Species and Trichothecene Genotypes of Fusarium Head Blight Pathogens in Nebraska, USA in 2015-2016

Esteban Valverde-Bogantes
University of Nebraska-Lincoln, esteban@huskers.unl.edu

Follow this and additional works at: http://digitalcommons.unl.edu/foodscidiss

Valverde-Bogantes, Esteban, "Species and Trichothecene Genotypes of Fusarium Head Blight Pathogens in Nebraska, USA in 2015-2016" (2017). Dissertations, Theses, & Student Research in Food Science and Technology. 80.
http://digitalcommons.unl.edu/foodscidiss/80

This Article is brought to you for free and open access by the Food Science and Technology Department at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Dissertations, Theses, & Student Research in Food Science and Technology by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.
SPECIES AND TRICHTHECENE GENOTYPES OF FUSARIUM HEAD BLIGHT
PATHOGENS IN NEBRASKA, USA IN 2015-2016

By

Esteban Valverde-Bogantes

A THESIS

Presented to the Faculty of
The Graduate College at the University of Nebraska
In Partial Fulfillment of Requirements
For the Degree of Master of Science

Major: Food Science and Technology

Under the Supervision of Professor Heather Hallen-Adams

Lincoln, Nebraska

May, 2017
Fusarium head blight (FHB) of wheat and other small grain cereals is a devastating and economically important disease caused by members of the *Fusarium graminearum* species complex (FGSC). FHB poses a threat to food security and food safety, due to yield and quality reduction, as well as mycotoxin contamination. To determine the diversity of species and trichothecene genotypes of the FGSC in Nebraska, 33 wheat samples were collected from FHB-affected fields during two consecutive years (2015 and 2016) and 50 *Fusarium* isolates were obtained from those wheat samples. Identification of *F. graminearum* was attempted by PCR using UBC85 and GO primers. However, these primers proved insufficient to accurately identify *F. graminearum*, because they were unable to discriminate between species within the FGSC. DNA was also subjected to a multilocus genotyping (MLGT) assay for the simultaneous determination of species identity. Inconclusive results from the MLGT assay prompted sequencing of the *RED* and *TRI101* genes from four isolates. Sequence analysis identified two of the isolates as *F. graminearum* and the other two isolates as *F. boothii*. Additionally, intragenomic heterogeneity of their *RED* and *TRI101* genes was detected.
The trichothecene genotype of the Nebraska isolates was determined using a multiplex PCR assay. The overwhelming majority of isolates had the 15-ADON genotype. However, two isolates produced inconclusive results with the trichothecene genotyping multiplex PCRs. Under greenhouse conditions, these two isolates failed to cause disease and accumulate mycotoxins in the grain of the FHB-susceptible wheat cultivar Samson.

This is the first study to report *F. boothii* isolates in Nebraska, as well as intrastrain *RED* and *TRI101* sequence heterogeneity in FHB pathogens. The data obtained from this study can be used as baseline data for future surveillance studies in Nebraska and North America and to better understand the biology and ecology of FHB pathogens. The findings will also be useful to lessen the risk of mycotoxin contamination in wheat and wheat-based products.
Acknowledgements

I would like to express my sincere gratitude to Dr. Heather Hallen-Adams for giving me the opportunity to join her lab. I am extremely grateful for your guidance, expertise, help and support.

I would also like to thank the remainder of my committee: Dr. Stephen Wegulo and Dr. Andreia Bianchini, who were willing to share their knowledge and expertise in order to support this project.

Thanks to Dr. Todd Ward from the USDA-ARS for providing the positive PCR controls used in this study and for his continuing support. Thanks to Dr. David Schmale III from Virginia Tech for providing reference DNA from isolates with a known trichothecene genotype and for performing the mycotoxin quantification.

Thanks to my family and friends for their unconditional support.
Declaration of Grant Information

This project is supported by USDA Hatch Multistate NC-1183: Mycotoxins: Biosecurity, Food Safety and Biofuels Byproducts.
TABLE OF CONTENTS

Acknowledgements ......................................................................................................................... iv
Declaration of Grant Information ........................................................................................................ v
Table of Contents ............................................................................................................................ vi
Table of Contents Tables and Figures .......................................................................................... viii
Chapter 1 ........................................................................................................................................ 10
Literature review ............................................................................................................................ 10
1.1 Fusarium head blight.............................................................................................................. 11
1.1.1 Importance to food security and food safety .............................................................. 12
1.2 Causal agents ..................................................................................................................... 13
1.2.1 Fusarium graminearum life cycle in wheat ............................................................... 15
1.3 Control of FHB .................................................................................................................. 16
1.4 Mycotoxin production and food safety .............................................................................. 19
1.4.1 Biosynthesis ............................................................................................................... 20
1.4.2 Chemotypes of FHB pathogens ................................................................................. 22
1.4.3 Effects on animals and humans .................................................................................. 24
1.4.4 Regulations in the USA.............................................................................................. 27
1.5 Conclusion ......................................................................................................................... 28
1.6 References .......................................................................................................................... 29
Chapter 2 ........................................................................................................................................ 35
Species of Fusarium head blight pathogens in Nebraska, USA in 2015-2016 .............................. 35
2.1 Abstract .............................................................................................................................. 36
2.2 Introduction ........................................................................................................................ 37
2.3 Materials and Methods ....................................................................................................... 39
2.3.1 Samples ...................................................................................................................... 39
2.3.2 Isolation of FHB pathogens from wheat kernels ........................................................ 42
2.3.3 DNA extraction from isolates and wheat kernels ...................................................... 43
2.3.4 F. graminearum PCR ................................................................................................. 45
2.3.5 Multilocus genotyping assay ...................................................................................... 45
2.3.6 Analysis of intragenomic heterogeneity of RED and TRI101 genes ......................... 49
2.3.6.1 PCR amplification and sequencing ........................................................................ 49
2.3.6.2 Cloning of RED & TRI101 genes ........................................................................ 49
TABLE OF CONTENTS TABLES AND FIGURES

Figures Chapter 1
Figure 1.1: Chemical structures of the most important Type B trichotheccenes.........................19

Tables Chapter 2
Table 2.1: Summary of the wheat heads sampled across Nebraska for this study and their location............................................................................................................................40
Table 2.2: Sequence of the primers used in this study........................................................................43
Table 2.3: ASPE probes used in this study............................................................................................46
Table 2.4: Median Fluorescent Intensity (MFI) values obtained from MLGT assay for reference isolates ........................................................................................................................................................................54
Table 2.5: Median Fluorescent Intensity (MFI) values obtained from MLGT assay for selected isolates ........................................................................................................................................................................55
Table 2.6: Intragenomic polymorphisms found in clones ..................................................................57
Table 2.7: Alignment of the sequences from RED clones, the sequences of their respective top five hits from GenBank and selected probe sequences designed by Ward et al. (2008) for the identification of FHB pathogens ........................................................................................................................................59
Table 2.8: Alignment of the sequences from TRI101 clones, the sequences of their respective top five hits from GenBank and selected probe sequences designed by Ward et al. (2008) for the identification of FHB pathogens ........................................................................................................................................60

Figures Chapter 2
Figure 2.1: Map showing the different wheat growing regions in Nebraska and sampling sites where wheat heads were obtained .................................................................40
Figure 2.2: Agarose gel showing fragments amplified with the UBC85 primers for isolates 1-5 from 2015 ..........................................................................................................................52
Figure 2.3: Agarose gel showing fragments amplified with the GO primers for isolates 1-5 from 2015 ..........................................................................................................................52
Figure 2.4: Agarose gel showing fragments amplified with the UBC85 and GO primers for reference Fusarium isolates ........................................................................................................53
Figure 2.5: Direct sequencing chromatogram of the PCR product of the RED gene from isolate 20

Figure 2.6: Phylogenetic tree inferred from RED sequence data representing the relationship of clones to their top hits from GenBank

Figure 2.7: Phylogenetic tree inferred from TRI101 sequence data representing the relationship of clones to their top hits from GenBank

Tables Chapter 3
Table 3.1: Sequence of the primers used in this study
Table 3.2: Results from trichothecene genotyping PCR for the Fusarium isolates used in this study
Table 3.3: Levels of deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON) and nivalenol (NIV) in grains from artificially inoculated wheat

Figures Chapter 3
Figure 3.1: Amplification products produced by multiplex PCR with the TRI3 primers
Figure 3.2: Amplification products produced by multiplex PCR with the TRI12 primers
Figure 3.3: Agarose gel showing fragments amplified with the individual trichothecene genotyping primer pairs
Chapter 1
LITERATURE REVIEW
FUSARIUM HEAD BLIGHT
1.1 Fusarium head blight

Fusarium Head Blight (FHB), also known as scab, is a disease that affects wheat, barley, oats and other small grain cereals. FHB was first described in 1884, but it was not characterized histologically and epidemiologically until the 1950s (Trail et al. 2005a). FHB is a devastating and economically important disease that has caused significant losses worldwide, primarily due to yield reduction and mycotoxin contamination (McMullen et al. 1997). From 1998 to 2000 alone, FHB epidemics caused losses of almost $3 billion, in the United States and Canada alone (Goswami and Kistler 2004), and all of the cereal-growing regions in the world have experienced a reemergence of FHB epidemics (Dean et al. 2012). Therefore, FHB is considered one of the major diseases currently known to plant pathologists (Dean et al. 2012).

The state of Nebraska has consistently been one of the top ten wheat producing states. In fact, according to data from the USDA, roughly 71 million bushels of wheat were produced in Nebraska in 2016 and, therefore, wheat production has a significant impact on the state’s economy (USDA 2017).

In order to ensure the safety of the grains produced, and to reduce economic losses, it is important to understand all aspects of FHB, including its importance to food security and food safety, the causal agents and how to control these plant pathogens, mycotoxins produced, and how these mycotoxins are regulated before the grains are released into the food chain. The following sections of this review will discuss these aspects and provide important background in support of the following chapters.
1.1.1 Importance to food security and food safety

Recent outbreaks from all over the world indicate that FHB is an emerging threat to the world’s grain supply (Starkey et al. 2007). This disease is particularly problematic because it poses a threat to both food security and food safety, due to yield reductions and mycotoxin contamination (Goswami and Kistler 2004). FHB also causes reduced test weight and lowers market grade (McMullen et al. 1997).

When *Fusarium* grows on the host plant, it causes a premature bleaching of spikes that are either sterile or produce shriveled and soft grains, also known as *Fusarium*-damaged kernels (FDK), scabby kernels, or “tombstones”, that have reduced weight and functional qualities (Trail 2009). Additionally, the FDK may be contaminated with trichothecene mycotoxins, mainly deoxynivalenol (DON) and nivalenol (NIV), which are harmful both to humans and animals (Wegulo 2012). DON is also known as vomitoxin due to its emetic effect, and it is very stable during processing and high temperature treatments (Rotter et al. 1996).

Grains contaminated with mycotoxins are unsafe for human consumption, animal consumption or malting (Dean et al. 2012). Mycotoxin contamination further reduces marketability and prices, in many instances forcing farmers to sell at animal feed prices and to be penalized with huge discounts (McMullen et al. 1997).

Due to the reasons mentioned, grains affected by FHB are difficult to market, export, process and feed. Low commodity prices coupled with the difficulties inherent in avoiding FHB and the economic losses it represents, make wheat cultivation unattractive
to farmers, who are discouraged to plant wheat. This situation has forced many U.S. farmers to shift to alternative less risky crops (McMullen et al. 2012; Bianchini et al. 2015).

1.2 Causal agents

Phylogeny:

Kingdom: Fungi
Subkingdom: Dikarya
Phylum: Ascomycota
Subphylum: Pezizomycotina
Class: Sordariomycetes
Subclass: Hypocreomycetidae
Order: Hypocreales
Family: Nectriaceae
Genus: *Fusarium*

(NCBI 2016b)

The causal agents of FHB are members of the genus *Fusarium*. During their sexual development, they produce flask-shaped perithecia, which are filled with tubular sacs called asci. Asci contain the sexual spores (ascospores), which are the products of meiosis (Trail 2009). *Fusarium* spp. may produce three types of asexual spores:
macroconidia, microconidia and chlamydospores. Whereas some species are able to produce all three types of spores, others are not (Nelson et al. 1994).

Even though FHB is caused by several species of *Fusarium*, *F. graminearum* is the predominant causal agent in North America, Europe and other regions of the world (Goswami and Kistler 2004). *F. graminearum* was thought to be a single panmictic species, but genealogical concordance/discordance phylogenetic species recognition studies showed that it actually contains 16 distinct species. Those 16 species are grouped within the *F. graminearum* species complex (FGSC) and its members are: *F. austroamericanum*, *F. boothii*, *F. meridionale*, *F. mesoamericanum*, *F. lousianense*, *F. acacia-mearnsii*, *F. brassilicum*, *F. cortaderiae*, *F. gerlachii*, *F. nepalense*, *F. graminearum*, *F. asiaticum*, *F. aethiopicum*, *F. vorosii*, *F. ussurianum*, and unnamed *Fusarium* sp. NRRL 34461. The members of the FGSC are self-fertile (=homothallic). In addition, *F. lunulosporum*, *F. pseudograminearum*, *F. cerealis* and *F. culmorum* are self-sterile (=heterothallic) FHB pathogens closely related to the FGSC. Members of the FGSC have a significant biogeographic structure, which suggests that independent allopatric speciation occurred in different parts of the world (Aoki et al. 2012). *F. dactylidis* and *F. praegraminearum*, two novel species able to cause FHB, have recently been described (Aoki et al. 2015; Gräfenhan et al. 2016).

*F. avenaceum*, *F. poae* and the non-toxigenic species *Microdochium nivale* are also commonly associated with the disease in different parts of the world (Edwards et al. 2001; Nielsen et al. 2014).

It has been reported that morphological and phenotypic characters are not sufficient to distinguish most of the species within the FGSC, due to their morphological simplicity
and overlapping conidial characters combined with within-species variability (Aoki et al. 2012). Thus, molecular methods are recommended for the identification of FHB pathogens.

