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

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SPECIES AND TRICHOTHECENE GENOTYPES OF FUSARIUM HEAD BLIGHT PATHOGENS IN NEBRASKA, USA IN 2015-2016

By

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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.

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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. 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. 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 asymptomatically 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; Mourelos 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). *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 *PFT*, a gene within *Fhb1*. Even though the biochemical mechanism for resistance mediated by 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.

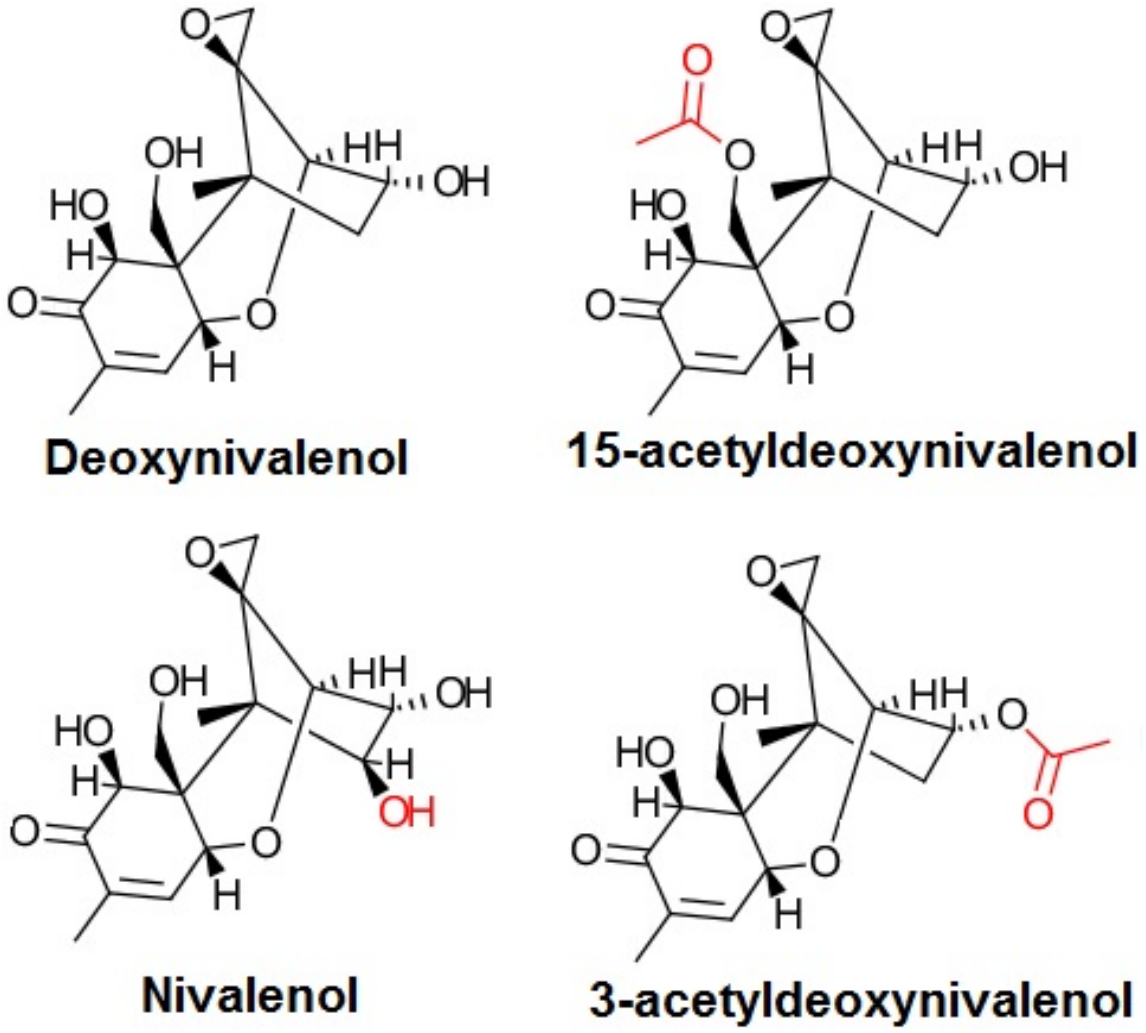

Figure 1.1: Chemical structures of the most important Type B trichothecenes. All type B trichothecenes have a keto (carbonyl) group at C-8. Differences between these four compounds are shown in red. Unlike DON and acetylated DON, NIV has an oxygen at C-4 (red). The acetylated derivatives of DON: 15-ADON 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 *TRI5* gene (Goswami and Kistler 2004; Alexander et al. 2009). Next, trichodiene undergoes four oxygenation reactions catalyzed by the cytochrome P450 monooxygenase encoded by *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 *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 *TRI11*. This hydroxyl group is then acetylated by an acetyltransferase encoded by *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 *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 *TRI13*. Conversely, DON-producing strains lack a functional *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 *TRI8*. Alexander and colleagues reported that the *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

Abramson, D., R. M. Clear, D. Gaba, D. M. Smith, S. K. Patrick, and D. Saydak. 2001. "Trichothecene and Moniliformin Production by *Fusarium* Species from Western Canadian Wheat." *Journal of Food Protection* 64 (8): 1220-1225.

Alexander, N. J., S. P. McCormick, and T. M. Hohn. 1999. "TRI12, a trichothecene efflux pump from *Fusarium sporotrichioides*: gene isolation and expression in yeast." *Molecular & General Genetics: MGG* 261 (6): 977-984.

Alexander, Nancy J., Susan P. McCormick, Troy M. Larson, and James E. Jurgenson. 2003. "Expression of Tri15 in *Fusarium sporotrichioides*." *Current Genetics* 45 (3): 157-162. doi:10.1007/s00294-003-0467-3.

Alexander, Nancy J., Susan P. McCormick, Cees Waalwijk, Theo van der Lee, and Robert H. Proctor. 2011. "The genetic basis for 3-ADON and 15-ADON trichothecene chemotypes in *Fusarium*." *Fungal Genetics and Biology* 48 (5): 485-495. doi:10.1016/j.fgb.2011.01.003.

Alexander, Nancy J., Robert H. Proctor, and Susan P. McCormick. 2009. "Genes, gene clusters, and biosynthesis of trichothecenes and fumonisins in *Fusarium*." *Toxin Reviews* 28 (2-3): 198-215. doi:10.1080/15569540903092142.

Aoki, Takayuki, Martha M. Vaughan, Susan P. McCormick, Mark Busman, Todd J. Ward, Amy Kelly, Kerry O'Donnell, Peter R. Johnston, and David M. Geiser. 2015. "*Fusarium dactylidis* sp. nov., a novel nivalenol toxin-producing species sister to *F. pseudograminearum* isolated from orchard grass (*Dactylis glomerata*) in Oregon and New Zealand." *Mycologia* 107 (2): 409-418. doi:10.3852/14-213.

Aoki, Takayuki, Todd J. Ward, H. Corby Kistler, and Kerry O'donnell. 2012. "Systematics, Phylogeny and Trichothecene Mycotoxin Potential of Fusarium Head Blight Cereal Pathogens." *JSM Mycotoxins* 62 (2): 91-102. doi:10.2520/myco.62.91.

Bai, Guihua and Gregory Shaner. 2004. "Management and Resistance in Wheat and Barley to Fusarium Head Blight." *Annual Review of Phytopathology* 42 (1): 135-161. doi:10.1146/annurev.phyto.42.040803.140340.

Bianchini, Andreia, Richard Horsley, Maia M. Jack, Brent Kobielush, Dojin Ryu, Sheryl Tittlemier, William W. Wilson, et al. 2015. "DON Occurrence in Grains: A North American Perspective." *Cereal Foods World* 60 (1): 32-56. doi:10.1094/CFW-60-1-0032.

Burden, Raymond S., David T. Cooke, and Geoffrey A. Carter. 1989. "Inhibitors of sterol biosynthesis and growth in plants and fungi." *Phytochemistry* 28 (7): 1791-1804. doi:10.1016/S0031-9422(00)97862-2.

Chiotta, M. L., M. S. Alaniz Zanon, G. Giaj-Merlera, D. Tessmann, G. G. Barros, and S. Chulze. 2015. "Phylogenetic analyses of the *Fusarium graminearum* species complex isolated from soybean in Argentina and Brazil." *Australasian Plant Disease Notes* 10 (1): 32. doi:10.1007/s13314-015-0182-1.

Dean, Ralph, Van Kan, Jan a L, Zacharias A. Pretorius, Kim E. Hammond-Kosack, Antonio Di Pietro, Pietro D. Spanu, Jason J. Rudd, et al. 2012. "The Top 10 fungal pathogens in molecular plant pathology." *Molecular Plant Pathology* 13 (4): 414-430. doi:10.1111/j.1364-3703.2011.00783.x.

Desjardins, A. E. and R. H. Proctor. 2007. "Molecular biology of *Fusarium* mycotoxins." *International Journal of Food Microbiology* 119 (1–2): 47-50. doi:10.1016/j.ijfoodmicro.2007.07.024.

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.

Desjardins, Anne E. 2006. *Fusarium mycotoxins: Chemistry, Genetics, and Biology*. St. Paul, Minn: APS Press.

Duvnjak, T., A. Sudarić, M. M. Kočar, J. Ćosić, and K. Vrandečić. 2016. "First Report of Soybean Fusarium Wilt Caused by *Fusarium graminearum* in Croatia." *Plant Disease* 100 (3): 648. doi:10.1094/PDIS-07-15-0748-PDN.

Edwards, S. G., S. R. Pirgozliev, M. C. Hare, and P. Jenkinson. 2001. "Quantification of trichothecene-producing *Fusarium* species in harvested grain by competitive PCR to determine efficacies of fungicides against Fusarium head blight of winter wheat." *Applied and Environmental Microbiology* 67 (4): 1575-1580. doi:10.1128/AEM.67.4.1575-1580.2001.

FDA, USA. 2010. "Guidance for Industry and FDA: Advisory Levels for Deoxynivalenol (DON) in Finished Wheat Products for Human Consumption and Grains and Grain By-Products used for Animal Feed." Accessed August 05, 2016, http://www.fda.gov/downloads/Food/GuidanceRegulation/UCM217558.pdf.

Filho, A. Bergamin, A. K. Inoue-Nagata, R. B. Bassanezi, J. Belasque, L. Amorim, M. A. Macedo, J. C. Barbosa, L. Willocquet, and S. Savary. 2016. "The importance of primary inoculum and area-wide disease management to crop health and food security." *Food Security* 8 (1): 221-238. doi:10.1007/s12571-015-0544-8.

Foroud, Nora A. and François Eudes. 2009. "Trichothecenes in Cereal Grains." *International Journal of Molecular Sciences* 10 (1): 147-173. doi:10.3390/ijms10010147.

Gale, L. R., T. J. Ward, V. Balmas, and H. C. Kistler. 2007. "Population Subdivision of *Fusarium graminearum* Sensu Stricto in the Upper Midwestern United States." *Phytopathology* 97 (11): 1434-1439. doi:10.1094/PHYTO-97-11-1434.

Gilbert, Jeannie and Steve Haber. 2013. "Overview of some recent research developments in Fusarium head blight of wheat." *Canadian Journal of Plant Pathology* 35 (2): 149-174. doi:10.1080/07060661.2013.772921.

Gong, Liang, Yueming Jiang, and Feng Chen. 2015. "Molecular strategies for detection and quantification of mycotoxin-producing *Fusarium* species: a review." *Journal of the Science of Food and Agriculture* 95 (9): 1767-1776. doi:10.1002/jsfa.6935.

Goswami, Rubella S. and H. Corby Kistler. 2004. "Heading for disaster: *Fusarium graminearum* on cereal crops." *Molecular Plant Pathology* 5 (6): 515-525. doi:10.1111/j.1364-3703.2004.00252.x.

Gräfenhan, Tom, Peter R. Johnston, Martha M. Vaughan, Susan P. McCormick, Robert H. Proctor, Mark Busman, Todd J. Ward, and Kerry O'Donnell. 2016. "*Fusarium praegraminearum* sp. nov., a novel nivalenol mycotoxin-producing pathogen from New Zealand can induce head blight on wheat." *Mycologia* 108 (6): 1229-1239. doi:10.3852/16-110.

