Qu *et al*., 2008b). It has been reported that *F. graminearum s.s* strains favor cooler regions (below 15 ºC) and *F. asiaticum* strains are more common in warmer regions (above 15 ºC). In addition to climatic conditions, crop rotation also plays a major role in FHB development and chemotype determination. Corn residues act as a good source for overwintering stages of the pathogen. Several species in the FGSC that are capable of producing NIV chemotype have been isolated from corn residues; such as *F. meridionale* and *F. austroamericanum*. In Argentina, NIV producers in the FGSC dominated in corn (Sampietro *et al*., 2012). Del Ponte *et al.* (2015) also reported that *F. meridionale* and *F. cortaderiae* of the NIV genotype accounted for the vast

majority (>96%) of strains from corn stubble in Brazil. Apart from corn, rice also provides a good source for overwintering stages of the pathogen. A predominance of *F. asaiticum* over *F. graminearum* in southern Korea was hypothesised to be due to better adaptability of the pathogen to rice than wheat (Lee *et al*., 2009). It is hypothesised that climate conditions that favour rice production also favour the emergence and survival of NIV chemotypes (Gale *et al*., 2011). In the southern USA, both NIV producing *F. graminearum* and *F. asiaticum* populations have been discovered in the rice growing areas in Louisiana (Gale *et al*., 2011). Therefore, the crops used in crop rotation practices provide a selective advantage for some species and chemotypes over others. Similarly, host prevalence in a particular region could also shape the distribution and survival of regional *Fusarium* populations. The prairie provinces Saskatchewan, Alberta and Manitoba are the major wheat producing provinces in Canada. Whereas, eastern provinces Ontario and Quebec are more contributing towards corn production. Therefore, distribution and availabilty of different host species could have a significant impact on *Fusarium* populations and chemotype distribution in those regions (Gale *et al*., 2011; Zhang *et al*., 2012; Kelly *et al*., 2015)

Our population genetic study with VNTR markers showed a significantly high genetic differentiation between the 3APOP and 15APOP at both locations. The gene flow between the two populations was also low. Therefore, these results suggest that the 3APOP is different from the native 15APOP and the 3APOP might have introduced recently. Also the results from the present study demonstrated that when strains were grouped according to their chemotypic origin they represent distinct populations although they co-exist in the same location. Similar observations have reported in other studies Zhang *et al.* (2012) observed high levels of population differentiation among the *F. graminearum* 15-ADON, *F. asiaticum* NIV and 3-

ADON strains. A study done by Karugia *et al*. (2009) grouped 478 *F. graminearum* strains from China and Japan in to three populations, each population showed a significant correlation with chemotype. It has been also reported that *F. graminearum* strains show significant geographic sub structure, the strains in the same location may consist of several sub populations (van der Lee *et al*., 2015). In this study we detected a substantial population structure within Manitoba based on the type of trichothecenes produced by the strains. Also, we observed an association between genetic population identity and type of trichothecenes produced by that population. Therefore, trichothecene types could be used as a marker to identify the *Fusarium* populations in Winnipeg and Carman, Manitoba. A better understanding of the factors responsible for the shift in population frequencies and differences in trichothecene chemotype composition may enable the prediction of future population distribution. *Fusarium* chemotype diversity may be driven by differences in local selective pressures within a region. Therefore, population level variation should be considered in developing disease management strategies and toxin control programs. Finally, this study shows the importance of continous monitoring of *Fusarium* populations in a specific field/region. Information on trichothecene chemotype in a specific region helps to characterize the changes in pathogen diversity. It may also provide opportunities to identify novel *Fusarium* populations and trichothecene chemotypes that may pose new threats to food and feed safety in Canada. Similarly, information from this study is important in developing disease forecast models which enable growers to make economic decisions about FHB disease management stratergies to protect their crops from *Fusarium* infection.

CHAPTER 5

MOLECULAR PHYLOGENETIC RELATIONSHIPS, TRICHOTHECENE CHEMOTYPE DIVERSITY AND AGGRESSIVENESS OF STRAINS IN THE *FUSARIUM GRAMINERAUM* SPECIES COMPLEX COLLECTED FROM DIFFERENT COUNTRIES

5.1 Abstract

Fusarium head blight (FHB), caused principally by the species in the *Fusarium graminearum* species complex (FGSC), is an important disease in wheat, barley and other small grain crops worldwide. Grain infected with species in the FGSC may be contaminated with trichothecene mycotoxins such as deoxynivalenol (DON) and nivalenol (NIV). In this study we characterized the phylogenetic relationships, chemotype diversity, phenotypic characters and aggressiveness of 150 strains in FGSC collected from eight different countries. Phylogenetic analysis based on portions of two genes *EF-1α* and *MAT1-1-3* from 150 strains revealed six species in the FGSC, *F. graminearum s.s*, *F. asiaticum*, *F. meridionale*, *F. cortaderiae*, *F. boothii* and *F. austroamericanum*. In this collection, 50% of the strains were 15-acetyldeoxynivalenol (15ADON), 35% were 3-acetyldeoxynivalenol (3-ADON) and 15% were NIV. Evaluation of strains on moderately resistant wheat cultivar Carberry indicated that there is no significant difference among the species for disease severity (DS), percent fusarium damaged kernel (FDK %) and deoxynivalenol (DON) production. However, significant differences were observed among the chemotypes. Results showed significantly higher FHB DS, FDK, DON production, growth rates and macroconidia production for the 3-ADON strains than the15-ADON and NIV

strains. Also significant differences for FHB response variables were observed among the strains from different countries. Our results demonstrate that, type and amount of trichothecene produced by a strain, play a key role in determining the level of aggressiveness of that particular strain regardless of the species.

5.2 Introduction

Presently fusarium head blight (FHB) is one of the most economically important diseases of wheat throughout the world. Epidemics of FHB over the past 15 years in North America have had a devastating economic impact on agriculture (McMullen *et al*., 1997; Goswami & Kistler, 2004). Recent FHB outbreaks in Canada, Asia, Europe, Australia and South America suggest that the disease is a growing threat to world grain production (Goswami & Kistler, 2004; Obanor *et al*., 2013; DelPonte *et al*., 2014). The cumulative losses due to FHB during the 1990s within North America were estimated to exceed $4 billion (McMullen *et al*., 1997). One of the major concerns attributed with FHB is the contamination of grains with trichothecene mycotoxins and other estrogenic compounds. The *F. graminearum* species are capable of producing various B-trichothecenes, particularly, deoxynivalenol (DON), its acetylated derivatives, 3-acetyl and 15-acetyl deoxynivalenol (3-ADON and 15-ADON) and nivalenol (NIV) and its acetylated derivative, 4-acetyl nivalenol (4ANIV) (Foroud & Eudes, 2009). Based on the trichothecenes profile, *F. graminearum* strains can be categorized into three main chemical groups or chemotypes namely, 3-ADON, 15-ADON and NIV. A 3-ADON chemotype produces DON and 3-ADON, while a 15-ADON chemotype produces DON and 15-ADON and a NIV chemotype produces NIV and 4-ANIV (Miller *et al*., 1991; Ward *et al*., 2002; O'Donnell *et al*., 2004). In North America, DON is the main mycotoxin in *Fusarium* infected grain, while in parts of Asia

and Europe both DON and NIV are the common contaminants of grain (van der Lee *et al*., 2015). These mycotoxins pose a significant threat to human and animal health (Ichinoe *et al*., 1983). Trichothecenes are also acutely phytotoxic and act as virulence factors on sensitive host plants (Foroud & Eudes, 2009). Fusarium head blight is caused by several *Fusarium* spp. such as *F. graminearum, F. sporotrichioides, F. culmorum, F. cerealis, F. avenaceum, F. equiseti* and *F. poae* (Goswami & Kistler, 2004). Among these species of *Fusarium*, members of the *F. graminearum* species complex (FGSC) are considered to be the major etiological agents of FHB worldwide (O'Donnell *et al*., 2000). Other *Fusarium* species play a minor role in FHB development. Every species within the FGSC is capable of producing B-trichothecenes *in planta* (Wang *et al*., 2011). Traditionally *Fusarium* species have been classified based on the morphological characteristics such as the shape and size of the macroconidia, shape of the apical and basal cells in macroconidia and presence/absence of chlamydospores (Leslie & Summerell, 2006). Until 2000, members of the FGSC were considered as single species as morphological species criterion could not accurately determine the species limits within the group (Wang *et al*., 2011). However, with the introduction of DNA sequencing technology, use of sequence variations at different loci has become an important tool in determining the species limits with *Fusarium* spp (Leslie & Summerell, 2006). The sequences most widely used to identify species of *Fusarium* are portions of the DNA sequences encoding translation elongation factor 1-α, β-tubulin, internally transcribed spacer regions in the ribosomal DNA repeat region (ITS1 and ITS2), histone H3, and trichothecene biosynthesis genes, especially *TRI101* (O'Donnell *et al*., 2000, 2004; Leslie & Summerell, 2006). O'Donnell *et al*. (2000) first identified seven phylogenetic species within the FGSC, using genealogical concordance phylogenetic species recognition (GCPSR). More recently, using a high-throughput multilocus genotyping (MLGT)

assay of portions of 13 housekeeping genes, coupled with GCPSR, another nine phylogenetically distinct, cryptic species have been identified within the FGSC (Starkey *et al*., 2007; Yli-Mattila *et al*., 2009; Gale *et al*., 2011; Sarver *et al*., 2011; Wang *et al*., 2011). The species designation *F. graminearum* will therefore be *sensu stricto* (*s. s.*) and retained for the species most commonly associated with FHB worldwide. For other species in the complex, different species designation will be used. So far, 16 monophyletic species have been identified within the FGSC and these include: *F. austroamericanum* (lineage 1), *F. meridionale* (lineage 2), *F. boothii* (lineage 3) *F. mesoamericanum* (lineage 4), *F. acacia-mearnsii* (lineage 5), *F. asiaticum*, *F. graminearum sensu stricto* (lineage 7) , *F. cortaderiae* (lineage 8), *F. brasilicum*, *F. vorosii* , *F. gerlachii*, *F. aethiopicum* , *F. ussurianum*, *F. nepalense*, *F. louisianense* and U.S. Gulf Coast population of *F. graminearum s.s* (O'Donnell *et al*., 2004; Starkey *et al*., 2007; Yli-Mattila *et al*., 2009; Gale *et al*., 2011; Sarver *et al*., 2011; Wang *et al*., 2011). Among the members of the FGSC, *F. graminearum s.s* has been found worldwide, while the distribution of other species is restricted to certain geographic areas.

The translation elongation factor-1α (*EF-1α*) gene that encodes an essential part of the protein translation machinery has been extensively used to differentiate *Fusarium* spp. It has been shown that *EF-1α* provides much better resolution of relationships among and within lineages than other loci such as β-tubulin, calmodulin and ITS region (O'Donnell *et al*., 2004; Desjardins & Proctor, 2011). Also, the absence of non-orthologous copies in the genus makes *EF-1α* a better candidate to resolve phylogenetic relationships among species. Similarly, mating type (*MAT*) genes have also been used as phylogenetic markers to detect species boundaries (O'Donnell *et al*., 2004). *MAT* genes have been shown to control sexual development and ascospore production in a number of ascomycete fungi (Desjardins *et al*., 2004). The *MAT* locus

consists of four genes: the *MAT1-1* idiomorph with three genes (*MAT1-1-1*, *MAT1-1-2*, and *MAT1-1-3*) and the *MAT1-2* idiomorph with one gene (*MAT1-2-1*). Among the four *MAT* genes, *MAT1-1-3* and *MAT1-1-2* are the most informative and least homoplasious (Desjardins *et al*., 2004; O'Donnell *et al*., 2004). The above mentioned genes, combined with other nuclear genes, have been used to investigate the species limits within the *F. graminearum* clade.

Recent studies have shown the emergence of new species within the FGSC and rapid population shifts that are characterized by different chemotypes (Gale *et al*., 2011; Varga *et al*., 2015). The displacement of native 15-ADON chemotypes by a population with 3-ADON chemotypes in North America and emergence of new NIV-type and NX-2 populations in the USA have shown the importance of continued assessment of population shifts in FGSC (Ward *et al*., 2008; Gale *et al*., 2011; Liang *et al*., 2014; Varga *et al*., 2015). Pathogenic and toxigenic differences between *F. graminearum* populations in combination with environmental factors are hypothesized to be the major reason for rapid shifts in pathogen populations. In recent years, many studies have been performed to examine species and trichothecene chemotypes diversity among FGSC strains (Obanor *et al*., 2013; Boutigny *et al*., 2014; DelPonte *et al*., 2014; Qiu & Shi, 2014). Most of these studies were restricted either to a country or a particular region in a country. So far, limited studies have attempted to examine and compare the genetic diversity and pathogenicity among strains of FGSC associated with FHB from different countries (Miedaner & Reinbrecht, 2001; Goswami & Kistler, 2005; Toth *et al*., 2005). These types of studies are important to determine the potential risk of accidental introduction of strains that are not native to particular wheat producing regions. Also, the findings from genetic diversity studies show merit when making decisions in developing durable FHB resistant wheat cultivars/lines. It has been reported that aggressiveness of *F. graminearum* strains collected from different regions

within a country and even within populations from individual fields are highly variable therefore; FHB resistant wheat cultivars/lines that are resistant in one region may not give consistent results in other regions (Miedaner & Schilling, 1996, Miedaner & Reinbrecht, 2010). The performance of a resistant cultivar mainly relies on the pathogen profile, environmental conditions and the interaction between these two variables in a specific location. Variation in pathogenicity and aggressiveness in pathogen populations can lead to host resistance being overcome (Akinsanmi *et al*., 2006; Miedaner *et al*., 2008). Therefore, a better understanding of the pathogen profile, chemotype diversity and aggressiveness is important to develop better disease control stratergies to overcome the potential risks from existing and changing pathogen population (Akinsanmi *et al*., 2006).

The members of the FGSC are highly variable in a number of morphological traits, including the size and shape of their conidia, growth rate on standard media, pathogenicity on wheat cultivars and the type of mycotoxins produced. Acquiring knowledge of the trichothecene mycotoxins produced by predominant FGSC populations in a cereal production region and further analysing their role in the pathogenesis are important to understand the factors affecting plant- pathogen interaction (Spolti *et al*., 2012). Also, this will offer new approaches to breeding for FHB resistance. Therefore, the objectives of this study were: (1) to determine the trichothecene chemotypes of *F. graminearum* strains based on *TRI3* and *TRI12* gene-specific polymerase chain reaction (PCR) assays; (2) to evaluate the molecular phylogenetic relationships of FGSC strains collected from different countries based on the *EF-1α* and *MAT* gene sequences ; (3) to determine the phenotypic characteristics of the strains in the FGSC; and (4) to determine the aggressiveness of strains from different species in the FGSC.

5.3 Materials and methods

5.3.1 *Fusarium* strain collection

One hundred and fifty *Fusarium* strains obtained from eight countries were included in this study (Appendix 1). All strains are stored in the culture collection maintained at Department of Plant Science, University of Manitoba, Canada. All Canadian strains are available upon request and other strains can be provided only with the permission from the donor listed in the acknowledgements.

5.3.2 DNA extraction

All strains were grown on potato dextrose agar (PDA) (Difco Laboratories, ON, Canada) plates at 25^0C, under fluorescent light for 7 days and genomic DNA was extracted from the freeze dried aerial mycelium using a CTAB based protocol described by Fernando *et al*. (2006). DNA was treated with RNase (0.75% vol/vol). DNA was quantified using the NanoDrop3300 (Thermo Fisher Scientific Inc., MA, USA). DNA was diluted using sterilized distilled water for a final concentration of 50ng/µL and stored at -20°C until further use.

5.3.3 PCR assay and DNA sequencing

Sequence analysis was performed for PCR-amplified fragments of two genes (*EF-1α* and *MAT1-1-3*) that have been used previously to discriminate between species in the FGSC. The primers used to amplify gene fragments are listed in Table 5.1. The PCR reaction was performed in a 25 µL volume containing 20 ng of template DNA, 2.0 mM $MgCl_2$, 50 mM KCl, 10 mM Tris HCl (pH 8.0), 0.2 mM each dNTP (Invitrogen Life Technologies, CA, USA), 0.4 µM each primer, and 0.75 units of Taq DNA polymerase (Invitrogen Life Technologies, CA, USA). The PCR

amplification protocol consisted of an initial denaturation at 94°C for 2 min, followed by 30 cycles of 30 s at 94°C, 60 s at annealing temperature, 1 min at 68°C, and a final extension of 68°C for 10 min. The annealing temperatures for the PCR amplification were 52 °C for *EF-1α* and 68 °C for *MAT1-1-3* genes. For sequence analysis, PCR amplified DNA fragments were purified with Exosap-IT (Affymetrix Inc., CA, USA) PCR product clean up kit according to the manufacturer's instructions. Sequencing reactions were prepared using the BigDye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA). Finally, the sequencing products were analyzed on an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, CA, USA). All sequencing reactions were performed at The University of Kentucky Advanced Genetic Technologies Center.

Table 5.1 List of primers and sequences used to identify the chemotypes of *Fusarium graminearum* species complex strains

Primer name	Gene	Sequence 5'-3'	Reference
3CON	*TRI3*	TGGCAAAGACTGGTTCAC	Ward *et al*., 2008
3NA	*TRI3*	GTGCACAGAATATACGAGC	Ward *et al*., 2008
3D15A	*TRI3*	ACTGACCCAAGCTGCCATC	Ward *et al*., 2008
3D3A	*TRI3*	CGCATTGGCTAACACATG	Ward *et al*., 2008
EF-1	*EF-1α*	ATGGGTAAGGARGACAAGAC	O'Donnell *et al*., 2000, 2004
EF-2	*EF-1α*	GGARGTACCAGTSATCATGTT	O'Donnell *et al*., 2000, 2004
MAT13-1	*MAT1-1-3*	ATGCCTCCTGAAGCCGAGGTCC	O'Donnell *et al*., 2004
MAT13-2	*MAT1-1-3*	CGCTAGTTATCGCGTCCTCCC	O'Donnell *et al*., 2004

5.3.4 Molecular phylogenetic analysis

EF-1α and *MAT1-1-3* gene sequences were assembled, trimmed and edited using GENEIOUS v. 5.4.5 (Drummond *et al*., 2011). Multiple sequence alignments were performed using BIOEDIT v. 7.1.3 sequence alignment editor (Hall, 1999) default settings and checked manually to find the correct open reading frame. The final data set had an aligned length of 679 bp for *EF-1α* gene and 655 bp for *MAT1-1-3* gene. Genetic distances and a test for base composition were performed using PAUP* v 4.0 b10 (Swofford, 2000). A chi-square test of homogeneity of base frequencies across taxa was used to determine the frequency distribution of observed number of substitutional changes per character. Base composition of aligned sequences was calculated in MEGA 5.1 beta version (Tamura *et al*., 2011). The Kimura 2-parameter plus Gamma (K80+G) and symmetrical (SYM) models were determined as the best fitting models of nucleotide substitution using the hierarchical likelihood ratio tests (hLRT) for *EF-1α* and *MAT1-1-3* genes respectively (Tamura & Nei, 1993). The model selection test was carried out using MODELTEST v. 3.7 (Posada & Crandall, 1998), and the MODELTEST SERVER (Posada, 2006). Bayesian phylogenetic inference was performed using MR. BAYES v.3.1.2 (Huelsenbeck, 2004) with two independent runs with four chains each with default priors, and run for 10,000,000 generations. Chains were sampled at every 1000th generation. Convergence of all parameters and correct mixing of chains were confirmed by examining the likelihood plots for each run and when the average standard deviation of split frequencies (potential scale reduction factor-PSRF) was less than 0.02 (Huelsenbeck *et al*., 2000; Huelsenbeck, 2004). Trees derived from two independent searches were summarized using majority rule consensus tree. First 25% of the samples were discarded for burn-in. Calculation of the consensus tree and of the posterior probabilities of clades were done based upon the trees sampled after the burn-in, and trees were compiled and drawn using

FIGTREE v. 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree). *F. pseudograminearum* strain NRRL 34426 and *F. culmorum* strain NRRL 52792 were used as out-groups for *EF-1α* gene phylogenetic tree reconstruction. *F. pseudograminearum* strain NRRL 28065 and *F. culmorum* strain NRRL 25475 were used as out-groups for *MAT1-1-3* gene. Also sequences from Agricultural Research Services- ARS (NRRL) culture collection reference strains were downloaded from GenBank and incorporated into each alignment and derived phylogenetic trees.

5.3.5 PCR assays to determine trichothecene chemotypes

All primers used for the PCR-based identification of mycotoxin biosynthetic genes, along with references are presented in Table 5.1. Chemotype identification of each strain was performed using multiplex PCR primers 3CON, 3NA, 3D3A, 3D15A described by Ward *et al*. (2008). The multiplex PCR primers amplified an 840 bp fragment from NIV producing strains, a 610 bp fragment from 15-ADON producers and a 243 bp fragment from 3-ADON producers, respectively. The PCR reaction was performed in a 15 µL volume containing 20 ng of template DNA, 2.0 mM $MgCl_2$, 50 mM KCl, 10 mM Tris HCl (pH 8.0), 0.2 mM each dNTP (Invitrogen Life Technologies, CA, USA), 0.4 µM each primer, and 0.75 units of Taq DNA polymerase (Invitrogen Life Technologies, CA, USA). The PCR cycling conditions for multiplex PCR consisted of an initial denaturation at 94°C for 4 min, followed by 35 cycles of 1 min at 94°C, 40 s at 52 °C, 40 s at 72°C, and a final extension of 72°C for 6min. The PCR amplicons were separated on a 2 % agarose gel stained with RedSafe nucleic acid staining solution (iNtRON Biotechnology Inc., ON, Canada).

5.3.6 Radial growth and macroconidia production

The radial growth rate of each strain was evaluated *in vitro* on PDA plates in three replications. A small agar plug (5mm in diameter) of each strain was placed in the center of a 9 cm PDA plate. The cultures were grown at 25^0C with a 24 h photoperiod. The average growth was measured every 48 h for six days from three replicates in two perpendicular directions. Average colony diameter was used to calculate the growth rate of the strain.

The macroconidia production was assessed based on the colonies growing on synthetic nutrient agar (SNA) media during seven days at 25^0C and a 24 h photoperiod. After seven days of growth, conidia were harvested by adding 1mL of sterilized distilled water and scraping the surface of SNA media with a sterilized scalpel. Number of conidia was determined using a haemocytometer and the conidial concentration was expressed in conidia/mL.

5.3.7 Aggressiveness experiments and mycotoxin analysis

All strains listed in Appendix 1 were individually inoculated on FHB resistant wheat cultivar Carberry (except for the strains obtained from Mexico). Wheat plants were grown in plastic pots containing Sunshine Mix™ (Sun Gro Horticulture Ltd, MA, USA). The pots were arranged in a completely randomized design with five replicates (one plant per pot). Inoculations were done using the dual floret point inoculation method. For each wheat plant, 4-5 spikes were inoculated once individual spikes were close to 50% anthesis. The point of inoculation on each spike was determined by calculating the total spikelets per spike and multiplied by two-thirds. Each spike was inoculated by injecting 10 µl of a macroconidial suspension ($5x10^4$ spores/mL) between the lemma and palea of a floret. To facilitate infection, high humidity was maintained around the inoculated spikes by placing a glassine bag over the spike. The bag was removed 48 h post-

inoculation. Disease severity (DS) was rated 14 days post-inoculation using a pictorial scale reported by Stack and McMullen (1995).

After maturity, inoculated spikes were hand harvested and threshed using a belt thresher. A 10 g random seed sample was selected from pooled replicates and fusarium damaged kernel (FDK) percentage was counted as the number of FDKs in the total number of seeds. A FDK was considered to be any seed that was shrivelled, had any mycelial growth, or a chalky white or pink discoloration.

Deoxynivalenol and nivalenol analysis was carried out using the same sample used for FDK determination. The samples were ground to a powder of similar consistency using a coffee grinder (Cuisinart model no: DCG20BKNC) for 5-8 minutes. DON was extracted using 50 mL of deionized water, and then quantified using Veratox® DON 5/5 kit (product no: 8331, Neogen Corp., Lansing, MI, USA) with a DON quantification limit of 0.1 ppm. The amount of NIV in infected grains were analysed by GC-MS according to the protocol described by Tittlemier *et al*. (2013).

5.3.8 Statistical analysis

Analysis of variance (ANOVA) for fusarium head blight disease severity (FHB DS), FDK, DON/NIV content, radial growth and macroconidia production was performed using 'PROC MIXED' procedure of the SAS software (SAS version 9.3, SAS Institute Inc., Cary, NC). Because this data set has an unequal distribution of sample sizes, it was not possible to analyse the data set including all effects and their interactions. Therefore, to determine the effect of species and chemotype on FHB disease variables, data were analysed using the following model statement; species country chemotype rep species*chemotype. A second data analysis was

performed to determine the effect of country of origin and chemotype on FHB disease variables. The model statement for this analysis consisted of species country chemotype rep country*chemotype. The species, country, chemotype and their interactions (species*chemotype and country*chemotype) were considered as fixed effects. The main effects of species, country, chemotype, and their interactions were tested for significance using residual error terms. The correlation between FHB response variables were analysed using the SAS PROC CORR (SAS version 9.3, SAS Institute Inc., Cary, NC) procedure.

5.4 Results

5.4.1 Identification of *Fusarium* strains to species level

Genetic polymorphisms in the DNA sequence of the *EF-1α* and mating type locus gene *MAT1-1-3* have been used to assist in the identification of 150 strains to species level. Similar phylogenetic relationships were observed from both genes. Seven thousand three hundred and seven trees were formed with *EF-1α* gene. According to the 50% majority rule consensus tree 111 strains formed a distinct clade with reference strain *F. graminearum s.s* NRRL28336 confirming the strains identity as *F. graminearum s.s* (Fig 5.1). This clade includes strains from Australia, Poland, UK, Germany, Brazil, China and Canada. Another 17 strains from Mexico formed a distinct clade with *F. boothii* reference strain NRRL 29105. Nine strains from China formed a distinct clade with *F. asiaticum* reference strains NRRL 26156 and 34578. Another ten strains from the collection formed a distinct clade with *F. meridionale* reference strain NRRL 34439. This clade consists of strains originating from Australia, Brazil, China and Mexico. Two strains from Brazil formed a distinct clade with *F. cortaderiae* reference strain 29306. One strain from Brazil also formed a distinct clade with *F. austroamericarnum* reference strain NRRL

36957. Although a similar phylogenetic relationship was obtained with *MAT1-1-3* sequences the level of species resolution was less than that from *EF-1α* gene (Fig 5.2). Based on *MAT1-1-3* gene analysis, six species were identified in the *Fusarium* strain collection obtained from different countries.

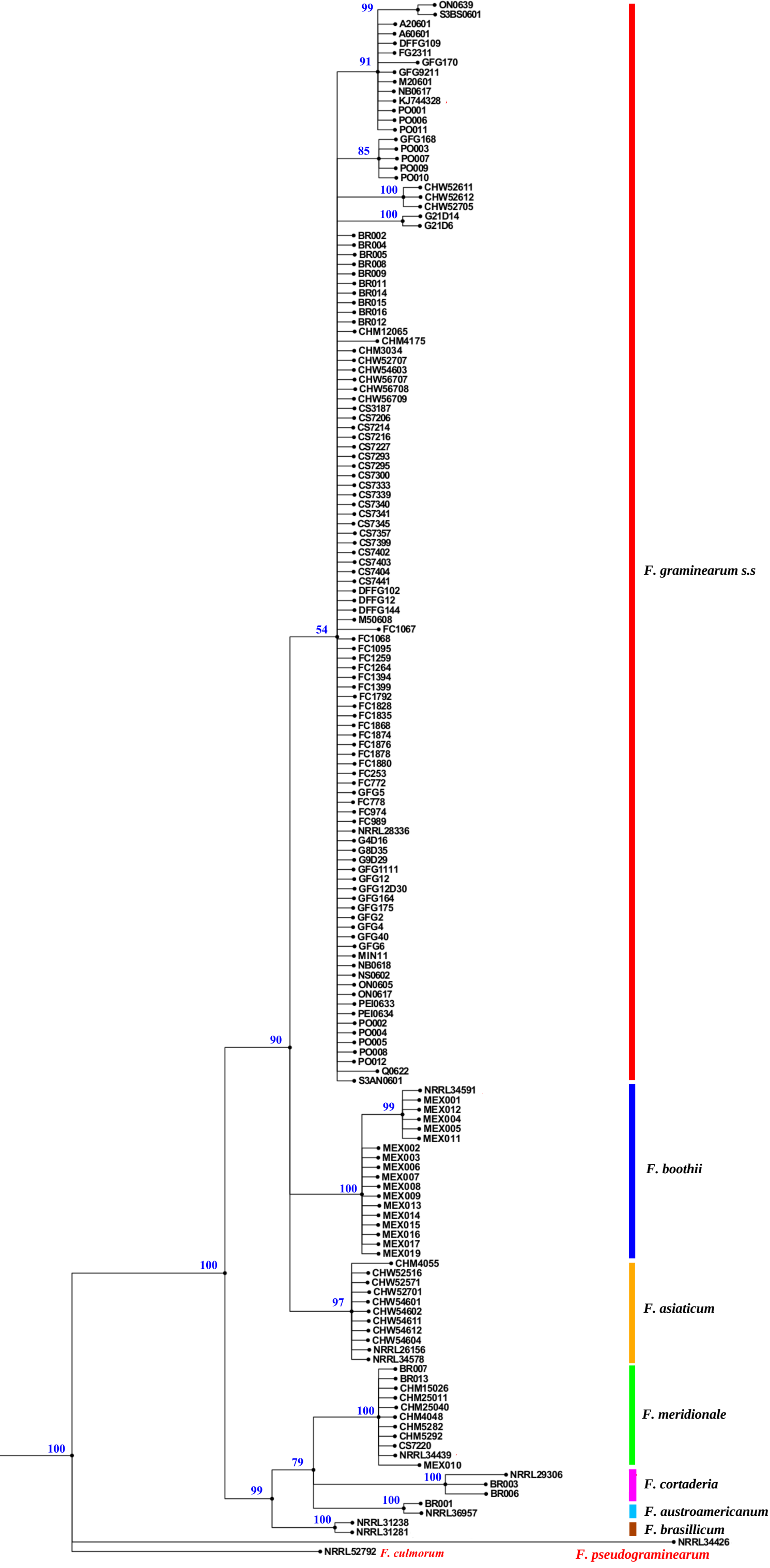

ON0639
S3BS0601
A20601
A60601
DFFG109
FG2311
GFG170
GFG9211
M20601
NB0617
KJ744328
PO001
PO006
PO011
GFG168
PO003
PO007
PO009
PO010
CHW52611
CHW52612
CHW52705
G21D14
G21D6
BR002
BR004
BR005
BR008
BR009
BR011
BR014
BR015
BR016
BR012
CHM12065
CHM4175
CHM3034
CHW52707
CHW54603
CHW56707
CHW56708
CHW56709
CS3187
CS7206
CS7214
CS7216
CS7227
CS7293
CS7295
CS7300
CS7333
CS7339
CS7340
CS7341
CS7345
CS7357
CS7399
CS7402
CS7403
CS7404
CS7441
DFFG102
DFFG12
DFFG144
M50608
FC1067
FC1068
FC1095
FC1259
FC1264
FC1394
FC1399
FC1792
FC1828
FC1835
FC1868
FC1874
FC1876
FC1878
FC1880
FC253
FC772
GFG5
FC778
FC974
FC989
NRRL28336
G4D16
G8D35
G9D29
GFG1111
GFG12
GFG12D30
GFG164
GFG175
GFG2
GFG4
GFG40
GFG6
MIN11
NB0618
NS0602
ON0605
ON0617
PEI0633
PEI0634
PO002
PO004
PO005
PO008
PO012
Q0622
S3AN0601
NRRL34591
MEX001
MEX012
MEX004
MEX005
MEX011
MEX002
MEX003
MEX006
MEX007
MEX008
MEX009
MEX013
MEX014
MEX015
MEX016
MEX017
MEX019
CHM4055
CHW52516
CHW52571
CHW52701
CHW54601
CHW54602
CHW54611
CHW54612
CHW54604
NRRL26156
NRRL34578
BR007
BR013
CHM15026
CHM25011
CHM25040
CHM4048
CHM5282
CHM5292
CS7220
NRRL34439
MEX010
NRRL29306
BR003
BR006
BR001
NRRL36957
NRRL31238
NRRL31281
NRRL34426
NRRL52792
F. culmorum
F. graminearum s.s
F. boothii
F. asiaticum
F. meridionale
F. cortaderia
F. austroamericanum
F. brasillicum
F. pseudograminearum

Figure 5.1 Phylogenetic tree based on a total of 679 bp of *EF-1α* gene sequences derived from Bayesian analysis. Numbers at the nodes represent the posterior probability values derived from the Bayesian analysis.

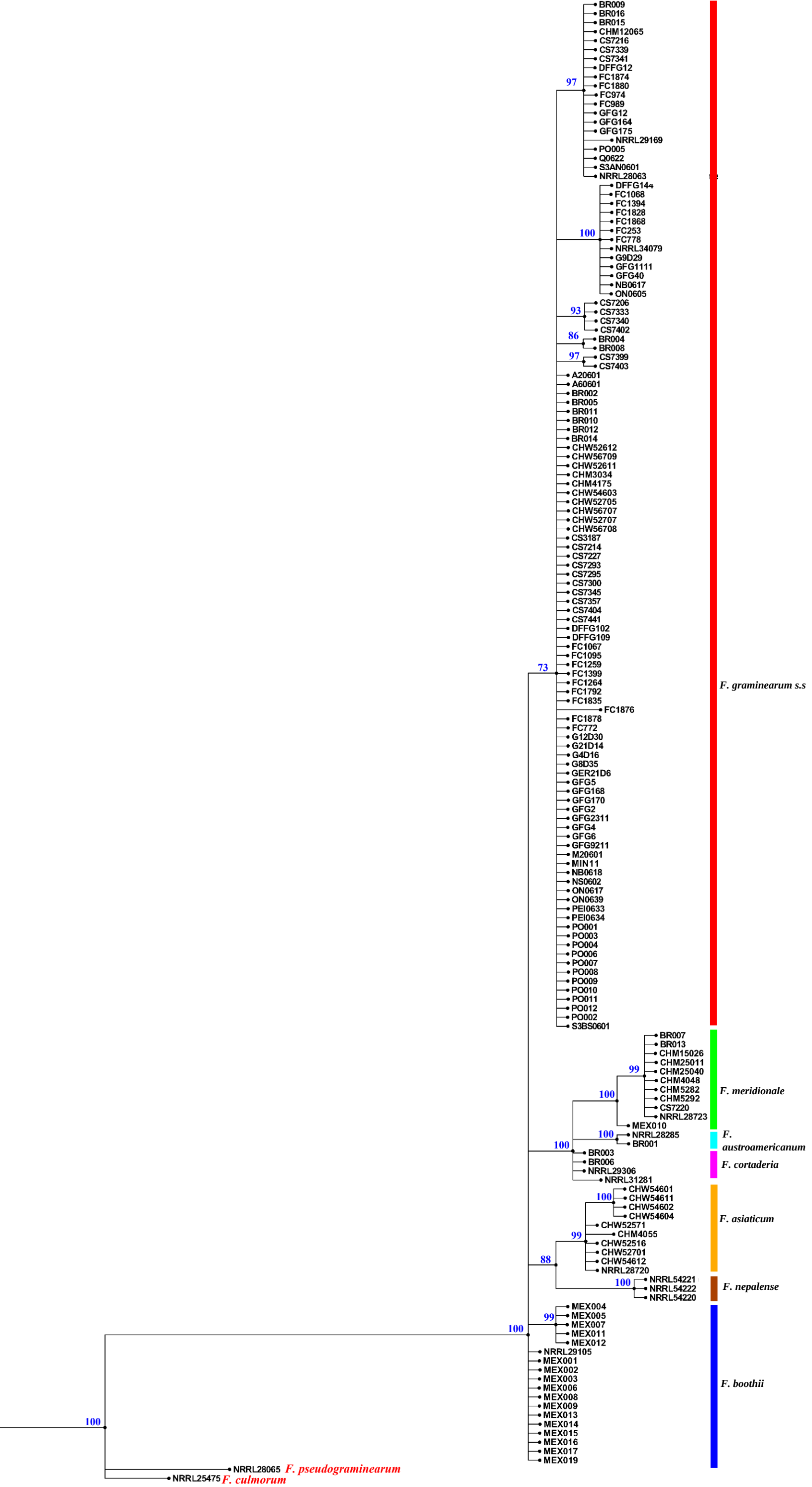

BR009
BR016
BR015
CHM12065
CS7216
CS7339
CS7341
DFFG12
FC1874
97
FC1880
FC974
FC989
GFG12
GFG164
GFG175
NRRL29169
PO005
Q0622
S3AN0601
NRRL28063
DFFG144
FC1068
FC1394
FC1828
FC1868
FC253
100
FC778
NRRL34079
G9D29
GFG1111
GFG40
NB0617
ON0605
CS7206
93
CS7333
CS7340
CS7402
86
BR004
BR008
97
CS7399
CS7403
A20601
A60601
BR002
BR005
BR011
BR010
BR012
BR014
CHW52612
CHW56709
CHW52611
CHM3034
CHM4175
CHW54603
CHW52705
CHW56707
CHW52707
CHW56708
CS3187
CS7214
CS7227
CS7293
CS7295
CS7300
CS7345
CS7357
CS7404
CS7441
DFFG102
DFFG109
FC1067
FC1095
73
FC1259
FC1399
FC1264
FC1792
FC1835
FC1876
FC1878
FC772
G12D30
G21D14
G4D16
G8D35
GER21D6
GFG5
GFG168
GFG170
GFG2
GFG2311
GFG4
GFG6
GFG9211
M20601
MIN11
NB0618
NS0602
ON0617
ON0639
PEI0633
PEI0634
PO001
PO003
PO004
PO006
PO007
PO008
PO009
PO010
PO011
PO012
PO002
S3BS0601
F. graminearum s.s
BR007
BR013
CHM15026
CHM25011
99
CHM25040
CHM4048
CHM5282
100
CHM5292
CS7220
NRRL28723
MEX010
100
NRRL28285
BR001
100
BR003
BR006
NRRL29306
NRRL31281
F. meridionale
F. austroamericanum
F. cortaderia
CHW54601
100
CHW54611
CHW54602
CHW54604
CHW52571
CHM4055
99
CHW52516
CHW52701
CHW54612
NRRL28720
88
NRRL54221
100
NRRL54222
NRRL54220
F. asiaticum
F. nepalense
MEX004
99
MEX005
MEX007
100
MEX011
MEX012
NRRL29105
MEX001
MEX002
MEX003
MEX006
MEX008
MEX009
MEX013
MEX014
MEX015
MEX016
MEX017
MEX019
F. boothii
100
NRRL28065
F. pseudograminearum
NRRL25475
F. culmorum
9.0E-4

Figure 5.2 Phylogenetic tree based on a total of 655 bp of *MAT1-1-3* gene sequences derived from Bayesian analysis. Numbers at the nodes represent the posterior probability values derived from the Bayesian analysis.

5.4.2 Trichothecene chemotypes

The PCR assays based on *TRI3* and *TRI12* genes produced amplified products of 840, 610 and 243 bp corresponding with NIV, 15-ADON and 3-ADON chemotypes, respectively. Among 111 *F. graminearum s.s* strains 50% of the strains were of 15-ADON chemotype, 35% were 3-ADON producers and 15% were NIV producers (Appendix 1). All NIV producing strains belonging to *F. graminearum s.s* were from Germany. Among the nine Chinese *F. asiaticum* strains, three strains were determined to be 3-ADON, three were NIV and another three were 15-ADON chemotypes. Among the 18 strains from Mexico, 17 strains were identified as *F. boothii* and all were 15-ADON producers. One strain was identified as *F. meridionale* and it was a NIV producer. All the *F. meridionale* strains in the collection (from Brazil, China and Australia) were identified as NIV producers. Also, the two strains of *F. cortaderiae* (from Brazil) were identified as NIV chemotype. One strain from Brazil, which identified as *F. austroamericanum* was determined to be a NIV chemotype.

5.4.3 Aggressiveness of strains on spring wheat

The aggressiveness of all 150 strains listed in Appendix 1 except for strains obtained from Mexico (strains from Mexico were received as DNA samples) was evaluated on the moderately resistant spring wheat cultivar Carberry. Analysis of variance on DS indicated that there was no significant difference among species. However, there were significant differences among the chemotypes. In addition, the two-way interaction species*chemotypes was not significant (Table

5.2). *F. graminearum s.s* and *F. asiaticum* 3-ADON strains showed a comparatively higher disease severity than other species and chemotypes (Fig 5.3a). When we analysed the DS data according to the country of origin of strains, and the trichothecene chemotypes, DS was significantly different among the strains originating from different countries. The interaction country*chemotype was also significant (data not shown). The 3-ADON chemotypes from Germany, UK, Poland and China showed higher DS followed by Canadian 3-ADON strains (Table 5.3). We could observe a similar pattern for FDK percentage, in which FDK percentage was not significantly different among the species, however, different among the chemotypes (Table 5.2). The species*chemotype interaction was not significant. However, *F. graminearum* 3-ADON and *F. asiaticum* 3-ADON strains showed a higher FDK percentage compared to other species and chemotypes (Fig 5.3b). In contrast to FHB DS, no significant difference was observed among the countries for FDK percentage (Table 5.2). The two-way interaction, country*chemotype was also not significantly different. However, 3-ADON chemotypes from UK, Germany, China, Canada and Poland showed higher FDK percentage than other chemotypes (Table 5.3).