1.2.1 *Fusarium graminearum* life cycle in wheat

After the growing season, *F. graminearum* overwinters on crop residue as saprophytic mycelia. During the spring, the fungus forms flask-shaped perithecia, which are quite ephemeral and contain ascospores (Trail 2009). Sexual development in this fungus is regulated by the mating type locus (*MAT*), which contains two alleles, *MAT* 1-1 and *MAT* 1-2 (Gilbert and Haber 2013).

The ascospores are forcibly discharged through a small opening at the apex of the perithecia called the ostiole (Gilbert and Haber 2013). Ascospore discharge generally occurs at the same time as the flowering of the cereal host plants (Trail et al. 2005b), when the florets are most susceptible to the infection (Goswami and Kistler 2004; Wegulo et al. 2015).

After being discharged, ascospores become airborne – although they can also be dispersed by rain or insects – and land on flowering spikelets, germinate and enter the plant through the anthers (Goswami and Kistler 2004). *F. graminearum* then spreads quickly and asymptptomatically to other parts of the wheat spikelet until it reaches the vascular system and the medulla of the rachis. From there, the fungus spreads to other spikelets and the culm, causing necrosis that is evident by the typical bleaching of the
tissue (Trail et al. 2005a). DON acts as a virulence factor in wheat, causing necrosis and facilitating the spread of the fungus, and its accumulation begins shortly after infection of the wheat florets (Trail 2009; Hallen-Adams et al. 2011).

The fungus produces sporodochia with abundant asexual spores (conidia), which produce a pinkish color on severely infected wheat heads (Pirgozliev et al. 2003). However, the conidia have a limited range of dispersal and are of minor epidemiological importance. As a consequence, FHB is considered to be a monocyclic disease, i.e. a disease in which the inoculum is not produced in the host and with only one infection event per year (Wegulo et al. 2015; Filho et al. 2016).

Finally, after harvest in a no-till system, F. graminearum remains in the crop debris and the cycle starts over (Trail 2009).

1.3 Control of FHB

Hitherto, no specific solution has been shown to fully control FHB. Even when the strategies available are used, some problems with yield reduction, grade reduction and mycotoxin contamination still persist, especially when the weather conditions are advantageous for FHB pathogens.

These strategies to prevent FHB and mycotoxin contamination of the grains include agricultural practices, for example, tillage to bury crop residue, thus reducing inoculum survival and production of perithecia and macroconidia (Wegulo et al. 2015).
Crop rotation with soybeans or other non-host plants, instead of corn or wheat, has been reported to reduce disease severity and mycotoxin levels (Marburger et al. 2015; Wegulo et al. 2015), although it was also reported that some non-host plants can serve as the inoculum source of FHB (Pioli et al. 2004; Mourellos et al. 2014; Chiotta et al. 2015; Duvnjak et al. 2016).

It is widely known that a warm and humid environment is an important factor for FHB disease development. For that reason, irrigation should be avoided during flowering and early grain fill periods, when the plants are more susceptible to infection (Wegulo et al. 2015).

As far as resistance to FHB in wheat, it involves a complex network of pathways and it is inherited quantitatively (Bai and Shaner 2004). The development of resistance has been difficult and, as of yet, only a few moderately resistant cultivars are available. However, efforts to develop and release cultivars with moderate resistance to FHB are continuing (Wegulo et al. 2015). \textit{Fhb1} is the most important quantitative trait locus (QTL) used for resistance breeding (Rawat et al. 2016). Recently, a chimeric lectin with agglutinin domains and a pore-forming toxin-like domain that confers qualitative disease resistance to plants against FHB was reported (Rawat et al. 2016). This protein is encoded by \textit{PFT}, a gene within \textit{Fhb1}. Even though the biochemical mechanism for resistance mediated by \textit{PFT} is still unknown, it is expected that this discovery will be used in the development of new disease-resistant cultivars (Rawat et al. 2016).

Another way to manage FHB is by using fungicides. Currently, the most commonly used fungicides are sterol biosynthesis inhibitors, which include metconazole, propiconazole, prothioconazole and tebuconazole (Wegulo et al. 2015). These fungicides inhibit the
biosynthesis of ergosterol, which is a structural constituent of the fungal cell membranes (Burden et al. 1989). However, the effectiveness of chemical control of FHB has been inconsistent (McMullen et al. 2012; Gilbert and Haber 2013), which has discouraged farmers from using fungicides to control FHB. It is important to remark that fungicides should be applied in an optimal and timely manner, during anthesis or up to 6 days after anthesis, and using FHB modeling and forecasting systems to determine the risk of disease outbreak (McMullen et al. 2012; Wegulo et al. 2015).

Microbial antagonists have also been proposed as a strategy to control FHB. Biocontrol agents can be useful during the pre-harvest interval when fungicides cannot be legally applied to the crops, or in organic farming (Wegulo et al. 2015). Many antagonists have been reported and they include bacterial organisms like *Bacillus* spp., *Pseudomonas* spp., *Lysobacter enzymogenes*, *Streptomyces* spp.; as well as fungal organisms including *Cryptococcus* spp., *Trichoderma* spp., *Clonostachys rosea* and *Aureobasidium pullulans* (Wegulo et al. 2015). These organisms are more effective when they are used in conjunction with chemical control, in order to reduce the use of fungicides, rather than completely replace them (Gilbert and Haber 2013). Additionally, *Microsphaeropsis* spp. have been shown to reduce *F. graminearum* production of perithecia when applied to crop residues in the field (Pirgozliev et al. 2003). Currently, none of these biological control agents are commercially available (Wegulo et al. 2015).

Overall, when controlling FHB and DON accumulation, the best results are obtained when two or more management practices are integrated (Wegulo 2012; Wegulo et al. 2015). Therefore, it is recommended that farmers in areas with a forecasted high risk of FHB should use multiple control strategies when feasible (Wegulo et al. 2015).
1.4 Mycotoxin production and food safety

Trichothecene mycotoxins are a group of related toxins produced by different species of *Fusarium* that have long been known to be harmful to animals and humans. Trichothecenes are secondary metabolites produced from the isoprenoid metabolism and have a tricyclic nucleus with an epoxide function (Alexander et al. 2009).

Four types of trichothecenes have been described: types A, B, C and D. Type A trichothecenes are very toxic, and include T-2 toxin and HT-2 toxin. Type B trichothecenes include DON, 15-acetyldeoxynivalenol (15-ADON), 3-acetyldeoxynivalenol (3-ADON), and NIV and its acetylated derivatives (Fig. 1.1). Type C and D trichothecenes are not associated with FHB (Foroud and Eudes 2009). Even though type B trichothecenes are not the most toxic trichothecenes, they are the most common trichothecenes contaminating wheat (Wegulo 2012), and therefore, the trichothecenes of most concern.
Figure 1.1: Chemical structures of the most important Type B trichothecenes. All type B trichothecenes have a keto (carbonyl) group at C-8. Differences between these four compounds are shown in red. Unlike DON and acetylated DON, NIV has an oxygen at C-4 (red). The acetylated derivatives of DON: 15-ADON and 3-ADON have an acetyl group at C-15 and C-3, respectively (red). Structures adapted from: NCBI (2016a).

1.4.1 Biosynthesis

The first step in the biosynthesis of type B trichothecenes is the sesquiterpene cyclization of farnesyl diphosphate to trichodiene. This reaction is catalyzed by the enzyme
trichodiene synthase, which is encoded by the \textit{TRI5} gene (Goswami and Kistler 2004; Alexander et al. 2009). Next, trichodiene undergoes four oxygenation reactions catalyzed by the cytochrome P450 monooxygenase encoded by \textit{TRI4}. This enzyme adds three oxygens at C-2, C-3, C-11, and the epoxidation at C-12/C-13 to form isotrichotriol (McCormick et al. 2011).

Isotrichotriol is non-enzymatically converted to isotrichodermol. During this step, the oxygen at the C-2 position becomes the pyran ring oxygen and the hydroxyl group at C-11 is lost (McCormick et al. 2011). Isotrichodermol undergoes an acetylation on the C-3 hydroxyl by the acetyltransferase encoded by \textit{TRI101} to produce isotrichodermin (Alexander et al. 2009).

The next step is the addition of another hydroxyl group to C-15 by a cytochrome P450 encoded by \textit{TRI11}. This hydroxyl group is then acetylated by an acetyltransferase encoded by \textit{TRI3} to form calonectrin (Alexander et al. 2009). Calonectrin is later modified by the addition of hydroxyl groups at the C-7 and C-8 positions by the product of \textit{TRI1}. Subsequently, the hydroxyl group at the C-8 position is converted to the keto form, producing 3,15-acetyldeoxynivalenol (3,15ADON) (McCormick et al. 2011). From here, the pathway branches and NIV-producing strains hydroxylate the C-4 position by a reaction catalyzed by the cytochrome P450 monooxygenase encoded by \textit{TRI13}.

Conversely, DON-producing strains lack a functional \textit{TRI13} and are unable to add a hydroxyl group at C-4 (Alexander et al. 2009).

The last step in the biosynthesis of type B trichothecenes is the selective removal of an acetyl group, either on C-3 or C-15, depending on the activity of the esterase encoded by \textit{TRI8}. Alexander and colleagues reported that the \textit{TRI8} gene from 3-ADON producer
strains encodes a C-15 deacetylase, whereas the TRI8 gene from 15-ADON producers encodes a C-3 deacetylase (Alexander et al. 2011).

Trichothecene biosynthesis also requires the expression of the TRI12 gene, which encodes for a trichothecene efflux pump (Alexander et al. 1999; Alexander et al. 2009). The genes TRI6 and TRI10 encode transcription factors that regulate trichothecene production, acting as positive regulators of the trichothecene biosynthesis genes (Goswami and Kistler 2004). Additionally, TRI15 encodes a transcription factor that has been proposed as a negative regulator of some of the trichothecene biosynthetic genes (Alexander et al. 2003; Desjardins and Proctor 2007).

1.4.2 Chemotypes of FHB pathogens

FHB pathogens are classified depending on their chemotype, i.e., the types of trichothecenes they produce. There are three different chemotypes: 3ADON (producing DON and 3-ADON), 15ADON (producing DON and 15-ADON) and NIV (producing nivalenol and its acetylated derivatives) (Pan et al. 2013). Differences in oxygenation and acetylation of the toxins can change their bioactivity and toxicity, which is relevant to strain fitness and ability to cause disease (Pasquali and Migheli 2014).

The purpose of determining the chemotype of strains is to obtain information on the population in a given area and identify the major toxigenic risks in the affected grains (Pasquali and Migheli 2014). In addition, the fact that, in some instances, isolates with
different chemotypes represent different genetic populations has been used in population
dynamics studies (Ward et al. 2008; Pasquali and Migheli 2014).

PCR-based methods have been developed to predict the chemotype of FHB pathogens.
These methods are based on differences in the alleles at some trichothecene biosynthesis
genes, for instance TRI3, TRI5, TRI7, TRI12 and TRI13; and rely on the amplification of
fragments with differing sizes in a multiplex PCR (Reynoso et al. 2011; Pasquali and
Migheli 2014; Gong et al. 2015). PCR assays have many advantages, like ease of use and
rapid results. These polymorphisms transcend species differences in their distribution;
however, they do not take into account all of the variation present in the target genes in
field populations. Additionally, discrepancies have been reported between the chemotype
determined by PCR and the chemotype obtained by chemical analysis (Reynoso et al.
2011; Pasquali and Migheli 2014; Kulik et al. 2016) and the genetic methods seem to be
insufficient to evaluate the ability of an isolate to produce the toxin (Pasquali and Migheli
2014). Hence, it was proposed that the results from PCR analysis should be referred to as
the trichothecene genotype, whereas chemotype should be reserved for the results from
chemical analysis (Desjardins 2008; Reynoso et al. 2011).

Previous reports showed that the 15ADON genotype is the dominant genotype in North
America (Abramson et al. 2001; Ward et al. 2008; Lee et al. 2015). Nevertheless, Ward
and colleagues reported a replacement of the existing 15ADON chemotype by a 3ADON-
producing F. graminearum that was able to produce more and larger conidia, grow more
rapidly and produce more trichothecene than the 15ADON chemotype (Ward et al. 2008).
They also reported that the 3ADON-producing strains were not different from a 3ADON
population identified in North Dakota and Minnesota by Gale and collaborators (Gale et
al. 2007; Ward et al. 2008). Furthermore, this genetically divergent population in North Dakota and Minnesota was reported to be expanding southwards in its distribution (Liang et al. 2014). Additional studies reported the presence of the NIV and the 3ADON chemotype in Louisiana and the eastern United States (Starkey et al. 2007; Schmale et al. 2011).

The location of Nebraska in the Midwest makes the introduction of these chemotypes into the state feasible and highlights the need for increased vigilance. Information on the circulating species and chemotypes is essential due to the fact that the rapid detection methods for mycotoxins that are being used in Nebraska have deficiencies in the detection and quantification of acetylated derivatives and NIV (Tangni et al. 2010). Hence, constant monitoring of the circulating chemotypes in Nebraska is vital to ensure the safety of the wheat produced and avoid the release of contaminated grain into the food chain by selecting suitable detection methods.

1.4.3 Effects on animals and humans

The main effect of DON, at the cellular level, is inhibition of protein synthesis by binding to the 60S subunit of the ribosomes and interfering with peptidyltransferase activity, which is necessary for polypeptide elongation and termination (Rotter et al. 1996; Desjardins 2006). It was also reported that alterations in signaling pathways, especially at the level of mitogen-activated protein kinases (MAPK) are important for trichothecene toxicity (Pestka and Smolinski 2005). This causes ribotoxic stress and consequently,
disruption of macromolecule synthesis, cell signaling, differentiation and proliferation (Pestka 2010).