Hallen-Adams, Heather E., Nancy Wenner, Gretchen A. Kuldau, and Frances Trail. 2011. "Deoxynivalenol biosynthesis-related gene expression during wheat kernel colonization by *Fusarium graminearum*." *Phytopathology* 101 (9): 1091-1096. doi:10.1094/PHYTO-01-11-0023.

Kulik, Tomasz, Maciej Buśko, Katarzyna Bilska, Anna Ostrowska-Kołodziejczak, Anne D. van Diepeningen, Juliusz Perkowski, and Sebastian Stenglein. 2016. "Depicting the Discrepancy between *Tri* Genotype and Chemotype on the Basis of Strain CBS 139514 from a Field Population of *F. graminearum* Sensu Stricto from Argentina." *Toxins* 8 (11): 330. doi:10.3390/toxins8110330.

Lee, Theo van der, Hao Zhang, Anne van Diepeningen, and Cees Waalwijk. 2015. "Biogeography of *Fusarium graminearum* species complex and chemotypes: a review." *Food Additives & Contaminants: Part A* 32 (4): 453-460. doi:10.1080/19440049.2014.984244.

Liang, J. M., H. Xayamongkhon, K. Broz, Y. Dong, S. P. McCormick, S. Abramova, T. J. Ward, Z. H. Ma, and H. C. Kistler. 2014. "Temporal dynamics and population genetic structure of *Fusarium graminearum* in the upper Midwestern United States." *Fungal Genetics and Biology* 73: 83-92. doi:10.1016/j.fgb.2014.10.002.

Marburger, David A., Muthusubramanian Venkateshwaran, Shawn P. Conley, Paul D. Esker, Joseph G. Lauer, and Jean-Michel Ané. 2015. "Crop Rotation and Management Effect on *Fusarium* spp. Populations." *Crop Science* 55 (1): 365-376. doi:10.2135/cropsci2014.03.0199.

McCormick, Susan P., April M. Stanley, Nicholas A. Stover, and Nancy J. Alexander. 2011. "Trichothecenes: From Simple to Complex Mycotoxins." *Toxins* 3 (7): 802-814. doi:10.3390/toxins3070802.

McMullen, Marcia, Gary Bergstrom, Erick De Wolf, Ruth Dill-Macky, Don Hershman, Greg Shaner, and Dave Van Sanford. 2012. "A Unified Effort to Fight an Enemy of Wheat and Barley: Fusarium Head Blight." *Plant Disease* 96 (12): 1712-1728. doi:10.1094/PDIS-03-12-0291-FE.

McMullen, Marcia, Roger Jones, and Dale Gallenberg. 1997. "Scab of Wheat and Barley: A Re-emerging Disease of Devastating Impact." *Plant Disease* 81 (12): 1340-1348. doi:10.1094/PDIS.1997.81.12.1340.

Mourelos, C. A., I. Malbrán, P. A. Balatti, P. D. Ghiringhelli, and G. A. Lori. 2014. "Gramineous and non-gramineous weed species as alternative hosts of *Fusarium graminearum*, causal agent of Fusarium head blight of wheat, in Argentina." *Crop Protection* 65: 100-104. doi:10.1016/j.cropro.2014.07.013.

NCBI, USA. 2016a. "PubChem Compound Database." Accessed August 05, 2016, https://pubchem.ncbi.nlm.nih.gov/.

NCBI, USA. 2016b. "Taxonomy browser (*Fusarium*)." Accessed August 05, 2016, https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=5506.

Nelson, P. E., M. C. Dignani, and E. J. Anaissie. 1994. "Taxonomy, biology, and clinical aspects of *Fusarium* species." *Clinical Microbiology Reviews* 7 (4): 479-504. doi:10.1128/CMR.7.4.479.

Nielsen, L. K., D. J. Cook, S. G. Edwards, and R. V. Ray. 2014. "The prevalence and impact of Fusarium head blight pathogens and mycotoxins on malting barley quality in UK." *International Journal of Food Microbiology* 179: 38-49. doi:10.1016/j.ijfoodmicro.2014.03.023.

Pan, Dinorah, Natalia Calero, Ana Mionetto, and Lina Bettucci. 2013. "Trichothecene genotypes of *Fusarium graminearum* from wheat in Uruguay." *International Journal of Food Microbiology* 162 (1): 120-123. doi:10.1016/j.ijfoodmicro.2013.01.002.

Pasquali, Matias and Quirico Migheli. 2014. "Genetic approaches to chemotype determination in type B-trichothecene producing Fusaria." *International Journal of Food Microbiology* 189: 164-182. doi:10.1016/j.ijfoodmicro.2014.08.011.

Pestka, James J. 2010. "Deoxynivalenol: mechanisms of action, human exposure, and toxicological relevance." *Archives of Toxicology* 84 (9): 663-679. doi:10.1007/s00204-010-0579-8.

Pestka, James J. and Alexa T. Smolinski. 2005. "Deoxynivalenol: toxicology and potential effects on humans." *Journal of Toxicology and Environmental Health. Part B, Critical Reviews* 8 (1): 39-69. doi:10.1080/10937400590889458.

Pioli, R. N., L. Mozzoni, and E. N. Morandi. 2004. "First Report of Pathogenic Association Between *Fusarium graminearum* and Soybean." *Plant Disease* 88 (2): 220-220. doi:10.1094/PDIS.2004.88.2.220A.

Pirgozliev, Stoyan R., Simon G. Edwards, Martin C. Hare, and Peter Jenkinson. 2003. "Strategies for the Control of Fusarium Head Blight in Cereals." *European Journal of Plant Pathology* 109 (7): 731-742. doi:1026034509247.

Rawat, Nidhi, Michael O. Pumphrey, Sixin Liu, Xiaofei Zhang, Vijay K. Tiwari, Kaori Ando, Harold N. Trick, et al. 2016. "Wheat *Fhb1* encodes a chimeric lectin with agglutinin domains and a pore-forming toxin-like domain conferring resistance to Fusarium head blight." *Nature Genetics* 48 (12): 1576-1580. doi:10.1038/ng.3706.

Reynoso, María Marta, María Laura Ramirez, Adriana Mabel Torres, and Sofía Noemí Chulze. 2011. "Trichothecene genotypes and chemotypes in *Fusarium graminearum* strains isolated from wheat in Argentina." *International Journal of Food Microbiology* 145 (2–3): 444-448. doi:10.1016/j.ijfoodmicro.2011.01.020.

Rotter, B. A., D. B. Prelusky, and J. J. Pestka. 1996. "Toxicology of deoxynivalenol (vomitoxin)." *Journal of Toxicology and Environmental Health* 48 (1): 1-34. doi:10.1080/713851046.

Schmale, D. G., A. K. Wood-Jones, C. Cowger, G. C. Bergstrom, and C. Arellano. 2011. "Trichothecene genotypes of *Gibberella zeae* from winter wheat fields in the eastern USA." *Plant Pathology* 60 (5): 909-917. doi:10.1111/j.1365-3059.2011.02443.x.

Sobrova, Pavlina, Vojtech Adam, Anna Vasatkova, Miroslava Beklova, Ladislav Zeman, and Rene Kizek. 2010. "Deoxynivalenol and its toxicity." *Interdisciplinary Toxicology* 3 (3): 94-99. doi:10.2478/v10102-010-0019-x.

Starkey, David E., Todd J. Ward, Takayuki Aoki, Liane R. Gale, H. Corby Kistler, David M. Geiser, Haruhisa Suga, Beáta Tóth, János Varga, and Kerry O'Donnell. 2007. "Global molecular surveillance reveals novel Fusarium head blight species and trichothecene toxin diversity." *Fungal Genetics and Biology* 44 (11): 1191-1204. doi:10.1016/j.fgb.2007.03.001.

Tangni, Emmanuel K., Jean-Claude Motte, Alfons Callebaut, and Luc Pussemier. 2010. "Cross-Reactivity of Antibodies in Some Commercial Deoxynivalenol Test Kits

against Some Fusariotoxins." *Journal of Agricultural and Food Chemistry* 58 (24): 12625-12633. doi:10.1021/jf103025e.

Trail, F., I. Gaffoor, J. C. Guenther, and H. E. Hallen. 2005a. "Using genomics to understand the disease cycle of the Fusarium head blight fungus, *Gibberella zeae* (anamorph *Fusarium graminearum*)." *Canadian Journal of Plant Pathology* 27 (4): 486-498. doi:10.1080/07060660509507250.

Trail, Frances. 2009. "For Blighted Waves of Grain: *Fusarium graminearum* in the Postgenomics Era." *Plant Physiology* 149 (1): 103-110. doi:10.1104/pp.108.129684.

Trail, Frances, Iffa Gaffoor, and Steven Vogel. 2005b. "Ejection mechanics and trajectory of the ascospores of *Gibberella zeae* (anamorph *Fusarium graminearum*)." *Fungal Genetics and Biology* 42 (6): 528-533. doi:10.1016/j.fgb.2005.03.008.

USDA, NASS. 2017. "Crop Production Annual Summary." Accessed February 17, 2017, http://usda.mannlib.cornell.edu/MannUsda/viewDocumentInfo.do?documentID=1047.

Ward, Todd J., Randall M. Clear, Alejandro P. Rooney, Kerry O'Donnell, Don Gaba, Susan Patrick, David E. Starkey, Jeannie Gilbert, David M. Geiser, and Tom W. Nowicki. 2008. "An adaptive evolutionary shift in Fusarium head blight pathogen populations is driving the rapid spread of more toxigenic *Fusarium graminearum* in North America." *Fungal Genetics and Biology* 45 (4): 473-484. doi:10.1016/j.fgb.2007.10.003.

Wegulo, Stephen N. 2012. "Factors Influencing Deoxynivalenol Accumulation in Small Grain Cereals." *Toxins* 4 (11): 1157-1180. doi:10.3390/toxins4111157.

Wegulo, Stephen N., P. Stephen Baenziger, John Hernandez Nopsa, William W. Bockus, and Heather Hallen-Adams. 2015. "Management of Fusarium head blight of wheat and barley." *Crop Protection* 73: 100-107. doi:10.1016/j.cropro.2015.02.025.

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.

