COMPARATIVE ANALYSIS OF FUSARIUM GRAMINEARUM STRAIN GENOTYPE
AND CHEMOTYPE FROM GRAINS SAMPLED DURING THE 2015-2017 ONTARIO
HARVESTS

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Abstract

Fusarium graminearum is a fungal pathogen of small grains and corn resulting in yield loss and more importantly the crops are contaminated with the regulated mycotoxin deoxynivalenol (DON). In recent years, genetic changes in the *F. graminearum* population have been occurring and potential concerns include the risk of more DON in grain with less visible disease, new toxins and an increase in pathogen fitness and virulence. The aim of this study was to isolate *F. graminearum* from wheat and corn collected during the 2015-2017 Ontario harvests, and compare genotype and chemotype analysis. From the 2015-2017, 228 corn fields and 118 wheat fields in Ontario were screened for the prevalence of *F. graminearum*. The genotypes of two known *F. graminearum* populations were assessed using multiplex PCR targeted to *TRI3* and *TRI12* gene sequences. *F. graminearum* isolates from 2015-2017 harvest wheat (n = 308) and corn (n = 160) were identified. Representative strains (n = 61) from each year were further examined using PCR-based assays to determine genotype and chemical analysis to determine chemotype. All wheat and corn isolates were the 15ADON genotype. However, chemical analysis of culture extracts showed co-production of 15ADON and the new trichothecene 3ANX in one or more culture media for 80% (n = 16), 59% (n = 13) and 73.7% (n = 14) for the years 2015, 2016, and 2017 respectively. None of the representative strains were of the 3ANX genotype. These findings demonstrate the existing probes to assess genotype cannot reliably predict chemotype of the population of *F. graminearum* in Ontario.

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List of Abbreviations

2 α -HDT = 2 α -hydroxytrichodiene

3ADON = 3-acetyldeoxynivalenol

3ANX = 3 α -acetoxy, 7 α , 15-dihydroxy-12,13-epoxytrichot-9-ene

15ADON = 15-acetyldeoxynivalenol

15ADON-3G = 15-acetyldeoxynivalenol-3-O- β -D-glucoside

15ANX = 15 α -acetoxy, 7 α , 15-dihydroxy-12,13-epoxytrichot-9-ene

BLAST = basic local alignment search tool

bp = base pair

CoA = coenzyme-A

DAOM = Canadian National Mycological Herbarium

DAOMC = Canadian Collection of Fungal Cultures

DON = deoxynivalenol

ddH₂O = distilled deionized water

DMAP = dimethyl phosphate

DMAPP = dimethylallyl diphosphate

DNA = deoxyribonucleic acid

ECCC = Environmental and Climate Change Canada

EF1- α = translation elongation factor 1- α

EtOAc = ethyl acetate

FPP = farnesyl pyrophosphate

FHB = Fusarium head blight

GYEP = glucose yeast extract peptone

HPLC = high performance liquid chromatography

IPP = isopentenyl diphosphate

ITS = internal transcribed spacer

JECFA = Joint Food and Agriculture Organization / World Health Organization, Expert
Committee on Food Additives

K_2HPO_4 = potassium phosphate dibasic

LC-MS = liquid chromatography mass spectrometry

LC-MS/MS = liquid chromatography tandem mass spectrometry

ME = malt extract

MEA = malt extract agar

MeOH = methanol

$\text{MgSO}_4 \cdot \text{H}_2\text{O}$ = magnesium sulphate heptahydrate

MS = mass spectrometry

NA1 = North American population 1 (15ADON)

NA2 = North American population 2 (3ADON)

NA3 = North American population 3 (3ANX)

NaClO = sodium hypochlorite

Na_2SO_4 = sodium sulphate

NOEL = no-observed-effect level

NPP = nerolidyl pyrophosphate

NX = 7 α -hydroxy, 3, 15-dideacetyl calonectrin

PCNB = pentachloronitrobenzene

PCR = polymerase chain reaction

PCR-RFLP = polymerase chain reaction restriction fragment length polymorphism

PDA = potato dextrose agar

PMTDI = provisional maximum tolerable daily intake

PTFE = polytetrafluoroethylene

PVDF = polyvinylidene fluoride

RFLP = restriction fragment length polymorphism

TEF = translation elongation factor gene

TDN = trichodiene

US-FDA = United States Food and Drug Administration

1.0 Introduction

1.1 *Fusarium graminearum*

F. graminearum comprises of a number of closely related clades (O'Donnell et al. 2004) which are part of the *Fusarium graminearum* species complex, however *F. graminearum sensu stricto* and *F. asiaticum* are most commonly reported. These fungi are important plant pathogens that cause Fusarium Head Blight in wheat and small grains, and Gibberella ear rot in corn (Bianchini et al. 2015). Morphologically identical isolates of *F. graminearum sensu lato* and *F. culmorum* can produce either deoxynivalenol (DON) and zearalenone or nivalenol and zearalenone. Strains of *F. graminearum* produce DON by one of two pathways after 3,15 diacetyl deoxynivalenol, through a 3-acetylated precursor (3ADON) or a 15-acetylated precursor (15ADON; Miller 2016). 30 years ago, there were two chemotypes in the Americas (Miller 2016; Miller et al. 1991). From 1983 to circa 2008, this determination was made by chemical analysis of infected cereals or culture material. After the development of genetic probes by Ward et al. (2008, see following), PCR tests were done and the term chemotype was increasingly made based solely on the genotype. Desjardins (2008) observed this was highly problematic. In this thesis, where this determination was made by PCR based tests, the term genotype will be used; if chemical analysis of culture material or infected grains was used, the term chemotype will be used.

Fusarium graminearum causes Fusarium head blight in small grains and Gibberella ear rot in corn in the USA and Canada. Fusarium head blight has been a major problem for agriculture since wheat production expanded across the continent in the late 19th century (Bai & Shaner 2010; Miller 1994; Sutton 1982). Because both wheat and corn are affected, normal

rotations in Ontario typically comprise soybeans, wheat and corn. *Fusarium* head blight incidence is most affected by high humidity and rainfall at anthesis at warmer temperatures (Hooker et al. 2002; Sutton 1982) as opposed to agronomic factors (Schaafsma et al. 2001).

Both ascospores and macroconidia infect wheat. In the early part of the season, ascospores can be released from crop residue, initiating infection followed by the production of macroconidia under favourable conditions, which serves to spread the disease (Fernando et al. 1997; 2000). Both ascospores and macroconidia of *F. graminearum* are present in high concentrations in outdoor air under epidemic conditions (Tschanz et al. 1975). Adjacent crops (non-host, corn, and wheat) affect the number of viable airborne propagules trapped and subsequent infection of seeds infected with *F. graminearum* (Hooker & Schaafsma 2005). Although a variety of non-host plants have been shown to be sources of *F. graminearum* inoculum (Martin & Johnston 1982; Miller et al. 1998) crop residue of the host plants in or near the fields that ultimately become affected is a critical factor in disease. There is evidence that the potential for the release of large amounts of ascospores in the spring has been increased by the widespread adoption of stay-green hybrids (Eli et al. 2018).

As with wheat, infection in corn results from ascospores and macroconidia of *F. graminearum* in the air landing on silks. The progression of Gibberella ear rot is most affected by moisture at silk emergence and prevalence is increased with wet weather later the season (Miller 1994; Sutton et al. 1982). Continuous rain in north-temperate areas is usually associated with cold temperatures not conducive to the growth of *F. graminearum* (Miller et al. 1998; Sutton 1982). As is the case elsewhere, in Ontario, damage to kernels by the European corn borer (*Ostrinia nubilalis*) increases ear rot incidence (Vigier et al. 1997). Western bean cutworm

(*Striacosta albicosta*) has recently emerged in Ontario as an increasingly serious problem, producing damage increasing ear rot and mycotoxin accumulation (Smith et al. 2018).

Although crop yield is reduced in both cereals when damaged by *F. graminearum*, the larger economic and public health issue is the presence of the mycotoxin DON and related compounds (Bianchini et al. 2015). Wheat damaged by Fusarium head blight resulted in what the Japanese called “Red mold poisoning” from the 1800s to 1950s (Miller 2008; Pitt & Miller 2017). Well-documented reports from this time describe human toxicosis such as nausea, vomiting and diarrhea from the consumption of contaminated grain (Yoshizawa 1983). DON was isolated from cultures of isolates of *F. graminearum* obtained from grain that had made people sick (Morooka et al. 1973; Yoshizawa & Morooka 1973). At the same time, DON was re-reported as “vomitoxin” from a *F. graminearum* isolate found on diseased corn (Vesonder et al. 1973). The corn had elicited an emetic response in pigs. Acute human toxicosis from DON contaminated grain has occurred in the Kashmir Valley in India (Bhat et al. 1989), China and Korea (Beardall & Miller 1994; Miller 2008).

In the 1920s, corn contaminated by *F. graminearum* was linked to estrogenic symptoms in pigs. Caldwell et al. (1970) isolated the active compound from corn kernels and determined the chemical structure of zearalenone. Zearalenone was a major contaminant in corn in North America, but largely disappeared by the mid-1980s due to climate change (Miller 2016; Pitt & Miller 2017). This was due to warmer climate and the use of corn hybrids that matured earlier (Miller 2016). *Fusarium* species grow on crops such as wheat, barley, triticale, oats and corn.

1.2 Chemotypes

Fusarium graminearum epidemics in North America during the 1980s led to a considerable amount of toxicological research on DON. To do these studies, the demand for

large quantities of DON and related compounds increased. Canadian researchers determined optimal fermentation conditions to increase the production of DON from *F. graminearum* in liquid fermentation. Parameters including medium, aeration, temperature, sugar concentration, pH and inoculum size were optimized (Miller et al. 1983; Miller & Blackwell 1986). This led to the discovery of two distinct DON producing chemotypes, one producing DON following 3, 15 diacetyl-deoxynivalenol via a 15-acetyldeoxynivalenol precursor (15ADON) and the other through a 3-acetyldeoxynivalenol precursor involving two different enzymes (3ADON; Miller 2016; Miller et al. 1983;1991).

In the early 1980s, almost all strains of *F. graminearum* in North and South America produced DON through the 15ADON precursor (Abbas et al. 1986; Miller et al. 1983; Miller et al. 1991; Mirocha et al. 1989; Piñeiro et al. 1995). In Asian countries such as Japan and China, the 3ADON precursor was most common (Luo et al. 1990; Miller et al. 1991). Strains from Asia and North America are genetically distinct (O'Donnell et al. 2000). In Europe, both chemotypes are present but the majority (82.9%) of *F. graminearum* strains are of the 15ADON type, and *F. culmorum* strains primarily produce DON through 3ADON (59.9%) and nivalenol (40.1%; Pasquali et al. 2016). At present, nivalenol producing strains of *F. graminearum* are rare in Canada and the USA (Gale et al. 2011; Kelly et al. 2015; Ward et al. 2008).

Recent studies on *F. graminearum* chemotypes have shown the 3ADON chemotype as more competitive based on shifts from 15ADON to 3ADON in North America (Gilbert et al. 2014; Spolti et al. 2014; Ward et al. 2008). Isolates from 3ADON populations had significantly higher fecundity and growth rates in comparison to isolates from the 15ADON population (Ward et al. 2008). A similar pattern was seen in field studies in Canada (Amarasinghe et al. 2015; Gilbert et al. 2010) and the USA. Aggressiveness, saprophytic fitness and pathogenic fitness

were similar between 15ADON and 3ADON chemotypes (Kuhnem et al. 2015; Spolti et al. 2014). Kuhnem et al. (2015) determined that host (wheat, corn) did not play a factor in population structure between the two chemotypes. These authors claimed that the 3ADON strains produced more DON than those of the 15ADON chemotype. However, this was shown to be incorrect; in corn, a portion of the 15ADON is converted to 15ADON-3-glucoside (Renaud & Sumarah 2016).

Figure 1. Structures of *Fusarium graminearum* mycotoxins. Deoxynivalenol (DON) and its monoacetylated precursors 3ADON and 15ADON, NX toxin and its monoacetylated precursors 3ANX and 15ANX, nivalenol (NIV), sambucinol (SAM) and zearalenone (ZEA).

1.2.1 Populations in North America

In the 1990s, *Fusarium* head blight reached epidemic proportions in North America. At the time, *Fusarium graminearum* was thought to be a single potentially panmitic species spanning up to six continents (O'Donnell et al. 2004). O'Donnell et al. (2000) used genealogical concordance phylogenetic species recognition, targeting six single-copy nuclear genes to investigate a global collection of *Fusarium* head blight strains. The authors found that all six genealogies recovered the same eight biogeographically structured lineages suggesting phylogenetically distinct species. The lineages being: [1] *Fusarium austroamericanum*, [2] *Fusarium meridionale*, [3] *Fusarium boothii*, [4] *Fusarium mesoamericanum*, [5] *Fusarium acacia-mearnsii*, [6] *Fusarium asiaticum*, [7] *Fusarium graminearum*, and [8] *Fusarium cortaderiae*. These strains belong to the *Fusarium graminearum* species complex, which now contains 16 phylogenetically distinct species (O'Donnell et al. 2004; Sarver et al. 2011). All lineages appeared to reach an advanced stage of genetic isolation because each was reciprocally monophyletic within these six genes (O'Donnell et al. 2000). Of these six nuclear genes, the translation elongation factor gene (*TEF*; *EF-1 α*) is commonly used to distinguish between the lineages (Burlakoti et al. 2008; Burlakoti et al. 2017; Ward et al. 2002; Ward et al. 2008). However, the nuclear genes used for phylogenetic analysis could not determine trichothecene metabolite production. Ward et al. (2002) sequenced the trichothecene gene cluster, which had allowed for the determination of chemospecific differences between Type B-producers (Starkey et al. 2007; Ward et al. 2008).

As noted, historically, the majority of DON-producing strains across North and South America were of the so-called native population, producing DON via the 15ADON precursor (Miller et al. 1983; Miller et al. 1991; Mirocha et al. 1989; Piñeiro et al. 1995). In Asian

countries such as China and Japan, the 3ADON precursor was most common (Miller et al. 1991; Yoshizawa & Jin 1995). The Asian and New World populations are genetically distinct (O'Donnell et al. 2000). As noted, nivalenol-producing strains of *Fusarium* are rare in Canada and the USA (Amarasinghe et al. 2015; Gale et al. 2011; Tanaka et al. 1988), but are more common in Japan and China due to the nivalenol-producing *F. asiaticum* (Gale et al. 2011).

The first 3ADON strain of *F. graminearum* isolated in Canada was deposited in 1969 in the Canadian National Mycological Herbarium (DAOM). In 1979, three strains of the 3ADON chemotype *F. graminearum* strains were isolated in Ontario (Ouellet & Seifert 1993). Within the past 10-15 years, surveys conducted in Canada and the USA have shown a shift from 15ADON chemotypes to 3ADON chemotypes (Ward et al. 2008). Ward et al. (2008), reported a significant shift in 1998 to 2004 where the 3ADON chemotype frequency in Western Canada increased by 14-fold. These authors reported the genotype of 492 *F. graminearum* complex strains collected in Canada between 1984-2004 using multiplex-PCR assays targeting *TRI3* and *TRI12*. Of these strains, 27.4% were of the 3ADON genotype and the remaining 72.6% were 15ADON genotype. No nivalenol producing strains were reported. The distribution of trichothecene genotypes revealed the highest frequency of 3ADON isolates in Prince Edward Island (100%), which decreased east to west: Quebec (50%), Manitoba (31%), Saskatchewan (10.7%), Alberta (5.9%). Analysis of *F. graminearum* isolates collected from 1999 and 2000 from Minnesota and North Dakota revealed a population of genetically divergent strains (Gale et al. 2007). These strains were genotyped 3ADON using *TRI3* and *TRI12* multiplex PCR assays (Starkey et al. 2007; Ward et al. 2008). Based on genetic variations at 10 restriction fragment length polymorphisms (RFLP), the 3ADON strains formed a distinct cluster with previously isolated Italian strains (Gale et al. 2007; Ward et al. 2008). Due to the similarities between the isolated 3ADON strains

and the Italian strains, the authors suggested that the 3ADON populations found in North America were most likely imported from Europe. Ward et al. (2008) indicated that eastern Canada was the original entry point into North America due to the high frequency of 3ADON genotypes, which decreases east to west.

In 2010, 155 *F.graminearum* isolates were collected from 24 wheat fields in southern Ontario, ranging from the counties of Chatham-Kent to Perth and Huron. Using the multiplex-PCR assay targeting *TRI3*, the majority (97%) of isolates were of the 15ADON genotype, the remainder (3%) were 3ADON genotype (Tamburic-Ilincic et al. 2015). Isolates of the 3ADON genotype were found in Perth County, at the northern part of the sampling area where spring wheat is produced. Burlakoti et al. (2017) conducted a survey from 2010 – 2012 on *F. graminearum* isolates collected from corn (n = 452) and wheat (n = 110) grown in Ontario. Using *TRI*-based multiplex-PCR assays targeting *TRI3* and *TRI12*, 96% of isolates recovered from corn were 15ADON genotype, and 4% resulted in 3ADON genotype. These samples were recovered from in the south-central region of New Hamburg (n = 2) in 2010 and northeast central region of Wingham (n = 16) in 2011. From wheat, 98% isolates were determined to be 15ADON genotypes. Two 3ADON genotypes were recovered from Kemptville in the east region of the sampling area.

In Upstate New York, the 15ADON genotype also predominated but there were a higher proportion of 3ADON genotype than reported in Ontario (Kuhnem et al. 2015a). Some 8% of isolates in Aurora near Lake Ontario, 22% of isolates from inland Belmont New York and 47% of isolates from Willsboro near Lake Champlain. A later reanalysis of these strains showed that a high percentage of the 3ADON genotype were of the NX genotype (Lofgren et al. 2018). Regardless, Kuhnem et al. (2015a) suggested that “regional populations may be differentiated

based on selection associated with climatic or landscape features not currently identified”. A subsequent study indicated that host (corn or wheat) did not have an effect on the structure of these populations (Kuhnem et al. 2015b).

A survey of 6,000 *F. graminearum* isolates conducted by (Gale et al. 2010) in Minnesota, North Dakota and South Dakota, reported that 400 isolates did not display population specific patterns. Further genotyping using PCR-RFLP assays revealed three populations; 15ADON, 3ADON, and a newly identified population named the Northland population. Of the 176 isolates identified from this new population, the majority originated from Minnesota (77%), followed by North Dakota (19%) and South Dakota (2%). Roughly 2/3 of the Northland population isolates resulted in 15ADON genotype, and the remainder were 3ADON genotype. Chemical analysis of wheat infected with the 15ADON-Northland isolates was contaminated by 15ADON and DON, However, wheat infected with strains which genotyped as 3ADON in the Northland population did not contain detectable levels of known trichothecenes.

Five years later, Varga et al. (2015) showed that when grown under laboratory conditions, strains of the 3ADON genotype Northland population produced two novel trichothecenes, they named NX-2 (3ANX) and NX-3 (NX). 3ANX (3 α -acetoxy, 7 α ,15-dihydroxy-12,13-epoxytrichothec-9-ene) and NX have analogous structures to 3ADON and DON, respectively, except for the keto-group on the C-8 position (Aitken et al. 2019). Using the Northland populations collected by Gale et al. (2010), Liang et al. (2014) developed a *TRII* PCR-RFLP assay capable of differentiating 3ADON and 3ANX chemotypes. This assay showed that 3ANX isolates collected from Minnesota, North and South Dakota were low in overall frequency (2.8%). Kelly et al. (2015) reported a study on *F. graminearum* isolates from eight Canadian provinces collected between 2005-2007. They found that the frequency of 3ANX

isolates was lower than 2% for any given year. Overall, 12 3ANX chemotypes were confirmed with chemical analysis (Quebec (n = 4); Ontario (n = 5); Prince Edward Island (n = 1); Saskatchewan (n = 2)). Each 3ANX isolate resulted in 3ADON genotype using *TRI3* and *TRI2*, and accounted 1% and 11% of the 3ADON genotypes in Quebec and Ontario, respectively. The authors showed that these cultures formed into two distinct genetic populations, NA1 that was dominated by 15ADON genotype (86%), and NA2 that mainly consisted of 3ADON genotype (88%).

However, the percentages of isolates with trichothecene genotypes that were not consistent with their genetic population assignment (recombinant genotypes) increased from 10% in 2005 to 17% in 2007. Kelly et al. (2015) subsequently reported that the frequency of recombinant genotypes is increasing rapidly and that trichothecene genotype is “*an increasingly unreliable marker of population identity*”. Kelly & Ward (2018) subsequently demonstrated that a third genetically distinct population was present, and that 3ANX isolates made the majority (60% (12/20)) of this group. The remaining 3ANX isolates had admixed genomes, sharing a majority of their ancestry with the NA1 (15ADON dominant) population.

1.3 Secondary metabolism

Fungi produce a large number of metabolites that are not required for cell growth but contribute to the survival of the colony (Demain & Fang 2000). Typically, such compounds are so-called secondary metabolites. Secondary metabolites produced by fungi include plant growth regulators, pharmaceuticals, and mycotoxins (Keller et al. 2005). There are four main classes of fungal secondary metabolites: polyketides, non-ribosomal proteins, indole alkaloids, and terpenes.

Secondary metabolism is closely tied to primary metabolism. The fundamental building blocks of secondary metabolites are derived from primary metabolite precursors; acetyl coenzyme A (acetyl-CoA), shikimic acid, mevalonic acid and various amino acids. Acetyl-CoA forms compounds such as phenols, protoginsins, and macrolide antibiotics. Shikimic acid is a precursor for phenols, cinnamic acid derivatives, lignans and alkaloids. A vast array of terpenoid and steroid metabolites are biosynthesized via the precursor mevalonic acid (Bu'Lock 1975).

Terpenoids are a large and structurally diverse family of natural products formed by units of isoprene (C_5) joined head to tail. Important fungal terpenes include aristolochenes, carotenoids, gibberellins, and trichothecenes (Keller et al. 2005). Terpenoids are synthesized from a condensation reaction with isopentenyl diphosphate and its isomer dimethylallyl diphosphate. Repeated condensation of isopentenyl diphosphate results in chain elongation facilitated by isoprenyl diphosphate synthases. Terpenoid compounds are classified based on the number of repeating isoprene units (C_5) in the carbon skeleton. Trichothecenes are part of the terpene class sesquiterpenes (C_{15}) which is derived from farnesyl pyrophosphate (Kimura et al. 2007). Dimethylallyl diphosphate and IPP react to produce geranyl pyrophosphate, and the addition of another isopentenyl diphosphate unit leads to the sesquiterpene precursor farnesyl pyrophosphate.