Sensitivity to DON varies between species: monogastric animals, especially swine, are the most sensitive; whereas chickens and turkeys, followed by ruminants, are more tolerant (Rotter et al. 1996; Desjardins 2006). Thus, feed ingredients intended for swine have a lower advisory tolerance limit than feed ingredients intended for other animals (Wegulo 2012).

The most common symptom after acute exposure to DON in sensitive animal species is emesis, but it also causes abdominal distress, increased salivation, malaise, fever, diarrhea and anorexia (Pestka and Smolinski 2005; Sobrova et al. 2010; Bianchini et al. 2015). DON’s strong emetic effects are caused by altered neuroendocrine signaling that might involve elevations of serotonin levels (Pestka 2010).

Consumption of extremely high DON doses (unlikely to be encountered in food, ≥27 mg/kg bw) by experimental animals can lead to severe tissue damage and even death (Pestka and Smolinski 2005; Sobrova et al. 2010). In contrast, chronic dietary exposure to low quantities of DON may lead to anorexia and weight gain suppression (Pestka and Smolinski 2005; Bianchini et al. 2015).

DON and other trichothecenes also have the ability to alter immune responses in different ways, depending on the dose. Whereas low levels of DON induce expression of early response and proinflammatory genes, high levels of DON promote leukocyte apoptosis (Pestka 2010; Bianchini et al. 2015). In addition, rodent studies have shown that
consumption of high levels of DON had negative effects on reproduction and development, which might reflect maternal toxicity (Pestka 2010).

Based on animal studies, it seems possible that DON might produce the same effects in humans. At the beginning of the 20th century, numerous outbreaks of human toxicosis associated with the consumption of grains contaminated by *Fusarium* ("red mold disease") were documented in China, Russia, Japan and Korea (Desjardins 2006). Primary symptoms included nausea, vomiting, diarrhea, headache, dizziness and trembling; which are very similar to the effects of DON consumption in animals (Pestka and Smolinski 2005). The consumption of products made from contaminated grains also produced similar symptoms and the disease could be controlled by stopping consumption of the implicated products (Desjardins 2006).

During the 1950s, *F. graminearum* was the most frequent species isolated from moldy grains implicated in an outbreak in Japan and the symptoms reported in humans were produced in mice after administration of wheat contaminated with the same *F. graminearum* isolates (Desjardins 2006). Nonetheless, DON content of the grains was not investigated due to the lack of analytical methods (Pestka and Smolinski 2005).

In 1989, the consumption of moldy wheat was associated with an outbreak of gastroenteritis in the Kashmir Valley of India. Some of the samples collected from the affected area had up to 8.4 ppm, however, a direct association between the samples and the disease could not be established because the samples were collected 4 months after the outbreak (Pestka and Smolinski 2005).
It was not until the 1990s that DON and other trichothecenes were detected in scabby cereals associated with several serious gastroenteritis outbreaks in China. Using the analytical methods available, the DON concentration in the samples was reported to range from 2 to 50 ppm (Pestka and Smolinski 2005).

DON consumption has also been linked epidemiologically with esophageal cancer in China and Africa (Rotter et al. 1996). However, it is difficult to establish this relationship taking into consideration that the carcinogenic mycotoxin fumonisin B1 – produced by different *Fusarium* species which also infect grains (particularly maize) – was reported as a contributing factor and most studies report that DON is noncarcinogenic (Pestka and Smolinski 2005).

### 1.4.4 Advisory levels in the USA

Due to its high prevalence and effects on human and animal health, many governments have regulatory standards to control DON contamination in foods. In the USA, the Food and Drug Administration has published a document that contains the following advisory levels for DON:

- 1 ppm in finished wheat products destined for human consumption.
- 10 ppm in grains and grains by-products and 30 ppm in distillers’ grains and brewers’ grains destined for ruminating beef and feedlot cattle older than 4 months and for chickens.
• 5 ppm in grains and grain by-products and 30 ppm in distillers’ grains and brewers’ grains destined for swine as long as these do not exceed 20% of their diet.

• 5 ppm in grains and grain by-products and 30 ppm in distillers’ grains and brewers’ grains destined for other animals as long as these do not exceed 40% of their diet.

(FDA 2010)

1.5 Conclusion

Numerous investigations have highlighted the impact of FHB on the economy as well as the risks it poses to human and animal health. In addition, strategies for the control of FHB are complex and inconsistently effective. Therefore, constant vigilance of FHB pathogens is important to monitor their distribution and ecology, in addition to detect population clines and the introduction of strains with novel phenotypes. For this purpose, trichothecene genotypes have been used in population studies and can be useful in monitoring potential threats to food safety.
1.6 References


Chapter 2

SPECIES OF FUSARIUM HEAD BLIGHT PATHOGENS IN NEBRASKA, USA IN 2015-2016
2.1 Abstract

Fusarium head blight (FHB) of wheat and other small grain cereals is a devastating and economically important disease caused by members of the *Fusarium graminearum* species complex (FGSC). FHB poses a threat to food security and food safety, due to yield and quality reduction, as well as mycotoxin contamination. To determine the diversity of species of the FGSC in Nebraska, 33 wheat samples were collected from affected fields during two consecutive years (2015 and 2016) and 50 *Fusarium* isolates were obtained from those wheat samples. Identification was attempted by PCR using UBC85 and GO primers, designed for the identification of *F. graminearum*. However, these primers proved insufficient to accurately identify *F. graminearum*, because they were unable to discriminate between species within the FGSC. DNA was also subjected to a multilocus genotyping (MLGT) assay for the simultaneous identification of several species. Inconclusive results from the MLGT assay prompted sequencing of the *RED* and *TRI101* genes from four selected isolates. The identities of those four isolates were confirmed to be *F. graminearum* (*n* = 2) and *F. boothii* (*n* = 2). To resolve initial sequence ambiguities, PCR products were cloned and intragenomic heterogeneity of *RED* and *TRI101* genes was detected. This is the first study to report that FHB pathogens can have intragenomic heterogeneity in the *RED* and *TRI101* genes, which are widely used for their species identification. Additionally, this is the first report of *F. boothii* isolates in Nebraska, which indicates that more studies are necessary to further understand the largely unknown ecology of this member of the FGSC.


2.2 Introduction

Fusarium head blight (FHB), also known as scab, is a devastating disease of wheat (*Triticum aestivum*) and other small grain cereals that has caused significant losses worldwide (Goswami and Kistler 2004). As a consequence, FHB has been proposed as an emerging threat to the world’s grain supply (Starkey et al. 2007). This disease is particularly problematic because it poses a threat to both food security and food safety, due to yield reductions and mycotoxin contamination (Goswami and Kistler 2004).

FHB is caused by several species of *Fusarium*, but the predominant causal agent in North America is *Fusarium graminearum* (teleomorph: *Gibberella zeae*) (Panthi et al. 2014), although other species have been reported (Aoki et al. 2012). *F. graminearum* was thought to be a single panmictic species, but genealogical concordance/discordance phylogenetic species recognition studies showed that it actually contains 16 distinct species. Those 16 species are grouped within the *F. graminearum* species complex (FGSC) and its members are: *F. austroamericanum*, *F. boothii*, *F. meridionale*, *F. mesoamericanum*, *F. louisianense*, *F. acacia-mearnsii*, *F. brasilicum*, *F. cortaderiae*, *F. gerlachii*, *F. nepalense*, *F. graminearum*, *F. asiaticum*, *F. aethiopicum*, *F. vorosii*, *F. ussurianum*, and unnamed *Fusarium* sp. NRRL 34461. Morphological and phenotypic characters are not sufficient to distinguish most of the species within the FGSC, due to their morphological simplicity and overlapping conidial characters combined with within-species variability (Aoki et al. 2012). As a consequence, molecular methods are recommended for the final identification of FHB pathogens.
Symptoms associated with FHB include premature bleaching of spikelets that are either sterile or produce shriveled and soft grains. Additionally, while the mold grows it can produce trichothecene mycotoxins that accumulate in the plant and are harmful to animals and humans. The most common trichothecenes contaminating wheat and, therefore, the trichothecenes of most concern, are type B (Wegulo 2012), which include deoxynivalenol (DON), 15-acetyldeoxynivalenol (15-ADON), 3-acetyldeoxynivalenol (3-ADON), and nivalenol (NIV) and its acetylated derivatives (Foroud and Eudes 2009). The most common symptom after acute exposure to DON in sensitive animal species is emesis (Pestka and Smolinski 2005). Besides, ingestion of low quantities of DON may lead to anorexia, weight gain suppression and impaired immune function (Pestka and Smolinski 2005; Bianchini et al. 2015).

Nebraska wheat fields were affected by severe FHB epidemics in 2007 and 2008. During the 2007 growing season, about one third of the fields were affected, especially in the south-central part of the state (USWBSI 2007). The 2008 epidemic was more severe, accounting for grain yield losses of up to 20% and discounts of up to $5.00 per bushel in the most affected areas (USWBSI 2008). More recently, the most severe FHB epidemic in recent memory affected southern Nebraska in 2015, causing a yield loss estimated at 30%. On top of that, mycotoxin contamination was noticeably high, which caused substantial economic losses due to price discounts or grain rejection at the elevators (USWBSI 2015).
This study was designed to acquire information on the species diversity of FHB pathogens in Nebraska wheat. Additionally, two different DNA extraction protocols (extraction from single-spore isolates and extraction directly from wheat kernels) used for the identification of FHB pathogens were compared.

2.3 Materials and Methods

2.3.1 Samples

A total of 33 wheat head samples were collected during the 2015 and 2016 growing seasons from randomly chosen FHB-affected fields in Nebraska and GPS coordinates for each sample were recorded (Fig. 2.1; Table 2.1). Twenty-three samples were collected in 2015 and the remaining 10 samples were collected in 2016.
Figure 2.1: Map showing the different wheat growing regions in Nebraska, as well as the sampling sites where wheat heads were obtained. Triangles (Δ) represent samples from 2015 and circles (○) represent samples from 2016.
Table 2.1: Summary of the wheat heads sampled across Nebraska for this study and their location. Sample names indicate the year of collection (15: 2015; 16: 2016).

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>GPS coordinates</th>
<th>County</th>
<th>Wheat growing region</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-15</td>
<td>40°11'01.8&quot;N 99°53'50.5&quot;W</td>
<td>Furnas</td>
<td>West Central</td>
</tr>
<tr>
<td>2-15</td>
<td>40°12'22.7&quot;N 100°58'00.2&quot;W</td>
<td>Hitchcock</td>
<td>West Central</td>
</tr>
<tr>
<td>3-15</td>
<td>40°26'27.9&quot;N 96°57'39.8&quot;W</td>
<td>Saline</td>
<td>Southeast</td>
</tr>
<tr>
<td>4-15</td>
<td>41°09'56.4&quot;N 96°44'43.8&quot;W</td>
<td>Saunders</td>
<td>Southeast</td>
</tr>
<tr>
<td>5-15</td>
<td>41°14'04.0&quot;N 96°31'46.1&quot;W</td>
<td>Saunders</td>
<td>Southeast</td>
</tr>
<tr>
<td>6-15</td>
<td>40°35'32.9&quot;N 96°58'16.3&quot;W</td>
<td>Saline</td>
<td>Southeast</td>
</tr>
<tr>
<td>7-15</td>
<td>41°08'44.0&quot;N 96°29'47.1&quot;W</td>
<td>Saunders</td>
<td>Southeast</td>
</tr>
<tr>
<td>8-15</td>
<td>41°01'50.0&quot;N 96°29'32.6&quot;W</td>
<td>Lancaster</td>
<td>Southeast</td>
</tr>
<tr>
<td>9-15</td>
<td>40°42'36.9&quot;N 98°03'29.4&quot;W</td>
<td>Clay</td>
<td>South Central</td>
</tr>
<tr>
<td>10-15</td>
<td>40°48'08.3&quot;N 98°75'26.2&quot;W</td>
<td>Kearney</td>
<td>South Central</td>
</tr>
<tr>
<td>11-15</td>
<td>40°45'23.7&quot;N 99°29'89.1&quot;W</td>
<td>Phelps</td>
<td>South Central</td>
</tr>
<tr>
<td>12-15</td>
<td>40°30'61.7&quot;N 99°71'57.4&quot;W</td>
<td>Harlan</td>
<td>West Central</td>
</tr>
<tr>
<td>13-15</td>
<td>40°25'22.1&quot;N 97°35'45.2&quot;W</td>
<td>Fillmore</td>
<td>South Central</td>
</tr>
<tr>
<td>14-15</td>
<td>40°50'24.2&quot;N 101°41'44.6&quot;W</td>
<td>Perkins</td>
<td>West Central</td>
</tr>
<tr>
<td>15-15</td>
<td>40°11'34.4&quot;N 100°59'01.8&quot;W</td>
<td>Hitchcock</td>
<td>West Central</td>
</tr>
<tr>
<td>16-15</td>
<td>40°38'12.6&quot;N 101°37'43.8&quot;W</td>
<td>Chase</td>
<td>West Central</td>
</tr>
<tr>
<td>17-15</td>
<td>40°49'18.4&quot;N 101°39'57.7&quot;W</td>
<td>Perkins</td>
<td>West Central</td>
</tr>
<tr>
<td>18-15</td>
<td>40°20'46.3&quot;N 100°95'16.3&quot;W</td>
<td>Hitchcock</td>
<td>West Central</td>
</tr>
<tr>
<td>19-15</td>
<td>40°27'36.0&quot;N 101°32'01.0&quot;W</td>
<td>Chase</td>
<td>West Central</td>
</tr>
<tr>
<td>20-15</td>
<td>41°09'06.9&quot;N 103°29'30.3&quot;W</td>
<td>Kimball</td>
<td>Panhandle</td>
</tr>
<tr>
<td>21-15</td>
<td>42°20'18.6&quot;N 103°04'22.1&quot;W</td>
<td>Box Butte</td>
<td>Panhandle</td>
</tr>
<tr>
<td>22-15</td>
<td>41°13'56.7&quot;N 103°01'13.0&quot;W</td>
<td>Cheyenne</td>
<td>Panhandle</td>
</tr>
<tr>
<td>23-15</td>
<td>41°15'52.7&quot;N 97°12'40.9&quot;W</td>
<td>Butler</td>
<td>Southeast</td>
</tr>
<tr>
<td>1-16</td>
<td>40°16'10.4&quot;N 99°79'17.8&quot;W</td>
<td>Furnas</td>
<td>West Central</td>
</tr>
<tr>
<td>2-16</td>
<td>40°24'24.5&quot;N 99°25'37.2&quot;W</td>
<td>Phelps</td>
<td>South Central</td>
</tr>
<tr>
<td>3-16</td>
<td>40°54'46.6&quot;N 98°84'55.4&quot;W</td>
<td>Kearney</td>
<td>South Central</td>
</tr>
<tr>
<td>4-16</td>
<td>40°57'54.2&quot;N 98°12'85.7&quot;W</td>
<td>Clay</td>
<td>South Central</td>
</tr>
<tr>
<td>5-16</td>
<td>41°22'86.0&quot;N 96°48'92.0&quot;W</td>
<td>Saunders</td>
<td>Southeast</td>
</tr>
<tr>
<td>6-16</td>
<td>41°05'23.9&quot;N 96°54'05.1&quot;W</td>
<td>Saunders</td>
<td>Southeast</td>
</tr>
<tr>
<td>7-16</td>
<td>40°43'11.4&quot;N 96°96'10.0&quot;W</td>
<td>Saline</td>
<td>Southeast</td>
</tr>
<tr>
<td>8-16</td>
<td>40°24'24.5&quot;N 99°25'37.2&quot;W</td>
<td>Nuckolls</td>
<td>South Central</td>
</tr>
<tr>
<td>9-16</td>
<td>40°48'03.8&quot;N 97°17'59.5&quot;W</td>
<td>Saline</td>
<td>Southeast</td>
</tr>
<tr>
<td>10-16</td>
<td>40°32'11.4&quot;N 98°44'25.3&quot;W</td>
<td>Webster</td>
<td>South Central</td>
</tr>
</tbody>
</table>
In addition, 97 mycelial samples were received in 2015 from Bayer Crop Science. These samples were collected from the Midwestern United States; however, no certain location data was available for those samples.