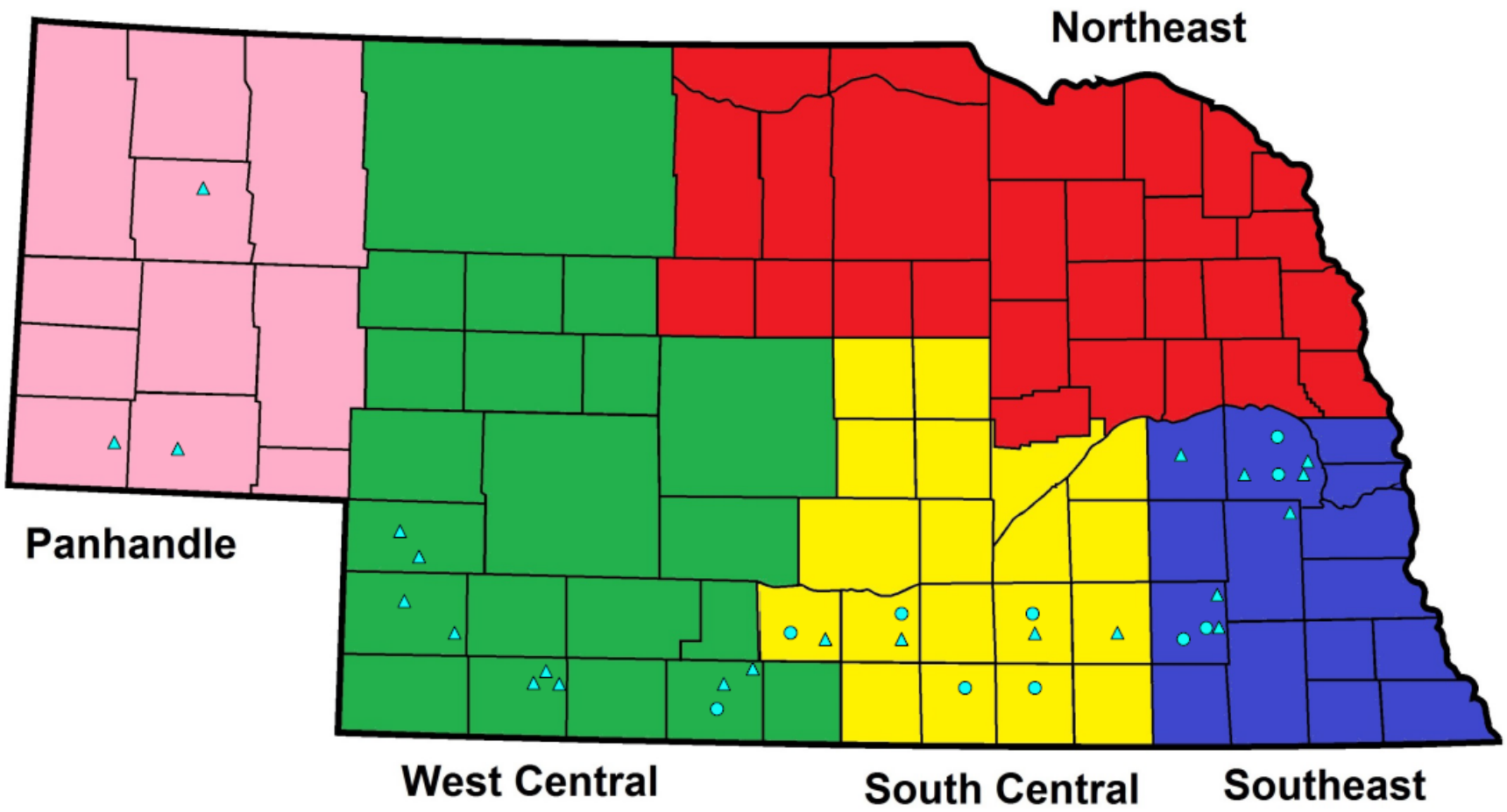

Figure 2.1: Map showing the different wheat growing regions in Nebraska, as well as the sampling sites where wheat heads were obtained. Triangles (Δ) represent samples from 2015 and circles (○) represent samples from 2016.

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).

Sample ID	GPS coordinates	County	Wheat growing region
1-15	40°11'01.8"N 99°53'30.5"W	Furnas	West Central
2-15	40°12'22.7"N 100°58'00.2"W	Hitchcock	West Central
3-15	40°26'27.9"N 96°57'39.8"W	Saline	Southeast
4-15	41°09'56.4"N 96°44'43.8"W	Saunders	Southeast
5-15	41°14'04.0"N 96°31'46.1"W	Saunders	Southeast
6-15	40°35'32.9"N 96°58'16.3"W	Saline	Southeast
7-15	41°08'44.0"N 96°29'47.1"W	Saunders	Southeast
8-15	41°01'50.0"N 96°29'32.6"W	Lancaster	Southeast
9-15	40°42'36.9"N 98°03'29.4"W	Clay	South Central
10-15	40°48'08.3"N 98°75'26.2"W	Kearney	South Central
11-15	40°45'23.7"N 99°29'89.1"W	Phelps	South Central
12-15	40°30'61.7"N 99°71'57.4"W	Harlan	West Central
13-15	40°25'22.1"N 97°35'45.2"W	Fillmore	South Central
14-15	40°50'24.2"N 101°41'44.6"W	Perkins	West Central
15-15	40°11'34.4"N 100°59'01.8"W	Hitchcock	West Central
16-15	40°38'12.6"N 101°37'43.8"W	Chase	West Central
17-15	40°49'18.4"N 101°39'57.7"W	Perkins	West Central
18-15	40°20'46.3"N 100°95'16.3"W	Hitchcock	West Central
19-15	40°27'36.0"N 101°32'01.0"W	Chase	West Central
20-15	41°09'06.9"N 103°29'30.3"W	Kimball	Panhandle
21-15	42°20'18.6"N 103°04'22.1"W	Box Butte	Panhandle
22-15	41°13'56.7"N 103°01'13.0"W	Cheyenne	Panhandle
23-15	41°15'52.7"N 97°12'40.9"W	Butler	Southeast
1-16	40°16'10.4"N 99°79'17.8"W	Furnas	West Central
2-16	40°24'24.5"N 99°25'37.2"W	Phelps	South Central
3-16	40°54'46.6"N 98°84'55.4"W	Kearney	South Central
4-16	40°57'54.2"N 98°12'85.7"W	Clay	South Central
5-16	41°22'86.0"N 96°48'92.0"W	Saunders	Southeast
6-16	41°05'23.9"N 96°54'05.1"W	Saunders	Southeast
7-16	40°43'11.4"N 96°96'10.0"W	Saline	Southeast
8-16	40°24'24.5"N 99°25'37.2"W	Nuckolls	South Central
9-16	40°48'03.8"N 97°17'59.5"W	Saline	Southeast
10-16	40°32'11.4"N 98°44'25.3"W	Webster	South Central

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 (dH_2O) 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 dH_2O 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 2.2: Sequence of the primers used in this study.

Primer	Sequence (5'-to-3')	Reference
ITS1F	TCCGTAGGTGAACCTGCGG	Gardes and Bruns (1993)
ITS4	TCCTCCGCTTATTGATATG	White et al. (1990)
GOFW	ACCTCTGTTGTTCTTCCAGACGG	de Biazio et al. (2008)
GORV	CTGGTCAGTATTAACCGTGTGTG	de Biazio et al. (2008)
UBC85F	GCAGGGTTTGAATCCGAGAC	Schilling et al. (1996)
UBC85R	AGAATGGAGCTACCAACGGC	Schilling et al. (1996)
RED-f	AGACTCATTCCAGCCAAG	Ward et al. (2008)
RED-r	TCGTGTTGAAGAGTTTGG	Ward et al. (2008)
TRI101-f	CAAGATACAGCTCGACACC	Ward et al. (2008)
TRI101-r	CTGGGTAGTTGTTCGAGA	Ward et al. (2008)

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; Chełkowski et al. 2012; Faria et al. 2012; Panthi et al. 2014; Nopsa 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. louisianense*) 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.

Probe name[a]	Gene	Sequence[b]	Target
T12-15(12)	*TRI12*	CATAATCAATTTCAACTTTCTACTtacagcggtcgcaacttc	15-ADON
T3-15 (34)	*TRI3*	ACTTATTTCTTCACTACTATATCAactgacccaagctgccatc	15-ADON
T12-3(13)	*TRI12*	CAAATACATAATCTTACATTCACTctttggcaagcccgtgca	3-ADON
T3-3(35)	*TRI3*	CATCTTCATATCAATTCTCTTATTcgcattggctaacacatg	3-ADON
T12-N(14)	*TRI12*	AATTTCTTCTCTTTCTTTCACAATtggtctcctcgttgtatctgg	NIV
T3-N(14)	*TRI3*	AATTTCTTCTCTTTCTTTCACAATgacaagtgcacagaatatacg	NIV
ATb(15)	*TRI101*	TACTTCTTTACTACAATTTACAACacggtgctatggatatgg	B-FHB clade
EFg(18)	*EF1α*	ACACTTATCTTTCAATTCAATTACtcatcatcacgtgtcaac	FGSC
ATg(19)	*TRI101*	ATACTTTACAAACAAATAACACACCcattcaccgaagaggaaat	FGSC
MAT6(27)	*MAT 1-1-1*	TAACTTACACTTAACTATCATCTTggctacttttctgagtactct	*F. asiaticum*
AT6(30)	*TRI101*	CTTAACATTTAACTTCTATAACACaagctgggcgttcttcaa	*F. asiaticum*
ATce(26)	*TRI101*	TACATTCAACACTCTTAAATCAAAgaggtagatcatcagattgtt	*F. cerealis*
REDce(29)	*RED*	TACTACTTCTATAACTCACTTAAAgttgcagacactacacaaa	*F. cerealis*
REDcu(22)	*RED*	CAAACAAACATTCAAATATCAATCgaagaaacgcttgtatcgaa	*F. culmorum*
ATcu(25)	*TRI101*	CTTTCTTAATACATTACAACATACaggacgttcctcgtgtta	*F. culmorum*
RED10(28)	*RED*	CACTTAATTCATTCTAAATCTATCgaactagaactagtcaatgcc	*F. gerlachii*
AT10(33)	*TRI101*	ACTACTTATTCTCAAACTCTAATAtgacgatgctctttcggcc	*F. gerlachii*
EF7(20)	*EF1α*	CTTTCTCATACTTTCAACTAATTTactcgagcgacaggcgtc	*F. graminearum*
AT7(21)	*TRI101*	TCAAACTCTCAATTCTTACTTAATatagttccttaccttgaaaactat	*F. graminearum*

[a] The Luminex xMAP fluorescent polystyrene microspheres sets (Luminex Corp.) used for hybridization reactions are indicated in parentheses.
[b] The 5' sequence tag portions of extension probes complementary to the anti-tag sequence attached to the surface of individual sets of microspheres are capitalized.

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 *Eco*RI, *Taq*I and *Hae*III (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).

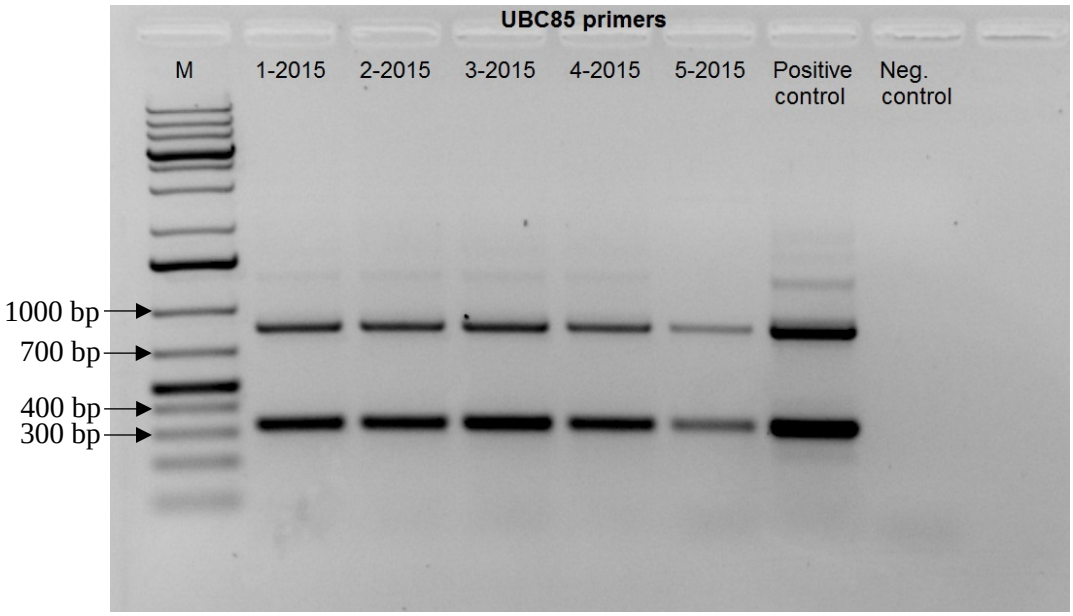

Figure 2.2: Agarose gel showing fragments amplified with the UBC85 primers for isolates 1-5 from 2015. Positive control: Reference *Fusarium graminearum*. Lane M, molecular weight marker (1 kb). Bands of around 800 bp were detected in several of the samples, as well as in the reference isolate.