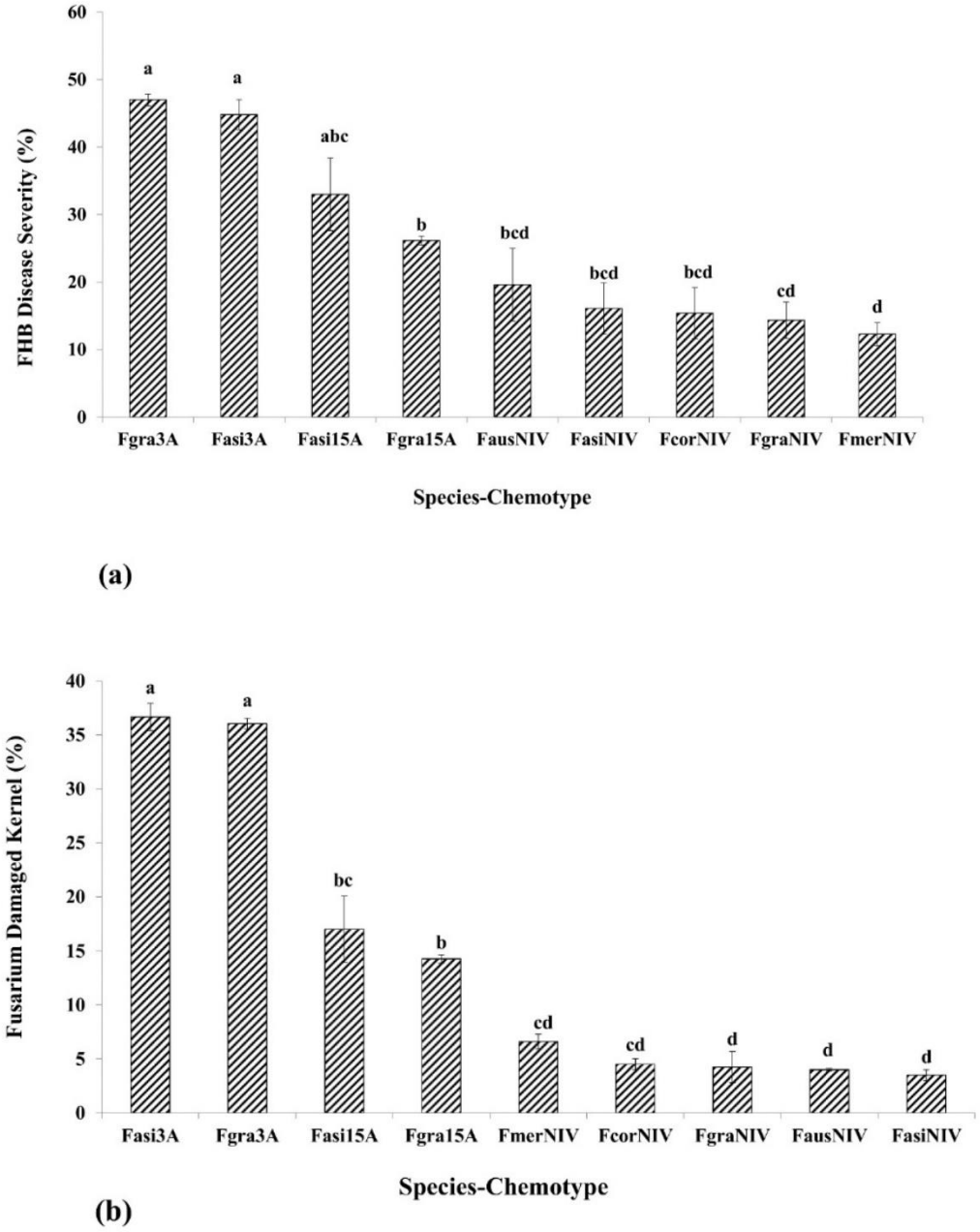


Figure 5.3 Comparison of mean (a) fusarium head blight disease severity and (b) percent fusarium damaged kernel by chemotype of different species in the *Fusarium graminearum* species complex on moderately resistant cultivar Carberry**.** Disease severity was measured 14 days post-inoculation. Means with the same letter for disease severity and Fusarium damaged kernels are not significantly different. (Fgra3A- *F. graminearum* 3-ADON chemotype; Fasi3A- *F. asiaticum* 3-ADON chemotype; Fasi15A- *F. asiaticum* 15-ADON chemotype; Fgra15A- *F. graminearum* 15-ADON chemotype; FausNIV- *F. austroamericanum* NIV chemotype; FasiNIV- *F. asiaticum* NIV chemotype; FcorNIV- *F. cortaderiae* NIV chemotype; FgraNIV- *F. graminearum* NIV chemotype; FmerNIV- *F. meridionale* NIV chemotype).

Table 5.2 Analysis of variance (ANOVA) table for fusarium head blight disease severity percentage, fusarium damaged kernel percentage, deoxynivalenol/nivalenol content, radial growth and macroconidia production in *Fusarium graminearum* species complex strains collected from different countries

Trait	Source	DF	MS	F	Pr >F
FHB DS %[1]	Species	4	108.82	0.89	0.4695
	Country	6	3358.81	27.47	<.0001
	Chemotype	2	12181.00	99.61	<.0001
	Species*Chemotype	8	327.51	2.69	0.0687
	Residual	636	122.28		
% FDK[2]	Species	4	11.90	1.18	0.3253
	Country	6	9.52	0.94	0.4694
	Chemotype	2	2481.38	245.05	<.0001
	Species*Chemotype	8	3.19	0.32	0.7303
	Residual	116	10.13		
DON/NIV (ppm)[3]	Species	4	37.08	0.34	0.8509
	Country	6	28.80	4.6	0.0003
	Chemotype	2	395.18	63.19	<.0001
	Species*Chemotype	8	7.01	1.12	0.3292
	Residual	116	6.25		
Growth (cm)	Species	4	4.00	6.35	<.0001
	Country	6	5.70	9.04	<.0001
	Chemotype	2	98.63	156.36	<.0001
	Species*Chemotype	8	0.36	0.57	0.5670
	Residual	376	0.63		
Macroconidia	Species	4	294.52	7.94	<.0001
	Country	2	270.51	7.29	<.0001
	Chemotype	6	8772.54	236.57	<.0001
	Species*Chemotype	8	393.36	10.61	0.0077
	Residual	376	37.08		

[1] %DS- Fusarium head blight disease severity percentage.
[2] %FDK- Fusarium damaged kernel percentage.
[3] DON/NIV (ppm)- Deoxynivalenol/nivalenol content in parts per million.

Table 5.3 Country of origin of strains, trichothecene chemotypes, mean values for disease severity, fusarium damaged kernel percentage, deoxynivalenol/ nivalenol content, radial growth, and macroconidia production of strains used in the study

Country of	Chemotype	% DS[1]	% FDK[2]	DON/NIV[3]	Growth (cm)	Macroconidia
Germany	3-ADON	53.2 a	38.0 a	17.3 a	8.5 a	69.1 ab
UK	3-ADON	48.1 ab	35.7 a	10.3 b	8.1 ab	66.2 b
Poland	3-ADON	46.9 abc	35.6 a	11.0 b	7.2 c	67.7 ab
China	3-ADON	44.8 abc	36.0 a	12.3 b	6.8 c	64.2 b
Canada	3-ADON	42.1 bcd	35.5 a	12.6 b	7.6 bc	70.9 a
China	15-ADON	39.1 cde	14.1 b	4.9 cd	4.6 fg	38.4 def
Germany	15-ADON	35.8 de	13.2 b	5.7 c	4.8 efg	43.0 cd
Canada	15-ADON	32.7 e	15.7 b	4.6 cd	5.4 de	43.6 cd
UK	15-ADON	23.5 fg	13.3 b	5.2 cd	5.0 def	45.0 c
Brazil	15-ADON	23.3 f	16.1 b	4.2 cd	5.6 d	38.4 def
Poland	15-ADON	18.8 fgh	13.8 b	4.0 cd	5.7 d	33.6 efg
Australia	15-ADON	16.6 gh	13.6 b	4.3 cd	4.5 fg	44.0 c
Brazil	NIV	15.8 fgh	5.8 c	0.4 d	4.1 gh	32.8 fg
Germany	NIV	14.3 fgh	4.2 c	0.3 d	4.0 gh	26.2 g
China	NIV	13.0 h	5.6 c	1.1 d	3.4 h	40.0 cde
Australia	NIV	9.8 fgh	7.0 bc	0.3 d	2.8 h	26.6 fg

[1] %DS- Fusarium head blight disease severity percentage.
[2] %FDK- Fusarium damaged kernel percentage.
[3] DON/NIV content (ppm)- Deoxynivalenol/nivalenol content in parts per million.
Means with the same letter in the column for each variable are not significantly different.

5.4.4 Deoxynivalenol /nivalenol content in infected grains

The ANOVA for total DON/NIV content in infected grains showed no significant difference among species, however, there were significant differences among the chemotypes (Table 5.2). The interaction species*chemotype was also not significant. However, F. *graminearum* 3-ADON and *F. asiaticum* 3-ADON strains produced higher amounts of DON in infected grains compared to other strains in the collection (Fig 5.4). In terms of DON/NIV content, significant differences were detected among countries and the country*chemotype interaction. The 3-ADON strains from Germany produced higher amounts of DON, followed by the 3-ADON strains from

Canada, China, Poland and UK. Nivalenol producing strains showed the lowest toxin contamination in infected grains (Table 5.3).

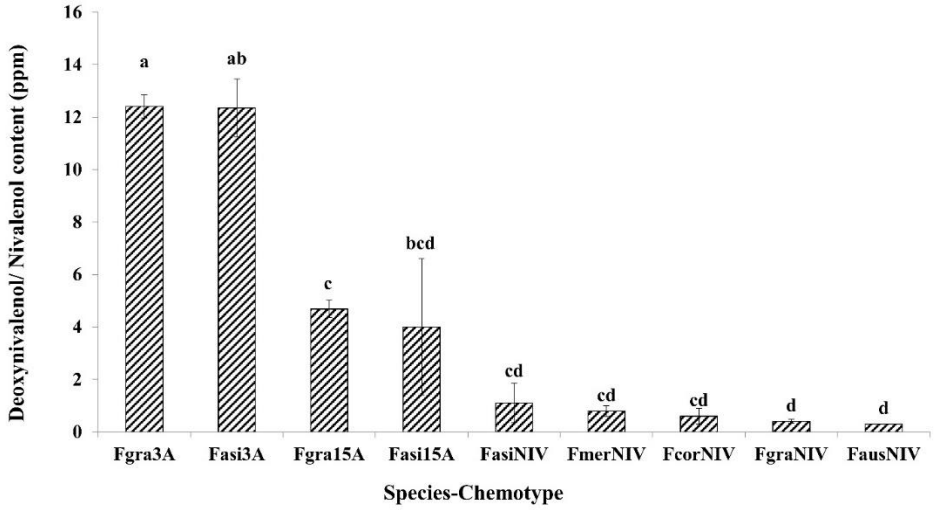


Figure 5.4 Comparison of mean deoxynivalenol/ nivalenol production by chemotype of different species in the *Fusarium graminearum* species complex on moderately resistant cultivar Carberry**.** Means with the same letter for deoxynivalenol/nivalenol content are not significantly different. (Fgra3A- *F. graminearum* 3-ADON chemotype; Fasi3A- *F. asiaticum* 3-ADON chemotype; Fgra15A- *F. graminearum* 15-ADON chemotype; Fasi15A- *F. asiaticum* 15-ADON chemotype; FasiNIV- *F. asiaticum* NIV chemotype; FmerNIV- *F. meridionale* NIV chemotype; FcorNIV- *F. cortaderiae* NIV chemotype; FgraNIV- *F. graminearum* NIV chemotype; FausNIV- *F. austroamericanum* NIV chemotype).

5.4.5 Radial growth and macroconidia production

In the present study, significant differences were observed among the species and chemotypes in the FGSC for growth rate (Table 5.2). The two-way interaction species*chemotype was not significant. *F. graminearum s.s* 3-ADON, *F. asiaticum* 3-ADON and *F. graminearum* 15-ADON showed significantly higher growth rates than other species (Fig 5.5). Also, significant differences in growth rates were observed among the strains originated from different countries (Table 5.2). The 3-ADON producing strains from Germany, UK, Canada, Poland and China showed higher growth rates than the other chemotypes from the same countries (Table 5.3).

Similar results were observed for macroconidia production, except the two-way interaction species*chemotype was significantly different (Table 5.2). *F. graminearum s.s* 3-ADON strains showed the highest macroconidia production on SNA media under *in vitro* conditions followed by *F. asiaticum* 3-ADON and *F. graminearum* 15-ADON strains (Fig 5.6). Canadian 3-ADON strains produced higher amounts of macroconidia under *in vitro* conditions followed by 3-ADON strains from Germany, Poland, UK and China (Table 5.3).

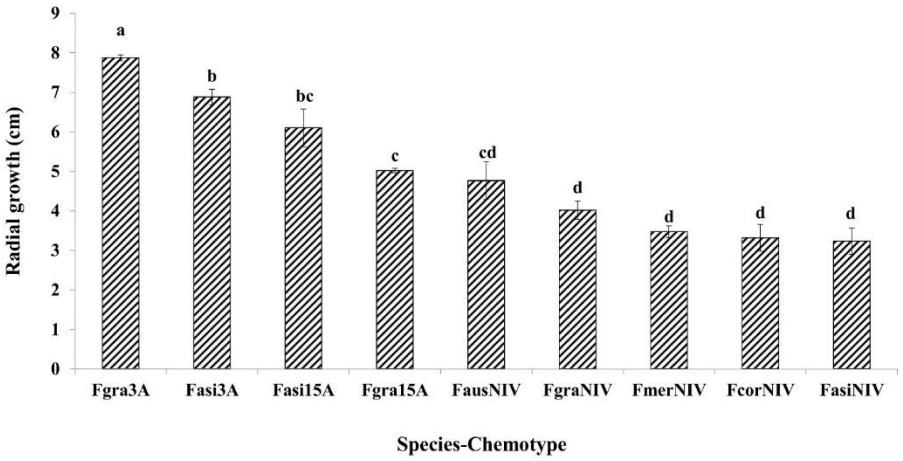


Figure 5.5 Comparison of mean radial growth by chemotype of different species in the *Fusarium graminearum* species complex on potato dextrose agar (PDA) media**,** seven days post-inoculation. Means with the same letter for mean radial growth are not significantly different. (Fgra3A- *F. graminearum* 3-ADON chemotype; Fasi3A- *F. asiaticum* 3-ADON chemotype; Fasi15A- *F. asiaticum* 15-ADON chemotype; Fgra15A- *F. graminearum* 15-ADON chemotype; FausNIV- *F. austroamericanum* NIV chemotype; FgraNIV- *F. graminearum* NIV chemotype; FmerNIV- *F. meridionale* NIV chemotype; FcorNIV- *F. cortaderiae* NIV chemotype; FasiNIV- *F. asiaticum* NIV chemotype).

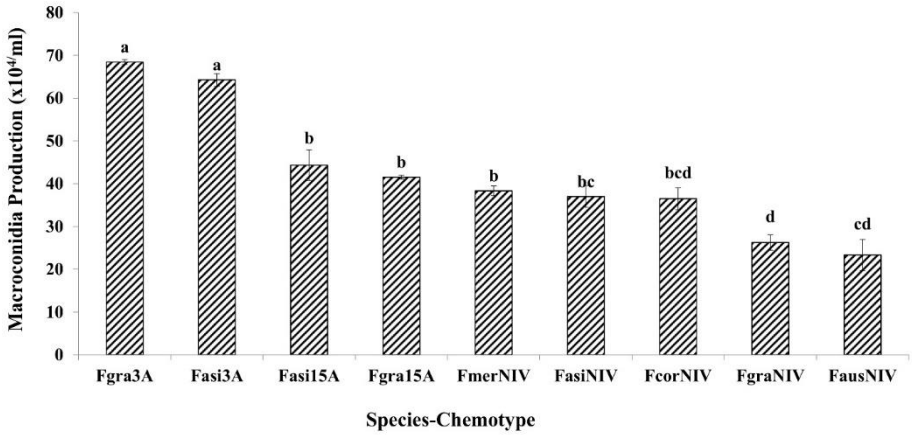


Figure 5.6 Comparison of mean macroconidia production by chemotype of different species in the *Fusarium graminearum* species complex on synthetic nutrient deficient agar (SNA) media**,** seven days post-inoculation. Means with the same letter for macroconidia production are not significantly different. (Fgra3A- *F. graminearum* 3-ADON chemotype; Fasi3A- *F. asiaticum* 3-ADON chemotype; Fasi15A- *F. asiaticum* 15-ADON chemotype; Fgra15A- *F. graminearum* 15-ADON chemotype; FmerNIV- *F. meridionale* NIV chemotype; FasiNIV- *F. asiaticum* NIV chemotype; FcorNIV- *F. cortaderiae* NIV chemotype, FgraNIV- *F. graminearum* NIV chemotype; FausNIV- *F. austroamericanum* NIV chemotype).

5.4.6 Correlation of FHB disease variables

To study the association between FHB disease variables, data on 132 strains were included in the correlation analysis. A significant positive correlation was observed between the FHB disease variables (Table 5.4). A highly significant positive correlation existed between FDK and DON/NIV content (R = 0.84, P <0.0001). A significant positive correlation was also identified between DS and FDK (R =0.72, P <0.0001). Among the examined FHB disease variables, the highest correlation was observed between macroconidia production and FDK (R =0.90, P <0.0001). The lowest correlation was observed between the DS and macroconidia production (R=0.64, P <0.0001).

Table 5.4 Pearson correlation coefficients of fusarium head blight response variables measured under controlled environment conditions and in the laboratory

	FHB Disease	**% FDK[1]**	**Radial**	**Macroconidia**	**DON/NIV**
FHB Disease severity	**1.000**	0.72*	0.67*	0.64*	0.66*
% FDK		**1.000**	0.84*	0.90*	0.84*
Radial growth			**1.000**	0.79*	0.75*
Macroconidia production				**1.000**	0.80*
DON/NIV content					**1.000**

[1] %FDK- Fusarium damaged kernel percentage.
[2] DON/NIV content - Deoxynivalenol/nivalenol content.
*Means correlation coefficient is significant at $p < 0.0001$

5.5 Discussion

In this study we conducted phylogenetic, *in vitro* and greenhouse experiments to compare the strains representing prevailing populations of the FGSC collected from different countries in relation to their trichothecene chemotype, phenotypic traits, and aggressiveness parameters. This type of comparative assessments for different species of the FGSC has not been exhaustively assessed since the subdivision of species in the FGSC has been proposed by O'Donnell *et al*. (2000). To our knowledge this is the first study that used *Fusarium* strains from eight different countries to analyse the phylogenetic relationships, chemotype diversity and aggressiveness. Therefore, this study helps to understand the population subdivision in FGSC based on differences in geographic location, chemotype diversity and aggressiveness.

Based on the *EF-1α* and *MAT1-1-3* gene sequences six species in the FGSC could be identified in our *Fusarium* strain collection. All the examined strains from Canada, UK, Germany and Poland, most of the strains from Australia and Brazil and few strains from China belong to the *F. graminearum s.s* clade. This is in agreement with other reports that show the wide geographical distribution of *F. graminearum s.s* among the other FHB species (Goswami &

Kistler, 2004; Wang *et al*., 2011; van der Lee *et al*., 2015). The strains from China belong to three different species, *F. asiaticum*, *F. graminearum s.s* and *F. meridionale*. In southern China, *F. asiaticum* is (approximately 97%) responsible for the major component of the FHB complex on wheat grown. However, in the north and northeast China, *F. graminearum s.s* (approximately 76%) was the most predominant species (Qu *et al*., 2008b; Zhang *et al*., 2012). This uneven distribution of *Fusarium* species in the FGSC is believed to depend on the different crop rotation practices and temperatures in those regions (Zhang *et al*., 2007, Zhang *et al*., 2012). A study done by Zhang *et al.* (2014) reported the occurrence of *F. meridionale* strains on maize in China for the first time. All the examined strains from Mexico belong to the *F. boothii* group except for one strain which belong to *F. meridionale*. The presence of *F. boothii* in Mexico has been previously reported by Malihipour *et al*. (2012). Similarly, all strains from Australia belong to the *F. graminearum s.s* except for one strain that was identified as *F. meridionale* (strain CS7220, isolated from wheat). *F. graminearum s.s* and *F. pseudograminearum* are considered to be the major pathogens causing FHB and fusarium root rot in Australia. In a recent outbreak of FHB in east Queensland and northern New South Wales *F. graminearum s.s* was found to be the cause (Obanor *et al*., 2014). Still there are no reports on *F. meridionale* strains from Australia. Therefore, the discovery of NIV producing *F. meridionale* from Australia may have a potential risk for Australian wheat industry (personnel communication with Dr. Friday Obanor).

Strains from Brazil consist of *F. graminearum s.s*, *F. cortaderiae*, *F. austroamericanum* and *F. meridionale*. Del Ponte *et al.* (2015) reported the co-occurrence of different species in the *F. graminearum* species complex in Brazil. They identified five species within FGSC in a multiyear survey of > 200 wheat fields in Paraná (PR) and Rio Grande do Sul (RS) states. These five species include *F. gramineraum s.s*, *F. meridionale*, *F. asiaticum, F. cortderiae* and *F.*

austroamericanum. Among these species, *F. graminearum s.s* and *F. meridionale* were the two most common species found in the regional collection of 671 strains.

In our collection 50% of the strains were of 15-ADON chemotype, 35% of the strains were 3-ADON and 15% of NIV chemotypes. All the strains in this collection from European countries such as Germany, UK and Poland were identified as *F. graminearum s.s*. In Europe for example, in Netherlands and Denmark, *F. culmorum* used to be the major causative agent of FHB before the year 2000, however, there was a population shift from *F. culmorum* to *F. graminearum* in Europe (Nielsen *et al*., 2001; Waalwijk *et al*., 2003). The dominance of *F. graminearum s.s* in European countries has also been reported by Jennings *et al*. (2004) and Talas *et al*. (2012). Among the different species in FGSC, all strains of *F. boothii* were determined to be 15-ADON producers. The PCR assays and chemical analysis done by Malihipour *et al*. (2012) and Sampietro *et al*. (2012) also reported that *F. boothii* strains are capable of producing DON and 15-ADON. All strains of *F. meridionale*, *F. cortaderiae* and *F. austroamericanum* were determined to be NIV producers. In contrast, 15-ADON producing *F. meridionale* strains, 3-ADON producing *F. cortaderiae* and *F. austroamericanum* have also been reported in Brazil and Uruguay (DelPonte *et al*., 2014). The trichothecene chemotypes detected in our collection are consistent with other reports of *F. meridionale*, *F. cortaderiae*, *F. austroamericanum* and *F. boothii* from other parts of the world including Europe, China, Brazil and Mexico (Scoz *et al*., 2009; Boutigny *et al*., 2011; Desjardins & Proctor, 2011).

Although many reports have been published on aggressiveness of *Fusarium* species and *F. graminearum* strains collected from a specific field or country, only a few studies have analysed and compared the aggressiveness in species within the FGSC (Toth *et al*., 2008;

Alvarez *et al*., 2010). In our study we have evaluated and compared the aggressiveness of strains representing five phylogenetic species of the FGSC collected from different geographical regions. We used the spring wheat cultivar Carberry which expresses moderate resistance to FHB and has marker alleles associated with *Fhb1* (DePauw *et al*., 2011). To date, most selections in FHB resistant wheat breeding programs in worldwide are concentrated on the *Qfhs.ndsu-3BS* (*Fhb1*). *Fhb1* is a major FHB resistance gene, which is essential to provide Type II resistance in wheat cultivars against *F. graminearum* (Cuthbert *et al*., 2006). Therefore, use of a cultivar having *Fhb1* is important to evaluate the stability of resistance under the influence of different species and chemotypes of *F. graminearum*. In our study, in terms of *F. graminearum* species origin, no significant differences were observed for FHB disease severity (DS), FDK percentage and DON/NIV content. However, significant differences were observed for radial growth and macroconidia production. A study done by Goswami & Kistler (2005) also reported a large variation among different strains in terms of aggressiveness and trichothecene production. This variation appeared to be strain-specific rather than species-specific characteristics. In terms of chemotype origin of strains, significant differences were observed for all analysed FHB response variables. The two-way interaction species*chemotype was not significantly different for all measured FHB response variables except for macroconidia production. When we analysed the data based on the country of origin of strains, significant differences were observed for all FHB response variables except for FDK percentage. A study done by Malihipour *et al.* (2010) also compared the aggressiveness of *F. graminearum* strains from Canada, Iran and Mexico and found significant differences in aggressiveness of the strains from different geographical regions. In the current study, country*chemotype interaction was also significantly different for FHB DS, DON/NIV content, radial growth and macroconidia production. However, country*chemotype

interaction was not significantly different for FDK percentage. Although there was no significant difference in species*chemotype interaction for FHB DS, *F. graminearum s.s* 3-ADON strains showed the highest FHB DS in MR cultivar Carberry, followed by *F. asiaticum* 3-ADON, *F. asiaticum* 15-ADON, *F. graminearum s.s* 15ADON and other species producing NIV toxin. The 3-ADON producing strains from Europe (Germany, UK and Poland) showed highest FHB DS in cultivar Carberry compared to other strains. When considered the total DON content in infected grains was considered, *F. graminearum s.s* 3-ADON showed the highest DON accumulation followed by *F. asiaticum* 3-ADON, *F. graminearum s.s* 15-ADON and *F. asiaticum* 15-ADON producing strains. Also, the 3-ADON producing strains from Germany showed the highest DON content followed by Canadian, Chinese and Poland strains. Apart from FHB DS and DON production, 3-ADON strains showed higher levels of FDK percentage, radial growth and macroconidia production compared to the 15-ADON and NIV strains. In our study, both *F. graminearum s.s* and *F. asiaticum* species showed higher aggressiveness than the other species of the FGSC. Other species in the FGSC such as *F. meridionale*, *F. austroamericanum*, *F. cortaderiae* showed lower aggressiveness towards the MR wheat cultivar, Carberry. One of the reasons may be the production of less phytotoxic NIV by these species. A study done by Eudes *et al*. (2000) compared the phytotoxicity of eight different trichothecenes and reported that NIV is less phytotoxic than DON. Taken together, the results obtained from this study showed that, 3-ADON producing strains had the highest aggressiveness followed by 15-ADON and NIV producing strains respectively. Similar results have been reported by Puri & Zhong (2010), Malihipour *et al*. (2012), Spolti *et al*. (2012) and Zhang *et al*. (2012) in which they also explained the higher aggressiveness of 3-ADON producing strains over 15-ADON and NIV strains. The lowest aggressiveness of NIV producing strains have also been reported in other

studies (Cumagun *et al*., 2004; Goswami & Kistler, 2005). The higher aggressiveness of 3-ADON producing strains in terms of DON production, mycelial growth and macroconidia production may provide a fitness advantage over other chemotypes, suggesting that selection was driving the rapid spread of 3ADON over 15ADON and NIV chemotypes. Therefore, the higher aggressiveness and possibility of production of higher amounts of DON by 3-ADON strains in wheat cultivars introgressed with *Fhb1*, is a concern due to the rapid increase in 3-ADON producing strains in Canada and North America (Guo *et al*., 2008; Ward *et al*., 2008; Amarasinghe *et al*., 2015). A significant correlation was observed between the amount of DON/NIV produced by each strain and its level of aggressiveness on wheat in terms of DS, FDK percentage, growth and macroconidia production. Therefore, this study suggests that the type and amount of trichothecenes produced by a strain functions as a major determinant of aggressiveness on wheat. Also our results are in agreement with previous reports showing that, trichothecenes, have a major role in determining the aggressiveness of the pathogen (McCormick, 2003; Talas *et al*., 2012). In terms of species origin of strains, we did not find a clear association between the species of a strain and its aggressiveness. However, the aggressiveness of strains may be partially based on species and population-specific features (Umpiérrez-Failache *et al*., 2013). Therefore, systematic testing of many more strains representing different species, populations, and toxin types within the FGSC is required to understand this relationship in more detail.

In this study we characterized the phylogenetic relationships, chemotype patterns and aggressiveness of *Fusarium* strains in the FGSC collected from different regions, and evaluated the association between their phylogenetic and/or chemotype patterns with aggressiveness. The existence of high variability in *Fusarium* strains may explain the reasons for different reactions

of wheat genotypes in different locations. Therefore, the results of the current study also suggest that screening for FHB resistance may require the use of highly aggressive strains or a mixture of strains representative of the FGSC diversity in order to develop durable FHB resistant wheat cultivars.

CHAPTER 6

NUCLEOTIDE AND AMINO ACID SEQUENCE VARIATIONS OF THE *TRI8* GENE AMONG THE STRAINS OF *FUSARIUM GRAMINEARUM* SPECIES COMPLEX

6.1 Abstract

Trichothecene chemotype variation is one of the key factors that is used to analyse the populations of *Fusarium* species that cause fusarium head blight. Determining the chemotype of a strain is important to gain epidemiological information on the *Fusarium* population distribution in a certain region/country. Information on trichothecene chemotype in a region/country will provide on the toxigenic risk associated with that chemotype in the given region/country. A better understanding of the DNA sequence variation in trichothecene biosynthesis genes, the quantitative variation of pathogenicity and deoxynivalenol (DON) production in *Fusarium graminearum* species complex (FGSC) is important for predicting the *Fusarium* population dynamics. It has been reported that the differential activity of the *TRI8* gene is a key determinant of the 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON) chemotypes in *Fusarium*. Therefore, in this study we have analysed the sequence variation of *TRI8* gene in different species in the FGSC. The Bayesian phylogenetic tree represented three monophyletic clades, each representing 3-ADON, 15-ADON and nivalenol (NIV) trichothecene chemotypes. *TRI8* haplotypes grouped according to chemotype rather than by species, indicating that NIV, 3-ADON and 15-ADON chemotypes have a single evolutionary origin. Also the unique DNA sequence polymorphisms observed in *TRI8* gene sequences suggest the potential use of *TRI8* gene as a marker to identify chemotypes within the FGSC.

6.2 Introduction

Trichothecenes are the most studied and harmful mycotoxins produced by *Fusarium* spp. They inhibit eukaryotic protein biosynthesis and cause severe toxicities in humans and other animals (Maresca, 2013). Trichothecenes are also phytotoxic and act as virulence factors on various host plants (Proctor *et al*., 2009; Suzuki *et al*., 2014). The species in the *Fusarium* genus are capable of producing different types of toxins depending on differences in the core trichothecene cluster (*TRI* cluster) genes (Pasquali & Migheli, 2014). The two main classes of trichothecenes are A-trichothecenes and B- trichothecenes. Type B trichothecens have a keto group at the C-8 position, whearas, Type A trichothecenes lack a keto group at the C-8 position (Ueno *et al*., 1973). Among Type B trichothecenes, deoxynivalenol (DON), nivalenol (NIV) and their acetylated derivatives 3-acetyldeoxynivalenol (3-ADON), 15- acetyldeoxynivalenol (15-ADON) and 4-acetylnivalenol (4ANIV) pose a significant impact on food and feed safety issues in many countries. Based on the type of trichothecenes produced, different chemotypes or trichothecene genotypes have been described for *Fusarium* species (Pasquali & Migheli, 2014). The two main chemotypes, 1) DON chemotype; consists of strains producing DON and its acetylated derivatives and 2) NIV chemotype; consists of strains producing NIV and its acetylated derivatives. The DON chemotype has been further classified into strains producing different combinations: the 3-ADON chemotype; strains producing DON and 3-ADON and the 15-ADON chemotype; strains producing DON and 15-ADON (Miller *et al*., 1991; Sydenham *et al*., 1991). The difference between the DON and NIV chemotypes are determined by the *TRI7* and *TRI13* genes in the core *TRI* cluster. DON, 3-ADON and 15-ADON producers lack functional *TRI7* and *TRI13* genes whereas NIV producers have functional copies of the above genes (Lee *et al*., 2002). The difference between 3-ADON and 15-ADON chemotypes is based on the presence or

absence of an acetyl group at C-3 and C-15 positions in the core trichothecene ring; 3-ADON has a C-3 acetyl group but does not have a C-15 acetyl group, whereas 15-ADON has a acetyl group at C-15 position, however lacks an acetyl group at C-3 position (Foroud & Eudes, 2009; Alexander *et al*., 2011). Nucleotide polymorphisms in the coding region of *TRI3* and *TRI12* have been used to differentiate the 3-ADON and 15-ADON chemotypes in *F. graminearum* species complex (Starkey *et al*., 2007). Apart from *TRI3* and *TRI12* genes, the DNA sequence polymorphisms in *TRI13* have also been used to distinguish the strains producing 3-ADON, 15-ADON and NIV (Wang *et al*., 2008). However, the PCR primers developed by Wang *et al*. (2008), based on the polymorphism of the *TRI13* gene was effective only for *F. asiaticum* strains and not effective for identifying chemotypes in other species in the FGSC (Amarasinghe *et al*., 2011; Pasquali *et al*., 2011). Alexander *et al*. (2011) have reported that the differential activity of the *TRI8* gene which encodes for a C-3 esterase determines the production of 3-ADON or 15-ADON in *F. graminearum* strains. In 3-ADON strains, C-3 esterase catalyzes deacetylation of 3, 15-diacetyldeoxynivalenol at the C-15 position to yield 3-ADON. Similarly, in 15-ADON strains, Tri8 catalyzes deacetylation of the 3, 15-diacetyldeoxynivalenol at C-3 position to yield 15-ADON (Alexander *et al*., 2011).

The fitness of a certain chemotype in a given environment may be detrmined by the structural differences among the trichothecene chemotypes. The different patterns of oxygenation and acetylation during the trichothecene biosynthesis process can modify the bioactivity and consequently the toxicity of trichothecenes on animal, human, or plant species (Pasquali & Migheli, 2014). Therefore, to date trichothecene chemotype variation has become an important tool to analyse the population structure of *Fusraium* species (Alexander *et al*., 2011). In North America, there are two major populations of *F. graminearum s.s* that have been

grouped according to their chemotypic origin, 3-ADON and 15-ADON populations. Recent trichothecene chemotype distribution studies in North America have shown a rapid shift of the previously common 15-ADON population by the 3-ADON population (Guo *et al*., 2008; Ward *et al*., 2008; Puri & Zhong, 2010; Amarasinghe *et al*., 2015). In China and Japan, a chemotype shift from NIV producing strains to DON producing strains has been reported (Zhang *et al*., 2012). Moreover, a study done by Waalwijk *et al*. (2003) reported an increase in NIV producing *F. graminearum* and *F. culmorum* strains rather than the DON producing strains in Europe. In order to understand the rapid and apparent shifts in trichothecene chemotype populations in *F. graminearum* species, it is important to determine the genetic basis of 3-ADON, 15-ADON and NIV chemotypes. Therefore, the objectives of this study were to: 1) characterize the DNA and amino acid sequence polymorphisms of *TRI8* genes in 3-ADON, 15-ADON and NIV strains of the *Fusarium graminearum* species complex and 2) determine the phylogenetic relationships among different chemotypes in the *F. graminearum* species complex based on the *TRI8* gene sequences.

6.3 Materials and methods

6.3.1 *Fusarium* strains

One hundred and forty-two strains from six species in the *Fusarium graminearum* species complex collected from eight different countries and six FGSC reference strains obtained from Agricultural Research Services- ARS (NRRL) culture collection in Peoria, Illinois, USA were included in this study (Appendix 2.1).

6.3.2 DNA extraction

All strains were grown on potato dextrose agar (PDA) (Difco Laboratories, ON, Canada) plates at 25^0C, under fluorescent light for 7 days and genomic DNA was extracted from the freeze dried aerial mycelium (~100 mg) using a CTAB based protocol described by Fernando *et al*. (2006). DNA was treated with RNase (0.75% vol/vol). DNA was quantified using the NanoDrop3300 (Thermo Fisher Scientific Inc., MA, USA). DNA was diluted using sterilized distilled water for a final concentration of 50 ng/µL and stored at -20°C until further use.

6.3.3 DNA sequencing

To evaluate the phylogenetic relationships of the strains based on the *TRI8* gene sequences, the *TRI8* gene was amplified with primers listed in Table 6.1 (Alexander *et al*., 2011). The partial sequence of *TRI8* was amplified from 15-ADON producers using primer pair 2036/2037; from 3-ADON producers using primer pair 2038/2039; and from NIV producers using primer pair 2040/2041. In order to compare the full length *TRI8* gene sequences and deduced amino acid sequences among the different species and chemotypes in the collection, representative strains from each chemotype and species were selected from the above collection. These selected strains include; *F. graminearum s.s* 15-ADON strain PH-1, *F. boothii* 15-ADON strain NRRL29105, *F. asiaticum* strain CHW52701, *F. graminearum* 3-ADON strain M50601, *F. asiaticum* 3-ADON strain CHW54601, *F. graminearum s.s* NIV strain GFG6, *F. asiaticum* NIV strain CHW52516, *F. cortaderiae* NIV strain NRRL 29306, *F. austroamericanum* NIV strain NRRL 28585, *F. acacciae-mearnsii* NIV strain NRRL 26754 and *F. meridionale* NIV strain NRRL 28436. To obtain full length *TRI8* gene sequences from 15-ADON producers, primer sets, TRI815A5PF/TRI815A5PR, 2036/2037 and TRI815A3PF/TRI815A3PR; from 3-ADON

producers, primer sets, TRI83A5PF/TRI83A5PR, 2038/2039 and TRI83A3PF/TRI83A3PR; and from NIV producers, primer sets, TRI8N5PF/TRI8N5PR, 2040/2041 and TRI8N3PF/TRI8N3PR were used (primer map is shown in Appendix 2.2). To analyse the full length of *TRI8* gene sequence, genomic DNA was used as a template. This is because, *TRI8* gene only consists with exons and so far, no introns have been reported (Alexander *et al*., 2011). All amplifications were performed using Platinum® Taq DNA polymerase high-fidelity enzyme (ThermoFisher Scientific Inc., MA, USA), following the manufacturer's recommendations. The PCR cycle consisted with following conditions, 95^0C, 2 min; followed by 30 cycles of 95^0C, 30 s; 52^0C, 30 s; 72^0C, 2 min; followed by 72^0C, 10 min. For sequence analysis, PCR amplified DNA fragments were purified with Exosap-IT (Affymetrix Inc., Santa Clara, USA) PCR product clean up kit according to the manufacturer's instructions. Sequencing reactions were prepared using the BigDye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA). Finally, the sequencing products were analyzed on an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). All sequencing reactions were performed at The University of Kentucky Advanced Genetic Technologies Center.

Table 6.1 Primer names and sequences used to amplify the *TRI8* gene sequences

Primer Name	5'----3' Sequence
2036	CAGAAGCTTGTTCACTCACTCAGTATGGCTCTCG
2037	CAGGAATTCTGTCAAGAAATGAAATTACCAGGCAG
2038	ACGAAGCTTATCCATCCATCCATTTAGTATGGTTCTCG
2039	CAGGAATTCGGCTCACATTTCTTATCACAACTCTC
2040	CAGAAGCTTCATTCATCCACTCAGCATGGCTCTCGATC
2041	CAGGAATTCAGGTCAAGAGATGAATTACCAGGC
TRI83A5PF	TCATCCATCCATCCATTCAG
TRI83A5PR	GGGAGTCATAAGGCACTTGG
TRI83A3PF	CTCAGCGACCCGAAGATTAC
TRI83A3PR	CTTCTCCCGTCACGTCTCTC
TRI815A5PF	CCTTAGCCCTTCCTCATTT
TRI815A5PR	GCGGGCTTGAAATGACTATC
TRI815A3PF	CACCTATAGCTGATACCGATGC
TRI815A3PR	ACACAGTATAAGACCACTACAGG
TRI8N5PF	AATCGCAGTACGTGTTGAGG
TRI8N5PR	TGATACCAGCGGTGAAAGC
TRI8N3PF	CAACCCAAGTGTCTTCATCC
TRI8N3PR	ACTGTACTGCAAGTTCTGATGC

6.3.4 Phylogeny reconstruction

The *TRI8* sequences obtained from different strains were assembled, trimmed and edited using GENEIOUS v. 5.4.5 (Drummond *et al*., 2011). The trimmed and edited sequences were aligned manually using BIOEDIT v. 7.1.3 sequence alignment editor (Hall, 1999). The final data set had an aligned length of 738 bp. According to the hierarchical likelihood ratio test (hLRT), the Kimura 2-parameter plus Gamma (K80+G), was selected as the best fitting model of nucleotide substitution for the analyzed data matrix (Tamura & Nei, 1993). The Bayesian inference of phylogeny was performed using MR. BAYES v. 3.2.1 on the CIPRES SCIENCE GATEWAY v. 3.1 as described by Amarasinghe *et al*. (2015). Phylogenetic tree was constructed using MR. BAYES v. 3.2.1 with *F. culmorum* (62A2) as the out-group.

The multiple sequence alignments for full length *TRI8* gene sequences obtained from selected strains mentioned above were performed by hand using BIOEDIT v. 7.1.3 (Hall 1999). Amino acid sequences were deduced from the aligned DNA sequences using the Ctrl+G function in BIOEDIT v. 7.1.3.

6.3.5 Haplotype network and population analysis

Haplotype analysis was carried out using DNASP v. 5.10.1 (Librado & Rozas, 2009). A haplotype network was generated using the NETWORK v. 4.6.12 program (fluxus-engineering.com). Sites with missing data and gaps were excluded from the analysis and all invariable sites were included in the analysis. Haplotype network was then constructed using a median joining approach with maximum parsimony.

6.4 Results

The final alignment length of *TRI8* gene sequence was 738 bp. The phylogenetic tree obtained from the Bayesian analysis is shown in Figure 6.1. According to the phylogenetic analysis, all strains were grouped in to three clusters with 100% posterior probability (shown in Fig 6.1 as 3-ADON, 15-ADON and NIV clusters). All strains which produce 15-ADON, grouped into one cluster and all 3-ADON strains grouped into another cluster. Similarly, all NIV producing strains grouped into another cluster. Therefore, all strains were grouped into clusters based on their chemotypic origin. The 15-ADON cluster was genetically closer to the NIV cluster and 3-ADON cluster was genetically more distant from both 15-ADON and NIV clusters (Fig 6.1).