### 2.3.2 Isolation of FHB pathogens from wheat kernels

For isolation of FHB pathogens, the protocol outlined by Panthi et al. (2014) was followed. Briefly, wheat kernels from each sample were disinfected in 70% ethanol, rinsed in sterile distilled water (dH₂O) and blotted dry. Subsequently, disinfected kernels were placed on Fusarium Selective Media (FSM) containing pentachloronitrobenzene (Nash and Snyder 1962), followed by incubation for 5 to 7 days at room temperature.

After incubation, plates were examined for typical *Fusarium* colonies, which were aseptically transferred to Potato Dextrose Agar (PDA; Becton Dickinson and Company, MD) and incubated for 7 days at room temperature. One isolate per wheat sample was obtained for the 2015 samples, whereas at least two isolates were obtained for the samples collected during 2016.

Single-spore isolation was performed by dilution plating. A spore suspension was prepared by adding 7 mL of sterile dH₂O to each PDA plate and scraping the surface with a sterile L-shaped rod. One mL of the spore suspension was transferred to a sterile 1.5-mL Eppendorf tube and serially diluted until 10⁻⁴. Serial dilutions were done by adding 100 µL of the spore suspension to 900 µL of sterile Peptone Water (0.1%) (Becton Dickinson and Company, MD). Three hundred microliters of each dilution was spread onto PDA plates, followed by incubation for 24 h at room temperature. Single spore
colonies were transferred to new PDA plates and grown for 7 days at room temperature. Isolates were stored in 35% glycerol (w/v) at -80°C.

2.3.3 DNA extraction from isolates and wheat kernels

For each wheat sample, 7-10 kernels (approximately 400 mg) were placed in a 1.5-mL tube. Prior to DNA extraction, the kernels were placed in a -80°C freezer for 10 min and then dried in a Vacufuge plus (Eppendorf AG, Hamburg, Germany) for 2 hours at 30°C using the vacuum-aqueous mode.

Single spore isolates were grown on PDA for 7 days at room temperature. Approximately 500 mg of mycelium from each isolate was transferred into a 1.5-mL tube using a sterile toothpick or a flame sterilized scalpel without disturbing the agar surface.

Both dried kernels and mycelia (including mycelial samples from Bayer Crop Science), were disrupted using a mortar and a pestle and DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method followed by a phenol/chloroform extraction as described by Panthi (2012), or using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) per manufacturer’s instructions.

The extracted DNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, DE) following the manufacturer’s instructions. Additionally, DNA quality was evaluated by performing a PCR reaction to amplify the internal transcribed spacer (ITS) regions of the nuclear ribosomal DNA gene using the ITS1F and ITS4 primers (Table 2.2). PCR was performed in a total volume of 25 µL prepared by combining 1 µL of template DNA, 2 µL of forward primer (ITS1F, 10 µM), 2 µL of
reverse primer (ITS4, 10 µM), 7.5 µL of molecular biology grade water and 12.5 µL of
DreamTaq Green PCR Master Mix (2X; Thermo Scientific, Vilnius, Lithuania).
Amplification was carried out in a T100 Thermal Cycler (Bio-rad, Hercules, CA) using
the following cycle parameters: 95°C for 3 min for denaturation, followed by 35 cycles at
95°C for 30 s, 55°C for 30 s, and 72°C for 1 min. Finally, extension was conducted at
72°C for 5 min. A negative control sample containing all reagents and primers but no
template DNA was included in each run. The amplification products were separated by
electrophoresis in 0.7% agarose gels with 0.5 µg/mL ethidium bromide at 80 V for 90
min. DNA was visualized under an UV transilluminator (GelDoc XR+, Bio-Rad,
Hercules, CA) and molecular weight markers (GeneRuler 1 kb Plus DNA ladder; Thermo
Scientific, Vilnius, Lithuania) were used to estimate the size of the amplicons.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-to-3')</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITS1F</td>
<td>TCCGTAGGTGAACCTGCGG</td>
<td>Gardes and Bruns (1993)</td>
</tr>
<tr>
<td>ITS4</td>
<td>TCCTCCGCTTTATTGATATG</td>
<td>White et al. (1990)</td>
</tr>
<tr>
<td>GOFW</td>
<td>ACCTCTGGTTTCTTCCAGACGG</td>
<td>de Biazio et al. (2008)</td>
</tr>
<tr>
<td>GORV</td>
<td>CTGGTCAGTATTAACCGTGTGTG</td>
<td>de Biazio et al. (2008)</td>
</tr>
<tr>
<td>UBC85F</td>
<td>GCAGGTTTTGAATCCGAGAC</td>
<td>Schilling et al. (1996)</td>
</tr>
<tr>
<td>UBC85R</td>
<td>AGAATGGAGCTACCAACGGC</td>
<td>Schilling et al. (1996)</td>
</tr>
<tr>
<td>RED-f</td>
<td>AGACTCATCCAGCCGGAAG</td>
<td>Ward et al. (2008)</td>
</tr>
<tr>
<td>RED-r</td>
<td>TCGTGGTTGAAGAGTTTGG</td>
<td>Ward et al. (2008)</td>
</tr>
<tr>
<td>TRI101-f</td>
<td>CAAGATACAGCTCGACACC</td>
<td>Ward et al. (2008)</td>
</tr>
<tr>
<td>TRI101-r</td>
<td>CTGGGTAGTTTGTCGAGA</td>
<td>Ward et al. (2008)</td>
</tr>
</tbody>
</table>
2.3.4 *F. graminearum* PCR

Both isolate-extracted DNA and plant-extracted DNA were subjected to PCR analysis using primers designed for the identification of *F. graminearum*. Two alternative sets of primers were used for this purpose, one set based on the galactose oxidase (GO) gene (GOFW/GORV) and the other set based on a fragment obtained from random amplification of polymorphic DNA unique to *F. graminearum* (UBC85F/UBC85R, Table 2.2). These sets of primers have been traditionally used to identify *F. graminearum* isolates (Yörük and Albayrak 2012; Chelkowski et al. 2012; Faria et al. 2012; Panthi et al. 2014; Nupsa et al. 2014; Kumar et al. 2015). PCR was performed as above, including the template-free control. Reference DNA from seven species of FHB pathogens (*F. graminearum*, *F. asiaticum*, *F. cerealis*, *F. culmorum*, *F. gerlachii*, *F. boothii* and *F. lousianense*) generously provided by Todd Ward (Agricultural Research Service, US Department of Agriculture) were included as controls. Fragments of 434 bp and 332 bp were expected from the GO primers and the UBC85 primers, respectively.

2.3.5 Multilocus genotyping assay

DNA was subjected to a multilocus genotyping (MLGT) assay described by Ward et al. (2008), with the exception that the assays were run on a Luminex Magpix (Luminex Corp., Austin, TX) and the allele-specific primer extension (ASPE) reactions were carried out using biotinylated probes instead of biotinylated dCTP.

A multiplex PCR was performed to amplify the following genes: reductase (*RED*), trichothecene 3-O-acetyltransferase (*TRI101*), translation elongation factor (*EF-1α*),
mating type protein 1-1-1 (*MAT 1-1-1*), 15-O-acetyltransferase (*TRI3*) and trichothecene efflux pump (*TRI12*). Amplifications were performed in 25 µL volumes with 4 µL of template DNA in a total volume of 25 µL containing 12.5 µL of DreamTaq Green PCR Master Mix (2X; Thermo Scientific, Vilnius, Lithuania), 1 µL of an equimolar solution of primers (approximately 0.8 µM) and 7.5 µL of molecular biology grade water. The PCR conditions were: 96°C for 1.5 min then 40 cycles of 94°C for 30 s, 50°C for 30 s and 68°C, 1.5 min. PCR products were purified using a Wizard SV Gel and PCR Clean-Up system (Promega, Madison, WI), per manufacturer’s instructions, prior to being used as a template for allele-specific primer extension (ASPE) reactions.

ASPE reactions were performed in order to obtain biotinylated products that have a 24-bp sequence tag on the 5’ end complementary to the anti-tag sequence attached to the surface of individual sets of Luminex xMAP fluorescent polystyrene microspheres (Luminex Corp., Austin, TX). A total of 19 probes were included (Table 2.3), targeting five *Fusarium* species that have been reported in North America (*F. graminearum*, *F. asiaticum*, *F. gerlachii*, *F. cerealis* and *F. culmorum*) and the three trichothecene genotypes (3-ADON, 15-ADON, NIV), as well as two probes specific to the *Fusarium graminearum* complex and one probe targeting the B-trichothecene lineage of FHB pathogens. The probes were designed and validated by Ward et al. (2008). Twenty-microliter reaction mixtures were prepared by combining 4 µL of 5X Phusion HF Buffer, 0.4 µL of 10 mM dNTPs, 0.2 µL of Phusion Hot Start II DNA Polymerase (2 U/µL, Thermo Scientific, Vilnius, Lithuania). A volume of 14.4 µL of purified multiplex PCR products was added as a template for the ASPE reactions, which consisted of an initial
denaturation at 96°C for 2 min then 40 cycles of 94°C for 30 s, 55°C for 1 min and 74°C for 2 min.

**Table 2.3: ASPE probes used in this study.**

<table>
<thead>
<tr>
<th>Probe name&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Gene</th>
<th>Sequence&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>T12-15(12)</td>
<td>TRI12</td>
<td>CATAATCAATTTCAACTTTCTACTTtacaggtgecgaacttc</td>
<td>15-ADON</td>
</tr>
<tr>
<td>T3-15 (34)</td>
<td>TRI3</td>
<td>ACTTATTTCTTACTACTATATACAccaccaacttcgctccat</td>
<td>15-ADON</td>
</tr>
<tr>
<td>T12-3(13)</td>
<td>TRI12</td>
<td>CAAATACTAACTTACATTCAATTTTCAACTTTCTACTTtacaggtgecgaacttc</td>
<td>3-ADON</td>
</tr>
</tbody>
</table>
| T3-3(35)               | TRI3 | CATCTTCATATCACCACACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTACTACTTAC...
Biotinylated ASPE products were hybridized with a mix of fluorescent microspheres specific to the extension probes. Hybridization was carried out in 50-μL volumes with 8 μL of extension product, 1000 microspheres from each set, and water. The samples were heated to 96°C for 1.5 min, followed by 45 min at 37°C. The microspheres were then centrifuged for 2 minutes at 2250 × g (Centrifuge 5810R, Eppendorf, AG, Hamburg, Germany) and then placed on a Luminex magnetic plate separator (Luminex Corp., Austin, TX) for 4 min. Keeping the plate on the magnet, the microspheres were washed with 75 μL of 1X TM buffer (0.2 M NaCl, 0.1 M Tris-HCl pH 8.0, 0.08% Triton X-100). Following the washes, the plate was removed from the magnetic plate and 75 μL of 1X TM buffer containing 2 μg/mL of streptavidin-R-phycoerythrin (SAPE) was added to each reaction well and the microspheres resuspended by pipetting. Samples were incubated at 37°C for 15 min prior to analysis by the Luminex Magpix (Luminex Corp., Austin, TX). Fifty microliters of biotinylated products hybridized to the microspheres was analyzed at a minimum microsphere count of 70 from each set. A template-free control was included in each run. In addition, DNA from the aforementioned reference isolates (F. graminearum, F. asiaticum, F. cerealis, F. culmorum, F. gerlachii, F. boothii and F. louisianense) was also included.
2.3.6 Analysis of intragenomic heterogeneity of RED and TRI101 genes

2.3.6.1 PCR amplification and sequencing

The DNA from single-spore *Fusarium* isolates 3, 19, 20 and 21 obtained in 2015 from Nebraska wheat affected by FHB was further used for the analysis of potential intrastrain heterogeneity of RED and TRI101 genes.