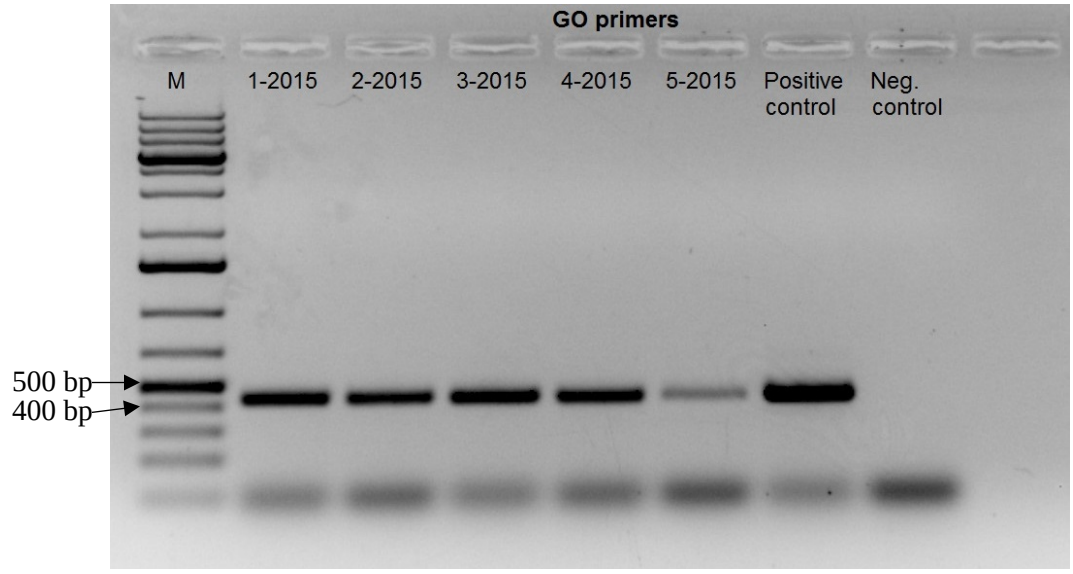

Figure 2.3: Agarose gel showing fragments amplified with the GO primers for isolates 1-5 from 2015. Positive control: Reference *Fusarium graminearum*. Lane M, molecular weight marker (1 kb).

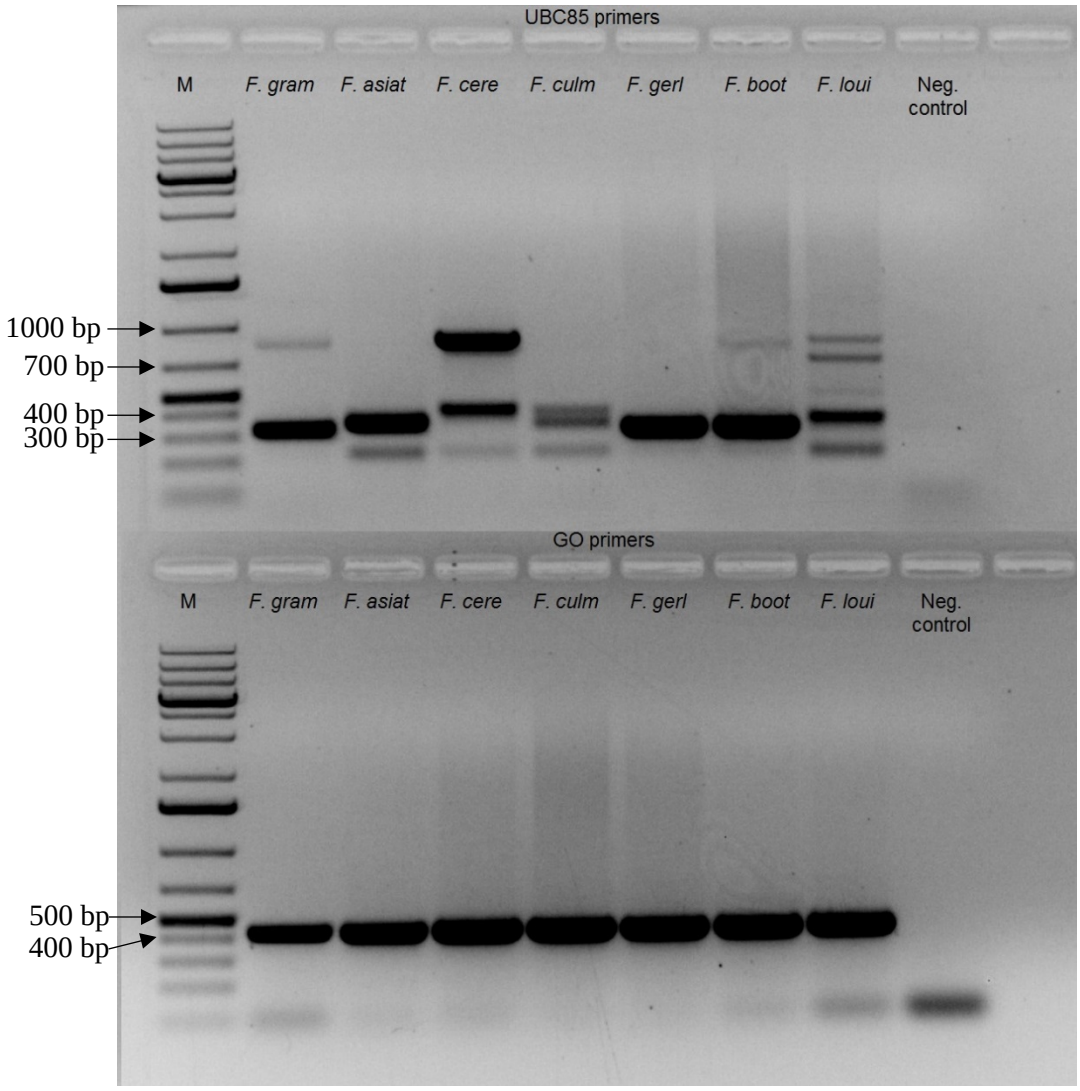

Figure 2.4: Agarose gel showing fragments amplified with the UBC85 and GO primers for reference *Fusarium* isolates. *F. gram*: *F. graminearum*, *F. asiat*: *F. asiaticum*, *F. cere*: *F. cerealis*, *F. culm*: *F. culmorum*, *F. gerl*: *F. gerlachii*, *F. boot*: *F. boothii*, *F. loui*: *F. louisianense*. 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.

Sample	T12-15	T12-3	T12-N	ATb	EFg	ATg	EF7	AT7	REDcu	ATcu	ATce	MAT6	RED10	REDce	AT6	AT10	T3-15	T3-3	Putative identification
***F. graminearum* 3-ADON**	197	214	195	606	341	383	2584	240	231	274	265	196	228	243	406	374	197	209	?
***F. graminearum* 3-ADON**	138	184	151	525	333	309	2521	184	159	219	163	123	175	201	339	307	144	156	?
***F. graminearum* 15-ADON**	159	173	179	554	333	310	2470	241	190	235	190	144	185	167	334	314	169	172	?
***F. graminearum* 15-ADON**	166	184	178	612	1061	320	1158	256	192	253	195	157	201	234	351	322	187	177	?
***F. graminearum* NIV**	172	187	179	587	342	357	2554	217	209	250	213	156	206	211	381	350	175	208	?
***F. graminearum* NIV**	184	216	192	527	384	333	2423	265	209	226	215	159	201	261	361	325	203	200	?
***F. cerealis* NIV**	208	229	193	718	196	214	224	222	229	350	476	184	225	316	493	439	193	221	?
***F. culmorum* 3-ADON**	216	279	224	544	193	205	251	239	236	605	267	181	235	232	295	625	229	278	?
Neg. control	353	350	372	388	616	321	2736	372	371	297	368	266	344	357	466	342	332	361	-

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.

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

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.

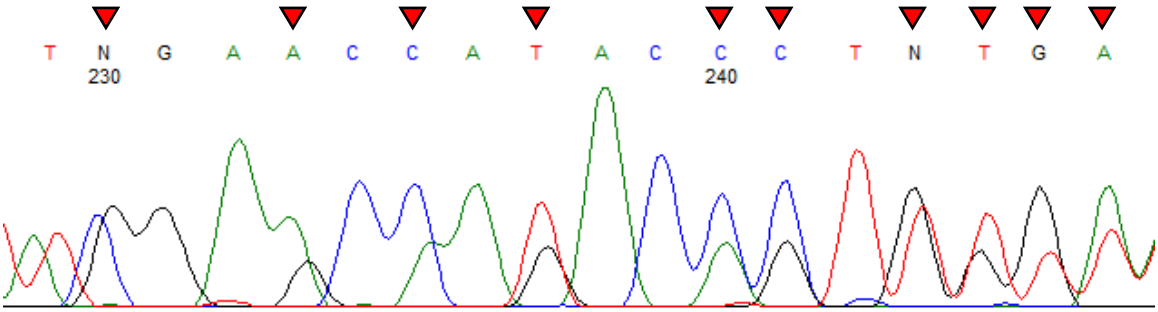

Figure 2.5: Direct sequencing chromatogram of the PCR product of the *RED* gene from isolate 20, showing double or multiple peaks denoted by a red arrow. Peaks of equivalent size called as 'N' can be seen at positions 230 and 243, whereas numerous peaks had higher than background signal for additional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

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 2.6: Intragenomic polymorphisms found in clones from each isolate in this study.

Isolate gene	Clone Name	Polymorphism
3-2015		
TRI101	TRI101 3-6	457C>G
19-2015		
TRI101	TRI101 19-1	208G>A
		241T>C
		584A>G
		659A>G
	TRI101 19-3	215A>G
20-2015		
RED	RED20-3	125A>G
		192T>C
		267A>G
		285T>C
		381T>C
TRI101	TRI101 20-11	602A>G
	TRI101 20-12	118A>G
		203C>A
21-2015		
RED	RED21-6	164T>C

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.

Sequence	23	43	97	334	352	460	546	606
Fusarium pseudograminearum NRRL 34261	---------..----C--...TG-----TT-----...----------T--T--T-----TA----T-----T..------C----------C...-------..------							
Gibberella zeae NRRL 34449	---------..----C--...TG-----TT-----...----------T--T--T-----TA----T-----T..------C----------C...-------..------							
Gibberella zeae NRRL 28063	---------..----C--...TG-----TT-----...----------T--T--T-----TA----T-----T..------C----------C...-------..------							
Fusarium graminearum NRRL 66037	---------..----C--...TG-----TT-----...----------T------A----------T..------C----------C...-------..------							
Fusarium graminearum NRRL 66033	---------..----C--...TG-----TT-----...----------T------A----------T..------C----------C...-------..------							
Fusarium graminearum NRRL 45380	---------..----C--...TG-----TT-----...----------T------A----------T...----------C...-------..------							
Fusarium graminearum NRRL 40567	---------..----C--...TG-----TT-----...----------T------A----------T...----------C...-------..------							
Fusarium nepalense NRRL 54222	---------..----C--...TG-----TT-----...----------T------A----------T...----------C...-------..------							
Gibberella zeae F10102005	---------..----C--...TG-----TT-----...----------T------A----------T...----------C...-------..------							
Gibberella zeae NRRL 31287	---------..----C--...TG-----TT-----...----------T------A----------T...----------C...-------..------							
RED3-1	---------..----C--...TG-----TT-----...----------T--T--T-----TA----T-----T..------C----------C...-------..------							
RED3-3	---------..-----C--...TG-----TT-----...----------T--T--T-----TA----T-----T..------C----------C...-------..------							
RED20-2	---------..-----C--...TG-----TT-----...----------T------A----------T...----------C...-------..------							
RED20-3	---------..-----C--...TG-----TT-----...----------T------A----------T...----------C...-------..------							
RED20-9	---------..-----C--...TG-----TT-----...----------T------A----------T...----------C...-------..------							
RED20-12	---------..-----C--...TG-----TT-----...----------T------A----------T...----------C...-------..------							
Fusarium boothii NRRL 34301	---------..----C--...TG-----TT-----...----------T------A----------T...----------C...-------..------							
Fusarium boothii NRRL 34351	---------..----C--...TG-----TT-----...----------T------A----------T...----------C...-------..------							
Fusarium boothii NRRL 29011	---------..----C--...TG-----TT-----...----------T------A----------T...----------C...-------..------							
Fusarium boothii strain NRRL 26916	---------..----C--...TG-----TT-----...----------T------A----------T...----------C...-------..------							
Fusarium boothii NRRL 29020	---------..----C--...TG-----TT-----...----------T------A----------T...----------C...-------..------							
RED19-1	---------..----C--...TG-----TT-----...----------T------A----------T...----------C...-------..------							
RED21-1	---------..----C--...TG-----TT-----...----------T------A----------T...----------C...-------..------							
RED21-6	---------..----C--...TG-----TT-----...----------T------A----------T...----------C...-------..------							
Fusarium boothii MLGT probe sequence				ATTGGTGTTGCCTTCGCC				
Fusarium gerlachii MLGT probe sequence			GAACTAGAACTAGT.....				.....CAATGCC	
Fusarium cerealis MLGT probe sequence						GTTGCAGACACTACACAAA		
Fusarium culmorum MLGT probe sequence	GAAGAAAC...GCTTGTA.....							.....TCGAA
Fusarium pseudograminearum MLGT probe sequence					CAAGCCGATGCCAAGTCC			

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.

