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Characterization of Chemotype and Aggressiveness of Nebraska Isolates of *Fusarium graminearum*

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CHARACTERIZATION OF CHEMOTYPE AND AGGRESSIVENESS OF
NEBRASKA ISOLATES OF *FUSARIUM GRAMINEARUM*

by

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Under the Supervision of Professors Stephen Wegulo and Heather Hallen-Adams

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CHARACTERIZATION OF CHEMOTYPE AND AGGRESSIVENESS OF NEBRASKA ISOLATES OF *FUSARIUM GRAMINEARUM*

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Fusarium head blight (FHB), caused mainly by *Fusarium graminearum*, is a devastating disease of wheat and other small grain cereals. FHB lowers grain yield and quality and contaminates grain with mycotoxins, predominantly deoxynivalenol (DON) and its acetylated derivatives 3-ADON and 15-ADON. Forty one *Fusarium* isolates collected from grain elevators and wheat fields in Nebraska in 2009 and 2010 were sequenced for molecular identification. Forty isolates were identified as *F. graminearum* and one isolate was identified as *F. culmorum*. Seventy seven *F. graminearum* isolates collected from grain elevators and wheat fields in Nebraska from 2007 to 2010 were tested for DON production *in vitro*. All isolates produced DON in variable amounts. A multiplexed PCR assay was carried out to identify the chemotype of the 77 isolates. All 51 isolates that were amplified belonged to the 15-ADON chemotype. Sixteen selected isolates varied widely in mycelial characteristics, and DON and spore production *in vitro*. Mycelia were sparse in some isolates and dense in others. Mycelial color ranged from white to yellow to pale orange. Pigments formed by the isolates ranged in color from dark red to bright red to yellow. DON and spore concentrations ranged from low to high. Under greenhouse conditions, eight selected isolates - four of which produced DON at high levels and...
four at low levels *in vitro* - differed significantly (*P* ≤ 0.05) in aggressiveness on spikes and DON production in grain of FHB-susceptible spring wheat cultivar Wheaton. High DON producers (*in vitro* and in grain) were more aggressive than low-DON producers. DON concentration *in vitro* accurately predicted aggressiveness on wheat spikes and DON production in grain. It is concluded that in Nebraska, i) *F. graminearum* is the major cause of FHB, ii) the 15-ADON chemotype of the pathogen predominates, iii) variation exists among isolates of the pathogen in DON production, in vitro cultural characteristics, and aggressiveness on wheat spikes, and iv) DON production *in vitro* is an accurate prediction of DON production in *planta* and of aggressiveness.
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CHAPTER I

INTRODUCTION

Fusarium head blight (FHB), also known as scab, is a destructive disease of wheat (*Triticum aestivum* L.) and other small grain crops. The disease, caused by several *Fusarium* species, is an important fungal disease of cereals especially in areas with warm and humid weather (28). FHB has emerged as a disease of economic importance affecting the production of wheat and barley worldwide (13). The disease has occurred sporadically in the United States and has been documented since the 19th century. Several recent epidemics have been reported, especially since the early 1990s (13, 53). The major *Fusarium* species causing scab in the U.S. is *Fusarium graminearum* Scwabe 1839 (teleomorph: *Gibberella zeae* (Schwein.) Petch 1936) (36, 58). Other species causing FHB worldwide are *F. culmorum* (W.G. Smith) Sacc. 1895, *F. poae* (Peck) Wollenw. 1914, *F. avenaceum* (Fr.) Sacc. 1886, *Microdochium nivale* (Fr.) Samuels & I.C. Hallett 1983, and *F. pseudograminearum* O’Donnell & T. Aoki, 1999 (36, 48). The disease is very important as it can affect both crop quantity and quality. FHB causes yield loss either by causing sterility in wheat spikes or by causing the production of light weight scabby seeds commonly referred as *Fusarium*-damaged kernels (FDK). In addition, it causes crop quality loss due to the contamination of grains by mycotoxins. These mycotoxins include deoxynivalenol (DON), nivalenol (NIV), T-2 toxin, and zearalenone (11, 15, 36). In North America, the predominant mycotoxin is DON (9, 11, 15). Mycotoxin-contaminated grains can
pose risks to human and animal health (9, 11). These mycotoxin-contaminated grains are usually discarded by the cereal industry thus causing huge economic losses.

1. HISTORY OF FHB EPIDEMICS IN U.S.A.

FHB occurs sporadically. Historically, several epidemics of the disease have occurred worldwide. In the U.S., the disease has become a persistent problem since the 19th century, especially for wheat and barley growers. Fusarium head blight was first described as wheat scab by W.G. Smith in 1884. He identified *Fusarium culmorum* as a causal agent of wheat scab (45). Later, Attanasoff (3) used the term “Fusarium head blight” for this disease. In the U.S., FHB was first reported in 1890 in the state of Ohio. During the early 1890s, FHB was observed in many states of the eastern and mid-western wheat growing regions including Delaware, Iowa, Indiana, Pennsylvania and Nebraska. In 1917 an outbreak of FHB occurred in the U.S., mainly in Ohio, Indiana, and Illinois, causing losses of over 10.6 million bushels (288,000 metric tons) (3). One of the most severe epidemics occurred in 1919 and caused a loss of 80 million bushels (2.18 million metric tons) of wheat. FHB outbreaks continued during the 1920s in the U.S. During these years, wheat fields in Minnesota and Missouri were affected seriously by FHB. In 1928, both wheat and barley were affected by FHB epidemics in Indiana causing 15-20% losses (12). The barley crop was affected again in 1932 in locations in North and South Dakota, Minnesota, and Iowa. FHB continued to occur in North America in the 1940s, 1950s, 1960s and 1970s. Major economic losses occurred again in the 1980s. One hundred million
bushels (2.72 million metric tons) of wheat were lost in 1982 alone (6, 24). FHB epidemics in the 1990s were very important in the history of FHB in the U.S. According to McMullen et al. (28) scab epidemics in 1993 caused huge losses in the tri-state area of Minnesota, North Dakota and South Dakota, and in the Canadian prairie province of Manitoba. Losses were estimated to be $1 billion. In 1996, scab occurred in almost all states of the U.S. where wheat and barley were grown. Illinois, Indiana, Michigan and Ohio were the most affected. In Ohio, the direct and indirect losses from FHB were estimated to be $100 million in 1996 (45). Johnson et al. (23) estimated that FHB caused direct losses in wheat and barley totaling more than $1.3 billion in the U.S. during the period from 1991 to 1997 (45).