The RED and TRI101 genes were amplified in separate reactions using RED and TRI101 primers (Table 2.2). Amplifications were carried out as above. Fragments of 702 bp and 911 bp were expected from the RED primers and the TRI101 primers, respectively.

PCR products were purified using the QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany) per manufacturer’s instructions. Subsequently, the purified products were sequenced from both directions by Sanger sequencing utilizing an ABI 3730xl platform at Michigan State University's Research Technology Support Facility (East Lansing, MI).

2.3.6.2 Cloning of RED & TRI101 genes

The purified PCR products were cloned using the TA Cloning® Kit, with pCR™2.1 Vector and transformed into One Shot® TOP10 Chemically Competent *Escherichia coli* (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Transformed *E. coli* cells were plated onto Luria-Bertani (LB) agar plates supplemented with 100 µg of ampicillin/mL and ChromoMax IPTG/X-Gal solution (Thermo Fisher Scientific, Waltham, MA) at the manufacturer's recommended concentration. The plates were
incubated overnight at 37°C. At least ten white colonies (β-galactosidase negative) were subcultured into tubes containing 5 mL of LB broth supplemented with 100 µg of ampicillin/mL, and incubated overnight at 37°C and 225 rpm. Plasmid DNA was extracted from transformed cells using the alkaline lysis with SDS protocol outlined by Sambrook and Russell (2001). Clones were digested with the restriction enzymes EcoRI, TaqI and HaeIII (Thermo Scientific, Waltham, MA) following the manufacturer’s instructions and those with different restriction patterns were sequenced from both directions as above.

2.3.6.3 Sequence alignment and phylogenetic analysis

The RED and TRI101 sequences obtained from the clones and the sequences of their respective top five hits from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) were assembled and aligned in MEGA7 (http://www.megasoftware.net/) using MUSCLE and corrected by manual inspection. Polymorphisms within the same fungal strain were recorded.

Phylogenetic trees were constructed in MEGA7 using the Neighbor-Joining method (Saitou and Nei 1987) and distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004). Statistical support for the branches was estimated by bootstrap analyses with 1000 replicates.
2.4 Results

2.4.1 *F. graminearum* PCR

Amplicons of the expected sizes were observed following amplification with either *F. graminearum* primer pair in single-spore isolates and plant-extracted DNA from Nebraska, as well as in 94 of the 97 mycelial samples from Bayer Crop Science (Figs. 2.2 and 2.3). The remaining 3 isolates repeatedly failed to yield amplicons with these primers; however, they successfully amplified with the ITS1F:ITS4 primers. DNA from the reference isolates used as controls also amplified PCR products of the size expected for *F. graminearum* (Fig. 2.4). The 434 bp product expected for *F. graminearum* with the GO primers amplified from all control isolates, regardless of species. The UBC85 primers amplified a 332 bp product from DNA of *F. graminearum*, *F. asiaticum*, *F. gerlachii* and *F. boothii*, while giving distinct results with DNA from *F. cerealis*, *F. culmorum* and *F. louisianense*. A fragment of about 800 bp was also obtained from DNA of *F. graminearum*, *F. cerealis* and *F. boothii*, with various degrees of intensity depending on species (Fig. 2.4). This 800 bp fragment was also detected in several of the Nebraska samples (Fig. 2.2). Taken together, these results show that these sets of primers are not sufficient to accurately identify *F. graminearum* isolates. Consequently, samples that yielded the expected amplicon with either set of primers were identified as members of the *Fusarium graminearum* species complex (FGSC).
**Figure 2.2:** Agarose gel showing fragments amplified with the UBC85 primers for isolates 1-5 from 2015. Positive control: Reference *Fusarium graminearum*. Lane M, molecular weight marker (1 kb). Bands of around 800 bp were detected in several of the samples, as well as in the reference isolate.

**Figure 2.3:** Agarose gel showing fragments amplified with the GO primers for isolates 1-5 from 2015. Positive control: Reference *Fusarium graminearum*. Lane M, molecular weight marker (1 kb).
Figure 2.4: Agarose gel showing fragments amplified with the UBC85 and GO primers for reference *Fusarium* isolates. *F. gram*: *F. graminearum*, *F. asiat*: *F. asiaticum*, *F. cere*: *F. cerealis*, *F. culm*: *F. culmorum*, *F. gerl*: *F. gerlachii*, *F. boot*: *F. boothii*, *F. loui*: *F. louisianense*. Lane M, molecular weight marker (1 kb).
2.4.2 MLGT assay

As of yet, the MLGT assay described has not produced the expected results. High median fluorescence intensity (MFI) background values have been obtained, which has complicated the discrimination between positive and negative signals (Table 2.4). In addition, strong signals were obtained during some of the trials from probes for different species, especially the probes designed based on the RED and TRI101 genes (Table 2.5). In the context of this assay, an unambiguous sample would only hybridize to the probes designed for one species. Strong signals from multiple probes indicate that the assay is identifying multiple species in one sample. This could be obtained when there is a mixture of DNA from multiple species in one sample. Also, positive results from multiple probes could be obtained from hybrids that contain genes from different species or if a DNA sequence (different from those used to develop the assay) contains multiple probe recognition sites. In order to determine the cause for this finding, the RED and TRI101 genes from four isolates were sequenced.

In order to optimize the reactions, some parameters were modified, including increasing or decreasing the target input, increasing the hybridization temperature to discourage non-specific probe binding and decreasing the amount of SAPE. Despite our efforts, the results obtained remain inconclusive and there is still room for improvement.
Table 2.4: Median Fluorescent Intensity (MFI) values obtained from MLGT assay for reference isolates. Highlighted values indicate the high MFI obtained for the template-free control.

<table>
<thead>
<tr>
<th>Sample</th>
<th>T12-15</th>
<th>T12-J</th>
<th>T12-N</th>
<th>ATb</th>
<th>EFg</th>
<th>ATg</th>
<th>EF7</th>
<th>AT7</th>
<th>REDeu</th>
<th>ATce</th>
<th>ATce</th>
<th>MAT6</th>
<th>RED10</th>
<th>REDce</th>
<th>AT6</th>
<th>AT10</th>
<th>T3-15</th>
<th>T3-J</th>
<th>Putative identification</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. graminearum</em> 3-ADON</td>
<td>197</td>
<td>214</td>
<td>195</td>
<td>606</td>
<td>341</td>
<td>383</td>
<td>2584</td>
<td>240</td>
<td>231</td>
<td>274</td>
<td>265</td>
<td>196</td>
<td>228</td>
<td>243</td>
<td>406</td>
<td>374</td>
<td>197</td>
<td>209</td>
<td>?</td>
</tr>
<tr>
<td><em>F. graminearum</em> 3-ADON</td>
<td>138</td>
<td>184</td>
<td>151</td>
<td>525</td>
<td>333</td>
<td>309</td>
<td>2521</td>
<td>184</td>
<td>159</td>
<td>219</td>
<td>163</td>
<td>123</td>
<td>175</td>
<td>201</td>
<td>339</td>
<td>307</td>
<td>144</td>
<td>156</td>
<td>?</td>
</tr>
<tr>
<td><em>F. graminearum</em> 15-ADON</td>
<td>159</td>
<td>173</td>
<td>179</td>
<td>554</td>
<td>333</td>
<td>310</td>
<td>2470</td>
<td>241</td>
<td>190</td>
<td>235</td>
<td>190</td>
<td>144</td>
<td>185</td>
<td>167</td>
<td>334</td>
<td>314</td>
<td>169</td>
<td>172</td>
<td>?</td>
</tr>
<tr>
<td><em>F. graminearum</em> 15-ADON</td>
<td>166</td>
<td>184</td>
<td>178</td>
<td>612</td>
<td>1061</td>
<td>320</td>
<td>1158</td>
<td>256</td>
<td>192</td>
<td>253</td>
<td>195</td>
<td>157</td>
<td>201</td>
<td>234</td>
<td>351</td>
<td>322</td>
<td>187</td>
<td>177</td>
<td>?</td>
</tr>
<tr>
<td><em>F. graminearum</em> NIV</td>
<td>172</td>
<td>187</td>
<td>179</td>
<td>587</td>
<td>342</td>
<td>357</td>
<td>2554</td>
<td>217</td>
<td>209</td>
<td>250</td>
<td>213</td>
<td>156</td>
<td>206</td>
<td>211</td>
<td>381</td>
<td>350</td>
<td>175</td>
<td>208</td>
<td>?</td>
</tr>
<tr>
<td><em>F. graminearum</em> NIV</td>
<td>184</td>
<td>216</td>
<td>192</td>
<td>527</td>
<td>384</td>
<td>333</td>
<td>2423</td>
<td>265</td>
<td>209</td>
<td>226</td>
<td>215</td>
<td>159</td>
<td>201</td>
<td>261</td>
<td>361</td>
<td>325</td>
<td>203</td>
<td>200</td>
<td>?</td>
</tr>
<tr>
<td><em>F. cerealis</em> NIV</td>
<td>208</td>
<td>229</td>
<td>193</td>
<td>718</td>
<td>196</td>
<td>214</td>
<td>224</td>
<td>222</td>
<td>229</td>
<td>350</td>
<td>476</td>
<td>184</td>
<td>225</td>
<td>316</td>
<td>493</td>
<td>439</td>
<td>193</td>
<td>221</td>
<td>?</td>
</tr>
<tr>
<td><em>F. culmorum</em> 3-ADON</td>
<td>216</td>
<td>279</td>
<td>224</td>
<td>544</td>
<td>193</td>
<td>205</td>
<td>251</td>
<td>239</td>
<td>236</td>
<td>605</td>
<td>267</td>
<td>181</td>
<td>235</td>
<td>232</td>
<td>295</td>
<td>625</td>
<td>229</td>
<td>278</td>
<td>?</td>
</tr>
<tr>
<td>Neg. control</td>
<td>353</td>
<td>350</td>
<td>372</td>
<td>388</td>
<td>616</td>
<td>321</td>
<td>2736</td>
<td>372</td>
<td>371</td>
<td>297</td>
<td>368</td>
<td>266</td>
<td>344</td>
<td>357</td>
<td>466</td>
<td>342</td>
<td>332</td>
<td>361</td>
<td>?</td>
</tr>
</tbody>
</table>
Table 2.5: Median Fluorescent Intensity (MFI) values obtained from MLGT assay for selected isolates from 2015 and *F. graminearum* reference isolates. Values highlighted in red indicate the high MFI obtained for some of the probes for different species designed based on RED and TRI101 genes.

<table>
<thead>
<tr>
<th>Sample</th>
<th>T12-15</th>
<th>T12-3</th>
<th>T12-N</th>
<th>Atb</th>
<th>EFg</th>
<th>Atg</th>
<th>EF7</th>
<th>AT7</th>
<th>REDcu</th>
<th>ATcu</th>
<th>ATee</th>
<th>MAT6</th>
<th>RED10</th>
<th>REDce</th>
<th>AT6</th>
<th>AT10</th>
<th>T3-15</th>
<th>T3-3</th>
<th>Putative identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>16-2015</td>
<td>432</td>
<td>88</td>
<td>50</td>
<td>534</td>
<td>1211</td>
<td>283</td>
<td>466</td>
<td>197</td>
<td>450</td>
<td>494</td>
<td>130</td>
<td>57</td>
<td>59</td>
<td>1690</td>
<td>192</td>
<td>291</td>
<td>942</td>
<td>48</td>
<td><em>F. cerealis</em></td>
</tr>
<tr>
<td>17-2015</td>
<td>445</td>
<td>290</td>
<td>56</td>
<td>46</td>
<td>2557</td>
<td>69</td>
<td>221</td>
<td>133</td>
<td>229</td>
<td>40</td>
<td>101</td>
<td>49</td>
<td>44</td>
<td>606</td>
<td>268</td>
<td>1786</td>
<td>923</td>
<td>82</td>
<td><em>F. graminearum</em></td>
</tr>
<tr>
<td>18-2015</td>
<td>660</td>
<td>159</td>
<td>55</td>
<td>185</td>
<td>1508</td>
<td>442</td>
<td>820</td>
<td>718</td>
<td>490</td>
<td>47</td>
<td>426</td>
<td>57</td>
<td>48</td>
<td>1342</td>
<td>392</td>
<td>731</td>
<td>954</td>
<td>285</td>
<td><em>F. cerealis</em></td>
</tr>
<tr>
<td>19-2015</td>
<td>586</td>
<td>147</td>
<td>57</td>
<td>790</td>
<td>1402</td>
<td>681</td>
<td>654</td>
<td>370</td>
<td>447</td>
<td>70</td>
<td>199</td>
<td>55</td>
<td>48</td>
<td>1683</td>
<td>274</td>
<td>454</td>
<td>981</td>
<td>49</td>
<td><em>F. cerealis</em></td>
</tr>
<tr>
<td>20-2015</td>
<td>563</td>
<td>132</td>
<td>58</td>
<td>99</td>
<td>2498</td>
<td>251</td>
<td>198</td>
<td>492</td>
<td>382</td>
<td>44</td>
<td>305</td>
<td>55</td>
<td>230</td>
<td>1062</td>
<td>452</td>
<td>1231</td>
<td>945</td>
<td>56</td>
<td><em>F. graminearum</em></td>
</tr>
<tr>
<td>21-2015</td>
<td>532</td>
<td>138</td>
<td>53</td>
<td>345</td>
<td>2638</td>
<td>603</td>
<td>142</td>
<td>456</td>
<td>454</td>
<td>135</td>
<td>276</td>
<td>57</td>
<td>51</td>
<td>1603</td>
<td>401</td>
<td>678</td>
<td>960</td>
<td>51</td>
<td><em>F. cerealis</em></td>
</tr>
<tr>
<td>22-2015</td>
<td>605</td>
<td>143</td>
<td>70</td>
<td>69</td>
<td>1888</td>
<td>151</td>
<td>571</td>
<td>419</td>
<td>452</td>
<td>42</td>
<td>398</td>
<td>54</td>
<td>46</td>
<td>1417</td>
<td>481</td>
<td>1187</td>
<td>929</td>
<td>294</td>
<td><em>F. cerealis</em></td>
</tr>
<tr>
<td><em>F. graminearum</em> 3-ADON</td>
<td>50</td>
<td>962</td>
<td>54</td>
<td>305</td>
<td>613</td>
<td>222</td>
<td>846</td>
<td>168</td>
<td>634</td>
<td>624</td>
<td>118</td>
<td>62</td>
<td>124</td>
<td>1403</td>
<td>182</td>
<td>288</td>
<td>77</td>
<td>937</td>
<td><em>F. cerealis</em></td>
</tr>
<tr>
<td><em>F. graminearum</em> 15-ADON</td>
<td>588</td>
<td>85</td>
<td>67</td>
<td>297</td>
<td>731</td>
<td>201</td>
<td>668</td>
<td>183</td>
<td>689</td>
<td>576</td>
<td>125</td>
<td>55</td>
<td>113</td>
<td>1429</td>
<td>177</td>
<td>280</td>
<td>846</td>
<td>53</td>
<td><em>F. cerealis</em></td>
</tr>
<tr>
<td><em>F. graminearum</em> NIV</td>
<td>51</td>
<td>50</td>
<td>709</td>
<td>612</td>
<td>705</td>
<td>308</td>
<td>1026</td>
<td>223</td>
<td>725</td>
<td>532</td>
<td>151</td>
<td>56</td>
<td>68</td>
<td>1744</td>
<td>220</td>
<td>389</td>
<td>99</td>
<td>59</td>
<td><em>F. cerealis</em></td>
</tr>
<tr>
<td>Neg. control</td>
<td>50</td>
<td>53</td>
<td>63</td>
<td>45</td>
<td>1448</td>
<td>45</td>
<td>69</td>
<td>105</td>
<td>53</td>
<td>44</td>
<td>231</td>
<td>48</td>
<td>44</td>
<td>52</td>
<td>56</td>
<td>87</td>
<td>149</td>
<td>51</td>
<td>-</td>
</tr>
</tbody>
</table>
2.4.3 Analysis of intrastrain heterogeneity of RED and TRI101 genes