1.4 Trichothecene biosynthesis

Trichothecenes are a large family of secondary metabolites and a major class of mycotoxins produced by several *Fusarium* species (Desjardins et al. 1993). More than 200 trichothecenes have been reported (Grove 2007). Trichothecenes share a tricyclic sesquiterpenoid 12,13-epoxytrichothec-9-ene backbone, which are further classified into four groups based on substituents at the C-8 position. Type A and B are more widely distributed in

cereal and feed products, whereas C and D occur less often in food and feed. Type B trichothecenes such as DON and nivalenol, are characterized by a carbonyl group at C-8. Type A trichothecenes lack the ketone group at C-8 being mainly represented by T-2 toxin, HT-2 toxin, and diacetoxyscirpenol. Type C has a second epoxy ring between C-7 and C-8 or C-9 and C-10 (crotocin and baccharin). Type D represented by satratoxin and roridin contain a macrocyclic ring between C-4 and C-15. The structural diversity between trichothecenes has been attributed to the genetic variation in the trichothecene biosynthetic pathway (Kamata et al. 2018).

Hohn and coworkers from the USDA began the genetic research of trichothecene biosynthesis of *Fusarium sporotrichioides* (Hohn & Vanmiddlesworth 1986). Since then, ten trichothecene biosynthetic genes (*TRI*) have been assigned to specific biosynthetic steps. Seven of the genes are located within a group (*TRI5* gene cluster) that spans 26 kb in the fungal genome. One of the remaining three genes is *TRII01*, is located between the phosphate permeases and UTP-ammonia ligase genes which are not related to trichothecene biosynthesis (Kimura et al. 1998). The other two, *TRII* and *TRII6* are located in a two-gene cluster (Meek et al. 2003).

Biosynthesis begins with the cyclization of farnesyl pyrophosphate to form trichodiene, facilitated by a terpene cyclase trichodiene synthase encoded by *TRI5* (Hohn & Vanmiddlesworth 1986). This begins with the isomerization of all-trans farnesyl pyrophosphate to nerolidyl pyrophosphate to bisabolyl cation. Cyclization, a 1,4-hydride shift and 1,2-methyl migration results in trichodiene synthase (Cane & Ha 1988) (Figure 2). Trichodiene then undergoes four oxygenations by a cytochrome P450 monooxygenase (*TRI4*) to form the intermediate isotrichotriol (Hohn et al. 1995; McCormick et al. 2006). The four oxygenation reactions occur sequentially beginning with the C-2 hydroxylation, C-12,13 epoxidation, C-11

hydroxylation and C-3 hydroxylation to yield 2 α -hydroxytrichodiene, 12,13-epoxy-9,10-trichoene-2 α -ol, isotrichodiol, and isotrichotriol respectively (McCormick et al. 1989; Zamir et al. 1991; Zamir et al. 1999) (Figure 3).

Figure 2. Isomerization of farnesyl pyrophosphate (FPP) to nerolidyl pyrophosphate (NPP), which undergoes cyclization to trichodiene (TDN). This is an enzyme catalyzed reaction by TDN synthase encoded by the *TR15* gene (after Kimura et al. 2007).

Figure 3. Sequential oxidation of trichodiene (TDN), 2 α -hydroxytrichodiene (2 α -HDT), 12,13-epoxy-9,10-trichoene-2 α -ol, isotrichodiol, and isotrichotriol. Oxidation at each step is dependent on cytochrome P450 monooxygenase encoded by the *TRI4* gene (after McCormick et al. 2011)

Cyclization of isotrichotriol then occurs through trichotriol to isotrichodermol (Hesketh et al. 1993), the first trichothecene in the biosynthetic pathway (Kimura et al. 2007). An acetyltransferase (*TRI101*) converts isotrichodermol to isotrichodermin through C-3 acetylation (Kimura et al. 1998) (Figure 4). After this step, there are oxygenations at C-7, C-8 and C-15 to produce 7-hydroxyisotrichodermin, 8-hydroxyisotrichodermin, and 15-deacetylcalonectrin, respectively. Two cytochrome P450 monooxygenases are responsible for these steps, *TRI11* gene encodes a C-15 hydroxylase, whereas *TRI1* hydroxylates either the C-7 or C-8 position to produce 7-hydroxyisotrichodermin or 8-hydroxyisotrichodermin, respectively (McCormick & Hohn 1997; McCormick et al. 2006). This biosynthetic step occurs in a grid. The major pathway occurs through 15-deacetylcalonectrin acetylation (*TRI3*) to calonectrin, which is then hydroxylated at the C-7 position to produce 7-hydroxycalonectrin or at the C-8 position to produce 8-hydroxycalonectrin (Zamir et al. 1991). Type A trichothecenes are not hydroxylated at these positions, but at the C-4 position by a cytochrome P450 monooxygenase encoded by *TRI7* gene. For Type B trichothecenes, cytochrome P450 monooxygenase encoded by *TRI1* is also responsible for the hydroxylation of calonectrin at C-7 and C-8 to produce 7,8-dihydroxycalonectrin (McCormick et al. 2004; McCormick et al. 2006) (Figure 5). The hydroxyl group at C-8 is transformed into a keto group to produce 3,15-acetyldeoxynivalenol. The gene responsible for this step has not been classified (McCormick et al. 2011). An esterase encoded by *TRI8* is responsible for the deacetylation at either C-3 or C-15 to produce 15ADON or 3ADON chemotypes, respectively (Alexander et al. 2011) (Figure 6).

Figure 4. The isomerization and cyclization of isotrichotriol to isotrichodermol, and acetylation to isotrichodermin. An acetyltransferase responsible for C-3 acetylation of isotrichodermol is encoded by *TRI101* gene (after Kimura et al. 2007; McCormick et al. 2011).

Figure 5. Biosynthetic grid from isotrichodermin to 7,8-dihydroxycalonectrin (DHC). (after Kimura et al. 2007; McCormick et al. 2011).

Figure 6. The oxidation of 7,8-dihydroxycalonectrin (DHC) to 3,15-acetyldeoxynivalenol (3,15-ADON), which is deacetylated either at the C-3 or C-15 position to produce 15ADON or 3ADON, respectively. (after Alexander et al. 2011)

1.5 Genotype

Genotype is the genetic composition or DNA sequence (Desjardins 2008). Since the development of DNA-based molecular biology, many fungal gene clusters for secondary metabolism have been identified. This includes agriculturally important mycotoxins such as alkaloids, fumonisins, ochratoxins, zearalenones and trichothecenes (Desjardins 2008). Sequencing these gene clusters has allowed for the determination of chemospecific differences between Type B trichothecene producers (Ward et al. 2002). As a result, PCR-based assays that target these genes have been developed as an alternative route to determining chemotype. Initially, PCR genotype screening of a large percentage of strains from cultures were strengthened by chemical analysis (Starkey et al. 2007; Ward et al. 2002, 2008). As noted above, today most studies report the use of PCR methods instead of chemical analysis. By comparison, PCR is rapid and inexpensive.

It has been known for some time that enzyme specificity of secondary metabolism is lower in comparison to primary metabolism in filamentous fungi (Bu'Lock 1975). The precursors and end-products of secondary metabolites are synthesized in metabolic grids (Bu'Lock 1965; Kimura et al. 2007). Genes encoding enzymes for biosynthetic reactions at branch points from primary metabolism are good candidates for genotyping assays because they are critical for the function of the entire metabolic pathway, and therefore are more conserved (Desjardins 2008). Between strains of the toxigenic *Fusarium* species, variations in secondary metabolite precursors can result in a variety of end-products. These variations are present between *F. sporotrichioides* and *F. graminearum* TRI1 enzyme (cytochrome P450 monooxygenase). The TRI1 enzyme in *F. graminearum* is responsible for hydroxylating the C-7 and C-8 positions of calonectrin, in *F. sporotrichioides* this enzyme only adds a hydroxyl to the

C-8 position (McCormick et al. 2004; Meek et al. 2003). This variation results in two separate classes of trichothecenes, Type B from *F. graminearum* and Type A from *F. sporotrichiodes*. The *TRI1* gene which encodes the TRI1 enzyme, shares as little as 66% identity at the amino acid level between species (Desjardins 2008; Proctor et al. 2009).

Most PCR genotyping assays are based on the absence or presence of a target gene. The most important step in developing these assays is synthesizing primers which are specific to the target gene. Primer specificity is crucial: if specificity is high, the risk of false negatives increases due to unexpected DNA polymorphisms at the target. One single nucleotide polymorphism in a 20 nucleotide primer can prevent primer binding and ultimately a lack of amplification (Desjardins 2008). Similarly, if the specificity of the primer is too low, the risk of false positives increases. Most PCR genotyping assays determine genotype through PCR product size comparison to a target sequence or control strain. However, PCR products similar in size can be differentiated through DNA sequencing.

Ward et al. (2002) sequenced the 19-kb region of the trichothecene gene cluster in 39 *F. graminearum* complex strains to compare mycotoxin production and *Fusarium graminearum* complex phylogeny. *TRI*-cluster haplotypes were grouped by chemotype (nivalenol, 3ADON and 15ADON) as opposed to species. *TRI3* (3-acetyltrichothecene 15-O-acetyltransferase) and *TRI12* (trichothecene efflux pump) were found to have distinct sequences for each chemotype across strains in the *F. graminearum* complex. Using the *TRI3* and *TRI12* sequences, two multiplex PCR assays were developed to differentiate each genotype (Starkey et al. 2007; Ward et al. 2002). For both the *TRI3* and *TRI12* multiplex PCR assays, there is one forward primer common to all genotypes, and three genotype specific reverse primers. These assays produce size-specific PCR products that are different for each resulting genotype.

Alexander et al. (2011) determined *TRI8* encoded the deacetylase that differentiates 3ADON and 15ADON in the biosynthetic pathway. It was found that the *TRI8* enzyme in 3ADON genotypes was responsible for deacetylating 3,15-ADON at C-15 to yield 3ADON. The *TRI8* enzyme in 15ADON strains deacetylate 3,15-ADON at C-3 to result in 15ADON. These authors concluded that the differentiation of 3ADON and 15ADON occurs with the *TRI8* enzyme. Consistent differences in *TRI8* DNA sequences were found for 15ADON and 3ADON genotypes.

Using strains collected from Minnesota, North Dakota and South Dakota, Liang et al. (2014) genotyped 82.3% as 15ADON and 14.9% as 3ADON genotype using the *TRI3* and *TRI12* multiplex PCR assays. All strains that were classified 3ANX producers by chemical analysis scored as 3ADON producers for these genotyping assays (Liang et al. 2014). Previous studies revealed that *F. graminearum TRI1* gene encodes the cytochrome P450 which hydroxylates calonectrin into 7-hydroxycalonectrin, 8-hydroxycalonectrin and ultimately 7,8-dihydroxycalonectrin (McCormick et al. 2004). The novel trichothecene 3ANX having only hydroxylation at C-7 led Liang et al. (2014) to believe that there were *TRI1* gene differences between 3ANX and DON producers. In order to differentiate between 3ADON and 3ANX strains, these authors developed a PCR-RFLP assay targeting the *TRI1* gene of 3ANX strains. Comparing the *TRI1* gene of 15ADON *F. graminearum* type strain PH-1 with a previously identified 3ANX producer revealed this polymorphism. The PCR-RFLP assay begins with amplifying the *TRI1* gene to result in a 1.7-kb PCR amplicon for 15ADON, 3ADON, nivalenol and 3ANX genotypes. The *TRI1* amplicon is then digested with the restriction endonuclease *ApoI*, which results in two fragments for ADON and nivalenol genotypes, and three fragments for 3ANX genotypes. This assay is based on the principle that ADON and nivalenol strains have

one restriction site for *ApoI* to cleave, and due to a polymorphism in the 3ANX *TRII* gene there is an additional restriction site resulting in three fragments as opposed to two.

Recently an assay developed by the Toomajian research group (Yue 2017; Prof. Chris Toomajian, Department of Plant Pathology, Kansas State University) developed a *TRII* allele-specific PCR amplification approach to rapidly distinguish 3ADON and 3ANX genotypes. The assay was designed to target the *TRII* gene with a common reverse primer, and two distinct forward primers for each genotype. The forward primers differ by four nucleotides to match four single nucleotide polymorphisms in the 3ANX *TRII* gene. Using this assay, an isolate is genotyped based on the presence of a PCR amplicon. Isolates of 3ANX chemotype will amplify in the 3ANX PCR assay, but not in the 3ADON assay. Isolates of 3ADON chemotype will amplify in the 3ADON PCR assay, but not in the 3ANX assay. This research group did not list the amplification result of 15ADON control strains.

1.6 Toxicity

Acute deoxynivalenol toxicosis in humans causes gastroenteritis and emesis. Outbreaks of these symptoms have been linked to grains highly contaminated with DON and other mycotoxins in Russia in the 1920s (Pitt & Miller 2017), Japan and Korea in the 1940s-1960s (Yoshizawa 2013; Yoshizawa & Morooka 1973), China in 1980s (Luo 1988; Yoshizawa 1983), and Kashmir Valley, India in 1987 (Bhat et al. 1989). In all cases, humans who had consumed contaminated grains complained of gastrointestinal disorder. In the Kashmir Valley, India incident, epidemiological investigations indicated that the outbreak was linked to bread made with highly contaminated wheat (Bhat et al. 1989). The incidence of acute toxicity from consumption of grain with high concentrations of DON is now rare. This is due to better global

regulation, crop management techniques, improved crop resistance to FHB, and advances in milling techniques (Bianchini et al. 2015).

The effects of chronic exposure to DON include weight gain suppression, anorexia, altered nutrient deficiency, and immunosuppression (EFSA 2013; Robbana-Barnat et al. 1988). DON is water soluble and is rapidly cleared from the body through urine, and at higher doses small amounts will excrete through the feces after conjugation (Miller 2016). In 2001, the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives (JECFA) established a provisional maximum tolerable daily intake (PMTDI) of 1 µg/kg bw for DON. This was based on a no-observed-effect level (NOEL) of 100 µg/kg bw per day for decreased body weight gain in 2 year feeding study with male and female B6C3F1 mice and a safety factor of 100 (Iverson et al. 1995; JECFA 2001). In 2010, the PMTDI was modified to include both DON monoacetates (3ADON and 15ADON) for a group PMTDI (JECFA 2011). Health Canada lists the maximum tolerable level of DON as 2.0 mg/kg in uncleaned soft wheat for use in non-staple foods, and 1.0 mg/kg in uncleaned soft wheat for use in baby foods. These levels have been under review for some time now.

At high doses, DON causes emesis in humans, mink, and swine. Swine appear to be the most affected, with the minimum oral dose of 50-100 µg/kg bw required for emesis (Pestka et al. 1987). Increased dosage was associated with decreased average time to vomition, increased duration of emesis and increased number of vomitions. The emetic effect on swine is where the American trivial name “vomitoxin” originated (Miller 2008). Wu et al. (2013) found a significant increase of the gut satiety hormone peptide YY₃₋₃₆ and the neurotransmitter 5-hydroxytryptamine in the blood plasma and DON-induced emesis in minks. Antagonists to both peptide YY₃₋₃₆ and 5-hydroxytryptamine resulted in reduced and complete suppression of

emesis, respectively. This suggested that both peptide YY₃₋₃₆ and 5-hydroxytryptamine play a role in the mechanism for DON-induced emesis. DON-induced anorexia and growth retardation involves a number of mechanisms. Amuzie & Pestka (2010) found that DON suppresses the insulin-like growth factor acid-labile subunit mRNA expression. The insulin-like growth factor acid-labile subunit is responsible for increasing the half-life of insulin growth factor 1 which is essential for postnatal growth and development. Depletion of insulin-like growth factor acid-labile subunit has been shown to severely reduce circulating insulin growth factor 1 and growth in mouse and humans (Heath et al. 2008; Ueki et al. 2009). Increased expression of pro-inflammatory cytokines observed in response to DON is proposed to drive the anorexic and reduced weight gain symptoms (Bonnet et al. 2012; Pestka 2010). During an infection or inflammation, pro-inflammatory cytokines interact with brain centers that control ingestion, such as the hypothalamus and dorsal vagus nerve to induce anorexia (Bonnet et al. 2012).

1.7 Management of DON in grains

Crop producers manage levels of DON by sowing resistant cultivars, using fungicides, crop rotation, tillage and disease forecasting. Sowing *Fusarium* resistant wheat, barley and corn cultivars has reduced disease severity and DON accumulation by more than 50% compared to the susceptible control cultivars (Bianchini et al. 2015). Dill-Macky & Jones (2000) reported that *Fusarium* head blight incidence and severity were increased when wheat followed corn, and least when wheat followed soybeans. DON level decreased when wheat followed soybeans in comparison to following wheat or corn. The authors also determined that disease incidence was lower in plots after conventional tillage in comparison to conservation tillage or no-till plots. Ascospore production on debris on fields after conservation or no-till plots increases the inoculum for the next growing season. Wegulo et al. (2011) showed that integrating tolerant

cultivars and fungicide application at anthesis is an effective strategy in managing *Fusarium* head blight and DON accumulation on winter wheat. Common fungicides with proven effectiveness are metconazole, tebuconazole and prothioconazole (Bianchini et al. 2015; Paul et al. 2008). Disease forecasting tools such as Weather Central (Weather Innovations) in Canada, predict regions for high potential of *Fusarium* infection. This system and similar US versions not only alert farmers if conditions are favourable for *Fusarium* infection, but also make suggestions for appropriate cultivars depending on temperature and risk of disease, appropriate timing for fungicide application.

1.8 Project aim

The purpose of this study was to isolate strains of *F. graminearum* across the grain growing regions of Ontario, Canada in 2015-2017. From the collection of 445 strains, a subset of 61 was chosen for chemical and genotypic analysis to determine chemotype and genotype, respectively. Chemotype was determined through comprehensive chemical analysis to distinguish among *F. graminearum* metabolites. Genotype was determined through multiple PCR-based assays to differentiate between 15ADON, 3ADON and 3ANX isolates. Comparisons between the two classification methods were made.

2.0 Materials and Methods

2.1 Isolates

Over the years 2015-2017, corn and wheat samples were collected from diverse cereal growing areas of southern, central, and eastern regions of Ontario. Grain samples were collected by the Ontario Corn Committee, Kent Corn Committee, experimental plots at the University of Guelph Ridgetown Campus, grain samples collected from annual ear rot and *Fusarium* Head

Blight/Gibberella ear rot surveys conducted by the Ontario Ministry of Food and Agriculture, and the University of Guelph, Ridgetown Campus. A total of 141 corn and 398 wheat samples were collected from the 2015 harvest. Corn samples were collected from 28 farms and wheat samples collected from 66 farms (Table 1A and 2A). A total of 299 corn and 191 wheat kernel samples were collected from the 2016 harvest. Corn samples were collected from 76 farms and wheat samples collected from 24 farms (Table 3A and 4A). A total of 333 corn and 490 wheat samples were collected from the 2017 harvest. Corn samples were collected from 124 farms and wheat samples collected from 28 farms (Table 5A and 6A). The kernels that were collected were stored in plastic bags at room temperature.

Three *F. graminearum* strains of known genotype and chemotype were obtained from Agriculture and Agri-Food Canada as positive controls. A 15ADON chemotype strain, *F. graminearum* DAOMC 180378, was isolated from corn in Ottawa, Ontario in 1979 by Dr. G. A. Neish (Miller et al. 1983; Neish et al. 1983). The 3ADON chemotype strain, *F. graminearum* strain DAOMC 242075, was isolated from red spring wheat in New Brunswick. The 3ANX chemotype strain, *F. graminearum* strain DAOMC 242077, was isolated from soft red winter wheat in Annapolis, Nova Scotia. The 3ADON and 3ANX strains were collected in 2011 by Dr. Randy Clear (Canadian Grain Commission, Winnipeg).

2.2 Isolation and preliminary identification

Fusarium graminearum strains were isolated from wheat and corn kernel samples from Ontario, Canada. Kernels from each sample bag were removed with forceps and surface sterilized by briefly submerging them in 70% ethanol (1 min), and then 5% NaClO (2 min). Kernels were then rinsed in ddH₂O, drained and blotted with Kimwipes. Kernels showing signs of *Fusarium* damage were preferentially plated. The kernels were plated onto PCNB media

(peptone (HI-Media Laboratories), 15 g; K₂HPO₄ (BioShop), 1 g; MgSO₄•7H₂O (BioShop), 0.5 g; pentachloronitrobenzene (Sigma-Aldrich), 0.75 g; streptomycin (Sigma-Aldrich), 0.3 g; neomycin (Sigma-Aldrich), 0.12 g; per L ultrapure water, Barnstead Nanopure Diamond) petri dishes (9 cm). The plates were incubated at 26 °C for 7 days in the dark. Fungal colonies were then transferred to Potato Dextrose Agar (PDA; Hardy Diagnostics) and incubated at 26 °C for 7 days in the dark, after which each *F. graminearum* culture was identified based on morphological characteristics. Cultures of *F. graminearum* were kept at 4 °C on 2% malt extract agar (BD Biosciences) plates for further analysis.

2.3 Molecular identification

Aerial mycelium from a fresh plate of each isolate was used to extract genomic DNA with the Macharey-Nagel Nucleospin 96 Plant-II kit (GmbH & Co. KG #740663.4). All PCR reactions were amplified in a T100 Thermal Cycler (Bio-Rad Technologies, Inc., CA, USA). 10 µL of amplified PCR products were run in a 1.5% (*wt/vol*) agarose (VWR) gels pre-stained with ethidium bromide (Invitrogen, Thermo Fisher Scientific, CA, USA) to confirm amplification. Gel photographs were taken on MultiImage™ Light Cabinet (Alpha Innotech, Thermo Fisher Scientific, CA, USA). Invitrogen™ PCR SuperMix (Thermo Fisher Scientific, CA, USA) was used for all PCR reactions. PCR primers were synthesized by Integrated DNA Technologies (Integrated DNA Technologies, Coralville, IA, USA). *F. graminearum* strains were confirmed by sequencing the *TEF* gene after O'Donnell et al. (2000). From the 2015 season, 77 strains were isolated from wheat from 25 farms and 9 strains of corn from two farms. A subset of 20 strains were selected for further examination, 18 isolates collected from wheat and two from corn (Table 1). From the 2016 season, 36 strains were isolated from wheat from 14 farms and 110 strains of corn from 8 farms. From this year, a subset of 22 strains were selected with 5 strains from wheat

and 17 from corn (Table 2). In the 2017 season, 195 strains were isolated from wheat from 8 farms and 40 strains from corn from 4 farms. A subset of 19 strains were selected, 13 strains from wheat and 6 from corn (Table 3).

Table 1. Crop and location details of representative strains from the 2015 harvest in Ontario.