Sequence	**1**	**50**	**397**	**433**	**501**	**597**
Fusarium graminearum 12002	G----G-A-T----...	-----------------G...	------------------------...	--------------------C...	-----------------C...	------------------G
Gibberella zeae PPRI10693	G----G-A-T----...	-----------------G...	------------------------...	--------------------C...	-----------------C...	------------------G
Gibberella zeae Z3639	G----G-A-T----...	-----------------G...	------------------------...	--------------------C...	-----------------C...	------------------G
Gibberella zeae NRRL 31084	G----G-A-T----...	-----------------G...	------------------------...	--------------------C...	-----------------C...	------------------G
Gibberella zeae NRRL 28063	G----G-A-T----...	-----------------G...	------------------------...	--------------------C...	-----------------C...	------------------G
Fusarium sp. NRRL 37605	G----G-A-T----...	-----------------G...	-----------------------C...	--------------------C...	-----------------C...	------------------G
Gibberella zeae isolate 42	G----G-A-T----...	-----------------G...	------------------------...	--------------------C...	-----------------C...	------------------G
Gibberella zeae isolate 30	G----G-A-T----...	-----------------G...	------------------------...	--------------------C...	-----------------C...	------------------G
Gibberella zeae NRRL 6394	G----G-A-T----...	-----------------G...	------------------------...	--------------------C...	-----------------C...	------------------G
Fusarium graminearum isolate FG2	G----G-A-T----...	-----------------G...	-----------------------C...	--------------------C...	-----------------C...	------------------G
Fusarium graminearum isolate FG1	G----G-A-T----...	-----------------G...	-----------------------C...	--------------------C...	-----------------C...	------------------G
TRI101 3-6	G----G-A-T----...	-----------------G...	------------------------...	--------------------C...	-----------------C...	------------------G
TRI101 20-11	G----G-A-T----...	-----------------G...	------------------------...	--------------------C...	-----------------C...	-----G------------G
TRI101 20-12	G----G-A-T----...	-----------------G...	------------------------...	--------------------C...	-----------------C...	------------------G
TRI101 20-15	G----G-A-T----...	-----------------G...	------------------------...	--------------------C...	-----------------C...	------------------G
Fusarium boothii isolate 53	G----G-A-T----...	-----------------G...	-----------------------C...	--------------------C...	-----------------C...	------------------G
Fusarium boothii NRRL 29020	G----G-A-T----...	-----------------G...	-----------------------C...	--------------------C...	-----------------C...	------------------G
Fusarium boothii isolate 52	G----G-A-T----...	-----------------G...	-----------------------C...	--------------------C...	-----------------C...	------------------G
Fusarium boothii isolate 56	G----G-A-T----...	-----------------G...	-----------------------C...	--------------------C...	-----------------C...	------------------G
TRI101 19-1	G----G-A-T----...	-----------------G...	-----------------------C...	--------------------C...	-----------------C...	------------------G
TRI101 19-2	G----G-A-T----...	-----------------G...	-----------------------C...	--------------------C...	-----------------C...	------------------G
TRI101 19-3	G----G-A-T----...	-----------------G...	-----------------------C...	--------------------C...	-----------------C...	------------------G
TRI101 21-1	G----G-A-T----...	-----------------G...	-----------------------C...	--------------------C...	-----------------C...	------------------G
TRI101 21-3	G----G-A-T----...	-----------------G...	-----------------------C...	--------------------C...	-----------------C...	------------------G
Fusarium boothii MLGT probe sequence	AAGGTCTTAAGCG....	....	....	....	............----G-	
Fusarium graminearum MLGT probe sequence			ATAGTTCCTTACCTTGAAAACTAT			
Fusarium gerlachii MLGT probe sequence						TGACGATGCTCTTTCGGCC
Fusarium cerealis MLGT probe sequence				GAGGTAGATCATCAGATTGTT		
Fusarium culmorum MLGT probe sequence		AGGACGTTCCTCGTGTTA				
Fusarium asiaticum MLGT probe sequence					AAGCTGGGCGTTCTTCAA	

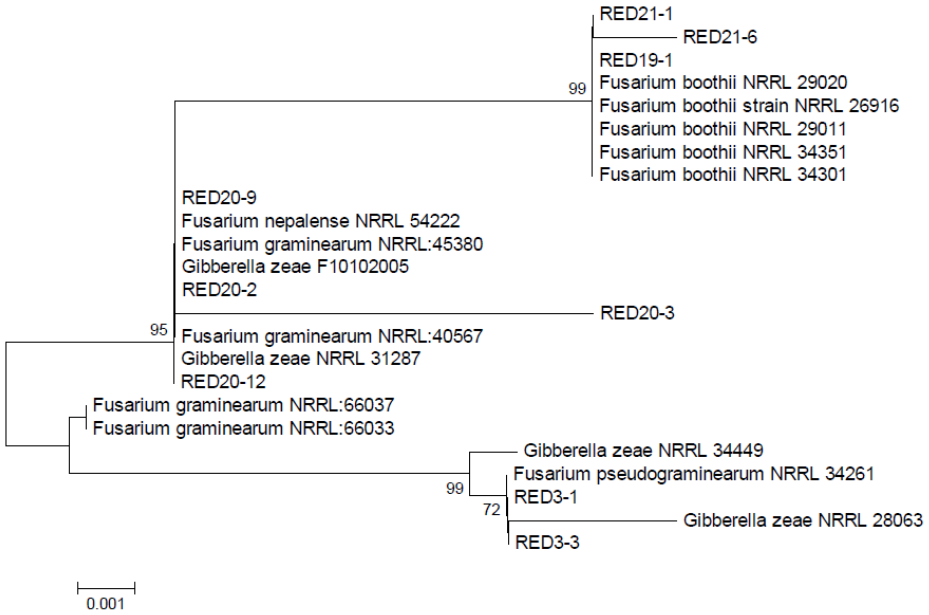

Figure 2.6: Phylogenetic tree representing the relationship of clones from four *Fusarium* isolates to their top hits from GenBank. The tree was inferred from *RED* sequence data and prepared by neighbor-joining. The scale bars represent the estimated numbers of substitutions per base. Numbers at the nodes represent bootstrap percentages and values lower than 70% are not shown.

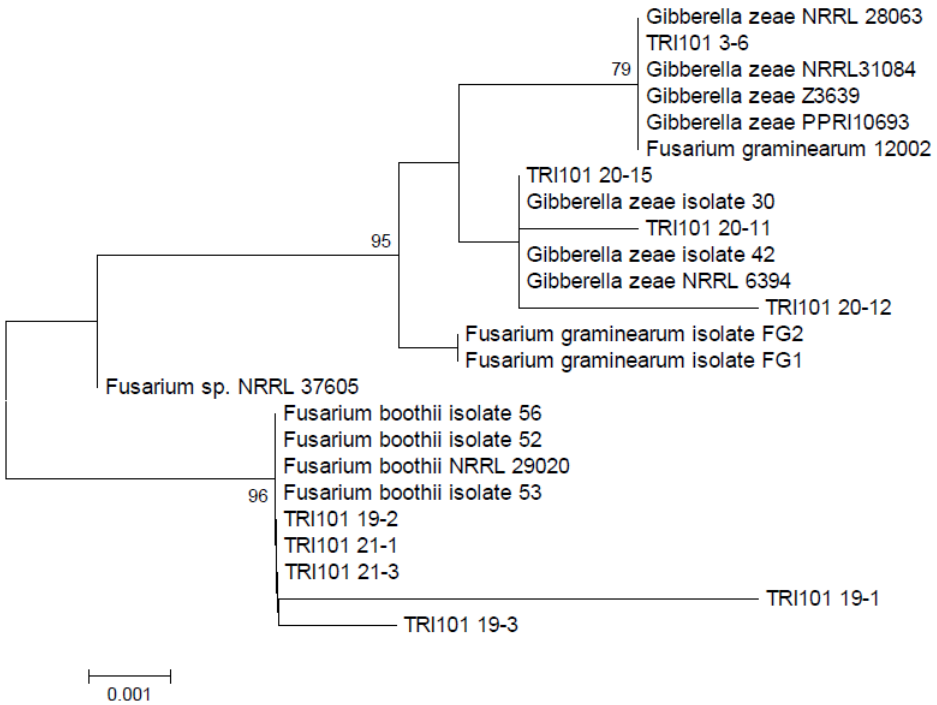

Figure 2.7: Phylogenetic tree representing the relationship of clones from four *Fusarium* isolates to their top hits from GenBank. The tree was inferred from *TRI101* sequence data and prepared by neighbor-joining. The scale bars represent the estimated numbers of substitutions per base. Numbers at 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 (Umpiérrez-Failache 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 *RED* and *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 *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; Vesgueiro 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 *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 *RED* and *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 illinoinensis*) 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

Aamot, H. U., T. J. Ward, G. Brodal, T. Vrålstad, G. B. Larsen, S. S. Klemsdal, A. Elameen, S. Uhlig, and I. S. Hofgaard. 2015. "Genetic and phenotypic diversity within the *Fusarium graminearum* species complex in Norway." *European Journal of Plant Pathology* 142 (3): 501-519. doi:10.1007/s10658-015-0629-4.

Abedi-Tizaki, Mostafa and Doustmorad Zafari. 2016. "Nucleotide polymorphisms and protein structure changes in the *Fg16* gene of *Fusarium graminearum sensu stricto*." *Meta Gene* 9: 62-69. doi:10.1016/j.mgene.2016.03.001.

Álvarez, I. and J. F. Wendel. 2003. "Ribosomal ITS sequences and plant phylogenetic inference." *Molecular Phylogenetics and Evolution* 29 (3): 417-434. doi:10.1016/S1055-7903(03)00208-2.

Aoki, Takayuki, Todd J. Ward, H. Corby Kistler, and Kerry O'donnell. 2012. "Systematics, Phylogeny and Trichothecene Mycotoxin Potential of Fusarium Head Blight Cereal Pathogens." *JSM Mycotoxins* 62 (2): 91-102. doi:10.2520/myco.62.91.

Balmas, V., B. Scherm, A. Marcello, M. Beyer, L. Hoffmann, Q. Migheli, and M. Pasquali. 2015. "*Fusarium* species and chemotypes associated with fusarium head blight and fusarium root rot on wheat in Sardinia." *Plant Pathology* 64 (4): 972-979. doi:10.1111/ppa.12337.

Bezzhonova, O. V. and I. I. Goryacheva. 2008. "Intragenomic heterogeneity of rDNA internal transcribed spacer 2 in *Anopheles messeae* (Diptera: Culicidae)." *Journal of Medical Entomology* 45 (3): 337-341.

Bianchini, Andreia, Richard Horsley, Maia M. Jack, Brent Kobielush, Dojin Ryu, Sheryl Tittlemier, William W. Wilson, et al. 2015. "DON Occurrence in Grains: A North American Perspective." *Cereal Foods World* 60 (1): 32-56. doi:10.1094/CFW-60-1-0032.

Boutigny, A. -L, I. Beukes, and A. Viljoen. 2011a. "Head blight of barley in South Africa is caused by *Fusarium graminearum* with a 15-ADON chemotype." *Journal of Plant Pathology* 93 (2): 321-329. doi:10.4454/jpp.v93i2.1186.

Boutigny, Anne-Laure, Todd J. Ward, Nicolas Ballois, Gabriela Iancu, and Renaud Ioos. 2014. "Diversity of the *Fusarium graminearum* species complex on French cereals." *European Journal of Plant Pathology* 138 (1): 133-148. doi:10.1007/s10658-013-0312-6.

Boutigny, Anne-Laure, Todd J. Ward, Gert J. Van Coller, Bradley Flett, Sandra C. Lamprecht, Kerry O'Donnell, and Altus Viljoen. 2011b. "Analysis of the *Fusarium graminearum* species complex from wheat, barley and maize in South Africa provides evidence of species-specific differences in host preference." *Fungal Genetics and Biology* 48 (9): 914-920. doi:10.1016/j.fgb.2011.05.005.