2.4.3.1 Direct sequencing of RED and TRI101 genes

The PCR-amplified RED and TRI101 products were represented by a single band on agarose gels for all four strains. However, when those amplicons were sequenced, the sequencing electropherograms showed double or multiple peaks (Fig. 2.5). Given that signal strength and other quality parameters indicated that the DNA was of appropriate quantity for sequencing and not degraded, such equivocal peaks suggest the presence of more than one DNA sequence.

![Figure 2.5: Direct sequencing chromatogram of the PCR product of the RED gene from isolate 20, showing double or multiple peaks denoted by a red arrow. Peaks of equivalent size called as ‘N’ can be seen at positions 230 and 243, whereas numerous peaks had higher than background signal for additional nucleotides (positions 233, 235, 237, 240, 241 and 243-246).](image)

2.4.3.2 Sequencing of cloned PCR products and phylogenetic analyses

To resolve ambiguities, the PCR amplicons were cloned. Unequivocal sequencing chromatograms were obtained from the clones. In total, there were 15 intragenomic differences across the entire spectrum of clone sequences (Table 2.6). Isolate 3 had two
TRI101 types, with only one polymorphism in the TRI101 gene, whereas the only polymorphism detected in isolate 21 was in the RED gene. Isolate 19 had three TRI101 types, with one type having four polymorphisms and the remaining type having just one. Isolate 20 had two RED types and three TRI101 types. The RED variant had 5 polymorphisms and the TRI101 variants had one and two polymorphisms. The polymorphism found in the first TRI101 variant was in the region used by Ward et al. (2008) to design a probe for the identification of F. gerlachii (Table 2.8).

<table>
<thead>
<tr>
<th>Isolate gene</th>
<th>Clone Name</th>
<th>Polymorphism</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-2015</td>
<td>TRI101</td>
<td>457C&gt;G</td>
</tr>
<tr>
<td>19-2015</td>
<td>TRI101</td>
<td>208G&gt;A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>241T&gt;C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>584A&gt;G</td>
</tr>
<tr>
<td></td>
<td></td>
<td>659A&gt;G</td>
</tr>
<tr>
<td></td>
<td>TRI101 19-3</td>
<td>215A&gt;G</td>
</tr>
<tr>
<td>20-2015</td>
<td>RED</td>
<td>125A&gt;G</td>
</tr>
<tr>
<td></td>
<td></td>
<td>192T&gt;C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>267A&gt;G</td>
</tr>
<tr>
<td></td>
<td></td>
<td>285T&gt;C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>381T&gt;C</td>
</tr>
<tr>
<td></td>
<td>TRI101</td>
<td>602A&gt;G</td>
</tr>
<tr>
<td></td>
<td></td>
<td>118A&gt;G</td>
</tr>
<tr>
<td></td>
<td></td>
<td>203C&gt;A</td>
</tr>
<tr>
<td>21-2015</td>
<td>RED</td>
<td>164T&gt;C</td>
</tr>
</tbody>
</table>
Sequence analysis showed that isolates 19 and 21 had *F. boothii* alleles at *RED* and *TRI101* (99.4 to 100% homology with the reference strains in the GenBank database). Additionally, these two isolates had *RED* sequences for the *F. boothii*-specific probe designed and validated by Ward et al. (2008) for the identification of FHB pathogens, thus confirming that in fact they correspond to *F. boothii* (Table 2.7). On the other hand, isolates 3 and 20 were confirmed to be *F. graminearum sensu stricto* based on their *RED* and *TRI101* homology (99.2 to 100%) with the reference strains in the GenBank database. These isolates had *TRI101* sequences for the *F. graminearum*-specific probe designed and validated by Ward et al. (2008), consequently confirming their identity (Table 2.8).

Phylogenetic analysis based on all intragenomic *RED* and *TRI101* copies of each strain showed that isolates 19 and 21 consistently clustered with *F. boothii*, whereas isolates 3 and 20 clustered with *F. graminearum* (Figs. 2.6 and 2.7).
Table 2.7: Alignment of the sequences from the 9 RED clones from Nebraska isolates, the sequences of their respective top five hits from GenBank and selected probe sequences designed by Ward et al. (2008) for the identification of FHB pathogens. Sequences from Nebraska isolates are denoted by RED followed by the isolate number and the clone number. Dashes indicate identity and dots indicate sequence break. Only nucleotides different from probe sequences are shown. Matches between a probe and a sequence are shaded.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>23</th>
<th>43</th>
<th>97</th>
<th>334</th>
<th>352</th>
<th>460</th>
<th>546</th>
<th>606</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fusarium pseudograminearum NRRL 34261</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>----</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Gibberella zeae NRRL 34449</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>----</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Gibberella zeae NRRL 28063</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>----</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Fusarium graminearum NRRL 66037</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>----</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Fusarium graminearum NRRL 66033</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>----</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Fusarium graminearum NRRL 45380</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>----</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Fusarium graminearum NRRL 40567</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>----</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Fusarium neapolense NRRL 54222</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>----</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Gibberella zeae F10102005</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>----</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Gibberella zeae NRRL 31287</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>----</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>RED3-1</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>----</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>RED3-3</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>----</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>RED20-2</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>----</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>RED20-3</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>----</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>RED20-9</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>----</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>RED20-12</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>----</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Fusarium boothii NRRL 34301</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>----</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Fusarium boothii NRRL 34351</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>----</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Fusarium boothii NRRL 29011</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>----</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Fusarium boothii strain NRRL 26916</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>----</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Fusarium boothii NRRL 29020</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>----</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>RED19-1</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>----</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>RED21-1</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>----</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>RED21-6</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>----</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Fusarium boothii MLGT probe sequence</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>----</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Fusarium gerlachi MLGT probe sequence</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>----</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Fusarium cerealis MLGT probe sequence</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>----</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Fusarium culmorum MLGT probe sequence</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>----</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Fusarium pseudograminearum MLGT probe sequence</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>----</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>
Table 2.8: Alignment of the sequences from the 9 TRI101 clones from Nebraska isolates, the sequences of their respective top five hits from GenBank and selected probe sequences designed by Ward et al. (2008) for the identification of FHB pathogens. Sequences from Nebraska isolates are denoted by TRI101 followed by the isolate number and the clone number. Dashes indicate identity and dots indicate sequence break. Only nucleotides different from probe sequences are shown. Matches between a probe and a sequence are shaded. Intrastrain polymorphism in clone TRI101 20-11 is underlined.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>1</th>
<th>50</th>
<th>397</th>
<th>433</th>
<th>501</th>
<th>597</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Fusarium graminearum</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12002</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gibberella zeae PPRI10693</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gibberella zeae Z3639</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gibberella zeae NRRL 31084</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gibberella zeae NRRL 28063</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Fusarium graminearum</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>isolate FG2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>isolate FG1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRI101 3-6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRI101 20-11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRI101 20-12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRI101 20-15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Fusarium boehmi</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>isolate 53</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>isolate 52</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>isolate 56</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRI101 19-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRI101 19-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRI101 19-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRI101 21-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRI101 21-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Fusarium boehmi</em> MLGT probe sequence</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Fusarium graminearum</em> MLGT probe sequence</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Fusarium gerlachi</em> MLGT probe sequence</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Fusarium cereale</em> MLGT probe sequence</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Fusarium culmorum</em> MLGT probe sequence</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Fusarium asiaticum</em> MLGT probe sequence</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.6: Phylogenetic tree representing the relationship of clones from four *Fusarium* isolates to their top hits from GenBank. The tree was inferred from RED sequence data and prepared by neighbor-joining. The scale bars represent the estimated numbers of substitutions per base. Numbers at the nodes represent bootstrap percentages and values lower than 70% are not shown.

Figure 2.7: Phylogenetic tree representing the relationship of clones from four *Fusarium* isolates to their top hits from GenBank. The tree was inferred from TRI101 sequence data and prepared by neighbor-joining. The scale bars represent the estimated numbers of substitutions per base. Numbers at the nodes represent bootstrap percentages and values lower than 70% are not shown.
2.5 Discussion

We evaluated the species diversity of FHB pathogens from Nebraska over a 2-year period. This study showed that the UBC85 primers and the GO primers are insufficient for accurate identification within the FGSC. Despite having been used traditionally for identification of *F. graminearum* (Nopsa et al. 2014; Panthi et al. 2014; Kumar et al. 2015), the UBC85 primers were designed in 1996 (Schilling et al. 1996) when all of the members of the FGSC were thought to be a single panmictic species. In 2004, the *F. graminearum* panmictic species was split into 16 different species (Aoki et al. 2012) amongst which the UBC85 primers are not able to distinguish. Hence, many authors have recently used the UBC85 primers as FGSC-specific primers (Suga et al. 2008; Karugia et al. 2009; Puri and Zhong 2010; Shen et al. 2012). The GO primers, despite having been designed following the description of the FGSC (de Biazio et al. 2008), were designed by aligning GO genes from *F. graminearum*, *Fusarium venenatum*, *Aspergillus oryzae* and *Fusarium sporotrichioides*, and validated using *Fusarium* species that are not part of the FGSC. We have demonstrated here that the GO primers are not sufficient to unambiguously confirm *F. graminearum*, due to the fact that DNA from all the different reference species included amplified the expected fragment.

Different methods have been proposed for the identification of FHB pathogens. Recent papers have identified isolates using primers designed based on the *Fg16* gene, which encodes for a hypothetical protein with an unknown function (Abedi-Tizaki and Zafari 2016). These primers amplify fragments of different size depending on the species: *F. graminearum* produces an amplicon of approximately 400 bp, *F. asiaticum* an amplicon
of about 550 bp and *F. meridionale* gives a product of about 500 bp (Castañares et al. 2014; Qiu et al. 2016; Pan et al. 2016). Qu et al. (2008) used the Fg16 primers and single-strand conformational polymorphism (SSCP) analysis to resolve isolates from *F. graminearum, F. asiaticum* and *F. meridionale*. However, none of these protocols are able to differentiate other species within the FGSC.

Another alternative is to use the MLGT protocol by Ward et al. (2008) that we are trying to implement in our laboratory. Many researchers have successfully used it to identify isolates from different parts of the world (Uméřičková et al. 2013; Machado et al. 2015; Kelly et al. 2015; Aamot et al. 2015). The main advantage in using the MLGT is a reduced test time, because it allows for simultaneous determination of species identity and trichothecene genotype.

Identification of FHB pathogens to species level can also be achieved by DNA sequencing. The genes that are most commonly used for identification are the *EF-1α*, *RED* and *TRI101* (Ward et al. 2008; Balmas et al. 2015; Castañares et al. 2016; Lee et al. 2016; Gryzenhout et al. 2016).

Previous studies have used DNA sequence data from nuclear genes encoding a putative reductase (*RED*) and the trichothecene 3-O-acetyltransferase (*TRI101*) to identify FHB pathogens and to infer phylogenetic relationships between them (O'Donnell et al. 2000; Scoz et al. 2009; Chiotta et al. 2015; Castañares et al. 2016). Additionally, these genes have been used to design probes for determination of species identity in a multilocus genotyping (MLGT) assay (Ward et al. 2008). The *RED* gene is considered a housekeeping gene, whereas the *TRI101* gene encodes an enzyme required for trichothecene biosynthesis (Boutigny et al. 2014).
In this study, we report for the first time intragenomic heterogeneity of the \textit{RED} and \textit{TRI101} sequences in FHB pathogens. So far, most reports of intrastrain heterogeneity in fungi have been in ITS sequences of several fungi, including basidiomycota (Wang and Yao 2005; Bovers et al. 2006; Vydryakova et al. 2012), zygomycota (Woo et al. 2010) and even one in \textit{Fusarium} species (O'Donnell and Cigelnik 1997). Intragenomic polymorphisms have also been reported in other eukaryotes, including plants (Álvarez and Wendel 2003; Wei et al. 2003), beetles (Vogler and DeSalle 1994), mosquitos (Bezzhonova and Goryacheva 2008; Veszgeiro et al. 2011) and sponges (Wörheide et al. 2004). Additionally, intrastrain heterogeneity in the 16S rRNA genes has been reported in bacteria in many investigations (Sun et al. 2013; LaFrentz et al. 2014; Chen et al. 2015).