2. CAUSAL ORGANISM

FHB in wheat and barley is caused by several Fusarium species (3, 28, 36, 45). Worldwide, F. graminearum, F. culmorum, F. avenaceum are major Fusarium species causing FHB in wheat and barley. The geographical distribution of these species is temperature dependent (36). It has been reported that F. graminearum tends to be more common in warmer and more humid regions and F. culmorum and F. avenaceum predominate in cooler regions of the world (31, 36). F. graminearum is the prominent causal agent of FHB in the United States, Canada, China, Australia, and central Europe. In past, F. graminearum was thought to comprise of single panmictic species spanning all over the world. However, recent study of worldwide collection of F. graminearum isolates has resulted division of original species to 16
phylogenetically distinct species based on genealogical concordance/discordance phylogenetic species recognition (GCPSR) analysis. These species are grouped under a complex known as *Fusarium graminearum* species complex (FGSC). These 16 species include *F. graminearum sensu stricto*, *F. gerlachii*, *F. louisianens*, *F. asiaticum*, *F. ussurianum*, *F. nepalense*, *F. vorosii*, *F. acaciae-mearnsii*, *Fusarium sp.*, *F. aethiopicum*, *F. boothii*, *F. mesoamericanum*, *F. austroamericanum*, *F. cortaderiae*, *F. brasilicum*, and *F. meridionale*. In the surveys conducted worldwide to date, *F. graminearum sensu stricto* (*F. graminearum s.s.*) is cosmopolitan in distribution and has been found in Asia, Africa, America and Europe while another species, *F. asiaticum*, is widespread in Asia including China, Japan and Korea (2).

3. SYMPTOMS

A distinctive symptom of Fusarium head blight is premature bleaching of one or more spikelets or the entire immature wheat head. Bleaching can start from any point on the head and progress until the entire head is whitened (12, 13, 53, 54). Prolonged wet and warm weather induces the fungus to produce salmon orange to pink spore masses on the infected area which are a diagnostic feature of head blight of wheat. Later in the growing season, sexual fruiting structures in the form of small black perithecia may appear on spikelets (12, 13). The infected spikelets usually become sterile or contain shriveled, undersized and chalky white or pink kernels commonly referred to as tombstones or *Fusarium*-damaged kernels (FDK). In
addition to causing FDK, the fungus also synthesizes substantial amounts of mycotoxins which accumulate in kernels (48, 53).

4. LIFECYCLE OF *Fusarium graminearum*

*Fusarium graminearum* overwinters as saprophytic mycelia or chlamydomospores on infected host residue and soil. With warm, wet and humid weather in spring, the fungus produces sexual fruiting structures called perithecia on these residues which serve as the primary source of inoculum. As temperatures warm up, perithecia release ascospores and these ascospores are carried by air currents or splashing water to wheat heads. Warm wet weather facilitates spore germination and spike tissue invasion. Infection can occur on glumes, flower parts and any other parts of the head (13, 54). Wheat heads are most susceptible at the anthesis or flowering stage. The extruding anthers are considered the primary site of infection by ascospores. If the fungus colonizes the wheat flower before grain development, sterile flowers will result and no grain will develop. However, if the fungus attacks later during grain development, then shrunken shriveled tombstone kernels will develop (13, 28, 54). Sometimes grain may appear normal but still contain significant amounts of DON. In many plant diseases, secondary infection may result from asexual spores called conidia. However, this type of infection is very limited in the case of FHB. Hence, FHB appears to be largely a monocyclic disease (13, 54). FHB symptoms may develop within 3 to 4 days after infection. If infected grain is used as seed for next season’s crop, seedling blights can occur (13, 53, 54).
5. TYPES OF RESISTANCE

Mesterhazy (29) proposed 5 types of resistance to FHB: Type I: resistance to initial infection (44), Type II: resistance to spread of the pathogen through the spike from the point of initial infection (37), Type III: resistance to kernel infection (29), Type IV: tolerance (44), Type V: resistance to mycotoxin accumulation. Type I resistance is assessed mostly by spray inoculation and Type II resistance by single floret point inoculation (44). In greenhouse experiments, Type II resistance is mostly quantified because it is less influenced by environmental factors and the greenhouse provides the best controlled environment (5).

6. MYCOTOXINS AND FOOD SAFETY

FHB is of great concern as a plant disease due to the fact that the pathogens not only damage kernels and cause yield loss but also contaminate grains with mycotoxins. Several Fusarium species are able to produce mycotoxins in grains, primarily type B trichothecenes (11, 36). Trichothecenes are sesquiterpenoid mycotoxins commonly found as food or feed contaminants and consumption of these mycotoxins can result in serious health issues. Trichothecene mycotoxins have been implicated in pathogen aggressiveness (10, 15, 20, 38). More than 200 trichothecenes have been reported to date and they are divided into four groups: types A, B, C and D, according to their characteristic functional groups at the C-8 position (9, 11, 49). Type A and type B are more common in cereals than type C and type D
trichothecenes. Type A trichothecenes include T-2 toxin and HT-2 toxin, both of which have a hydrogen, a hydroxyl, or an ester group at the C-8 position. F. sporotrichioides and F. poae are some of the major Fusarium species known to produce Type A trichothecenes. Type B toxins are represented by deoxynivalenol, nivalenol and fusarenone X, which have a keto group at C-8. Deoxynivalenol and nivalenol are most frequently associated with FHB. F. graminearum and F. culmorum are two major Fusarium species producing DON and NIV mycotoxins in wheat, barley and other small cereal grains (9, 11, 15, 19, 20). Trichothecene profiles known as chemotypes are used to analyze pathogen populations in any geographical region. There are three main strain-specific profiles of trichothecene metabolites (chemotypes) known to be present in Fusarium species associated with FHB (32, 35). Isolates with the nivalenol chemomtype produce nivalenol and its acetylated derivative 4-acetyl nivalenol. Hence, this chemotype is commonly known as the 4A-NIV chemotype. Isolates with the deoxynivalenol chemotype produce deoxynivalenol and either of its acetylated derivatives, 3-acetyl DON or 15-acetyl DON. Isolates with the 3-ADON chemotype produce DON and 3-ADON, and isolates with the 15-ADON chemotype produce DON and 15-ADON (32). Both DON and NIV are found in grains which, when ingested in high doses, can cause alimentary hemorrhage and vomiting (7).
7. TRICHOTHECENE EFFECTS ON ANIMALS AND PLANTS

Trichothecene exposure in animals can lead to growth retardation, reduced ovarian function and reproductive disorders, immunocompromise, feed refusal and vomiting (40, 49). Trichothecenes such as T-2 toxin, DON and NIV inhibit eukaryotic protein synthesis by inhibiting peptidyl transferase activity of ribosomes. DON occurs most frequently in cereal grains and is associated with feed refusal and induction of vomiting (14, 20, 40, 49). Trichothecenes affect plants by inhibiting seedling growth. They may induce programmed cell death in plants (40). A very low trichothecene concentration can cause wilting, chlorosis and necrosis in plants. Generally, NIV is considered more toxic than DON in humans and animals (41). However, DON is more phytotoxic than NIV (15, 49). Eudes et al. (14) reported that high concentrations of DON and 3-ADON inhibited wheat coleoptile growth and showed that they were more toxic than T-2 toxin, HT-2 toxin, and NIV. These toxins are considered as virulence factors and are implicated in pathogen aggressiveness (6, 14, 15, 27, 38).