DAOMC	Crop	Region	County	Latitude	Longitude
251903	Wheat	Sarnia	Lambton	42.9357	-82.2496
251904	Wheat	Essex	Essex	42.1291	-82.9093
251905	Wheat	Palmerston	Wellington	43.8725	-80.8346
251906	Wheat	Belle River	Essex	42.2941	-82.6414
251907	Wheat	Denfield	Middlesex	43.1412	-81.2558
251908	Wheat	Inwood	Lambton	42.8053	-81.9101
251909	Wheat	Muirkirk	Chatham-Kent	42.5253	-81.7382
251910	Wheat	Duart	Chatham-Kent	42.4972	-81.7625
251911	Wheat	Ridgetown	Chatham-Kent	42.4445	-81.8797
251912	Wheat	Blenheim	Chatham-Kent	42.3616	-82.0730
251913	Wheat	Melbourne	Middlesex	42.8570	-81.5464
251914	Wheat	Dresden	Chatham-Kent	42.5849	-82.1254
251915	Wheat	Lakeshore	Essex	42.2964	-82.6439
251916	Wheat	Exeter	Huron	43.1347	-81.2377
251917	Wheat	Huron East	Huron	43.4700	-81.4517
251918	Wheat	Dublin	Perth	43.4683	-81.2497
251919	Wheat	Mitchell	Perth	43.1347	-81.2377
251920	Wheat	Lucan	Middlesex	43.2277	-81.4009
251921	Corn	Florence	Lambton	42.7052	-81.9636
251922	Corn	Rodney	Elgin	42.5458	-81.6216

Table 2. Crop and location details of representative strains from the 2016 harvest across Ontario.

Strain	Crop	Region	County	Latitude	Longitude
24553	Wheat	Bryanston	Middlesex	43.1347	-81.2353
24558	Wheat	Mitchell	Perth	43.4175	-81.1808
24590	Wheat	Ridgetown	Chatham-Kent	42.4069	-81.9281
24782	Wheat	Dresden	Chatham-Kent	42.5849	-82.1265
24893	Wheat	Sarnia	Lambton	42.5560	-82.1517
25173	Corn	Harwich	Chatham-Kent	42.3536	-81.9145
25178	Corn	Bothwell	Chatham-Kent	42.6380	-81.8732
25191	Corn	Bothwell	Chatham-Kent	42.6380	-81.8732
25192	Corn	Bothwell	Chatham-Kent	42.6380	-81.8732
25213	Corn	Bothwell	Chatham-Kent	42.6380	-81.8732
25219	Corn	West Elgin	Elgin	42.6026	-81.7456
25232	Corn	Ridgetown	Chatham-Kent	42.4478	-81.8787
25241	Corn	Ridgetown	Chatham-Kent	42.4478	-81.8787
25250	Corn	Ridgetown	Chatham-Kent	42.4478	-81.8787
25268	Corn	Ridgetown	Chatham-Kent	42.4478	-81.8787
25281	Corn	Ridgetown	Chatham-Kent	42.4478	-81.8787
25291	Corn	Ridgetown	Chatham-Kent	42.4478	-81.8787
25432	Corn	Ridgetown	Chatham-Kent	42.4478	-81.8787
25437	Corn	Ridgetown	Chatham-Kent	42.4478	-81.8787
25442	Corn	Ridgetown	Chatham-Kent	42.4478	-81.8787
25450	Corn	Ridgetown	Chatham-Kent	42.4478	-81.8787
25457	Corn	Ridgetown	Chatham-Kent	42.4478	-81.8787

Table 3. Crop and location details of representative strains from the 2017 harvest across Ontario and St-Hyacinthe, Québec.

Strain	Crop	Region	County	Latitude	Longitude
26203	Wheat	Lucan	Middlesex	43.1566	-81.4688
26223	Wheat	Lucan	Middlesex	43.2277	-81.4009
26233	Wheat	Kingsville	Essex	42.0952	-82.6854
26292	Wheat	Kemptville	Leeds and Grenville	45.0064	-75.6329
26295	Wheat	Kemptville	Leeds and Grenville	45.0064	-75.6329
26296	Wheat	Kemptville	Leeds and Grenville	45.0064	-75.6329
26301	Wheat	Palmerston	Wellington	43.8725	-80.8346
26314	Wheat	St-Hyacinthe	Montréal	45.6282	-72.9568
26320	Wheat	St-Hyacinthe	Montréal	45.6282	-72.9568
26326	Wheat	St-Hyacinthe	Montréal	45.6282	-72.9568
26330	Wheat	St-Hyacinthe	Montréal	45.6282	-72.9568
26331	Wheat	St-Hyacinthe	Montréal	45.6282	-72.9568
26335	Wheat	St-Hyacinthe	Montréal	45.6282	-72.9568
26348	Corn	Muirkirk	Chatham-Kent	42.5041	-81.7765
26349	Corn	Harwich	Chatham-Kent	42.3536	-81.9145
26359	Corn	Londesborough	Huron	43.6755	-81.4661
26361	Corn	Londesborough	Huron	43.6755	-81.4661
26362	Corn	Londesborough	Huron	43.6755	-81.4661
36368	Corn	Londesborough	Huron	43.6755	-81.4661

Two multiplex-PCR assays that target the *TRI3* and *TRI2* genes developed by Ward et al. (2002) were used to differentiate between 15ADON, 3ADON and nivalenol producers of *F. graminearum* following the protocol described by Starkey et al. (2007). Each assay consists of one forward primer and three reverse primers that bind to each genotype (15ADON, 3ADON and nivalenol) respectively. The *TRI3* assay involves the forward primer common to all genotypes, 3CON (5'-TGGCAAAGACTGGTTCAC-3'), and three genotype-specific reverse primers: 3NA (5'-GTGCACAGAATATACGAGC-3'), 3D15A (5'-ACTGACCCAAGCTGCCATC-3'), and 3D3A (5'-CGCATTGGCTAACACATG-3'). This multiplex assay produces amplicons of approximately 840-, 610- and 243-bp for isolates with

nivalenol, 15ADON and 3ADON genotypes respectively. The *TRI2* assay involves a forward primer common to all genotypes, 12CON (5'-CATGAGCATGGTGATGTC-3'), and three genotype-specific reverse primers: 12NF (5'-TCTCCTCGTTGTATCTGG-3'), 12-15F (5'-TACAGCGGTCGCAACTTC-3'), and 12-3F (5'-CTTTGGCAAGCCCGTGCA-3'). This assay produces amplicons of approximately 840-, 670- and 410-bp for isolates with nivalenol, 15ADON and 3ADON genotypes respectively. Thermal cycling conditions were 94 °C for 2 min, 40 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min. Each PCR reaction had a total volume of 33 µL containing 22.5 µL Supermix, 2 µL of each primer, and 2.5 µL of fungal genomic DNA. Molecular size of amplification products were scored relative to GeneRuler 100-bp DNA ladder (Thermo Fisher Scientific, CA, USA).

A *TRI8* PCR assay developed by Alexander et al. (2011), was used to confirm *F. graminearum* genotype. This assay involves a separate PCR reaction for 15ADON and 3ADON genotypes. The 15ADON *TRI8* sequence was amplified using the forward primer 2036 (5'-CAGAAGCTTGTTCACTCACTCAGTATGGCTCTCG-3') and the reverse primer 2037 (5'-CAGGAATTCTGTCAAGAAATGAAATTACCAGGCAG-3'). The 3ADON *TRI8* sequence was amplified using the forward primer 2038 (5'-ACGAAGCTTATCCATCCATCCATTTAGTATGGTTCTCG-3') and reverse primer 2039 (5'-CAGGAATTCGGCTCACATTTCTTATCACAACCTCTC-3'). Thermal cycling conditions were 95 °C for 2 min, 30 cycles of 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 2 min, followed by a final extension at 72°C for 10 min. Each 29 µL PCR reaction consisted of 22.5 µL Supermix, 2 µL 10 µM of each forward and reverse primer, and 2.5 µL of fungal genomic DNA. Each *TRI8* PCR assay resulted in amplicons of approximately 1338-bp and 1299-bp for 15ADON and

3ADON assays, respectively. A 1-kb DNA Ladder (New England BioLabs, MA, USA) was used to score the molecular size of each amplicon.

A *TRII* PCR-RFLP assay developed by Liang et al. (2014), was used to differentiate 3ANX producers from other *F. graminearum* strains. The *TRII* gene was first amplified using the forward primer *TRIIF* (5'-ATGGCTCTCATCACCAG-3') and reverse primer *TRIIR* (5'-CAATTCCAATCGCAGACAA-3'). Each 29 µL PCR reaction consisted of 22.5 µL Supermix, 2 µL 10 µM of each forward and reverse primer, and 2.5 µL of fungal genomic DNA. Thermal cycling conditions were 94 °C for 2 min, 25 cycles of 94 °C for 1 min, 50.3 °C for 1 min, extension at 72 °C for 1:45 min, and a final extension at 72 °C for 10 min. A 1-kb DNA Ladder (New England BioLabs, MA, USA) was used to score the molecular size of each amplicon. Isolates that resulted in 1740-bp amplicons were then digested with the restriction endonuclease *ApoI* (New England Bio Labs, Inc., MA, USA), which is designed to produce two restriction fragments of approximately 888- and 851-bp for 15ADON, 3ADON and nivalenol genotypes, and three fragments of 407-, 482-, and 851-bp for 3ANX genotype. Amplicon digestion was performed in 15.15 µL volumes with 10 µL of *TRII* PCR amplicon, 5 µL 1X NEB Buffer 3.1, and 0.15 µL *ApoI* (5U/µL). Digestion was carried out at 50°C for 2 h. PCR products were separated by electrophoresis and scored manually using the 1-kb DNA Ladder (New England BioLabs, MA, USA).

Because some 3ANX and 3ADON isolates scored similarly in the *TRI3* and *TRI12* assays of Ward et al. (2002), Professor Christopher Toomajian (Department of Plant Pathology, Kansas State University) developed a *TRII* allele-specific PCR amplification approach to differentiate the two genotypes (Yue 2017). This approach uses two PCR assays with a constant reverse primer (*TRII-R*; 5'-TTCCTGCAGGGGCTTGATG-3') and differing forward primers (*TRII-F*-

3ADON; 5'-AATGCTCGCGAACTAATCAC-3', *TRII*-F-NX2; 5'AATGCTAGCGAAATGATCAA-3'). The first assay uses a forward primer that targets 3ADON genotypes, while the second assay uses a forward primer that targets 3ANX genotypes. These forward primers differ by four nucleotides, which Prof. Toomajian suggested match four single nucleotide polymorphisms in the 3ANX *TRII* gene. Each 29 µL PCR reaction consisted of 22.5 µL Supremix, 2 µL 10 µM of each forward and reverse primer, and 2.5 µL of fungal genomic DNA. The thermal cycling conditions for both assays were 94 °C for 2 min, 40 cycles of 94 °C for 30 s, 49.5 °C for 30 s, 68 °C for 1 min, and a final extension at 68 °C for 5 min. A GeneRuler 100-bp DNA ladder (Thermo Fisher Scientific, CA, USA) was used to score the molecular size of each amplicon. It is expected that isolates of the 3ADON genotype will only amplify in the 3ADON PCR assay and isolates of the 3ANX genotype will only amplify in the 3ANX PCR assay.

Subsamples of isolates from each year were chosen to represent a region in which *F. graminearum* was detected, the identity of these isolates were confirmed using ITS sequencing. Fungal strains were identified by their ITS region as described by Schoch et al. (2012). Fungal genomic DNA was extracted (Macharey-Nagel Nucleospin 96 Plant-II kit) from fresh cultures on 2% MEA. The ITS sequences were amplified using the forward ITS5 (5'-TCCTCCGCTTATTGATATGC-3') and the reverse primer ITS4 (5'-GGAAGTAAAAGTCGTAACAAGG-3') (Schoch et al. 2012). Thermal cycling conditions were 95 °C for 10 min, 35 cycles of 95 °C for 30 s, 52 °C for 30 s and 72 °C for 1.5 s, with a final extension of 72 °C for 7 min. Each 58 µL PCR reaction consisted of 45 µL Supremix, 4 µL 10 µM of each forward and reverse primer, and 5 µL of fungal genomic DNA. 10 µL of amplified PCR products were run in a 1.5% (*wt/vol*) agarose gel to confirm amplification. The remaining

48 µL of PCR product from each sample were sent to McGill University and Génome Québec Innovation Centre (Montreal, Québec, Canada) for sequencing. Sequencing of the ITS gene was performed using Sanger sequencing in both sense and antisense directions. The raw sequences were aligned using BioEdit and the identities were confirmed using BLAST (blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4 Liquid culture fermentation and extraction

The toxigenic potential of each of the 61 strains was determined from analysis of extracts of culture filtrates from liquid cultures and from agar plugs. The three control isolates were tested for comparison.

Fermentations in liquid culture followed the protocols outlined in Miller et al. (1983; 1991). Media were autoclaved at 121 °C for 12 min at 1.25 kg/cm². A 2% MEA plug from a plate of each strain was macerated in sterile distilled water (27 mL) under aseptic conditions. An aliquot (2.5 mL) was used to inoculate each of two 250 mL Erlenmeyer flasks each containing 50 mL of starter media (molasses (Selection, Metro) 20 g; glucose (BioShop) 30 g; fish-meal 15 g; Pharmamedia (ADM, Decatur, IL) 15 g per L of ultrapure water, Barnstead Nanopure Diamond). The cultures were placed on a rotary shaker (3.81 cm throw) at 220 rpm at 28 °C for 72 h in the dark. The resulting mycelium from the starter culture was macerated and 2.5 mL aliquots were added to six 250 mL Erlenmeyer flasks containing 50 mL of Glucose Yeast Extract Peptone (GYEP) medium (glucose (BioShop), 10 g; yeast extract (VWR), 1 g; peptone (Hi-Media Laboratories), 1 g per L ultrapure water, Barnstead Nanopure Diamond; pH 6.5). These cultures were incubated at 28 °C in still culture for 10 days in the dark.

After incubation, the liquid cultures were combined to give three solutions of 100 mL for each solution (i.e. two 250 ml flasks containing 50 mL liquid culture combined). The solutions

were filtered by suction through Whatman #4 (240 mm) filter papers (Whatman GE Healthcare, UK). 100 mL of culture filtrate was loaded on to a 100 mL Chem Elut column (Agilent Technologies, CA, USA) and extracted using three 100 mL washes of reagent grade ethyl acetate (CFS Chemicals). The extracts were dried on a rotary evaporator, then re-dissolved in methanol to transfer into 7 mL amber vials with PTFE lids. The resulting extracts were dried under nitrogen and stored at -20 °C until analysis.

To confirm that the original isolates were not mixed, four single-spore isolates were produced from DAOMC 251912. These isolates were fermented in liquid culture, extracted, and analyzed as described above.

2.5 Agar plate fermentation and extraction

The toxigenic potential of each of the 61 representative strains was also determined by analysis of extracts obtained from agar plate cultures after Visagie et al. (2016). All strains were grown on two 9 cm polystyrene Petri dishes on both GYEP agar (glucose (BioShop), 10 g; yeast extract (Difco), 1 g; peptone (Hi-Media Laboratories, 1 g; agar (BioShop), 18 g; per L ultrapure water, Barnstead Nanopure Diamond) and 2% MEA (malt extract (BD Biosciences) 20 g; agar (BioShop) 18 g; per L ultrapure water). The plates were incubated at 26 °C for 7 days in the dark. Three agar plugs were removed with a sterilized cork borer (s#4) from each of the three colonies on the plate for a total of nine plugs. The plugs were collected into a 20 mL scintillation vial and submerged in 2 mL ethyl acetate. The sample vials were then vortexed for 30 s, sonicated for 15 min at 25 °C followed by vortexing for another 30 s. The supernatants were transferred to clean amber vials and dried under a gentle stream of nitrogen. Extracts were reconstituted in 1 mL of 8:2 methanol:water and filtered into 2 mL amber glass HPLC vials

using a 0.45 μ m PVDF syringe filter. Fungal extracts were dried under nitrogen and stored at -20 °C until analysis.

2.6 LC-MS/MS chemical analysis

Chemical standards of nivalenol, 4-acetylnivalenol (fusarenone-X), 3ADON and 15ADON were purchased from Romer Labs (BC, Canada). 3ANX was isolated from liquid fermentations of DAOMC 242077. NX was produced by hydrolysis of the purified 3ANX (Aitken et al. 2019). All solvents used for MS analysis were Optima LC-MS grade (Fisher Scientific, ON, Canada). Extracts of fungal cultures were dissolved in methanol, 1 mg was transferred into 2 mL HPLC vials (PTFE lid) and dried under nitrogen. The samples were reconstituted by the addition of 500 μ L of LC-MS grade methanol and vortexed for 10 s. 500 μ L of LC-MS grade water was then added and samples were syringe filtered through a 0.45 μ m PTFE filter (ChromeSpec Inc., ON, Canada) into 2 mL HPLC vials. All MS data were collected with a Q-Exactive™ Quadrupole Orbitrap mass spectrometer (Thermo Scientific, MA, USA) coupled to an Agilent 1290 high-performance liquid chromatography system. Five μ L injections were resolved on a Zorbax Eclipse Plus RRHD C18 column (2.1 x 100 mm, 1.8 μ m; Agilent Technologies, CA, USA) maintained at 35 °C (Renaud & Sumarah, 2016). Separation was achieved using a flow rate of 0.3 mL/min with a mobile phase of water with 0.1% formic acid (A), and acetonitrile with 0.1% formic acid (B). Mobile phase B was held at 5% for 30 seconds, before increasing to 22% over 30 seconds. Mobile phase B was then increased to 37% over 3 minutes before increasing to 100% over 1.5 minutes. Mobile phase B was held at 100% for 2 minutes before returning to 5% over 45 seconds. The following conditions were used for atmospheric pressure chemical ionization (APCI): spray current, 3.5 kV; capillary temperature, 300 °C; sheath gas, 32 arbitrary units; auxiliary gas, 10 arbitrary units; probe heater temperature,

250 °C, S-Lens rf level, 50%. Compounds were analyzed using full MS, and MS/MS at a resolution of 17, 500, automatic gain control target of 1×10^6 , maximum injection time (IT) of 64 ms and isolation width of 1.2 amu. Between the mass range m/z 100-1000, full MS scans were acquired throughout the entire LC run to allow for retrospective analysis and detection of other fungal metabolites.

3.0 Results

3.1 *F. graminearum* contamination on corn and wheat kernels

A total of 141 samples of corn were collected from 28 farms from the 2015 crop year. *F. graminearum* was detected in 6.3% ($n = 9$) of corn kernel samples and 7.1% ($n = 2$) of corn producing farms (Table 1A). A total of 398 wheat samples were collected from 66 wheat-producing farms in 2015. *F. graminearum* was detected in 19.3% ($n = 77$) of wheat samples overall and 39% ($n = 26$) of wheat producing farms, all from counties in southern Ontario (Table 2A). Each isolate was preliminarily identified as *F. graminearum* based on morphological characteristics, which was confirmed using a PCR assay targeting the *TEF* gene after O'Donnell et al. (2000). In this crop year, most isolates of *F. graminearum* that were recovered came from the most southern counties of Ontario between Wellington and Essex Counties (43.8725° N, 80.8346° W to 42.1291° N, 82.9093° W), excepting one isolate recovered from wheat in Kemptville (45.0064° N, 75.6329° W) (Figure 7). In the month of June, precipitation was higher than normal for all counties in which *F. graminearum* contaminated samples were recovered (Table 4).

Figure 7. Sites with *Fusarium graminearum* contaminated wheat and corn kernels collected across Ontario in 2015.

Table 4. Total precipitation (mm) and mean temperature (°C) in June for grain growing counties across Ontario from 2015-2017. Data obtained from Environment and Climate Change Canada (ECCC) historical data for each region.

Year		Essex	Kent	Lambton	Middlesex	Huron	Wellington	Kemptville
2015	Total precipitation (mm)	132.4	52.7	100.4	173.1	164.9	139.5	141.1
	Mean temperature (°C)	19.2	20.9	17.2	18	16.3	16.2	16.9
2016	Total precipitation (mm)	26.4	97.7	87.5	63.1	48.2	48.7	54.3
	Mean temperature (°C)	21.6	18.1	18.4	18.4	16.8	16.5	18.2
2017	Total precipitation (mm)	86.5	51.1	35.8	67.6	192	245.1	144.1
	Mean temperature (°C)	20.9	18.9	20.1	19.3	18.1	17.2	17.6

From the 2016 crop year, a total of 299 shelled corn samples were collected from 76 farms. *F. graminearum* was detected in 36.7% (n = 110) of corn kernel samples and 9.2% (n = 7)

of corn-producing farms in 2016 (Table 3A). A total of 191 grain samples were collected from 24 wheat-producing farms in 2016. *F. graminearum* was detected in 18.8% (n = 36) of grain samples overall and 58.3% (n = 14) of wheat producing farms, all from counties in southern Ontario (Table 4A). For the year 2016, all *F. graminearum* isolates were recovered from the southern counties of Ontario, between Perth and Chatham-Kent (43.4175° N, 81.4492° W to 42.3536° N, 81.9145° W) (Figure 8). In the month of June, precipitation was recorded as lower than normal for sites where *F. graminearum* was recovered (Table 4).

Figure 8. Sites with *Fusarium graminearum* contaminated wheat and corn kernels collected across Ontario in 2016.

From the 2017 crop year, a total of 333 shelled corn samples were collected from 124 farms. *F. graminearum* was detected in 12.3% (n = 41) of corn kernel samples and 3.2% (n = 4) of corn producing farms in 2017 (Table 5A). A total of 490 grain samples were collected from 28 wheat-producing farms in 2017. *F. graminearum* was detected in 39.8% (n = 195) of grain

samples overall and 28.6% (n = 8) of wheat producing farms, all from counties in southern Ontario (Table 6A). Isolates from 2017 were distributed across Ontario, with the majority in Southern Ontario between Wellington and Essex counties (43.9200° N, 80.0° W to 42.0952° N, 82.6854° W). Isolates were also recovered from wheat in the eastern county Kemptville (45.0064° N, 75.6329° W) and in St-Hyacinthe, Québec (45.6282° N, 72.9568° W) (Figure 9). For the month of June, precipitation was lower than normal for the more southern sampling locations such as Essex, Chatham-Kent, Middlesex and Lambton. Huron, Wellington and eastern Ontario received higher than normal precipitation (Table 4).

Figure 9. Sites with *Fusarium graminearum* contaminated wheat and corn kernels collected across Ontario and St-Hyacinthe, Quebec in 2017.

Representative isolates were chosen for further testing based on the proportion to the total within a county each crop year while making sure to include isolates from across the sampling area. From 2015, 20 representative isolates were chosen with 18 and 2 recovered from wheat and

corn respectively (Table 1). From the 2016 harvest, 22 representative isolates were selected with 5 and 17 isolates recovered from wheat and corn, respectively (Table 2). Some 19 isolates were selected to represent the 2017 harvest, with 13 and 6 samples from wheat and corn respectively (Table 3).