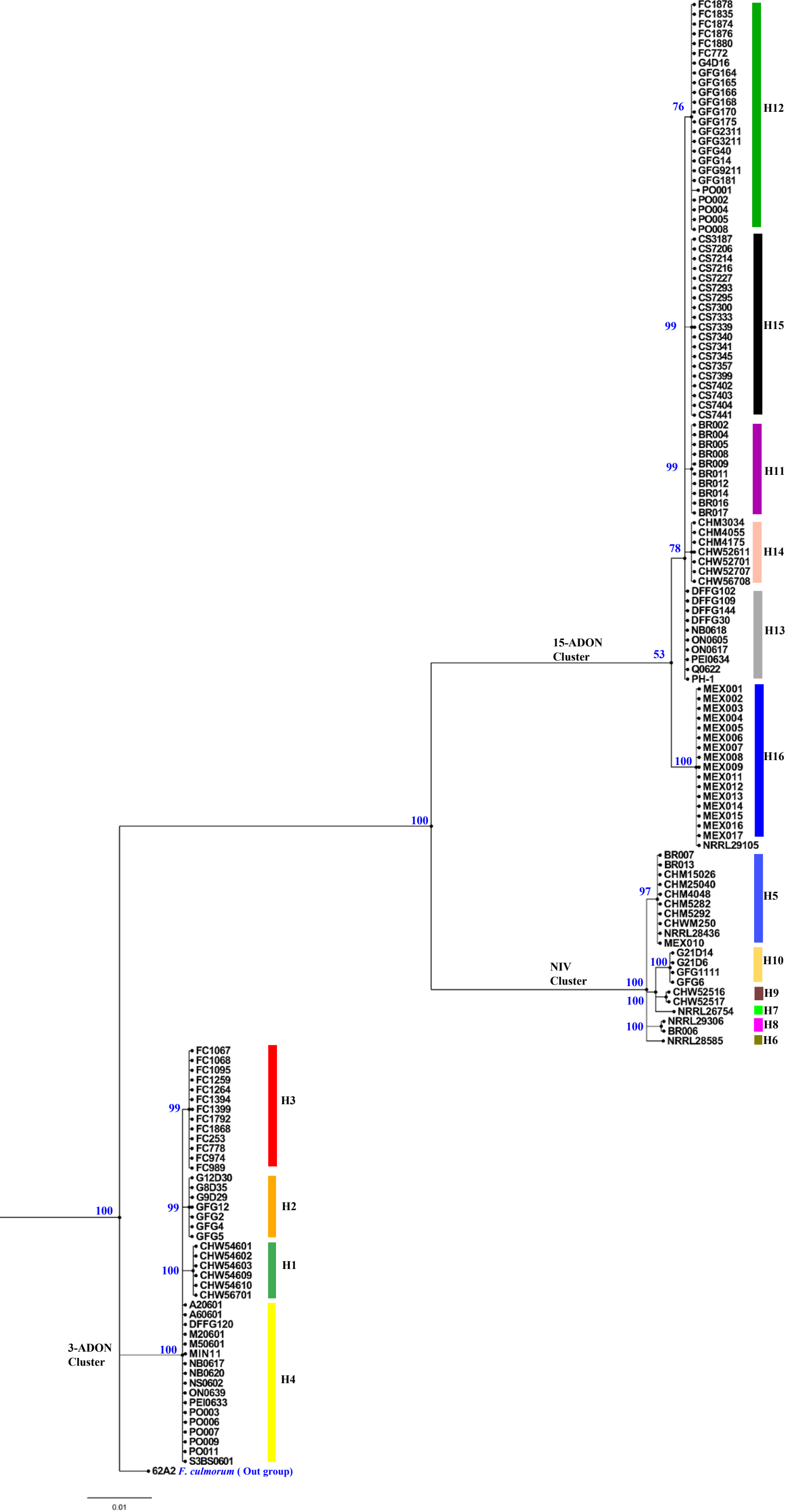

FC1878
FC1835
FC1874
FC1876
FC1880
FC772
G4D16
GFG164
GFG165
GFG166
GFG168
GFG170
GFG175
GFG2311
GFG3211
GFG40
GFG14
GFG9211
GFG181
PO001
PO002
PO004
PO005
PO008
CS3187
CS7206
CS7214
CS7216
CS7227
CS7293
CS7295
CS7300
CS7333
CS7339
CS7340
CS7341
CS7345
CS7357
CS7399
CS7402
CS7403
CS7404
CS7441
BR002
BR004
BR005
BR008
BR009
BR011
BR012
BR014
BR016
BR017
CHM3034
CHM4055
CHM4175
CHW52611
CHW52701
CHW52707
CHW56708
DFFG102
DFFG109
DFFG144
DFFG30
NB0618
ON0605
ON0617
PEI0634
Q0622
PH-1
MEX001
MEX002
MEX003
MEX004
MEX005
MEX006
MEX007
MEX008
MEX009
MEX011
MEX012
MEX013
MEX014
MEX015
MEX016
MEX017
NRRL29105
BR007
BR013
CHM15026
CHM25040
CHM4048
CHM5282
CHM5292
CHWM250
NRRL28436
MEX010
G21D14
G21D6
GFG1111
GFG6
CHW52516
CHW52517
NRRL26754
NRRL29306
BR006
NRRL28585
FC1067
FC1068
FC1095
FC1259
FC1264
FC1394
FC1399
FC1792
FC1868
FC253
FC778
FC974
FC989
G12D30
G8D35
G9D29
GFG12
GFG2
GFG4
GFG5
CHW54601
CHW54602
CHW54603
CHW54609
CHW54610
CHW56701
A20601
A60601
DFFG120
M20601
M50601
MIN11
NB0617
NB0620
NS0602
ON0639
PEI0633
PO003
PO006
PO007
PO009
PO011
S3BS0601
62A2 F. culmorum (Out group)
H12
H15
H11
H14
H13
H16
H5
H10
H9
H7
H8
H6
H3
H2
H1
H4
15-ADON Cluster
NIV Cluster
3-ADON Cluster
76
99
99
78
53
100
100
97
100
100
100
99
100
100
0.01

Figure 6.1 Phylogenetic tree based on a total of 738 bp of *TRI8* gene sequences derived from Bayesian analysis. Numbers at the nodes represent the posterior probability values derived from the Bayesian analysis. The colored vertical bars represent the *TRI8* haplotypes in each chemotype group. The colored vertical bars correspond with the haplotypes shown in the haplotype network (Figure 6.2).

The median-joining haplotype network of the *TRI8* gene sequences of all strains is shown in Figure 6.2. Similar to the phylogenetic tree, three main clusters can be identified in the haplotype network representing each chemotype. Within the 3-ADON cluster, four haplotypes were identified (H1, H2, H3 and H4). These haplotypes represented strains of different geographical origin. Six haplotypes were identified within the NIV producing cluster (H5, H6, H7, H8, H9 and H10) and six haplotypes within the 15-ADON cluster (H11, H12, H13, H14, H15 and H16). In the NIV cluster, each haplotype represents a different species within the FGSC. Similar to the 3-ADON cluster, haplotypes within the 15-ADON cluster represented strains of different geographical origin (Fig 6.2). Moreover, the 3-ADON producing strains from Poland and Canada belonged to the same haplotype (Fig 6.1: H4, from strain A20601 to S3BS0601, strains from Poland were designated using the code 'PO' and the remaining strains were from Canada). This result indicated that 3-ADON producers in Canada are more closely related to the 3-ADON producers in Poland than to the 15-ADON producers in Canada.

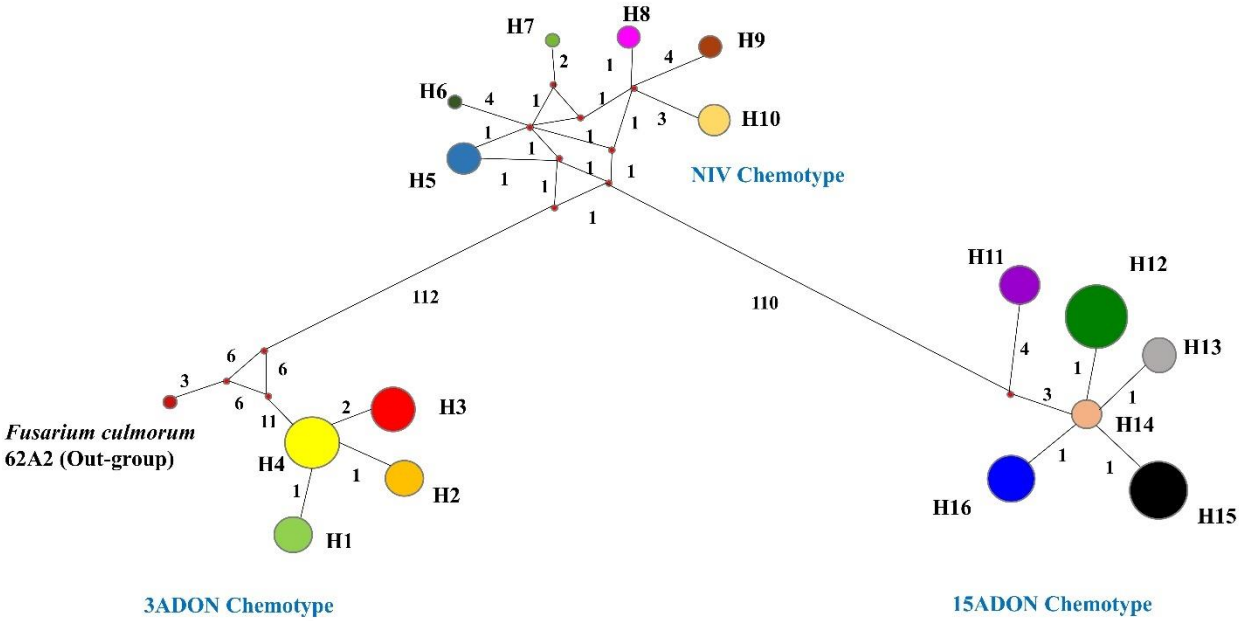


Figure 6.2 Haplotype network derived based on the *TRI8* gene sequences among the strains of *Fusarium graminearum* species complex**.** Each colored circle represents the corresponding haplotypes within a chemotype. The size of the circles represents the number of strains in each haplotype. Haplotypes are numbered starting with letter 'H'. The numbers along the edges are for the number of mutations separating haplotypes.

When we analysed the full length *TRI8* gene from selected strains representing different species and chemotypes in the FGSC, the length of the coding region was different among the chemotypes. The 15-ADON producing strains had the longest coding region with 1338 bp in length followed by NIV producing strains (1335 bp). The 3-ADON producing strains had the shortest coding region, which was 1299 bp in length. The variations in length were due to the insertion and/or deletion at the 3' end of the coding region. No predictive introns were found in *TRI8* gene sequences from all the analysed species and chemotypes. The amino acid sequences deduced from the *TRI8* gene sequences were also different among the chemotypes. Amino acid sequences from 15-ADON producing strains were more similar to NIV producing strains than the 3-ADON producing strains. When analysed the deduced amino acid sequence of the *TRI8*

gene among the *F. graminearum s.s* 3-ADON, 15-ADON and NIV strains was 85% similar between 15-ADON and NIV chemotype, however, the similarity between 3-ADON and 15-ADON was only 78% (Fig 6.3). The conserved amino acid sequence "GYSSG" was found at at amino acid positions 201-205 in 15-ADON chemotypes, 200-204 in 3-ADON and 199-203 in NIV chemotype. Within the same chemotypes, the species did not show very high amino acid variations. For example, the amino acid sequence similarity was 99.3% between 15-ADON producers, *F. graminearum s.s* strain PH-1 and *F. boothii* strain NRRL29105. Similarly, the 3-ADON producers, *F. graminearum s.s* strain M50601 and *F. asiaticum* strain CHW54601 showed 96.5% similarity between the *TRI8* amino acid sequences. Among NIV producers, *F. graminearum s.s* strain GFG6 and *F. asiaticum* strain CHW52516 showed 99.7 % amino acid sequence similarity (Fig 6.3).

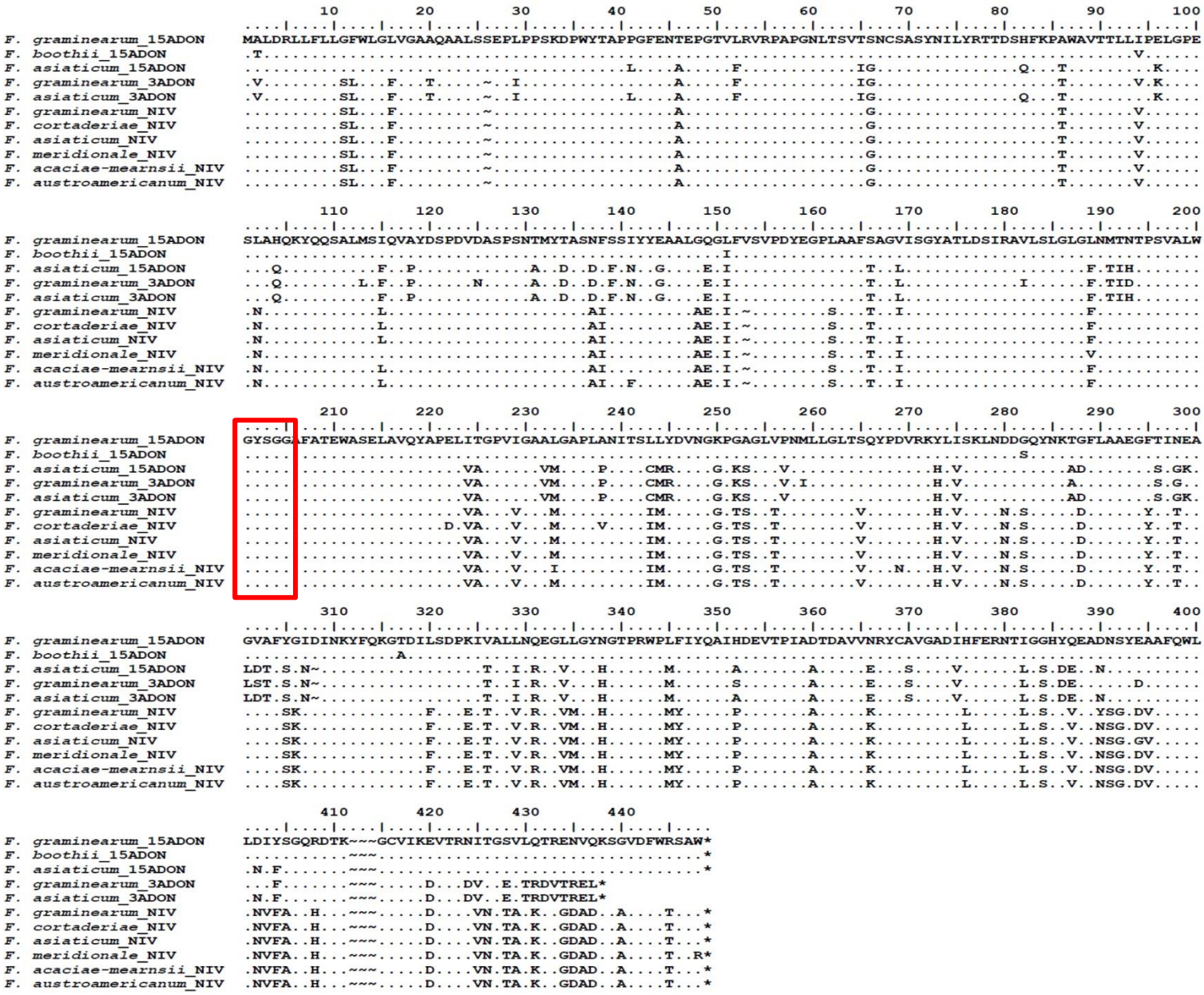


Figure 6.3 Amino acid sequences of *TRI8* from different species and chemotypes in the *Fusarium graminearum* species complex. Identical sequences are indicated by a period (.) and gaps are indicated by a tilde (~). The nucleophilic binding site "GYSGG" is represented within the red colored rectangle.

6.5 Discussion

Genetic polymorphisms within the trichothecene biosynthesis related genes are responsible for determining different chemotypes of *F. graminearum*. Therefore, in this study we have analysed the genetic polymorphism of *TRI8* gene in different species and chemotypes in FGSC. The current study has shown considerable polymorphisms and variations in DNA and amino acid sequences of the *TRI8* gene among the different species and chemotypes in FGSC. When we analysed both the phylogenetic tree and the haplotype network derived from *TRI8* gene sequences, all strains originating from different geographical regions and species clustered according to their chemotypic origin. These results are in agreement with studies by Ward *et al*. (2002), in which they explained that 3-ADON, 15-ADON and NIV each have multiple independent evolutionary origins. Ward *et al*. (2002) have analysed other *TRI*- cluster genes such as *TRI3*, *TRI4*, *TRI5*, *TRI6*, *TRI10*, *TRI11* and *TRI12*. Therefore, this study, based on *TRI8* gene phylogeny, further confirms the findings by Ward *et al*. (2002), in which they explained that, polymorphisms within the *TRI* cluster genes were trans-specific and maintained by balancing selection acting on chemotype differences. Trans-species polymorphism is the occurrence of similar alleles in related species. This can occur when an ancestral species carries multiple alleles of a gene and the alleles are inherited differentially during subsequent speciation events (Proctor *et al*., 2009). Separation between groups may continue for a long period of time and the differences between their alleles can become more and more pronounced due to differences in climate, host and disease management practices, eventually leading to the formation of new species with novel characteristics. Interestingly, we have found that the 3-ADON producing strains from Canada and Poland belong to the same haplotype. Therefore, these results further validate the evidence that 3-ADON population in Canada/North America are the result of a

transcontinental introduction. The studies by Gale *et al*. (2007) and Ward *et al*. (2008) have also reported that 3-ADON populations in North America are more closely related to the Italian population than to sympatric 15-ADON populations. Taken together, these results strongly suggest that, 3-ADON populations in Canada/North America was introduced from Europe to eastern Canada and have subsequently spread west across Canada. The phylogenetic tree derived from *TRI8* gene sequences demonstrated that 15-ADON producing strains are genetically more related to NIV producing strains than 3-ADON producing strains. A comparative analysis among the chemotypes revealed significant differences in the DNA and deduced amino acid sequences of *TRI8* gene from 3-ADON, 15-ADON and NIV strains. The overall sequence identity of 3-ADON and 15-ADON in *F. graminearum s.s* was 84%, 15-ADON and NIV was 88% and 3-ADON and NIV was 85%. The main sequence differences among chemotypes were based on the 3' end of the *TRI8* gene. The 15-ADON and NIV producers have 21 base pairs at the 3'- end of *TRI8* gene that were absent from the 3-ADON producers. This 21 bp difference at the 3' end was also observed in *F. asiaticum* 3-ADON producers. In this study, we have also included other species in the FGSC such as *F. boothii*, *F. asiaticum*, *F. meridionale*, *F. cortaderiae*, *F. austroamericanum* and *F. acacia-mearnsii*. When we consider the deduced amino acid sequences from all three chemotypes, a conserved amino acid motif "GYSGG" was present in all chemotypes in the same approximate location. This conserved amino acid motif was present in all the analysed species in the FGSC and represents the characteristic esterase activity. For most esterase enzymes, the active-site serine residue is located within the "GXSXG" motif, forming a 'nucleophile elbow' (Ollis *et al*., 1992). Alexander *et al*. (2011) have reported that, the differences in the 3' end of the coding region of *TRI8* gene might not be responsible for the differences in Tri8 esterase function. However, the sequence differences between amino acid

positions 216 and 312 of the protein may regulate the Tri8 activity in 3-ADON and 15-ADON chemotypes. As reported by many authors, 3-ADON producing strains have emerged recently in the environment and they are rapidly replacing the older populations of 15-ADON and NIV (Ward *et al*., 2008, Guo *et al*., 2008; Puri & Zhong, 2010). The 3-ADON producers were shown to be the predominant chemotype in many environments and tend to be more aggressive and produce higher levels of toxins in culture and in planta (Ward *et al*., 2008; Puri & Zhong, 2010; von der Ohe *et al*., 2010). The increased virulence of 3-ADON producers may be explained by the sequence variations in the *TRI8* gene. Alexander *et al*. (2011) showed that differences in the *TRI8* sequence play an important role in determining whether a certain isolate of *Fusarium* either produces 3-ADON or 15-ADON. Results from the current study further expanded the previous findings by including other species in the FGSC. We have observed that sequence variations of *TRI8* genes in all three chemotypes occur across all the analysed species in the FGSC.

Finally, this study showed that, sequence differences in the *TRI8* gene are more distinct to the chemotype rather than the species within the FGSC. Therefore, sequence varaitions in the *TRI8* gene are useful in developing molecular markers to differentiate chemotypes in the FGSC. Also *TRI8* gene phylogeny from different species in the FGSC further confirms the trans-species polymorphism within the *TRI* cluster genes. The DNA and amino acid polymorphism in *TRI8* gene sequences may have important fitness consequences in *Fusarium* strains.

CHAPTER 7

COMPARATIVE ANALYSIS OF DEOXYNIVALENOL BIOSYNTHESIS RELATED GENE EXPRESSION AMONG DIFFERENT CHEMOTYPES OF *FUSARIUM GRAMINEARUM* IN SPRING WHEAT

7.1 Abstract

Fusarium mycotoxins, deoxynivalenol (DON) and nivalenol (NIV) act as virulence factors and are essential for symptom development after initial infection in wheat. To date, 16 genes have been identified in the deoxynivalenol biosynthesis pathway. However, a comparative gene expression analysis in different chemotypes of *F. graminearum* in response to FHB infection remains to be explored. Therefore, in this study, nine genes belonging to the trichothecene biosynthesis pathway were compared at the transcriptional level among 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON) and nivalenol (NIV) producing *F. graminearum* strains in a time course study. Real time quantitative polymerase chain reaction revealed that the expression of all examined *TRI* gene transcripts initiated at two days post-inoculation (dpi), peaked at three to four dpi and gradually decreased at seven dpi. The early induction of *TRI* genes indicates that high levels of *Tri* transcript accumulation at early stages, is important to initiate the biosynthetic pathway of DON and NIV. Comparison of gene expression among the three chemotypes showed that relative expression of *TRI* genes was higher in 3-ADON producing strains compared with 15-ADON and NIV strains. Comparatively higher levels of gene expression may contribute to the higher levels of DON produced by 3-ADON strains in infected grains.

7.2 Introduction

Fusarium mycotoxins are the largest group of mycotoxins, which contains over 140 known metabolites. The exposure risk to human and animals is directly through foods of plant origin or indirectly through foods of animal origin (Sobravo *et al*., 2010). These mycotoxins are synthesized mainly by the members in the *Fusarium graminearum* species complex (FGSC) (Foroud & Eudes, 2009). The fungi in the FGSC have the potential to devastate a crop by reducing grain quality and quantity. After *Fusarium* infection, t he grains become contaminated with mycotoxins such as deoxynivalenol (DON), produced by the pathogen, making the crop unsuitable for food and feed. The mycotoxin deoxynivalenol acts as a virulence factor in wheat plants and is necessary for symptom development after initial infection (Audenaert *et al*., 2013). It has been reported that DON is important for the spread and establishment of *F. graminearum* within the host plant. Non-DON producing strains of *F. graminearum* can initiate the infection, but not spread within the host tissue (Bai *et al*., 2002). A study done by Diamond *et al*. (2013) found that DON is capable of inhibiting the apoptosis–like programmed cell death in *Arabidopsis* cell cultures subjected to heat stress. So far, 16 genes have been characterized in the DON biosynthesis pathway. These genes reside at four different loci on different chromosomes; the core *TRI* cluster consists of 12 genes located on chromosome 2, the *TRI1-TRI16* loci on chromosome 1, *TRI101* on chromosome 4, and *TRI15* on chromosome 3, respectively (Gale *et al*., 2005; Alexander *et al*., 2009; Merhej *et al*., 2011). The first step in the DON biosynthesis pathway consists of the cyclization of farnesyl pyrophosphate (FPP) to produce trichodiene, and this step is catabolized by the trichodiene synthase encoded by *TRI5* (Hohn & Beremand, 1989). This step is followed by nine reactions mediated by the enzymes encoded by *TRI4*, *TRI101*, *TRI11* and *TRI3*, respectively and leading to the formation of calonectrin (Fig 2.6, literature

review) (Alexander *et al*., 2009; Merhej *et al*., 2011). The genes *TRI7* and *TRI13* are functional only in *F. graminearum* strains that are capable of producing NIV (Brown *et al*., 2001; Lee *et al*., 2002). The products from these genes mediate two common steps following calonectrin. In nivalenol producing *F. graminearum* strains, the pathway continues with the product of *TRI1* to produce 4-acetyl NIV and the final step mediated by *TRI8* to give NIV. The *TRI7* and *TRI13* genes are not active in DON producers, therefore, DON biosynthesis proceeds directly from calonectrin with the products of *TRI1* and *TRI8* and leads to the formation of either 3-ADON or 15-ADON followed by DON (McCormick & Alexander 2002; Alexander *et al*., 2011; Merhej *et al*., 2011). The formation of 3-ADON or 15-ADON is strain specific and decided by the esterase coding sequence of *TRI8* gene (Alexander *et al*., 2011). To date, limited research has been done on expression of *TRI* genes in different chemotypes of *F. graminearum* during wheat colonization. Among the different *TRI* genes, *TRI5* gene has received more attention and so far the majority of studies have focused on the expression of the *TRI5* gene during *Fusarium*-wheat colonization. A study done by Hallen-Adams *et al*. (2011) examined the expression of the *TRI5* gene during wheat spike infection of susceptible and resistant cultivars and susceptible cultivars treated with strobilurin fungicides. The highest expression of the *TRI5* gene was observed at the infection front. Gardiner *et al*. (2009) reported that *TRI5* gene is strongly expressed in the rachis tissue of wheat. In this study they used a *F. graminearum* strain constructed by fusing a green fluorescent protein (GFP) marker to the promoter of *TRI5* gene. Zhang *et al*. (2009) examined the expression of the *TRI5* gene between carbendazim-resistant and sensitive *F. graminearum* in shake culture and found a significant exponential relationship between trichothecene production and *TRI5* gene expression. More recently Lee *et al*. (2014) compared the expression of *TRI* cluster genes in DON vs NIV producing *F. graminearum* strains in liquid cultures. No study has

been done to compare the level of expression of *TRI* genes in different chemotypes of *F. graminearum* during wheat colonization.

Therefore, in this study we have compared the level of expression of nine *TRI* genes in 3-ADON, 15-ADON and NIV-producing *F. graminearum* strains in a time course study both in resistant and susceptible wheat cultivars. The objective of this study was to evaluate the chemotype specific gene expression patterns in trichothecene biosynthesis related genes in different chemotypes of *F. graminearum* during wheat infection and colonization.

7.3 Materials and methods

7.3.1 Greenhouse experiment and RNA isolation

Two wheat (*Triticum aestivum* L.) genotypes with contrasting levels of resistance to fusarium head blight (FHB) were used in this study. A spring wheat cultivar, Roblin, which is highly susceptible (S) to FHB, along with a FHB moderately resistant (MR) cultivar, Carberry, with resistance originating from the Chinese cultivar Sumai3 which is known for Type I and II FHB resistance (Bai & Shaner, 1994) were used in the study. To prepare inoculum, two *F. graminearum* strains from each chemotype were cultured on synthetic nutrient agar (SNA) medium. *F. graminearum* strains used in this study were consist of; Q-06-11 (designated as: 3-ADON1), A6-06-01 (3-ADON2), PH1 (15-ADON1), M2-06-02 (15-ADON2), W52516 (NIV1) and W56604 (NIV2). To produce liquid inoculum, 1.5 L of carboxymethyl cellulose (CMC) liquid media was prepared and four SNA plates from each strain were divided into sections and added into each flask. Seven days after incubation at 25ºC under fluorescent light, the number of conidiospores per milliliter was determined by counting spores using a haemocytometer and adjusted to the desired spore concentration of 50,000 conidia spores/mL with distilled water.

Seeds of spring wheat cultivars; Carberry and Roblin were planted in 15-cm plastic pots and maintained at 22-24 ºC in the greenhouse at the Department of Plant Science, University of Manitoba, Winnipeg. Inoculations were conducted at 30-50% anthesis. A 10 µL of *F. graminearum* suspension was injected between the palea and lemma of spikelets per each spike according to the protocol described by Cuthbert *et al*. (2006). Five biological replicates for each strain and time point were conducted following a complete randomized design. Four to five spikes were inoculated per plant. FHB disease severity (DS) ratings were taken at 2, 3, 4, 7, 10 and 14 days post-inoculation (dpi) using the FHB disease scale by Stack & McMullen (1995). FHB DS readings were taken from five inoculated spikes for each replicate. The infected spikes were collected for RNA isolation at 2, 3, 4, 7, 10 and 14 dpi. The mock inoculation was made by distilled water in both Roblin and Carberry for all time points. Immediately, the sampled spikes were placed on liquid nitrogen and transferred into a -80˚C freezer for storage until RNA extraction. The lemma, palea and subtending section of the rachis from five replicates were pooled and ground into fine powder in liquid nitrogen using a mortar and pestle. Total RNA was isolated using TRIzol® reagent according to manufacturer's instructions (Invitrogen Life Technologies, CA, USA). Extracted RNA was quantified using the NanoDrop 3300 (Thermo Fisher Scientific Inc., DE, USA) and its quality was verified by 1% agarose gel electrophoresis. RNA was treated with TURBO™ DNaseI (Invitrogen Life Technologies, CA, USA) to remove DNA contamination before cDNA synthesis according to manufacturer's instructions. The first strand of cDNA was synthesized from 2 µg total RNA as the template using SuperScript™ III First-Strand Synthesis System for reverse transcription-polymerase chain reaction (Invitrogen Life Technologies, CA, USA).

7.3.2 FDK, DON and NIV analysis

Kernels were harvested from inoculated spikes from both cultivars Carberry and Roblin, at 14 dpi. The percentage of fusarium damaged kernels (FDK) was estimated by taking a pooled sample of 10 g from all replicates. The same kernels used for FDK analysis were used for DON or NIV analysis. Wheat kernels of each strain were pooled, ground and analysed by Veratox® DON 5/5 kit (product no: 8331, Neogen Corp., Lansing, MI, USA) for DON analysis. NIV analysis was done using GC-MS according to the protocol described by Tittlemier *et al.* (2012).

7.3.3 Quantitative real-time PCR

A total of nine genes in the DON biosynthetic pathway were examined using quantitative real time PCR (qPCR) (Table 7.1). To analyse the level of expression of each gene, a set of gene specific primers designed from the coding regions of each gene were used as described by Lee *et al*. (2014). As a house-keeping gene, translation elongation factor 1 alpha (*EF-1α*) from *F. graminearum* was selected (Kim & Yun, 2011). Quantitative real-time PCR reactions were performed in a CFX96 Touch™ Real Time PCR Detection System according to the protocol described by Lee *et al*. (2014) (Bio-Rad, Hercules, CA, USA). The qPCR reaction cycles were as follows: 95°C for 3 min, followed by 45 cycles at 95°C for 10 s, 60°C for 20 s, 72°C for 20 s, and finally 95°C for 10 s and 65°C for 5 s. The qPCR reaction mixture contained 10 µL of 2× iQ SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA), 0.5 µL of each primer (10 pM), 1 µL of template cDNA (10 ng), and RNase free water to a final volume of 20 µL. Quantification values were analysed using the Bio-Rad CFX Manager v1.6, and the threshold cycle (Ct) values were determined. In all reactions, a non-template control (NTC) was set up to avoid any DNA contaminations in the reaction mixtures. Each reaction sample was amplified three times in every

experiment, and final Ct values are presented as an average of three replicates. The relative transcript abundance of the target genes was determined by ΔΔCt (Livak & Schmittgen, 2001) using *EF-1α* as the endogenous reference. Other house keeping genes such as *F. graminearum* Actin and β-tubulin were also tested with *EF-1α* gene. Among the analysed house keeping genes, *EF-1α* gene was selected as the reference gene due to the consistent Ct values in all analysed samples. The genes analysed and primer sequences are listed in Table 7.1.

7.3.4 Statistical analysis

Analysis of variance (ANOVA) for gene expression data of *FPP*, *TRI3*, *TRI4*, *TRI5*, *TRI6*, *TRI8*, *TRI12*, and *TRI101* genes in the cultivars Carberry (MR) and Roblin (S) was performed using the PROC Mixed procedure of SAS software (SAS version 9.3, SAS Institute Inc., NC, USA) (Appendix 3). Days post-inoculation (dpi), cultivar, strain, the two-way interactions, dpi*strain and cultivar *strain and the three-way interaction dpi* cultivar *strain were considered as sources of variation with fixed effects. Similarly, ANOVA for FHB DS at seven and 14 dpi was performed using the PROC Mixed procedure of SAS software (SAS version 9.3, SAS Institute Inc., NC, USA). Cultivar, strain and cultivar*strain were considered as fixed effects. The Bonferroni method was used to compare statistically significant differences in least squares (LS) means of all variables. The type 3 test of fixed effects was determined and those with $p \leq 0.05$ were considered significant.

Table 7.1 Genes analysed, their function, forward and reverse primer sequences

Gene (Ref)	Function	Forward primer (5′→3′) Reverse primer (5′→3′)
FPP (Desjardins, 2006)	Farnesyl pyrophosphate synthase	TTTGGCAAGCCCGAACACATT GCGGATCTGGCCAACAACCTTCT
*TRI*3 (Kimura *et al*., 2007)	Trichothecene 15-O-acetyltransferase	CTTGCAGGGATATCAAGAAATGTTACGA CTCGCCTGTTGTAGTTCGCTTGATTT
TRI4 (Kimura *et al*., 2007)	Cyctochrome P450 monooxygenase	TCGAGGCACAACAGAAGGGTATCC AATGTCGGCCTTGGTGGTGTC
*TRI*5 (Desjardins, 2006)	Trichodiene synthase	CCAGGAAACCCTACACTCGTCTAAG TGGCCGCCTGCTCAAAGAAC
*TRI*6 (Kimura *et al*., 2007)	Transcriptional regulator	GGCATTACCGGCAACACTTCAA CATGTTATCCACCCTGCTAAAGACC
TRI8 (Kimura *et al*., 2007)	Trichothecene 3-O-esterase	GCTACTTTGGACTCAATTCG CATACTGTACYGCAAGTTCTG
*TRI*9 (Kimura *et al*., 2007)	Unknown	AGCCGCTAAACTGATCGACTCATA GCTTTGGCTGCGACCCATAT
TRI12 (Kimura *et al*., 2007)	Trichothecene efflux pump	TCCACAGTCATCTTTCCCCAGTCT CTCCCAGTGCCATAGCGAAGTAGT
TRI101 (Kimura *et al*., 2007)	Trichothecene 3-O-acetyltransferase	GTGGGACTCTGGGATTACGACTTT GTCCACTCCTTATCCGCCTTCAA

7.4 Results

Nine genes from the *F. graminearum* trichothecene biosynthesis pathway, *TRI4*, *TRI5*, *TRI6*, *TRI3*, *TRI8*, *TRI101*, *TRI9*, *TRI12* and *FPP* along with the housekeeping gene *EF-1α*, were selected for gene expression analysis. Each selected gene was analysed by real time quantitative PCR to examine the changes in transcript levels at different time intervals post-inoculation. Transcript accumulations of the *TRI* genes showed an early induction in most strains as early as two dpi. Significant differences were observed for cultivar, strain, and dpi for all the analysed genes. Similarly, the two-way interactions, cultivar *strain and dpi*strain and the three-way interaction cultivar*strain*dpi were also significantly different for all the genes examined

(Appendix 3.1). The qPCR analysis showed that the *FPP* transcript accumulation initiated at 2 dpi, peaked at 4 dpi and rapidly decreased at 7 dpi in moderately resistant (MR) cultivar Carberry (Fig 7.1a). A similar transcript accumulation pattern was observed in the susceptible (S) cultivar Roblin, however, at 10 dpi there was a slight increase in transcript accumulation and then gradually decreased at 14 dpi (Fig 7.1b). In both MR cultivar and S cultivar, the abundance of *FPP* transcripts was significantly higher in 3-ADON producing *F. graminearum* strains than 15-ADON and NIV producing strains (Appendix 3.2).

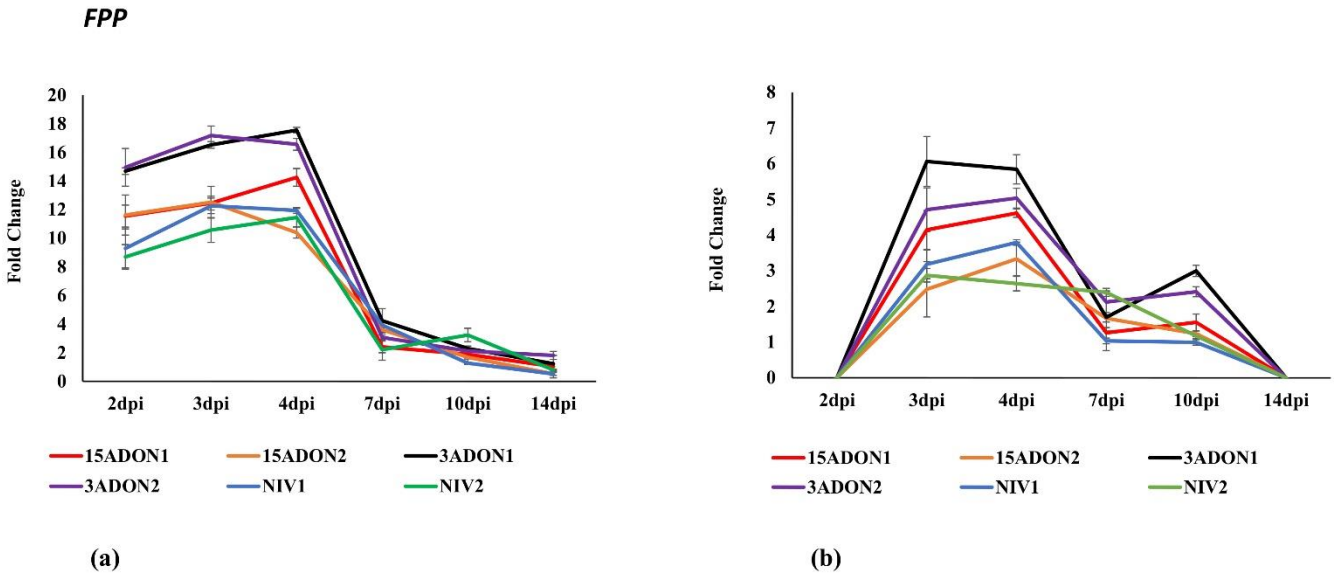


Figure 7.1 Quantitative real-time PCR analyses of farnesyl pyrophosphate synthase (*FPP*) gene. Fold change in transcript levels in (a) moderately resistant cultivar Carberry and (b) susceptible cultivar Roblin. All data were normalized to the *EF-1α* expression level. Values are means ± SE of three replicates.

The qPCR analysis for *TRI5*, *TRI4*, *TRI6* and *TRI8* genes showed a similar transcript accumulation pattern during the time course study in MR cultivar, Carberry. In all genes, transcript accumulation initiated at 2 dpi, peaked at day 4, abruptly decreased at day 7, and stayed low until day 14 (Fig 7.2a, 7.3a, 7.4a and 7.5a). In S cultivar, Roblin, *TRI5* and *TRI6*

genes showed a similar transcript accumulation pattern as in MR cultivar (Fig 7.2b and 7.4b). However, in the *TRI4* gene, transcript accumulation was peaked at 3 dpi, decreased at 7 dpi and again increased at 10 dpi in R cultivar Roblin (Fig 7.3b). For *TRI8* gene, transcript accumulation was peaked at 3 dpi in 3-ADON and 15-ADON strains whereas for NIV strains it was at 4 dpi (Fig 7.5b). With respect to *TRI5* gene, 3-ADON producing strains maintained significantly higher expression levels than 15-ADON and NIV strains in both cultivars (Fig 7.2; Appendix 3.2). A similar pattern was observed for other genes *TRI4*, *TRI6* and *TRI8* (Fig 7.3, 7.4, 7.5; Appendix 3.2).

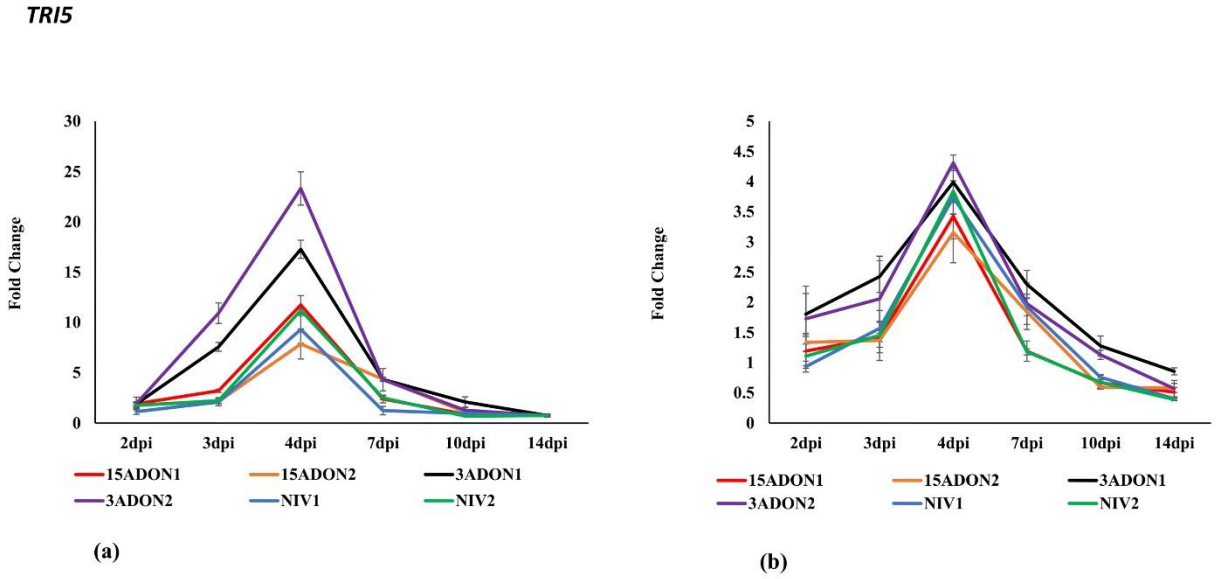


Figure 7.2 Quantitative real-time PCR analyses of trichodiene synthase (*TRI5*) gene**.** Fold change in transcript levels in (a) moderately resistant cultivar Carberry and (b) susceptible cultivar Roblin.

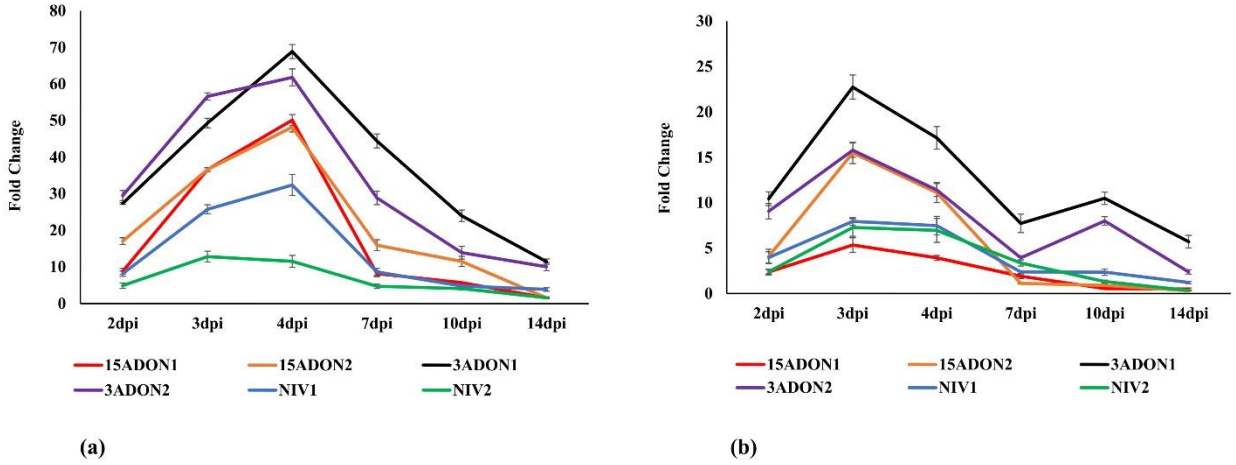


Figure 7.3 Quantitative real-time PCR analyses of trichodiene oxygenase (*TRI4*) gene**.** Fold change in transcript levels in (a) moderately resistant cultivar Carberry and (b) susceptible cultivar Roblin.

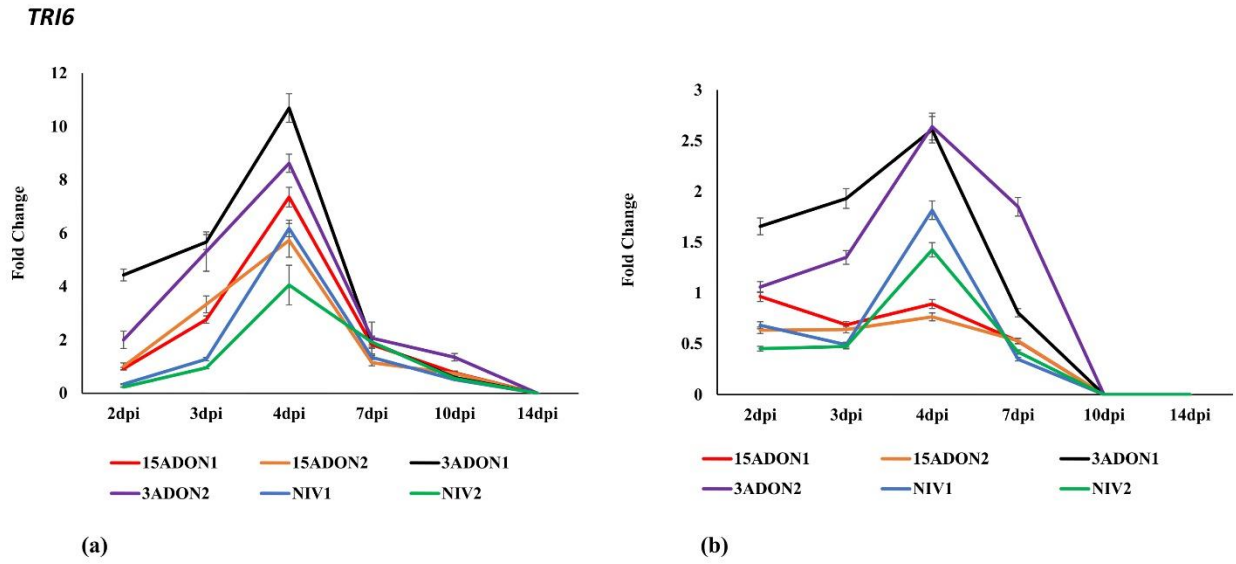


Figure 7.4 Quantitative real-time PCR analyses of transcription factor *TRI6* gene**.** Fold change in transcript levels in (a) moderately resistant cultivar Carberry and (b) susceptible cultivar Roblin.