8. TRICHOTHECENES AS VIRULENCE FACTORS

Trichothecenes play an important role in fungal pathogenesis as virulence factors. Virulence, also referred as aggressiveness, is the relative ability of a pathogen to colonize and cause damage to plants. Aggressiveness is quantitative and conditioned by several genes. DON is one of the factors influencing aggressiveness
Desjardins et al. (10) found mutants of trichotheccene non-producing strains of *F. graminearum* when inoculated on wheat spikes to result in low disease severity compared to their wild type genotype. Non-trichotheccene producing mutants of *F. graminearum* were created by disrupting the *Tri5* gene which is responsible for encoding the first enzyme in the trichotheccene biosynthesis pathway. Although *Tri5* mutants are less virulent, they are still pathogenic to wheat, rye and corn (6). Similarly, Proctor et al. (38) demonstrated that virulence of *F. graminearum* mutants lacking a functional *Tri5* gene was significantly reduced in wheat seedlings. Bai et al. (6) found that a *Tri 5* deficient strain of *F. graminearum* was unable to colonize wheat heads beyond initial infection. Therefore, it is suggested that DON and other trichotheccenes can cause greater virulence by enabling pathogen spread within a spike of wheat; however, they are not required for initial infection (6, 11). Adams et al. (1) reported DON and 15-ADON are not virulence factor in corn and carnation plant. Hence, trichotheccene are virulence factors in wheat but not necessarily in all crops infected with FHB.

9. PRODUCTION OF DON, NIV AND THEIR ACETYL DERIVATIVES

Trichotheccenes are synthesized through complex biosynthesis pathways and are encoded by *Tri* genes. Most of the *Tri* genes are located in a cluster known as the *Tri* cluster. Production of DON versus NIV is determined by the core *Tri* cluster genes *Tri13* and *Tri7*. Functional *Tri13* and *Tri7* will result in the NIV chemotype whereas nonfunctional *Tri13* and *Tri7* will result one of the DON chemotypes. During
the synthesis of NIV, these genes are responsible for oxygenation and acetylation, oxygen at the C-4 position. The Tri13 and Tri7 genes are nonfunctional in DON-producing strains because of multiple insertions and deletions in the genes’ coding regions and, consequently, the C-4 hydroxyl group is absent from DON (25). DON producers are further divided into 3-ADON and 15-ADON producers due to the presence of the acetyl group at the C-3 and C-15 positions, respectively. 3-ADON and 15-ADON also lack the C-4 hydroxyl group. The production of 3-ADON and 15-ADON depends on the Tri8 gene from the Tri cluster which, in 3-ADON strains, catalyzes deacetylation of 3, 15-diacetyldeoxynivalenol at carbon 15 to yield 3-ADON. Similarly, in 15-ADON strains, Tri8 catalyzes deacetylation of 3, 15-diacetyldeoxynivalenol at carbon 3 to yield 15-ADON (4).

10. REGULATORY STANDARDS FOR DON

DON is found in naturally infected grains worldwide. Due to the harmful effects of DON and other mycotoxins in humans and animals, many countries have set regulatory limits for mycotoxins in food and feed. The U.S. Food and Drug Administration (FDA) has established advisory levels for DON-contaminated grain and grain-based products as 1 ppm in finished wheat products for human consumption, 10 ppm for grain and grain byproducts destined for ruminating beef and feedlot cattle older than 4 months and for chickens, 5 ppm in grain and grain by-products destined for swine, and 5 ppm in grain and grain by-products destined for all other animals (50).
11. DON DETECTION METHODS

There are several methods available for DON detection. These methods can be divided into two categories, those that are destructive to the grain samples and those that are nondestructive. Major destructive methods include thin layer chromatography (TLC) (57), high performance liquid chromatography (HPLC) and gas chromatography (GC) (43, 57) and enzyme linked immunosorbent assays (ELISA) (26). Thin layer chromatography, high performance liquid chromatography, gas chromatography are conventional analytical methods for DON detection which are used for multi mycotoxins analysis. The immunological methods, ELISA are used for rapid screening and quantifying DON (42). The nondestructive methods are the electronic nose (22), near infrared spectroscopy (NRS) (37).

12. CHEMOTYPE DISTRIBUTIONS AND POPULATION BIOLOGY

*Fusarium* isolates causing FHB tend to produce different trichotheccene profiles (chemotypes). Some strains of *Fusarium* produce DON and 3-ADON, some produce DON and 15-ADON, while others produce NIV and its acetylated derivative (32). Some studies reported that chemotype diversity depends on geographical distribution. Both DON and NIV chemotypes are reported from several countries in Asia, Africa, Europe, and South and North America. Previous studies reported the DON chemotype as the major chemotype present in North America while the NIV chemotype was not detected. The NIV chemotype was most frequently isolated from
some Asian and European counties (34, 35, 58). DON and its derivatives are produced mostly by *F. graminearum* and *F. culmorum*, whereas NIV is produced by *F. asiaticum*, *F. cerealis* and *F. crookwellense* (33, 47, 55).

All three chemotypes may be present in the same geographical location; however, only one is predominant. For example, in North America, 15-ADON is the predominant chemotype (17). In Europe, much variability was observed. For example, in Northwestern Russia and Finland, 3-ADON was the major chemotype; however, in southern Russia and Germany, 15-ADON was most frequently isolated (55). In China, all three chemotypes are present and chemotype occurrence seems to be temperature dependent (60). Zhang et al. (60) reported that *F. graminearum* produces 15-ADON as a major chemotype and the majority of this chemotype occurs in cooler regions of China, whereas *F. asiaticum* yields predominantly 3-ADON along with 15-ADON. In contrast, the NIV chemotype occurred in warmer regions. Similar results were reported from Japan, where *F. asiaticum* strains produced NIV, 3-ADON and 15-ADON. NIV was not detected from *F. graminearum* strains, either in China or Japan (47). Yoruk et al. (56) reported *F. graminearum* and *F. culmorum* as the two major species causing FHB in Turkey, where all except one *F. graminearum* isolate produced 15-ADON. The one different chemotype was NIV, produced by *F. graminearum*; and *F. culmorum* produced 3-ADON.

Zeller et al. (58, 59) reported significant levels of sexual recombination to occur within populations of an *F. graminearum* clade and found low genetic differentiation among them. However, several recent studies indicated genetically differentiated populations of *F. graminearum* emerged in Canada and North America. For example, Gale et al. (17) reported co-existence of the 3-ADON and 15-ADON chemotypes in
Minnesota and North Dakota. Starkey et al. (46) identified six *F. graminearum* isolates with the NIV chemotype, which had not been reported before in North America. Gale et al. (18) reported an *F. asiaticum* population for the first time in and identified the presence of the NIV chemotype in 79% of *F. graminearum* isolates in Louisiana State of the United States.