3.2 Chemical analysis

The 61 representative strains of *F. graminearum* were grown under three conditions (GYEP agar, 2% ME agar, and liquid culture) and screened using our LC-MS/MS method to detect targeted trichothecenes. The extracts were compared to standards of nivalenol, 4-acetylnivalenol, 3ADON, 15ADON, sambucinol, zearalenone and 3ANX. For the liquid fermentation cultures, the presence of each analyte (DON, 15ADON, 3ADON, and 3ANX) was confirmed. The 15ADON, 3ADON and 3ANX control strains produced the expected monoacetate in all conditions. The 15ADON control strain (DAOMC 180378) had a 15ADON peak at the retention time 3.78 minutes, and confirmed by the intensity ratio of m/z 137.1/231.1 > 1 indicating 15ADON using the MS/MS spectrum (Figure 10 and 11). The 3ADON control strain (DAOMC 242075) had a 3ADON peak at the retention time 3.91, which was confirmed by the intensity ratio of m/z 137.1/231.1 < 1 indicating 3ADON using the MS/MS spectrum (Figure 12 and 13). The 3ANX control strain (DAOMC 242077) had a 3ANX peak at 4.05 minutes, the presence of the product ions m/z 199.1 and 137.1 in the MS/MS spectrum confirmed the presence (Figure 14 and 15).

Figure 10. LC-MS/MS extracted ion chromatogram of 15ADON detected in culture filtrate extracts from the 15ADON chemotype DAOMC 180378.

Figure 11. MS/MS spectrum of 15ADON detected in culture extracts from the 15ADON chemotype DAOMC 180378. Presence confirmed by a retention time of 3.78 mins and product ion intensity ratio of m/z 137.1/231.1 > 1 indicating 15ADON.

Figure 12. LC-MS/MS extracted ion chromatogram of 3ADON detected in culture filtrate extracts from the 3ADON strain DAOMC 242075.

Figure 13 MS/MS spectrum of 3ADON detected in culture extracts from the 3ADON strain DAOMC 242075. Presence confirmed by the retention time of 3.91 mins and the product ion intensity ratio of m/z 137.1/231.1 < 1 indicating 3ADON.

Figure 14. LC-MS/MS extracted ion chromatogram of 3ANX detected in culture filtrate extracts from the 3ANX strain DAOMC 242077.

Figure 15 MS/MS spectra of 15ADON detected in culture extracts from the 3ANX strain DAOMC 242077. Presence was confirmed by the retention time of 4.05 min, and the presence of product ions m/z 199.1 and 137.1.

Figure 16. LC-MS/MS extracted ion chromatograms of (a) 15ADON and (b) 3ANX detected in culture filtrate extracts from the DAOMC 251918.

Figure 17. MS/MS spectra of (a) 15ADON and (b) 3ANX detected in culture extracts from DAOMC 251918.

3.2.1 Chemical analysis of 2015-2017 *F. graminearum* strains tested

In all three media, 15% (n = 3) of the 20 representative strains from 2015 produced 15ADON and no 3ANX, while only 5% (n = 1) produced 3ANX and no 15ADON. Interestingly, we found that 80% (n = 16) of the isolates examined, co-produced detectable levels of both 15ADON and 3ANX in at least one medium (Figure 16 and 17). Some differences were observed with the strain grown on different growth media. Under the growth conditions where there was co-production of 15ADON and 3ANX, the amount of 15ADON was greater than the amount of 3ANX in 88%, 100% and 63% for 2% ME agar, GYEP agar and liquid culture respectively.

Of the 22 strains from 2016, 31.8% (n = 7) produced solely 15ADON. No strains were found to produce only 3ANX or 3ADON. The majority of the strains (59.1%, n = 13) produced both 15ADON and 3ANX in at least one medium. As seen with the 2015 strains, where there was co-production, the amount of 15ADON produced was higher than 3ANX. Two strains (9.1%), 25178 and 25437, did not produce the toxins.

From the 2017 representative isolates (n = 19), 15.8% (n = 3) produced 15ADON and no 3ANX, and only 5.3% (n = 1) produced solely 3ANX. As with 2015 and 2016, the majority (73.6%, n = 14) of strains produced both 15ADON and 3ANX on at least one medium. One strain 26330 was found to produce 3ADON and 3ANX separately in different media. Chemical analysis of the plated cultures on both GYEP agar and 2% ME agar resulted in 3ADON production, where liquid cultures produced 3ANX.

3.2.2 Confirmation of 15ADON and 3ANX co-production

To confirm that 3ANX was present in the same fermentation cultures as 15ADON, the MS/MS spectra of a 3ANX standard were compared to 3ANX detected in strain DAOMC

251912 at two different collision energies. Not only were the accurate masses of the product ions observed in MS/MS spectra from the standard and the compound in the extracts identical (< 3 ppm), their relative intensity ratios matched at both collision energies. To confirm that the co-detection of both 15ADON and 3ANX in the majority of strains was not the result of mixed cultures, single spore isolates of DAOMC 251907 were produced and confirmed the co-production of both compounds (Figure 9). The majority of strains also produced zearalenone and sambucinol under one or more of the growth conditions tested (Tables 5-7).

Table 5. Chemical analysis of culture extracts from 20 representative strains collected across Ontario in 2015.

DAOMC	Chemical assay Analytes detected				
	15ADON	3ADON	3ANX	Sambucinol	Zearalenone
<i>180378*</i>	+			+	+
<i>242075*</i>		+		+	+
<i>242077*</i>			+	+	+
<i>251903</i>	+		+	+	+
<i>251904</i>	+			+	+
<i>251905</i>	+		+	+	+
<i>251906</i>	+		+	+	+
<i>251907</i>	+		+	+	+
<i>251908</i>	+		+	+	+
<i>251909</i>	+		+	+	+
<i>251910</i>	+				
<i>251911</i>	+		+	+	+
<i>251912</i>	+		+	+	+
<i>251913</i>	+		+	+	+
<i>251914</i>	+		+	+	
<i>251915</i>	+		+		+
<i>251916</i>	+		+	+	
<i>251917</i>	+			+	
<i>251918</i>	+		+	+	+
<i>251919</i>	+		+	+	+
<i>251920</i>	+		+	+	+
<i>251921</i>	+		+	+	+
<i>251922</i>	+		+	+	

Table 6. Chemical analysis of culture extracts from 22 representative strains collected across Ontario in 2016.

Sample ID	Chemical assay Analytes detected				
	15ADON	3ADON	3ANX	Sambucinol	Zearalenone
180378*	+			+	+
242075*		+		+	+
242077*			+	+	+
24553	+		+	+	+
24558	+		+	+	+
24590	+				+
24782	+		+	+	+
24893	+		+	+	+
25173	+		+	+	+
25178				+	+
25191	+			+	+
25192	+		+	+	+
25213	+				+
25219	+			+	+
25232	+		+	+	+
25241	+			+	+
25250	+		+	+	+
25268	+				+
25281	+		+		+
25291	+		+	+	+
25432	+		+	+	+
25437				+	+
25442				+	+
25450	+		+	+	+
25457	+		+	+	+

Table 7. Chemical analysis of culture extracts from 19 representative strains collected across Ontario in 2017.

Sample ID	Chemical assay Analytes detected				
	15ADON	3ADON	3ANX	Sambucinol	Zearalenone
<i>180378*</i>	+			+	+
<i>242075*</i>		+		+	+
<i>242077*</i>			+	+	+
<i>26203</i>	+		+		+
<i>26223</i>	+				+
<i>26233</i>	+		+		+
<i>26292</i>	+		+	+	+
<i>26295</i>	+		+	+	+
<i>26296</i>	+		+		
<i>26301</i>	+		+	+	+
<i>26314</i>	+		+		+
<i>26320</i>	+		+	+	+
<i>26326</i>			+	+	+
<i>26330</i>		+	+		+
<i>26331</i>	+		+		+
<i>26335</i>	+		+		+
<i>26348</i>	+		+		
<i>26349</i>	+				+
<i>26359</i>	+		+		+
<i>26361</i>	+				+
<i>26362</i>	+		+		+
<i>36368</i>	+		+		+

3.3 Molecular identification

Contaminated kernels with cultures that morphologically resembled *F. graminearum* were confirmed by sequencing of the *TEF* gene after O'Donnell et al. (2000). Isolates not identified as *F. graminearum* were omitted from further analysis. The identity of the 61 representative strains from 2015-2017 were further confirmed by sequencing the *ITS* region after Schoch et al. (2012). Five PCR assays that target the trichothecene core gene cluster were used to predict trichothecene genotype. The genotype analysis of 15ADON (DAOMC 180378), 3ADON (DAOMC 242075) and 3ANX (DAOMC 242077) strains resulted in the expected monoacetate. Genotype analysis using multiplex PCR targeting genetic variation in *TRI3* and *TRI12* genes were used on all 468 isolates identified as *F. graminearum* (Figure 18). For 2015 and 2016, 100% of isolates were identified as 15ADON genotype. In 2017, this percentage lowered to 88.9% (n = 209) and 11.1% (n = 26) for 15ADON and 3ADON genotypes, respectively. Of the 26 isolates identified as 3ADON genotype using this assay, three originated from St. Hyacinthe, Québec (250 km east of Ottawa) and the other 23 were from inoculation trials in Ridgeway in southern Ontario.

Figure 18. Amplification products of control strains and 4 representative *F. graminearum* strains using *TRI3* multiplex-PCR assay. **L**, 100-bp DNA ladder; **C1**, 15ADON control strain (DAOMC 180378); **C2**, 3ADON control strain (DAOMC 242075); **C3**, 3ANX control strain (DAOMC 242077); **Lanes 1-4**, DAOMC 251903, DAOMC 251904, DAOMC 251905 and DAOMC 251906.

Further genotype analyses using *TRI8* PCR assay, *TRII* RFLP-PCR assay, and *TRII* PCR assay were performed only on the control strains and representative strains from each year. The *TRI8* PCR assay resulted in 15ADON amplification for 75% ($n = 15$) of the representative strains in 2015 (Figure 19). The remaining 25% ($n = 5$) did not result in amplification in either 15ADON or 3ADON *TRI8* assay (Figure 20). In the next year 2016, the number of strains that resulted in amplification in either assay decreased to 18.18% ($n = 4$), the remaining 81.81% ($n = 18$) resulted in no amplification. In 2017, only 10.5% ($n = 2$) of the representative strain tested resulted in 15ADON amplification. The remaining 89.5% ($n = 17$) could not be assigned to a

genotype. Using this assay, the percentage of strains where genotype could not be assigned increased from 25% to 89.5% from 2015-2017.

Figure 19. Amplification products of control strains and 6 representative *F. graminearum* strains using *TRI8* (15ADON) PCR assay. **L**, 1-kb DNA ladder; **C1**, 15ADON control strain (DAOMC 180378); **C2**, 3ADON control strain (DAOMC 242075); **C3**, 3ANX control strain (DAOMC 242077); **Lanes 1-6**, DAOMC 251903, DAOMC 251904, DAOMC 251905, DAOMC 251906, DAOMC 251907 and DAOMC 251908.

Figure 20. Amplification products of control strains and 4 representative *F. graminearum* strains using *TRI8* (3ADON) PCR assay. **L**, 1-kb DNA ladder; **C1**, 15ADON control strain (DAOMC 180378); **C2**, 3ADON control strain (DAOMC 242075); **C3**, 3ANX control strain (DAOMC 242077); **Lanes 1-4**, DAOMC 251903, DAOMC 251904, DAOMC 251905 and DAOMC 251906.

Figure 21 Amplification products of control strains and 4 representative *F. graminearum* strains using *TRII* RFLP-PCR assay. **L**, 1-kb DNA ladder; **C1**, 15ADON control strain (DAOMC 180378); **C2**, 3ADON control strain (DAOMC 242075); **C3**, 3ANX control strain (DAOMC 242077); **Lanes 1-4**, DAOMC 251903, DAOMC 251904, DAOMC 251905 and DAOMC 251906.

Figure 22. ApoI digestion products of control strains and 4 representative *F. graminearum* strains using *TRII* RFLP-PCR assay described by Liang et al. (2014). **L**, 100-bp DNA ladder; **C1**, 15ADON control strain (DAOMC 180378); **C2**, 3ADON control strain (DAOMC 242075); **C3**, 3ANX control strain (DAOMC 242077); **Lanes 1-4**, DAOMC 251903, DAOMC 251904, DAOMC 251905 and DAOMC 251906.

Figure 23. Amplification products of control strains and 4 representative *F. graminearum* strains using *TRII* (ADON) PCR assay described by Prof. Toomajian. **L**, 100-bp DNA ladder; **C1**, 15ADON control strain (DAOMC 180378); **C2**, 3ADON control strain (DAOMC 242075); **C3**, 3ANX control strain (DAOMC 242077); **Lanes 1-4**, DAOMC 251915, DAOMC 251916, DAOMC 251917 and DAOMC 251919.

Figure 24. Amplification products of control strains and 4 representative *F. graminearum* strains using *TRII* (3ANX) PCR assay described by Prof. Toomajian. **L**, 100-bp DNA ladder; **C1**, 15ADON control strain (DAOMC 180378); **C2**, 3ADON control strain (DAOMC 242075); **C3**, 3ANX control strain (DAOMC 242077); **Lanes 1-4**, DAOMC 251915, DAOMC 251916, DAOMC 251917 and DAOMC 251919.

3.3.1 Concordance with probe results

We found that all probes tested were consistent with the expected results for the control strains used in this study. The probes for *TRI3* and *TRII2* reported by Ward et al. (2002) and Starkey et al. (2007) as well as the *TRI8* assay reported by Alexander et al. (2011) indicated that the well-studied 15ADON producing strain DAOMC 180378 represented the 15ADON genotype correctly. The 3ADON producing strain DAOMC 242075 also tested correctly as a 3ADON genotype for *TRI3*, *TRII2*, and *TRI8* assays. As expected, the 3ANX control strain DAOMC 242077 tested as a 3ADON genotype for *TRI3*, *TRII2*, and *TRI8* assays. The PCR-RFLP assay targeting *TRII* (Liang et al. 2014) and a separate *TRII* assay developed by Prof. Toomajian

believed to discriminate between 3ANX and ADON genotypes (15ADON or 3ADON). Both the PCR-RFLP and Toomajian assays resolved DAOMC 242077 as a 3ANX genotype. When applied to DAOMC 180378 (15ADON) and DAOMC 242075 (3ADON) strains, the PCR-RFLP assay indicated the ADON genotype (Figure 22). The Toomajian *TRI1* assay correctly distinguished the 3ADON and 3ANX strains however, suggested the 15ADON control strain was both an ADON and 3ANX genotype (Figure 23 and 24).

In contrast to the agreement observed in the positive control strains, genotypes based on the literature probes of the strains collected in the present study did not align with the genetic assays for the control strains. For 55% (n = 11) of 2015 strains, the combined probe results provided no genotype consensus with any of the positive control strains. The 3ANX producer, DAOMC 251911, indicated a 15ADON genotype for the *TRI3*, *TRI2* and *TRI8* assays. The PCR-RFLP assay targeting *TRI1* resulted in ADON genotype, whereas the assay developed by Prof. Chris Toomajian suggested that DAOMC 251911 was both an ADON and 3ANX genotype, consistent with the 15ADON positive control strain (DAOMC 180378). The three strains producing only 15ADON were DAOMC 251904, 251910 and 251917. The genetic analysis performed in this study obtained a consensus match with the 15ADON type strain for DAOMC 251904, 251910. However, the PCR-RFLP assay could not identify DAOMC 251917 as an ADON nor a 3ANX genotype.

Strains that could produce both 15ADON and 3ANX in liquid culture were isolated from both wheat (n = 14) and corn (n = 2) including DAOMC 251906, 251907, 251908, 251913, 251916 and 251921. All strains resulted in 15ADON amplification for *TRI3* and *TRI2* assay. Although these six strains produced both 15ADON and 3ANX in liquid culture, the PCR results matched those obtained from the 15ADON strain (DAOMC 180378). For the remaining 10

strains that produced both 15ADON and 3ANX in liquid culture, there was no evident genotype compared to the control strains (Table 8).

For 95% (n = 20) of 2016 strains, the combined probe results did not result in a genotype consensus with any of the type strains. Of these strains, there were none that produced only ANX. The combined probe results obtained a consensus match with the 15ADON type strain for strain 24552 only. All strains that were of the 15ADON chemotype resulted in 15ADON genotype for both *TRI3* and *TRI2* assays, and ADON genotype for the *TRI1* PCR-RFLP assay. Strains 24590, 25219 and 25268 resulted in no amplification for the *TRI8* assay, and the ADON genotype by the Toomajian *TRI1* PCR assay. Strain 24191 resulted in 15ADON amplification for the *TRI8* assay, and no amplification for either ADON or 3ANX genotype using the Toomajian assay. The remaining strains 25213 and 25241 resulted in no amplification for the *TRI8* assay, whereas the Toomajian *TRI1* PCR assay suggested both ADON and 3ANX genotype.

Extracts from strains 25178 and 25437 did not have detectable levels of 15ADON or 3ANX in either plate or liquid cultures. The combined probe results for each strain were identical. Both strains resulted in 15ADON for *TRI3* and *TRI2* PCR assays, ADON genotype for *TRI1* PCR-RFLP assay, ADON and 3ANX genotype for the Toomajian *TRI1* PCR assay, no amplification by the *TRI8* PCR assay.

Strains that could produce both 15ADON and 3ANX were isolated from both wheat (n = 4) and corn (n = 9). Although these strains produced both 15ADON and 3ANX, the strain 25281 obtained a consensus match with the 15ADON control strain (DAOMC 180378). The remaining 12 strains that produced both 15ADON and 3ANX did not match genotype consensus with any of the control strains (Table 9).

For all 2017 representative strains, the overall probe results provided no genotype consensus with any of the control strains (Table 10). Of these strains there was one 3ANX producer, three 15ADON producers, fourteen 15ADON and 3ANX producers, and one 3ADON and 3ANX producer. The 3ANX producer, 26326, indicated a 3ADON genotype for the *TRI3* and *TRI12* assays. The *TRI8* resulted in 15ADON genotype, the *TRI1* PCR-RFLP assay resulted in ADON genotype, and no amplification by the Toomajian *TRI1* PCR assay. The 3ADON and 3ANX producer, 26330, resulted in a 15ADON genotype for *TRI3* and *TRI12* assays. The *TRI1* PCR-RFLP assay and Toomajian *TRI1* PCR assay resulted in the ADON genotype, with no amplification in the *TRI8* assay. The three strains producing only 15ADON were 26223, 26361 and 26349. These strains resulted in 15ADON genotype by *TRI3* and *TRI12*, ADON genotype using the Toomajian *TRI1* PCR assay, and no amplification in the *TRI8* PCR assay. The strains 26223 and 26361 obtained an ADON genotype using the *TRI1* PCR-RFLP assay, where strain 26349 resulted in no amplification.

Strains that could produce both 15ADON and 3ANX were isolated from both wheat (n = 10) and corn (n = 4). All except for two strains that resulted in a determination of the 15ADON genotype using the *TRI3* and *TRI12* assays. The two remaining strains, 26314 and 26335 resulted in 3ADON genotype for the *TRI3* and *TRI12* assays. Strain 26314 resulted in the ADON genotype based on the *TRI1* PCR-RFLP assay, however, there was no amplification in the *TRI8* and *TRI1* PCR assays. Strain 26335 resulted in ADON genotype for both *TRI1* PCR and PCR-RFLP assays, and 15ADON by the *TRI8* PCR assay. The remaining strains that resulted in 15ADON genotype using *TRI3* and *TRI12* assays, did not match genotype results to the control strains (Table 10).

Table 8. Genotype and chemotype of 2015 strains tested.

DAOMC	Location	Chemical analysis ¹	Genetic analysis					Chemical analysis		
			Starkey ² <i>TRI3/TRI12</i>	Alexander ³ <i>TRI8</i>	Liang ⁴ <i>TRI1</i>	Toomajian ⁵ (<i>TRI1</i>)		analytes detected		
						ADON	ANX	GYEP	MEA	liquid
180378*	Ottawa, ON	15ADON	15ADON	15ADON	ADON	ADON	3ANX	15ADON	15ADON	15ADON
242075*	New Brunswick	3ADON	3ADON	3ADON	ADON	ADON	-	3ADON	3ADON	3ADON
242077*	Annapolis, NS	3ANX	3ADON	3ADON	ANX	-	3ANX	3ANX	3ANX	3ANX
<i>251904</i>	Essex	15ADON	15ADON	15ADON	ADON	ADON	3ANX	15ADON	15ADON	15ADON
<i>251910</i>	Chatham-Kent	15ADON	15ADON	15ADON	ADON	ADON	3ANX	15ADON	nd	15ADON
<i>251917</i>	Huron	15ADON	15ADON	15ADON	-	ADON	3ANX	15ADON	15ADON	15ADON
<i>251908</i>	Lambton	15ADON,3ANX	15ADON	15ADON	ADON	ADON	3ANX	15ADON, 3ANX	nd	15ADON, 3ANX
<i>251906</i>	Essex	15ADON,3ANX	15ADON	15ADON	ADON	ADON	3ANX	15ADON, 3ANX	15ADON, 3ANX	15ADON, 3ANX
<i>251907</i>	Middlesex	15ADON,3ANX	15ADON	15ADON	ADON	ADON	3ANX	15ADON, 3ANX	nd	15ADON, 3ANX
<i>251921</i>	Lambton	15ADON,3ANX	15ADON	15ADON	ADON	ADON	3ANX	15ADON	15ADON	15ADON, 3ANX
<i>251913</i>	Middlesex	15ADON,3ANX	15ADON	15ADON	ADON	ADON	3ANX	15ADON	15ADON	15ADON, 3ANX
<i>251916</i>	Huron	15ADON,3ANX	15ADON	15ADON	ADON	ADON	3ANX	15ADON	15ADON, 3ANX	15ADON, 3ANX
<i>251918</i>	Perth	15ADON,3ANX	15ADON	15ADON	-	-	3ANX	15ADON, 3ANX	15ADON, 3ANX	15ADON, 3ANX
<i>251909</i>	Chatham-Kent	15ADON,3ANX	15ADON	15ADON	ADON	-	3ANX	15ADON	nd	15ADON, 3ANX
<i>251914</i>	Chatham-Kent	15ADON,3ANX	15ADON	-	ADON	ADON	3ANX	15ADON	3ANX	15ADON, 3ANX
<i>251912</i>	Chatham-Kent	15ADON,3ANX	15ADON	-	ADON	ADON	-	15ADON	15ADON	15ADON, 3ANX
<i>251903</i>	Lambton	15ADON,3ANX	15ADON	15ADON	ADON	ADON	-	15ADON, 3ANX	15ADON	15ADON, 3ANX
<i>251920</i>	Middlesex	15ADON,3ANX	15ADON	-	ADON	ADON	3ANX	15ADON, 3ANX	15ADON, 3ANX	15ADON
<i>251915</i>	Essex	15ADON,3ANX	15ADON	-	ADON	ADON	3ANX	15ADON, 3ANX	15ADON	15ADON, 3ANX
<i>251922</i>	Elgin	15ADON,3ANX	15ADON	15ADON	ADON	-	-	15ADON	15ADON, 3ANX	15ADON, 3ANX
<i>251919</i>	Perth	15ADON,3ANX	15ADON	-	ADON	ADON	-	15ADON	15ADON	15ADON, 3ANX
<i>251905</i>	Wellington	15ADON,3ANX	15ADON	15ADON	ADON	ADON	-	15ADON, 3ANX	nd	3ANX
<i>251911</i>	Chatham-Kent	3ANX	15ADON	15ADON	ADON	ADON	3ANX	nd	nd	3ANX

* genotype/chemotype strain; ¹ Total range of target analytes detected in all three growth conditions; ² Starkey et al. (2007); ³ Alexander et al. (2011); ⁴ Liang et al. (2014); ⁵ see text.