Bovers, Marjan, Ferry Hagen, Eiko E. Kuramae, Mara R. Diaz, Lodewijk Spanjaard, Françoise Dromer, Hans L. Hoogveld, and Teun Boekhout. 2006. "Unique hybrids between the fungal pathogens *Cryptococcus neoformans* and *Cryptococcus gattii*." *FEMS Yeast Research* 6 (4): 599-607. doi:10.1111/j.1567-1364.2006.00082.x.

Castañares, E., M. I. Dinolfo, E. M. Del Ponte, D. Pan, and S. A. Stenglein. 2016. "Species composition and genetic structure of *Fusarium graminearum* species complex populations affecting the main barley growing regions of South America." *Plant Pathology* 65 (6): 930-939. doi:10.1111/ppa.12470.

Castañares, Eliana, Diana Ramirez Albuquerque, María Inés Dinolfo, Virginia Fernandez Pinto, Andrea Patriarca, and Sebastián Alberto Stenglein. 2014. "Trichothecene genotypes and production profiles of *Fusarium graminearum* isolates obtained from barley cultivated in Argentina." *International Journal of Food Microbiology* 179: 57-63. doi:10.1016/j.ijfoodmicro.2014.03.024.

Chełkowski, J., K. Gromadzka, Ł Stępień, L. Lenc, M. Kostecki, and F. Berthiller. 2012. "*Fusarium* species, zearalenone and deoxynivalenol content in preharvest scabby wheat heads from Poland." *World Mycotoxin Journal* 5 (2): 133-141. doi:10.3920/WMJ2011.1304.

Chen, Jiazhen, Xinyu Miao, Meng Xu, Junlin He, Yi Xie, Xingwen Wu, Gang Chen, Liying Yu, and Wenhong Zhang. 2015. "Intra-Genomic Heterogeneity in 16S rRNA Genes in Strictly Anaerobic Clinical Isolates from Periodontal Abscesses." *Plos One* 10 (6): e0130265. doi:10.1371/journal.pone.0130265.

Chiotta, M. L., M. S. Alaniz Zanon, G. Giaj-Merlera, D. Tessmann, G. G. Barros, and S. Chulze. 2015. "Phylogenetic analyses of the *Fusarium graminearum* species complex isolated from soybean in Argentina and Brazil." *Australasian Plant Disease Notes* 10 (1): 32. doi:10.1007/s13314-015-0182-1.

de Biazio, Gleison Ricardo, Gabriela Guimarães Sousa Leite, Dauri José Tessmann, and Ione Parra Barbosa-Tessmann. 2008. "A new PCR approach for the identification of *Fusarium graminearum*." *Brazilian Journal of Microbiology* 39 (3): 554-560. doi:10.1590/S1517-838220080003000028.

Desjardins, Anne E. and Robert H. Proctor. 2011. "Genetic diversity and trichothecene chemotypes of the *Fusarium graminearum* clade isolated from maize in Nepal and identification of a putative new lineage." *Fungal Biology* 115 (1): 38-48. doi:10.1016/j.funbio.2010.10.002.

Duan, Canxing, Zihui Qin, Zhihuan Yang, Weixi Li, Suli Sun, Zhendong Zhu, and Xiaoming Wang. 2016. "Identification of Pathogenic *Fusarium* spp. Causing Maize Ear Rot and Potential Mycotoxin Production in China." *Toxins* 8 (6): 186. doi:10.3390/toxins8060186.

Faria, Carla, Giovana Almeida-Ferreira, Karina Gagliardi, Tatiane Cristina Alves, Dauri José Tessmann, Miguel Machinski-Junior, and Ione Barbosa-Tessmann. 2012. "Use of the polymerase chain reaction for detection of *Fusarium graminearum* in bulgur wheat." *Ciência E Tecnologia De Alimentos* 32 (1).

Foroud, Nora A. and François Eudes. 2009. "Trichothecenes in Cereal Grains." *International Journal of Molecular Sciences* 10 (1): 147-173. doi:10.3390/ijms10010147.

Gardes, M. and T. D. Bruns. 1993. "ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts." *Molecular Ecology* 2 (2): 113-118. doi:10.1111/j.1365-294X.1993.tb00005.x.

Gomes, AAM, A. R. Machado, APS Ferreira, D. C. Dutra, and O. L. Pereira. 2015. "Postharvest Fruit Rot of Tomato in Brazil Caused by *Fusarium boothii*." *Plant Disease* 99 (4): 555. doi:10.1094/PDIS-09-14-0903-PDN.

Goswami, Rubella S. and H. Corby Kistler. 2004. "Heading for disaster: *Fusarium graminearum* on cereal crops." *Molecular Plant Pathology* 5 (6): 515-525. doi:10.1111/j.1364-3703.2004.00252.x.

Goswami, Rubella S. and H. Corby Kistler. 2005. "Pathogenicity and In Planta Mycotoxin Accumulation Among Members of the *Fusarium graminearum* Species Complex on Wheat and Rice." *Phytopathology* 95 (12): 1397-1404. doi:10.1094/PHYTO-95-1397.

Gryzenhout, M., B. Khooa, and L. Landman. 2016. "First report of *Fusarium boothii* from pecan (*Carya illinoinensis*) and camel thorn (*Vachellia erioloba*) trees in South Africa." *South African Journal of Botany* 105: 158-162. doi:10.1016/j.sajb.2016.03.003.

Karugia, G. W., H. Suga, L. R. Gale, T. Nakajima, K. Tomimura, and M. Hyakumachi. 2009. "Population Structure of the *Fusarium graminearum* Species Complex from a Single Japanese Wheat Field Sampled in Two Consecutive Years." *Plant Disease* 93 (2): 170-174. doi:10.1094/PDIS-93-2-0170.

Kelly, Amy C., Randall M. Clear, Kerry O'Donnell, Susan McCormick, T. Kelly Turkington, Andy Tekauz, Jeannie Gilbert, H. Corby Kistler, Mark Busman, and Todd J. Ward. 2015. "Diversity of Fusarium head blight populations and trichothecene toxin types reveals regional differences in pathogen composition and temporal dynamics." *Fungal Genetics and Biology* 82: 22-31. doi:10.1016/j.fgb.2015.05.016.

Kumar, Arun, Shailesh Karre, Dhananjay Dhokane, Udaykumar Kage, Shivappa Hukkeri, and Ajjamada C. Kushalappa. 2015. "Real-time quantitative PCR based method for the quantification of fungal biomass to discriminate quantitative resistance in barley and wheat genotypes to fusarium head blight." *Journal of Cereal Science* 64: 16-22. doi:10.1016/j.jcs.2015.04.005.

LaFrentz, B. R., G. C. Waldbieser, T. J. Welch, and C. A. Shoemaker. 2014. "Intragenomic heterogeneity in the 16S rRNA genes of *Flavobacterium columnare* and standard protocol for genomovar assignment." *Journal of Fish Diseases* 37 (7): 657-669. doi:10.1111/jfd.12166.

Lee, Theresa, Ji-Seon Paek, Kyung Ah Lee, Soohyung Lee, Jung-Hye Choi, Hyeonheui Ham, Sung Kee Hong, and Jae-Gee Ryu. 2016. "Occurrence of Toxigenic *Fusarium vorosii* among Small Grain Cereals in Korea." *The Plant Pathology Journal* 32 (5): 407-413. doi:10.5423/PPJ.OA.05.2016.0123.

Machado, F. J., P. A. Möller, C. P. Nicolli, E. M. Del Ponte, and T. J. Ward. 2015. "First Report of *Fusarium graminearum*, *F. asiaticum*, and *F. cortaderiae* as Head Blight Pathogens of Annual Ryegrass in Brazil." *Plant Disease* 99 (12): 1859-1859. doi:10.1094/PDIS-04-15-0376-PDN.

Malihipour, Ali, Jeannie Gilbert, Michele Piercey-Normore, and Sylvie Cloutier. 2012. "Molecular Phylogenetic Analysis, Trichothecene Chemotype Patterns, and Variation in Aggressiveness of *Fusarium* Isolates Causing Head Blight in Wheat." *Plant Disease* 96 (7): 1016-1025. doi:10.1094/PDIS-10-11-0866-RE.

Nash, S. M. and W. C. Snyder. 1962. "Quantitative and estimations by plat counts of propagules of the bean rot *Fusarium* in field soils." *Phytopathology* 52: 567-572.

Nebraska Wheat Board. 2011. "Wheat Production Handbook." Accessed March 30, 2017, http://www.nebraskawheat.com/wp-content/uploads/2014/01/WheatProductionHandbook.pdf.

Nopsa, Hernandez, John F, Stephen N. Wegulo, Anita Panthi, Heather E. Hallen-Adams, Steven D. Harris, and P. Stephen Baenziger. 2014. "Characterization of Nebraska Isolates of Causing Head Blight of Wheat." *Crop Science* 54 (1): 310-317. doi:10.2135/cropsci2013.04.0229.

O'Donnell, Kerry and Elizabeth Cigelnik. 1997. "Two Divergent Intragenomic rDNA ITS2 Types within a Monophyletic Lineage of the Fungus *Fusarium* Are Nonorthologous." *Molecular Phylogenetics and Evolution* 7 (1): 103-116. doi:10.1006/mpev.1996.0376.

O'Donnell, Kerry, H. Corby Kistler, Beth K. Tacke, and Howard H. Casper. 2000. "Gene genealogies reveal global phylogeographic structure and reproductive isolation among lineages of *Fusarium graminearum*, the fungus causing wheat scab." *Proceedings of the National Academy of Sciences* 97 (14): 7905-7910. doi:10.1073/pnas.130193297.

Pan, D., A. Mionetto, N. Calero, M. M. Reynoso, A. Torres, and L. Bettucci. 2016. "Population genetic analysis and trichothecene profiling of *Fusarium graminearum* from wheat in Uruguay." *Genetics and Molecular Research* 15 (1). doi:10.4238/gmr.15017270.

Panthi, Anita. 2012. "Characterization of Chemotype and Aggressiveness of Nebraska Isolates of *Fusarium graminearum*." MS thesis, University of Nebraska-Lincoln.

Panthi, Anita, Heather Hallen-Adams, Stephen N. Wegulo, John Hernandez Nopsa, and P. Stephen Baenziger. 2014. "Chemotype and aggressiveness of isolates of *Fusarium graminearum* causing head blight of wheat in Nebraska." *Canadian Journal of Plant Pathology* 36 (4): 447-455. doi:10.1080/07060661.2014.964775.

Pestka, James J. and Alexa T. Smolinski. 2005. "Deoxynivalenol: toxicology and potential effects on humans." *Journal of Toxicology and Environmental Health. Part B, Critical Reviews* 8 (1): 39-69. doi:10.1080/10937400590889458.

Puri, Krishna D. and Shaobin Zhong. 2010. "The 3ADON Population of *Fusarium graminearum* Found in North Dakota Is More Aggressive and Produces a Higher Level of DON than the Prevalent 15ADON Population in Spring Wheat." *Phytopathology* 100 (10): 1007-1014. doi:10.1094/PHYTO-12-09-0332.

Qiu, Jian-bo, Jing-Tao Sun, Ming-Zheng Yu, Jian-Hong Xu, and Jian-Rong Shi. 2016. "Temporal dynamics, population characterization and mycotoxins accumulation of *Fusarium graminearum* in Eastern China." *Scientific Reports* 6: 36350. doi:10.1038/srep36350.

Qu, B., H. P. Li, J. B. Zhang, T. Huang, J. Carter, Y. C. Liao, and P. Nicholson. 2008. "Comparison of genetic diversity and pathogenicity of fusarium head blight pathogens from China and Europe by SSCP and seedling assays on wheat." *Plant Pathology* 57 (4): 642-651. doi:10.1111/j.1365-3059.2008.01824.x.

Saitou, N. and M. Nei. 1987. "The neighbor-joining method: a new method for reconstructing phylogenetic trees." *Molecular Biology and Evolution* 4 (4): 406-425.

Sambrook, J. and D. Russell. 2001. *Molecular Cloning: A Laboratory Manual*. Third ed. New York: Cold Spring Harbor Laboratory Press.

Schilling, A. G., E. M. Moller, and H. H. Geiger. 1996. "Polymerase chain reaction-based assays for species-specific detection of *Fusarium culmorum*, *F. graminearum*, and *F. avenaceum*." *Phytopathology* 86 (5): 515-522. doi:10.1094/Phyto-86-515.