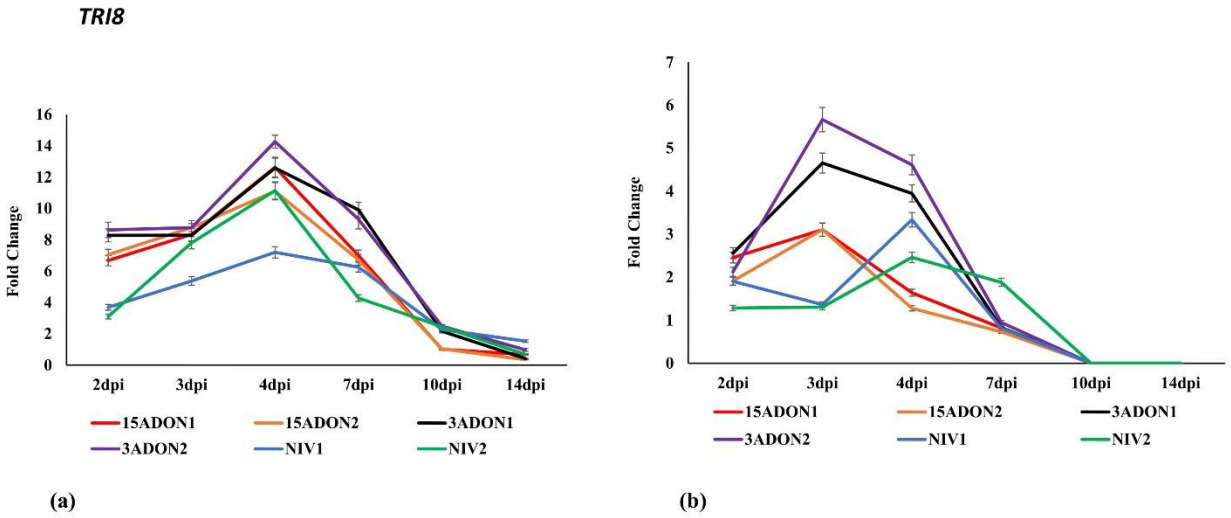


Figure 7.5 Quantitative real-time PCR analyses of trichothecene 3-*O*-esterase (*TRI8*) gene**.** Fold change in transcript levels in (a) moderately resistant cultivar Carberry and (b) susceptible cultivar Roblin.

The expression of the *TRI101* gene initiated at 2 dpi and remained relatively constant during the early time intervals at 2-7 dpi and started decreasing at 10 dpi in MR cultivar, Carberry (Fig 7.6a). A similar pattern was observed in S cultivar, however, some peaks were observed for 3ADON2, 15ADON2 and NIV1 strains (Fig 7.6b). Significant differences were observed among the strains for *TRI101* transcript accumulation (Appendix 3.1). Similar to other genes, the accumulation of *TRI3* transcripts initiated at 2 dpi and peaked at 3 dpi in most strains in both cultivars; however, the transcript abundance started decreasing at 4 dpi (Fig 7.7). With respect to *TRI3* gene, significant differences were observed among the strains for transcript accumulation (Appendix 3.1).

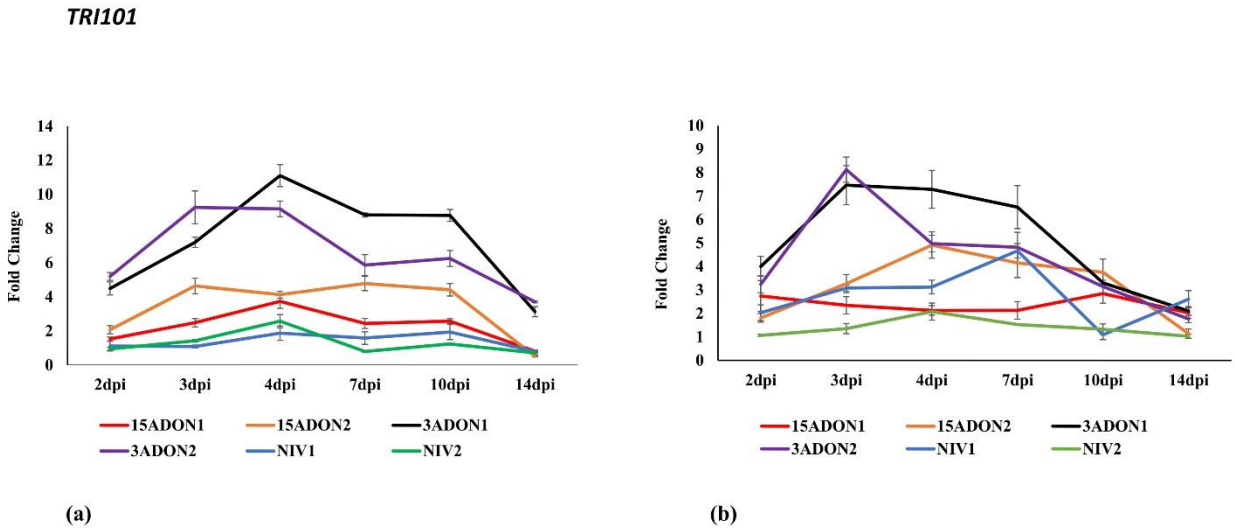


Figure 7.6 Quantitative real-time PCR analyses of trichothecene 3-*O*-acetyltransferase (*TRI101*) gene. Fold change in transcript levels in (a) moderately resistant cultivar Carberry and (b) highly susceptible cultivar Roblin.

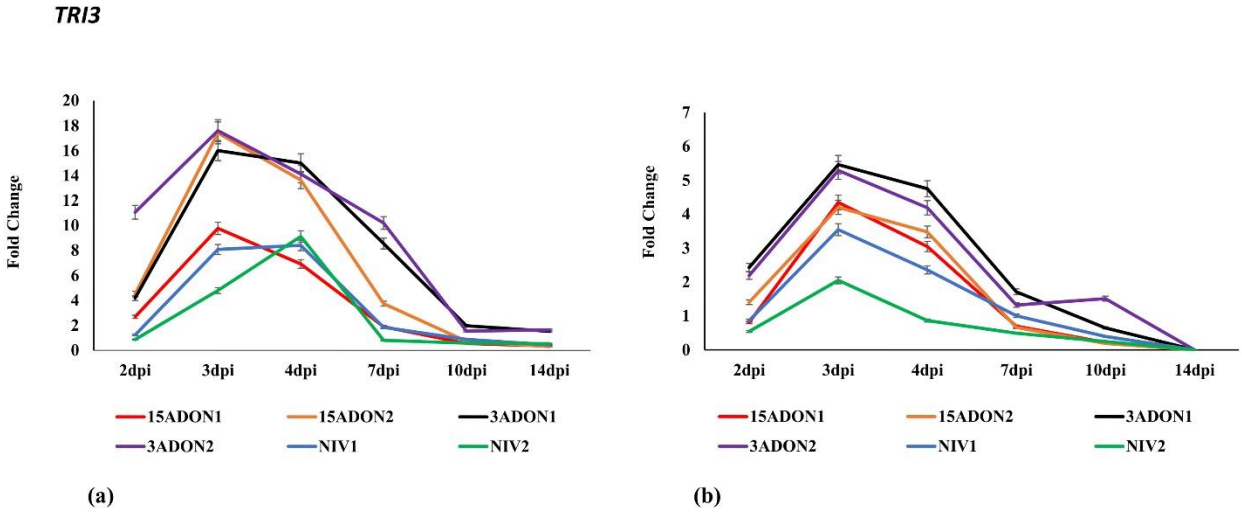


Figure 7.7 Quantitative real-time PCR analyses of trichothecene 15-*O*-acetyltransferase (*TRI3*) gene. Fold change in transcript levels in (a) moderately resistant cultivar Carberry and (b) highly susceptible cultivar Roblin.

Despite the earlier induction (2 dpi), the level of expression of *TRI*9 gene peaked at 7 dpi (in cultivar Carberry) and 10 dpi (in cultivar Roblin) and gradually decreased in both cultivars starting at 10 dpi (Fig 7.8). Transcript accumulation of the *TRI12* gene also initiated at 2 dpi, peaked at 4 dpi and gradually decreased at 7 dpi in most of the strains (Fig 7.9). Significant differences were observed among the strains for *TRI12* transcript accumulation (Appendix 3.1). Based on the qPCR data, *F. graminearum* 3-ADON strains showed a higher level of *TRI* gene expression compared to the other strains for all genes at most time points (*FPP*, *TRI3*, *TRI4*, *TRI5*, *TRI6*, *TRI8*, *TRI12*, and *TRI101).* The level of gene expression in 15-ADON and NIV producing strains showed no specific pattern of higher or lower expression. In some genes and time points the level of transcript accumulation was higher in 15-ADON strains and lower in NIV strains and vice versa. Among the analysed genes, the highest abundance of transcripts was observed for *TRI4* and *TRI12* genes for all the examined strains (Fig 7.3 and 7.9). Our data showed that relative expression of *TRI* genes was significantly higher in wheat cultivar Carberry (MR) compared with Roblin (S).

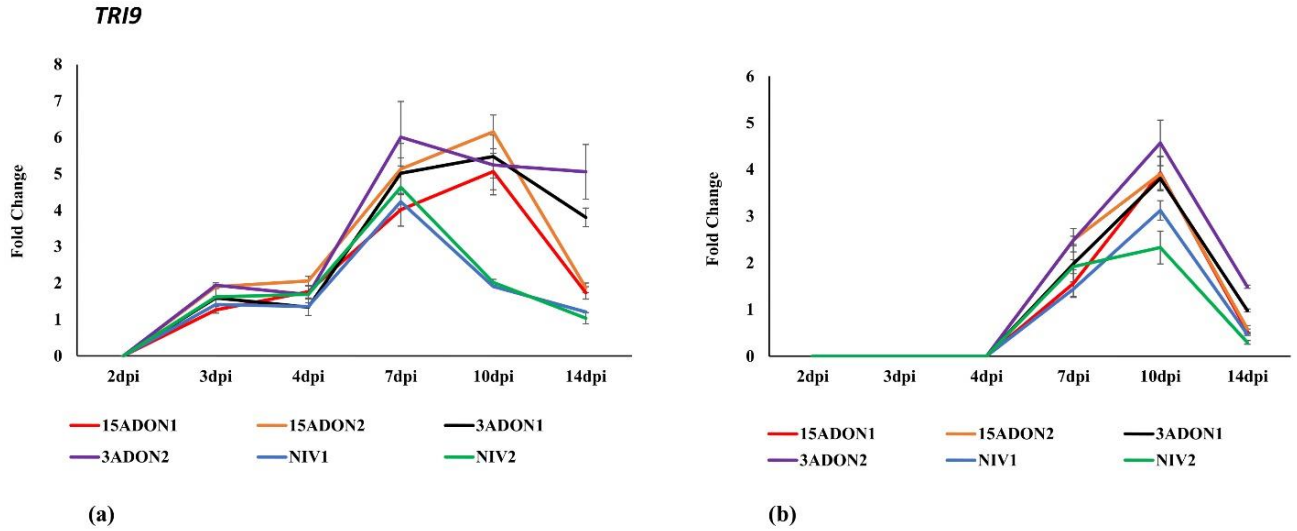


Figure 7.8 Quantitative real-time PCR analyses of *TRI9* gene**.** Fold change in transcript levels in (a) moderately resistant cultivar Carberry and (b) highly susceptible cultivar Roblin.

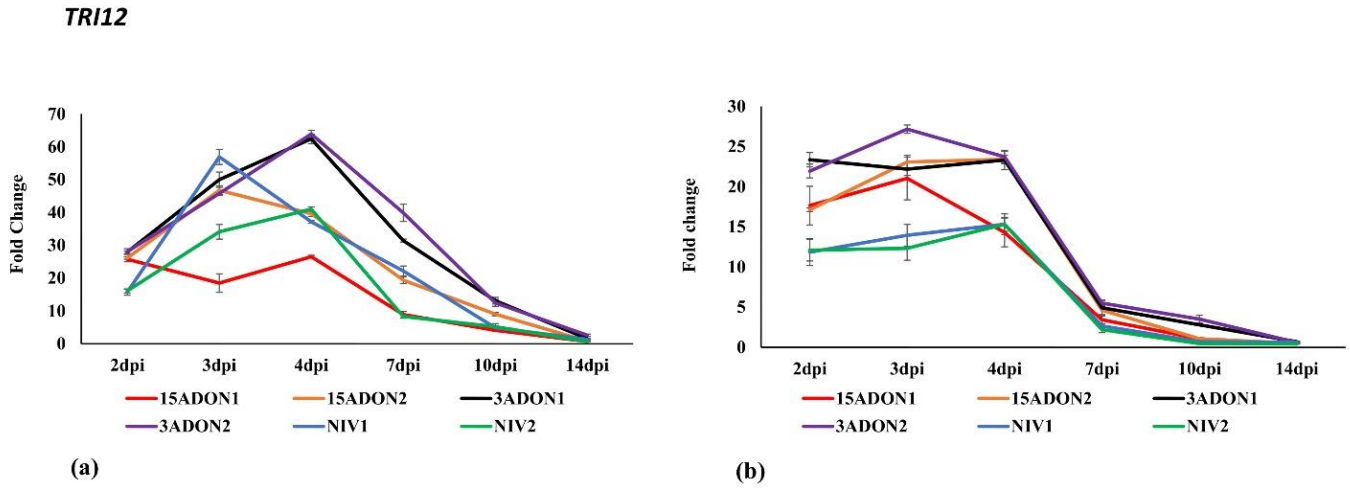


Figure 7.9 Quantitative real-time PCR analyses of *TRI12* gene**.** Fold change in transcript levels in (a) moderately resistant cultivar Carberry and (b) highly susceptible cultivar Roblin.

The FHB DS was analysed at 7 dpi and 14 dpi, terminal FDK and DON/NIV content were analysed at 14 dpi. When FHB DS was considered, there were significant differences between the cultivars and among the strains. The two-way interaction cultivar*strain was significantly different (Table 7.2). The highest FHB DS was shown by cultivar Roblin inoculated by 3-ADON strains followed by 15-ADON and NIV strains (Fig 7.10b). FHB disease severity caused by 3-ADON strains was significantly different from the 15-ADON producing strains and NIV strains. A similar trend was observed in the MR cultivar Carberry, however, the FHB symptom development was slower than in cultivar Roblin which is highly susceptible to FHB (Fig 7.10a). The percentage of FDK was higher in cultivar Roblin than in cultivar Carberry (Fig 7.11). Similarly, a higher total DON content was observed in cultivar Roblin inoculated with 3-ADON strains than the 15-ADON strains (Fig 7.11). Cultivars inoculated with 3-ADON strains showed higher levels of FDK and DON content than 15-ADON strains. NIV producing strains showed the lowest FDK percentage and toxin contamination (Fig 7.11).

Table 7.2 Analysis of variance (ANOVA) table for cultivar, strain and their interaction for fusarium head blight disease severity at 7 and 14 days post-inoculation

Days post-inoculation	Source	DF	MS	F value	Pr>F
7 dpi	Cultivar	1	34556	694.69	<.0001
	Strain	5	1858.88	37.37	<.0001
	Cultivar*Strain	5	478.35	9.62	0.0001
	Rep	14	60.10	1.20	0.2806
	Error	154	49.74		
14 dpi	Cultivar	1	59405	585.26	<.0001
	Strain	5	4323.23	42.59	<.0001
	Cultivar*Strain	5	2804.13	5.53	0.0001
	Rep	14	58.06	0.57	0.8836
	Error	154	101.5		

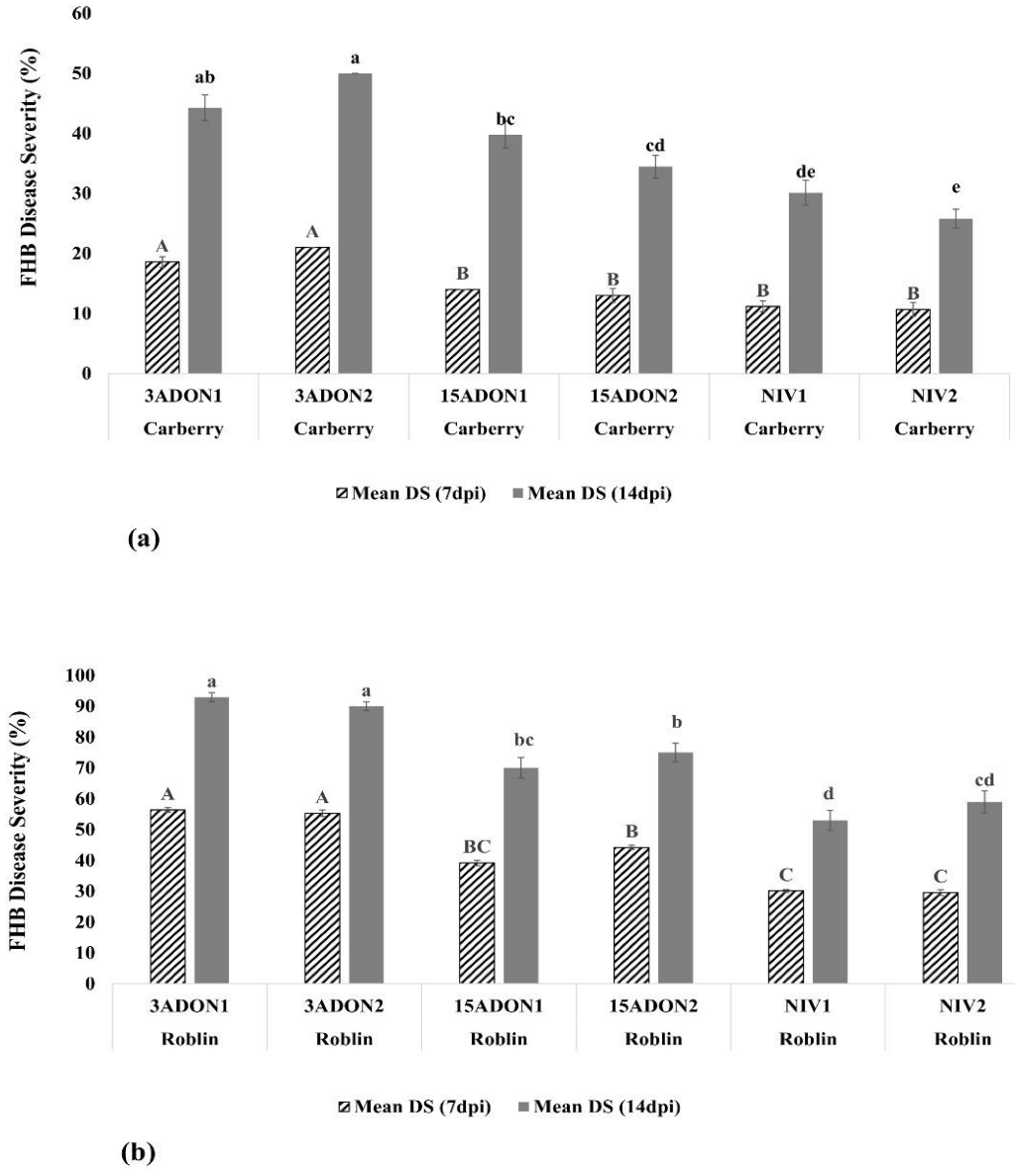
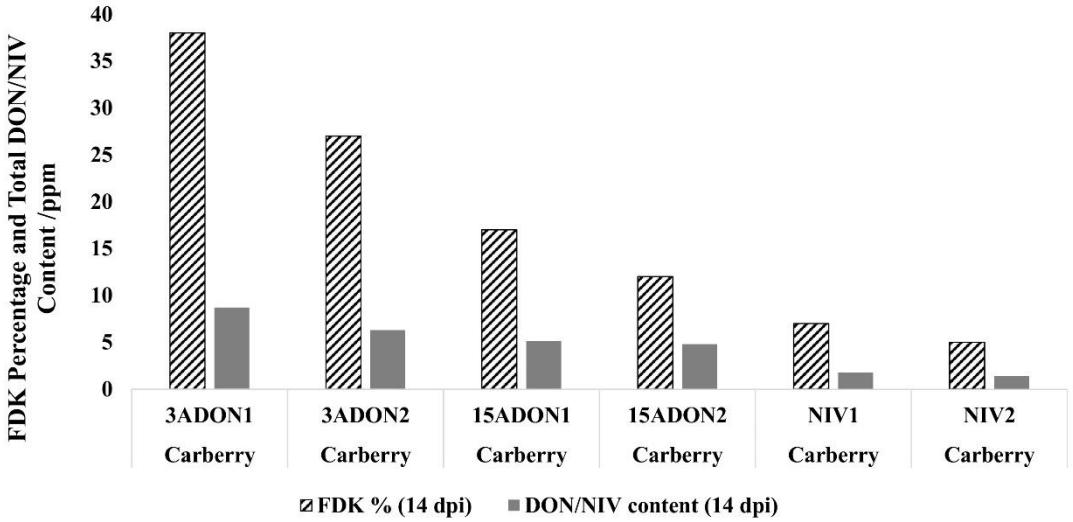
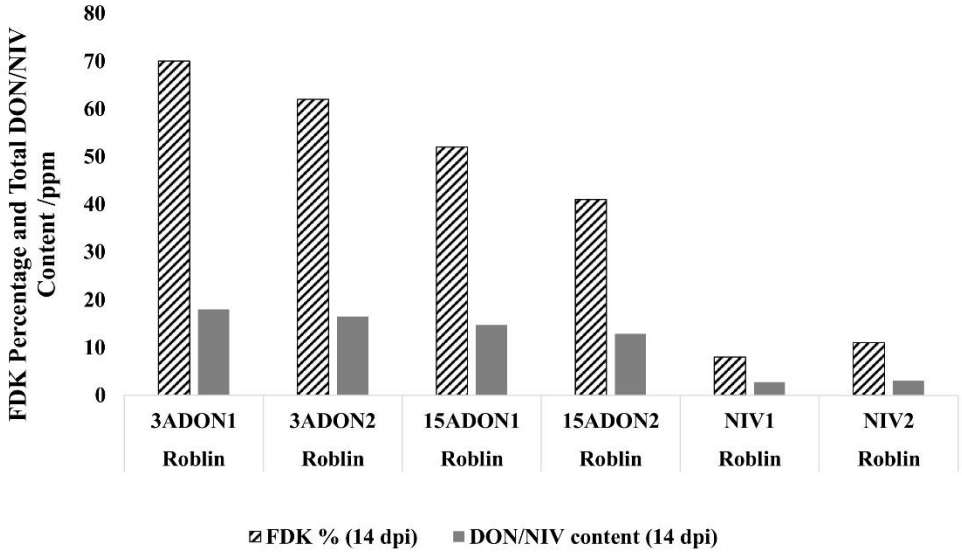


Figure 7.10 Mean fusarium head blight disease severity in (a) moderately resistant cultivar Carberry and (b) susceptible cultivar Roblin after inoculating with different chemotypes of *Fusarium graminearum* at 7 and 14 days post-inoculation.



(a)



(b)

Figure 7.11 Terminal fusarium damaged kernel (FDK) percentage and total terminal deoxynivalenol (DON) or nivalenol (NIV) content in (a) moderately resistant cultivar Carberry and (b) susceptible cultivar Roblin after inoculating with different chemotypes of *Fusarium graminearum* at 14 days post-inoculation.

7.5 Discussion

The objective of this study was to identify the potential chemotype-specific gene expression patterns of the *TRI* genes during wheat- *F. graminearum* infection and colonization. The expression of most *TRI* genes required for trichothecene production in *F. graminearum* were strongly induced at early time points after infection (i.e., 2- 4 dpi) and the expression levels gradually decreased at 7 dpi. Also 3-ADON producing strains showed a comparatively higher level of gene expression than 15-ADON and NIV producing strains, confirming their ability to produce higher amounts of toxin in infected wheat kernels.

DON biosynthesis related gene expression profiling indicated that, the expression of most *TRI* genes were initiated at 2 dpi. This shows that a high level of *TRI* transcript accumulation is essential for initiating the biosynthetic pathway of DON or NIV during wheat infection and colonization. The early expression patterns of five *TRI* genes along with *FPP* gene (*TRI4*, *TRI5*, *TRI6*, *TRI8* and *TRI3*) strongly suggested that *TRI6* gene which encodes a transcriptional regulator, positively regulates the expression of other *TRI* genes in the DON biosynthesis pathway, leading to the synthesis of enzymes and proteins essential for DON production in *F. graminearum*. Similar observations have reported by Lee *et al.* (2014) in liquid culture media. The level of *TRI* gene expression was significantly different among the three chemotypes analysed. The level of expression of *TRI5* gene was higher in 3-ADON producing strains in both cultivars compared to the 15-ADON producing strains and NIV producing strains. The *TRI5* gene encodes for trichodiene synthase enzyme, which mediates the first step in the trichothecenes biosynthesis pathway. Also, we could observe a similar gene expression pattern in *TRI6* gene. *TRI6* gene acts as a transcription regulator encoding gene (Desjardins, 2006; Kimura *et al*., 2007). It has been reported that 3-ADON strains produce more

trichothecenes than 15-ADON and NIV strains; therefore, the higher levels of expression of *TRI5* and *TRI6* during colonization may mediate the production of high amounts of toxins (Ward *et al*., 2008). According to the total DON content at 14 dpi, kernels infected with 3-ADON strains showed higher total DON content than the 15-ADON strains in both cultivars. Also, in this study the level of transcript accumulation of *TRI4* gene was comparatively higher than other genes (except for *TRI12* gene). The *TRI4* gene regulates multiple steps (four steps) in the trichothecene biosynthesis pathway (McCormick *et al*., 2006). The accumulation of *TRI4* transcripts in higher amounts could be explained by the involvement of this gene in multiple steps during trichothecene production. The level of expression of the *TRI12* gene initiated at 2 dpi, peaked at 4 dpi and gradually decreased at 7 dpi, which was similar to the other analysed *TRI* genes in the present study. It has been reported that *TRI12* gene encodes for trichothecenes efflux pump, which gives self-protection for the fungus from the produced trichothecenes (Alexander *et al*., 1999). Therefore, coherent gene expression patterns of *TR112* genes with other analysed *TRI* genes further supports the role of *TRI12* gene as a self-protector against the produced trichothecenes.

The level of expression of *TRI* genes was significantly higher in the MR cultivar Carberry than in the S cultivar Roblin. Similar results have been reported in other studies (Boddu *et al*., 2007; Brown *et al*., 2011; Hallen-Adams, 2011). Still there is no clear reason to explain the higher levels of expression in trichothecene biosynthesis genes in MR cultivar compared to the S cultivar. However, when we analysed the total DON content at 14 dpi it was higher in the susceptible cultivar Roblin than in the MR cultivar Carberry. Deoxynivalenol is a virulence

factor in wheat, necessary for the spread beyond the infected floret (Bai *et al*., 2002; Audenaert *et al*., 2013). Audenaert *et al*. (2013) have observed that, in susceptible cultivars, DON acts in some unknown manner on the plant. As a result, the fungus is able to escape the infection point and spread, while resistant cultivars (having *Fhb1* resistance) are unresponsive to DON and the fungus does not spread from the point of infection. Based on the previous observations, the course of events in a resistant plant upon *F. graminearum* infection can be explained as follows: fungus enters the plant (inoculation, natural infection); fungus grows at infection point; in response to (unknown) plant signals, fungus begins producing DON; fungus "attempts" to grow into adjacent spikelet but is prevented by an unknown resistance mechanism in the plant. The plant's lack of response to DON, prevents the fungus from leaving the infection point, then as a response to this inability to grow further, the fungus increases DON production. In order to increase the DON production, the fungus increases the level of expression of DON biosynthesis related genes in resistant cultivars. Investigations are in progress to further understand the reasons for higher levels of *TRI* gene expression in MR cultivars than in S cultivars.

Although the level of expression of DON biosynthetic genes were higher in the MR cultivar than in the S cultivar the final DON content is higher in the S cultivar. It has been reported that during *Fusarium* infection there is a broad expression of genes related to the DON detoxification process (Muhovski *et al*., 2012). This may explain the low levels of DON contamination in the MR cultivar compared to S cultivar. Gene expression studies have shown that the expression of DON detoxification transcripts such as UGTs, CYP450s, ABC transporters and MRP were more highly abundant in FHB resistant cultivars than in susceptible cultivars during *Fusarium* infection (Muhovski *et al*., 2012; Al-Taweel *et al*., 2012; Kosaka *et al*., 2015).

Therefore, it can be hypothesised that, although the level of *TRI* gene expression is higher in the MR cultivar, the resistance mechanisms within the cultivar can more efficiently detoxify the produced DON than the susceptible cultivar.

This study provides evidence on the chemotype specific gene expression patterns in the DON biosynthesis pathway during wheat infection and colonization. The results from this study indicated that 3-ADON producing strains showed higher levels of gene expression compared to 15-ADON and NIV producing strains. However, use of only two strains representing a chemotype may not be sufficient to draw definitive conclusions. Therefore, this study suggests the use of more strains from each chemotype group to gain a more comprehensive understanding of chemotype specific gene expression patterns during *F. graminearum* infection and colonization.

CHAPTER 8

ANALYSIS OF DEOXYNIVALENOL AND DEOXYNIVALENOL-3-GLUCOSIDES CONTENT IN CANADIAN SPRING WHEAT CULTIVARS INOCULATED WITH DIFFERENT CHEMOTYPES OF *FUSARIUM GRAMINEARUM*

8.1 Abstract

Mycotoxins are a chemically diverse group of toxic secondary metabolites produced by fungi and can occur in a wide range of commodities including human food and animal feed. In order to cope with these mycotoxins, plants have the potential to modify the chemical structures as part of their defense mechanism. The resulting metabolic products are neither routinely screened at grain elevators, nor regulated by proper legislations, but can be reactivated in the digestive tract of infected animals and humans. The objective of this study was to analyse the deoxynivalenol (DON) and deoxynivalenol-3-glucosides (D3G) content in Canadian spring wheat cultivars grown in two locations, inoculated with a mixture of 3-acetyldeoxynivalenol (3-ADON) producing *F. graminearum* strains and a mixture of 15-acetlyldeoxynivalenol (15-ADON) producing *F. graminearum* strains. According to the analysis of variance, significant differences were observed among the cultivars for all the measured fusarium head blight response variables such as FHB disease index, fusarium damaged kernel percentage (%FDK), DON content and D3G content. When the effect of chemotype considered, the D3G content and D3G/DON ratio were not significantly different between the chemotypes. Correlation analysis showed a strong positive correlation between DON and D3G at both locations. The total DON content was higher in susceptible cultivars, Roblin, CDC Teal and Harvest compared to moderately

resistant/intermediate cultivars used in the study. The highest D3G/DON ratio was observed in moderately resistant cultivars Carberry in Carman and CDC Kernen in Winnipeg. The susceptible cultivars showed lower D3G/DON ratio compared to the moderately resistant cultivars. The current study indicated that Canadian spring wheat cultivars produce D3G upon *Fusarium* infection and the level of resistance in a wheat cultivar may have an effect on the conversion of DON into D3G.

8.2 Introduction

Fusarium head blight (FHB) continues to threaten the wheat industry in Canada and other major wheat growing countries. The contamination of wheat products with *Fusarium* mycotoxins, mainly deoxynivalenol (DON) poses a major issue for global food and feed safety. DON acts as a virulence factor in disease development and facilitates the spread of the fungus from the point of invasion (Desjardins, 2006). Upon production in the infected tissue these mycotoxins inhibit protein synthesis, alter cell signalling and membrane permeability, and ultimately cause cell death (Bai *et al*., 2002; McCormick 2003). Plants have multifaceted detoxification systems to cope with a wide variety of xenobiotics such as mycotoxins (Boutigny *et al*., 2008; Audenaert *et al*., 2013; Berthiller *et al*., 2013). Plants are equipped with two main detoxification mechanisms; chemical modification and compartmentation (Coleman *et al*., 1997). Chemical modification consists of two main phase reactions. Phase I involves hydrolysis or oxidation of xenobiotics; the resulting compounds are either more toxic than or as toxic as the parent compound. Phase II involves conjugation of xenobiotics. In these reactions, residues such as glucose, malonic acid, and glutathione are bound to the functional groups of xenobiotics. The final converted products from phase II reactions are either nontoxic or less phytotoxic than the parent compounds

(Coleman *et al*., 1997; Cole & Edward, 2000). These mycotoxin derivatives are not screened by routine analytical techniques as their structure has changed in the plants. However, they can be reactivated or reconverted to the parent compound in the digestive tract of humans and animals. Therefore, these compounds are known as "masked mycotoxins" (Berthiller *et al*., 2013). Recently Rychlik *et al.* (2014) proposed to restrict the usage of the term "masked mycotoxins" only to the fraction of biologically modified mycotoxins that were conjugated by plants. One major pathway of detoxifying DON is the conjugation of DON to a glucose moiety. This reaction gives rise to deoxynivalenol-3-glucoside (D3G), which exhibits reduced ability to inhibit protein synthesis of wheat ribosomes *in vitro* compared to its parent compound, DON (Poppenberger *et al*., 2003). The occurrence of D3G in *Fusarium* infected wheat and maize was first reported by Berthiller *et al.* (2005). Since then, a worldwide occurrence of D3G in different cereal crops has been reported (Berthiller *et al*., 2009; Li *et al*., 2011; De Boevre *et al*., 2012; Streit *et al*., 2013). Considerable amounts of D3G were also detected in different cereal based food products such as bread, breakfast cereals, snacks and beers (Kostelanska *et al*., 2009; Malachova *et al*., 2011). It has been shown that formation of D3G from DON is a detoxification reaction mediated by the UDP- glucosyl transferase enzyme. This enzyme catalyses the transfer of glucose from UDP-glucose to the hydroxyl group at the carbon 3 of DON. In a study done by Lemmens *et al.* (2005) the QTL for DON resistance has been mapped to the major QTL for FHB resistance on chromosome 3B. Therefore, it was hypothesized that *Qfhs.ndsu-3BS* QTL (*Fhb1*) may encode a DON-glucosyltransferase or regulate the expression of such an enzyme. Lemmens *et al*. (2005) also found that DON resistant wheat lines are more efficient in converting DON to D3G than the DON susceptible wheat lines. Therefore, it is expected that incorporation of glycosylation based detoxification mechanisms in *Fusarium* susceptible cultivars, will increase FHB resistance.

Schweiger *et al.* (2010) have identified the first monocot DON-glucosyltransferase gene, HvUGT13248, in barley. Recently a transgenic wheat line expressing barley UDP glucosyl transferase gene has been developed, and it shows a high level of resistance to FHB (Li *et al*., 2015).

In Canada, there is no routine testing done for D3G at grain mills or elevators. The fate of D3G after digestion by mammals has not yet been fully understood, and the concern is that this compound may be cleaved to DON and glucose by lactic acid bacteria in the digestive tract of mammals (Dall'Erta *et al*., 2013; Gratz *et al*., 2013; Nagl *et al*., 2013). A study done by Gratz *et al*. (2013) analysed the effect of human intestinal bacteria on D3G *in vitro* and found that fecal microbiota efficiently released DON from D3G. Similarly, Warth *et al*. (2013) conducted an *in vivo* study to determine the fate of D3G and 3-ADON during human metabolism. Masked forms were not detected in any of the analysed urine samples suggesting that, they might be hydrolysed back into DON as shown in pigs (Warth *et al*., 2013). Therefore, the knowledge about the natural occurrence of masked mycotoxins in *Fusarium* infected wheat is important to evaluate the potential health risks associated with masked mycotoxin contamination. The objective of this study was to analyse the DON and D3G content in spring wheat cultivars in Canada, after inoculating with *F. graminearum* 3-Acetyldeoxynivalenol (3-ADON) and 15-Acetlyldeoxynivalenol (15-ADON) chemotypes. This study also attempted to determine if there is a correlation between the DON, D3G, fusarium damaged kernel (FDK) and FHB disease index in spring wheat cultivars grown in Manitoba, Canada.

8.3 Materials and methods

8.3.1 Field experiment

Ten different spring wheat cultivars (Roblin-S, Harvest-S, CDC Teal-S, Glenn-I, CDC Kernen-I, AAC Elie-I, AAC Iceberg-I, Carberry-MR, 5602HR-MR, and Waskada-MR) showing different levels of resistance to FHB were grown at the research field stations of University of Manitoba in Winnipeg and Carman in Manitoba, Canada. All cultivars belong to the Canada Western Red Spring (CWRS) class except for AAC Iceberg, which belongs to Canada Western Hard White Spring (CWHWS) class. A three replicate split plot design was used. The main plot effect was *F. graminearum* strains (a mixture of 3-ADON strains and a mixture of 15ADON strains) plus a water control and wheat cultivar was the sub plot effect. Main plots were separated by buffer plots of the wheat cultivar Amazon, to reduce potential inoculum drift among main plots. Plots at both locations consisted of 1 m rows with 17 cm row spacing. Sowing density was approximately 80 seeds per row. At the 50% flowering stage, the spikes of the entire row were spray-inoculated with a 50 mL inoculum mixture of 3-ADON producing *F. graminearum* strains (M2-06-01, Q-06-11 and S3BS-06-01) and a mixture of 15-ADON producing *F. graminearum* strains (S3AN-06-01, NB-06-18 and A1-06-01) using a CO_2 backpack sprayer calibrated at 30 psi. The *F. graminearum* inoculum concentration was adjusted to $5x10^4$ spores/mL using water. Re-inoculation of the same rows was performed 3 days following the first inoculation. Control plots were sprayed with 50 mL of distilled water per row. The plots were mist irrigated after each inoculation using a sprinkler system for 5 minutes every hour for 10-12 hours to increase the relative humidity.

8.3.2 FHB disease index and FDK percentage

Fusarium head blight disease incidence and severity of each row were rated 21 days post-inoculation using the FHB disease scale described by Stack & McMullen (1995). At maturity rows were hand harvested and threshed using the Wintersteiger® Elite combine. The wind speed was set at 'very low' to retain as many FDK as possible. Harvested wheat samples were placed in paper bags and air dried for one week at 36ºC. Threshed samples were again cleaned using a belt thresher and a blower. Fusarium damaged kernel percentage was calculated from a 10 g subsample from each row and recorded as a percentage of weight.

8.3.3 Total DON and D3G content

The same subsample taken for FDK assessment was ground to a powder of similar consistency using a coffee grinder (Cuisinart model no: DCG20BKNC) for 5-8 minutes and prepared for DON and D3G testing. DON and D3G testing was done using LC-MS according to the protocol described by Ovando-Martinez *et al*. (2013). The DON and D3G testing was done at the hard red spring wheat quality lab, North Dakota State University, Fargo, USA.

8.3.4 Statistical analysis

First, Leven's test for homogeneity of variances was conducted to ensure that the data could be combined over the two locations. However, the test of homogeneity of variances indicated that error variances were not homogeneous when data were combined over locations. Therefore, analysis of variance (ANOVA) was performed individually for data from two locations using the PROC MIXED procedure in SAS 9.3 (SAS version 9.3, SAS Institute Inc., Cary, NC). The treatments (3-ADON +15-ADON+Control), wheat cultivars and their interactions were

considered as fixed effects; rep and rep*treatment were considered as random effects. The model statement used in the analysis was: variable= treatment cultivar treatment*cultivar. The treatment effects were partitioned into: between chemotypes (3-ADON vs. 15-ADON) and inoculated vs. water control lines (DON vs. Control). The reason for partitioning the sources of variation within treatment effects was to provide a comparison between the chemotypes and between the control and inoculated lines. The model statements used for analyzing variation between chemotypes and inoculated vs. control were the same as in the complete analysis. The ANOVA table for between chemotypes (as treatment effect, 3-ADON vs. 15-ADON) is shown in Appendix 4. The correlations between FHB response variables at each location were analysed using the SAS PROC CORR (SAS version 9.3, SAS Institute Inc., Cary, NC) procedure.

8.4 Results

8.4.1 FHB disease index and FDK percentage

In Carman, significant differences were observed among the cultivars and treatments for FHB disease index. The two-way interaction, cultivar*treatment was also significantly different (Table 8.1). Also, significant differences were observed between the chemotypes (3-ADON vs. 15-ADON). However, the two-way interaction, cultivar*chemotype was not significantly different for FHB index (Appendix 4). Similar results were observed in Winnipeg (Table 8.2). In both locations, cultivars Roblin and AAC Iceberg inoculated with a mixture of 3-ADON strains showed higher FHB disease index (96.7% in Carman and 80% in Winnipeg) than the other cultivars and chemotype combinations. The lowest FHB disease index were shown by cultivars Carberry, 5602HR and CDC Kernen (23.3%) inoculated with a mixture of 15-ADON strains in Carman (Fig 8.1). In Carman due to high FHB disease pressure, plots inoculated with distilled

water also showed a low percentage of FHB disease severity and incidence. In Winnipeg, the lowest FHB disease index was observed in cultivars 5602HR, CDC Kernen and AAC Elie (5%) inoculated with a mixture of 15-ADON strains (Fig 8.2).

Table 8.1 Analysis of variance (ANOVA) table for fusarium head blight disease index, fusarium damaged kernel percentage, deoxynivalenol content, deoxynivalenol-3-glucoside content and ratio between deoxynivalenol-3-glucoside to deoxynivalenol content in Carman

Trait	Source	DF	MS	F value	Pr >F
FHB Disease Index[1]	Treatment (15ADON +3ADON+Control)	2	13266.00	133.77	0.0002
	15ADON vs 3ADON	1	4420.41	54.97	0.0177
	DON vs Control	1	22111.00	187.52	0.0053
	Cultivar	9	2816.08	0.16	<0.0001
	Cultivar* Treatment	18	379.41	1.85	<0.0001
	Rep	2	15.83	0.16	0.8576
	Treatment*Rep	4	99.17	1.85	0.1320
	Error	54	53.49		
% FDK[2]	Treatment (15ADON +3ADON+Control)	2	331.55	309.17	<.0001
	15ADON vs 3ADON	1	99.71	275.62	0.0036
	DON vs Control	1	563.39	315.97	0.0031
	Cultivar	9	82.39	24.79	<.0001
	Cultivar* Treatment	18	12.05	3.63	0.0001
	Rep	2	7.69	7.17	0.0500
	Treatment*Rep	4	1.07	0.32	0.8616
	Error	54	3.32		
DON (ppm)[3]	Treatment (15ADON +3ADON+Control)	2	974.34	88.19	0.0005
	15ADON vs 3ADON	1	80.75	11.58	0.0166
	DON vs Control	1	1867.92	123.54	0.0080
	Cultivar	9	219.59	17.72	<.0001
	Cultivar* Treatment	18	31.71	2.56	0.0040
	Rep	2	42.18	3.82	0.1182
	Treatment*Rep	4	11.05	0.89	0.4754
	Error	54	12.39		
D3G (ppm)[4]	Treatment (15ADON +3ADON+Control)	2	81.73	124.56	0.0002
	15ADON vs 3ADON	1	1.86	18.91	0.0500
	DON vs Control	1	161.59	133.13	0.0074
	Cultivar	9	7.58	6.21	<.0001
	Cultivar* Treatment	18	1.87	1.54	0.1134
	Rep	2	0.23	0.35	0.7244
	Treatment*Rep	4	0.66	0.54	0.7088
	Error	54	1.22		
D3G/DON Ratio	Treatment (15ADON +3ADON+Control)	2	1573.23	7.91	0.0407
	15ADON vs 3ADON	1	55.75	0.29	0.6428
	DON vs Control	1	3090.76	14.93	0.0609
	Cultivar	9	412.74	1.99	0.0580
	Cultivar* Treatment	18	185.69	0.90	0.5846
	Rep	2	445.92	2.24	0.2222
	Treatment*Rep	4	198.80	0.96	0.4369
	Error	54	207.05		

[1]FHB Disease Index- Fusarium head blight disease index.
[2]% FDK- Fusarium damaged kernel percentage.
[3]DON (ppm)- Deoxynivalenol content in parts per million.
[4]D3G (ppm)- Deoxynivalenol-3-glucosides content in parts per million.