Table 9. Genotype and chemotype of 2016 strains tested.

Isolate	Location	Chemical analysis ¹	Genetic analysis					Chemical analysis		
			Starkey ² <i>TRI3/TRI12</i>	Alexander ³ <i>TRI8</i>	Liang ⁴ <i>TRI1</i>	Toomajian ⁵ (<i>TRI1</i>)		analytes detected		
						ADON	3ANX	GYEP	MEA	liquid
180378*	Ottawa, ON	15ADON	15ADON	15ADON	ADON	ADON	3ANX	15ADON	15ADON	15ADON
242075*	New Brunswick	3ADON	3ADON	3ADON	ADON	ADON	-	3ADON	3ADON	3ADON
242077*	Annapolis, NS	3ANX	3ADON	3ADON	ANX	-	3ANX	3ANX	3ANX	3ANX
24590	Chatham-Kent	15ADON	15ADON	-	ADON	ADON	-	15ADON	15ADON	nd
25191	Chatham-Kent	15ADON	15ADON	15ADON	ADON	-	-	15ADON	15ADON	nd
25213	Chatham-Kent	15ADON	15ADON	-	ADON	ADON	3ANX	15ADON	nd	nd
25219	Elgin	15ADON	15ADON	-	ADON	ADON	-	15ADON	15ADON	nd
25241	Chatham-Kent	15ADON	15ADON	-	ADON	ADON	3ANX	15ADON	15ADON	nd
25268	Chatham-Kent	15ADON	15ADON	-	ADON	ADON	-	15ADON	nd	nd
25442	Chatham-Kent	15ADON	15ADON	15ADON	ADON	ADON	3ANX	15ADON	nd	nd
24553	Middlesex	15ADON, 3ANX	15ADON	-	ADON	ADON	-	15ADON, 3ANX	nd	nd
24558	Perth	15ADON, 3ANX	15ADON	15ADON	ADON	-	3ANX	15ADON	nd	15ADON, 3ANX
24782	Chatham Kent	15ADON, 3ANX	15ADON	-	ADON	ADON	-	15ADON	15ADON, 3ANX	15ADON, 3ANX
24893	Lambton	15ADON, 3ANX	15ADON	-	ADON	-	3ANX	15ADON	nd	15ADON, 3ANX
25173	Chatham-Kent	15ADON, 3ANX	15ADON	-	ADON	ADON	3ANX	15ADON	15ADON	15ADON, 3ANX
25192	Chatham-Kent	15ADON, 3ANX	15ADON	-	ADON	ADON	3ANX	nd	15ADON, 3ANX	3ANX
25232	Chatham-Kent	15ADON, 3ANX	15ADON	-	ADON	-	3ANX	15ADON	15ADON, 3ANX	15ADON, 3ANX
25250	Chatham-Kent	15ADON, 3ANX	15ADON	-	ADON	ADON	3ANX	15ADON	3ANX	nd
25281	Chatham-Kent	15ADON, 3ANX	15ADON	15ADON	ADON	ADON	3ANX	15ADON	15ADON, 3ANX	15ADON
25291	Chatham-Kent	15ADON, 3ANX	15ADON	-	ADON	ADON	3ANX	15ADON	15ADON	15ADON, 3ANX
25432	Chatham-Kent	15ADON, 3ANX	15ADON	-	ADON	ADON	3ANX	15ADON, 3ANX	15ADON	nd
25450	Chatham-Kent	15ADON, 3ANX	15ADON	-	ADON	ADON	3ANX	15ADON	15ADON, 3ANX	nd
25457	Chatham-Kent	15ADON, 3ANX	15ADON	-	ADON	ADON	3ANX	15ADON, 3ANX	15ADON, 3ANX	15ADON, 3ANX
25178	Chatham-Kent	-	15ADON	-	ADON	ADON	3ANX	nd	nd	nd
25437	Chatham-Kent	-	15ADON	-	ADON	ADON	3ANX	nd	nd	nd

* genotype/chemotype strain; ¹ Total range of target analytes detected in all three growth conditions; ² Starkey et al. (2007); ³ Alexander et al. (2011); ⁴ Liang et al. (2014); ⁵ see text.

Table 10. Genotype and chemotype of 2017 strains tested.

Isolate	Location	Chemical analysis ¹	Genetic analysis					Chemical analysis		
			Starkey ² <i>TRI3/TRI12</i>	Alexander ³ <i>TRI8</i>	Liang ⁴ <i>TRI1</i>	Toomajian ⁵ (<i>TRI1</i>)		analytes detected		
						ADON	ANX	GYEP	MEA	liquid
180378*	Ottawa, ON	15ADON	15ADON	15ADON	ADON	ADON	3ANX	15ADON	15ADON	15ADON
242075*	New Brunswick	3ADON	3ADON	3ADON	ADON	ADON	-	3ADON	3ADON	3ADON
242077*	Annapolis, NS	3ANX	3ADON	3ADON	ANX	-	3ANX	3ANX	3ANX	3ANX
26223	Middlesex	15ADON	15ADON	-	ADON	ADON	-	15ADON	15ADON	15ADON
26349	Chatham-Kent	15ADON	15ADON	-	-	ADON	-	15ADON	15ADON	-
26361	Huron	15ADON	15ADON	-	ADON	ADON	-	15ADON	15ADON	15ADON
26203	Middlesex	15ADON,3ANX	15ADON	-	-	-	-	15ADON	15ADON	15ADON, 3ANX
26233	Essex	15ADON,3ANX	15ADON	-	ADON	-	-	15ADON	nd	3ANX
26292	Leeds and Grenville	15ADON,3ANX	15ADON	-	ADON	ADON	-	15ADON, 3ANX	3ANX	15ADON, 3ANX
26295	Leeds and Grenville	15ADON,3ANX	15ADON	-	ADON	ADON	-	15ADON, 3ANX	15ADON	15ADON, 3ANX
26296	Leeds and Grenville	15ADON,3ANX	15ADON	-	ADON	ADON	-	15ADON, 3ANX	nd	3ANX
26301	Wellington	15ADON,3ANX	15ADON	-	ADON	ADON	-	15ADON	nd	15ADON, 3ANX
26314	Montréal	15ADON,3ANX	3ADON	-	ADON	-	-	15ADON	nd	15ADON, 3ANX
26320	Montréal	15ADON,3ANX	15ADON	-	ADON	-	-	15ADON	15ADON	15ADON, 3ANX
26331	Montréal	15ADON,3ANX	15ADON	-	-	ADON	-	15ADON, 3ANX	15ADON, 3ANX	3ANX
26335	Montréal	15ADON,3ANX	3ADON	15ADON	ADON	ADON	-	15ADON	nd	3ANX
26348	Elgin	15ADON,3ANX	15ADON	-	ADON	ADON	-	15ADON	nd	15ADON, 3ANX
26359	Huron	15ADON,3ANX	15ADON	-	ADON	ADON	-	15ADON	15ADON	15ADON, 3ANX
26362	Huron	15ADON,3ANX	15ADON	-	ADON	ADON	-	15ADON, 3ANX	15ADON	15ADON, 3ANX
36368	Huron	15ADON,3ANX	15ADON	-	ADON	ADON	-	15ADON	nd	3ANX
26326	Montréal	3ANX	3ADON	15ADON	ADON	-	-	3ANX	-	3ANX
26330	Montréal	3ADON, 3ANX	15ADON	-	ADON	ADON	-	3ADON	3ADON	3ANX

* genotype/chemotype strain; ¹ Total range of target analytes detected in all three growth conditions; ² Starkey et al. (2007); ³ Alexander et al. (2011); ⁴ Liang et al. (2014); ⁵ see text.

4.0 Discussion

The results presented in this thesis compare and contrast standard methods of assessing the genotype and the chemotype of 61 representative *F. graminearum* strains collected across Ontario from 2015-2017. Overall, isolates of *F. graminearum* were mainly isolated from the southern grain-growing regions of Ontario. Consistent with previous research in Ontario, the strains studied in detail were primarily of the 15ADON genotype. However, unexpectedly more than half of the strains from each year coproduced 15ADON and the recently described 3ANX toxin (Aitken et al. 2019; Varga et al. 2015). Strains that were capable of producing detectable amounts of 3ANX and 15ADON were genotyped as 15ADON producers and not 3ANX producers. Two strains, one from 2015 and 2017, chemotyped as 3ANX producers. This new information highlights concerns with the use of the common genetic probes to infer chemotype when classifying *F. graminearum* isolates (Desjardins 2008).

Epidemics of *F. graminearum* diseases, such as Fusarium head blight in wheat and Gibberella ear rot in corn, reduces crop yield and quality. Grain damaged by *F. graminearum* cannot be processed into food or feed if regulatory levels of DON are exceeded. In Ontario, Fusarium head blight epidemics in 1996 and 2000 resulted in estimated losses of \$2019 150 million and \$2019 36 million, respectively (Hooker et al. 2002). To place this in perspective, the 1996 wheat crop harvest was reduced by a third (Bianchini et al. 2015). A survey by the Ontario Ministry of Food & Rural Affairs reported that 40% of corn samples collected from the 2018 crop had > 2 mg/kg DON. Of the samples examined, 25% had DON concentrations greater than 5 mg/kg (Rosser & Tenuta 2018), high enough to discount or render the corn worthless. The cause of this epidemic was above average rainfall experienced during the growing season.

Ontario grain farmers produce 50% of the corn and most of the soft wheat produced in Canada. Every year grain samples from both corn and wheat are collected across the grain growing regions of Ontario, which covers approximately 200, 000 km². The distance from the most southern to eastern tip of Ontario is roughly 1000 km. Within this area there are variations in climate such a rainfall, humidity, and temperature, which are factors in *F. graminearum* infection. For the month of June, southern Ontario experiences corn heat units averaging 3500 with relative humidity of 91% in the morning to 80% in the afternoon. Central and Eastern Ontario experience corn heat units as low as 2700-2900 with a relative humidity of 81% in the morning and 53% in the afternoon. Over the years 2015-2017, corn and wheat samples were systematically collected across Ontario. Samples were collected to represent grain growing regions from the southern county Essex (42.1233° N, 83.0401° W) to the most eastern county Prescott and Russell (45.4085° N, 74.7915° W). For the month of June in both 2015 and 2017, precipitation was higher than normal for all counties in which *F. graminearum* contaminated samples were recovered. The mean temperature for June increased over each year for each county. The most southern counties such as Essex and Chatham-Kent experienced mean temperatures 1-2 °C higher (20-18 °C) than Middlesex, Huron and Wellington (18-16 °C). *F. graminearum* was isolated from 6.3%, 36.7% and 12.3% of corn samples overall and 7.1%, 9.2% and 3.2% of farms from 2015, 2016 and 2017, respectively. *F. graminearum* was isolated from 19.3%, 19.2% and 39.8% of wheat samples overall and 39.4%, 58.3% and 28.6% of farms from 2015, 2016 and 2017, respectively.

4.1 Genotype

In this context, ‘genotype’ is determined with PCR primers for short sequences from the known biosynthetic genes developed by researchers at the National Center for Agricultural

Utilization Research of the USDA. The chemotype is the metabolic profile or chemical phenotype (Desjardins 2008). Initially, PCR-based screening of the toxigenic potential (chemotype) of all or a high percentage of strains from cultures or infected wheat were also assessed by chemical analysis (Starkey et al. 2007; Ward et al. 2002; 2008). Recent studies of *F. graminearum* populations in Canada (Kelly et al. 2015), France (Boutigny et al. 2014), Norway (Aamot et al. 2015), the UK (Pasquali et al. 2016) and Uruguay (Garmendia et al. 2018; Umpiérrez-Failache et al. 2013) reported no chemical data. Other studies performed chemotype analysis on a modest percentage of strains examined e.g. from Brazil (12% of strains; Gomes et al. 2015), Japan (3%; Suga et al. 2008), Ontario (19%; Burlakoti et al. 2017) and the USA (10%; Gale et al. 2011).

PCR genotyping assays in common use are based on the presence or absence of a target gene. Desjardins (2008) stated that “*In the simplest system, a gene would be present when the corresponding metabolite is present and absent when the metabolite is absent. Systems are necessarily more complex for natural products, which are synthesized by pathways comprising multiple genes and gene products*”. *Fusarium* trichothecenes are produced along metabolic grids (Kimura et al. 2007). Alternative pathways have arisen in nature through recombination during sexual reproduction (Kim et al. 2003), mutations, gains or losses in key biosynthetic genes such as *TRI1* (Proctor et al. 2018), mutations that change precursor pools (Kamata et al. 2018; Kimura et al. 2007) or events unrelated to trichothecene production (Nakajima et al. 2015).

In the present study, trichothecene genotypes of *F. graminearum* strains were identified using *TRI3*, *TRI12*, *TRI8* and two *TRI1* PCR-based assays (Starkey et al. 2007; Ward et al. 2002; Ward et al. 2007; Alexander et al. 2011; Liang et al. 2014; Prof. Chris Toomajian, personal communication). The genotype determination for all PCR-assays of the three positive control

strains was consistent with expectations. Using the *TRI3* and *TRI2* PCR-assays, all *F. graminearum* strains recovered from 2015 and 2016 were of the 15ADON genotype. In 2017, 88.9% *F. graminearum* strains were 15ADON and 11.1% (n = 26) of the strains were identified as the 3ADON genotype. No nivalenol-producing strains were detected. The 3ADON strains originated from St.Hyacinthe, Montérégie, Québec (250 km east of Ottawa) and the remaining strains were from inoculation trials in Ridgetown, Chatham-Kent (Tables 11, 12 and 13).

The *TRI8* PCR assay resulted in amplification indicating the 15ADON genotype for 75% (n = 15), 18.18% (n = 4) and 10.5% (n = 2) of the representative strains in 2015, 2016 and 2017, respectively. For the strains that produced solely 15ADON, only 38.5% (n = 5) resulted in the 15ADON genotype (Table 11). Strains that co-produced 15ADON and 3ANX, 32.5% (n = 14) resulted in the 15ADON genotype (Table 12). Both 3ANX chemotypes resulted in the 15ADON genotype, and the one 3ADON and 3ANX producer did not amplify in either the 15ADON or 3ADON *TRI8* assay (Table 13). To differentiate the 3ADON and 3ANX genotypes, a PCR-RFLP assay was developed by Liang et al (2014), designed to target polymorphisms on the *TRI1* gene of 3ANX producers. Of the strains tested, all but four resulted in ADON genotype over 2015-2017. Of the four strains that did not result in a genotype determination, one was of the 15ADON chemotype and the other three were 15ADON and 3ANX co-producers (Table 11 and 12).

Table 11. Genotype of the 15ADON chemotype representative strains.

Sample ID	Location	Lat.	Long.	Starkey ¹ <i>TRI3</i> <i>TRI12</i>	Alexander ² <i>TRI8</i>	Liang ³ <i>TRI1</i>	Toomajian ⁴ <i>TRI1</i> - ADON <i>TRI1</i> - 3ANX		Chemotype
251904	Essex	42.13	-82.91	15ADON	15ADON	ADON	ADON	3ANX	15ADON
26349	Chatham	42.35	-81.91	15ADON	-	-	ADON	-	15ADON
24590	Chatham	42.41	-81.93	15ADON	-	ADON	ADON	-	15ADON
25241	Chatham	42.45	-81.88	15ADON	-	ADON	ADON	3ANX	15ADON
25268	Chatham	42.45	-81.88	15ADON	-	ADON	ADON	-	15ADON
25442	Chatham	42.45	-81.89	15ADON	15ADON	ADON	ADON	3ANX	15ADON
251910	Chatham	42.50	-81.76	15ADON	15ADON	ADON	ADON	3ANX	15ADON
25219	Elgin	42.60	-81.75	15ADON	-	ADON	ADON	-	15ADON
25191	Chatham	42.64	-81.87	15ADON	15ADON	ADON	-	-	15ADON
25213	Chatham	42.64	-81.87	15ADON	-	ADON	ADON	3ANX	15ADON
26223	Middlesex	43.15	-81.42	15ADON	-	ADON	ADON	-	15ADON
251917	Huron	43.47	-81.45	15ADON	15ADON	-	ADON	3ANX	15ADON
26361	Huron	43.68	-81.47	15ADON	-	ADON	ADON	-	15ADON

¹ Starkey et al. (2007); ² Alexander et al. (2011); ³ Liang et al. (2014); ⁴ see text.

The *TRI1* PCR assay developed by Prof. Toomajian used two separate PCR assays to target ADON and 3ANX genotypes, based on polymorphisms in the *TRI1* gene. The assay was designed so that 3ADON strains would only amplify in the ADON assay, and the 3ANX strains would only amplify in the 3ANX assay. The 15ADON control strain (DAOMC 180378) used throughout this thesis resulted in positive amplification for both assays consistently.

Table 12. Genotype of the 15ADON-3ANX chemotype representative strains.

Sample ID	Location	Lat.	Long.	Starkey ¹ <i>TRI3</i> <i>TRI12</i>	Alexander ² <i>TRI8</i>	Liang ³ <i>TRI1</i>	Toomajian ⁴ <i>TRI1</i> - ADON	<i>TRI1</i> - 3ANX	Chemotype
26233	Essex	42.10	-82.69	15ADON	-	ADON	-	-	15ADON, 3ANX
251906	Essex	42.29	-82.64	15ADON	15ADON	ADON	ADON	3ANX	15ADON, 3ANX
251915	Essex	42.30	-82.64	15ADON	-	ADON	ADON	3ANX	15ADON, 3ANX
25173	Chatham	42.35	-81.91	15ADON	-	ADON	ADON	3ANX	15ADON, 3ANX
251912	Chatham	42.36	-82.07	15ADON	-	ADON	ADON	-	15ADON, 3ANX
25232	Chatham	42.45	-81.88	15ADON	-	ADON	-	3ANX	15ADON, 3ANX
25250	Chatham	42.45	-81.88	15ADON	-	ADON	ADON	3ANX	15ADON, 3ANX
25281	Chatham	42.45	-81.88	15ADON	15ADON	ADON	ADON	3ANX	15ADON, 3ANX
25291	Chatham	42.45	-81.88	15ADON	-	ADON	ADON	3ANX	15ADON, 3ANX
25432	Chatham	42.45	-81.89	15ADON	-	ADON	ADON	3ANX	15ADON, 3ANX
25450	Chatham	42.45	-81.89	15ADON	-	ADON	ADON	3ANX	15ADON, 3ANX
25457	Chatham	42.45	-81.89	15ADON	-	ADON	ADON	3ANX	15ADON, 3ANX
26348	Chatham	42.50	-81.78	15ADON	-	ADON	ADON	-	15ADON, 3ANX
251909	Chatham	42.53	-81.74	15ADON	15ADON	ADON	-	3ANX	15ADON, 3ANX
251922	Elgin	42.55	-81.62	15ADON	15ADON	ADON	-	-	15ADON, 3ANX
24893	Lambton	42.56	-82.15	15ADON	-	ADON	-	3ANX	15ADON, 3ANX
251914	Chatham	42.58	-82.13	15ADON	-	ADON	ADON	3ANX	15ADON, 3ANX
24782	Chatham	42.58	-82.13	15ADON	-	ADON	ADON	-	15ADON, 3ANX
25192	Chatham	42.64	-81.87	15ADON	-	ADON	ADON	3ANX	15ADON, 3ANX
251921	Lambton	42.71	-81.96	15ADON	15ADON	ADON	ADON	3ANX	15ADON, 3ANX
251908	Lambton	42.81	-81.91	15ADON	15ADON	ADON	ADON	3ANX	15ADON, 3ANX
251913	Middlesex	42.86	-81.55	15ADON	15ADON	ADON	ADON	3ANX	15ADON, 3ANX
251903	Lambton	42.94	-82.25	15ADON	15ADON	ADON	ADON	-	15ADON, 3ANX
24553	Middlesex	43.13	-81.24	15ADON	-	ADON	ADON	-	15ADON, 3ANX
251916	Huron	43.13	-81.24	15ADON	15ADON	ADON	ADON	3ANX	15ADON, 3ANX
251919	Perth	43.13	-81.24	15ADON	-	ADON	ADON	-	15ADON, 3ANX
251907	Middlesex	43.14	-81.26	15ADON	15ADON	ADON	ADON	3ANX	15ADON, 3ANX
26203	Middlesex	43.16	-81.47	15ADON	-	-	-	-	15ADON, 3ANX
251920	Middlesex	43.23	-81.40	15ADON	-	ADON	ADON	3ANX	15ADON, 3ANX
24558	Perth	43.42	-81.18	15ADON	15ADON	ADON	-	3ANX	15ADON, 3ANX
251918	Perth	43.47	-81.25	15ADON	15ADON	-	-	3ANX	15ADON, 3ANX
26359	Huron	43.68	-81.47	15ADON	-	ADON	ADON	-	15ADON, 3ANX
26362	Huron	43.68	-81.47	15ADON	-	ADON	ADON	-	15ADON, 3ANX
26368	Huron	43.68	-81.47	15ADON	-	ADON	ADON	-	15ADON, 3ANX
251905	Wellington	43.87	-80.83	15ADON	15ADON	ADON	ADON	-	15ADON, 3ANX
26301	Wellington	43.87	-80.83	15ADON	-	ADON	ADON	-	15ADON, 3ANX
26292	Leeds	45.01	-75.63	15ADON	-	ADON	ADON	-	15ADON, 3ANX
26295	Leeds	45.01	-75.63	15ADON	-	ADON	ADON	-	15ADON, 3ANX
26296	Leeds	45.01	-75.63	15ADON	-	ADON	ADON	-	15ADON, 3ANX
26314	Montréal	45.64	-72.97	3ADON	-	ADON	-	-	15ADON, 3ANX
26320	Montréal	45.64	-72.97	15ADON	-	ADON	-	-	15ADON, 3ANX
26331	Montréal	45.64	-72.97	15ADON	-	-	ADON	-	15ADON, 3ANX
26335	Montréal	45.64	-72.97	3ADON	15ADON	ADON	ADON	-	15ADON, 3ANX

¹ Starkey et al. (2007); ² Alexander et al. (2011); ³ Liang et al. (2014); ⁴ see text.