Scoz, L. B., P. Astolfi, D. S. Reartes, D. G. Schmale III, M. G. Moraes, and E. M. Del Ponte. 2009. "Trichothecene mycotoxin genotypes of *Fusarium graminearum sensu stricto* and *Fusarium meridionale* in wheat from southern Brazil." *Plant Pathology* 58 (2): 344-351. doi:10.1111/j.1365-3059.2008.01949.x.

Shen, Cheng-mei, Ying-chun Hu, Hai-yan Sun, Wei Li, Jian-hua Guo, and Huai-gu Chen. 2012. "Geographic Distribution of Trichothecene Chemotypes of the *Fusarium graminearum* Species Complex in Major Winter Wheat Production Areas of China." *Plant Disease* 96 (8): 1172-1178. doi:10.1094/PDIS-11-11-0974-RE.

Starkey, David E., Todd J. Ward, Takayuki Aoki, Liane R. Gale, H. Corby Kistler, David M. Geiser, Haruhisa Suga, Beáta Tóth, János Varga, and Kerry O'Donnell. 2007.

"Global molecular surveillance reveals novel Fusarium head blight species and trichothecene toxin diversity." *Fungal Genetics and Biology* 44 (11): 1191-1204. doi:10.1016/j.fgb.2007.03.001.

Suga, H., G. W. Karugia, T. Ward, L. R. Gale, K. Tomimura, T. Nakajima, A. Miyasaka, S. Koizumi, K. Kageyama, and M. Hyakumachi. 2008. "Molecular Characterization of the *Fusarium graminearum* Species Complex in Japan." *Phytopathology* 98 (2): 159-166. doi:10.1094/PHYTO-98-2-0159.

Sun, Dong-Lei, Xuan Jiang, Qinglong L. Wu, and Ning-Yi Zhou. 2013. "Intragenomic heterogeneity in 16S rRNA genes causes overestimation of prokaryotic diversity." *Applied and Environmental Microbiology*: 13. doi:10.1128/AEM.01282-13.

Tamura, Koichiro, Masatoshi Nei, and Sudhir Kumar. 2004. "Prospects for inferring very large phylogenies by using the neighbor-joining method." *Proceedings of the National Academy of Sciences of the United States of America* 101 (30): 11030-11035. doi:10.1073/pnas.0404206101.

Umpiérrez-Failache, M., G. Garmendia, S. Pereyra, A. Rodríguez-Haralambides, T. J. Ward, and S. Vero. 2013. "Regional differences in species composition and toxigenic potential among Fusarium head blight isolates from Uruguay indicate a risk of nivalenol contamination in new wheat production areas." *International Journal of Food Microbiology* 166 (1): 135-140. doi:10.1016/j.ijfoodmicro.2013.06.029.

USWBSI. 2015. "FHB in 2015: A Vexing Year, Overall." Accessed November 11, 2016, http://scabusa.org/pdfs/USWBSI-Article_2015FHB-Summary_10-26-15.pdf.

USWBSI. 2007. "Fusarium Focus." Accessed November 11, 2016, http://scabusa.org/pdfs/fus-focus_10-07_newsletter.pdf.

USWBSI. 2008. "Fusarium Focus." Accessed November 11, 2016, http://scabusa.org/pdfs/fus-focus_10-08_newsletter.pdf.

Vesgueiro, Fabiana Tavares, Bruna Demari-Silva, Rosely dos Santos Malafronte, Maria Anice Mureb Sallum, and Mauro Toledo Marrelli. 2011. "Intragenomic variation in the second internal transcribed spacer of the ribosomal DNA of species of the genera *Culex* and *Lutzia* (Diptera: Culicidae)." *Memorias do Instituto Oswaldo Cruz* 106 (1): 1-8.

Vogler, A. P. and R. DeSalle. 1994. "Evolution and phylogenetic information content of the ITS-1 region in the tiger beetle *Cicindela dorsalis*." *Molecular Biology and Evolution* 11 (3): 393-405.

Vydryakova, Galina A., Dao T. Van, Parivash Shoukouhi, Nadezhda V. Psurtseva, and John Bissett. 2012. "Intergenomic and intragenomic ITS sequence heterogeneity in

Neonothopanus nambi (Agaricales) from Vietnam." *Mycology* 3 (2): 89-99. doi:10.1080/21501203.2011.637085.

Wang, D. -M and Y. -J Yao. 2005. "Intrastrain internal transcribed spacer heterogeneity in *Ganoderma* species." *Canadian Journal of Microbiology* 51 (2): 113-121. doi:10.1139/w04-118.

Ward, Todd J., Randall M. Clear, Alejandro P. Rooney, Kerry O'Donnell, Don Gaba, Susan Patrick, David E. Starkey, Jeannie Gilbert, David M. Geiser, and Tom W. Nowicki. 2008. "An adaptive evolutionary shift in Fusarium head blight pathogen populations is driving the rapid spread of more toxigenic *Fusarium graminearum* in North America." *Fungal Genetics and Biology* 45 (4): 473-484. doi:10.1016/j.fgb.2007.10.003.

Wegulo, Stephen N. 2012. "Factors Influencing Deoxynivalenol Accumulation in Small Grain Cereals." *Toxins* 4 (11): 1157-1180. doi:10.3390/toxins4111157.

Wei, Xiao-Xin, Xiao-Quan Wang, and De-Yuan Hong. 2003. "Marked Intragenomic Heterogeneity and Geographical Differentiation of nrDNA ITS in *Larixpotaninii* (Pinaceae)." *Journal of Molecular Evolution* 57 (6): 623-635. doi:10.1007/s00239-003-2512-8.

White, T. J., T. D. Bruns, S. B. Lee, and J. W. Taylor. 1990. "Amplification and direct sequencing of fungal ribosomal RNA Genes for phylogenetics." In *PCR Protocols: A Guide to Methods and Applications*, edited by M. A. Innis, D. H. Gelfans, J. J. Sninsky and T. J. White, 315-322. San Diego, CA: Academic Press.

Woo, Patrick C. Y., Shui-Yee Leung, Kelvin K. W. To, Jasper F. W. Chan, Antonio H. Y. Ngan, Vincent C. C. Cheng, Susanna K. P. Lau, and Kwok-Yung Yuen. 2010. "Internal Transcribed Spacer Region Sequence Heterogeneity in *Rhizopus microsporus*: Implications for Molecular Diagnosis in Clinical Microbiology Laboratories." *Journal of Clinical Microbiology* 48 (1): 208-214. doi:10.1128/JCM.01750-09.

Wörheide, Gert, Scott A. Nichols, and Julia Goldberg. 2004. "Intragenomic variation of the rDNA internal transcribed spacers in sponges (Phylum Porifera): implications for phylogenetic studies." *Molecular Phylogenetics and Evolution* 33 (3): 816-830. doi:10.1016/j.ympev.2004.07.005.

Yörük, Emre and Gülruh Albayrak. 2012. "Chemotyping of *Fusarium graminearum* and *F. culmorum* Isolates from Turkey by PCR Assay." *Mycopathologia* 173 (1): 53-61. doi:10.1007/s11046-011-9462-2.

Zhang, H., B. Brankovics, W. Luo, J. Xu, J. s. Xu, C. Guo, J. g. Guo, et al. 2016. "Crops are a main driver for species diversity and the toxigenic potential of *Fusarium*

isolates in maize ears in China." *World Mycotoxin Journal* 9 (5): 701-715. doi:10.3920/WMJ2015.2004.

Chapter 3

TRICHOTHECENE 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.

Primer	Sequence (5'-to-3')	Reference
3CON	TGGCAAAGACTGGTTCAC	Starkey et al. (2007)
3D3A	CGCATTGGCTAACACATG	Starkey et al. (2007)
3D15A	ACTGACCCAAGCTGCCATC	Starkey et al. (2007)
3NA	GTGCACAGAATATACGAGC	Starkey et al. (2007)
12CON	CATGAGCATGGTGATGTC	Starkey et al. (2007)
12-3F	CTTTGGCAAGCCCGTGCA	Starkey et al. (2007)
12-15F	TACAGCGGTCGCAACTTC	Starkey et al. (2007)
12NF	TCTCCTCGTTGTATCTGG	Starkey et al. (2007)

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 NH_4NO_3, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 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 (dH_2O) 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 dH_2O. 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 5×10^5 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 (5×10^5 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*.

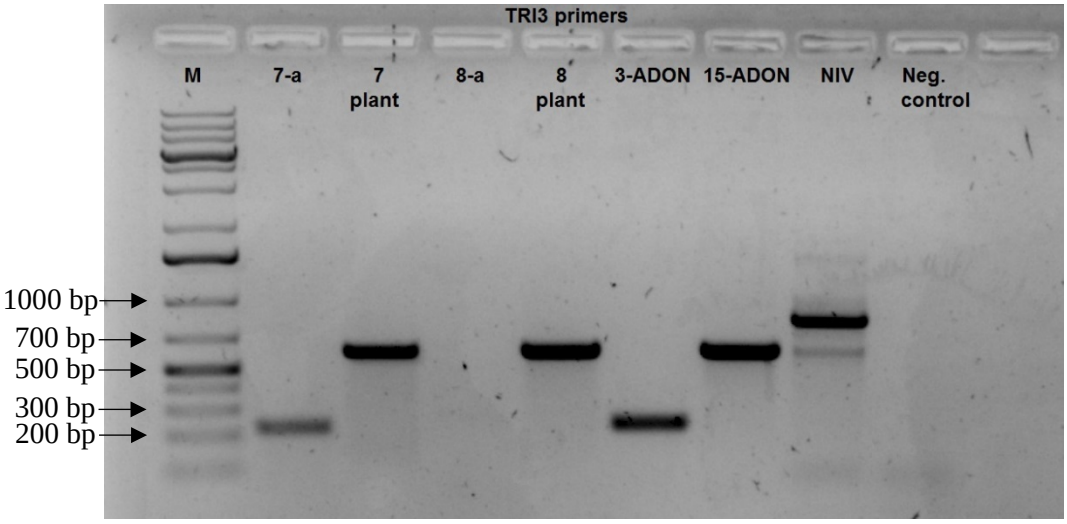

Figure 3.1: Amplification products produced by multiplex PCR with the TRI3 primers for single-spore isolates 7-a and 8-a from 2016, as well as for kernel-extracted DNA from samples 7 and 8. Lane M, molecular weight marker (1 kb).

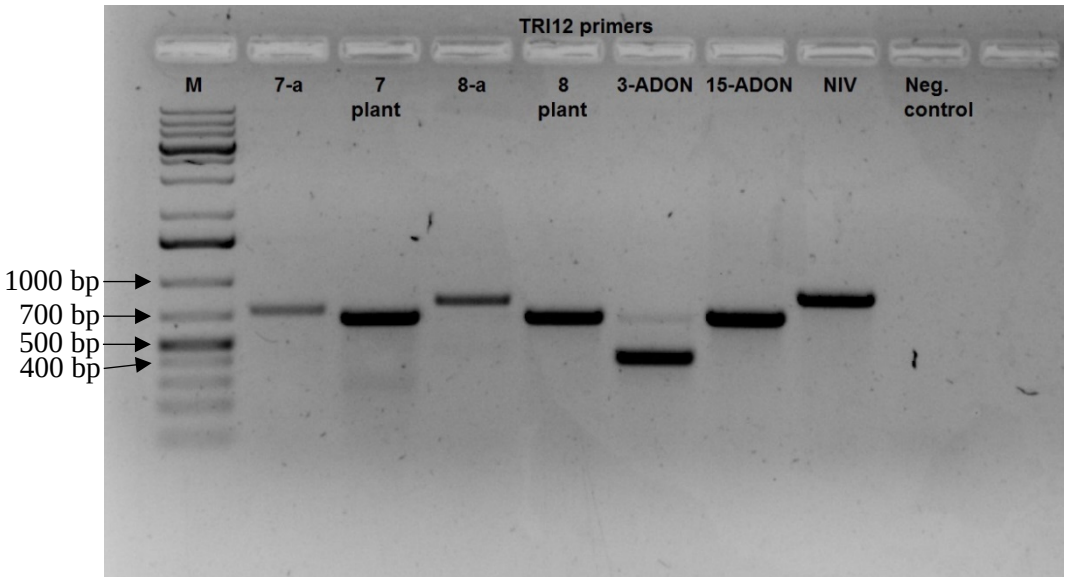

Figure 3.2: Amplification products produced by multiplex PCR with the TRI12 primers for single-spore isolates 7-a and 8-a from 2016, as well as for kernel-extracted DNA from samples 7 and 8. Lane M, molecular weight marker (1 kb).