Table 8.2 Analysis of variance (ANOVA) table for fusarium head blight disease index, fusarium damaged kernel percentage, deoxynivalenol content, deoxynivalenol-3-glucoside content and ratio between deoxynivalenol-3-glucoside to deoxynivalenol content in Winnipeg

Trait	Source	DF	MS	F value	Pr >F
FHB Disease Index[1]	Treatment (15ADON +3ADON+Control)	2	7355.83	275.84	<.0001
	15ADON vs 3ADON	1	1706.66	110.70	0.0089
	DON vs Control	1	13005.00	342.99	0.0029
	Cultivar	9	2917.16	87.11	<.0001
	Cultivar* Treatment	18	520.95	15.56	<.0001
	Rep	2	50.83	1.91	0.2621
	Treatment*Rep	4	26.67	0.80	0.5328
	Error	54	33.49		
% FDK[2]	Treatment (15ADON +3ADON+Control)	2	266.44	41.66	0.0021
	15ADON vs 3ADON	1	81.92	7.77	0.1082
	DON vs Control	1	450.96	200.99	0.0049
	Cultivar	9	31.29	17.80	<.0001
	Cultivar* Treatment	18	6.98	3.97	<.0001
	Rep	2	4.01	0.63	0.5799
	Treatment*Rep	4	6.40	3.64	0.0107
	Error	54	1.76		
DON (ppm)[3]	Treatment (15ADON +3ADON+Control)	2	815.63	97.29	0.0004
	15ADON vs 3ADON	1	65.00	9.89	0.0880
	DON vs Control	1	1566.27	153.62	0.0064
	Cultivar	9	65.88	15.61	<.0001
	Cultivar* Treatment	18	8.34	1.98	0.0279
	Rep	2	26.93	3.21	0.1472
	Treatment*Rep	4	8.38	1.99	0.1097
	Error	54	4.22		
D3G (ppm)[4]	Treatment (15ADON +3ADON+Control)	2	115.93	77.98	0.0006
	15ADON vs 3ADON	1	8.05	5.02	0.1544
	DON vs Control	1	222.84	163.58	0.0061
	Cultivar	9	8.21	8.42	<.0001
	Cultivar* Treatment	18	1.51	1.55	0.1089
	Rep	2	5.66	3.80	0.1187
	Treatment*Rep	4	1.49	1.52	0.2082
	Error	54	0.98		
D3G/DON Ratio	Treatment (15ADON +3ADON+Control)	2	12542	401.22	<.0001
	15ADON vs 3ADON	1	87.74	3.48	0.2030
	DON vs Control	1	24995.00	669.61	0.0015
	Cultivar	9	641.70	3.18	0.0037
	Cultivar* Treatment	18	402.46	2.00	0.0263
	Rep	2	170.03	5.44	0.0723
	Treatment*Rep	4	31.26	0.16	0.9599
	Error	54	201.60		

[1]FHB Disease Index- Fusarium head blight disease index.
[2]% FDK- Fusarium damaged kernel percentage.
[3]DON (ppm) - Deoxynivalenol content in parts per million.
[4]D3G (ppm) - Deoxynivalenol-3-glucosides content in parts per million.

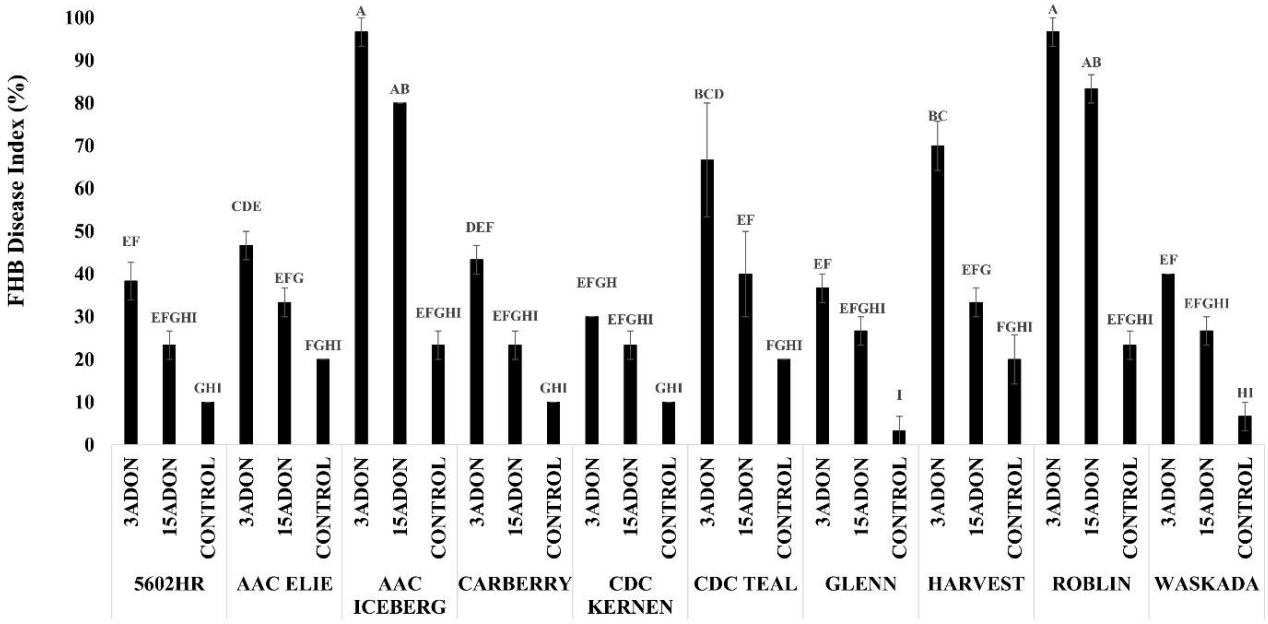


Figure 8.1 Fusarium head blight disease index in spring wheat cultivars inoculated with a mixture of 3-acetyldeoxynivalenol producing or 15-acetlyldeoxynivalenol producing *Fusarium graminearum* strains in Carman**.**

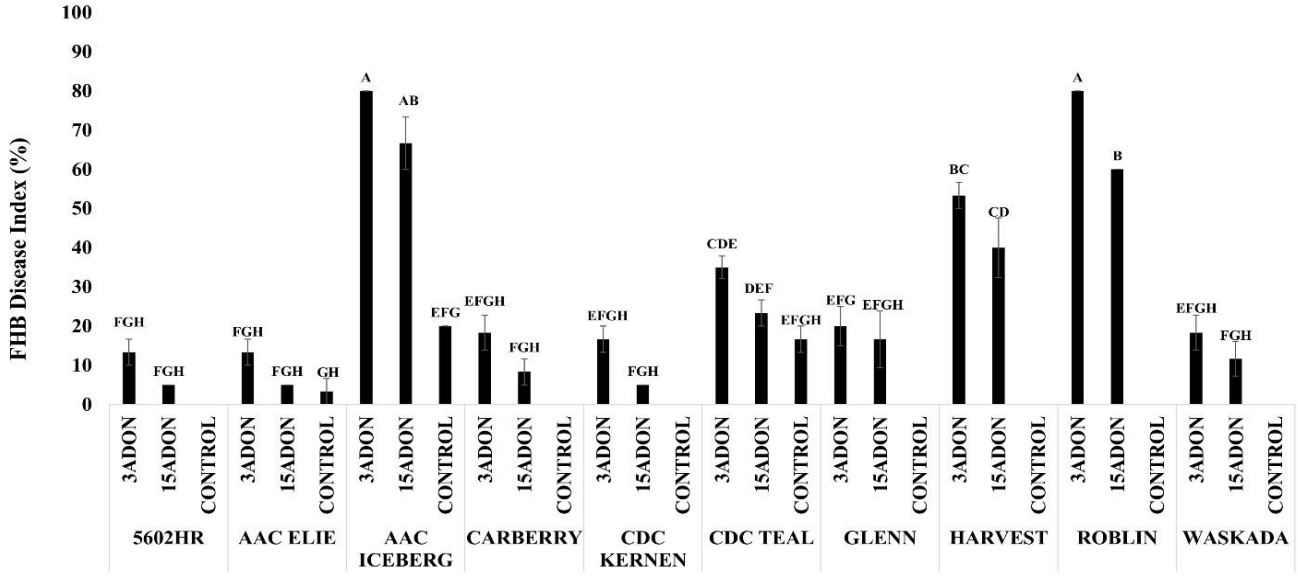


Figure 8.2 Fusarium head blight disease index in spring wheat cultivars inoculated with a mixture of 3-acetyldeoxynivalenol producing or 15-acetlyldeoxynivalenol producing *Fusarium graminearum* strains in Winnipeg**.**

When FDK percentage was considered, there were significant differences among the cultivars and the treatments at both locations (Table 8.1 and 8.2). However, at Winnipeg location, significant differences were not observed between the two chemotypes. The two-way interaction, cultivar*treatment was significantly different at both locations. Similar to FHB index, the two-way interaction, cultivar*chemotype was not significantly different at both locations (Appendix 4). The highest percentage of FDK was observed in cultivar Roblin inoculated with a mixture of 3-ADON strains at both locations (18% in Carman and 11.5% in Winnipeg) (Fig 8.3 and 8.4). The lowest FDK percentage was observed in cultivar 5602HR inoculated with a mixture of 15-ADON strains in Carman (2.3%) and cultivar Glenn inoculated with a mixture of 15-ADON strains in Winnipeg (2.2%) (Fig 8.3 and 8.4).

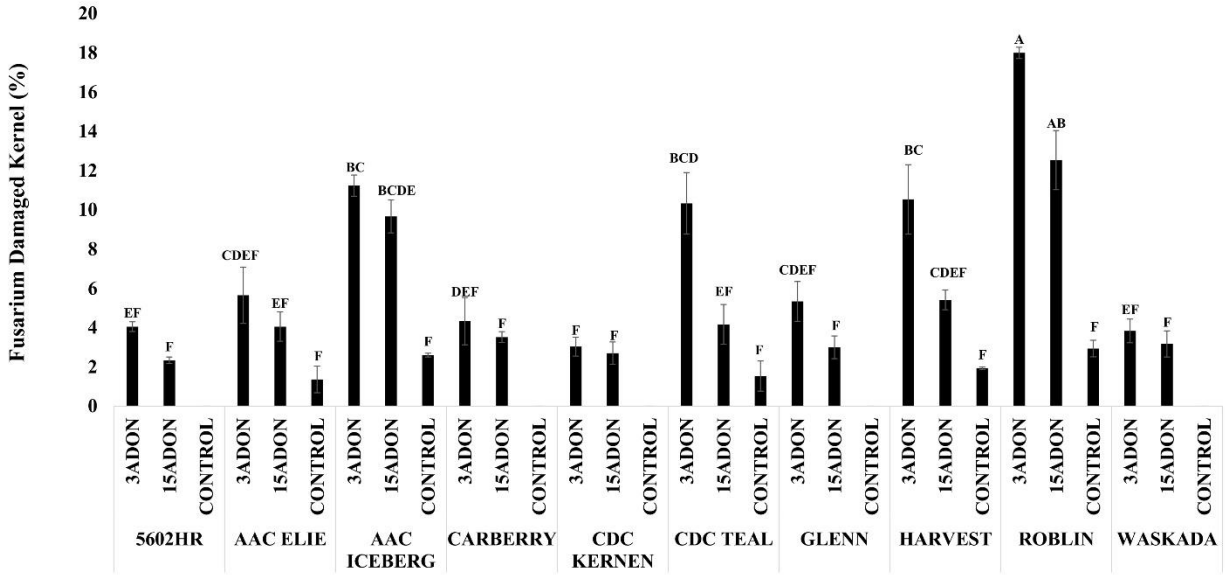


Figure 8.3 Fusarium damaged kernel percentage in spring wheat cultivars inoculated with a mixture of 3-acetyldeoxynivalenol producing or 15-acetlyldeoxynivalenol producing *Fusarium graminearum* strains in Carman**.**

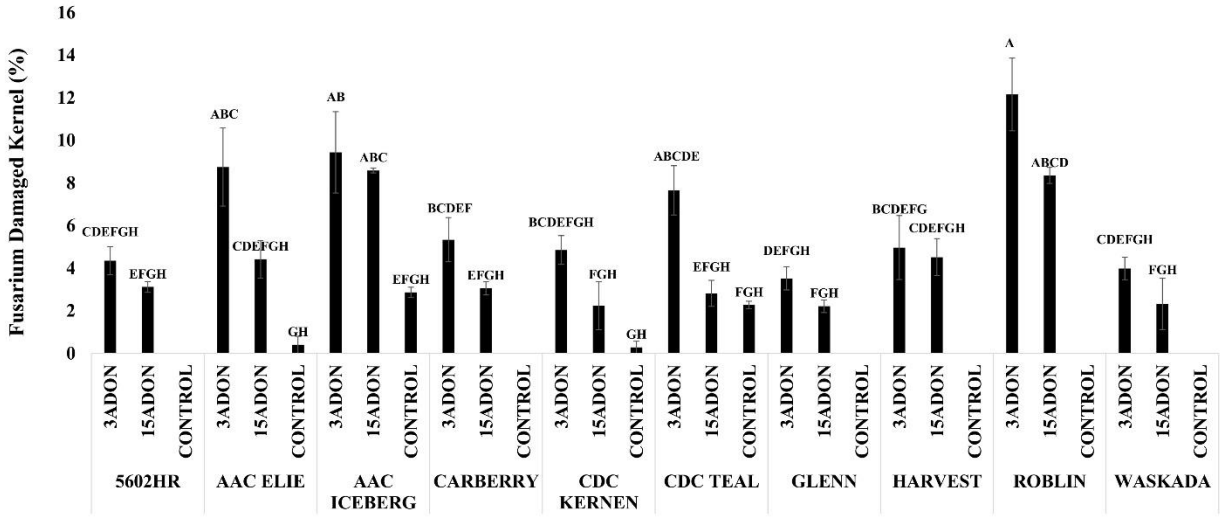


Figure 8.4 Fusarium damaged kernel percentage in spring wheat cultivars inoculated with a mixture of 3-acetyldeoxynivalenol producing or 15-acetlyldeoxynivalenol producing *Fusarium graminearum* strains in Winnipeg**.**

8.4.2 The total DON and D3G content

Similar to other FHB variables represented thus far, the total DON content was significantly different among cultivars and treatments at both locations (Table 8.1 and 8.2). Although there were significant differences between the two chemotypes at Carman location, significant differences were not observed at Winnipeg location for total DON content (Table 8.2). Similar to FHB index and FDK percentage, the two-way interaction, cultivar*treatment was significantly different for total DON content at both locations. The total DON content in different cultivars inoculated with *F. graminearum* strains ranged from 5.6 to 34.4 ppm (Fig 8.5 and 8.6). The highest DON content was observed in cultivar Roblin inoculated with a mixture of 3-ADON strains in Carman (34.4 ppm) (Fig 8.5). In Winnipeg, the highest total DON content was found in cultivar AAC Iceberg inoculated with a mixture of 3-ADON strains (20.4 ppm) (Fig 8.6). The

lowest total DON content was observed in cultivar Glenn inoculated with a mixture of 15-ADON in Carman (6.0 ppm) and cultivar 5602HR (5.6 ppm) in Winnipeg. Due to high FHB disease pressure in Carman, all wheat cultivars inoculated with distilled water also showed DON contamination. In water inoculated wheat cultivars the DON content ranged between 2.1 to 6.1 ppm. In Winnipeg location, cultivars AAC Elie, AAC Iceberg and CDC Teal showed DON contaminations in water control lines.

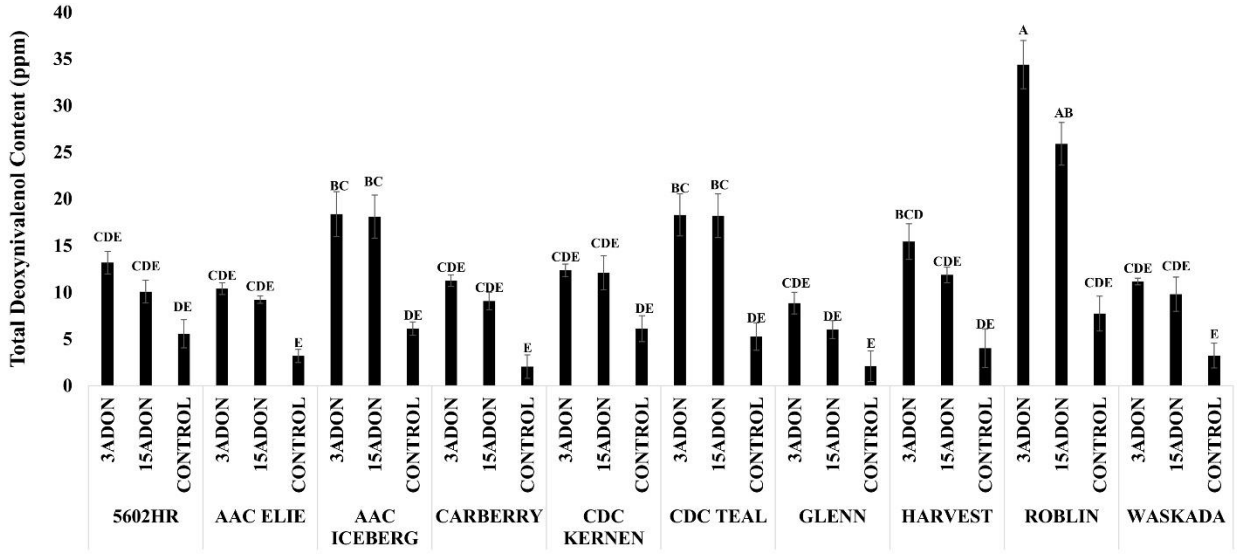


Figure 8.5 Total deoxynivalenol (DON) content in spring wheat cultivars inoculated with a mixture of 3-acetyldeoxynivalenol producing or 15-acetlyldeoxynivalenol producing *Fusarium graminearum* strains in Carman**.**

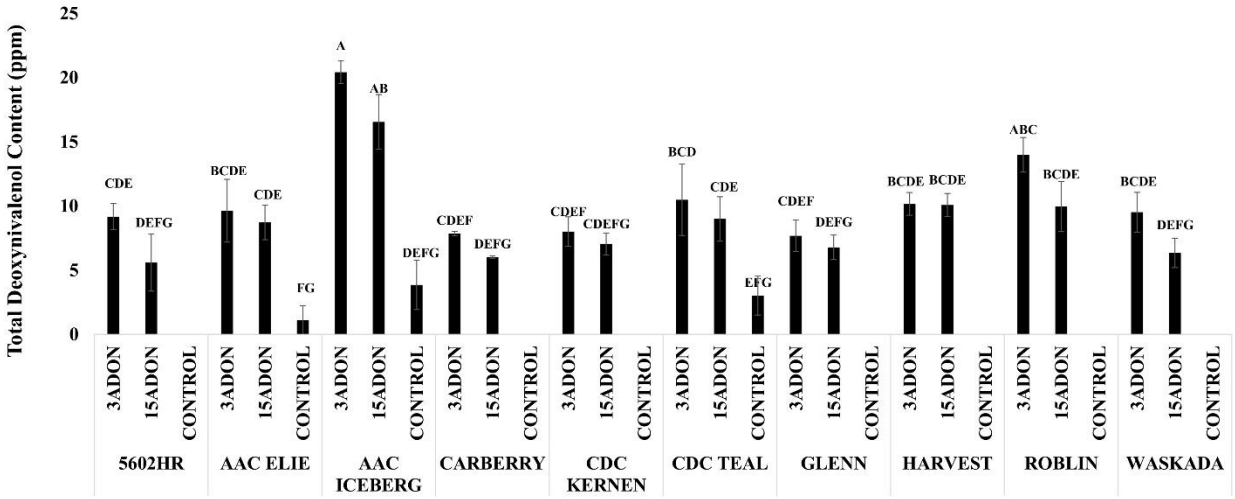


Figure 8.6 Total deoxynivalenol (DON) content in spring wheat cultivars inoculated with a mixture of 3-acetyldeoxynivalenol producing or 15-acetlyldeoxynivalenol producing *Fusarium graminearum* strains in Winnipeg**.**

As with other variables presented, significant differences were observed among the cultivars for D3G content at both locations (Table 8.1 and 8.2). Significant differences were not observed between the chemotypes for D3G content at both locations. Also the two-way interaction of cultivar*treatment was not significant (Table 8.1). The D3G content ranged between 0 to 6.9 ppm in different wheat cultivars (Fig 8.7 and 8.8). The highest D3G content was observed in cultivar AAC Iceberg inoculated with a mixture of 3-ADON strains at both locations (6.9 ppm in Winnipeg and 6.3 ppm in Carman) (Fig 8.7 and 8.8). The D3G/DON ratio was significantly different among the cultivars at Winnipeg location but not at Carman location. No significant differences were observed between the chemotypes at both locations (Table 8.1 and 8.2). The two-way interaction cultivar*chemotype was also not significantly different at both locations. The highest D3G/ DON ratio was observed in cultivar Carberry inoculated with a mixture of 15-ADON strains in Carman (44%) (Fig 8.9). In Winnipeg, the highest D3G/DON

ratio was observed in cultivar CDC Kernen inoculated with a mixture of 3-ADON strains (63.8%) (Fig 8.10). The D3G/ DON ratio was low in susceptible cultivars such as CDC Teal, Harvest and Roblin.

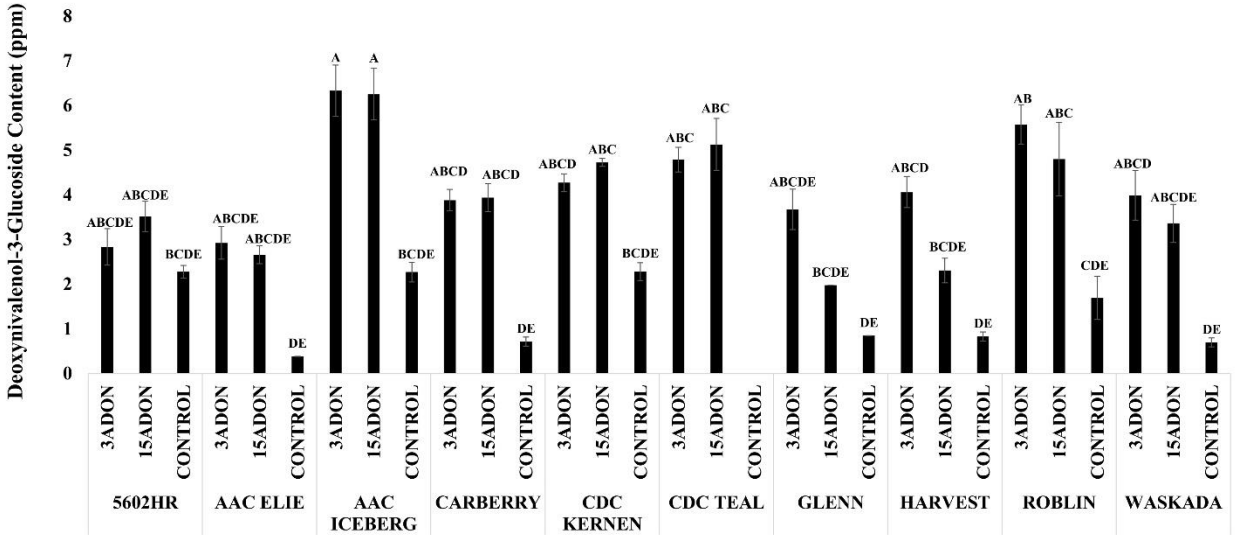


Figure 8.7 The deoxynivalenol-3-glucoside (D3G) content in spring wheat cultivars inoculated with a mixture of 3-acetyldeoxynivalenol producing or 15-acetlyldeoxynivalenol producing *Fusarium graminearum* strains in Carman**.**

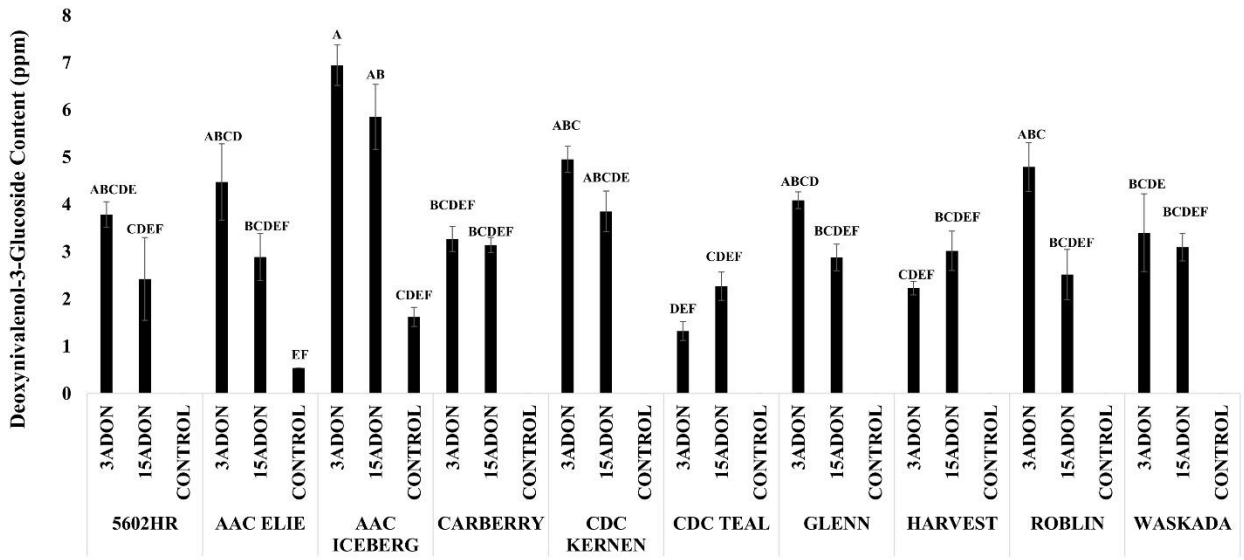


Figure 8.8 The deoxynivalenol-3-glucoside (D3G) content in spring wheat cultivars inoculated with a mixture of 3-acetyldeoxynivalenol producing or 15-acetlyldeoxynivalenol producing *Fusarium graminearum* strains in Winnipeg**.**

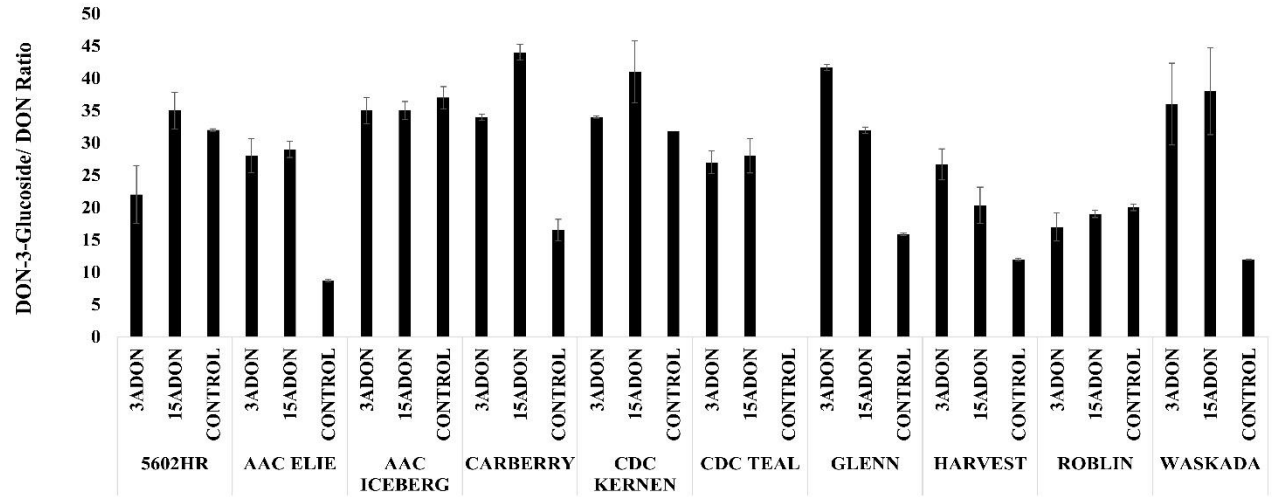


Figure 8.9 Deoxynivalenol-3-glucoside / Deoxynivalenol ratio in spring wheat cultivars inoculated with a mixture of 3-acetyldeoxynivalenol producing or 15-acetlyldeoxynivalenol producing *Fusarium graminearum* strains in Carman**.**

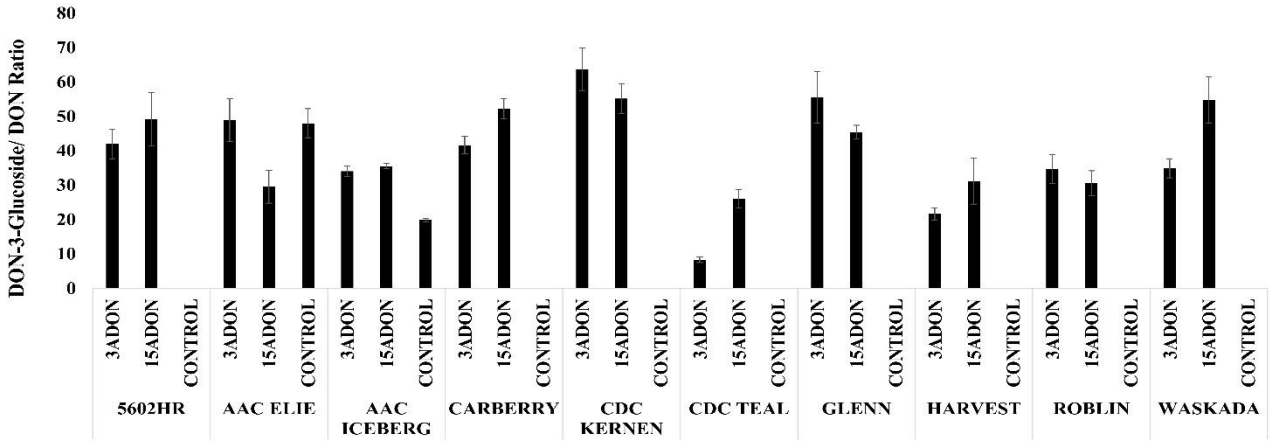


Figure 8.10 Deoxynivalenol-3-glucoside / Deoxynivalenol ratio in spring wheat cultivars inoculated with a mixture of 3-acetyldeoxynivalenol producing or 15-acetlyldeoxynivalenol producing *Fusarium graminearum* strains in Winnipeg**.**

8.4.3 Correlation between FHB disease variables

Significant positive correlations were observed for all FHB disease variables analysed (Table 8.3 and 8.4). A strong positive correlation was observed between DON and D3G content at both locations. When two locations were analysed separately, the highest correlation between DON and D3G was observed at the Winnipeg location (Table 8.4). The correlation between DON and D3G was 0.84. The correlation between FDK percentage and D3G percentage was 0.66. In Carman, the correlation between DON and D3G was 0.77. The correlation between FDK and D3G was 0.61 (Table 8.3).

Table 8.3 Pearson correlation coefficients between fusarium head blight disease Index, fusarium damaged kernel percentage, total deoxyninvalenol content and deoxynivalenol-3-glucoside content in Carman, Manitoba

	FHB Index[1]	**% FDK[2]**	**DON (ppm)[3]**	**D3G (ppm)[4]**
FHB Index	**1.00**	0.92*	0.79*	0.69*
% FDK		**1.00**	0.78*	0.61*
DON (ppm)			**1.00**	0.77*
D3G (ppm)				**1.00**

[1]FHB Index- Fusarium head blight disease index.
[2]% FDK- Fusarium damaged kernel percentage.
[3]DON (ppm)- Deoxynivalenol content in parts per million.
[4]D3G (ppm)- Deoxynivalenol-3-glucosides content in parts per million.
*Means correlation coefficient is significant at $p< 0.0001$
Number of samples=n=90

Table 8.4 Pearson correlation coefficients between fusarium head blight disease index, fusarium damaged kernel percentage, total deoxynivalenol content and deoxynivalenol-3-glucoside content in Winnipeg, Manitoba

	FHB Index[1]	**% FDK[2]**	**DON (ppm)[3]**	**D3G (ppm)[4]**
FHB Index	**1.00**	0.79*	0.79*	0.56*
% FDK		**1.00**	0.81*	0.66*
DON (ppm)			**1.00**	0.84*
D3G (ppm)				**1.00**

[1]FHB Index- Fusarium head blight disease index.
[2]% FDK- Fusarium damaged kernel percentage.
[3]DON (ppm)- Deoxynivalenol content in parts per million.
[4]D3G (ppm)- Deoxynivalenol-3-glucosides content in parts per million.
*Means correlation coefficient is significant at $p< 0.0001$
Number of samples=n=90

8.5 Discussion

In this study we have observed significant differences for FHB disease index, FDK percentage, DON and D3G content between the two locations. The FHB disease severity and incidence was higher at the Carman site than at the Winnipeg site. This may be explained by the amount of inoculum present at the location and the climatic conditions at the flowering time. The Carman experimental location has been used for a FHB disease nursery for more than 15 years and may have more inoculum compared to the Winnipeg experimental site. At the Carmen site, even in

control plots, DON contamination was observed in all the analysed cultivars. This can be due to the inoculum drift from the adjacent plots or natural inoculum present at the site. In this study, significant differences were observed among the cultivars for all of the FHB response variables measured except for D3G/DON ratio at Carman location. This indicates that there were differences in the FHB disease progression for the cultivars. This was to be expected since the cultivars used in this study ranged in reaction to FHB from susceptible, intermediate to moderately resistant. The relative rankings of cultivars remained the same for all FHB disease variables except for cultivar AAC Iceberg. The highest FHB disease index, FDK percentage, DON and D3G content was shown by cultivar Roblin and AAC Iceberg inoculated with a mixture of 3-ADON strains. The cultivar Roblin is rated as highly FHB susceptible and is usually included as a check in disease nursery trials. Although AAC Iceberg rated as intermediate resistance to FHB, we could observe higher levels of disease at both locations. The moderately resistant cultivars such as Carberry, Waskada and 5602HR showed lower FHB index, FDK percentage and DON content compared to the susceptible cultivars. The intermediate resistant cultivars, Glenn, CDC Kernen and AAC Elie also showed lower FHB index, FDK percentage and DON content compared to susceptible cultivars. Therefore, these findings further confirm that, the use of resistant cultivars is one of the major management strategies to combat fusarium head blight in wheat. When chemotype origin of strains is considered, significant differences were observed between 3-ADON and 15-ADON strains for FHB index at both locations, FDK percentage at Carman, and total DON content at Carman. However, there were no significant differences between the two chemotypes for D3G content and D3G/DON ratio. Although the cultivar*treatment interaction was significantly different for most of the FHB disease variables analysed (except for D3G and D3G/DON ratio at Carman location), the

cultivar*chemotype interaction was not significant. Therefore, these results indicate that wheat cultivars used in this study may be ranked similarly for both 3-ADON strains and 15-ADON strains under field environmental conditions. However, the magnitude of the difference in all measured FHB disease variables among cultivars differed with the chemotype used. The wheat cultivars inoculated with a mixture of 3-ADON strains always showed higher FHB disease index, FDK percentage and DON content. Similar results have been reported in other studies, in which 3-ADON producing strains showed higher disease severity and DON accumulation compared to the 15-ADON producing strains (Ward *et al*., 2008; Puri & Zhong *et al*., 2010).

Both DON and D3G contents were measured simultaneously using LC-MS. There was a strong positive correlation between the DON and D3G content. Our results are in agreement with results by Ovando- Martínez *et al.* (2013). They have reported a positive correlation between DON and D3G in hard red spring wheat inoculated with *F. graminearum* strains. The ratios of D3G/DON were higher in MR cultivars CDC Kernen and Carberry. Lemmens *et al.* (2005) have also shown that DON resistant lines with *Qfhs.ndsu-3BS* QTL (*Fhb1* gene) have higher D3G to DON ratio. It is believed that *Qfhs.ndsu-3BS* QTL encodes a DON-glucosyltransferase or regulates the expression of such an enzyme. The cultivars CDC Kernen and Carberry used in this study, have been bred by introgressing the *Qfhs.ndsu-3BS* QTL. This may explain the higher D3G/DON ratio in these cultivars compared to other cultivars in the study. The D3G/DON ratio at both locations, were not significantly different between the chemotypes used in the study. The amount of D3G content in the infected wheat kernels were maintained by the resistance mechanisms within the wheat cultivars, not by the chemotypic origin of the *F. graminearum* strain. Therefore, these results showed that level of resistance in the wheat cultivar plays a key role in detoxification of DON to D3G during the infection. DON acts as a virulence factor in

FHB disease development; hence the detoxification of DON to less toxic D3G can reduce the virulence of the pathogen (Mesterházy, 2002). This may lead to lower FHB symptoms and lower DON contamination in resistant wheat lines compared to susceptible lines.

According to our knowledge, this is the first study done in Canada to determine the amount of D3G in Canadian spring wheat cultivars after inoculating with different chemotypes of *F. graminearum*. The findings from this study help to understand the occurrence of D3G in commonly grown spring wheat cultivars in Canada. So far, D3G content is not assessed in routine food and feed safety protocols in many countries. Therefore, this study shows the importance of testing D3G in food and feed safety assessments in Canada, as these masked mycotoxins might be converted back to the toxic forms inside human/animal body.

In conclusion, this study shows that Canadian spring wheat cultivars produce D3G upon *F. graminearum* infection and there is a positive correlation between the total DON content and the D3G content. The moderately resistant wheat cultivars such as CDC Kernen and Carberry have shown a higher D3G/DON ratio suggesting that detoxification of DON by conjugating with glucose molecules may be one of the mechanisms to reduce DON content in moderately resistant wheat cultivars. Further, this study shows the importance of a more detailed analysis of D3G in Canadian spring wheat cultivars using more locations and years. Finally, it is important to attain a more complete understanding of how a plant's defence system is able to convert DON into other non or less toxic compounds in order to develop cultivars with improved FHB resistance.

CHAPTER 9

GENERAL DISCUSSION AND CONCLUSIONS

The current study has provided novel insights into a better understanding of the complex relationships in the *F. graminearum*-wheat pathosystem as listed below:

- First study to report the presence of nivalenol producing *F. cerealis* in winter wheat in Manitoba, Canada.
- Provided information on *Fusarium* species distribution and trichothecene chemotype diversity in wheat collected from Winnipeg and Carman in Manitoba, Canada.
- Identified the phylogenetic relationships, trichothecene chemotype diversity, phenotypic characteristics and aggressiveness of FGSC strains from eight countries.
- Characterized the chemotype specific trichothecene biosynthesis related gene expression patterns during *F. graminearum*- wheat infection and colonization.
- Analysed the effect of cultivar and chemotype D3G production in ten Canadian spring wheat cultivars and showed that D3G content significantly different among the cultivars but not between the chemotypes.

Due to the increasing concerns regarding food and feed safety, contamination of wheat products with *Fusarium* mycotoxins has become a major topic of social concern. The mycotoxin contamination profile and associated toxicological risk can vary significantly with the *Fusarium* species and the type of trichothecenes present in the contaminated wheat products. Therefore, disease management strategies must be developed to encounter a range of *Fusarium* species,

each with unique ecological, epidemiological, and population identities. Hence the accurate, reliable and rapid diagnosis of *Fusarium* spp. and trichothecene chemotypes in wheat is a major challenge for researcher's work in the *Fusarium* field. An increasing acceptance of a phylogenetic species concept, the availability of sequencing techniques coupled with PCR flexibility, allowed researchers to postulate and develop tools to identify different species and chemotypes in the FGSC (Stępień, 2014). The most commonly used method to detect strains is from infected wheat tissue. This method involves the isolation of single spore colonies from the infected wheat spikes and analysing the *Fusarium* species and trichothecene chemotype on the isolated strains (Pasquali & Migheli, 2014). Recently, the phylogenetic species recognition criterion and genetic chemotyping have provided tools to acquire information on the distribution of a certain species or a chemotype on a seed sample, within a field, a region, or a country (Sarver *et al*., 2011; Starkey *et al*., 2011; Astolfi *et al*., 2012; Zhang *et al*., 2012; Qiu & Shi, 2014).

Shifts in *Fusarium* species and chemotype populations have been reported from many surveys (Guo *et al*., 2008; Ward *et al*., 2008; Lindblad *et al*., 2013; Beyer *et al*., 2014). As not all *Fusarium* species are capable of producing all trichothecene chemotypes, a shift in *Fusarium* species population could also be associated with the shift in type of trichothecene type. Among the *Fusarium* spp, *F. graminearum s.s* is the major causal agent of FHB in wheat in Manitoba, Canada. The finding of nivalenol producing *F. cerealis* in winter wheat in Manitoba, Canada poses a potential threat to the wheat industry in Canada (Chapter 3). Routine chemical analysis for nivalenol is not conducted at mills or elevators. Therefore, presence of NIV in infected wheat samples represents a major concern for safety of wheat products in Canada. Guo *et al*. (2008) investigated the trichothecene chemotype distribution of *F. graminearum* strains in Manitoba,

Canada from 2004 to 2005. A rapid chemotype shift was observed from 15-ADON producing strains to 3-ADON strains. This chemotype shift is of concern, because, both field and laboratory studies have shown that 3ADON producing strains are sometimes more aggressive, cause severe disease symptoms and produce higher levels of DON in infected wheat grains compared to 15ADON producing strains (Ward *et al*., 2008; Puri & Zhong, 2010; von der Ohe *et al*., 2010). This study showed that chemotype shift from 15-ADON to 3-ADON was more apparent during the 2010 and 2013 in Winnipeg and Carman locations in Manitoba, Canada (Chapter 4). So far, no clear reasons have been provided to explain the rapid increase in the frequency of 3-ADON producing strains over the 15-ADON strains. One possible explanation is the fitness characteristics such as growth and the capacity to produce toxins, which is associated with a chemotype, may favour its establishment and survival in a given area (Ward *et al*., 2008; Puri & Zhong, 2010; Zhang *et al*., 2012). The chemotype distribution in a given area may also be driven by the environmental conditions and cultural practices. Recent migration events and gene introgression were also suggested to play key roles in establishing a novel chemotype with adequate fitness in resident *Fusarium* populations (Gale *et al*., 2011; Zhang *et al*., 2012; Pasquali & Migheli, 2014). Among the other factors, the type of fungicides used, may also contribute in determining the distribution of certain trichothecene chemotypes in a given area. Some studies have shown that use of azoles, and strobilurins have no effect on the *F. graminearum* chemotype (Kulik *et al*., 2012; Amarasinghe *et al*., 2013; Beyer *et al*., 2014). The studies on carbendazim sensitivity have shown mixed results. Zhang *et al*. (2009) explained a difference between 3-ADON and NIV strains towards carbendazim sensitivity where as, Qiu & Shi (2014) did not find any difference between the two chemotypes.