Several studies in Canada and the U.S. revealed that the 3-ADON chemotype of *F. graminearum* is now widely prevalent and there has been a significant population shift of the FHB pathogen population towards 3-ADON producers (21, 51). Puri et al. (39) found a 15 fold increase of 3-ADON isolates in North Dakota. They found the 3-ADON chemotype to be more aggressive both in terms of disease severity and DON production. Between 1998 and 2004, the frequency of 3-ADON producers in western Canada increased more than 14-fold. In addition, 3-ADON isolates produced more trichothecenes and grew more rapidly. This result suggested that 3-ADON chemotype has a selective advantage over the 15-ADON chemotype which is more toxigenic in nature (52). Foroud et al. (16) named newly emerged 15-ADON for 15-ADON chemotypes that share genetic similarities with 3-ADON and found newly emerged 15-ADON and 3-ADON isolates are more aggressive in terms of FDK production and trichothecene accumulation in wheat than traditional 15-ADON and NIV chemotypes. This leads to the conclusion that 3-ADON is the most aggressive chemotype among all.
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CHAPTER II

MOLECULAR IDENTIFICATION OF SPECIES AND CHEMOTYPING OF NEBRASKA ISOLATES CAUSING FUSARИУМ HEAD BLIGHT DISEASE OF WHEAT

1. INTRODUCTION

Fusarium head blight (FHB) is a devastating disease that can affect all small grain crops including wheat and barley. The disease is caused by several Fusarium species as well as Microdochium nivale. The most common Fusarium species are Fusarium graminearum, F. culmorum, F. avenaceum and F. poae. 1

The geographical distribution of these Fusarium species depends on their temperature requirements. In warmer regions including the United States, Canada, Australia, and central Europe, F. graminearum is the most important causal agent of FHB, whereas in cooler regions of northwestern Europe, F. culmorum predominates. F. avenaceum has been isolated from diseased grain ears over a range of climatic zones. This species is usually predominant in the colder areas of northern Europe and Canada but is also of high prevalence in central Europe. 3,2,4 In England, F. poae was most frequently isolated when fields were surveyed in 1989 and 1990 by Polly et al. 5 Similarly, F. pseudograminearum caused severe FHB epidemics in Australia. 2
Several studies have revealed that *F. graminearum* and *F. culmorum* were the most aggressive among these species. Some studies report *F. avenaceum* as equally pathogenic as *F. culmorum* and *F. graminearum*. 2

FHB is favored by hot and humid weather with an extended period of moisture at the flowering stage of the crop. FHB has caused multibillion dollar losses over the past two decades worldwide. 6 Losses are realized in the form of direct yield and quality loss due to contamination of grains by B-trichothecene mycotoxins as well as price discounts associated with toxin contaminated grains. Trichothecene-contaminated grains pose a serious threat to food safety as these toxins are associated with toxicosis in humans and animals. 7, 8 Many *Fusarium* species are known to produce B-trichothecenes while causing FHB and are members of B-trichothecene lineage. In the past, before genealogical concordance/discardance phylogenetic species recognition (GCPSR) was used to analyze FHB populations, the primary causal agent of FHB was thought to comprise a single panmictic species, *F. graminearum*. GCPSR analysis has since been used to split into 16 phylogenetically distinct species now known as the *Fusarium graminearum* species complex (FGSC). 9 *F. graminearum* and *F. asiaticum* are the two most important species in the FGSC known to cause FHB based on surveys of FHB genetic diversity throughout the world. Like the name suggests, *F. asiaticum* is largely responsible for FHB in Asian countries such as China, Japan and Korea, whereas *F. graminearum* is distributed globally including North America, South America, Europe and Australia. In North America, *F. graminearum* accounts for close to 100% of FHB, with the exception of some recent findings of existence of *Fusarium* species such as *F. asiaticum* in Pennsylvania and Louisiana, *F. boothii* in Texas and *F. mesoamericanum* in
Pennsylvania. Although, these are found at low levels, the recent identification of these species is very interesting regarding population diversity in North America. 

Species within the FGSC are known to produce B-trichothecene mycotoxins. These mycotoxins include deoxynivalenol (DON) and its acetylated derivatives (3-acetyl deoxynivalenol (3-ADON) and 15-ADON), nivalenol (NIV) and its acetylated derivative (4-ANIV; fusarenone X). FHB-causing fungi can be classed into three different chemotypes based on trichothecene profiles: 3-ADON, 15-ADON (both of which also produce DON), and NIV. Whether a strain possesses a DON versus a NIV chemotype depends upon the trichothecene cluster genes Tri13 and Tri7. These genes are required for the NIV chemotype, where they are responsible for oxygenation and acetylation, respectively, of the C-4 carbon. The Tri13 and Tri7 genes possess multiple insertion and deletions in the coding region and are thus are nonfunctional in DON producers. While only a slight difference exists in the pattern of hydroxylation or acetylation between DON and NIV, their toxicity and activity differ greatly with reference to the health of both humans and animals. Generally, NIV is considered a more toxic than DON in humans and animals. However, NIV is less phytotoxic than DON in plants.

It has been reported that phylogenetic analysis does not correlate to the type of trichothecene production, as genes traditionally used for species recognition and trichothecene cluster genes produce different phylograms. Hence, knowledge of the species prevalent in any area is at best an imperfect predictor of chemotype. For example, F. louisiannaense belong to only NIV-chemotype and F. asiaticum predominantly of NIV chemotype while F. graminearum have all 3-ADON or 15-ADON or NIV chemotype. In North America, 15-ADON is the predominant
chemotype detected from *Fusarium* isolates infecting wheat and barley. However, recent studies suggested a shift towards the 3-ADON chemotype in the mid-western States such as North Dakota and western Canada. 3-ADON is considered more aggressive both in terms of disease severity and trichothecene production than either the 15-ADON or the NIV chemotype. 13, 14

Molecular methods such as DNA sequencing and species-specific PCR are used for correct identification of species. Molecular methods are preferable over morphological identification, as they are fast and reliable. *Fusarium* species produce different sizes and shapes of conidia. Some *Fusarium* species produce both micro- and macroconidia, while some *Fusarium* species such as *F. graminearum* and *F. culmorum* produce only macroconidia. Morphological identification of *Fusarium* is further complicated by the importance of spore morphology, which is to a great extent influenced by cultural and environmental factors. Morphological identification alone cannot provide positive identification. Additionally, microscopic observation can be stressful and tedious. 15 Hence, molecular identification of *Fusarium* species is preferred over morphological diagnosis. For chemotyping of the isolates, various methods are available. 15 Starkey et al. 2007 16 used multiplex PCR to distinguish 3-ADON, 15-ADON and NIV chemotypes, using primers previously validated by Ward et al. 2002. 17 The technique described by Starkey and colleagues is simple, straightforward, and rapid, and allows large numbers of strains to be screened.