The different intragenomic copies observed in this study were due to substitutions that were observed as overlapping or multiple peaks if the PCR products were sequenced directly. Cloning and subsequent sequencing of the cloned genes showed that some strains can contain up to three alleles differing in at least one nucleotide site.

Some of the reasons that previous authors have proposed to explain intragenomic heterogeneity include hybridization, gene duplication and heterokaryotic status (Wang and Yao 2005). O'Donnell and Cigelnik (1997) suggested that two divergent intragenomic ITS types found in \textit{Fusarium} were due to ancient interspecific hybridization or gene duplication. Multiple forms of a gene could be found if independent mutations occur in genes that are duplicated within the same nucleus. However, more information would be needed to support any of these hypotheses.

The \textit{RED} and \textit{TRI101} genes are among the most frequently used for the molecular identification of FHB pathogens and have been used for the development of a MLGT
assay for the simultaneous identification of isolates causing FHB (Ward et al. 2008). Most of the polymorphisms found in this study are not in the regions used to design the probes for the MLGT assay. However, one of the intrastrain polymorphisms in the TRI101 gene was in the region used by Ward et al. (2008) to design one of the F. gerlachii-specific probes. Although this intragenomic polymorphism would not have affected the results from this study, it is important to be aware of intragenomic heterogeneity when using RED and TRI101 sequences as targets for the identification of isolates.

This is the first report of the cereal pathogen F. boothii in Nebraska. Isolate 19 was from the West Central region of the state, whereas isolate 21 was obtained from the Panhandle region of Nebraska. Due to the small number of isolates identified to species level, no broad conclusions can be drawn regarding location; however, it is notable that the two isolates were separated by about 200 miles, and it would not be unreasonable to infer a broader distribution.

F. boothii has also been isolated from barley, corn and wheat in different parts of the world, including South Africa, Asia, Mexico, South America and Europe (Desjardins and Proctor 2011; Boutigny et al. 2011a; Boutigny et al. 2011b; Malihipour et al. 2012; Boutigny et al. 2014; Duan et al. 2016; Zhang et al. 2016). Previous surveys of FHB in the USA have isolated F. boothii from corn in Texas (Aoki et al. 2012). Moreover, F. boothii has also been isolated from other, unrelated hosts, including soybean (Glycine max) (Chiotta et al. 2015) tomato (Solanum lycopersicum) (Gomes et al. 2015), pecan (Carya illinoiensis) and camel thorn (Vachellia erioloba) (Gryzenhout et al. 2016). Nevertheless, most of the studies have isolated F. boothii from maize, causing Gibberella
ear rot (GER). Boutigny et al. (2011b) reported *F. boothii* as the almost exclusive pathogen causing GER of maize in South Africa, suggesting that *F. boothii* may be more fit to infect corn than *F. graminearum*. However, pathogenicity studies on wheat have shown that some *F. boothii* isolates can be as aggressive to wheat as *F. graminearum* isolates (Goswami and Kistler 2005). Living up to the nickname “Cornhuskers”, corn is one of the top commodities produced in Nebraska. Additionally, corn and wheat are usually included in crop rotation systems in Nebraska (Nebraska Wheat Board 2011), which could be associated with the presence of *F. boothii* in wheat.

The present study was initiated by the conflicting results obtained from the MLGT protocol, where high intensity values were obtained from some of the probes for different species designed based on the *RED* and *TRI101* genes. As it was mentioned before, strong signals from multiple probes indicate that the assay is identifying multiple species in one sample. Mixtures of DNA from multiple species in one sample could explain this finding. Additionally, hybrids that contain alleles from different species or DNA sequences not included in the development of the assay containing multiple probe recognition sites could also hybridize multiple probes. Even though the reason for those high values remains unclear, the identification of *F. boothii* isolates in Nebraska indicates that probes for its identification should be included in future studies.

DNA amplifiable with the UBC85 and GO primers was obtained regardless of whether extracted directly from the wheat or from single-spore isolates from the same samples. Since these primers have proven insufficient for accurate identification of FHB pathogens, it would be premature to declare the two DNA extraction methods equivalent.
for FHB pathogen identification; however, the ease of obtaining DNA suitable for PCR amplification and sequencing directly from infected plant tissue is promising.

In summary, the fact that the UBC85 and GO primers are not able to discriminate between species within the FGSC indicates that a different method should be used to identify FHB pathogens. Alternative methods include sequencing of selected genes or the MLGT developed by Ward et al. (2008). Furthermore, the development of new primers for the final identification of FHB pathogens would be valuable. The results from this study also show that FHB pathogens can have intragenomic heterogeneity in the RED and TRI101 genes, which are widely used for their species identification. Additionally, the identification of *F. boothii* isolates in Nebraska shows that more studies are necessary to further understand the largely unknown ecology of this member of the FGSC.

2.6 References


Chapter 3

TRICHTHECENE GENOTYPE OF FUSARIUM HEAD BLIGHT PATHOGENS
IN NEBRASKA, USA IN 2015-2016
3.1 Abstract

Members of the *Fusarium graminearum* species complex (FGSC) cause Fusarium head blight (FHB) of wheat and other small grain cereals. This devastating disease poses a threat to food security and food safety, due to yield and quality reduction, as well as mycotoxin contamination. To determine the diversity of trichothecene genotypes of the FGSC in Nebraska, 33 wheat samples were collected from affected fields during two consecutive years (2015 and 2016) and 50 *Fusarium* isolates were obtained from those wheat samples. A PCR-based assay was used to determine the potential toxigenic capacity of FHB pathogens in Nebraska. In agreement with previous studies, the overwhelming majority of isolates had the 15-ADON genotype. However, two isolates produced inconclusive results with the trichothecene genotyping multiplex PCRs. Additional reactions showed that the PCR products obtained for both isolates in the multiplex reactions were amplified by primers designed to yield a different fragment. In order to determine their chemotype, plants of the FHB-susceptible wheat cultivar Samson were inoculated with the inconclusive trichothecene genotype isolates. However, these isolates failed to cause disease and accumulate mycotoxins in the grains. Additional studies should be done to evaluate their ability to produce mycotoxins *in vitro* and to further understand the underlying genetics. The population genetic data obtained from this study can be used as baseline data for future surveillance of FHB pathogens populations in Nebraska and North America and to better understand their biology and ecology. The findings will also be useful to lessen the risk of mycotoxin contamination in wheat and wheat-based products.
3.2 Introduction

Fusarium head blight (FHB) is a devastating and economically important disease of wheat and other small grain cereals. From 1998 to 2000 alone, FHB caused losses of almost $3 billion in the United States and Canada (Goswami and Kistler 2004). Economic losses can be attributed to low yield and quality, and price discounts resulting from mycotoxin contamination, posing a threat to food security and food safety.

FHB is caused by several species of *Fusarium*, but the predominant causal agent in North America is *Fusarium graminearum* (teleomorph: *Gibberella zeae*) (Panthi et al. 2014), although other species have been reported (Aoki et al. 2012). Symptoms associated with FHB include premature bleaching of spikelets that are either sterile or produce shriveled and soft grains. Additionally, while the mold grows it can produce trichothecene mycotoxins that accumulate in the plant and are harmful to animals and humans.

Trichothecenes are secondary metabolites produced from the isoprenoid metabolism and have a tricyclic nucleus with an epoxide function (Alexander et al. 2009). Four types of trichothecenes have been described: types A, B, C and D. However, the most common trichothecenes contaminating wheat and, therefore, the trichothecenes of most concern, are type B (Wegulo 2012). Type B trichothecenes include deoxynivalenol (DON), 15-acetyldeoxynivalenol (15-ADON), 3-acetyldeoxynivalenol (3-ADON), and nivalenol (NIV) and its acetylated derivatives (Foroud and Eudes 2009).

The main effect of DON, at the cellular level, is inhibition of protein synthesis (Rotter et al. 1996). DON is also known as vomitoxin, due to its emetic effect after acute exposure
in sensitive animal species (Rotter et al. 1996; Pestka and Smolinski 2005). Moreover, ingestion of low quantities of DON may lead to anorexia, weight gain suppression and impaired immune function (Pestka and Smolinski 2005; Bianchini et al. 2015).

Depending on the toxins they produce, FHB pathogens are classified into three different chemotypes: 3-ADON (producing DON and 3-ADON), 15-ADON (producing DON and 15-ADON) and NIV (producing nivalenol and its acetylated derivatives) (Pan et al. 2013). Due to their ease of use and rapid results, PCR assays have been developed to predict the chemotype of *Fusarium* isolates. Some authors have referred to the results from the PCR assays as chemotypes, while results from these assays should be referred to as trichothecene genotype due to the fact that disagreements between genotypes and chemotypes have been reported (Desjardins 2008).

Previous studies have reported that the 15-ADON genotype is predominant in Nebraska (Panthi et al. 2014). However, the other two genotypes have been recently reported in North America, including a more aggressive and toxigenic 3-ADON population in Canada that is replacing the existing 15-ADON population (Ward et al. 2008). Additionally, this 3-ADON population has been identified in North Dakota and Minnesota (Gale et al. 2007) showing a southward expansion of its distribution (Liang et al. 2014). Also, NIV and 3-ADON isolates have been reported in Louisiana and the eastern United States (Starkey et al. 2007; Schmale et al. 2011; Gale et al. 2011). Therefore, constant monitoring is important to recognize the potential toxigenic risk of the wheat produced in Nebraska.

This study was designed to acquire information on the predicted toxigenic capacity of FHB pathogens in Nebraska and compare two different DNA extraction protocols
(extraction from single-spore isolates and extraction directly from wheat kernels) in determining trichothecene genotype of FHB pathogens.

3.3 Materials and Methods

3.3.1 Samples

The samples used in this study were the same samples described in Chapter 2 of this thesis, consisting DNA extracted directly from 33 wheat samples collected from Nebraska fields during the 2015 and 2016 growing seasons, as well as from 50 single spore isolates obtained from those wheat samples. GPS coordinates for each sample were obtained (Table 2.1).

In addition, DNA from the 97 mycelial samples received in 2015 from Bayer Crop Science was also included. These samples were collected from the Midwestern United States; however, no certain location data was available for those samples.

3.3.2 Trichothecene genotyping PCR

The protocol outlined by Starkey et al. (2007), as implemented by Panthi et al. (2014) was followed. Briefly, a multiplex reaction was used to determine the trichothecene genotype of the isolates. Additionally, the plant-extracted DNA was subjected to the same reaction. Two alternative sets of primers were used, which amplify portions of the TRI3 and TRI12 genes. The primers based on the TRI3 (TRI3 primers) gene include
3CON, 3D3A, 3D15A and 3NA; whereas the primers based on the TRI12 gene (TRI12 primers) are 12CON, 12-3F, 12-15F and 12NF (Table 3.1). Multiplex PCR was conducted using 1 µL of template DNA in a total volume of 25 µL containing 12.5 µL of DreamTaq Green PCR Master Mix (2X; Thermo Scientific, Vilnius, Lithuania), 0.5 µL of each primer (10 µM) and 9.5 µL of molecular biology grade water. Amplification was carried out in a T100 Thermal Cycler (Bio-rad, Hercules, CA) using the following cycle parameters: 95°C for 3 min then 35 cycles of 95°C for 30 s, 52°C for 30 s and 72°C, 1 min, followed by a final extension step of 5 min at 72°C. A template-free control was included in each run. Reference DNA from isolates with a known trichothecene genotype generously provided by David Schmale (Virginia Tech University) were included as controls.

The amplification products were separated by electrophoresis in 0.7% agarose gels with 0.5 µg/mL ethidium bromide at 80 V for 90 min. DNA was visualized under an UV transilluminator (GelDoc XR+, Bio-Rad, Hercules, CA) and molecular weight markers (GeneRuler 1 kb Plus DNA ladder; Thermo Scientific, Vilnius, Lithuania) were used to estimate the size of the amplicons. The TRI3 primers produce bands of 243, 610 and 840 bp, whereas the TRI12 primers yield bands of 410, 670 and 840 bp for the 3-ADON, 15-ADON and NIV genotypes, respectively.
### Table 3.1: Sequence of the primers used in this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-to-3')</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>3CON</td>
<td>TGGCAAAGACTGGGTTTCAC</td>
<td>Starkey et al. (2007)</td>
</tr>
<tr>
<td>3D3A</td>
<td>CGCATTTGGCTAACACATG</td>
<td>Starkey et al. (2007)</td>
</tr>
<tr>
<td>3D15A</td>
<td>ACTGACCCAAGCTGCCATC</td>
<td>Starkey et al. (2007)</td>
</tr>
<tr>
<td>3NA</td>
<td>GTGCACAGAATATACGAGC</td>
<td>Starkey et al. (2007)</td>
</tr>
<tr>
<td>12CON</td>
<td>CATGAGCATGGGTGATGTC</td>
<td>Starkey et al. (2007)</td>
</tr>
<tr>
<td>12-3F</td>
<td>CTTTGGAAGCCCGTGCA</td>
<td>Starkey et al. (2007)</td>
</tr>
<tr>
<td>12-15F</td>
<td>TACAGCGGTCGCAACTTC</td>
<td>Starkey et al. (2007)</td>
</tr>
<tr>
<td>12NF</td>
<td>TCTCCTCGTTGATCTGG</td>
<td>Starkey et al. (2007)</td>
</tr>
</tbody>
</table>

#### 3.3.3 Multilocus genotyping assay

DNA was also subjected to a multilocus genotyping (MLGT) assay for the identification and trichothecene genotyping of FHB pathogens developed by Ward et al. (2008), as described in chapter 2 of this thesis.