In the PCR-assays described above, 15ADON genotype was indicated for the majority of the *F. graminearum* strains tested. For the 15ADON chemotype strains, 92.3% (n = 12) resulted in amplification for the ADON assay, and 46.1% (n = 6) in the 3ANX assay. The strains genotyped 3ANX according to this assay, also amplified in the ADON assay (Table 11). For the 15ADON and 3ANX co-producers, 76.7% (n = 33) resulted in ADON genotype, and 51.1% (n = 22) in 3ANX genotype. 39.5% (n = 17) of the co-producing strains resulted in amplification for both assays (Table 12). One of the two 3ANX chemotype strains amplified for both *TRII* ADON and 3ANX assays. The other 3ANX chemotype strain did not amplify using these assays (Table 13).

Table 13. Genotype of the 3ANX and the 3ADON-3ANX chemotype representative strains.

Sample ID	Location	Lat.	Long.	Starkey ¹ <i>TRI3</i> <i>TRII2</i>	Alexander ² <i>TRI8</i>	Liang ³ <i>TRII</i>	Toomajian ⁴ <i>TRII</i> - ADON <i>TRII</i> - 3ANX		Chemotype
251911	Chatham	42.44	-81.88	15ADON	15ADON	ADON	ADON	3ANX	3ANX
26326	Montérégie	45.64	-72.97	3ADON	15ADON	ADON	-	-	3ANX
26330	Montérégie	45.64	-72.97	15ADON	-	ADON	ADON	-	3ADON, 3ANX

¹ Starkey et al. (2007); ² Alexander et al. (2011); ³ Liang et al. (2014); ⁴ see text.

In general, genotype results from the *F. graminearum* representative strains did not align with the genotype of the positive control strains for all PCR-assays. Of the representative strains chosen, only 18% (n = 11) matched the overall genotype results of the 15ADON control strain. The remaining strains resulted in 15ADON genotype in two or more assays. The present data suggests that these *F. graminearum* strains have *TRI* gene clusters more similar to 15ADON strains than the 3ANX strain used to develop the *TRII* PCR-assays.

4.2 Chemotype

Chemical analysis using LC-MS/MS, excepting the 15ADON and 3ADON isomers allowed all targeted metabolites could be detected by mass. 15ADON and 3ADON are positional isomers, sharing the same mass and mass fragmentation pattern. The MS/MS fragmentation pattern result in different intensities for the product ions m/z 137.1 and 231.1. The presence of 15ADON and 3ADON was confirmed using the intensity ratios of product ions m/z 137.1 and 231.1. If the intensity of the m/z 137.1 product ion was higher, this was consistent with the presence of 15ADON. If the intensity of the m/z 231.1 product ion was higher, this was consistent with the presence of 3ADON (Renaud & Sumarah 2016). 15ADON and 3ADON were further resolved by the use of a shallow gradient, starting with 22% organic phase at 1 minute increasing to 37% over 3 minutes.

Chemical analysis showed co-production of 15ADON and 3ANX in one or more media for 80% ($n = 16$), 59% ($n = 13$) and 73.7% ($n = 14$) for the years 2015, 2016, and 2017 respectively (Table 11). Most strains also produced minor amounts of 3ADON, sambucinol and zearalenone as well. From the year 2017, strain 26330 was shown to produce 3ADON on solid media, and 3ANX in liquid fermentation. This is the first study reports strains that produce both 15ADON and 3ANX.

4.3 Genotype vs. chemotype

Strains of *F. graminearum* are defined by chemotype based on the ability to produce 15ADON, 3ADON, nivalenol and now 3ANX. *F. graminearum* strains that that produce unexpected patterns of dominant trichothecenes are uncommon. Sugiura et al. (1990) were the first to report a DON producing strain of *F. graminearum* from Japan that is capable of co-

producing DON and nivalenol. *F. graminearum* strains isolated from grains in South Korea, Kim et al. (2003) reported DON genotyped strains were capable of producing nivalenol, also nivalenol genotyped strains capable of producing DON. The authors suggested that this was a result of recombination between the two chemotypes. De Kuppler et al. (2011) reported *F. graminearum* strains from Germany that produced both 15ADON and material amounts of 3ADON in culture (as opposed to trace amounts as was the case for a strain in the present study). Consistent with the South Korean study, these European authors also reported DON chemotype strains capable of producing nivalenol and vice versa, not detected by genotype analysis. Castañares et al. (2014) reported strains from Argentina producing solely 3ADON, although the strains were determined 15ADON genotype. Kulik et al. (2016) sequenced the *TRI* gene cluster from the Castañares et al. (2014) work comparing its sequence to the *TRI* sequence data of other type B *TRI* genotypes. The authors reported an identical sequence match within the entire *TRI* gene cluster to a 15ADON genotype. The present data and the work of other described above, reflects that *Fusarium* trichothecenes are biosynthesized along metabolic grids (Kimura et al. 2007).

Variations in precursors and end-products of secondary metabolites are synthesized in metabolic grids (Bu'Lock 1965; Kimura et al. 2007). Between strains and chemotypes of *Fusarium*, differences at the DNA sequence level result in end-product variations. For example, differences in *TRI1* gene between *F. sporotrichioides* and *F. graminearum*, result in two separate classes of trichothecene. For the strains described in this thesis, one possible reason for the co-production of 15ADON and 3ANX is a lowered specificity in *TRI1* and *TRI8* enzymes. In *F. graminearum*, the *TRI1* enzyme is responsible for the hydroxylating the C-7 and C-8 positions on calonectrin (McCormick et al. 2004). Lowered specificity of *TRI1* enzyme could produce 7-

hydroxycalonectrin, the suspected precursor to 3ANX. This would occur if only the C-7 position is hydroxylated. To result in 3ANX, the C-3 position would be deacetylated by the TRI8 enzyme.

Figure 25. Biosynthetic pathway of calonectrin (CAL) to 15ADON, 3ADON and a putative pathway for 3ANX synthesis. The ADON pathway includes 7-hydroxycalonectrin (7-CAL); 8-hydroxycalonectrin (8-CAL); 7,8-dihydroxycalonectrin (DHC) and 3,15-acetyldeoxynivalenol (3,15-ADON).

Alexander et al. (2011) demonstrated that the TRI8 enzyme is responsible for the deacetylation of C-3 or C-15 on 3,15-diacetyldeoxynivalenol to produce 15ADON or 3ADON, respectively. The authors reported 15ADON producers were capable of co-producing low levels of 3ADON, and 3ADON producers were capable of co-producing low levels of 15ADON. This result may suggest of lowered specificity in the TRI8 enzyme. The strains examined in this thesis produce primarily 15ADON, therefore it can be assumed that the TRI8 enzyme would deacetylate 7-hydroxycalonectrin at C-15 to produce 15ANX. Without a chemical standard for 15ANX, we cannot conclude that these strains do not also produce 15ANX. However, the ability of these strains to produce 3ANX supports with a lowered specificity of TRI8 enzyme due to the C-3 deacetylation.

F. graminearum populations in North and South America have been predominantly of the 15ADON chemotype since its discovery in 1983 (Miller et al. 1983; Miller et al. 1991; Mirocha et al. 1989; Piñeiro et al. 1995). Within the past 10-15 years, surveys conducted in Canada and the USA have shown a shift towards the 3ADON chemotype which is more common in Asian countries such as Japan and China (Ward et al. 2008). Ward et al. (2008), reported a significant shift during 1994-2004 where the 3ADON chemotype frequency in Prince Edward Island increased to 100%, and 14-fold in Western Canada. However, the prevalence of the 15ADON population in Ontario remains high. *F. graminearum* strains collected from Ontario harvests during 2010 resulted in the 97% 15ADON and 3% 3ADON genotype (Tamburic-Ilincic et al. 2015). Working with samples from southern Ontario from 2010-2012, Burlakoti et al. (2017) reported that 96% and 98% of isolates collected from corn and wheat, respectively, were of the 15ADON genotype. The remainder were the 3ADON genotype. However, it is likely the genotype in these studies has not correctly predicted the chemotype since it is likely that the

majority of strains in Ontario also produce 3ANX. In nearby New York, the 15ADON genotype was the dominant population, but there was a higher percentage of 3ADON genotype than found in Ontario (Kuhnem et al. 2015). Reanalysis of these strains resulted in a high percentage of the 3ADON genotype that were actually 3ANX genotype (Lofgren et al. 2018).

After the discovery of 3ANX, Liang et al. (2015) conducted population analysis on strains from the upper Midwestern states. Two distinct populations were detected, NA1 and NA2 which were primarily composed of 15ADON and 3ADON genotypes, respectively. 3ANX producing strains did not fit into either NA1 or NA2 population with high confidence. The authors suggested that it is possible for 3ANX producing strains to fall within the NA2 population based on the *TRI3* PCR result, which indicates 3ADON genotype. Otherwise, the 3ANX strains may belong to a distinct population. Kelly et al. (2015) showed that strains collected in 2005-2007 across Canada had a similar population structure (NA1 and NA2). However, 3ANX strains were placed with the NA1 population. This assignment was unexpected because all 3ANX strains were of the 3ADON genotype using the *TRI3* and *TRI12* assays, which is characteristic of the NA2 population. It was shown that isolates with genotypes not predictive of genetic population assignment increased over the sampling period. The authors stated that the frequency of genotype recombination is increasing rapidly, therefore suggesting that trichothecene genotype is an increasingly unreliable marker of population identity.

In the past two decades, Ontario corn producers have switched to stay-green corn hybrids that have higher yields. This is due to its ability to continue filling the ear later in the growing season (Tollenaar et al. 1997). However, when the crop is harvested the stalks remain green in the field. The crop residue provides a substrate for *F. graminearum* ascocarp production (Eli et al. 2018). Increased use of this hybrid over time may explain the stability of the 15ADON

population in Ontario, and higher frequencies of genotype recombination. This agrees with the findings of Kelly & Ward (2018). These authors reported a third population (NA3) which is comprised primarily of 3ANX. Of the 3ANX strains examined, strains that did not fall into the NA3 with high confidence were shown to have admixed genomes that infer a history of recombination with the NA1 population. Kelly & Ward (2018) reported that full genome scans found frequently recombining regions notably with high genomic divergence of the trichothecene toxin gene cluster. Yue (2017) reported a similar phenomenon.

As new fungal metabolites are characterized, the demand for screening methods such as LC-MS/MS to target these metabolites in extracts has increased. Targeted LC-MS/MS is a powerful tool to detect multiple mycotoxin present in an extract. However, this method is limited to compounds for which there is a standard available. It remains that there are no commercial standard available for 3ANX thus failure to report 3ANX or NX from chemical analyses of grain or culture samples does not mean absence. In our lab, Aitken et al. (2019) isolated 3ANX from the *F. graminearum* strain DAOMC 242077 and produced very pure chemical standards for 3ANX and NX toxin. The present data were compared to these standards to confirm the production of 3ANX in culture extracts.

Strains examined in this project capable of producing 3ANX were not identified using the *TRII* assays developed to detect 3ANX strains (Liang et al. 2014). In the present work, strains that produced both 15ADON and 3ANX were determined 15ADON producers in one or all of the assays used. Although the genotype assays were correct in determining the ability to produce 15ADON, they did not provide a complete picture as to the toxigenic potential of *F. graminearum* strains.

5.0 Conclusion

These experiments contributed to a better understanding of *Fusarium graminearum* genotype and chemotype. The present data showed that over 50% of Ontario *F. graminearum* strains collected from 2015-2017, co-produced both 15ADON and 3ANX. Although a potential metabolic pathway for 3ANX in these strains was inferred, co-production of other mycotoxins has been previously reported. Lowered specificity in both *TRI1* and *TRI8* may result in the accumulation of 3ANX. Excepting strains from St. Hyacinthe, QB, all *F. graminearum* strains were 15ADON genotype using PCR-based assays. Without chemical analysis and chemical standards, the co-production of 15ADON and 3ANX would have been overlooked. Therefore, I argue that genotype analysis should only be used to complement chemical analysis in future studies of *F. graminearum*. Future research on the nature of the 15ADON/3ANX strains should include making standards of 15ANX and related metabolites.

6.0 References

- Aamot, H. U., Ward, T. J., Brodal, G., Vrålstad, T., Larsen, G. B., Klemsdal, S. S., Elameen, A., Uhlig, S., Hofgaard, I. S. (2015). Genetic and phenotypic diversity within the *Fusarium graminearum* species complex in Norway. *European Journal of Plant Pathology*, 142(3), 501–519.
- Abbas, H. K., Mirocha, C. J., & Tuite, J. (1986). Natural occurrence of deoxynivalenol, 15-acetyl-deoxynivalenol, and zearalenone in refusal factor corn stored since 1972. *Applied and Environmental Microbiology*, 51(4), 841–843.
- Aitken, A., Miller, J. D., & McMullin, D. R. (2019). Isolation, chemical characterization and hydrolysis of the trichothecene 7 α -hydroxy, 15-deacetylcalonecitrin (3ANX) from *Fusarium graminearum* DAOMC 242077. *Tetrahedron Letters*, 60(12), 852–856.

- Alexander, N. J., McCormick, S. P., Waalwijk, C., van der Lee, T., & Proctor, R. H. (2011). The genetic basis for 3-ADON and 15-ADON trichothecene chemotypes in *Fusarium*. *Fungal Genetics and Biology*, 48(5), 485–495.
- Amarasinghe, C. C., Tittlemier, S. A., & Fernando, W. G. D. (2015). Nivalenol-producing *Fusarium cerealis* associated with fusarium head blight in winter wheat in Manitoba, Canada. *Plant Pathology*, 64(4), 988–995.
- Amuzie, C. J., & Pestka, J. J. (2010). Suppression of Insulin-Like Growth Factor Acid-Labile Subunit Expression—A Novel Mechanism for Deoxynivalenol-Induced Growth Retardation. *Toxicological Sciences*, 113(2), 412–421.
- Bai, G., & Shaner, G. (2010). Scab of Wheat: Prospects For Control. *Plant Disease*, 78(8), 760-766.
- Beardall, J., & Miller, J. D. (1994). Natural occurrence of Mycotoxins other than Aflatoxin in Africa, Asia and South America. *Mycotoxin Research*, 10, 21–40.
- Bhat, R. V., Ramakrishna, Y., Beedu, S. R., & Munshi, K. L. (1989). Outbreak of Trichothecene Mycotoxicosis Associated With Consumption of Mould-Damaged Wheat Products in Kashmir Valley, India. *The Lancet*, 333(8628), 35–37.
- Bianchini, A., Horsley, R., Jack, M. M., Kobielski, B., Ryu, D., Tittlemier, S., Wilson, W.W., Abbas, H.K., Abel, S., Harrison, G., Miller, J.D., Shier, W.T., Weaver, G., Katzke, D., Maier, D.E., Pestka, J., Boroughs, B., & Bridges, A. R. (2015). DON occurrence in grains: A North American perspective. *Cereal Foods World*, 60(1), 32–56.
- Bonnet, M. S., Roux, J., Mounien, L., Dallaporta, M., & Troadec, J. D. (2012). Advances in Deoxynivalenol Toxicity Mechanisms: The Brain as a Target. *Toxins*, 4(11), 1120–1138.

- Boutigny, A. L., Ward, T. J., Ballois, N., Iancu, G., & Ioos, R. (2014). Diversity of the *Fusarium graminearum* species complex on French cereals. *European Journal of Plant Pathology*, 138, 133–148.
- Bu'Lock, J. D. (1965). The biosynthesis of natural products: an introduction to secondary metabolism. London, UK: McGraw-Hill.
- Bu'Lock, J. D. (1975). Secondary metabolism. In J. E. Smith & D. R. Berry (Eds.), *The Filamentous Fungi* (pp. 33–58). New York, USA: Academic Press.
- Burlakoti, R.R., Ali, S., Secor, G.A, Neate, S.M., McMullen, M.P., & Adhikari, T.B. (2008). Genetic Relationship Among Populations of *Gibberella zeae* from Barley, Wheat, Potato, and Sugar Beet in the Upper Midwest of the United States. *Phytopathology*, 98(9), 969-976.
- Burlakoti, R. R., Tamburic-Ilincic, L., Limay-Rios, V., & Burlakoti, P. (2017). Comparative population structure and trichothecene mycotoxin profiling of *Fusarium graminearum* from corn and wheat in Ontario, central Canada. *Plant Pathology*, 66(1), 14–27.
- Caldwell, R. W., Tuite, J., Stob, M., & Baldwin, R. (1970). Zearalenone production by *Fusarium* species. *Applied Microbiology*, 20(1), 31–34.
- Cane, D. E., & Ha, H. J. (1988). Trichodiene Biosynthesis and the Role of Nerolidyl Pyrophosphate in the Enzymatic Cyclization of Farnesyl Pyrophosphat. *Journal of the American Chemical Society*, 110(20), 6865–6870.
- Castañares, E., Albuquerque, D. R., Dinolfo, M. I., Pinto, V. F., Patriarca, A., & Stenglein, S. A. (2014). Trichothecene genotypes and production profiles of *Fusarium graminearum* isolates obtained from barley cultivated in Argentina. *International Journal of Food Microbiology*, 179, 57–63.

- De Kuppler, A. L. M., Steiner, U., Sulyok, M., Krska, R., & Oerke, E. C. (2011). Genotyping and phenotyping of *Fusarium graminearum* isolates from Germany related to their mycotoxin biosynthesis. *International Journal of Food Microbiology*, 151(1), 78–86.
- Demain, A. L., & Fang, A. (2000). The Natural Functions of Secondary Metabolites. *Advances in Biochemical Engineering/Biotechnology*, 69, 1–39.
- Desjardins, A. E. (2008). Natural product chemistry meets genetics: When is a genotype a chemotype? *Journal of Agricultural and Food Chemistry*, 56(17), 7587–7592.
- Desjardins, A. E., Hohn, T. M., & McCormick, S. P. (1993). Trichothecene biosynthesis in *Fusarium* species: chemistry, genetics, and significance. *Microbiological Reviews*, 57(3), 595–604.
- Dill-Macky, R., & Jones, R. K. (2000). The Effect of Previous Crop Residues and Tillage on *Fusarium* Head Blight of Wheat. *Plant Disease*, 84(1), 71–76.
- Eli, K., Hooker, D. C., & Schaafsma, A. W. (2018). Is increased inoculum for *Fusarium graminearum* an unintended consequence of stay green maize?. *Phytopathology*, 108(10), 3340.
- Fernando, W. G. D., Miller, J. D., Seaman, W. L., Seifert, K., & Paulitz, T. C. (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, W. G. D., Paulitz, T. C., Seaman, W. L., Dutilleul, P., & Miller, J. D. (1997). Head Blight Gradients Caused by *Gibberella zeae* from Area Sources of Inoculum in Wheat Field Plots. *Phytopathology*, 87(4), 414–421.
- EFSA. (2013). Deoxynivalenol in food and feed: occurrence and exposure. *EFSA Journal*, 11(10).

- Gale, L. R., Harrison, S. A., Ward, T. J., O'Donnell, K., Milus, E. A., Gale, S. W., & Kistler, H. C. (2011). Nivalenol-type populations of *Fusarium graminearum* and *F. asiaticum* are prevalent on wheat in southern Louisiana. *Phytopathology*, 101(15), 124–134.
- Gale, L. R., Ward, T. J., Balmas, V., & Kistler, H. C. (2007). Population Subdivision of *Fusarium graminearum* Ssensu Stricto in the Upper Midwestern United States. *Phytopathology*, 97, 1434–1439.
- Gale, L. R., Ward, T. J., & Kistler, H. C. (2010). A subset of the newly discovered Northland population of *Fusarium graminearum* from the U.S. does not produce the B-type trichothecenes DON, 15ADON, 3ADON or NIV. In S. Canty, A. Clark, A. Anderson-Scully, D. Ellis, & D. Van Sanford (Eds.), *Proceedings of the National Fusarium Head Blight Forum* (pp. 48–49). Milwaukee, WI, USA: University of Kentucky.
- Garmendia, G., Pattarino, L., Negrín, C., Martínez-Silveira, A., Pereyra, S., Ward, T. J., & Vero, S. (2018). Species composition, toxigenic potential and aggressiveness of *Fusarium* isolates causing Head Blight of barley in Uruguay. *Food Microbiology*, 76, 426–433.
- Gilbert, J., Brûlé-Babel, A., Guerrieri, A. T., Clear, R. M., Patrick, S., Slusarenko, K., & Wolfe, C. (2014). Ratio of 3-ADON and 15-ADON isolates of *Fusarium graminearum* recovered from wheat kernels in Manitoba from 2008 to 2012. *Canadian Journal of Plant Pathology*, 36(1), 54–63.
- Gilbert, J., Clear, R. M., Ward, T. J., Gaba, D., Tekauz, A., Turkington, T. K., Woods, S.M., Nowicki, T., & O'Donnell, K. (2010). Relative aggressiveness and production of 3- or 15-acetyl deoxynivalenol and deoxynivalenol by *Fusarium graminearum* in spring wheat. *Canadian Journal of Plant Pathology*, 32(2), 146–152.

- Gomes, L. B., Ward, T. J., Badiale-Furlong, E., & Del Ponte, E. M. (2015). Species composition, toxigenic potential and pathogenicity of *Fusarium graminearum* species complex isolates from southern Brazilian rice. *Plant Pathology*, 64(4), 980–987.
- Grove, J. F. (2007). The Trichothecenes and Their Biosynthesis. *Progress in the Chemistry of Organic Natural Products*. 88, 63-130.
- Heath, K. E., Argente, J., Barrios, V., Pozo, J., Díaz-González, F., Martos-Moreno, G. A., Caimari, M., Gracia, R., & Campos-Barros, Á. (2008). Primary acid-labile subunit deficiency due to recessive IGFALS mutations results in postnatal growth deficit associated with low circulating insulin growth factor (IGF)-I, IGF binding protein-3 levels, and hyperinsulinemia. *Journal of Clinical Endocrinology and Metabolism*, 93(5), 1616–1624.
- Hesketh, A. R., Bycroft, B. W., Dewick, P. M., & Gilbert, J. (1993). Revision of the stereochemistry in trichodiol, trichotriol and related compounds, and concerning their role in the biosynthesis of trichothecene mycotoxins. *Phytochemistry*, 32(1), 105–116.
- Hohn, T. M., Desjardins, A. E., & McCormick, S. P. (1995). The Tri4 gene of *Fusarium sporotrichioides* encodes a cytochrome P450 monooxygenase involved in trichothecene biosynthesis. *MGG Molecular & General Genetics*, 248(1), 95–102.
- Hohn, T. M., & Vanmiddlesworth, F. (1986). Purification and Characterization of the Sesquiterpene Cyclase Trichodiene Synthetase from *Fusarium sporotrichioides*. *Arch. Biochem. Biophys.*, 251(2), 756–761.
- Hooker, D. C., & Schaafsma, A. W. (2005). Agronomic and environmental impacts on concentrations of deoxynivalenol and fumonisin B1 in corn across Ontario. *Canadian Journal of Plant Pathology*, 27(3), 347–356.