The pathogenicity, aggressiveness, distribution, chemotypes and mycotoxin production all vary among *Fusarium* species causing FHB. 18 The accurate identification of species and their chemotypes is essential to characterize any specific population of FHB-causing *Fusarium* species.
In the central Great Plains, FHB has occurred sporadically due to variable climate and has been documented since the 19th century. Nebraska is one of the major wheat-producing states in the U.S. In Nebraska, FHB has occurred many times in history with major recent epidemics in 2007 and 2008. We might assume that Nebraska FHB is caused by *F. graminearum* as this species is predominant in North America; however, this has not been verified in Nebraska. In addition, there are some recent studies which showed presence of *Fusarium* species other than *F. graminearum* such as *F. asistantum*, *F. gerlachii* and *F. louisianenense* elsewhere in the U.S. It has been suggested that knowledge of *Fusarium* chemotype distribution may help in forecasting schemes for disease development and mycotoxin contamination on a regional basis. Knowing the major *Fusarium* species present in any location will help breeders to deploy resistant cultivars. Similarly, the geographical distribution of the trichothecene chemotypes is important in predicting the potential impact of these pathogens on food safety. As there is little information available on FHB in Nebraska both in terms of *Fusarium* species identification and mycotoxin/chemotype profile, the objectives of this study were to identify the major *Fusarium* species involved in FHB and to determine their trichothecene chemotypes using molecular methods.
2. MATERIALS AND METHODS

2.1. Single spore isolation.

Samples of wheat grains were collected from elevators and fields in Nebraska in 2009 and 2010. *Fusarium* damaged kernels (FDK) were selected and surface sterilized using 1% sodium hypochlorite for 1 minute, rinsed with double distilled water, disinfected with 70% ethyl alcohol followed by another rinse with double distilled water. The disinfected kernels were placed on Nash & Snyder peptone PCNB medium (NS) in Petri plates with two FDK per plate. The plates were incubated at 25°C in 12 h light and 12 h dark for 5 to 7 days in a Precision Incubator, Model 818, (Thermo Fisher Scientific, Waltham, MA). A 10 mm diameter mycelial plug from the actively growing edge of the fungus was transferred to potato dextrose agar (PDA) plates. After one week of fungal growth, 7 ml of sterilized double distilled water was placed on each plate and all the aerial mycelia were scraped with cell scraper. One ml of this mycelia-water mixed suspension was placed into an Eppendorf tube and serial dilution was carried out. For serial dilution, 100 µl suspension was used and dilutions were made with the ratio of 1:10, 1:100, 1:1000, and 1:10000. Three hundred µl from each dilution was spread and incubated on PDA for 12-48 h under the same incubation conditions as described above. A single spore colony was picked and transferred into a new PDA plate. After 7 days of growth, single spore cultures were stored in 15% glycerol suspension and kept at -80°C until needed. A total of 41 pure isolates were prepared: 21 from wheat samples collected in year 2009 (NE-166 to NE-190) and 20 from samples collected in 2010 (NE-191 to NE-210). A total of 77
isolates were used for chemotyping including an additional 36 isolates collected from year 2007 and 2008 and provided by John Nopsa, which were already identified as *F. graminearum*.

### 2.2. Genomic DNA extraction.

Pure isolates were grown on PDA for one week. A 10 mm mycelial plug from actively growing mycelia was placed in a test tube containing 4 ml Yeast Extract Sucrose (YES) medium and grown for 2 days at room temperature at 200 rpm. Fungal mycelia were harvested and lyophilized and DNA extraction was carried out using a CTAB extraction procedure described by O’Donnell et al. as follows. Approximately 15 µg of mycelia were placed in a 1.5 ml microfuge tube and the tube was chilled at -80°C to break the cells, followed by grinding with a pellet pestle. After that, 500 µl CTAB was added and mycelia were macerated continuously until they were dispersed thoroughly in the solution. An additional 500 µl CTAB and 1 µl 2-mercaptoethanol were added to the previous suspension followed by incubation at 65°C for 20 min. Three hundred fifty µl of tris-saturated phenol and 150 µl chloroform:isoamyl alcohol (24:1) were added and vortexed. Samples were centrifuged at 14,000 rpm for 5 min. The upper (aqueous) layer was transferred to a new tube and 300 µl phenol and 100 µl chloroform:isoamyl alcohol were added and vortexed. The samples were then centrifuged at a higher speed (14,000 rpm) for 5 min. The upper aqueous layer was transferred to a new tube, followed by the addition of 1 ml ice-cold 100% ethanol. The samples were vortexed and centrifuged again, and ethanol was poured off from the tube. The remaining pellet at the base of the tube was
washed with 1 ml ice-cold 70% ethanol and again centrifuged for 5 min. Alcohol
from the tube was poured off and the pellet was dried by inverting the tubes and
incubating it at room temperature. After drying the pellet, 50 µl of molecular biology
grade water was added and the DNA was stored at -20°C.

2.3. Molecular identification of *Fusarium* species causing Fusarium head blight
disease.

2.3.1. Primers and Polymerase Chain Reaction.

To amplify the Internal Transcribed Spacer (ITS) region of the fungus, two
oligonucleotide fungal primers as described by White et al. were used. The ITS
region primers ITS 1 (5’-TCC GTA GGT GAA CCT GCG G- 3’) and ITS 4 (5’-TCC
TCC GCT TAT TGA TAT G-3’) were used to amplify the ITS 1 and 2 regions and
the intervening 5.8S gene. The PCR mixture consisted of 1µl forward primer (ITS
1F), 1 µl reverse primer (ITS4), 1µl fungal DNA sample, 12.5 µl Red Taq ReadyMix
(Sigma-Aldrich, St. Louis, MO) and 9.5 µl of molecular biology grade water for each
sample. PCR water was used instead of DNA in one reaction as a negative control.
PCR was performed on a Mastercycler Pro (Eppendorf) and the PCR program
consisted of an initial denaturing step for 5 min at 95°C, followed by 30 cycles of
denaturation at 95°C for 45 sec, annealing at 57°C for 45 sec and extension at 72°C
for 2 min, with a final step at 72°C for 10 min.
2.3.2. Determination of DNA yield and quality.

After PCR amplification, DNA samples were first run on 0.7% agarose gels in
0.5 M Tris acetate EDTA (TAE) buffer (pH 8) at room temperature at a constant
voltage of 80 V for 60 minutes to determine the quality. The PCR products were
quantified based on their staining intensity.

2.3.3. Sequencing of ITS region.

DNA sequencing was performed by the Michigan State University Research
Technology Support Facility, using dye-terminated capillary electrophoresis on an
ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA). The ITS
sequences were BLASTed for comparison to NCBI GeneBank database

2.3.4. F. culmorum specific primers.

There were 6 isolates whose ITS sequences failed to yield an exact match with
a known F. graminearum and had a major hit as F. culmorum. These isolates were
further identified using F. culmorum-specific primers. The primers used for F.
culmorum identification were OPT18 F (5’-GATGCCAGAC-3’) and OPT18 R (5’-
GATGCCAGACGCACCTAAGAT-3’). PCR was performed as above.
2.4. Chemotyping of *Fusarium* isolates.