Environmental factors may have an effect on the establishment and survival of a chemotype in a given area. Some chemotypes are more prevalent under specific temperature conditions. For example, Zhang *et al*. (2007) reported that 3-ADON producing strains were more prevalent in warmer regions where the annual average temperatures were above 15 ºC, and 15-ADON producers were more common in cooler regions (annual average temperature less than 15 ºC). However, based on an examination of weather variables over a five-year period (2008-2012) no correlation between recovery of a chemotype and temperature or precipitation was observed (Gilbert *et al*. 2014). Therefore, it is difficult to draw conclusions on effect of climatic factors on chemotype selection. Chemotype selection in a given area may also be driven by a certain host species. NIV chemotypes were found to be more aggressive toward maize than DON chemotypes (Carter *et al*., 2002; Qu *et al*., 2008b; Lee *et al*., 2009). In North America, the recovery of NIV chemotype has been associated with rice cultivation (Gale *et al*., 2011). Under northern European climatic conditions, Nielsen *et al*. (2012) recovered higher frequency of 3-ADON producing strains in oats and barley compared to wheat. A better understanding of the toxigenic potential of *Fusarium* strains collected from a given area is important to prescribe risk assessment on toxin contamination at the regional scale (Pasquali & Migheli, 2014). Therefore, the results from chapter 3 and 4 suggest the continuous monitoring of *F. graminearum* chemotype situation in a given region, because this may well inform on the potential risk associated with food and feed safety in that region.

Chapter 5 evaluated the phylogenetic relationships, trichothecene chemotype diversity, and aggressiveness of *F. gramineraum* strains collected from different countries. The phylogenetic relationships among the strains were recovered based on the *EF-1α* and *MAT1-1-3* genes. *EF-1α* gene has gained the most research interest as the sequence divergence revealed by

phylogenetic analysis, appeared to be high enough to differentiate the *Fusarium* populations to a sub-species level (Geiser *et al*., 2004; Kristensen *et al*., 2005; Stępień, 2014). To date, information on chemotype distribution of FGSC is available from all continents (Wang *et al*., 2011, van der Lee *et al*., 2015). Some studies have stated a relationship between the geographic origin of strains and the type of trichothecenes produced (Astolfi *et al*., 2012; Zhang *et al*., 2012; Backhouse, 2014; Qiu & Shi, 2014). The nivalenol producing species such as *F. austroamericanum*, *F. meridionale* and *F. acacia-mearnsii* were more prevalent in South America. *F. asiaticum* species, also capable of producing NIV, were more common in China (Chapter 5). This situation may be due to the adaptation to the environmental conditions and the host species in those geographical regions. Also, in this study the aggressiveness of FGSC strains were assessed in terms of FHB disease severity, FDK percentage, amount of toxins produced, growth rate, and macroconidia production. Based on our knowledge, this study provides the first detailed analysis of aggressiveness of FGSC strains which includes six species in the FGSC. Gaining knowledge of the trichothecene chemotype diversity in prevailing FGSC populations from major wheat producing regions/countries, and further assessing their role in the aggressiveness are essential for understanding factors affecting plant-pathogen interactions and offering new approaches to breeding for resistance. Testing local and global genetic diversity and toxin producing abilities of *F. graminearum* strains in wheat breeding programmes are important to develop locally adapted cultivars. Differences in the characteristics of a species/strain used in a breeding program may influence the reaction of host genotypes and may provide erroneous conclusions. Therefore, this study suggests the use of a diverse collection of strains from different geographical zones in current breeding programs to develop more durable and stable resistant cultivars (Chapter 5). Also in this study, the aggressiveness parameters were assessed

based on the species and the trichothecene chemotype of the strain. The results obtained from this study suggest that the capacity of strains within the FGSC to spread and cause FHB on wheat is more likely to be trichothecene chemotype specific rather than species-specific. Of course, trichothecene chemotype cannot only explain all phenotypic or aggressiveness differences observed among different species. Therefore, more detailed analysis is required to clarify the level of variability in strains within a species.

The trichothecene biosynthesis related genes are often clustered at a single locus and involved exclusively in the trichothecene biosynthesis (Kimura *et al*., 2007). Therefore, environmental factors and the overall evolution of the population strongly influence the divergence of the coding region of trichothecene biosynthesis related genes. Because the secondary metabolites are frequently associated with the infection and pathogenesis process, the selection pressure is much lower than on housekeeping genes (Stępień, 2014). This allows faster evolution of secondary biosynthesis genes than for the housekeeping genes. Nucleotide polymorphisms in multiple genes (*TRI3*, *TRI7*, *TRI8, TRI12* and *TRI3*) in the trichothecene biosynthesis pathway lead to separate chemotypes. As mentioned before the selection may occur on the end products of the mycotoxin biosynthesis pathway. Therefore, recombination between genes that encodes for different trichothecene chemotypes might result in combinations that produce no toxins or toxins with different biological activity (Ward *et al*., 2002; Rep & Kistler, 2010). The use of secondary metabolite synthesis genes for *Fusarium* phylogeny reconstruction has been discussed in many studies (Ward *et al*., 2002; Proctor *et al*., 2003; Waalwijk *et al*., 2004; Proctor *et al*., 2009). The phylogeny reconstructed based on the trichothecene biosynthesis genes (*TRI* cluster genes), vary in the topology from those based on the housekeeping genes. The topologies obtained from individual *TRI* genes (*TRI3*, *TRI4, TRI5, TRI6, TRI10, TRI11* and

TRI12) of *Fusarium* reflected the clusters based on the chemotype and not the lineages of *F. graminearum* (Ward *et al*., 2002). This study uses the *TRI8* gene for the first time in *Fusarium* phylogenetic reconstruction. The topology recovered by the Bayesian analysis, performed for *TRI8* gene of *F. graminearum* belonging to different trichothecene chemotypes, also reflected the groupings based on the chemotype (Chapter 6). Therefore, *TRI8* gene performed similar to other *TRI*-cluster genes in recovering phylogenetic relationships among *F. graminearum* strains from different geographic regions. The conflict between the phylogenies, reconstructed based on *TRI* cluster genes and non-*TRI* cluster genes can be explained by the trans-species polymorphism (Ward *et al*., 2002). The correlation between trans-species polymorphisms and chemotype, suggests that diversification of trichothecene cluster appeared prior to species divergence (Rep & Kistler, 2010). Trans-species polymorphism is believed to be maintained via balancing selection acting on chemotype differences (Ward *et al*., 2002). Balancing selection refers to selective processes by which different alleles are kept in the gene pool of a population at frequencies above that of gene mutation. Because chemotypes may confer different fitness in different environments and host species, the chemotype diversity may be maintained by balancing selection in a given environment. Use of trichothecene biosynthesis genes for phylogeny reconstruction is advantageous in identifying different chemotypes in *Fusarium* populations. Also phylogenetic studies on *TRI* cluster genes between trichothecene producing and non-producing species will provide very important insight into the evolution of the *TRI* cluster genes.

Limited studies have been done on trichothecene biosynthesis related gene expression in different chemotypes during wheat infection and colonization (Hallen-Adams *et al*., 2011; Lee *et al*., 2014). The availability of structures and function of important trichothecene biosynthesis related genes enable the chemotype specific gene expression studies. The expression levels of

most *TRI* genes essential for trichothecene production were strongly induced at early time intervals (2-3 dpi) (Chapter 7). The reasons for this observation may be explained from gene expression studies from both host and pathogen. Kosaka *et al*. (2015) have observed a high expression of DON detoxification genes such as multidrug resistant proteins, multidrug resistance-associated protein, UDP-glycosyltransferase and ABC transporters at early stages of infection in the cultivar Nobeokabouzu-komugi. Therefore, to counter-attack the resistance response by host, the pathogen may induce the *TRI* gene expression at early stages of infection. The level of *TRI* gene expression was higher in moderately resistant wheat cultivars than in susceptible wheat cultivars. Similar results were reported by Hallen-Adams *et al*. (2011). They have also observed the higher levels of *TRI* gene expression in the moderately resistant cultivar 'Alsen'. So far, no clear reasons have been provided for the higher levels of *TRI* gene expression in moderately resistant cultivars. One possible explanation is that the pathogen has to put more energy in infecting and colonizing the wheat cultivars with moderate level of resistance than the low levels of resistance. Therefore, the pathogen has to withstand the resistance response by host, so they have to express either more genes or/and increase the level of gene expression. However, more studies, with cultivars having different levels of resistance to FHB are needed to obtain clearer conclusions. The level of *TRI* gene expression was higher in 3-ADON producing strains compared to 15-ADON and NIV strains (Chapter 7). Based on our knowledge, no studies have compared the *TRI* gene expression levels in different chemotypes during wheat colonization. In this study we have used two strains representing one chemotype. Although similar gene expression patterns were observed in both strains representing each chemotype, this study stresses the need of analysing more strains from each chemotype to obtain more detailed chemotype specific *TRI*- gene expression patterns.

The final study of this thesis was focused on analysing the DON and D3G content in spring wheat cultivars after inoculating with *F. graminearum* 3-ADON and 15-ADON strains (Chapter 8). Significant differences were observed among the cultivars in their ability to convert DON into D3G. The use of yeast, *Arabidopsis* and wheat germ *in vitro* systems have shown that the formation of D3G significantly associated with *Fusarium* resistance in wheat (Lemmens *et al*., 2005). However, toxicological studies on D3G in animal and human systems indicated that D3G can be effectively hydrolysed in the intestinal tract, and, hence pose a potential health risk to exposed animals and humans (Dall'Erta *et al*., 2013; Gratz *et al*., 2013; Warth *et al*., 2013; Nagl *et al*., 2014). Therefore, the inclusion of masked mycotoxins in future toxin risk assessment schemes is warranted. A study done by Lemmens *et al*. (2005) observed a close relationship between DON resistance data and the ratio of D3G to DON in infected wheat spikes. The DON resistance and major QTL for FHB resistance, *Qfhs.ndsu-3BS* is mapped to the same region on chromosome 3B. These observations, lead to the speculation that *Qfhs.ndsu-3BS* may either encode for UDP- glucosyl transferase or regulate the expression of such an enzyme (Lemmens *et al*., 2005; Kluger *et al*., 2015). Therefore, FHB resistant wheat cultivars, developed by introgressing the *Qfhs.ndsu-3BS* may be more efficient in converting DON into D3G during detoxification process.

In conclusion, the present thesis showed that better understanding of the genetic structure, trichothecene chemotype diversity and toxigenic ability of the *Fusarium* strains collected from a given field or region may help in assessing potential risk at the field /regional sacle due to mycotoxin contamination. Because *Fusarium* spp. are continuously evolving with novel mechanisms to combat the FHB disease resistance further continental scale surveys are justified using more strains from different geographical regions. Furthermore, substantial progress in

understanding the *F. graminearum*-wheat pathosystem will depend on rigorous phylogeographic surveys, molecular characterization of chemotypes/strains and comprehensive analysis of toxin biosynthesis pathways. To date, the advancement of sequencing technology, transcriptomics, proteomics and metabolomics approaches have made it feasible to uncover the unknown behaviours in this pathosystem. Recovery of new mycotoxins and chemotypes suggest that *F. graminearum* species have the genetic potential to produce many more toxins than can be detected by chemical analyses. Therefore, as future studies are conducted, it will be important to focus on phylogenomic studies of *F. graminearum* to uncover the origin and diversity of toxic secondary metabolites and identify phylogenetically informative genes. Also there is an emerging need to develop new forward genetic approaches to discover the genes that detoxify mycotoxins, which enable breeders to develop cultivars having broad based resistance to fusarium head blight disease.

REFERENCES

Aamot HU, Ward TJ, Brodal G *et al*., 2015. Genetic and phenotypic diversity within the *Fusarium graminearum* species complex in Norway. *European Journal of Plant Pathology*, 501-19.

Abramson D, Clear RM, Smith DM, 1993. Trichothecene production by *Fusarium* spp. isolated from Manitoba grain. *Canadian Journal of Plant Pathology*. **15**, 147-52.

Abramson D, Clear RM, Gaba D, Smith DM, Patrick SK, Saydak D, 2001. Trichothecene and moniliformin production by *Fusarium* species from western Canadian wheat. *Journal of Food Protection* **64**, 1220-5.

Akinsanmi OA, Backhouse D, Simpfendorfer S, Chakraborty S, 2006. Genetic diversity of Australian *Fusarium graminearum* and *F. pseudograminearum*. *Plant Pathology* **55**, 494-504.

Alexander NJ, Hohn TM, McCormick SP, 1998. The *TRI11* gene of *Fusarium sporotrichioides* encodes a cytochrome P450 monooxygenase required for C-15 hydroxylation in trichothecene biosynthesis. *Applied and Environmental Microbiology* **64**, 221-5.

Alexander NJ, Hohn TM, McCormick SP, 1999. *TRI12*, a trichothecene efflux pump from *Fusarium sporotrichioides*: gene isolation and expression in yeast. *Molecular Genetics and Genomics* **261**, 977-84.

Alexander NJ, McCormick SP, Larson TM, Jurgenson JE, 2004. Expression of Tri15 in *Fusarium sporotrichiodides*. *Current Genetics* **45**, 157-62.

Alexander NJ, McCormick SP, Waalwijk C, van der Lee T, Proctor RH, 2011. The genetic basis for 3-ADON and 15-ADON trichothecene chemotypes in *Fusarium graminearum*. *Fungal Genetics and Biology* **48**, 485-95.

Alexander NJ, Proctor RH, McCormick SP, 2009. Genes, gene clusters, and biosynthesis of trichothecenes and fumonisins in *Fusarium*. *Toxin reviews* **28**, 198-215.

Alvarez CL, Somma S, Moretti A, Fernández Pinto V, 2010. Aggressiveness of *Fusarium graminearum sensu stricto* strains in wheat kernels in Argentina. *Journal of Phytopathology* **158**, 173-81.

Al-Taweel K, Fernando WGD, Brûlé-Babel AL, 2014. Transcriptome profiling of wheat differentially expressed genes exposed to different chemotypes of *Fusarium graminearum*. *Theoretical and Applied Genetics* **127**, 1703-18.

Amarasinghe C, Wang JH, Liao YC, Dilantha Fernando WG, 2011. Difference in *TRI13* gene sequences between the 3-acetyldeoxynivalenol producing *Fusarium graminearum* chemotypes from Canada and China. *International Journal of Molecular Sciences* **12**, 6164 -75.

Amarasinghe CC, Tamburic-Ilincic L, Gilbert J, Brûlé-Babel AL, Fernando WGD, 2013. Evaluation of different fungicides for control of fusarium head blight in wheat inoculated with 3-ADON and 15-ADON chemotypes of *Fusarium graminearum* in Canada. *Canadian Journal Plant Pathology* **35**, 1-9.

Amarasinghe CC, Tittlemier SA, Fernando WGD, 2015. Nivalenol-producing *Fusarium cerealis* associated with fusarium head blight in winter wheat in Manitoba, Canada. *Plant Pathology* **64**, 988-95.

Aoki T, O'Donnell K, Geiser DM, 2014. Systematics of key phytopathogenic *Fusarium* species: Current status and future challenges. *Journal of General Plant Pathology* **80**, 189 -201.

Astolfi P, Reynoso MM, Ramirez ML *et al*., 2012. Genetic population structure and trichothecene genotypes of *Fusarium graminearum* isolated from wheat in southern Brazil. *Plant Pathology* **61**, 289-95.

Audenaert K, Callewaert E, Hofte M, De Saeger S, Haesaert G, 2010. Hydrogen peroxide induced by the fungicide prothioconazole triggers deoxynivalenol (DON) production by *BMC Microbiology* **10,** 1-14.

Audenaert K, Vanheule A, Höfte M, Haesaert G, 2013. Deoxynivalenol: A major player in the multifaceted response of Fusarium to its environment. *Toxins* **6**, 1-19.

Backhouse D, 2014. Global distribution of *Fusarium graminearum, F. asiaticum* and *F. boothii* from wheat in relation to climate. *European Journal of Plant Pathology* **139**, 161-73.

Bacon CW, Porter JK, Norred WP, Leslie JE, 1996. Production of fusaric acid by *Fusarium* species. *Applied and Environmental Microbiology* **62**, 403-43.

Bai GH, Desjardins AE, Plattner RD, 2002. Deoxynivalenol-nonproducing *Fusarium graminearum* causes initial infection, but does not cause disease spread in wheat spikes. *Mycopathologia* **153**, 91-8.

Bai GH, Plattner R, Desjardins A, Kolb F, 2001. Resistance to fusarium head blight and deoxynivalenol accumulation in wheat. *Plant Breeding* **120**, 1-6.

Bai GH, Shaner G, 1994. Scab of wheat: Prospect for control. *Plant Disease* **78**, 760-6.

Bai GH, Shaner G, 2004. Management and resistance in wheat and barley to fusarium head blight. *Annual Review of Phytopathology* **42**, 135-61.

Berthiller F, Crews C, Dall'Asta C *et al*., 2013. Masked mycotoxins: A review. *Molecular Nutrition and Food Research* **57**, 165-86.

Berthiller F, Dall'Asta C, Schuhmacher R, Lemmens M, Adam G, Krska AR, 2005. Masked mycotoxins: Determination of a deoxynivalenol glucoside in artificially and naturally contaminated wheat by liquid chromatography-tandem mass spectrometry. *Journal of Agriculture and Food Chemistry* **53**, 3421-5.

Berthiller F, Krska R, Domig KJ, Kneifel W, Juge N, Schuhmacher R, Adam, G, 2011. Hydrolytic fate of deoxynivalenol-3-glucoside during digestion. *Toxicology Letters* **206**, 264-7.

Beyer M, Pogoda F, Pallez M, Lazic J, Hoffmann L, Pasquali M, 2014. Evidence for a reversible drought induced shift in the species composition of mycotoxin producing Fusarium head blight pathogens isolated from symptomatic wheat heads. *International Journal of Food Microbiology* **182-183**, 51-6.

Beyer M, Verreet, JA, 2005. Germination of *Gibberella zeae* ascospores as affected by age of spores after discharge and environmental factors. *European Journal of Plant Pathology* **111**, 381-9.

Binder EM, 2007. Managing the risk of mycotoxins in modern feed production. *Animal Feed Science and Technology* **133**, 149-66.

Boddu J, Cho S, Muehlbauer GJ, 2007. Transcriptome analysis of trichothecene-induced gene expression in barley. *Molecular plant-microbe interactions* **20**, 1364-75.

Bottalico A, Perrone G, 2002. Toxigenic *Fusarium* species and mycotoxins associated with head blight in small grain cereals in Europe. *European Journal of Plant Pathology* **108**, 611-64.

Boutigny AL, Atanasova-Penichon V, Benet M, Barreau C, Richard-Forget F, 2010. Natural phenolic acids from wheat bran inhibit *Fusarium culmorum* trichothecene biosynthesis in vitro by repressing Tri gene expression. *European Journal of Plant Pathology* **127**, 275-86.

Boutigny AL, Richard-Forget F, Barreau C, 2008. Natural mechanisms for cereal resistance to the accumulation of *Fusarium* trichothecenes. *European Journal of Plant Pathology* **121**, 411-23.

Boutigny AL, Ward TJ, Ballois N, Iancu G, Ioos R, 2014. Diversity of the *Fusarium graminearum* species complex on French cereals. *European Journal of Plant Pathology* **138**, 133-48.

Boutigny AL, Ward TJ, Van Coller GJ *et al*., 2011. Analysis of the *Fusarium graminearum* species complex from wheat, barley and maize in South Africa provides evidence of species-specific differences in host preference. *Fungal Genetics and Biology* **48**, 914-20.

Brown NA, Bass C, Baldwin TK *et al*., 2011. Characterisation of the *Fusarium graminearum*-Wheat Floral Interaction. *Journal of pathogens* **2011**, 1-9.

Brown DW, McCormick SP, Alexander NJ, Proctor RH, Desjardins AE. 2001. A genetic and biochemical approach to study trichothecene diversity in *Fusarium sporotrichioides* and *Fusarium graminearum*. *Fungal Genetics and Biology* **32**, 121-33.

Brown DW, Proctor RH, Dyer RB, Plattner RD, 2003. Characterization of a Fusarium 2-Gene Cluster Involved in Trichothecene C-8 Modification. *Journal of Agricultural and Food Chemistry* **51**, 7936-44.

Brian PW, Dawkins AW, Grove JF, Hemming HG, Lowe D, Norris GLF, 1961. Phytotoxic Compounds produced by *Fusarium equiseti*, *Journal of Experimental Botany* **12**, 1-12.

Bruins MBM, Karsai I, Schepers J, Snijders CHA, 1993. Phytotoxicity of deoxynivalenol to wheat tissue with regard to in vitro selection for Fusarium head blight resistance. *Plant Science* **94**, 195-206.

Bushnell WR, Hazen BH, Pritsch C, 2003. Histology and Physiology of Fusarium Head Blight. In: Leonard KJ, Bushnell WR, eds. *Fusarium Head Blight of Wheat and Barley*. St. Paul, USA: The American Phytopathological Society.44-83.

Buerstmayr H, Ban T, Anderson JA, 2009. QTL mapping and marker-assisted selection for Fusarium head blight resistance in wheat: A review. *Plant Breeding* **128**, 1-26.

Cai L, Giraud T, Zhang N, Begerow D, Cai G, Shivas RG, 2011. The evolution of species concepts and species recognition criteria in plant pathogenic fungi. *Fungal Diversity* **50**, 121-33.

Carter JP, Rezanoor HN, Holden D, Desjardins a E, Plattner RD, Nicholson P, 2002. Variation in pathogenicity associated with the genetic diversity of. *European Journal of Plant Pathology*, 573-83.

Castanares E, Dinilfo MI, Moreno MV, Beron C, Stenglein SA, 2013. *Fusarium cerealis* associated with barley seeds in Argentina. *Journal of Phytopathology* **161**, 586-9.

Cativelli M, Appendino ML, Lewis S, 2011. A novel QTL for type II Fusarium head blight resistance mapped on chromosome 7D of wheat. In: Dreisigacker S, Singh S, eds. *21st International Triticeae Mapping Initiative Workshop*, Mexico City, Mexico: CIMMYT. 76.

Chan Y, Savard ME, Reid LM, Cyr T, McCormick WA, Seguin C, 2009. Identification of lipopeptide antibiotics of a *Bacillus subtilis* isolate and their control of *Fusarium graminearum* diseases in maize and wheat. *Bio Control* **54**, 567-74.

Chandler EA, Simpson DR, Thomsett MA, Nicholson P, 2003. Development of PCR assays to *Tri7* and *Tri13* trichothecene biosynthetic genes, and characterisation of chemotypes of *Fusarium graminearum*, *Fusarium culmorum* and *Fusarium cerealis*. *Physiological and Molecular Plant Pathology* **62**, 355-67.

Chen P, Liu W, Yuan J *et al*., 2005. Development and characterization of wheat- *Leymus racemosus* translocation lines with resistance to Fusarium Head Blight. *Theoretical and Applied Genetics* **111**, 941-8.

Cole RA, Jarvis BB, Schweikert MA. 2003. Handbook of secondary metabolites, New York, USA: Academic press. 199-560.

Cole DJ, Edwards R, 2000. Secondary Metabolism of Agrochemicals in Plants, In: Roberts T. eds, *Metabolism of Agrochemicals in Plants*. NY, USA: Wiley. 107-54.

Coleman JOD, Blake-Kalff MMA, Davies TGE, 1997. Detoxification of xenobiotics by plants: Chemical modification and vacuolar compartmentation. *Trends in Plant Science* **2**, 144-51.

Cowger C, Arellano C, 2013. *Fusarium graminearum* infection and deoxynivalenol concentrations during development of wheat spikes. *Phytopathology* **103**, 460-71.

Cracraft J, 1983. Species Concepts and Speciation Analysis. *Current Ornithology* **1**, 159-187.

Cumagun CJR, Bowden RL, Jurgenson JE, Leslie JF, Miedaner T, 2004.Genetic mapping of pathogenicity and aggressiveness of *Gibberella zeae Fusarium graminearum* toward wheat. *Phytopathology* **94**, 520-6.

Cuthbert PA, Somers DJ, Thomas J, Cloutier S, Brule-Babel A, 2006. Fine mapping *Fhb1*, a major gene controlling fusarium head blight resistance in bread wheat (*Triticum aestivum* L.). *Theoretical and Applied Genetics* **112**, 1465-72.

D'Mello JPF, Placinta CM, Macdonald a. MC, 1999. *Fusarium* mycotoxins: A review of global implications for animal health, welfare and productivity. *Animal Feed Science and Technology* **80**, 183-205.

Dall'Erta A, Cirlini M, Dall'Asta M, Del Rio D, Galaverna G, Dall'Asta C, 2013. Masked mycotoxins are efficiently hydrolyzed by human colonic microbiota releasing their aglycones. *Chemical Research in Toxicology* **26**, 305-12.

De Boevre M, Di Mavungu JD, Maene P *et al.*, 2012. Development and validation of an LC-MS/MS method for the simultaneous determination of deoxynivalenol, zearalenone, T-2-toxin and some masked metabolites in different cereals and cereal-derived food. *Food Additives & Contaminants: Part A* **29**, 819-35.

De Nijs M, Stentor P, DelfgouVan Asch E, Kamphuis H, Rombouts FM, Notermans SHW, 1996. Fungal infection and presence of DON and zearalenone in cereals grown in the Netherlands. *Journal of Food Protection* **59**, 772-7.

Del Ponte E, Spolti P, Ward T *et al*., 2014. Regional and field-specific factors affect the composition of Fusarium head blight pathogens in subtropical no-till wheat agroecosystem of Brazil. *Phytopathology* **105(2)**, 246-54.

Del Ponte EM, Tessmann DJ, Spolti P, Kuhnem PR, da Silva CN. 2013. Species Identification, Genetic Diversity and Phenotypic Variation Studies on the *Fusarium graminearum* Complex Populations from Brazil. In: Magliano TMA, Chulze, SN, eds. *Fusarium head blight in Latin America*. Dordrecht: Springer Science Business Media. 15-30.

Demeke T, Clear RM, Patrick SK, Gaba D, 2005. Species-specific PCR-based assays for the detection of *Fusarium* species and a comparison with the whole seed agar plate method and trichothecene analysis. *International Journal of Food Microbiology* **103**, 271-84.

DePauw RM, Singh A, Singh AK, Knox RE, Singh D, Bhavani S, Fetch T, 2011. The presence of SrCad and Sr2 influences reaction to stripe rust and Fusarium head blight. BGRI

Technical Workshop, Oral Presentations Poster Abstracts Participants and Program; Minnesota (USA); 13-16 Jun 2011.

Desjardins AE, 2006. Fusarium Mycotoxins Chemistry, Genetics and Biology. St. Paul, MN: APS Press.

Desjardins AE, Jarosz AM, Plattner RD, Alexander NJ, Brown DW, Jurgenson JE, 2004. Patterns of trichothecene production, genetic variability, and virulence to wheat of *Fusarium graminearum* from smallholder farms in Nepal. *Journal of Agricultural and Food Chemistry* **52**, 6341-6.

Desjardins AE, Proctor RH, 2007. Molecular biology of *Fusarium* mycotoxins. *International Journal of Food Microbiology* **119**, 47-50.

Desjardins AE, Proctor RH, 2011. Genetic diversity and trichothecene chemotypes of the *Fusarium graminearum* clade isolated from maize in Nepal and identification of a putative new lineage. *Fungal Biology* **115**, 38-48.

Desjardins AE, Proctor RH, Bai GH, McCormick SP, Shaner G, Buechley G, Hohn TM, 1996. Reduced virulence of trichothecene-nonproducing mutants of *Gibberella zeae* in wheat field tests. *Molecular Plant-Microbe Interaction* **9**, 775-81.

Di Menna ME, Lauren DR, Hardacre A. 1997. *Fusaria* and *Fusarium t*oxins in New Zealand maize plants. *Mycopathologia* **139**, 165-73.

Di Menna ME, Lauren DR, Smith WA, 1991. Effect of incubation temperature on zearalenone production by strains of *Fusarium crookwellense*. *Mycopathologia* **116**, 81-6.

Diamond M, Reape TJ, Rocha O, Doyle SM, Kacprzyk J, Doohan FM, McCabe PF, 2013.The *Fusarium* mycotoxin deoxynivalenol can inhibit plant apoptosis-like programmed cell death. *PloS ONE* **8**, 1-8.

Dill-Macky R, Jones RK, 2000. The effect of previous crop residues and tillage on *Fusarium* head blight of wheat. *Plant Disease* **84**, 71-6.

Drummond AJ, Ashton B, Buxton S, Cheung M, Cooper A, Duran C, Field M, Heled J, Kearse M, Markowitz S, Moir R, Stones-Havas S, Sturrock S, Thierer T, Wilson A,2011. GENEIOUS v. 5.4.5. [http://www.geneious.com].

Dusabenyagasani, M., Dostaler, D. and Hamelin, R.C. 1999. Genetic diversity among *Fusarium graminearum* strains from Ontario and Quebec. *Canadian Journal of Plant Pathology* **21**, 308-14.

Dyer RB, Plattner RD, Kendra DF, Brown DW, 2005. *Fusarium graminearum TRI1*4 is required for high virulence and DON production on wheat but not for DON synthesis *in vitro*. *Journal of Agriculture and Food Chemistry* **53**, 9281-7.

Earl DA & vonHoldt BM, 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetics Resources* **4**, 359-61.

Edwards SG, Godley NP, 2010. Reduction of Fusarium head blight and deoxynivalenol in wheat with early fungicide applications of prothioconazole. *Food Additives and Contaminants Part A* **27**, 629-35.

Edwards SG, Pirgozliev SR, Hare MC, Jenkinson P, 2001. Quantification of trichothecene producing *Fusarium* species in harvested grain by competitive PCR to determine efficacies of fungicides against fusarium head blight of winter wheat. *Applied and environmental microbiology* **67**, 1575-80.

Eudes F, Comeau A, Rioux S, Collin J, 2000. Phytotoxicity of eight mycotoxins associated with the fusariosis of wheat spikelets. *Canadian Journal of Plant Pathology* **22**, 286-92.

Faris JD, 2014. Wheat domestication: Key to agricultural revolutions past and future. In: Tuberosa R, Graner A, Frison E, eds. *Genomics of plant genetic resources*. Netherlands: Springer. 439-64.

Fernando WGD, Miller JD, Seaman WL, Seifert K, Paulitz TC, 2000. Daily and seasonal dynamics of airborne spores of *Fusarium graminearum* and other *Fusarium* species sampled over wheat plots. *Canadian Journal of Botany* **78(4)**, 497-505.

Fernando WG, Paulitz TC, Seaman WL, Dutilleul P, Miller JD, 1997. Head Blight Gradients Caused by *Gibberella zeae* from Area Sources of Inoculum in Wheat Field Plots. *Phytopathology* **87**, 414-21.

Fernando WGD, Zhang JK, Dusabenyagasani M, Guo XM, Ahmed H, McCallum B, 2006. Genetic diversity *of Gibberella zea* strains from Manitoba. *Plant Disease* **90**, 1337-42.

Foroud NA, Eudes F, 2009. Trichothecenes in cereal grains. *International Journal of Molecular Sciences* **10**, 147-73.

Gale LR, Bryant JD, Calvo S, *et al*, 2005. Chromosome Complement of the Fungal Plant Pathogen *Fusarium graminearum* Based on Genetic and Physical Mapping and Cytological Observations. *Genetics* **171**, 985-1001.

Gale LR, Harrison SA, Ward TJ, O'Donnell K, Milus EA, Gale SW and Kistler, HC, 2011. Nivalenol type populations of *Fusarium graminearum* and *F. asiaticum* areprevalent on wheat in Southern Louisiana. *Phytopathology* **101**, 124-34.

Gale LR, Ward TJ, Balmas V, Kistler HC. 2007. Population subdivision of *Fusarium graminearum sensu stricto* in the upper Midwestern United States. *Phytopathology* **97**, 1434 -9.

Gale LR, Chen L-F, Hermick CA, Takamura K, Kistler HC, 2002. Population analysis of *Fusarium graminearum* from wheat fields in Eastern China. *Phytopathology* **92**, 1315-22.

Gareis M, Bauer J, Thiem J, Plank G, Grabley S, Gedek, B, 1990. Cleavage of zearalenone-glycoside, a "masked" mycotoxin during digestion in swine. *Journal of Veterinary Medicine* **37**, 236 -40.

Garvey GS, McCormick SP, Rayment I, 2008. Structural and functional characterization of the TRI101 trichothecene 3-O-acetyltransferase from *Fusarium sporotrichioides* and *Fusarium graminearum*: kinetic insights to combating Fusarium head blight. *The Journal of Biological Chemistry* **283**, 1660-9.

Geiser DM, Aoki T, Bacon CW, Baker SE, Bhattacharyya MK, Brandt ME, Brown DW, Burgess LW, Chulze S, Coleman JJ, *et al.* 2013. One fungus, one name: defining the genus *Fusarium* in a scientifically robust way that preserves longstanding use. *Phytopathology* **103**, 400-8.

Geiser DM, Jiménez-Gasco MDM, Kang S *et al*., 2004. FUSARIUM-ID v. 1.0: A DNA sequence database for identifying *Fusarium*. *European Journal of Plant Pathology* **110**, 473-9.

Gilbert J, Brûlé-Babel A, Guerrieri AT *et al*., 2014. Ratio of 3-ADON and 15-ADON strains of *Fusarium graminearum* recovered from wheat kernels in Manitoba from 2008 to 2012. *Canadian Journal of Plant Pathology* **36**, 54-63.

Gilbert J, Haber S, 2013. Overview of some recent research developments in fusarium head blight of wheat. *Canadian Journal of Plant Pathology* **35**, 149-74.

Gilbert J, Woods SM, Kromer U, 2008. Germination of ascospores of *Gibberella zeae* after exposure to various levels of relative humidity and temperature. *Phytopathology* **98**, 504-8.

Gilbert J, Tekauz A, 2000. Review: Recent developments in research on fusarium head blight of wheat in Canada. *Canadian Journal of Plant Pathology* **22**, 1-8.

Glienke C, Pereira OL, Stringari D, Fabris J, Kava-Cordeiro V, GalliTerasawa L, Cunnington J, Shivas RG, Groenewald JZ, Crous PW, 2011. Endophytic and pathogenic *Phyllosticta* species, with reference to those associated with Citrus Black Spot. *Persoonia* **26**,47-56

Goswami RS, Kistler HC, 2005. Pathogenicity and In Planta Mycotoxin Accumulation Among Members of the *Fusarium graminearum* Species Complex on Wheat and Rice. *Phytopathology* **95**, 1397-404.

Goswami RS, Kistler, HC, 2004. Heading for disaster: *Fusarium graminearum* on cereal crops. *Molecular Plant Pathology* **5**, 515-25.

Gratz SW, Duncan G, Richardson AJ, 2013. The Human Fecal Microbiota Metabolizes Deoxynivalenol and Deoxynivalenol-3-Glucoside and May Be Responsible for Urinary Deepoxy-Deoxynivalenol. *Applied and Environmental Microbiology* **79**, 1821-5.

Grove JF. 2007. The trichothecens and their biosynthesis. *Progress in the Chemistry of Organic Natural Products* **88**, 63-130.

Guo XW, Fernando WGD, Seow-Brock HY, 2008. Population Structure, Chemotype Diversity, and Potential Chemotype Shifting of *Fusarium graminearum* in Wheat Fields of Manitoba. *Plant Disease* **92**, 756-62.

Hall TA, 1999. BIOEDIT: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**, 95-8

Hallen-Adams HE, Wenner N, Kuldau GA, Trail F, 2011. Deoxynivalenol biosynthesis-related gene expression during wheat kernel colonization by *Fusarium graminearum*. *Phytopathology* **101**, 1091-6.

Harris LJ, Desjardins a. E, Plattner RD *et al*., 1999. Possible Role of Trichothecene Mycotoxins in Virulence of *Fusarium graminearum* on Maize. *Plant Disease* **83**, 954-60.

Henriksen B, Elen O, 2005. Natural Fusarium grain infection level in wheat, barley and oat after early application of fungicides and herbicides. *Journal of Phytopathology* **220**, 214-20.

Hohn TM, Beremand PD, 1989. Isolation and nucleotide sequence of a sesquiterpene cyclase gene from the trichothecene-producing fungus *Fusarium sporotrichioides*. *Gene* **79**, 131-8.

Hohn TM, Desjardins AE, McCormick SP, 1995. The Tri4 gene of *Fusarium sporotrichioides* encodes a cytochrome P450 monooxygenase involved in trichothecene biosynthesis. *Molecular Plant-Microbe Interactions* **248**, 95-102.

Hohn TM, Vanmiddlesworth F, 1986. Purification and characterization of the sesquiterpene cyclase trichodiene synthetase from *Fusarium sporotrichioides*. *Archives of Biochemistry and Biophysics* **251**, 756-61.

Hollingsworth CR, Motteberg CD, Thompson WG, 2006. Assessing fungicide efficacies for the management of Fusarium head blight on spring wheat and barley. Plant Health Progress, http://www.plantmanagementnetwork.org/pub/php/research/2006/fusarium/.

Huang Z, White DG, Payne GA, 1997. Corn seed proteins inhibitory to *Aspergillus flavus* and aflatoxin biosynthesis. *Phytopathology* **87**, 622-7.

Huelsenbeck JP, 2004. Bayesian Phylogenetic Model Selection Using Reversible Jump Markov chain Monte Carlo. *Molecular Biology and Evolution* **21**, 1123-33.

Huelsenbeck JP, Larget B, Miller RE, Ronquist F, 2002. Potential applications and pitfalls of Bayesian inference of phylogeny. *Systemic Biology* **51**, 673-88.

Ichinoe M, Kurata H, Sugiura Y, Ueno Y, 1983. Chemotaxonomy of *Gibberella zeae* with special reference to production of trichothecenes and zearalenone. *Applied and Environmental Microbiology* **46**, 1346-69.

Inch S, Gilbert J, 2003. The incidence of Fusarium species recovered from inflorescences of wild grasses in southern Manitoba. *Candian Journal of Plant Pathology* **25**,379-83.

Inch S, Gilbert J, 2007. Effect of *Trichoderma harzianum* on perithecial production of *Gibberella zeae* on wheat straw. *Biocontrol Science and Technology* **17**, 635-46.

Inch S, Gilbert J, 2011. Scanning electron microscopy observations of the interaction between Trichoderma harzianum and perithecia *of Gibberella zeae*. *Mycologia* **103**, 1-9.

Jansen C, Von Wettstein D, Schafer W, Kogel KH, Felk A, Maier FJ, 2005. Infection patterns in barley and wheat spikes inoculated with wild-type and trichodiene synthase gene disrupted *Fusarium graminearum*. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 16892-7.

Jennings P, Coates ME, Walsh K, Turner JA, Nicholson P, 2004. Determination of deoxynivalenol- and nivalenol-producing chemotypes of *Fusarium graminearum* isolated from wheat crops in England and Wales. *Plant Pathology* **53**, 643-52.

Jones P, Vogt T, 2001. Glycosyltransferases in secondary plant metabolism: tranquilizers and stimulant controllers. *Planta* **213**, 164-74.

Jonkers W, Dong Y, Broz K, Kistler HC, 2012. The Wor1-like protein Fgp1 regulates pathogenicity, toxin synthesis and reproduction in the phytopathogenic fungus *Fusarium graminearum*. *PLoS Pathogens* **8**, 1-18.

Kang Z, Buchenauer H, 2000. Cytology and ultrastructure of the infection of wheat spikes by *Fusarium culmorum*. *Mycology Research* **104**, 1083-93.

Karugia GW, Suga H, Gale LR, Nakajima T, Ueda A, Hyakumachi M, 2009. Population structure of *Fusarium asiaticum* from two Japanese regions and eastern China. *Journal of General Plant Pathology* **75**, 110-8.

Kelly AC, Clear RM, O'Donnell K *et al*., 2015. Diversity of Fusarium head blight populations and trichothecene toxin types reveals regional differences in pathogen composition and temporal dynamics. *Fungal genetics and biology* **82**, 22-31.

Kerenyi Z, Moretti A, Waalwijk C, Olah B, Hornok L, 2004. Mating type sequences in asexually reproducing *Fusarium* species. *Applied and Environmental Microbiology* **70**, 4419 -4423.

Kim H, Yun S, 2011. Evaluation of Potential Reference Genes for Quantitative RT-PCR Analysis in *Fusarium graminearum* under Different Culture Conditions. *Plant Pathology Journal* **27**, 301-9.

Kimura M, Kaneko I, Komiyama M, Takatsuki A, Koshino H, Yoneyama K et al, 1998. Trichothecene 3-O-acetyltransferase protects both the producing organism and transformed yeast from related mycotoxins -Cloning and characterization of Tri101. *Journal of Biological Chemistry* **273**, 1654-61.

Kimura M, Takahashi-Ando N, Nishiuchi T, Ohsato S, Tokai T, Ochiai N et al, 2006. Molecular biology and biotechnology for reduction of Fusarium mycotoxin contamination. *Pesticide Biochemistry and Physiology* **86**, 117-23.

Kimura M, Tokai T, Takahashi-Ando N, Ohsato S, Fujimura M, 2007. Molecular and Genetic Studies of *Fusarium* Trichothecene Biosynthesis: Pathways, Genes, and Evolution. *Bioscience, Biotechnology and Biochemistry* **71**, 2105-23.

Klix BM, Verreet JA, Beyer M. 2007. Comparison of the declining triazole sensitivity of *Gibberella zeae* and increased sensitivity achieved by advances in triazole fungicide development. *Crop protection* **26(4)**, 683-90.

Kluger, B., Bueschl, C., Lemmens, M., Michlmayr, H., Malachova, A., Koutnik, A., Maloku, I., Berthiller, F., Adam, G., Krska, R., Schuhmacher, R., 2015. Biotransformation of the Mycotoxin Deoxynivalenol in *Fusarium* Resistant and Susceptible Near Isogenic Wheat Lines. *PLoS One* **10(3)**, e0119656. doi:10.1371/journal.pone.0119656.

Kohl J, Fokkema NJ.1998. Stratergies for biocontrol of nectrotrophic fungal foliar pathogens. In: Boland G, Bolis L, eds. *Plant-microbe interactions and biological control*. NY, USA: Marcel Dekker Inc.49-55.

Kosaka A, Manickavelu A, Kajihara D, Nakagawa H, Ban T, 2015. Altered Gene Expression Profiles of Wheat Genotypes against Fusarium Head Blight. *Toxins* **7**, 604-20.

Kostelanska M, Hajslova J, Zachariasova M *et al.*, 2009. Occurrence of deoxynivalenol and its major conjugate, deoxynivalenol-3- glucoside, in beer and some brewing intermediates. *Journal of Agricultural and Food Chemistry* **57**, 3187-94.