- Hooker, D. C., Schaafsma, A. W., & Tamburic-Ilincic, L. (2002). Using Weather Variables Pre- and Post-heading to Predict Deoxynivalenol Content in Winter Wheat. *Plant Disease*, 86(6), 611–619.
- Iverson, F., Armstrong, C., Nera, E., Truelove, J., Fernie, S., Scott, P., Stapley, R., Hayward, S., & Gunner, S. (1995). Chronic Feeding Study of deoxynivalenol in B6C3F1 male and female mice. *Teratogenesis, Carcinogenesis, and Mutagenesis*, 15(6), 283–306.
- JECFA. (2001) Evaluation of certain mycotoxins in food: *Fifty-sixth meeting*. *FAO Food and nutrition paper 74*. Retrieved December 19, 2018 from: <http://apps.who.int/food-additives-contaminants-jecfa-database/chemical.aspx?chemID=2947>
- JECFA. (2011). Evaluation of Certain Contaminants in Food: *Seventy-second meeting*. Retrieved December 19, 2018 from: http://apps.who.int/iris/bitstream/10665/44514/1/WHO_TRS_959_eng.pdf
- Kamata, K., Sato, H., Maeda, K., Furihata, K., Aikawa, S., Adachi, K., Tanaka, A., Tokai, T., Nakajima, Y., Yoshida, Y., Sakuda, S., Kimura, M., & Takahashi-Ando, N. (2018). Exploring an Artificial Metabolic Route in *Fusarium sporotrichioides*: Production and Characterization of 7-Hydroxy T-2 Toxin. *Journal of Natural Products*, 81, 1041–1044.
- Keller, N. P., Turner, G., & Bennett, J. W. (2005). Fungal secondary metabolism - From biochemistry to genomics. *Nature Reviews Microbiology*, 3(12), 937–947.
- Kelly, A. C., Clear, R. M., O'Donnell, K., McCormick, S., Turkington, T. K., Tekauz, A., Gilbert, J., Kistler, H.C., Busman, M., & Ward, T. J. (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.

- Kelly, A. C., & Ward, T. J. (2018). Population genomics of *Fusarium graminearum* reveals signatures of divergent evolution within a major cereal pathogen. *PLoS ONE*, *13*(3), 1–32.
- Kim, H. S., Lee, T., Dawlatana, M., Yun, S. H., & Lee, Y. W. (2003). Polymorphism of trichothecene biosynthesis genes in deoxynivalenol- and nivalenol-producing *Fusarium graminearum* isolates. *Mycological Research*, *107*(2), 190–197.
- Kimura, M., Matsumoto, G., Shingu, Y., Yoneyama, K., & Yamaguchi, I. (1998). The mystery of the trichothecene 3- O -acetyltransferase gene. *FEBS Letters*, *435*(2–3), 163–168.
- 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*(9), 2105–2123.
- Kuhnem, P. R., Del Ponte, E. M., Dong, Y., & Bergstrom, G. C. (2015a). *Fusarium graminearum* Isolates from Wheat and Maize in New York Show Similar Range of Aggressiveness and Toxigenicity in Cross-Species Pathogenicity Tests. *Phytopathology*, *105*(4), 441–448.
- Kuhnem, P. R., Spolti, P., Del Ponte, E. M., Cummings, J. A., & Bergstrom, G. C. (2015b). Trichothecene Genotype Composition of *Fusarium graminearum* Not Differentiated Among Isolates from Maize Stubble, Maize Ears, Wheat Spikes, and the Atmosphere in New York. *Phytopathology*, *105*(5), 695–699.
- Kulik, T., Buśko, M., Bilska, K., Ostrowska-Kołodziejczak, A., van Diepeningen, A., Perkowski, J., & Stenglein, S. (2016). Depicting the Discrepancy between Tri Genotype and Chemotype on the Basis of Strain CBS 139514 from a Field Population of *F. graminearum* Ssensu Stricto from Argentina. *Toxins*, *8*(11), 330.

- Liang, J. M., Xayamongkhon, H., Broz, K., Dong, Y., McCormick, S. P., Abramova, S., Ward, T.J., Ma, Z.H., Kistler, H. C. (2014). Temporal dynamics and population genetic structure of *Fusarium graminearum* in the upper Midwestern United States. *Fungal Genetics and Biology*, 73, 83–92.
- Lofgren, L., Riddle, J., Dong, Y., Kuhnem, P. R., Cummings, J. A., Del Ponte, E. M., Bergstrom, G.C. Kistler, H. C. (2018). A high proportion of NX-2 genotype strains are found among *Fusarium graminearum* isolates from northeastern New York State. *European Journal of Plant Pathology*, 150(3), 791–796.
- Luo, X. Y. (1988). Outbreaks of moldy cereals poisoning in China. In: Issues in Food Safety, Washington D.C.: Toxicology Forum, Inc. pp. 56-63.
- Luo, Y., Yoshizawa, T., & Katayama, T. (1990). Comparative study on the natural occurrence of *Fusarium* mycotoxins (trichothecenes and zearalenone) in corn and wheat from high- and low-risk areas for human esophageal cancer in China. *Applied and Environmental Microbiology*, 56(12), 3723–3726.
- Martin, R. A., & Johnston, H. W. (1982). Effects and control of *Fusarium* disease of cereal grains in the Atlantic provinces. *Canadian Journal of Plant Pathology*, 4, 210–216.
- McCormick, S. P., Alexander, N. J., & Proctor, R. H. (2006). Heterologous expression of two trichothecene P450 genes in *Fusarium verticillioides*. *Canadian Journal of Microbiology*, 52(3), 220–226.
- McCormick, S. P., Harris, L. J., Alexander, N. J., Ouellet, T., Saparno, A., Allard, S., & Desjardins, A. E. (2004). Tri1 in *Fusarium graminearum* Encodes a P450 Oxygenase. *Applied and Environmental Microbiology*, 70, 2044–2051.

- McCormick, S. P., & Hohn, T. M. (1997). Accumulation of trichothecenes in liquid cultures of a *Fusarium sporotrichioides* mutant lacking a functional trichothecene C-15 hydroxylase. *Applied and Environmental Microbiology*, 63(5), 1685–1688.
- McCormick, S. P., Stanley, A. M., Stover, N. A., & Alexander, N. J. (2011). Trichothecenes: From simple to complex mycotoxins. *Toxins*, 3(7), 802–814.
- McCormick, S. P., Taylor, S. L., Plattner, R. D., & Beremand, M. N. (1989). New modified trichothecenes accumulated in solid culture by mutant strains of *Fusarium sporotrichioides*. *Applied and Environmental Microbiology*, 55(9), 2195–2199.
- Meek, I. B., Peplow, A. W., Ake, C., Phillips, T. D., & Beremand, M. N. (2003). Tri1 encodes the cytochrome P450 monooxygenase for C-8 hydroxylation during trichothecene biosynthesis in *Fusarium sporotrichioides* and resides upstream of another new Tri gene. *Applied and Environmental Microbiology*, 69, 1607–1613.
- Miller, J. D. (1994). Epidemiology of *Fusarium graminearum* diseases of Wheat and Corn. In *Eagan Press* (pp. 19–33).
- Miller, J. D. (2008). Mycotoxins in small grains and maize: Old problems, new challenges. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*, 25(2), 219–230.
- Miller, J. D. (2016). Mycotoxins in Food and Feed: A Challenge for the Twenty-First Century. In: Li, D.W. (Eds) *Biology of Microfungi. Fungal Biology*. Springer Cham. 469–488.
- Miller, J. D., & Blackwell, B. A. (1986). Biosynthesis of 3-acetyldeoxynivalenol and other metabolites by *Fusarium culmorum* HLX 1503 in a stirred jar fermentor. *Canadian Journal of Botany*, 64(1), 1–5.

- Miller, J. D., Culley, J., Fraser, K., Hubbard, S., Meloche, F., Ouellet, T., Seaman, W.L., Seifert, K.A., Turkington, K., & Voldeng, H. (1998). Effect of tillage practice on *Fusarium* head blight of wheat. *Canadian Journal of Plant Pathology*, 20(1), 95–103.
- Miller, J. D., Greenhalgh, R., Wang, Y., & Lu, M. (1991). Trichothecene Chemotypes of Three *Fusarium* Species. *Mycologia*, 83, 121–130.
- Miller, J. D., Taylor, A., & Greenhalgh, R. (1983). Production of deoxynivalenol and related compounds in liquid culture by *Fusarium graminearum*. *Canadian Journal of Microbiology*, 29(9), 1171–1178.
- Mirocha, C. J., Abbas, H. K., Windels, C. E., & Xie, W. (1989). Variation in deoxynivalenol, 15-acetyldeoxynivalenol, 3-acetyldeoxynivalenol, and zearalenone production by *Fusarium graminearum* isolates. *Applied and Environmental Microbiology*, 55, 1315–1316.
- Morooka, N., Uratsuji, N., Yoshizawa, T., & Yamamoto, H. (1972). Studies on the toxic substances in barley infected with *Fusarium* spp. *Canadian Journal of Botany*, 13, 368–375.
- Nakajima, Y., Koseki, N., Sugiura, R., Tominaga, N., Maeda, K., Tokai, T., Izawa, M., Kanamaru, T., Koboyashi, T., Nishiuchi, T., Yoshida, M., & Kimura, M. (2015). Effect of disrupting the trichothecene efflux pump encoded by FgTri12 in the nivalenol chemotype of *Fusarium graminearum*. *The Journal of General and Applied Microbiology*, 61(3), 93–96.
- Neish, G. A., Farnworth, E. R., Greenhalgh, R., & Young, J. C. (1983). Observations on the occurrence of *Fusarium* species and their toxins in corn in eastern ontario. *Canadian Journal of Plant Pathology*, 5, 11–16.

- O'Donnell, K., Kistler, H. C., Tacke, B. K., & Casper, H. H. (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*, 97, 7905–7910.
- O'Donnell, K., Ward, T. J., Geiser, D. M., Kistler, H. C., & 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(6), 600–623.
- Ouellet, T., & Seifert, K. A. (1993). Genetic Characterization of *Fusarium graminearum* Strains Using RAPD and PCR Amplification. *Phytopathology*, 83(9), 1003–1007.
- Pasquali, M., Beyer, M., Logrieco, A., Audenaert, K., Balmas, V., Basler, R., Boutigny, A.L., Chrpova, J., Czembor, E., Gagkaeva, T., Gonzalez-Jaen, M.T., Hofgaard, I.S., Koycu, N.D., Hoffman, L., Levic, J., Marin, P., Miedaner, T., Migheli, Q., Moretti, A., Muller, M. E. H., Munaut, F, Parikka, P., Pallez-Barthel, M., Picc, J., Scauflaire, J., Scherm, B., Stankovic, S., Thrane, U., Uhlig, S., Vanheule, A., Yli-Mattila, T., & Vogelgsang, S. (2016). A European Database of *Fusarium graminearum* and *F. culmorum* Trichothecene Genotypes. *Frontiers in Microbiology*, 7, 406.
- Pasquali, M., Cocco, E., Guignard, C., & Hoffmann, L. (2016). The effect of agmatine on trichothecene type B and zearalenone production in *Fusarium graminearum* , *F. culmorum* and *F. poae*. *PeerJ*, 4, e1672.

- Paul, P. A., Lipps, P. E., Hershman, D. E., McMullen, M. P., 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(9), 999–1011.
- Pestka, J. J. (2010). Deoxynivalenol: Mechanisms of action, human exposure, and toxicological relevance. *Archives of Toxicology*, 84(9), 663–679.
- Pestka, J. J., Lin, W. S., & Miller, E. R. (1987). Emetic activity of the trichothecene 15-acetyldeoxynivalenol in swine. *Food and Chemical Toxicology*, 25(11), 855–858.
- Piñeiro, M. S., Scott, P. M., & Kanhere, S. R. (1995). Mycotoxin producing potential of *Fusarium graminearum* isolates from Uruguayan barley. *Mycopathologia*, 132, 167–172.
- Pitt, J. I., & Miller, J. D. (2017). A Concise History of Mycotoxin Research. *Journal of Agricultural and Food Chemistry*, 65(33), 7021–7033.
- Proctor, R. H., McCormick, S. P., Alexander, N. J., & Desjardins, A. E. (2009). Evidence that a secondary metabolic biosynthetic gene cluster has grown by gene relocation during evolution of the filamentous fungus *Fusarium*. *Molecular Microbiology*, 74(5), 1128–1142.
- Proctor, R. H., McCormick, S. P., Kim, H.-S., Cardoza, R. E., Stanley, A. M., Lindo, L., Kelly, A., Brown, D.W., Lee, T., Vaughan, M. M., Alexander, N. J., Busman, M., & Gutiérrez, S. (2018). Evolution of structural diversity of trichothecenes, a family of toxins produced by plant pathogenic and entomopathogenic fungi. *PLOS Pathogens*, 14, e1006946.
- Renaud, J. B., & Sumarah, M. W. (2016). Data independent acquisition-digital archiving mass spectrometry: Application to single kernel mycotoxin analysis of *Fusarium graminearum* infected maize. *Analytical and Bioanalytical Chemistry*, 408(12), 3083–3091.

- Robbana-Barnat, S., Lafarge-Frayssinet, C., Cohen, H., Neish, G. A., & Frayssinet, C. (1988). Immunosuppressive properties of deoxynivalenol. *Toxicology*, 48(2), 155–166.
- Rosser, B., & Tenuta, A. (2018, October 12). 2018 Grain Corn Ear Mould and Vomitoxin (DON) Survey. *Field Crop News*. Retrieved on April 1st, 2019 from <https://fieldcropnews.com/2018/10/2018-grain-corn-ear-mould-and-vomitoxin-don-survey/>
- Sarver, B.A., Ward, T.J., Gale, L.R., Broz, K., Kistler, H.C., Aoki, T., Nicholson, P., Carter, J., & O'Donnell, K. (2011). Novel *Fusarium* head blight pathogens from Nepal and Louisiana revealed by multilocus genealogical concordance. *Fungal Genetics and Biology*, 48(12), 1096-1107.
- Schaafsma, A. W., Ilinic, L. T., Miller, J. D., & Hooker, D. C. (2001). Agronomic considerations for reducing deoxynivalenol in wheat grain. *Canadian Journal of Plant Pathology*, 23(3), 279–285.
- Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., Chen, W., & Schindel, D. (2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for *Fungi*. *Proceedings of the National Academy of Sciences*, 109(16), 6241–6246.
- Smith, J. L., Limay-Rios, V., Hooker, D. C., & Schaafsma, A. W. (2018). *Fusarium graminearum* Mycotoxins in maize associated with *striacosta albicosta* (lepidoptera: Noctuidae) Injury. *Journal of Economic Entomology*, 111(3), 1227–1242.
- Spolti, P., Del Ponte, E. M., Cummings, J. A., Dong, Y., & Bergstrom, G. C. (2014). Fitness Attributes of *Fusarium graminearum* Isolates from Wheat in New York Possessing a 3-ADON or 15-ADON Trichothecene Genotype. *Phytopathology*, 104(5), 513–519.

- Starkey, D. E., Ward, T. J., Aoki, T., Gale, L. R., Kistler, H. C., Geiser, D. M., 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(11), 1191–1204.
- Suga, H., Karugia, G. W., Ward, T., Gale, L. R., 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(2), 159–166.
- Sugiura, Y., Watanabe, Y., Tanaka, T., Yamamoto, S., & Ueno, Y. (1990). Occurrence of *Gibberella zeae* strains that produce both nivalenol and deoxynivalenol. *Applied and Environmental Microbiology*, 56(10), 3047–3051.
- Sutton, J. C. (1982). Epidemiology of wheat head blight and maize ear rot caused by *Fusarium graminearum*. *Canadian Journal of Plant Pathology*, 4(2), 195–209.
- Tamburic-Ilincic, L., Wragg, A., & Schaafsma, A. (2015). Mycotoxin accumulation and *Fusarium graminearum* chemotype diversity in winter wheat grown in southwestern Ontario. *Canadian Journal of Plant Science*, 95, 931–938.
- Tanaka, T., Hasegawa, A., Yamamoto, S., Lee, U. S., Sugiura, Y., & Ueno, Y. (1988). Worldwide contamination of cereals by the *Fusarium* mycotoxins nivalenol, deoxynivalenol, and zearalenone. 1. survey of 19 countries. *Journal of Agricultural and Food Chemistry*, 36(5), 979–983.
- Tollenaar, M., Nissanka, S. P., Rajcan, I., & Bruulsema, T. W. (1997). Yield response of old and new corn hybrids to nitrogen. *Better Crop*, 81, 3–5.
- Tschanz, A. T., Horst, R. K., & Nelson, P. E. (1975). Ecological Aspects of Ascospore Discharge in *Gibberella zeae*. *Phytopathology*.

- Ueki, I., Giesy, S. L., Harvatine, K. J., Jin, W. K., & Boisclair, Y. R. (2009). The acid-labile subunit is required for full effects of exogenous growth hormone on growth and carbohydrate metabolism. *Endocrinology*, *150*(7), 3145–3152.
- Umpiérrez-Failache, M., Garmendia, G., Pereyra, S., Rodríguez-Haralambides, A., Ward, T. J., & Vero, S. (2013). Regional differences in species composition and toxigenic potential among *Fusarium* head blight isolates from Uruguay indicate a risk of nivalenol contamination in new wheat production areas. *International Journal of Food Microbiology*, *166*, 135–140.
- Varga, E., Wiesenberger, G., Hametner, C., Ward, T. J., Dong, Y., Schöffbeck, D., McCormick, S., Broz, K., Stuckler, R., Schuhmacher, R., Krska, R., Kistler, H. C., Berthiller, F., & Adam, G. (2015). New tricks of an old enemy: Isolates of *Fusarium graminearum* produce a type A trichothecene mycotoxin. *Environmental Microbiology*, *17*, 2588–2600.
- Vesonder, R. F., Ciegler, A., & Jensen, A. H. (1973). Isolation of the emetic principle from *Fusarium*-infected corn. *Applied Microbiology*, *26*(6), 1008–1010.
- Vigier, B., Reid, L. M., Seifert, K. A., Stewart, D. W., & Hamilton, R. I. (1997). Distribution and prediction of *Fusarium* species associated with maize ear rot in Ontario. *Canadian Journal of Plant Pathology*, *19*(1), 60–65.
- Visagie, C. M., Renaud, J. B., Burgess, K. M. N., Malloch, D. W., Clark, D., Ketch, L., Urb, M., Louis-Seize, G., Assabgui, R., Sumarah, M. W., & Seifert, K. A. (2016). Fifteen new species of *Penicillium*. *Persoonia*, *36*, 247–280.
- Ward, T. J., Bielawski, J. P., Kistler, H. C., Sullivan, E., & O'Donnell, K. (2002). Ancestral polymorphism and adaptive evolution in the trichothecene mycotoxin gene cluster of phytopathogenic *Fusarium*. *PNAS*, *99*(14), 9278–9283.

- Ward, T. J., Clear, R. M., Rooney, A. P., O'Donnell, K., Gaba, D., Patrick, S., Starkey, D. E., Gilbert, J., Geiser, D. M., & Nowicki, T. W. (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*, 45(4), 473–484.
- Wegulo, S. N., Bockus, W. W., Nopsa, J. H., De Wolf, E. D., Eskridge, K. M., Peiris, K. H. S., & Dowell, F. E. (2011). Effects of Integrating Cultivar Resistance and Fungicide Application on *Fusarium* Head Blight and Deoxynivalenol in Winter Wheat. *Plant Disease*, 95(5), 554–560.
- Wu, W., Bates, M. A., Bursian, S. J., Flannery, B., Zhou, H.-R., Link, J. E., Zhang, H., & Pestka, J. J. (2013). Peptide YY3–36 and 5-Hydroxytryptamine Mediate Emesis Induction by Trichothecene Deoxynivalenol (Vomitoxin). *Toxicological Sciences*, 133(1), 186–195.
- Yoshizawa, T. (1983). Red-mold disease and natural occurrence. In: *Developments in Food Science 4, Trichothecenes - Chemical, Biological and Toxicological Aspects* (pp. 195–209). Amsterdam, The Netherlands: Elsevier.
- Yoshizawa, T. (2013). Thirty-five Years of Research on Deoxynivalenol, a Trichothecene Mycotoxin: with Special Reference to Its Discovery and Co-occurrence with Nivalenol in Japan. *Food Safety*, 1(1), 2013002–2013002.
- Yoshizawa, T., & Jin, Y. (1995). Natural occurrence of acetylated derivatives of deoxynivalenol and nivalenol in wheat and barley in Japan. *Food Additives and Contaminants*, 12(5), 689–694.
- Yoshizawa, T., & Morooka, N. (1973). Deoxynivalenol and its monoacetate: New mycotoxins from *Fusarium roseum* and moldy barley. *Agricultural and Biological Chemistry*, 37(12), 2933–2934.

Yue, W. (2017). *Characterization of genetic variation in secondary metabolites in Fusarium*.

Ph.D. Dissertation. Kansas State University.

Zamir, L. O., Devor, K. A., Morin, N., & Sauriol, F. (1991). Biosynthesis of Trichothecenes:

Oxygenation Steps Post-trichodiene. *Journal of the Chemical Society, Chemical*

Communications, 1033–1034.

Zamir, L. O., Nikolakakis, A., Huang, L., St-Pierre, P., Sauriol, F., Sparace, S., & Mamer, O.

(1999). Biosynthesis of 3-Acetyldeoxynivalenol and Sambucinol. *Journal of Biological*

Chemistry, 274(18), 12269–12277.

Appendix

Table 1A. Details of corn sampling locations, number of samples and incidence of *Fusarium graminearum* contamination across Ontario in 2015. Locations organized from west to east.

Region	County	Latitude	Longitude	Total samples	<i>F. graminearum</i> positive samples
Pain Court	Chatham-Kent	42.3470	-82.3215	4	0
Muirkirk	Chatham-Kent	42.4671	-81.7298	3	0
Rodney	Elgin	42.5455	-81.6221	28	7
Dresden	Chatham-Kent	42.5531	-82.1483	4	0
Bothwell	Chatham-Kent	42.6312	-81.8722	8	0
Florence	Lambton	42.7052	-81.9636	28	2
Alvinston	Lambton	42.7731	-81.8704	1	0
Port Dover	Norfolk	42.8121	-80.1967	1	0
Tillsonburg	Oxford	42.8270	-80.7025	1	0
Strathroy	Middlesex	42.8586	-81.5646	1	0
Malahide	Elgin	42.8704	-81.0732	4	0
Norwich	Oxford	42.9083	-80.5503	1	0
Norwich	Oxford	42.9877	-80.5971	8	0
Wingham	Huron	43.0040	-81.7994	1	0
Watford	Lambton	43.1010	-81.4576	4	0
Woodstock	Oxford	43.1426	-80.7961	4	0
Woodstock	Oxford	43.1562	-80.7782	1	0
Woodstock	Oxford	43.2362	-80.8134	1	0
Brant	Brant	43.2454	-80.3944	1	0
Exeter	Huron	43.2752	-81.4623	4	0
Tavistock	Oxford	43.3347	-80.7908	1	0
Mitchell	Perth	43.5273	-81.1384	2	0
Elora	Wellington	43.6457	-80.4034	1	0
Blyth	Huron	43.6611	-81.3924	4	0
Wingham	Huron	43.8654	-81.2958	3	0
Joyceville	Frontenac	44.3626	-76.3350	3	0
Chesterville	Stormont,	45.0779	-75.2149	1	0
Chesterville	Stormont	45.0894	-75.2286	2	0
Williamstown	Stormont	45.1059	-74.6519	1	0
Carleton Place	Lanark	45.1407	-76.0506	4	0
Winchester	Stormont	45.1793	-75.4559	1	0
Crysler	Stormont	45.2013	-75.2686	4	0
Moose Creek	Stormont	45.2916	-74.9593	4	0
St. Isidore	Prescott and Russell	45.3882	-74.8662	1	0
Dunvegan	Prescott and Russell	45.4085	-74.7915	1	0
Total	28 farms			141	9

Table 2A. Details of wheat sampling locations, number of samples and incidence of *Fusarium graminearum* contamination across Ontario in 2015. Locations organized from west to east.