Multiplex PCR was conducted to determine the chemotype of pure *Fusarium* isolates. Two alternative sets of multiplexing primers were available, based on *Tri3* and *Tri12* sequences. The *Tri3* multiplexing primers included 3CON (5′-TGGCAAGACTGGTTCAC-3′), 3NA (5′-GTGCACAGAATATACGAGC-3′), 3D15A (5′-ACTGACCCAAGCTGCCATC-3′), and 3D3A (5′-CGCATTGGCTAACACATG-3′). This reaction produced amplicons of approximately 840-, 610-, and 243-bp for isolates that had NIV, 15ADON, and 3ADON chemotypes, respectively. The primers used in the *Tri12* multiplexing included 12CON (5′-CATGAGCATGGTGATGTC-3′), 12NF (5′-TCTCCTCGTTGTATCTGG-3′), 12-15F (5′-TACAGCGGTCGCAACTTC-3′), and 12-3F (5′-CTTTGGCAAGCCCGTGCA-3′). This reaction produced amplicons of approximately 840-bp, 670-bp, and 410-bp with isolates that had NIV, 15ADON, and 3ADON chemotypes, respectively. We used the *Tri3* multiplex PCR version consisting of 0.5µl of 3CON, 0.5µl of 3D3A, 0.5µl of 3D15A, 0.5µl of 3NA, 12.5µl of RedTaq (Sigma-Aldrich, St. Louis, MO) and 8.5µl of PCR water. PCR was performed on a Mastercycler Pro (Eppendorf) and the PCR program consisted of an initial denaturing step for 5 min at 95°C, followed by 30 cycles of denaturation at 95°C for 45 sec, annealing at 57°C for 45 sec and extension at 72°C for 2 min, with a final step at 72°C for 10 min. The resulting PCR products were run on 0.7% agarose gels in TAE buffer at room temperature at a constant voltage of 80 V for 90 minutes. For negative control, PCR water was used. The PCR products were visualized under UV light.
3. RESULTS

3.1. Species identification.

A total of 41 *Fusarium* isolates were used for identification. Using universal fungal primers (ITS1F and ITS4), PCR products of 550-570 bp were generated from all pure isolates. This confirmed the quality of DNA which was further used for sequencing. All 41 sequences “passed” the ABI quality qualification and we concluded that all sequences obtained were of good quality (See appendix I and appendix II). Use of BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) confirmed most of the isolates as *Gibberella zeae* (*F. gramineaum*), along with some for which the closely related *F. culmorum* was also detected in the BLAST search with slightly less sequence similarity. All the isolates had *Gibberella zeae* as the top hit except isolates NE 189, NE199, NE198, NE200, NE206, and NE209, for which *F. culmorum* was the top hit, while *Gibberella zeae* showed slightly less sequence similarity (See appendix III). Of these 6 isolates, NE198 was confirmed as *F. culmorum* using *F. culmorum* specific primers, while the others failed to amplify with these primers, indicating that they did not represent *F. culmorum*.

Figure 1: DNA band amplified at 472 bp using *F. culmorum* specific primers showing NE198 *Fusarium* isolate amplified as *F. culmorum*, and M as 100 bp DNA ladder.
3.2. Chemotyping of fungal isolates.

The PCR assay based on the Tri3 gene will produce amplicons of approximately 243 bp, 610 bp and 840 bp corresponding with 3-ADON, 15-ADON and NIV chemotypes, respectively.\textsuperscript{16, 17} Results showed 51 isolates were amplified at 610 bp indicating they did of the 15-ADON chemotype (Fig. 2). The results were approximate as DNA bands amplified between 650 bp and 500 bp are considered as 15-ADON with amplicon size of 610 bp. The remaining 26 isolates did not amplify. We tried to amplify all the isolates more than five or six times, however, only 51 isolates were amplified. The reason for no amplification was not clear as the DNA used for multiplex PCR was of good quality. Good quality DNA was already determined from ITS amplification results.

Thus, only 15-ADON producing isolates were identified among collected Nebraska isolates. The 3-ADON and NIV chemotype were not detected among these isolates.
Figure 2: Amplification products of 840bp, 610 bp, 243 bp produced by multiplex PCR using 3CON, 3D3A, 3D15A and 3NA corresponding to NIV, 15-ADON and 3-ADON chemotype. Figure A, B, C, D corresponds to all 51 isolates amplified at 610 bp representing 15-ADON chemotype. Lane M represents a 100 bp ladder and lanes S1, S2 and S3 represents Standard 3-ADON chemotype, Standard 15-ADON chemotype and Standard NIV chemotype. Lanes 1 to 51 represents 51 F. graminearum isolates from year 2007-2010 and lane N as negative control water.
4. DISCUSSION

Accurate identification of species followed by correct management procedures is essential for a successful disease management program. In our study, *Fusarium* species causing Fusarium head blight disease were identified using molecular techniques. ITS sequencing results revealed *F. graminearum* as the primary causal agent of FHB in Nebraska from 2009 and 2010-collected isolates. Our lab previously sequenced 40 Nebraska isolates from 2007 and 2008 and found all isolates to be *F. graminearum*. This finding was consistent with other findings for North America, which accounts for close to 100% FHB disease in U.S. In our study, surprisingly, one isolate was identified as *F. culmorum*. Usually, *F. graminearum* is more important than *F. culmorum* as it is more common cause of head blight disease worldwide. *F. culmorum* is most frequently associated with cereal root rot and head blight found particularly in cooler parts of Europe. *F. culmorum* is also a problem in the Great Plains region of the United States including Nebraska, causing crown rot disease in winter wheat. Thus, while our results confirm *F. graminearum* as the dominant FHB species in Nebraska, other species cannot be ruled out, and multiple FHB organisms may co-occur. Recent findings on population diversity in the U.S. revealed the presence of minor *Fusarium* species as causal agents of FHB such as *F. gerlachii* in the northern and *F. lousianenese* in the southern parts of the U.S. Gale et al. isolated *F. asiaticum* for the first time in the U.S. from fields of Louisiana State.
We used ITS1 and ITS4 to amplify the ITS region of the fungal genome. The ITS region is commonly sequenced in fungi because there is sufficient variation to yield identification to the species level in many instances, and the datasets are sufficiently large to allow the use of ITS to identify unknowns. However, ITS is not always sufficient for distinguishing very closely related species, and therefore we complemented the ITS sequencing with the *F. culmorum*-specific primer in cases in which there was any doubt about the sequence. Unfortunately we do not have exact locale data for the *F. culmorum* isolate, as these isolates were prepared from scabby grain samples collected from Nebraska fields and grain elevators.