Kristensen R, Torp M, Kosiak B, Holst-Jensen A, 2005. Phylogeny and toxigenic potential is correlated in *Fusarium* species as revealed by partial translation elongation factor 1 alpha gene sequences. *Mycological research* **109**, 173-86.

Kulik T, LŁojko M, Jestoi M, Perkowski J, 2012. Sublethal concentrations of azoles induce tri transcript levels and trichothecene production in *Fusarium graminearum*. *FEMS Microbiology Letters* **335**, 58-67.

Lauren DR, Di Minna ME, 1999. *Fusaria* and *Fusarium* mycotoxins in leaves and ears of maize plants. 2. A time course study made in the Waikato region, New Zealand, in 1997. *New Zealand Journal of Crop and Horticultural Science* **27**, 215-23.

Lauren DR, Sayer ST, di Menna ME, 1992. Trichothecene production by *Fusarium* species isolated from grain and pasture throughout New Zealand. *Mycopathologia* **120**, 167-76.

Lee J, Chang I-Y, Kim H, Yun S-H, Leslie JF, Lee Y-W, 2009. Genetic diversity and fitness of *Fusarium graminearum* populations from rice in Korea. *Applied and Enviromental Microbiology* **75**, 3289-95.

Lee T, Han Y, Kim K *et al*., 2002. Tri13 and Tri7 Determine Deoxynivalenol- and Nivalenol-Producing Chemotypes of *Gibberella zeae*. *Applied and Environmental Microbiology* **68**, 2148-54.

Lee T, Lee S-H, Shin JY *et al*., 2014. Comparison of Trichothecene Biosynthetic Gene Expression between *Fusarium graminearum* and *Fusarium asiaticum*. *The plant pathology journal* **30**, 33-42.

Lee T, Oh DW, Kim HS *et al*., 2001. Identification of deoxynivalenol-and nivalenol-producing chemotypes of *Gibberella zeae* by using PCR. *Applied and Environmental Microbiology* **67**, 2966-72.

Leeson S, Diaz GJ, Summers JD, 1995. Poultry Metabolic Disorders and Mycotoxins. University Book. Ontario, Canada. 190-226.

Lemmens M, Scholz U, Berthiller F, Dall'Asta C, Koutnik A, Schuhmacher R, Adam G, Buerstmayr H, Mesterházy A, Krska R, Ruckenbauer P, 2005. The ability to detoxify the mycotoxin deoxynivalenol colocalizes with a major quantitative trait locus for Fusarium head blight resistance in wheat. *Molecular Plant Microbe Interactions* **18**, 1318-24.

Leslie JF, 1995. *Gibberella fujikuroi*: available populations and variable traits. *Canadian Journal of Botany* **73**, 282-91.

Leslie JF, Summerell BA, 2006. The Fusarium laboratory manual. Iowa, USA: Wiley-Blackwell.81-96.

Li FA, Yu CC, Shao B, Wang W, Yu HX, 2011. Natural occurance of masked deoxynivalenol and multi-mycotoxins in cereals from China harvested in 2007 and 2007. *Chinese Journal of Preventive Medicine* **45**,57-63.

Li X, Sanghyun S, Heinen S, Dill-Macky R, Berthiller F, Nersesian N, Clemente T, McCormick S, Muehlbauer G, 2015. Transgenic wheat expressing a barley UDP-glucosyltransferase detoxifies deoxynivalenol and provides high levels of resistance to *Fusarium graminearum*. *Molecular Plant Microbe Interactions* 28, 1237-46.

Liang JM, Xayamongkhon H, Broz K *et al*., 2014. Temporal dynamics and population genetic structure of *Fusarium graminearum* in the upper Midwestern United States. *Fungal Genetics and Biology* **73**, 83-92.

Librado P & Rozas J, 2009. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**, 1451-2.

Liddell CM, 2003. Systematics of *Fusarium* species and allies associated with Fusarium head blight, In: Leonard KJ & Bushnell WR, eds. *Fusarium Head Blight of Wheat and Barley*. St. Paul, USA: The American Phytopathological Society, 35-43.

Lindblad M, Gidlund A, Sulyok M *et al*., 2013. Deoxynivalenol and other selected *Fusarium* toxins in Swedish wheat — Occurrence and correlation to specific *Fusarium* species. *International Journal of Food Microbiology* **167**, 284-91.

Livak KJ, Schmittgen TD, 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ÄÄCT method. *Methods* **25**, 402-8.

Logrieco A, Bottalico A, Mule G, Moretti A, Perrone G, 2003. Epidemiology of toxigenic fungi and their associated mycotoxins for some Mediterranean crops. *European Journal of Plant Pathology* **109**, 645-67.

Logrieco AF, Visconti A, 2014. An Introduction to the MycoRed Project 1. In: Leslie JF, Logrieco A, eds. *Mycotoxin reduction in grain chains*, Oxford, UK: Wiley Blackwell, 1-7.

Luongo L, Galli M, Corazza L, Meekes E, De Haas L, Van Der Plas CL, et al, 2005. Potential of fungal antagonists for biocontrol of *Fusarium* spp. in wheat and maize through competition in crop debris. *Biocontrol Science and Technology* **15**, 229-42.

Lysoe E, Pasquali M, Breakspear A, Kistler HC, 2011. The transcription factor FgStuAp The transcription factor FgStuAp influences spore development, pathogenicity, and secondary metabolism in *Fusarium graminearum*. *Molecular Plant Microbe Interactions* **24**, 54-67.

Ma LJ, Geiser DM, Proctor RH *et al*., 2013. *Fusarium* Pathogenomics. *Annual Review of Microbiology* **67**, 399-416.

Malachova A, Dzuman Z, Veprikova Z, Vaclavikova M, Zachariasova M, Hajslova J, 2011. Deoxynivalenol, deoxynivalenol-3-glucoside, and enniatins: The major mycotoxins found in cereal-based products on the Czech market. *Journal of Agricultural and Food Chemistry* **59**, 12990-7.

Malbrán I, Mourelos CA, Girotti JR, Balatti PA, Lori GA. 2014. Toxigenic capacity and trichothecene production by *Fusarium graminearum* strains from Argentina and their relationship with aggressiveness and fungal expansion in the wheat spike. *Phytopathology* **104**, 357-64.

Malihipour A, Gilbert J, Piercey-Normore M, Cloutier S, 2012. Molecular Phylogenetic Analysis, Trichothecene Chemotype Patterns, and Variation in Aggressiveness of *Fusarium* Strains Causing Head Blight in Wheat. *Plant Disease* **96**, 1016-25.

Mardi M, Pazouki L, Delavar H *et al*., 2006. QTL analysis of resistance to Fusarium head blight in wheat using a 'Frontana' -derived population. *Plant Breeding* **125(4)**, 313-7.

Maresca M, 2013. From the Gut to the Brain: Journey and Pathophysiological Effects of the Food-Associated Trichothecene Mycotoxin Deoxynivalenol. *Toxins* **5**, 784-820.

Markell S, Francl L, 2003. Fusarium head blight inoculum: species prevalence and *Gibberella zeae* spore type. *Plant disease* **87**, 814-20.

Matarese F, Sarrocco S, Gruber S, Seidl-Seiboth V, Vannacci G, 2012. Biocontrol of Fusarium head blight: interactions between Trichoderma and mycotoxigenic *Fusarium*. *Microbiology* **158**, 98-106.

Mayr E, 1940. Speciation phenomena in birds. *The American Naturalist* **74**, 249-78

Mayr E, 1963. Animal species and evolution. Harvard University Press, Cambridge.

McCormick SP, Alexander NJ, 2002. Fusarium Tri8 encodes a trichothecene C-3 esterase. *Applied and environmental microbiology* 68, 2959-64.

McCormick SP, Alexander NJ, Proctor RH, 2006. *Fusarium* Tri4 encodes a multifunctional oxygenase required for trichothecene biosynthesis. *Canadian Journal of Microbiology* **52**, 636-42.

McCormick SP, Alexander NJ, Trapp SE, Hohn TM, 1999. Disruption of *TRI101*, the gene encoding trichothecene 3-O-acetyltransferase, from *Fusarium sporotrichioides*. *Applied and Environmental Microbiology* **65**, 5252-6.

McCormick SP, Harris LJ, Alexander NJ, Ouellet T, Saparno A, Allard S, Desjardins AE, 2004. Tri1 in *Fusarium graminearum* encodes a P450 oxygenase. *Applied and Environmental Microbiology* **70**, 2044-51.

McCormick SP, Hohn TM, Desjardins AE, 1996. Isolation and characterization of Tri3, a gene encoding 15-O-acetyltransferase from *Fusarium sporotrichioides*. *Applied and Environmental Microbiology* **62**, 353-9.

McCormick SP, Stanley AM, Stover NA, Alexander NJ, 2011. Trichothecenes: From simple to complex mycotoxins. *Toxins* **3**, 802-14.

McCormick SP, Taylor, SL, Plattner RD, BeremanD MN, 1990. Bioconversion of possible T-toxin precursors by a mutant strain of *Fusarium sporotrichioides*. *Applied and Environmental Microbiology* **56**, 702-6.

McCormick S, 2003. The role of DON in pathogenicity. In: Leonard KJ, Bushnell WR.eds. *Fusarium Head Blight of Wheat and Barley*. St. Paul, USA: The American Phytopathological Society. 35-43.

McMullen M, Halley S, Schatz B, Meyer S, Jordahl J, Ransom J, 2008. Integrated strategies for Fusarium head blight management in the United States. *Cereal Research Communications* **36**, 563-8.

McMullen M, Jones R, Gallenberg D, 1997. Scab of Wheat and Barley: A Re-emerging Disease of Devastating Impact. *Plant Disease,* **81**, 1340-8.

Merhej J, Richard-Forget F, Barreau C, 2011. Regulation of trichothecene biosynthesis in Fusarium: Recent advances and new insights. *Applied Microbiology and Biotechnology* **91**, 519-28.

Mesterházy A, 1995. Types and components of resistance to Fusarium head blight of wheat. *Plant Breeding* **114**, 377-86.

Mesterhazy, A., 1997. Breeding for resistance to Fusarium head blight of wheat. In: Dubin J, eds. *Fusarium head scab: global status and future prospects*. El Batan, Mexico: CIMMYT.79-85.

Mesterházy Á, 2003. Breeding wheat for fusarium head blight resistance in Europe. In: Leonard KJ, Bushnell WR, eds. *Fusarium HeadBlight of Wheat and Barley*. St. Paul, USA: The American Phytopathological Society. 363-81.

Mesterházy Á, Bartók T, Lamper C, 2003. Influence of wheat cultivar, species of *Fusarium,* and isolate aggressiveness on the efficacy of fungicides for control of Fusarium head blight. *Plant Disease* **87**, 1107-15.

Mesterházy Á, Bartók T, Mirocha CG, Komoróczky R, 1999. Nature of resistance to Fusarium head blight and the role of deoxynivalenol for breeding. *Plant Breeding* **118**, 97-110.

Mesterházy Á, 2002. Role of deoxynivalenol in aggressiveness of *Fusarium graminearum* and *F. culmorum* and in resistance to Fusarium head blight. *European Journal of Plant Pathology* **108**, 675-84.

Miedaner T, Cumagun CJR, Chakraborty S, 2008. Population genetics of three important head blight pathogens *Fusarium graminearum*, *F. pseudograminearum* and *F. culmorum*. *Journal of Phytopathology* **156**, 129-39.

Miedaner T, Schilling AG, 1996. Genetic variation of aggressiveness in individual field populations of *Fusarium graminearum* and *Fusarium culmorum* tested on young plants of winter rye. *European Journal of Plant Pathology* **102**, 823-30.

Miedaner T, Reinbrecht C, 2001. Trichothecene content of rye and wheat genotypes inoculated with a deoxynivalenol- and a nivalenol-producing isolate of *Fusarium culmorum*. *Journal of Phytopathology* **149**, 245-51.

Miller JD, 1994. Epidemiology of *F. graminearum* diseases of wheat and corn. In: Miller JD, Trenholm HL, eds. *Mycotoxins in grain: compounds other than aflatoxin*. St. Paul. MN: Eagan press, 19-36.

Miller JD, Greenhalgh R, Wang Y, Lu M, 1991. Trichothecene chemotypes of three *Fusarium* species. *Mycologia* **83(2),** 121-30.

Miller JD, Young JC, Sampson DR, 1985. Deoxynivalenol and Fusarium head blight resistance in spring cereals. *Phytopathology* **113**, 359-67.

Mishra PK, Tewari JP, Turkington T. K, Clear RM, 2009. Genetic evidence for a recent geographic expansion of 15-acetyldeoxynivalenol chemotypes of *Fusarium graminearum* in Canada. *Canadian Journal of Plant Pathology* **31**, 468-74.

Monds RD, Cromey MG, Lauren DR, di Menna M, Marshall J, 2005. *Fusarium graminearum*, *F. cortaderiae* and *F. pseudograminearum* in New Zealand: molecular phylogenetic analysis, mycotoxin chemotypes and co-existence of species. *Mycological research* **109**, 410-20.

Muhovski Y, Batoko H, Jacquemin JM, 2012. Identification, characterization and mapping of differentially expressed genes in a winter wheat cultivar (Centenaire) resistant to *Fusarium graminearum* infection. *Molecular Biology Reports* **39**, 9583-600.

Nagl V, Schwartz H, Krska R, Moll W-D, Knasmuller S, Ritzmann M, Adam G, Berthiller F, 2012. Metabolism of the masked mycotoxin deoxynivalenol-3-glucoside in rats. *Toxicology Letters* **213**, 367-73.

Nelson PE, 1991. History of *Fusarium* systematics. *Phytopathology* **81**, 1045-8.

Nelson PE, Toussoun TA, Marasas WFO, 1983. *Fusarium* species: an illustrated manual for identification. Pennsylvania State University Press, University Park.

Nicholson P, Chandler E, Draeger RC *et al*., 2003. Molecular tools to study epidemiology and toxicology of fusarium head blight of cereals. *European Journal of Plant Pathology* **109**, 691-703.

Nielsen LK, Jensen JD, Nielsen GC, Jensen JE, Spliid NH, Thomsen IK, Justesen AF, Collinge DB, Jørgensen LN. 2011. Fusarium head blight of cereals in Denmark: species complex and related mycotoxins. *Phytopathology* **101**, 960-9.

Nielsen LK, Jensen JD, Rodríguez A, Jørgensen LN, Justesen AF, 2012. *TRI12* based quantitative real-time PCR assays reveal the distribution of trichothecene genotypes of *F. graminearum* and *F. culmorum* strains in Danish small grain cereals. *International Journal of Food Microbiology* **157**, 384-92.

Nishiuchi T, Masuda D, Nakashita H *et al*., 2006. Fusarium phytotoxin trichothecenes have an elicitor-like activity in Arabidopsis thaliana, but the activity differed significantly among their molecular species. *Molecular plant-microbe interactions* **19**, 512-20.

O'Donnell K, Kistler HC, Tacke BK, Casper HH, 2000. Gene genealogies reveal global phylogeographic structure and reproductive isolation among lineages of *Fusarium graminearum*, the fungus causing wheat scab. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 7905-10.

O'Donnell K, Rooney AP, Proctor RH, Brown DW, McCormick SP, Ward TJ, Frandsen RJN, Lysøe E, Rehner SA, Aoki T, Robert, VARG, Crous PW, Groenewald JZ, Kang S, Geiser DM, 2013. Phylogenetic analyses of RPB1 and RPB2 support a middle Cretaceous origin

for a clade comprising all agriculturally and medically important fusaria. *Fungal Genetics and Biology* **52**, 20-31

O'Donnell K, Ward TJ, Geiser DM, Kistler HC, Aoki T, 2004. Genealogical concordance between the mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade. *Fungal Genetics and Biology* **41**, 600-23.

Obanor F, Neate S, Simpfendorfer S, Sabburg R, Wilson P, Chakraborty S. 2013. *Fusarium graminearum* and *Fusarium pseudograminearum* caused the 2010 head blight epidemics in Australia. *Plant Pathology* **62**, 79-91.

Ohsato S, Ochiai-Fukuda T, Nishiuchi T, Takahashi N, Koizumi S, Hamamoto H, *et al.* 2007. Transgenic rice plants expressing trichothecene 3-O-acetyltransferase show resistance to the *Fusarium* phytotoxin deoxynivalenol. *Plant Cell Reports* **26**, 531-8.

Okubara PA, Blechl AE, McCormick SP, Alexander NJ, Dill-Macky R, Hohn TM, 2002. Engineering deoxynivalenol metabolism in wheat through the expression of a fungal trichothecene acetyltransferase gene. *Theoretical and Applied Genetics* **106**, 74-83.

Ollis DL, Cheah E, Cygler M, Dijkstra BW, Frolow F, Franken SM, Harel M, Remington SJ, Silman I et al., 1992. The a/b hydrolase fold. *Protein Engineering, Design and Selection* **5**, 197-211.

Osborne LE & Stein JM, 2007. Epidemiology of *Fusarium* head blight on small-grain cereals. *International Journal of Food Microbiology* **119,** 103-8.

Ovando-Martínez, M., Ozsisli, B., Anderson, J., Whitney, K., Ohm, J.B., Simsek, S., 2013. Analysis of deoxynivalenol and deoxynivalenol-3-glucoside in hard red spring wheat inoculated with *Fusarium graminearum*. *Toxins* **5**, 2522-32.

Palazzini JM, Ramirez ML, Torres AM, Chulze SN, 2007. Potential biocontrol agents for Fusarium head blight and deoxynivalenol production in wheat. *Crop Protection* **26**, 1702-10.

Park B, Park J, Cheong K-C, Choi J, Jung K, Kim D, Lee Y-H, Ward TJ, O'Donnell K, Geiser DM, Kang S, 2011. Cyber infrastructure for *Fusarium*: three integrated platforms supporting strain identification, phylogenetics, comparative genomics and knowledge sharing. *Nucleic Acids Research* **39**, 640-6.

Parry D.W, Jenkinson P, McLeod L, 1995. Fusarium ear blight scab. in small grain cereals- a review. *Plant Pathology* **44**, 207-38.

Pasquali M, Beyer M, Bohn T, Hoffmann L, 2011. Comparative Analysis of Genetic Chemotyping Methods for *Fusarium*: Tri13 Polymorphism Does not discriminate between 3- and 15-acetylated Deoxynivalenol Chemotypes in *Fusarium graminearum*. *Journal of Phytopathology* **159**, 700-4.

Pasquali M, Migheli Q, 2014. Genetic approaches to chemotype determination in type B-trichothecene producing Fusaria. *International Journal of Food Microbiology* **189**, 164-82.

Paul PA, Lipps PE, Hershman DE, McMullen MP, Draper M a, Madden L V, 2008. Efficacy of triazole-based fungicides for fusarium head blight and deoxynivalenol control in wheat: a multivariate meta-analysis. *Phytopathology* **98**, 999–1011.

Paulitz TC. 1996. Diurnal release of ascospores by *Gibberella zeae* in inoculated wheat plots. *Plant Disease* **80**, 674-8.

Peakall R & Smouse PE, 2012. GenAlEx6.5: genetic analysis in Excel. Population genetic software for teaching and research – an update. *Bioinformatics* **28**, 2537-9.

Peplow AW, Meek IB, Wiles MC, Phillips TD, Beremand MN, 2003. Tri16 is required for esterification of position C-8 during trichothecene mycotoxin production by *Fusarium sporotrichioides*. *Applied and Environmental Microbiology* **69**, 5935-40.

Perkowski J, Chelkowski J, Wakulinski W, 1990. Deoxynivalenol and 3-acetyl-deoxynivalenol in wheat kernels and chaff with head fusariosis symptoms. *Nahrung Food* **34**, 325-8.

Pestka JJ, 2007. Deoxynivalenol: Toxicity, mechanisms and animal health risks. *Animal Feed Science and Technology* **137**, 283-98.

Pestka JJ, Zhou HR, 2000. Interleukin-6-deficient mice refractory to IgA dysregulation but not anorexia induction by vomitoxin (deoxynivalenol) ingestion. *Food Chemistry and Toxicology* **38**, 565-75.

Pirgozliev SR, Edwards SG, Hare MC, Jenkinson P, 2002. Effect of dose rate of azoxystrobin and metconazole on the development of fusarium head blight and the accumulation of deoxynivalenol (DON) in wheat grain. *European Journal of Plant Pathology* **108**, 469-78.

Pirgozliev SR, Edwards SG, Hare MC, Jenkinson P, 2003. Strategies for the control of *Fusarium* head blight in cereals. *European Journal of Plant Pathology* **109**, 731-42.

Ponts N, Pinson-Gadais L, Verdal-Bonnin MN, Barreau C, Richard-Forget F, 2006. Accumulation of deoxynivalenol and its 15-acetylated form is significantly modulated by oxidative stress in liquid cultures of *Fusarium graminearum*. *FEMS Microbiology Letters* **258**, 102-7.

Poppenberger B, Berthiller F, Lucyshyn D, Sieber T, Schuhmacher R, Krska R, Kuchler K, Glössl J, Luschnig C, Adam G, 2003. Detoxification of the *Fusarium* Mycotoxin Deoxynivalenol by a UDP-glucosyltransferase from *Arabidopsis thaliana*. *Journal of Biology and Chemistry* **278**, 47905-14.

Posada D, Crandall KA, 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14**, 817-8.

Posada D, 2006. MODELTEST Server: a web-based tool for the statistical selection of models of nucleotide substitution online. Nucleic Acids Research **34**,700-3.

Prelusky DB, Rotter BA, Rotter RG, 1994. Toxicology of mycotoxins. In: Miller JD, Trenholm HL, eds. *Mycotoxins in Grain. Compounds Other Than Aflatoxin*, St. Paul , USA: Eagen Press. 359-403.

Pringle A, Baker DM, Platt JK, Wares JP, Latgé JP, Taylor JW, 2005. Cryptic speciation in the cosmopolitan and clonal human pathogenic fungus *Aspergillus fumigatus*. *Evolution* **59**, 1886-99.

Pritchard J, Stephens M and Donnelly P. 2000. Inference of population structure using multilocus genotype data. *Genetics* **155**, 945-59.

Pritsch C, Muehlbauer GJ, Bushnell WR, Somers DA, Vance CP, 2000. Fungal development and induction of defence response genes during early infection of wheat spikes by *Fusarium graminearum*. *Molecular Plant -Microbe Interactions* **12**, 159-69.

Proctor RH, Brown DW, Plattner RD, Desjardins AE, 2003. Co-expression of 15 contiguous genes delineates a fumonisin biosynthetic gene cluster in *Gibberella moniliformis*. *Fungal Genetics and Biology* **38**, 237-49.

Proctor RH, Hohn TM, McCormick SP, Desjardins AE, 1995**a**. Tri6 encodes an unusual zinc finger protein involved in regulation of trichothecene biosynthesis in *Fusarium sporotrichioides*. *Applied and Environmental Microbiology* **61**, 1923-30.

Proctor RH, Hohn TM, McCormick SP, 1995**b**. Reduced virulence of *Giberella zeae* caused by disruption of a trichothecene toxin biosynthetic gene. *Molecular Plant -Microbe Interactions* **8,** 593-601.

Proctor RH, McCormick SP, Alexander NJ, Desjardins AE, 2009. Evidence that a secondary metabolic biosynthetic gene cluster has grown by gene relocation during evolution of the filamentous fungus *Fusarium*. *Molecular Microbiology* **74**, 1128-42.

Prodi A, Purahong W, Tonti S, Salomoni D, Nipoti P, Covarelli L, Pisi A. 2011. Difference in chemotype composition of *Fusarium graminearum* populations isolated from durum wheat in adjacent areas separated by the Apennines in Northern-Central Italy. *Plant Pathology Journal* **27**, 354-9.

Prodi A, Tonti S, Nipoti P, Pancaldi D, Pisi A. 2009. Identification of deoxynivalenol and nivalenol producing chemotypes of *Fusarium graminearum* strains from durum wheat in a restricted area of northern Italy. *Journal of Plant Pathology* **91**, 727-31.

Przemieniecki SW, Kurowski TP, Korzekwa K, 2014. Chemotypes and geographic distribution of the *Fusarium graminearum* species complex. *Environmental Biotechnology* **10**, 45 -54.

Puri KD, Zhong S, 2010. The 3-ADON population of *Fusarium graminearum* found in North Dakota is more aggressive and produces a higher level of DON than the prevalent 15-ADON population in spring wheat. *Phytopathology* **100**, 1007-14.

Qiu J, Shi J, 2014. Genetic relationships, Carbendazim sensitivity and mycotoxin production of the *Fusarium graminearum* populations from maize, wheat and rice in eastern china. *Toxins* **6**, 2291-309.

Qu B, Li HP, Zhang JB *et al*., 2008**a**. Comparison of genetic diversity and pathogenicity of fusarium head blight pathogens from China and Europe by SSCP and seedling assays on wheat. *Plant Pathology* **57**, 642-51.

Qu B, Li HP, Zhang JB *et al*., 2008**b**. Geographic distribution and genetic diversity of *Fusarium graminearum* and *F. asiaticum* on wheat spikes throughout China. *Plant Pathology* **57**, 15-24.

Rep M, Kistler HC, 2010. The genomic organization of plant pathogenicity in *Fusarium* species. *Current Opinion in Plant Biology* **13**, 420-6.

Reynoso MM, Ramirez ML, Torres AM, Chulze SN. 2011.Trichothecene genotypes and chemotypes in *Fusarium graminearum* strains isolated from wheat in Argentina. *International Journal of Food Microbiology* **145**, 444-8.

Ribichich, KF, Lopez SE, Vegetti AC, 2000. Histopathological spikelet changes produced by *Fusarium graminearum* in susceptible and resistant wheat varieties. *Plant Disease* **84**, 794-802.

Rocha O, Ansari K and Doohan FM. 2005. Effects of trichothecene mycotoxins on eukaryotic cells: a review. *Food Additives and Contaminants* **22(4),** 369-78.

Rychlik M, Humpf H, Marko D *et al*., 2014. Proposal of a comprehensive definition of modified and other forms of mycotoxins including “masked” mycotoxins. *Mycotoxin Research* **30**. 197-205.

Sampietro DA, Aristimuño ME, Jimenez CM, Vattuone MA, Catalán CA, 2012. Trichothecene genotypes and chemotypes in *Fusarium graminearum* complex strains isolated from maize fields of northwest Argentina. *International Journal of Food Microbiology* **153**, 229-33.

Sampietro DA, Marín P, Iglesias J, Presello DA, Vattuone MA, Catalan CAN, Gonzalez-Jaen MT. 2010. A molecular based strategy for rapid diagnosis of toxigenic *Fusarium* species associated to cereal grains from Argentina. Fungal Biology **114**, 74-81.

Sarver BA, Ward TJ, Gale LR *et al*., 2011. Novel Fusarium head blight pathogens from Nepal and Louisiana revealed by multilocus genealogical concordance. *Fungal Genetics and Biology* **48**, 1096-107.

Sato I, Ito M, Ishizaka M, Ikunaga Y, Sato Y, Yoshida S, *et al.* 2012. Thirteen novel deoxynivalenol-degrading bacteria are classified within two genera with distinct degradation mechanisms. *FEMS Microbiology Letters* **327**, 110-7.

Savard, ME, 1991. Deoxynivalenol fatty acid and glucoside conjugates. *Journal of Agricultural and Food Chemistry* **39**, 570-4.

Schaafsma AW, Tamburic-Ilincic L, Hooker DC, 2005. Effect of previous crop, tillage, field size, adjacent crop, and sampling direction on airborne propagules of *Gibberella zeae*/*Fusarium graminearum*, fusarium head blight severity, and deoxynivalenol accumulation in winter wheat. *Canadian Journal of Plant Pathology* **27**, 217-24.

Schaafsma AW, Tamburic-Ilinic L, Miller JD, Hooker DC, 2001. Agronomic considerations for reducing deoxynivalenol in wheat grain. *Canadian Journal of Plant Pathology* **23**, 279-85.

Schmale DG, Wood-Jones AK, Cowger C, Bergstrom GC, Arellano C, 2011, Trichothecene genotypes of *Gibberella zea* from winter wheat fields in the eastern USA. *Plant Pathology* **60**, 909-17.

Schoneberg A, Musa T, Voegele RT, Vogelgsang S, 2015. The potential of antagonistic fungi for control of *Fusarium graminearum* and *Fusarium crookwellense* varies depending on the experimental approach. *Journal of Applied Microbiology* **118**, 1165-78.

Schroeder HW, Christensen JJ. 1963. Factors affecting resistance of wheat to scab caused by *Gibberella zeae*. *Phytopathology* **53**, 83-8.

Schweiger W, Boddu J, Shin S, Poppenberger B, Berthiller F, Lemmens M, Muehlbauer GJ, Adam G, 2010. Validation of a candidate deoxynivalenol-inactivating UDP-glucosyltransferase from barley by heterologous expression in yeast. *Molecular Plant Microbe Interactions* **23**, 977-86.

Scoz LB, Astolfi P, Reartes DS, Schmale DG, Moraes MG, Del Ponte EM, 2009. Trichothecene mycotoxin genotypes of *Fusarium graminearum sensu stricto* and *Fusarium meridionale* in wheat from southern Brazil. *Plant Pathology* **58**, 344-51.

Seed Manitoba, 2015. Variety Selection & Growers Source Guide, Manitoba Co-operator.

Shaner G, 2003. Epidemiology of fusarium head blight of small cereals in North America. In: Leonard KJ, Bushnell WR, eds. *Fusarium Head Blight of Wheat and Barley*. St. Paul, USA: The American Phytopathological Society. 84-119.

Shank RA, Foroud NA, Hazendonk P, Eudes F, Blackwell BA, 2011. Current and future experimental strategies for structural analysis of trichothecene mycotoxins-A prospectus, *Toxins* **3**, 1518-53.

Simpson DR, Weston GE, Turner JA, Jennings P, Nicholsonm P, 2001. Differential control of head blight pathogens of wheat by fungicides and consequences for mycotoxin contamination of grain. *European Journal of Plant Pathology* **107**, 421-31.

Sobrova P, Adam V, Vasatkova A, Beklova M, Zeman L, Kizek R, 2010. Deoxynivalenol and its toxicity. *Interdisciplinary toxicology* **3**, 94-9.

Somma S, Petruzzella AL, Logrieco AF, Meca G, Cacciola OS, Moretti A. 2014. Phylogenetic analyses of *Fusarium graminearum* strains from cereals in Italy, and characterisation of their molecular and chemical chemotypes. *Crop Science* **65**, 52-60.

Spolti P, Barros NC, Gomes LB, dos Santos J, Del Ponte EM, 2012. Phenotypic and pathogenic traits of two species of the *Fusarium graminearum* complex possessing either 15-ADON or NIV genotype. *European Journal of Plant Pathology* **133**, 621-9.

Šrobárová A, Šliková S, Šudyova V, 2008. Diversity of the *Fusarium* species associated with head and seedling blight on wheat in Slovakia. *Biologia* **63**, 332-7.

Stack R, McMullen M, 1995. A visual scale to estimate severity of Fusarium head blight of wheat. http://www.ag.ndsu.edu/pubs/plantsci/smgrains/pp1095.pdf

Starkey DE, Ward TJ, Aoki T, Gale LR, Kistler HC, Geiser DM, Suga H, Toth B, Varga, J, O'Donnell K, 2007. Global molecular surveillance reveals novel Fusarium head blight species and trichothecene toxin diversity. *Fungal Genetics and Biology* **44**, 1191-204.

Steenkamp ET, Wingfield BD, Coutinho TA, Zeller KA, Wingfield MJ *et al*, 2000. PCR-based identification of MAT-1 and MAT-2 in the *Gibberella fujikuroi* species complex. Applied and Environmental Microbiology **66**, 4378-82.

Stępień L, 2014. The use of *Fusarium* secondary metabolite biosynthetic genes in chemotypic and phylogenetic studies. *Critical reviews in microbiology* **40**, 176-85.

Stepień Ł, Popiel D, Koczyk G, Chełkowski J, 2008. Wheat-infecting *Fusarium* species in Poland--their chemotypes and frequencies revealed by PCR assay. *Journal of applied genetics* **49**, 433-41.

Streit E, Schwab C, Sulyok M, Naehrer K, Krska R, Schatzmayr G, 2013. Multi-mycotoxin screening reveals the occurrence of 139 different secondary metabolites in feed and feed ingredients. *Toxins* **5**, 504-23.

Suga H, Gale LR, Kistler HC, 2004. Development of VNTR markers for two *Fusarium graminearum* clade species. *Molecular Ecology Notes* **4**, 468-70.

Suga H, Karugia GW, Ward TJ, Gale LR, Tomimura K, Nakajima T, Miyasaka A, Koizumi S, Kageyama K, Hyakumachi M. 2008. Molecular characterization of the *Fusarium graminearum* species complex in Japan. *Phytopathology* **98**, 159-66.

Sugiura Y, Saito H, Tanaka T, Tanaka T. Ichinoe M, Ueno Y, 1994. *Fusarium crookwellense*, a newly isolated fungus from wheat in Japan: its mycotoxin production and pathogenicity to wheat and barley. *Mycoscience* **35**. 77-82.

Summerell BA, Laurence MH, Liew ECY, Leslie JF, 2010. Biogeography and phylogeography of *Fusarium*: A review. *Fungal Diversity* **44**, 3-13.

Sutton JC, 1982. Epidemiology of wheat head blight and maize ear rot caused by *Fusarium graminearum*. *Canadian Journal of Plant Pathology* **4**, 195-209.

Suzuki T, Iwahashi Y, 2014. Phytotoxicity Evaluation of Type B Trichothecenes Using a *Chlamydomonas reinhardtii* Model System. *Toxins* **6**, 453-63.

Swofford, D.L. 2000. PAUP: phylogenetic analysis using parsimony (and other methods), Version 4.0. Sinauer Associates, Sunderland, Massachusetts.

Sydenham EW, Marasas WFO, Thiel PG, Shephard GS, Nieuwenhuis JJ, 1991.Production of mycotoxins by selected *Fusarium graminearum* and *F. crookwellense* strains. *Food Additives and Contaminants* **8**, 31-41.

Szécsi Á, Bartók T, Varga M, Magyar D, Mesterházy Á, 2005. Determination of trichothecene chemotypes of *Fusarium graminearum* strains isolated in Hungary. *Journal of Phytopathology* **153**, 445-8.

Tag AG, Garifullina GF, Peplow AW *et al*., 2001. A novel regulatory gene, Tri10, controls trichothecene toxin production and gene expression. *Applied and Environmental Microbiology* **67**, 5294-302.

Talas F, Kalih R, Miedaner T, 2012. Strains for Aggressiveness and Deoxynivalenol Production in Wheat Head Blight. *Phytopathology* **102**, 128-34.

Talas F, Parzies HK, Miedaner T. 2011. Diversity in genetic structure and chemotype composition of *Fusarium graminearum sensu stricto* populations causing wheat head blight in individual fields in Germany. *European Journal of Plant Pathology* **131**, 39-48.

Tamura K, Nei M, 1993. Estimation of the number of base nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* **10**, 512-26.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S, 2011. MEGA 5: molecular genetics analysis using maximum likelihood, evolutionary distance and maximum parsimony methods. *Molecular Biology and Evolution* **28**, 2731-39.

Tan DC, Flematti GR, Ghisalberti EL, Sivasithamparam K, Chakraborty S, Obanor F, Jayasena K, Barbetti MJ. 2012. Mycotoxins produced by *Fusarium* spp. associated with Fusarium head blight of wheat in Western Australia. *Mycotoxin Research* **28**, 89-96.

Tan MK, Simpfendorfer S, Backhouse D, and Murray GM, 2004. Occurrence of fusarium head blight (FHB) in southern NSW in 2000: identification of causal fungi and determination of

putative chemotype of *Fusarium graminearum* strains by PCR. *Australian Plant Pathology* **33**, 385-92.

Taylor JW, Jacobson DJ, Kroken S *et al*., 2000. Phylogenetic species recognition and species concepts in fungi. *Fungal genetics and biology:* **31**, 21-32.

Teich AH, Nelson K, 1984. Survey of Fusarium head blight and possible effects of cultural practices in wheat fields in Lambton County in 1983. *Canadian Plant Disease Survey* **6**, 11-13.

Tittlemier SA, Gaba D, Chan JM, 2013. Monitoring of *Fusarium* trichothecenes in Canadian cereal grain shipments from 2010 to 2012. *Journal of Agricultural and Food Chemistry* **61**, 7412-8.

Tokai T, Koshino H, Kawasaki T *et al*., 2005. Screening of putative oxygenase genes in the *Fusarium graminearum* genome sequence database for their role in trichothecene biosynthesis. *FEMS Microbiology Letters* **251**, 193-201.

Tokai T, Koshino H, Takahashi-Ando N, Sato M, Fujimura M, Kimura M, 2007. Fusarium Tri4 encodes a key multifunctional cytochrome P450 monooxygenase for four consecutive oxygenation steps in trichothecene biosynthesis. *Biochemical and Biophysical Research Communications* **353**, 412-7.

Tóth B, Kászonyi G, Bartók T, Varga J, Mesterházy Á, 2008. Common resistance of wheat to members of the *Fusarium graminearum* species complex and *F. culmorum*. *Plant Breeding* **127**, 1-8.

Tóth B, Mesterházy Á, Horváth Z, Bartók T, Varga M, Varga J, 2005. Genetic variability of central European isolates of the *Fusarium graminearum* species complex. *European Journal of Plant Pathology* **113**, 35-45.

Trail F, 2009. For blighted waves of grain: *Fusarium graminearum* in the postgenomics era. *Plant Physiology* **149**,103-10.

Tunali B, Obanor F, Erginbas G, Westecott RA, Nicol J, Hakraborty S, 2012. Fitness of three *Fusarium* pathogens of wheat. *FEMS Microbiology and Ecology* **81**, 596-609.

Ueno Y,1977. Mode of action of trichothecenes. *Pure and Applied chemistry* **3**, 1737-45.

Ueno, Y., Nakajima, M., Sakai, K., Ishii, K., Sato, N., Shimada, N., 1973. Comparative toxicology of trichothecene mycotoxins: Inhibition of protein synthesis in animal cells. *Journal of Biochemistry* **74**, 285-96.

Umpiérrez-Failache M, Garmendia G, Pereyra S, Rodríguez-Haralambides a., Ward TJ, Vero S, 2013. Regional differences in species composition and toxigenic potential among Fusarium head blight strains from Uruguay indicate a risk of nivalenol contamination in new wheat production areas. *International Journal of Food Microbiology* **166**, 135-40.

Van der Lee T, Zhang H, van Diepeningen A, Waalwijk C, 2015. Biogeography of *Fusarium graminearum* species complex and chemotypes: a review. *Food Additives & Contaminants*: Part A, 1-8.

Varga E, Wiesenberger G, Hametner C *et al*., 2015. New tricks of an old enemy: strains of *Fusarium graminearum* produce a type A trichothecene mycotoxin. *Environmental Microbiology* **17**. 2588-600.

Visconti A, Minervini F, Lucivero G, Gambatesa V, 1991. Cytotoxic and immunotoxic effects of *Fusarium* mycotoxins using a rapid colorimetric bioassay. *Mycopathologia* **113**, 181-6.

Vogelgsang S, Widmer F, Jenny E, Enkerli J, 2009. Characterisation of novel *Fusarium graminearum* microsatellite markers in different *Fusarium* species from various countries. *European Journal of Plant Pathology* **123**, 477-82.

von der Ohe C, Gauthier V, Tamburic-Ilincic L *et al*., 2010. A comparison of aggressiveness and deoxynivalenol production between Canadian *Fusarium graminearum* strains with 3-acetyl and 15-acetyldeoxynivalenol chemotypes in field-grown spring wheat. *European Journal of Plant Pathology* **127**, 407-17.

Waalwijk C, de Vries PHM, Köhl J, Xu X, van der Lee TAJ, Kema GHJ. 2008. Development of quantitative detection methods for *Fusarium* in cereals and their application. In: Leslie J, Bandyopadhyay R, Visconti A, eds. *Mycotoxins: detection methods, management, public health and agricultural trade.* Wallingford: CAB International. 195-205.

Waalwijk C, Kastelein P, de Vries I *et al*., 2003. Major changes in *Fusarium* spp. in wheat in the Netherlands. *European Journal of Plant Pathology* **109**, 743-54.

Waalwijk C, Van Der Lee T, De Vries I, Hesselink T, Arts J, Kema GHJ, 2004. Synteny in toxigenic *Fusarium* species: The fumonisin gene cluster and the mating type region as examples. *European Journal of Plant Pathology* **110**, 533-44.

Wakulinski W, 1989. Phytotoxicity of the secondary metabolites of fungi causing wheat head fusariosis (head blight). *Acta Physiologiae Plantarum* **11**, 301-6.

Wang J, Liu J, Chen H, Yao J, 2007. Characterization of *Fusarium graminearum* inhibitory lipopeptide from *Bacillus subtilis* IB. *Applied Microbiology and Biotechnology* **76**, 889-94.

Wang JH, Li H-P, Qu B *et al*., 2008. Development of a Generic PCR Detection of 3-Acetyldeoxynivalenol-, 15-Acetyldeoxynivalenol- and Nivalenol-Chemotypes of *Fusarium graminearum* Clade. *International Journal of Molecular Sciences* **9**, 2495-504.

Wang JH, Ndoye M, Zhang J-B, Li H-P, Liao Y-C, 2011. Population Structure and Genetic Diversity of the *Fusarium graminearum* Species Complex. *Toxins* **3**, 1020-37.

Wang YZ, Miller JD, 1988. Effects of *Fusarium graminearum* metabolites on wheat tissue in relation to Fusarium head blight resistance. *Journal of Phytopathology* **122**, 118-25.

Ward TJ, Bielawski JP, Kistler HC, Sullivan E, O'Donnell K, 2002. Ancestral polymorphism and adaptive evolution in the trichothecene mycotoxin gene cluster of phytopathogenic *Fusarium*. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 9278-83.

Ward TJ, Clear RM, Rooney AP, O'Donnell K, Gaba D, Patrick S, Starkey D, Gilbert J, Geiser D, Nowicki T, 2008. An adaptive evolutionary shift in Fusarium head blight pathogen populations is driving the rapid spread of more toxigenic *Fusarium graminearum* in North America. *Fungal Genetics and Biology* **25**, 473-84.

Warth B, Sulyok M, Berthiller F, Schuhmacher R, Krska R, 2013. New insights into the human metabolism of the Fusarium mycotoxins deoxynivalenol and zearalenone. *Toxicology Letters* **220**, 88-94.

Wegulo SN, Bockus WW, Hernandez Nopsa J, De Wolf ED, Eskridge KM, Peiris KHS, *et al.* 2011. Effects of integrating cultivar resistance and fungicide application on Fusarium head blight and deoxynivalenol in winter wheat. *Plant Disease* **95**, 554-60.

Xue AG, Voldeng HD, Savard ME, Fedak G, Tian X, Hsiang T, 2009. Biological control of fusarium head blight of wheat with *Clonostachys rosea* strain ACM941. *Canadian Journal of Plant Pathology* **31**, 169-79.

Yli-Mattila T, Gagkaeva T, 2010. Molecular chemotyping of *Fusarium graminearum*, *F. culmorum* and *F. cerealis* strains from Finland and Russia. In: Gherbawy Y, Voigt K, eds. *Molecular Identification of Fungi*. Berlin, Germany: Springer Verlag, 159-77.