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.

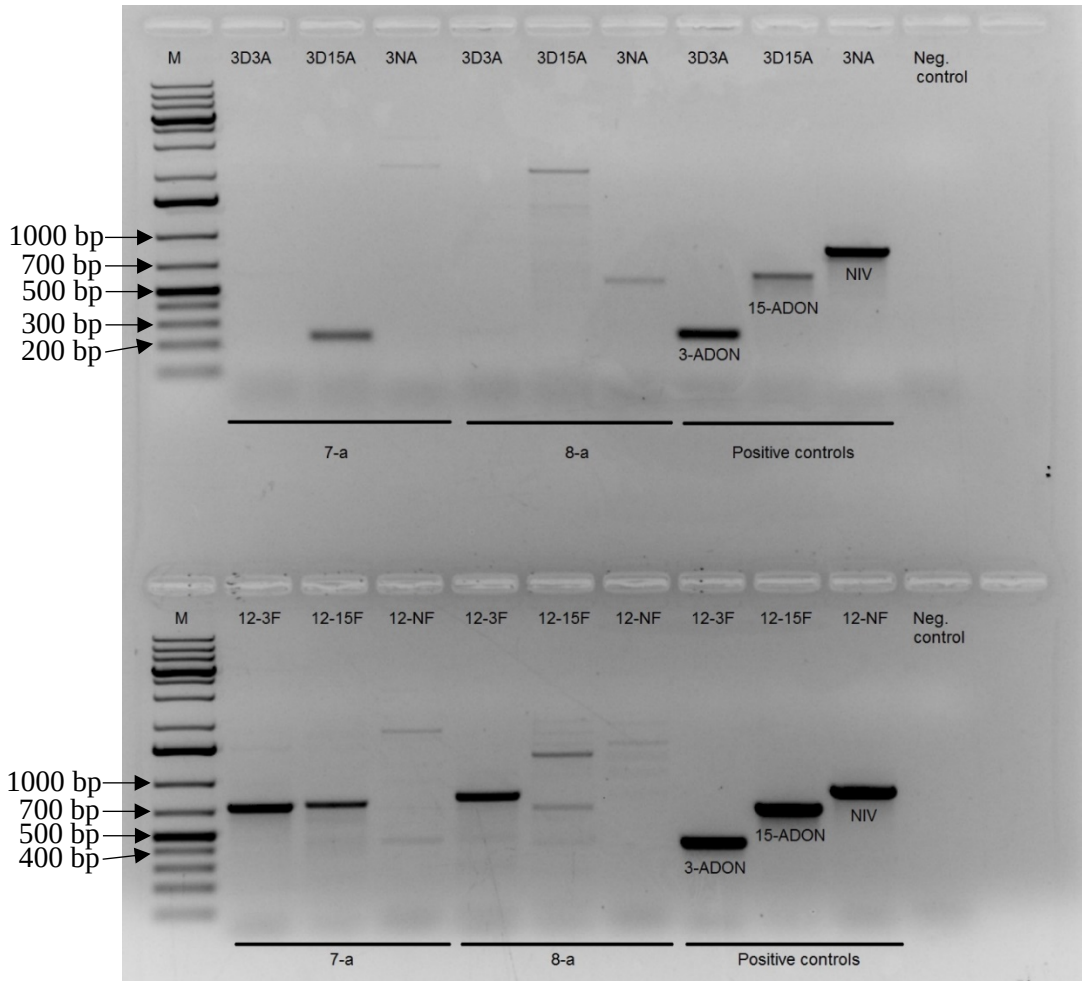

Figure 3.3: Agarose gel showing fragments amplified with the individual trichothecene genotyping primer pairs for isolates 7-a and 8-a from 2016. Lane M, molecular weight marker (1 kb).

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).

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.

Sample group	Trichothecene genotype			
	3-ADON	**15-ADON**	**NIV**	**Inconclusive**
2015 Nebraska single-spore isolates	-	23/23	-	-
2015 DNA extracted directly from kernels	-	23/23	-	-
2016 Nebraska single-spore isolates	-	25/27	-	2/27
2016 DNA extracted directly from kernels	-	10/10	-	-
Bayer Crop Science isolates	9/97	66/97	-	22/97

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.

	Mycotoxin (ppm)			
Sample	**DON**	**3-ADON**	**15-ADON**	**NIV**
1-c	18.71 ± 4.89	-	0.29 ± 0.10	-
7-a	-	-	-	-
8-a	-	-	-	-
Non-inoculated	-	-	-	-

-: 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

Alexander, Nancy J., Robert H. Proctor, and Susan P. McCormick. 2009. "Genes, gene clusters, and biosynthesis of trichothecenes and fumonisins in *Fusarium*." *Toxin Reviews* 28 (2-3): 198-215. doi:10.1080/15569540903092142.

Aoki, Takayuki, Todd J. Ward, H. Corby Kistler, and Kerry O'donnell. 2012. "Systematics, Phylogeny and Trichothecene Mycotoxin Potential of Fusarium Head Blight Cereal Pathogens." *JSM Mycotoxins* 62 (2): 91-102. doi:10.2520/myco.62.91.

Bianchini, Andreia, Richard Horsley, Maia M. Jack, Brent Kobielush, Dojin Ryu, Sheryl Tittlemier, William W. Wilson, et al. 2015. "DON Occurrence in Grains: A North American Perspective." *Cereal Foods World* 60 (1): 32-56. doi:10.1094/CFW-60-1-0032.

Ceron-Bustamante, M., V. Ayala-Escobar, C. Nava-Diaz, T. J. Ward, G. Leyva-Mir, and H. E. Villasenor-Mir. 2016. "First Report of *Fusarium meridionale* Causing Fusarium Head Blight of Wheat in Mexico." *Plant Disease* 100 (8): 1790. doi:10.1094/PDIS-11-15-1315-PDN.

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.

Foroud, Nora A. and François Eudes. 2009. "Trichothecenes in Cereal Grains." *International Journal of Molecular Sciences* 10 (1): 147-173. doi:10.3390/ijms10010147.

Gale, L. R., T. J. Ward, V. Balmas, and H. C. Kistler. 2007. "Population Subdivision of *Fusarium graminearum* Sensu Stricto in the Upper Midwestern United States." *Phytopathology* 97 (11): 1434-1439. doi:10.1094/PHYTO-97-11-1434.

Gale, Liane Rosewich, Stephen A. Harrison, Todd J. Ward, Kerry O'Donnell, Eugene A. Milus, Samuel W. Gale, and H. Corby Kistler. 2011. "Nivalenol-Type Populations of *Fusarium graminearum* and *F. asiaticum* Are Prevalent on Wheat in Southern Louisiana." *Phytopathology* 101 (1): 124-134. doi:10.1094/PHYTO-03-10-0067.

Goswami, Rubella S. and H. Corby Kistler. 2004. "Heading for disaster: *Fusarium graminearum* on cereal crops." *Molecular Plant Pathology* 5 (6): 515-525. doi:10.1111/j.1364-3703.2004.00252.x.

Gräfenhan, Tom, Peter R. Johnston, Martha M. Vaughan, Susan P. McCormick, Robert H. Proctor, Mark Busman, Todd J. Ward, and Kerry O'Donnell. 2016. "*Fusarium*

praegraminearum sp. nov., a novel nivalenol mycotoxin-producing pathogen from New Zealand can induce head blight on wheat." *Mycologia* 108 (6): 1229-1239. doi:10.3852/16-110.

Hallen-Adams, Heather E., Brad L. Cavinder, and Frances Trail. 2011. "*Fusarium graminearum* from Expression Analysis to Functional Assays." Chap. 6, In *Fungal Genomics: Methods and Protocols*, edited by Jin-Rong Xu and Burton H. Bluhm. Vol. 722, 79-101. New York: Humana Press.

Liang, J. M., H. Xayamongkhon, K. Broz, Y. Dong, S. P. McCormick, S. Abramova, T. J. Ward, Z. H. Ma, and H. C. Kistler. 2014. "Temporal dynamics and population genetic structure of *Fusarium graminearum* in the upper Midwestern United States." *Fungal Genetics and Biology* 73: 83-92. doi:10.1016/j.fgb.2014.10.002.

Pan, Dinorah, Natalia Calero, Ana Mionetto, and Lina Bettucci. 2013. "Trichothecene genotypes of *Fusarium graminearum* from wheat in Uruguay." *International Journal of Food Microbiology* 162 (1): 120-123. doi:10.1016/j.ijfoodmicro.2013.01.002.

Panthi, Anita, Heather Hallen-Adams, Stephen N. Wegulo, John Hernandez Nopsa, and P. Stephen Baenziger. 2014. "Chemotype and aggressiveness of isolates of *Fusarium graminearum* causing head blight of wheat in Nebraska." *Canadian Journal of Plant Pathology* 36 (4): 447-455. doi:10.1080/07060661.2014.964775.

Pasquali, Matias and Quirico Migheli. 2014. "Genetic approaches to chemotype determination in type B-trichothecene producing Fusaria." *International Journal of Food Microbiology* 189: 164-182. doi:10.1016/j.ijfoodmicro.2014.08.011.

Pestka, James J. and Alexa T. Smolinski. 2005. "Deoxynivalenol: toxicology and potential effects on humans." *Journal of Toxicology and Environmental Health. Part B, Critical Reviews* 8 (1): 39-69. doi:10.1080/10937400590889458.

Rotter, B. A., D. B. Prelusky, and J. J. Pestka. 1996. "Toxicology of deoxynivalenol (vomitoxin)." *Journal of Toxicology and Environmental Health* 48 (1): 1-34. doi:10.1080/713851046.

Schmale, D. G., S. D. Ross, T. L. Fetters, P. Tallapragada, A. K. Wood-Jones, and B. Dingus. 2012. "Isolates of *Fusarium graminearum* collected 40–320 meters above ground level cause Fusarium head blight in wheat and produce trichothecene mycotoxins." *Aerobiologia* 28 (1): 1-11. doi:10.1007/s10453-011-9206-2.

Schmale, D. G., A. K. Wood-Jones, C. Cowger, G. C. Bergstrom, and C. Arellano. 2011. "Trichothecene genotypes of *Gibberella zeae* from winter wheat fields in the eastern USA." *Plant Pathology* 60 (5): 909-917. doi:10.1111/j.1365-3059.2011.02443.x.

Starkey, David E., Todd J. Ward, Takayuki Aoki, Liane R. Gale, H. Corby Kistler, David M. Geiser, Haruhisa Suga, Beáta Tóth, János Varga, and Kerry O'Donnell. 2007. "Global molecular surveillance reveals novel Fusarium head blight species and trichothecene toxin diversity." *Fungal Genetics and Biology* 44 (11): 1191-1204. doi:10.1016/j.fgb.2007.03.001.

von der Ohe, Christiane, Victoria Gauthier, Lily Tamburic-Ilincic, Anita Brule-Babel, W. G. Dilantha Fernando, Randy Clear, Todd J. Ward, and Thomas Miedaner. 2010. "A comparison of aggressiveness and deoxynivalenol production between Canadian *Fusarium graminearum* isolates with 3-acetyl and 15-acetyldeoxynivalenol chemotypes in field-grown spring wheat." *European Journal of Plant Pathology* 127 (3): 407-417. doi:10.1007/s10658-010-9607-z.

Ward, Todd J., Randall M. Clear, Alejandro P. Rooney, Kerry O'Donnell, Don Gaba, Susan Patrick, David E. Starkey, Jeannie Gilbert, David M. Geiser, and Tom W. Nowicki. 2008. "An adaptive evolutionary shift in Fusarium head blight pathogen populations is driving the rapid spread of more toxigenic *Fusarium graminearum* in North America." *Fungal Genetics and Biology* 45 (4): 473-484. doi:10.1016/j.fgb.2007.10.003.

Wegulo, Stephen N. 2012. "Factors Influencing Deoxynivalenol Accumulation in Small Grain Cereals." *Toxins* 4 (11): 1157-1180. doi:10.3390/toxins4111157.