To differentiate mycotoxin chemotypes of Nebraska *F. graminearum* isolates, *Tri3* multiplex PCR was used. Our study used 77 isolates collected from 2007 to 2010 for chemotyping and only 51 isolates amplified. The result placed all 51 isolates in the 15-ADON chemotype. No NIV or 3-ADON chemotype was detected from isolates collected in Nebraska. As 3-ADON is more phytotoxic and NIV is more toxigenic for humans and animals, it seems 15-ADON is a little safer among these chemotypes. Like many states of North America, 15-ADON is predominant in Nebraska. The lack of detectable NIV-producing isolates in Nebraska isolates was expected, as this chemotype is not common in the U.S. However, Gale et al. reported presence of NIV chemotype in Southern Louisiana from isolates of *F. graminearum* collected in year 2001-2007. In addition, they detected presence of *F. asiaticum* species with NIV chemotype in Louisiana State. Both of these findings were unique in North America, as NIV chemotype and *F. asiaticum* species were not reported before.

The relationship between the presence of *Fusarium* species and the associated mycotoxin is interesting. This is due to differences in tendency to produce differing
chemicals by these species thus belonging to different chemotypes. For example, *F. asiaticum* produces all three chemotypes, predominantly the NIV chemotype; *F. gerlachii*, *F. louisianense* and *F. meridionale* predominantly produce only the NIV chemotype; and *F. boothii*, *F. nepalense*, *F. aethiopicum* and *F. vorosii* predominantly produce only the 15-ADON chemotype. Similarly, *F. graminearum* belong to all three chemotypes, predominantly 15-ADON chemotype is major chemotype present in North America. However, recent studies suggest replacement of the 15-ADON chemotype by the 3-ADON chemotype in some states of North Dakota. Research findings from Canada also showed 3-ADON was displacing the previously predominant 15-ADON chemotype in western Canada. The newly predominant 3-ADON chemotype is considered to be more aggressive and more toxic than the (15-ADON and NIVchemotypes). In addition, the difference in chemotype distribution may result in the shift in toxin accumulation in grains. These findings suggest the necessity for regular monitoring of *Fusarium* populations in any location.

Contamination of grains by mycotoxins is of great concern, especially for the food industry. Mycotoxin-contaminated barley is rejected by the brewing industry and DON-contaminated wheat grains get a discounted price due to their effect on the safety and baking quality of food. These mycotoxins have a great impact on the health of animals and humans, due to its cytotoxic activity and immunosuppressive effects. As a consequence, FHB and DON contamination are responsible for serious direct and indirect economic losses.

Since Nebraska is one of the top states producing wheat, it is very important to have knowledge on the population biology of Nebraska *Fusarium* species causing FHB. This is the first time that the FHB and chemotype profile has been characterized in Nebraska. This finding can be of great significance
as the dominant chemotype in the FHB fungal population provides important information about the type of toxin contamination in food.
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CHAPTER III

VARIATION IN FUSARIUM GRAMINEARUM ISOLATES ASSOCIATED WITH AGGRESSIVENESS AND MYCOTOXIN PRODUCTION

1. INTRODUCTION

Fusarium head blight (FHB), also known as scab, is a destructive disease of wheat, barley and other small grain crops. FHB reduces the yield and quality of grain. *Fusarium* species contaminate grain with trichothecene mycotoxins such as deoxynivalenol (DON) and nivalenol (NIV), and estrogenic compounds such as zearalenone (17, 29). FHB is caused by several *Fusarium* species. The major species are *F. graminearum* (teleomorph *Gibberella zeae*), *F. culmorum*, and *F. avenaceum* (4, 5). In the United States, *F. graminearum* is the major species reported to cause head blight of wheat and barley (8, 21, 29). Past studies revealed that this single *Fusarium* species dominates any one location and the local population represents a subset of a larger panmictic population in North America. This is due to lower genetic differentiation and high sexual recombination among *Fusarium* isolates that occur in any area (37, 38). However, genealogical concordance and phylogenetic species recognition approaches were used to delineate this morphospecies and identified at least 16 phylogenetically distinct species groups now referred as the *Fusarium graminearum* species complex (FGSC). Other minor species include *F. gerlachii*, *F. louisianense*, *F. boothii*, *F. mesoamericanum*, *F. meridionale* and *F. asiaticum* which are considered to be distributed regionally (1, 12, 34). *Fusarium* species under the
FGSC produce the B trichothecenes DON and NIV and their acetylated derivatives 3-acetyl deoxynivalenol (3-ADON), 15-acetyl deoxynivalenol (15-ADON) and 4-acetyl nivalenol (4-ANIV) (6, 10, 28). FHB causes mycotoxin accumulation in cereal grains. These mycotoxins are harmful to humans and animals if consumed in high doses (30, 31).

Many studies have reported a positive correlation between FHB severity and trichothecene production (13, 19, 32). Disease development and toxin accumulation are influenced by several environmental factors such as rainfall, relative humidity and temperature (8, 29). Desjardins et al. (7) found mutants of trichothecene non-producing strains of *F. graminearum* resulted in low disease severity on wheat spikes compared to their wild type counterparts. Non-trichothecene producing mutants of *F. graminearum* were created by disrupting the *Tri5* gene which is responsible for encoding the first enzyme in the trichothecene biosynthesis pathway (32). Proctor et al. (32) demonstrated that virulence of *F. graminearum* mutants lacking a functional *Tri5* gene was significantly reduced in wheat seedlings. Bai et al. (2) found that a *Tri5* deficient strain of *F. graminearum* was unable to colonize wheat heads beyond initial infection. Therefore, it is suggested that DON and other trichothecenes can cause greater virulence by enabling pathogen spread within a spike; however, they are not required for initial infection (2, 20).

Several studies reported variation in aggressiveness among *F. graminearum* isolates sampled from various parts of the world (3, 26), within a country or state, and even within populations from individual fields (24). Aggressiveness of *F. graminearum* is not geographically structured since isolates with low, medium, and high levels of aggressiveness make up the population in a single location. Large
genetic variation in terms of aggressiveness also exists among these isolates collected from different continents (25). Fernando et al. (9) reported that diversity among isolates was more closely related with aggressiveness and toxin production than with geographic location or type of the host. The high level of genotypic diversity is consistent among isolates from different countries and continents displaying a unique haplotype (11, 26). Most studies revealed a high level of genetic diversity in F. graminearum within individual field populations or populations sampled across a definite geographical scale. Knowledge of isolate characteristics in terms of aggressiveness and mycotoxin production in any location is necessary for predicting the pathogenic potential of FHB pathogens and for the deployment of resistance in a given location.