#### 3.3.4 *In planta* mycotoxin production

A greenhouse experiment was conducted in order to determine the chemotype of inconclusive trichothecene genotype isolates.

#### 3.3.4.1 Plant material

The FHB-susceptible hard red spring wheat cultivar Samson was used for this experiment. Four seeds were planted per 4½” square plastic pot in potting soil consisting
of 33% clay loam soil, 33% peat soil, 16.5% sand, and 16.5% vermiculite. The temperature in the greenhouse ranged between 18°C minimum (nighttime) and 24°C maximum (daytime), with 14 hours of light and 10 hours of darkness. Plants were watered as needed and fertilized with Peter’s Peat Lite Special Fertilizer, 20-10-20 (Everris, Marysville, Ohio) at 250 ppm five days a week.

3.3.4.2 Inoculum preparation

Isolates were grown on Potato Dextrose Agar (PDA; Becton Dickinson and Company, MD) for 5 days at room temperature. Spore suspensions were prepared according to the following protocol outlined by Hallen-Adams et al. (2011). A mycelial plug from PDA was inoculated into 100 mL of carboxymethyl cellulose medium (CMC; 15 g/L carboxymethyl cellulose sodium salt, 1 g/L NH4NO3, 1 g/L KH2PO4, 0.5 g/L MgSO4·7H2O, 1 g/L yeast extract). The flasks were incubated for 4 days at room temperature and 200 rpm. The cultures grown on CMC were filtered through sterile miracloth (Millipore, Bedford, MA) into sterile 250-mL centrifuge bottles. The bottles were centrifuged at 4000×g for 5 minutes in a Beckman J2-21 centrifuge (Beckman, Fullerton, CA) to precipitate the spores. The pellet was resuspended in 1 mL of sterile distilled water (dH2O) and transferred into 1.5-mL microcentrifuge tubes. The tubes were centrifuged at 4500×g for 5 minutes in an Eppendorf 5424 centrifuge (Eppendorf, AG, Hamburg, Germany). The supernatant was decanted and the spores were washed twice with 1 mL of dH2O. The spore pellet was resuspended in 1 mL of 35% glycerol and the spore concentration was calculated using a hemocytometer. The suspensions were diluted
in 35% glycerol as needed to a final concentration of $5 \times 10^5$ conidia/mL and stored at -80°C until needed for inoculation.

### 3.3.4.3 Wheat infection

The protocol described by Hallen-Adams et al. (2011) was used with slight modifications. Briefly, plants were inoculated just prior to anthesis – when the plants are most susceptible to infection. A spikelet in the middle of each head was selected for inoculation. Gently, the lemma and lower glume were peeled back and the pipette tip was inserted beside the ovary. Ten microliters of the appropriate spore suspension ($5 \times 10^5$ conidia/mL) was slowly injected into the floret. After removing the pipette tip, the glume and lemma were gently returned to position. Inoculation points were marked with a black marker pen on the outside of the glume. After inoculation, the wheat heads were covered with a transparent plastic bag for 72 h with the purpose of maintaining high humidity and favor infection.

Experimental units consisted of the totality of spikes in four pots, with three repetitions for each isolate. Additionally, non-inoculated plants were used as negative controls.

### 3.3.4.4 Mycotoxin quantification

Spikes were manually harvested when the grain moisture content was below 15%. Subsequently, the spikes were threshed and the grains were ground to flour. Mycotoxin quantification was generously performed by David Schmale’s lab (Virginia Tech...
University) using an Agilent 6890/5975 gas chromatographer coupled with a mass spectrometer for detection and quantification of DON, 15-ADON, 3-ADON and NIV.

3.4 Results

3.4.1 Trichothecene genotyping PCR

PCR assays with the trichothecene genotyping primer sets revealed that all the DNA from the 2015 isolates and wheat kernels produced the amplicons expected for the 15-ADON genotype. Additionally, DNA extracted directly from the 2016 wheat kernels, as well as from 25 single spore isolates produced fragments consistent with the 15-ADON genotype (Table 3.2). However, 2 of the 27 single-spore isolates from 2016 showed inconclusive results (Figs. 3.1 and 3.2). Isolate 7-a (2016) had the 3-ADON genotype with the TRI3 set of primers, but had the 15-ADON genotype with the TRI12 set of primers. For the other isolate, 8-a (2016), no amplification was obtained with the primer set from TRI3, and a fragment consistent with the NIV genotype was obtained with the primers from TRI12.
Figure 3.1: Amplification products produced by multiplex PCR with the TRI3 primers for single-spore isolates 7-a and 8-a from 2016, as well as for kernel-extracted DNA from samples 7 and 8. Lane M, molecular weight marker (1 kb).

Figure 3.2: Amplification products produced by multiplex PCR with the TRI12 primers for single-spore isolates 7-a and 8-a from 2016, as well as for kernel-extracted DNA from samples 7 and 8. Lane M, molecular weight marker (1 kb).
Additional PCRs were carried out using the individual primer sets from the multiplex reactions separately in order to determine the primers that amplified the fragments obtained with the inconclusive genotype isolates. For the TRI3 primers, the 3CON primer was used as the reverse primer, whereas the 12CON primer was used for the TRI12 primers. The 3D3A and 12-3F primers target the 3-ADON genotype, the 3D15A and 12-15F primers target the 15-ADON genotype, and the 3NA and 12-NF primers target the NIV genotype. The results from these individual reactions are shown in Fig. 3.3. For isolate 7-a, the 3D15A primer that was supposed to amplify the 15-ADON fragment, produced the amplicon size expected for the 3-ADON genotype. Furthermore, the 12-3F primer that was designed to amplify the 3-ADON fragment, produced fragments consistent with the 15-ADON and the NIV genotype for samples 7-a and 8-a, respectively.
Among the mycelial samples received from Bayer Crop Science, 66 isolates had the 15-ADON genotype and 9 isolates belonged to the 3-ADON genotype. For the remaining 22 isolates, no amplification was obtained with either of the trichothecene genotyping sets of primers. The reason for the absence of an amplicon is unclear, since these isolates successfully amplified with ITS1F:ITS4, UBC85 and GO primers (Chapter 2 of this thesis).

**Figure 3.3:** Agarose gel showing fragments amplified with the individual trichothecene genotyping primer pairs for isolates 7-a and 8-a from 2016. Lane M, molecular weight marker (1 kb).
Table 3.2: Results from trichothecene genotyping PCR for the *Fusarium* isolates used in this study. Table contains the number of isolates/samples that amplified the expected fragment for each reaction.

<table>
<thead>
<tr>
<th>Sample group</th>
<th>Trichothecene genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3-ADON</td>
</tr>
<tr>
<td>2015 Nebraska single-spore isolates</td>
<td></td>
</tr>
<tr>
<td>2015 DNA extracted directly from kernels</td>
<td></td>
</tr>
<tr>
<td>2016 Nebraska single-spore isolates</td>
<td></td>
</tr>
<tr>
<td>2016 DNA extracted directly from kernels</td>
<td></td>
</tr>
<tr>
<td>Bayer Crop Science isolates</td>
<td>9/97</td>
</tr>
</tbody>
</table>

3.4.2 MLGT assay

As it was mentioned in chapter two of this thesis, the MLGT assay described has not produced the expected results yet, largely because high median fluorescent intensity (MFI) background values have been obtained. This has complicated the discrimination between positive and negative signals (Table 2.4). Some parameters have been modified, but the results obtained remain inconclusive.

3.4.3 Mycotoxin production *in planta*

The isolates used for the controlled inoculations correspond to isolates 7-a and 8-a obtained in 2016. These isolates were selected because they had inconclusive results with the trichothecene genotyping PCRs. Isolate 1-c from 2016, identified as 15-ADON by the genotyping assay, was included for comparison.

Typical FHB symptoms were observed in plants inoculated with isolate 1-c. Furthermore, isolate 1-c was able to accumulate DON and 15-ADON, in agreement with its genotype.
However, isolates 7-a and 8-a failed to cause FHB symptoms and failed to produce mycotoxins in planta (Table 3.3). Therefore, a chemotype for these isolates could not be obtained.

### Table 3.3: Levels of deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON) and nivalenol (NIV) in grains from artificially inoculated wheat (Samson cultivar). Values reported as mean ± standard deviation.

<table>
<thead>
<tr>
<th>Mycotoxin (ppm)</th>
<th>Sample</th>
<th>DON</th>
<th>3-ADON</th>
<th>15-ADON</th>
<th>NIV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1-c</td>
<td>18.71 ± 4.89</td>
<td>-</td>
<td>0.29 ± 0.10</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>7-a</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>8-a</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Non-inoculated</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*: value below limit of quantification (0.05 ppm).

### 3.5 Discussion

We evaluated the trichothecene genotype diversity of FHB pathogens from Nebraska over a 2-year period. Our results show that the overwhelming majority of isolates from Nebraska have the 15-ADON genotype. This finding is in line with previous studies that detected only the 15-ADON genotype among Nebraska isolates from 2007 to 2010 (Panthi et al. 2014). No NIV or 3-ADON genotype was detected in Nebraska during 2015-2016 and two isolates had inconclusive genotyping results. While more toxic to
animals and humans than DON, the NIV genotype has a low prevalence in North America (Ceron-Bustamante et al. 2016). Isolates with the NIV genotype have been reported in Mexico (Ceron-Bustamante et al. 2016), Louisiana (Gale et al. 2011), New York, North Carolina (Schmale et al. 2011) and Virginia (Schmale et al. 2012). The fact that no 3-ADON genotype was found in Nebraska is beneficial for the wheat growers in the state, since many studies have pointed out that 3-ADON isolates are more aggressive and produce more DON than 15-ADON isolates (Ward et al. 2008; von der Ohe et al. 2010). Nonetheless, the 3-ADON genotype was detected in 9 of the Bayer Crop Science mycelial samples. Even though no specific location is available for these isolates, they were obtained from the Midwest. This finding is not surprising given the fact that the 3-ADON genotype has been reported in North Dakota and Minnesota (Gale et al. 2007); however, it demonstrates that the introduction of this genotype into Nebraska is feasible. In order to guarantee the safety of the grains produced, it is important to know which toxigenic hazards might be present and prevalent in Nebraska, as chemotypes may change over time (Ward et al. 2008; Pasquali and Migheli 2014). Moreover, the detection of spores of the 3-ADON and the NIV genotypes in the atmospheric boundary layer could facilitate their introduction into new areas (Schmale et al. 2012), which highlights the importance of ongoing surveillance.

In the present study, two Nebraska isolates produced inconclusive results with the trichothecene genotyping multiplex PCRs. Isolate 7-a was obtained from Saline county (Southeast region) and isolate 8-a from Nuckolls county (South central region). However, no conclusions can be drawn about their location due to the small number of isolates with inconclusive genotypes. Isolate 7-a (2016) gave conflicting results the trichothecene
genotype sets of primers. This isolate had the 3-ADON genotype with the TRI3 primers, but had the 15-ADON genotype with the TRI12 primers. Isolate 8-a (2016) failed to amplify with the primer set from TRI3, but a fragment corresponding to the NIV genotype was obtained with the primers from TRI12. Nonetheless, the PCR products obtained for both isolates in the multiplex reactions were amplified by primers designed to yield a different fragment. The underlying genetics in both cases remain unclear.

The strains with inconclusive genotype were unable to cause disease and accumulate toxins in planta. Based on these findings, more experiments should be conducted to further understand the toxigenic capacity of the isolates with inconclusive trichothecene genotype. It would be important to first identify those strains to species level, as the trichothecene genotyping primers were designed to work only with members of the Fusarium graminearum species complex (FGSC). Additionally, it would be important to sequence the amplicons obtained in the genotyping PCRs, as well as other genes in the trichothecene biosynthetic gene cluster. Alternative methods to determine the chemotype of these isolates involve growing them in liquid culture, on rice kernels or on cracked maize, to subsequently detect the mycotoxins using liquid chromatography coupled with mass spectrometry, as it was described by Gräfenhan et al. (2016).

Of the 97 mycelial samples received from Bayer Crop Science, 22 failed to yield amplification with the trichothecene genotyping primers. Previous studies have reported strains for which the trichothecene genotyping multiplex PCRs fail. Panthi et al. (2014) isolated four strains that repeatedly failed to amplify with the genotyping primers. The authors hypothesized that the lack of amplification was possibly due to mutations in the TRI3 and TRI12 genes as part of multiple deletions, and these strains did not produce
detectable DON in vitro. Nevertheless, the genetic basis for these results remains unknown.

DNA amplifiable with the trichothecene genotyping primers was obtained both when the DNA was extracted from single-spore isolates and when extracted directly from the wheat kernels that gave rise to the isolates. However, inconclusive genotypes were not detected when the plant kernel DNA was used and were only found using DNA from isolates. This outcome is possibly due to differences in abundance of the strains with indeterminate trichothecene genotypes. If indeterminate genotype strains are atoxigenic, they may be present in low levels in plant tissue, but be outcompeted in active infection by toxin-producing strains.

In summary, the results from this study give a significant insight into the mycotoxin profiles of FHB pathogens in Nebraska. Other studies should be done to further understand the basis of inconclusive trichothecene genotypes and possibly improve the current methods. Species identification of the isolates with inconclusive trichothecene genotypes would be valuable, as well as additional studies to evaluate their ability to produce mycotoxins in vitro.

The data obtained from this study can be used as baseline data for future surveillance of FHB pathogen populations in Nebraska and North America and to better understand their biology and ecology. The findings will also be useful to lessen the risk of mycotoxin contamination in wheat and wheat-based products.
3.6 References


Desjardins, Anne. 2008. "Natural product chemistry meets genetics: when is a genotype a chemotype?" **Journal of Agricultural and Food Chemistry** 56 (17): 7587-7592. doi:10.1021/jf801239j.