Region	County	Latitude	Longitude	Total samples	<i>F. graminearum</i> positive samples
Harrow	Essex	42.0412	-82.8639	2	0
Essex	Essex	42.1291	-82.9093	4	1
St. Joachim	Essex	42.2930	-82.6177	2	0
Belle River	Essex	42.2941	-82.6414	13	3
St. Joachim	Essex	42.2956	-82.6172	2	0
Lakeshore	Essex	42.2964	-82.6439	40	16
Blenheim	Chatham-Kent	42.3409	-81.8809	2	0
Blenheim	Chatham-Kent	42.3616	-82.0730	1	1
Howard	Chatham-Kent	42.4071	-81.9297	10	2
Dover	Norfolk	42.4286	-82.3222	2	0
Ridgetown	Chatham-Kent	42.4291	-81.9250	3	0
Ridgetown	Chatham-Kent	42.4445	-81.8797	1	1
Kent	Chatham-Kent	42.4488	-81.7513	4	0
Thamesville	Chatham-Kent	42.4565	-81.7917	9	0
Ridgetown	Chatham-Kent	42.4648	-81.8925	4	1
Muirkirk	Chatham-Kent	42.4737	-81.7382	3	1
Duart	Chatham-Kent	42.4972	-81.7625	2	1
Muirkirk	Chatham-Kent	42.4996	-81.7802	1	0
Muirkirk	Chatham-Kent	42.5028	-81.7763	1	0
Muirkirk	Chatham-Kent	42.5253	-81.7382	2	1
Dresden	Chatham-Kent	42.5544	-82.1446	2	0
Dresden	Chatham-Kent	42.5834	-82.1235	4	0
Dresden	Chatham-Kent	42.5849	-82.1254	15	11
Wallaceburg	Chatham-Kent	42.5984	-82.3203	3	0
Bothwell	Chatham-Kent	42.6256	-81.9824	3	0
Glencoe	Middlesex	42.7858	-81.7787	4	0
Inwood	Lambton	42.8053	-81.9101	3	1
Melbourne	Middlesex	42.8386	-81.5677	3	0
Melbourne	Middlesex	42.8544	-81.5468	3	0
Melbourne	Middlesex	42.8570	-81.5464	10	4
Mt Brydges	Middlesex	42.8582	-81.4802	4	0
Mt Brydges	Middlesex	42.8679	-81.4811	3	0
Sarnia	Lambton	42.9357	-82.2496	6	1
Wellandport	Lincoln	43.0138	-79.4426	1	0
Ilderton	Middlesex	43.0182	-81.4750	3	0
Wellandport	Lincoln	43.0238	-79.5224	4	0
Strathroy	Middlesex	43.0240	-81.5889	2	0
Exeter	Huron	43.0683	-81.3222	3	0
Exeter	Huron	43.0759	-81.4882	1	0
Exeter	Huron	43.0931	-81.5144	2	0

Thornedale	Middlesex	43.1035	-81.1805	4	0
Exeter	Huron	43.1347	-81.2377	5	3
Mitchell	Perth	43.1347	-81.2377	10	4
Denfield	Middlesex	43.1412	-81.2558	1	1
Ilderton	Middlesex	43.1419	-81.2237	2	0
Caledonia	Haldimand	43.1484	-79.9344	3	0
Lucan	Middlesex	43.1502	-81.4692	3	1
Exeter	Huron	43.1533	-81.2629	2	0
Exeter	Huron	43.1652	-81.2685	1	1
Grand Bend	Lambton	43.2147	-81.7926	5	0
Lucan	Middlesex	43.2277	-81.4009	11	2
Lucan	Middlesex	43.2278	-81.3986	5	0
Grand Bend	Lambton	43.2348	-81.7638	4	2
Grand Bend	Lambton	43.2397	-81.7699	2	1
Zurich	Huron	43.4243	-81.6770	2	0
Huron East	Huron	43.4607	-81.5015	3	0
Dublin	Perth	43.4683	-81.2497	5	1
Huron East	Huron	43.4700	-81.4517	27	7
Huron East	Huron	43.5059	-81.3805	2	0
West Perth	Perth	43.5167	-81.1885	2	0
West Perth	Perth	43.5191	-81.1840	2	0
Huron East	Huron	43.5209	-81.4243	2	0
Central Huron	Huron	43.6180	-81.4265	4	0
Palmerston	Wellington	43.8725	-80.8346	64	4
Kemptville	Grenville	45.0064	-75.6329	28	2
Unspecified	-	-	-	12	3
Total	66 farms			398	77

Table 3A. Details of corn sampling locations, number of samples and incidence of *Fusarium graminearum* contamination across Ontario in 2016. Locations organized from west to east.

Region	County	Latitude	Longitude	Total samples	<i>F. graminearum</i> positive samples
Leamington	Essex	42.0562	-82.5103	1	0
Lakeshore	Essex	42.2346	-82.4840	1	0
Tillbury	Chatham-Kent	42.3489	-82.3226	4	0
Harwich	Chatham-Kent	42.3536	-81.9145	6	6
Blenheim	Chatham-Kent	42.3873	-81.8684	3	0
Ridgetown	Chatham-Kent	42.4478	-81.8787	121	74
Ridgetown	Chatham-Kent	42.4534	-81.8792	4	0
Howard	Chatham-Kent	42.4689	-81.8912	3	3
Dresden	Chatham-Kent	42.5566	-82.1516	4	1
Orford	Chatham-Kent	42.5587	-81.8178	1	1
West Elgin	Elgin	42.5905	-81.6161	1	0
West Elgin	Elgin	42.6026	-81.7456	5	5
Wallacetown	Elgin	42.6251	-81.4613	1	0
West Elgin	Elgin	42.6329	-81.7424	1	0
Bothwell	Chatham-Kent	42.6380	-81.8732	40	20
Port Stanley	Elgin	42.6919	-81.2520	1	0
Oil Springs	Lambton	42.7603	-82.1244	1	0
Dehli	Norfolk	42.8338	-80.5491	1	0
Strathroy	Middlesex	42.8537	-81.5478	1	0
Watford	Lambton	42.8559	-81.8919	1	0
Belmont	Elgin	42.8714	-81.0744	4	0
Lowbanks	Haldimand	42.8790	-79.5224	1	0
Tillsonburg	Oxford	42.8798	-80.8131	1	0
Watford	Lambton	42.9255	-81.8248	1	0
Hagersville	Haldimand	42.9490	-80.1151	1	0
Strathroy	Middlesex	42.9811	-81.6414	2	0
Duncrief	Middlesex	43.0602	-81.4892	1	0
Iderton	Chatham-Kent	43.1267	-81.4058	4	0
Lambton Shores	Lambton	43.1371	-81.9127	1	0
Woodstock	Oxford	43.1433	-80.7959	4	0
Lucan	Middlesex	43.2018	-81.3982	1	0
St. Mary's	Perth	43.2206	-81.1637	1	0
Brant	Brant	43.2440	-80.3953	1	0
Exeter	Huron	43.2693	-81.4901	4	0
West Perth	Perth	43.4044	-81.3925	1	0
Mennonite	Perth	43.4402	-80.8131	1	0
Woolwich	Waterloo	43.4988	-80.4526	4	0
Bornholm	Perth	43.5280	-81.1383	1	0
Wellesley	Waterloo	43.5496	-80.7898	1	0
Elora	Wellington	43.6493	-80.3977	4	0
Blyth	Huron	43.6543	-81.3833	4	0

Alma	Wellington	43.7174	-80.4673	4	0
Mapleton	Wellington	43.7569	-80.8150	1	0
Wingham	Huron	43.8505	-81.2521	4	0
Orangeville	Dufferin	43.9009	-80.1528	4	0
Clarington	Durham	43.9370	-78.6487	1	0
Harriston	Wellington	43.9487	-80.9020	1	0
Bewdley	Northumberland	43.9965	-78.2976	1	0
Cramahe	Northumberland	44.0000	-77.8589	2	0
Oshawa	Durham	44.0189	-78.9250	1	0
New Tecumseth	Simcoe	44.0281	-79.8563	1	0
Ameliasburg	Prince Edward	44.0708	-77.4712	1	0
Dundalk	Grey	44.1018	-80.3689	4	0
Walkerton	Bruce	44.1143	-81.1646	1	0
Brighton	Northumberland	44.1169	-77.7151	1	0
Greater Napanee	Lennox	44.1227	-77.0265	1	0
Kincardine	Bruce	44.1374	-81.6371	1	0
Thorton	Simcoe	44.2666	-79.7447	1	0
Tyendinaga	Hastings	44.2851	-77.2188	1	0
Lindsay	Kawartha Lakes	44.3219	-78.7980	4	0
Minesing	Simcoe	44.3993	-79.9652	1	0
Prescott	Leeds	44.6998	-75.5710	1	0
Williamsburg	Stormont	44.9886	-75.2545	1	0
Williamsburg	Stormont	44.9956	-75.2595	1	0
Winchester	Stormont	45.0600	-75.3398	4	0
Chesterville	Stormont	45.0934	-75.2208	1	0
Munster	Ottawa-Carleton	45.1141	-75.7602	1	0
Ashton	Lanark	45.1579	-76.0336	1	0
Osgoode	Ottawa-Carleton	45.1697	-75.6067	1	0
Moose Creek	Stormont	45.2485	-75.0252	2	0
Stittsville	Ottawa-Carleton	45.2500	-75.8757	1	0
Metcalfe	Ottawa-Carleton	45.2852	-75.4557	1	0
Pakenham	Lanark	45.3323	-76.2907	1	0
Mississippi Mills	Lanark	45.3323	-76.2907	1	0
Kinburn	Ottawa-Carleton	45.3923	-76.1904	1	0
Arnprior	Renfrew	45.4314	-76.3543	1	0
Total	76 farms			299	110

Table 4A. Details of wheat sampling locations, number of samples and incidence of *Fusarium graminearum* contamination across Ontario in 2016. Locations organized from west to east.

Region	County	Latitude	Longitude	Total samples	<i>F. graminearum</i> positive samples
Wheatley	Chatham-Kent	42.1146	-82.4236	4	0
Belle River	Essex	42.2964	-82.6413	13	1
Ridgetown	Chatham-Kent	42.4069	-81.9281	4	1
Ridgetown	Chatham-Kent	42.4317	-81.7882	2	0
Sarnia	Lambton	42.5560	-82.1517	7	3
Dresden	Chatham-Kent	42.5849	-82.1265	17	1
Inwood	Lambton	42.7759	-81.9372	2	0
Melbourne	Middlesex	42.8526	-81.5505	12	1
Sarnia	Lambton	42.9357	-82.2496	14	14
Wyoming	Lambton	42.9815	-82.2220	14	2
Dunfield	Middlesex	43.0446	-81.5094	14	4
London	Middlesex	43.0788	-81.3419	6	0
Ailsa Craig	Middlesex	43.1034	-81.5268	12	2
Ilderton	Middlesex	43.1193	-81.2876	4	1
Bryanston	Middlesex	43.1347	-81.2353	3	1
Grand Bend	Lambton	43.1431	-81.4725	7	2
Lucan	Middlesex	43.2274	-81.4053	9	0
Seaforth	Huron	43.2315	-81.7917	7	0
Mitchell	Perth	43.2634	-81.8266	8	0
Mitchell	Perth	43.4175	-81.1808	17	2
Kippen	Huron	43.4698	-81.4492	5	0
Mitchell	Perth	43.4765	-81.2659	3	0
Bryanston	Middlesex	43.8584	-81.1353	3	1
Lucan	Middlesex	43.9237	-81.2867	4	0
Total	24 farms			191	36

Table 5A. Details of corn sampling locations, number of samples and incidence of *Fusarium graminearum* contamination across Ontario in 2017. Locations organized from west to east.

Region	County	Latitude	Longitude	Total samples	<i>F. graminearum</i> positive samples
Wheatley	Leamington	42.0231	-82.5554	1	0
Kingsville	Essex	42.0537	-82.7208	1	0
Amherstburg	Essex	42.1233	-83.0401	1	0
Lakeshore	Essex	42.1708	-82.5945	2	0
Romney	Chatham-Kent	42.1807	-82.2461	2	0
Emeryville	Essex	42.2448	-82.8041	1	0
Tillbury	Chatham-Kent	42.2598	-82.4320	4	0
Lakeshore	Essex	42.2786	-82.4990	1	0
Blenheim	Chatham-Kent	42.3344	-81.9935	3	0
Harwich	Chatham-Kent	42.3536	-81.9145	15	3
Dover	Chatham-Kent	42.3952	-82.2965	1	0
Chatham	Chatham-Kent	42.4062	-82.1906	8	0
Dover	Chatham-Kent	42.4119	-82.2716	1	0
Dover	Chatham-Kent	42.4204	-82.2575	1	0
Dover	Chatham-Kent	42.4408	-82.3544	4	0
Ridgetown	Chatham-Kent	42.4478	-81.8787	88	30
Duart	Chatham-Kent	42.4756	-81.6985	2	0
Dover	Chatham-Kent	42.4894	-82.4065	1	0
Muirkirk	Chatham-Kent	42.5041	-81.7765	4	2
Howard	Chatham-Kent	42.5050	-81.9997	1	0
Orford	Chatham-Kent	42.5170	-81.7100	1	0
Ridgetown	Chatham-Kent	42.5257	-81.9089	1	0
West Elgin	Chatham-Kent	42.5425	-81.7135	6	0
Thamesville	Chatham-Kent	42.5449	-81.9331	1	0
Thamesville	Chatham-Kent	42.5682	-81.9461	1	0
Camben	Chatham-Kent	42.5686	-82.0124	1	0
Camben	Chatham-Kent	42.6023	-82.2128	1	0
Wallaceburg	Chatham-Kent	42.6045	-82.3584	1	0
Wallacetown	Elgin	42.6088	-81.4338	1	0
Wallacetown	Elgin	42.6306	-81.4630	5	0
St. Clair	Chatham-Kent	42.6570	-82.4583	1	0
Port Stanley	Elgin	42.6760	-81.2187	3	0
Dunwich	Elgin	42.7136	-81.5394	6	0
St. Thomas	Elgin	42.7594	-81.1002	4	0
Mabee's Corners	Norfolk	42.7607	-80.6453	1	0
St. Thomas	Elgin	42.7723	-81.2513	1	0
St. Thomas	Elgin	42.7770	-81.0555	4	0
London	Middlesex	42.8350	-81.1098	2	0
Dehli	Norfolk	42.8362	-80.5545	1	0
Watford	Lambton	42.8571	-81.8938	1	0
Dunnville	Haldimand	42.8594	-79.7818	1	0
Watford	Lambton	42.8699	-81.8768	1	0

St. Clair	Lambton	42.8715	-82.2589	1	0
Dehli	Norfolk	42.8724	-80.4772	1	0
Simcoe	Norfolk	42.8729	-80.2917	1	0
Simcoe	Norfolk	42.8937	-80.1638	1	0
Wainfleet	Welland	42.9054	-79.4312	1	0
London	Middlesex	42.9092	-81.3005	3	0
Jarvis	Haldimand	42.9113	-80.0157	1	0
Watford	Lambton	42.9141	-81.8260	1	0
Norwich	Oxford	42.9872	-80.7044	4	0
Warner	Lincoln	43.0374	-79.6361	1	0
Exeter	Huron	43.0435	-81.4657	1	0
Arva	Middlesex	43.0443	-81.3244	1	0
Wyoming	Lambton	43.0542	-82.1018	1	0
Ailsa Craig	Middlesex	43.0605	-81.5882	1	0
Woodstock	Oxford	43.0660	-80.7654	3	0
Arkona	Lambton	43.0735	-81.8342	3	0
Lambton Shores	Lambton	43.0833	-81.8782	1	0
Carlisle	Hamilton-	43.1025	-81.4558	4	0
Hamilton	Hamilton	43.1095	-79.7603	1	0
Woodstock	Oxford	43.1513	-80.7891	3	0
Hamilton	Hamilton	43.1662	-79.9722	1	0
Lucan	Middlesex	43.2236	-81.4562	9	0
Flamborough	Hamilton	43.2324	-80.1214	1	0
Mount Carmel	Middlesex	43.2557	-81.5722	1	0
Woolwich	Chatham-Kent	43.4962	-80.4285	4	0
Bornholm	Perth	43.5268	-81.1375	2	0
Guelph	Wellington	43.6588	-80.2266	1	0
Blyth	Huron	43.6683	-81.3865	4	0
Londesborough	Huron	43.6755	-81.4661	18	5
Mapleton	Wellington	43.7578	-80.8139	1	0
Arthur	Wellington	43.8194	-80.5410	1	0
Caledon	York	43.8400	-79.8559	1	0
Bluevale	Huron	43.8478	-81.2416	4	0
East Garafraxa	Dufferin	43.9009	-80.1552	4	0
Clarington	Durham	43.9098	-78.6356	1	0
Schomberg	York	43.9750	-79.6720	1	0
Teeswater	Bruce	43.9909	-81.2266	2	0
Port Hope	Northumberland	43.9915	-78.3487	1	0
Haldimand	Northumberland	43.9955	-77.9611	1	0
South bruce	Bruce	44.0052	-81.2361	1	0
Oshawa	Durham	44.0213	-78.9201	1	0
Port Perry	Durham	44.0891	-78.9649	1	0
Blackstock	Durham	44.0931	-78.8169	1	0
Bradford	Simcoe	44.0940	-79.6392	1	0
Shelburne	Dufferin	44.0998	-80.3705	4	0
Brockton	Bruce	44.1528	-81.2919	1	0

Uxbridge	Durham	44.1534	-79.2158	1	0
Alliston	Simcoe	44.1902	-79.8374	1	0
Tyendinaga	Hastings	44.2000	-77.1200	1	0
Brock	Durham	44.2151	-79.0446	1	0
Sunderland	Kawartha Lakes	44.2192	-78.9466	1	0
Greater Napanee	Lennox	44.2347	-76.8690	1	0
Greater Napanee	Lennox	44.2500	-76.9900	1	0
Little Britain	Kawartha Lakes	44.2659	-78.8244	4	0
Greater Napanee	Lennox	44.2700	-76.9500	1	0
Kingston	Frontenac	44.2771	-76.3519	1	0
Creemore	Simcoe	44.3197	-80.0764	1	0
South frontenac	Frontenac	44.3436	-76.5233	1	0
Stayner	Simcoe	44.3977	-79.9457	1	0
Stayner	Simcoe	44.4039	-80.0820	1	0
Augusta	Leeds and Grenville	44.7108	-75.5376	1	0
Shanly	Leeds and Grenville	44.8910	-75.4705	1	0
Manotick	Ottawa-Carleton	45.1498	-75.7392	1	0
Martintown	Stormont	45.1545	-74.7105	1	0
Finch	Stormont	45.1597	-75.0450	1	0
Winchester	Stormont	45.1820	-75.2881	1	0
Monkland	Stormont	45.2105	-74.8955	1	0
Nepean/Kanata	Ottawa-Carleton	45.2703	-75.8321	1	0
Maxville	Stormont	45.2887	-74.8558	1	0
Alexandria	Stormont	45.2950	-74.7634	1	0
Carp	Ottawa-Carleton	45.3370	-76.0503	1	0
Dunvegan	Stormont	45.3619	-74.8186	1	0
Kinburn	Ottawa-Carleton	45.3627	-76.2235	1	0
Lochiel	Stormont	45.3778	-74.6235	1	0
Mississippi	Lanark	45.3855	-76.3732	1	0
Fournier	Prescott and Russell	45.3964	-74.9653	1	0
Arnprior	Renfrew	45.3985	-76.2939	1	0
Dunvegan	Stormont,	45.4069	-74.8378	1	0
Dunvegan	Stormont	45.4074	-74.8218	1	0
St-Bernardin	Prescott and Russell	45.4407	-74.8570	1	0
Bromley	Renfrew	45.5480	-76.9017	1	0
Haley Station	Renfrew	45.5914	-76.7087	1	0
Unspecified	-	-	-	5	0
Total	124 farms			333	40

Table 6A. Details of wheat sampling locations, number of samples and incidence of *Fusarium graminearum* contamination across Ontario in 2017. Locations organized from west to east.

Region	County	Latitude	Longitude	Total samples	<i>F. graminearum</i> positive samples
Kingsville	Essex	42.0952	-82.6854	2	1
Romney	Chatham-Kent	42.1448	-82.4228	4	0
Belle River	Essex	42.2964	-82.6421	26	2
Lakeshore	Essex	42.2964	-82.6413	15	0
Ridgetown	Chatham-Kent	42.4443	-81.8799	216	164
Dresden	Chatham-Kent	42.5848	-82.1251	37	0
Melbourne	Middlesex	42.8537	-81.5484	5	0
Melbourne	Middlesex	42.8538	-81.5487	12	0
Watford	Lambton	42.8793	-81.9851	1	0
Sarnia	Lambton	42.9331	-82.2503	1	0
Wyoming	Lambton	42.9562	-82.1932	5	0
Sarnia	Lambton	42.9647	-82.2833	7	0
London	Middlesex	43.0640	-81.3298	17	0
Ilderton	Middlesex	43.1207	-81.2872	14	0
Bryanston	Middlesex	43.1346	-81.2360	8	0
Bryanston	Middlesex	43.1487	-81.2348	6	0
Parkhill	Middlesex	43.1564	-81.7035	2	0
Lucan	Middlesex	43.1566	-81.4688	3	1
Parkhill	Middlesex	43.1585	-81.7180	3	0
Lucan	Middlesex	43.2175	-81.4034	11	0
Lucan	Middlesex	43.2277	-81.4009	11	1
Grand Bend	Lambton	43.2680	-81.7724	22	1
Mitchell	Perth	43.4183	-81.1953	5	0
Mitchell	Perth	43.4974	-81.2641	2	0
Mitchell	Perth	43.5185	-81.1854	2	0
Palmerston	Wellington	43.8725	-80.8346	15	2
Kemptville	Leeds	45.0064	-75.6329	14	3
St-Hyacinthe	Monteregie	45.6282	-72.9568	24	20
Total	28 farms			490	195