Yli-Mattila T, Gagkaeva T, Ward TJ, Aoki T, Kistler HC, O'Donnell K. 2009. A novel Asian clade within the *Fusarium graminearum* species complex includes a newly discovered cereal head blight pathogen from the Russian Far East. *Mycologia* **101,** 841-52.

Yoshida M, Nakajima T, Tomimura K, Suzuki F, Arai M, Miyasaka A, 2012. Effect of the timing of fungicide application on Fusarium head blight and mycotoxin contamination in wheat. *Plant Disease* **96**, 845-51.

Yoshizawa T, Takeda H, Ohi T, 1983. Structure of a novel metabolite from deoxynivalenol, a trichothecene mycotoxin, in animals. *Agricultural and Biological Chemistry* **47**, 2133-5.

Yu JB, Bai GH, Zhou WC, Dong YH, Kolb FL, 2008: Quantitative trait loci for Fusarium head blight resistance in a recombinant inbred population of Wangshuibai/Wheaton. *Phytopathology* **98**, 87-94.

Zamir LO, Nikolakakis A, Devor KA, Sauriol F, 1996. Biosynthesis of the trichothecene 3-acetyldeoxynivalenol. Is isotrichodermin a biosynthetic precursor?. *Journal of Biological Chemistry* **271**, 27353-9.

Zeller, K.A., Bowden, R.L. and Leslie, J.F. 2003. Diversity of epidemic populations of *Gibberella zeae* from small quadrats in Kansas and North Dakota. *Phytopathology* **93**, 874-80.

Zhang H, Chen WQ, Xu J, Xu SJ, Feng J, 2011. First report of *Fusarium cerealis* causing fusarium head blight on barley in China. *Plant Disease* **95**, 774.

Zhang H, Luo W, Pan Y, Xu J, Xu SJ, Chen WQ, Feng J, 2014. First report of Fusarium Maize Ear Rot caused by *Fusarium meridionale* in China. *Plant Disease* **98 (8**). 1156.

Zhang H, van der Lee T, Waalwijk C *et al*., 2012. Population analysis of the *Fusarium graminearum* species complex from wheat in China show a shift to more aggressive strains. *PLoS ONE* **7**, 1-13.

Zhang H, Zhang Z, van der Lee T *et al*., 2010. Population genetic analyses of *Fusarium asiaticum* populations from barley suggest a recent shift favoring 3-ADON producers in southern China. *Phytopathology* **100**, 328-36.

Zhang JB, Li HP, Dang FJ *et al*., 2007. Determination of the trichothecene mycotoxin chemotypes and associated geographical distribution and phylogenetic species of the *Fusarium graminearum* clade from China. *Mycological Research* **111**, 967-75.

Zhang Y-J, Yu J-J, Zhang Y-N *et al*., 2009. Effect of carbendazim resistance on trichothecene production and aggressiveness of *Fusarium graminearum*. *Molecular plant-microbe interactions* **22**, 1143-50.

Web pages cited in the literature review:

http://www.fao.org/worldfoodsituation/csdb/en/, Accessed 10 October, 2015

http://www.agr.gc.ca/, Accessed 10 October, 2015

APPENDICES

APPENDIX 1: Strain code, species, geographic origin, host and trichothecene chemotype of *Fusarium graminearum* species complex strains used in Chapter 5.

Strain code	Species	Geographic origin	Host	Trichothecene chemotype
CS7402	*F. graminearum s.s*	Australia	Wheat	15-ADON
CS7300	*F. graminearum s.s*	Australia	Wheat	15-ADON
CS7399	*F. graminearum s.s*	Australia	Wheat	15-ADON
CS7403	*F. graminearum s.s*	Australia	Wheat	15-ADON
CS7293	*F. graminearum s.s*	Australia	Wheat	15-ADON
CS7295	*F. graminearum s.s*	Australia	Wheat	15-ADON
CS7333	*F. graminearum s.s*	Australia	Wheat	15-ADON
CS7340	*F. graminearum s.s*	Australia	Wheat	15-ADON
CS7206	*F. graminearum s.s*	Australia	Wheat	15-ADON
CS7404	*F. graminearum s.s*	Australia	Wheat	15-ADON
CS7227	*F. graminearum s.s*	Australia	Wheat	15-ADON
CS7214	*F. graminearum s.s*	Australia	Wheat	15-ADON
CS7216	*F. graminearum s.s*	Australia	Wheat	15-ADON
CS7345	*F. graminearum s.s*	Australia	Wheat	15-ADON
CS7341	*F. graminearum s.s*	Australia	Wheat	15-ADON
CS7357	*F. graminearum s.s*	Australia	Wheat	15-ADON
CS3187	*F. graminearum s.s*	Australia	Wheat	15-ADON
CS7339	*F. graminearum s.s*	Australia	Wheat	15-ADON
CS7441	*F. graminearum s.s*	Australia	Wheat	15-ADON
CS7220	*F. meridionale*	Australia	Wheat	NIV
BR002	*F. graminearum s.s*	Brazil	Wheat	15-ADON
BR004	*F. graminearum s.s*	Brazil	Wheat	15-ADON
BR005	*F. graminearum s.s*	Brazil	Wheat	15-ADON
BR008	*F. graminearum s.s*	Brazil	Wheat	15-ADON
BR009	*F. graminearum s.s*	Brazil	Wheat	15-ADON
BR011	*F. graminearum s.s*	Brazil	Wheat	15-ADON
BR012	*F. graminearum s.s*	Brazil	Wheat	15-ADON
BR014	*F. graminearum s.s*	Brazil	Wheat	15-ADON
BR016	*F. graminearum s.s*	Brazil	Wheat	15-ADON
BR017	*F. graminearum s.s*	Brazil	Wheat	15-ADON
BR001	*F.austroamericanum*	Brazil	Wheat	NIV
BR003	*F. cortaderiae*	Brazil	Wheat	NIV
BR006	*F. cortaderiae*	Brazil	Wheat	NIV
BR007	*F. meridionale*	Brazil	Wheat	NIV
BR013	*F. meridionale*	Brazil	Wheat	NIV
NB0618	*F. graminearum s.s*	Canada	Wheat	15-ADON
Q0622	*F. graminearum s.s*	Canada	Wheat	15-ADON

DFFG109	*F. graminearum s.s*	Canada	Wheat	15-ADON
DFFG144	*F. graminearum s.s*	Canada	Wheat	15-ADON
DFFG102	*F. graminearum s.s*	Canada	Wheat	15-ADON
S3AN0601	*F. graminearum s.s*	Canada	Wheat	15-ADON
ON0617	*F. graminearum s.s*	Canada	Wheat	15-ADON
DFFG30	*F. graminearum s.s*	Canada	Wheat	15-ADON
ON0605	*F. graminearum s.s*	Canada	Wheat	15-ADON
PEI0634	*F. graminearum s.s*	Canada	Wheat	15-ADON
M50601	*F. graminearum s.s*	Canada	Wheat	3-ADON
M20601	*F. graminearum s.s*	Canada	Wheat	3-ADON
A20601	*F. graminearum s.s*	Canada	Wheat	3-ADON
NB0617	*F. graminearum s.s*	Canada	Wheat	3-ADON
A60601	*F. graminearum s.s*	Canada	Wheat	3-ADON
NS0602	*F. graminearum s.s*	Canada	Wheat	3-ADON
ON0639	*F. graminearum s.s*	Canada	Wheat	3-ADON
PEI0633	*F. graminearum s.s*	Canada	Wheat	3-ADON
MIN11	*F. graminearum s.s*	Canada	Wheat	3-ADON
NB0617	*F. graminearum s.s*	Canada	Wheat	3-ADON
S3BS0601	*F. graminearum s.s*	Canada	Wheat	3-ADON
DFFG120	*F. graminearum s.s*	Canada	Wheat	3-ADON
CHW52707	*F. graminearum s.s*	China	Wheat	15-ADON
CHW52611	*F. graminearum s.s*	China	Wheat	15-ADON
CHM4175	*F. graminearum s.s*	China	Wheat	15-ADON
CHM4055	*F. graminearum s.s*	China	Wheat	15-ADON
CHW52701	*F. asiaticum*	China	Wheat	15-ADON
CHW56708	*F. graminearum s.s*	China	Wheat	15-ADON
CHW56707	*F. graminearum s.s*	China	Wheat	15-ADON
CHM3034	*F. graminearum s.s*	China	Maize	15-ADON
CHM1265	*F. graminearum s.s*	China	Maize	15-ADON
CHW54601	*F. asiaticum*	China	Wheat	3-ADON
CHW54603	*F. asiaticum*	China	Wheat	3-ADON
CHW54611	*F. asiaticum*	China	Wheat	15-ADON
CH54604	*F. asiaticum*	China	Wheat	NIV
CHW54612	*F. asiaticum*	China	Wheat	15-ADON

CHW54602	*F. asiaticum*	China	Wheat	3-ADON
CHW52516	*F. asiaticum*	China	Wheat	NIV
CHWM25011	*F. meridionale*	China	Maize	NIV
CHM15026	*F. meridionale*	China	Maize	NIV
CHW52517	*F. asiaticum*	China	Wheat	NIV
CHM5282	*F. meridionale*	China	Maize	NIV
CHM5292	*F. meridionale*	China	Maize	NIV
CHM25040	*F. meridionale*	China	Maize	NIV
CHM4048	*F. meridionale*	China	Maize	NIV
GFG175	*F. graminearum s.s*	Germany	Wheat	15-ADON
G4D16	*F. graminearum s.s*	Germany	Wheat	15-ADON
GFG170	*F. graminearum s.s*	Germany	Wheat	15-ADON
GFG164	*F. graminearum s.s*	Germany	Wheat	15-ADON
GFG40	*F. graminearum s.s*	Germany	Wheat	15-ADON
GFG9211	*F. graminearum s.s*	Germany	Wheat	15-ADON
GFG2311	*F. graminearum s.s*	Germany	Wheat	15-ADON
GFG164	*F. graminearum s.s*	Germany	Wheat	15-ADON
GFG168	*F. graminearum s.s*	Germany	Wheat	15-ADON
G9D29	*F. graminearum s.s*	Germany	Wheat	3-ADON
GFG4	*F. graminearum s.s*	Germany	Wheat	3-ADON
GFG5	*F. graminearum s.s*	Germany	Wheat	3-ADON
GFG2	*F. graminearum s.s*	Germany	Wheat	3-ADON
G12D30	*F. graminearum s.s*	Germany	Wheat	3-ADON
GFG12	*F. graminearum s.s*	Germany	Wheat	3-ADON
G8D35	*F. graminearum s.s*	Germany	Wheat	3-ADON
G21D14	*F. graminearum s.s*	Germany	Wheat	NIV
G21D6	*F. graminearum s.s*	Germany	Wheat	NIV
GFG6	*F. graminearum s.s*	Germany	Wheat	NIV
GFG1111	*F. graminearum s.s*	Germany	Wheat	NIV
MEX001	*F. boothii*	Mexico	Wheat	15-ADON
MEX002	*F. boothii*	Mexico	Wheat	15-ADON
MEX003	*F. boothii*	Mexico	Wheat	15-ADON
MEX004	*F. boothii*	Mexico	Wheat	15-ADON
MEX005	*F. boothii*	Mexico	Barley	15-ADON

MEX006	*F. boothii*	Mexico	Wheat	15-ADON
MEX007	*F. boothii*	Mexico	Wheat	15-ADON
MEX008	*F. boothii*	Mexico	Wheat	15-ADON
MEX009	*F. boothii*	Mexico	Wheat	15-ADON
MEX011	*F. boothii*	Mexico	Wheat	15-ADON
MEX012	*F. boothii*	Mexico	Wheat	15-ADON
MEX013	*F. boothii*	Mexico	Wheat	15-ADON
MEX014	*F. boothii*	Mexico	Wheat	15-ADON
MEX015	*F. boothii*	Mexico	Wheat	15-ADON
MEX016	*F. boothii*	Mexico	Wheat	15-ADON
MEX017	*F. boothii*	Mexico	Wheat	15-ADON
MEX019	*F. boothii*	Mexico	Wheat	15-ADON
MEX010	*F. meridionale*	Mexico	Wheat	NIV
PO001	*F. graminearum s.s*	Poland	Wheat	15-ADON
PO002	*F. graminearum s.s*	Poland	Wheat	15-ADON
PO004	*F. graminearum s.s*	Poland	Wheat	15-ADON
PO005	*F. graminearum s.s*	Poland	Wheat	15-ADON
PO008	*F. graminearum s.s*	Poland	Wheat	15-ADON
PO003	*F. graminearum s.s*	Poland	Wheat	3-ADON
PO006	*F. graminearum s.s*	Poland	Wheat	3-ADON
PO007	*F. graminearum s.s*	Poland	Wheat	3-ADON
PO009	*F. graminearum s.s*	Poland	Wheat	3-ADON
PO010	*F. graminearum s.s*	Poland	Wheat	3-ADON
PO011	*F. graminearum s.s*	Poland	Wheat	3-ADON
PO012	*F. graminearum s.s*	Poland	Wheat	3-ADON
FC772	*F. graminearum s.s*	UK	Wheat	15-ADON
FC1878	*F. graminearum s.s*	UK	Wheat	15-ADON
FC1876	*F. graminearum s.s*	UK	Wheat	15-ADON
FC1874	*F. graminearum s.s*	UK	Wheat	15-ADON
FC1880	*F. graminearum s.s*	UK	Wheat	15-ADON
FC1835	*F. graminearum s.s*	UK	Wheat	15-ADON
FC778	*F. graminearum s.s*	UK	Wheat	3-ADON
FC1792	*F. graminearum s.s*	UK	Wheat	3-ADON
FC1068	*F. graminearum s.s*	UK	Wheat	3-ADON

FC253	*F. graminearum s.s*	UK	Wheat	3-ADON
FC1067	*F. graminearum s.s*	UK	Wheat	3-ADON
FC989	*F. graminearum s.s*	UK	Wheat	3-ADON
FC974	*F. graminearum s.s*	UK	Wheat	3-ADON
FC1868	*F. graminearum s.s*	UK	Wheat	3-ADON
FC1394	*F. graminearum s.s*	UK	Wheat	3-ADON
FC1095	*F. graminearum s.s*	UK	Wheat	3-ADON
FC1828	*F. graminearum s.s*	UK	Wheat	3-ADON
FC1264	*F. graminearum s.s*	UK	Wheat	3-ADON
FC1259	*F. graminearum s.s*	UK	Wheat	3-ADON
FC1399	*F. graminearum s.s*	UK	Wheat	3-ADON

APPENDIX 2.1: Strain code, species, geographic origin, host and trichothecene chemotype of *Fusarium graminearum* species complex strains used in Chapter 6.

Strain code	Species	Geographic origin	Host	Trichothecene chemotype
CS3187	*F. graminearum s.s*	Australia	Wheat	15-ADON
CS7206	*F. graminearum s.s*	Australia	Wheat	15-ADON
CS7214	*F. graminearum s.s*	Australia	Wheat	15-ADON
CS7216	*F. graminearum s.s*	Australia	Wheat	15-ADON
CS7227	*F. graminearum s.s*	Australia	Wheat	15-ADON
CS7293	*F. graminearum s.s*	Australia	Wheat	15-ADON
CS7295	*F. graminearum s.s*	Australia	Wheat	15-ADON
CS7300	*F. graminearum s.s*	Australia	Wheat	15-ADON
CS7333	*F. graminearum s.s*	Australia	Wheat	15-ADON
CS7339	*F. graminearum s.s*	Australia	Wheat	15-ADON
CS7340	*F. graminearum s.s*	Australia	Wheat	15-ADON
CS7341	*F. graminearum s.s*	Australia	Wheat	15-ADON
CS7345	*F. graminearum s.s*	Australia	Wheat	15-ADON
CS7357	*F. graminearum s.s*	Australia	Wheat	15-ADON
CS7399	*F. graminearum s.s*	Australia	Wheat	15-ADON
CS7402	*F. graminearum s.s*	Australia	Wheat	15-ADON
CS7403	*F. graminearum s.s*	Australia	Wheat	15-ADON
CS7441	*F. graminearum s.s*	Australia	Wheat	15-ADON
BR002	*F. graminearum s.s*	Brazil	Wheat	15-ADON
BR004	*F. graminearum s.s*	Brazil	Wheat	15-ADON
BR005	*F. graminearum s.s*	Brazil	Wheat	15-ADON
BR008	*F. graminearum s.s*	Brazil	Wheat	15-ADON
BR009	*F. graminearum s.s*	Brazil	Wheat	15-ADON
BR011	*F. graminearum s.s*	Brazil	Wheat	15-ADON
BR012	*F. graminearum s.s*	Brazil	Wheat	15-ADON
BR014	*F. graminearum s.s*	Brazil	Wheat	15-ADON
BR016	*F. graminearum s.s*	Brazil	Wheat	15-ADON
BR017	*F. graminearum s.s*	Brazil	Wheat	15-ADON
BR006	*F. cortaderiae*	Brazil	Wheat	NIV
BR007	*F. meridionale*	Brazil	Wheat	NIV
BR013	*F. meridionale*	Brazil	Wheat	NIV
NB0618	*F. graminearum s.s*	Canada	Wheat	15-ADON
Q0622	*F. graminearum s.s*	Canada	Wheat	15-ADON
DFFG109	*F. graminearum s.s*	Canada	Wheat	15-ADON
DFFG144	*F. graminearum s.s*	Canada	Wheat	15-ADON
DFFG102	*F. graminearum s.s*	Canada	Wheat	15-ADON

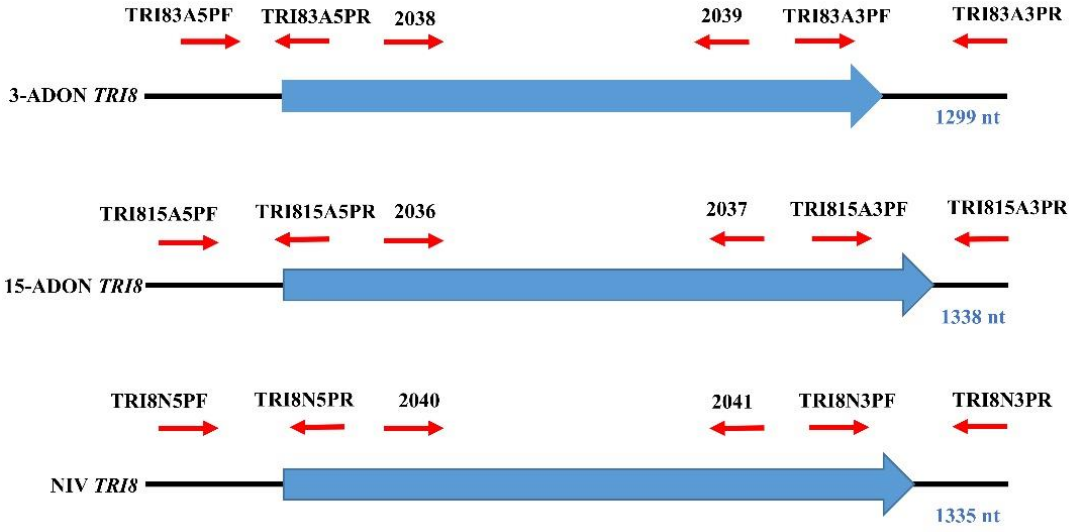
ON0617	*F. graminearum s.s*	Canada	Wheat	15-ADON
ON0605	*F. graminearum s.s*	Canada	Wheat	15-ADON
PEI0634	*F. graminearum s.s*	Canada	Wheat	15-ADON
M50601	*F. graminearum s.s*	Canada	Wheat	3-ADON
M20601	*F. graminearum s.s*	Canada	Wheat	3-ADON
A20601	*F. graminearum s.s*	Canada	Wheat	3-ADON
NB0617	*F. graminearum s.s*	Canada	Wheat	3-ADON
A60601	*F. graminearum s.s*	Canada	Wheat	3-ADON
NS0602	*F. graminearum s.s*	Canada	Wheat	3-ADON
ON0639	*F. graminearum s.s*	Canada	Wheat	3-ADON
PEI0633	*F. graminearum s.s*	Canada	Wheat	3-ADON
MIN11	*F. graminearum s.s*	Canada	Wheat	3-ADON
NB0617	*F. graminearum s.s*	Canada	Wheat	3-ADON
S3BS0601	*F. graminearum s.s*	Canada	Wheat	3-ADON
DFFG120	*F. graminearum s.s*	Canada	Wheat	3-ADON
CHW52707	*F. graminearum s.s*	China	Wheat	15-ADON
CHW52611	*F. graminearum s.s*	China	Wheat	15-ADON
CHM4175	*F. graminearum s.s*	China	Wheat	15-ADON
CHM4055	*F. graminearum s.s*	China	Wheat	15-ADON
CHW52701	*F. asiaticum*	China	Wheat	15-ADON
CHW56708	*F. graminearum s.s*	China	Wheat	15-ADON
CHW56707	*F. graminearum s.s*	China	Wheat	15-ADON
CHW54601	*F. asiaticum*	China	Wheat	3-ADON
CHW54603	*F. asiaticum*	China	Wheat	3-ADON
CHW54609	*F. asiaticum*	China	Wheat	3-ADON
CHW56701	*F. asiaticum*	China	Wheat	3-ADON
CHW54610	*F. asiaticum*	China	Wheat	3-ADON
CHW54602	*F. asiaticum*	China	Wheat	3-ADON
CHW52516	*F. asiaticum*	China	Wheat	NIV
CHWM250	*F. meridionale*	China	Maize	NIV
CHM15026	*F. meridionale*	China	Maize	NIV
CHW52517	*F. asiaticum*	China	Wheat	NIV
CHM5292	*F. meridionale*	China	Maize	NIV
CHM25040	*F. meridionale*	China	Maize	NIV

CHM4048	*F. meridionale*	China	Maize	NIV
G4D16	*F. graminearum s.s*	Germany	Wheat	15-ADON
GFG164	*F. graminearum s.s*	Germany	Wheat	15-ADON
GFG165	*F. graminearum s.s*	Germany	Wheat	15-ADON
GFG166	*F. graminearum s.s*	Germany	Wheat	15-ADON
GFG168	*F. graminearum s.s*	Germany	Wheat	15-ADON
GFG170	*F. graminearum s.s*	Germany	Wheat	15-ADON
GFG175	*F. graminearum s.s*	Germany	Wheat	15-ADON
GFG2311	*F. graminearum s.s*	Germany	Wheat	15-ADON
GFG3211	*F. graminearum s.s*	Germany	Wheat	15-ADON
GFG40	*F. graminearum s.s*	Germany	Wheat	15-ADON
GFG14	*F. graminearum s.s*	Germany	Wheat	15-ADON
GFG9211	*F. graminearum s.s*	Germany	Wheat	15-ADON
GFG181	*F. graminearum s.s*	Germany	Wheat	15-ADON
G12D30	*F. graminearum s.s*	Germany	Wheat	3-ADON
G8D35	*F. graminearum s.s*	Germany	Wheat	3-ADON
G9D29	*F. graminearum s.s*	Germany	Wheat	3-ADON
GFG12	*F. graminearum s.s*	Germany	Wheat	3-ADON
GFG2	*F. graminearum s.s*	Germany	Wheat	3-ADON
GFG4	*F. graminearum s.s*	Germany	Wheat	3-ADON
GFG5	*F. graminearum s.s*	Germany	Wheat	3-ADON
G21D14	*F. graminearum s.s*	Germany	Wheat	NIV
G21D6	*F. graminearum s.s*	Germany	Wheat	NIV
GFG1111	*F. graminearum s.s*	Germany	Wheat	NIV
GFG6	*F. graminearum s.s*	Germany	Wheat	NIV
MEX001	*F. boothii*	Mexico	Wheat	15-ADON
MEX002	*F. boothii*	Mexico	Wheat	15-ADON
MEX003	*F. boothii*	Mexico	Wheat	15-ADON
MEX004	*F. boothii*	Mexico	Wheat	15-ADON
MEX005	*F. boothii*	Mexico	Barley	15-ADON
MEX006	*F. boothii*	Mexico	Wheat	15-ADON
MEX007	*F. boothii*	Mexico	Wheat	15-ADON
MEX008	*F. boothii*	Mexico	Wheat	15-ADON
MEX009	*F. boothii*	Mexico	Wheat	15-ADON

MEX011	*F. boothii*	Mexico	Wheat	15-ADON
MEX012	*F. boothii*	Mexico	Wheat	15-ADON
MEX013	*F. boothii*	Mexico	Wheat	15-ADON
MEX014	*F. boothii*	Mexico	Wheat	15-ADON
MEX015	*F. boothii*	Mexico	Wheat	15-ADON
MEX016	*F. boothii*	Mexico	Wheat	15-ADON
MEX017	*F. boothii*	Mexico	Wheat	15-ADON
MEX010	*F. meridionale*	Mexico	Wheat	NIV
PO001	*F. graminearum s.s*	Poland	Wheat	15-ADON
PO002	*F. graminearum s.s*	Poland	Wheat	15-ADON
PO004	*F. graminearum s.s*	Poland	Wheat	15-ADON
PO005	*F. graminearum s.s*	Poland	Wheat	15-ADON
PO008	*F. graminearum s.s*	Poland	Wheat	15-ADON
PO003	*F. graminearum s.s*	Poland	Wheat	3-ADON
PO006	*F. graminearum s.s*	Poland	Wheat	3-ADON
PO007	*F. graminearum s.s*	Poland	Wheat	3-ADON
PO009	*F. graminearum s.s*	Poland	Wheat	3-ADON
PO011	*F. graminearum s.s*	Poland	Wheat	3-ADON
FC772	*F. graminearum s.s*	UK	Wheat	15-ADON
FC1878	*F. graminearum s.s*	UK	Wheat	15-ADON
FC1876	*F. graminearum s.s*	UK	Wheat	15-ADON
FC1874	*F. graminearum s.s*	UK	Wheat	15-ADON
FC1880	*F. graminearum s.s*	UK	Wheat	15-ADON
FC1835	*F. graminearum s.s*	UK	Wheat	15-ADON
FC778	*F. graminearum s.s*	UK	Wheat	3-ADON
FC1792	*F. graminearum s.s*	UK	Wheat	3-ADON
FC1068	*F. graminearum s.s*	UK	Wheat	3-ADON
FC253	*F. graminearum s.s*	UK	Wheat	3-ADON
FC1067	*F. graminearum s.s*	UK	Wheat	3-ADON
FC989	*F. graminearum s.s*	UK	Wheat	3-ADON
FC974	*F. graminearum s.s*	UK	Wheat	3-ADON
FC1868	*F. graminearum s.s*	UK	Wheat	3-ADON
FC1394	*F. graminearum s.s*	UK	Wheat	3-ADON
FC1095	*F. graminearum s.s*	UK	Wheat	3-ADON

FC1264	*F. graminearum s.s*	UK	Wheat	3-ADON
FC1259	*F. graminearum s.s*	UK	Wheat	3-ADON
FC1399	*F. graminearum s.s*	UK	Wheat	3-ADON
FC1364	*F. graminearum s.s*	UK	Wheat	3-ADON
PH1/NRRL31084	*F. graminearum s.s*	USA	Corn	15-ADON
NRRL29105	*F.boothii*	Nepal	Corn	15-ADON
NRRL28436	*F.meridionale*	New Caledonia	Orange twig	NIV
NRRL26754	*F. acacia-mearnsii*	South Africa	*A.mearnsii*	NIV
NRRL29306	*F. cortaderiae*	New Zealand	Orchard grass	NIV
NRRL28585	*F.austroamericanum*	Venuzuela	Herbaceous vine	NIV

APPENDIX 2.2: Primer map showing the location and direction of primer sequences used to amplify the full length of *TRI8* gene in 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON) and nivalenol (NIV) producing *Fusarium* strains (Chapter 6).



Note: nt indicates nucleotide number.
A graphical representation only. Not drawn to the propotion.

APPENDIX 3.1: Analysis of variance (ANOVA) for expression data of *FPP*, *TRI4*, *TRI5*, *TRI6*, *TRI8*, *TRI101*, *TRI3*, *TRI9* and *TRI12* genes using the PROC Mixed procedure of SAS 9.3 software (Chapter 7).

Gene	Source	DF	SS	MS	F value	Pr >F
FPP	dpi	5	2423.05	484.61	916.05	<.0001
	Cultivar	1	1694.89	1694.89	3203.82	<.0001
	Strain	5	183.91	36.78	69.53	<.0001
	Rep	2	0.53	0.26	0.51	0.6043
	dpi*Strain	25	116.23	4.64	8.79	<.0001
	Cultivar*Strain	5	42.374	8.47	16.02	<.0001
	Cultivar*dpi	5	1241.59	248.31	469.39	<.0001
	Cultivar*Strain*dpi	25	70.36	2.81	5.32	<.0001
	Error	142	75.121	0.52		
TRI4	dpi	5	16104	3220.88	1055.24	<.0001
	Cultivar	1	13263	13263	4345.24	<.0001
	Strain	5	10125	2024.92	663.41	<.0001
	Rep	2	11.42	5.71	1.87	0.1576
	dpi*Strain	25	2337.22	93.48	30.63	<.0001
	Cultivar*Strain	5	3146.12	629.22	206.15	<.0001
	Cultivar*dpi	5	6077.96	1215.59	398.26	<.0001
	Cultivar*Strain*dpi	25	2339.72	93.58	30.66	<.0001
	Error	142	433.42	3.05		
TRI5	dpi	5	1571.76	314.35	485.80	<.0001
	Cultivar	1	331.45	331.45	512.23	<.0001
	Strain	5	197.13	39.42	60.93	<.0001
	Rep	2	0.27	0.13	0.21	0.8093
	dpi*Strain	25	239.48	9.57	14.80	<.0001
	Cultivar*Strain	5	111.76	22.35	34.54	<.0001
	Cultivar*dpi	5	618.53	123.70	191.18	<.0001
	Cultivar*Strain*dpi	25	208.43	8.33	12.88	<.0001
	Error	142	91.88	0.64		
TRI6	dpi	5	442.70	88.541	370.10	<.0001
	Cultivar	1	158.15	158.15	661.09	<.0001
	Strain	5	79.57	15.91	66.52	<.0001
	Rep	2	0.40	0.20	0.84	0.4351
	dpi*Strain	25	73.33	2.93	12.26	<.0001
	Cultivar*Strain	5	21.98	4.39	18.38	<.0001
	Cultivar*dpi	5	175.61	35.12	146.81	<.0001
	Cultivar*Strain*dpi	25	29.67	1.18	4.96	<.0001
	Error	142	33.97	0.23		
TRI8	dpi	5	1251.30	250.26	388.39	<.0001
	Cultivar	1	1030.00	1030.00	1598.50	<.0001
	Strain	5	103.65	20.73	32.17	<.0001
	Rep	2	1.840	0.92	1.43	0.2432
	dpi*Strain	25	105.48	4.21	6.55	<.0001
	Cultivar*Strain	5	24.37	4.87	7.56	<.0001
	Cultivar*dpi	5	364.93	72.98	113.27	<.0001
	Cultivar*Strain*dpi	25	107.62	4.30	6.68	<.0001
	Error	142	91.49	0.64		

TRI101	dpi	5	276.08	55.21	118.95	<.0001
	Cultivar	1	9.40	9.40	20.27	<.0001
	Strain	5	723.40	144.68	311.69	<.0001
	Rep	2	0.23	0.11	0.25	0.7792
	dpi*Strain	25	189.24	7.56	16.31	<.0001
	Cultivar*Strain	5	107.181	21.43	46.18	<.0001
	Cultivar*dpi	5	37.92	7.58	16.34	<.0001
	Cultivar*Strain*dpi	25	82.55	3.30	7.10	<.0001
	Error	142	65.91	0.46		
TRI3	dpi	5	1874.39	374.87	501.83	<.0001
	Cultivar	1	776.79	776.79	1039.86	<.0001
	Strain	5	420.18	84.03	112.49	<.0001
	Rep	2	0.02	0.01	0.02	0.9829
	dpi*Strain	25	197.95	7.91	10.60	<.0001
	Cultivar*Strain	5	136.42	27.28	36.52	<.0001
	Cultivar*dpi	5	510.33	102.06	136.63	<.0001
	Cultivar*Strain*dpi	25	116.46	4.65	6.24	<.0001
	Error	142	106.077	0.74		
TRI9	dpi	5	453.49	90.69	400.70	<.0001
	Cultivar	1	93.18	93.18	411.67	<.0001
	Strain	5	25.86	5.17	22.86	<.0001
	Rep	2	0.33	0.16	0.74	0.4776
	dpi*Strain	25	41.50	1.66	7.33	<.0001
	Cultivar*Strain	5	4.23	0.84	3.75	0.0032
	Cultivar*dpi	5	42.78	8.55	37.80	<.0001
	Cultivar*Strain*dpi	25	11.27	0.45	1.99	0.0064
	Error	142	32.14	0.22		
TRI12	dpi	5	29699	5939.82	1842.79	<.0001
	Cultivar	1	6498.95	6498.95	2016.26	<.0001
	Strain	5	3366.73	673.34	208.90	<.0001
	Rep	2	6.75	3.37	1.05	0.3533
	dpi*Strain	25	2715.62	108.62	33.70	<.0001
	Cultivar*Strain	5	921.40	184.28	57.17	<.0001
	Cultivar*dpi	5	3281.55	656.31	203.62	<.0001
	Cultivar*Strain*dpi	25	1624.64	64.98	20.16	<.0001
	Error	142	457.70	3.22		

APPENDIX 3.2: Bonefferoni mean comparisons of the relative fold change of *FPP*, *TRI4*, *TRI5*, *TRI6*, *TRI8*, *TRI101*, *TRI3*, *TRI9* and *TRI12* genes for different strains analysed in chapter 7 ($p \leq 0.05$).

Gene	Cultivar	Strain	Mean Fold Change	Std. Error	Letter Group
FPP	CARBERRY	3ADON1	9.42	0.20	A
	CARBERRY	3ADON2	9.28	0.20	A
	CARBERRY	15ADON1	7.26	0.20	B
	CARBERRY	15ADON2	6.72	0.20	BC
	CARBERRY	NIV1	6.53	0.20	BC
	CARBERRY	NIV2	5.88	0.20	C
	ROBLIN	3ADON1	2.76	0.14	A
	ROBLIN	3ADON2	2.37	0.14	AB
	ROBLIN	15ADON1	1.92	0.14	BC
	ROBLIN	NIV1	1.51	0.14	C
	ROBLIN	NIV2	1.46	0.14	C
	ROBLIN	15ADON2	1.45	0.14	C
TRI4	CARBERRY	3ADON1	37.47	0.51	A
	CARBERRY	3ADON2	32.78	0.51	B
	CARBERRY	15ADON2	21.15	0.51	C
	CARBERRY	15ADON1	18.41	0.51	D
	CARBERRY	NIV1	14.43	0.51	E
	CARBERRY	NIV2	6.60	0.51	F
	ROBLIN	3ADON1	12.80	0.27	A
	ROBLIN	3ADON2	8.42	0.27	B
	ROBLIN	15ADON2	5.26	0.27	C
	ROBLIN	NIV1	4.22	0.27	CD
	ROBLIN	NIV2	3.70	0.27	D
	ROBLIN	15ADON1	2.43	0.27	E
TRI5	CARBERRY	3ADON2	7.08	0.25	A
	CARBERRY	3ADON1	5.69	0.25	B
	CARBERRY	15ADON1	3.48	0.25	C
	CARBERRY	NIV2	3.09	0.25	C
	CARBERRY	15ADON2	2.97	0.25	C
	CARBERRY	NIV1	2.48	0.25	C
	ROBLIN	3ADON1	2.10	0.11	A
	ROBLIN	3ADON2	1.96	0.11	AB
	ROBLIN	NIV1	1.55	0.11	BC
	ROBLIN	15ADON2	1.47	0.11	C
	ROBLIN	NIV2	1.44	0.11	C
	ROBLIN	15ADON1	1.40	0.11	C
TRI6	CARBERRY	3ADON1	3.88	0.15	A
	CARBERRY	3ADON2	3.30	0.15	A
	CARBERRY	15ADON1	2.29	0.15	B
	CARBERRY	15ADON2	2.05	0.15	BC
	CARBERRY	NIV1	1.58	0.15	CD
	CARBERRY	NIV2	1.29	0.15	D
	ROBLIN	3ADON1	1.15	0.06	A
	ROBLIN	3ADON2	1.10	0.06	A
	ROBLIN	NIV1	0.57	0.06	B
	ROBLIN	15ADON1	0.45	0.06	B
	ROBLIN	15ADON2	0.44	0.06	B
	ROBLIN	NIV2	0.39	0.06	B

TRI8	CARBERRY	3ADON2	7.17	0.25	A
	CARBERRY	3ADON1	6.94	0.25	AB
	CARBERRY	15ADON1	6.05	0.25	BC
	CARBERRY	15ADON2	5.85	0.25	CD
	CARBERRY	NIV2	4.91	0.25	DE
	CARBERRY	NIV1	4.38	0.25	E
	ROBLIN	3ADON2	2.24	0.09	A
	ROBLIN	3ADON1	1.97	0.09	A
	ROBLIN	15ADON1	1.33	0.09	B
	ROBLIN	NIV1	1.22	0.09	B
	ROBLIN	15ADON2	1.18	0.09	B
	ROBLIN	NIV2	1.15	0.09	B
TRI101	CARBERRY	3ADON1	7.95	0.14	A
	CARBERRY	3ADON2	6.06	0.14	B
	CARBERRY	15ADON2	3.41	0.14	C
	CARBERRY	15ADON1	2.25	0.14	D
	CARBERRY	NIV1	1.42	0.14	E
	CARBERRY	NIV2	1.21	0.14	E
	ROBLIN	3ADON1	5.29	0.17	A
	ROBLIN	3ADON2	4.34	0.17	B
	ROBLIN	15ADON2	3.17	0.17	C
	ROBLIN	NIV1	3.08	0.17	CD
	ROBLIN	15ADON1	2.36	0.17	D
	ROBLIN	NIV2	1.56	0.17	E
TRI3	CARBERRY	3ADON2	8.68	0.27	A
	CARBERRY	3ADON1	7.87	0.27	A
	CARBERRY	15ADON2	5.80	0.27	B
	CARBERRY	NIV1	4.09	0.27	C
	CARBERRY	15ADON1	3.68	0.27	CD
	CARBERRY	NIV2	2.77	0.27	D
	ROBLIN	3ADON1	2.50	0.09	A
	ROBLIN	3ADON2	2.45	0.09	A
	ROBLIN	15ADON2	1.65	0.09	B
	ROBLIN	15ADON1	1.49	0.09	B
	ROBLIN	NIV1	1.35	0.09	B
	ROBLIN	NIV2	0.69	0.09	C
TRI9	CARBERRY	3ADON2	2.95	0.14	A
	CARBERRY	15ADON2	2.85	0.14	AB
	CARBERRY	3ADON1	2.55	0.14	AB
	CARBERRY	15ADON1	2.30	0.14	BC
	CARBERRY	NIV2	1.83	0.14	CD
	CARBERRY	NIV1	1.68	0.14	D
	ROBLIN	3ADON2	1.43	0.07	A
	ROBLIN	15ADON2	1.17	0.07	AB
	ROBLIN	3ADON1	1.09	0.07	BC
	ROBLIN	15ADON1	1.00	0.07	BCD
	ROBLIN	NIV1	0.83	0.07	CD
	ROBLIN	NIV2	0.75	0.07	D
TRI12	CARBERRY	3ADON1	31.73	0.45	A
	CARBERRY	3ADON2	25.22	0.45	B
	CARBERRY	15ADON2	22.08	0.45	C
	CARBERRY	NIV1	17.92	0.45	D
	CARBERRY	NIV2	16.16	0.45	DE
	CARBERRY	15ADON1	15.24	0.45	E
	ROBLIN	3ADON1	12.98	0.39	A
	ROBLIN	3ADON2	12.93	0.39	A
	ROBLIN	15ADON2	12.41	0.39	A
	ROBLIN	15ADON1	9.65	0.39	B
	ROBLIN	NIV2	8.20	0.39	B
	ROBLIN	NIV1	6.35	0.39	C

APPENDIX 4: Mean square values of cultivar, chemotype and their interaction for fusarium head blight disease index, fusarium damaged kernel percentage, deoxynivalenol content, deoxynivalenol-3-glucoside content and ratio between deoxynivalenol-3-glucoside to deoxynivalenol content in Carman and Winnipeg locations (Chapter 8).

Location	Source	df	Mean square value and significance (Type III)				
			FHB Index (%)[1]	FDK %[2]	DON (ppm)[3]	D3G (ppm)[4]	D3G/DON Ratio (%)
Carman	Chemotype **(3ADON vs. 15ADON)**	1	4420.42 *	99.72 *	80.75 *	1.86 ns	55.76 ns
	Cultivar	9	3295.60 *	95.26 *	262.63*	8.02 *	294.53 *
	Cultivar*Chemotype	9	114.87 ns	7.04 ns	9.49 ns	1.12 ns	68.81 ns
	Rep	2	90.42 ns	9.15 ns	56.56 ns	1.00 ns	253.78 ns
	Rep*Chemotype	2	80.42 ns	0.36 ns	6.98 ns	0.09 ns	190.56 ns
	Error	36	69.67	4.79	15.08	0.86	71.54
Winnipeg	Chemotype **(3ADON vs. 15ADON)**	1	1706.67 *	81.92 ns	65.00 ns	8.05 ns	87.74 ns
	Cultivar	9	3752.04 *	38.36 *	73.15 *	8.81 *	920.38 *
	Cultivar*Chemotype	9	30.74 ns	3.49 ns	3.13 ns	1.61 ns	246.10 ns
	Rep	2	78.75 ns	6.18 ns	32.13 ns	6.67 ns	58.76 ns
	Rep*Chemotype	2	15.42 ns	10.54 *	6.57 ns	1.60 ns	25.19 ns
	Error	36	47.08	2.58	5.45	1.27	217.78

[1]FHB Index- Fusarium head blight disease index.
[2]% FDK- Fusarium damaged kernel percentage.
[3]DON (ppm)- Deoxynivalenol content in parts per million.
[4]D3G (ppm)- Deoxynivalenol-3-glucosides content in parts per million.

APPENDIX 5: List of Abbreviations

15-ADON	15-Acetyldeoxynivalenol
3-ADON	3-Acetyldeoxynivalenol
4-ANIV	4-Acetylnivalenol
ANOVA	Analysis of Variance
ARS	Agricultural Research Service
D3G	Deoxynivalenol-3-Glucosides
DAS	Diacetoxyscirpenol
DNA	Deoxyribonucleic Acid
DON	Deoxynivalenol
DPI	Days Post-Inoculation
DS	Disease Severity
FDK	Fusarium Damaged Kernels
FGSC	*Fusarium graminearum* Species Complex
FHB	Fusarium Head Blight
FUS-X	Fusareon-X
GC-MS	Gas Chromatography Mass Spectrometry
GCPSR	Genealogical Concordance Phylogenetic Species Recognition
MCMC	Markov Chain Monte Carlo
MLGT	Multilocus Genotyping
MR	Moderately Resistant
NIV	Nivalenol
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PP	Posterior Probability
qPCR	Quantitative Real Time Polymerase Chain Reaction
QTL	Quantitative Trait Locus
RFLP	Restriction Fragment Length Polymorphisms
RNA	Ribonucleic Acid
S	Susceptible
SNA	Synthetic Nutrient Agar
SSR	Simple Sequence Repeats
VNTR	Variable Number Tandem Repeats
WA	Water Agar