Information is lacking on the variation of aggressiveness among Nebraska isolates of F. graminearum. Such information can be used to estimate the potential loss from FHB and its associated mycotoxins in growing seasons during which environmental conditions favor the development of FHB epidemics. Hence, the purpose of this work was to determine the degree to which F. graminearum isolates collected from Nebraska wheat fields and grain elevators differed in aggressiveness and mycotoxin production.
2. METHODS

2.1. Preliminary screening of isolates for DON production.

To induce trichothecene production in liquid culture, a two stage medium was used based on the protocol of Miller and Blackwell (27). In a preliminary experiment, 77 *F. graminearum* isolates collected from wheat fields and grain elevators in Nebraska from 2007 to 2010 were screened for in vitro DON production. The isolates were grown on potato dextrose agar (PDA) for 7-10 days. A 1 cm square mycelial plug was cut from the outer, actively growing region of the PDA plate of each isolate and used to inoculate 50 ml of first stage medium (3 g NH₄Cl, 2 g MgSO₄·7 H₂O, 0.2 g of FeSO₄·7H₂O, 2 g KH₂PO₄, 2 g peptone, 2 g yeast extract, 2 g malt extract, and 20 g glucose in 1 L distilled water), in 125-ml Erlenmeyer flasks. The cultures were grown at 28 °C on a rotary shaker at 220 rpm in the dark for 3 days. The mycelial culture with a small amount of liquid medium was ground using a mortar and pestle. A 3.5 ml concentrated suspension of ground mycelia was used to inoculate 50 ml of second stage medium consisting of 1g (NH₄)₂HPO₄, 3 g KH₂PO₄, 0.2 gMgSO₄·7H₂O, 5 g NaCl, 40 g sucrose, and 10 g glycerol in 1 L of distilled water in 125 ml Erlenmeyer flasks, grown under the same conditions as the first stage medium for 8 days. After 8 days, the culture was harvested by filtration through a 1 mm Whatman filter paper with a Buchner funnel and lyophilized for 2 days. The dried mycelium was ground using a mortar and pestle. The finely ground powder of fungal mycelium was used for DON quantification.
2.1.1. Competitive direct enzyme linked immunosorbent assay (CD-ELISA).

DON was quantified as follows: Each sample was weighed and dissolved in distilled water at the rate of 1 ml to 100 mg and vortexed for 5 minutes. Each sample was filtered using a 1 mm Whatman qualitative filter paper. The filtered solution was used for DON quantification using a competitive direct enzyme linked immunosorbent assay for deoxynivalenol (Veratox DON 5/5, cat. no. 8331, Neogen Corporation, Lansing, MI) following manufacturer’s instructions. Briefly, 100 µl conjugate was added to 100 µl either of DON control (0, 0.5, 1, 2 and 6 ppm) or of sample, and mixed by pipetting. One hundred µl of this conjugate-sample mixture was added to an antibody coated well, mixed by gently shaking for 1 min, and incubated 5 min at room temperature. Liquid was discarded and wells were rinsed 5 times with deionized water. One hundred µl of substrate was added to each antibody coated well and incubated 5 minute at room temperature. One hundred µl stop solution was added to each well and absorbance was read using a Neogen microwell reader with a 650 nm filter.

2.2. In vitro DON production.

An in vitro experiment was carried out to evaluate DON production of 16 selected isolates, four isolates from each year’s (2007-2010) collection. The isolates were selected based on high and low DON production in the trichothecene screening experiment described above. Hence, two of each year’s isolates were high DON producers and two were low DON producers. The isolates were NE90, NE99, NE102, NE110, NE121, NE157, NE160, NE161, NE167, NE168, NE170, NE172, NE196,
The experiment was run in triplicate using the same methodology as described above for the DON production screening experiment.

2.3. Culture appearance and spore production.

The 16 isolates previously used for DON production \textit{in vitro} were further evaluated for morphological characteristics and spore production on PDA in 9-cm-diameter Petri plates (20 ml of PDA per plate). A ten-millimeter-diameter mycelial plug from an actively growing edge of each isolate was transferred to a PDA plate. The plates were incubated at 25ºC in a 12 h light/dark cycle. Treatments (isolates) were arranged in a randomized complete block design with three replications. After 20 days, the plates were examined for differences in culture appearance. Then, 5 ml of sterile distilled water was poured on each plate and spores were dislodged with an L-shaped cell spreader. The spore and mycelia suspension was filtered through four layers of cheese cloth. Spore concentration in the filtered suspension was determined with a haemacytometer. The experiment was conducted twice.

2.4. Aggressiveness on wheat spikes.

A greenhouse experiment was conducted to evaluate the aggressiveness of eight selected \textit{F. graminearum} isolates. The isolates were a subset of the 16 isolates described above: one high DON producer and one low DON producer from each of the four years’ (2007-2010) isolate collections. The isolates were NE90, NE110, NE121, NE157, NE168, NE172, NE201 and NE210.
2.4.1. Plant material.

Seed of FHB-susceptible spring wheat cultivar “Wheaton” was planted in 15-cm-diameter pots, four seeds per pot. The potting mix consisted of 33% clay loam soil, 33% peat soil, 16.5% sand, and 16.5% vermiculite. Plants were maintained on a greenhouse bench. Temperature in the greenhouse ranged between 21°C minimum (nighttime) and 27°C maximum (daytime). The lighting regimen was 14-h light and 10-h dark. Plants were watered twice daily, in the morning and in the evening, and were fertilized with a water soluble 20-10-20, N-P-K fertilizer (Peter's Professional Peat-Lite special) (Everris, Marysville, Ohio) at 250 ppm five days a week.

2.4.2. Growth of fungal cultures and inoculum preparation.

Each isolate was grown on PDA in 9-cm-diameter Petri plates for 3 weeks in a low temperature illuminated incubator set at 25°C and a 12 h light/dark cycle. Five ml of dd water was poured onto each plate and conidia were dislodged from the surface of the agar with a plastic L-shaped cell spreader and filtered through 2 layers of sterile cheesecloth. Spores were quantified and adjusted to the final concentration of $1 \times 10^4$ spores/ml and kept in a 50 ml Falcon tube at 4°C until needed for inoculation.

2.4.5. Inoculation.

Inoculation was done within 6 h of inoculum preparation. Wheat spikes were inoculated at anthesis with spores of each isolate using the single-floret injection method (41). The central floret of individual spikes was inoculated with 10 μl of
inoculum using a micropipette and 10-12 spikes per pot (experimental unit) were inoculated. Inoculated spikes were covered with a transparent plastic bag for 72 h to maintain high humidity and tagged with colored tape for identification during harvesting. Treatments were the eight *F. graminearum* isolates and were arranged in a randomized complete block design with three replications. Disease severity (percent of symptomatic spikelets) was visually assessed on each spike 5, 7, 10, 14, and 21 days after inoculation. Spikes were hand-harvested when grain moisture content dropped below 15%. The experiment was conducted twice. Aggressiveness was quantified as FHB severity and area under the disease progress curve (AUDPC) on spikes. AUDPC was calculated using the formula:

\[
\text{AUDPC} = \sum_{i=1}^{n-1} [(0.5)(Y_i + Y_{i+1})](t_{i+1} - t_i)
\]

Where \(Y_i\) is disease severity at the \(i\)th assessment, \(t_i\) is the time (days) since inoculation at the \(i\)th assessment and \(n\) is the number of assessment times.

2.4.6. DON measurement in grain.

The harvested spikes were threshed and the grain was ground to a fine powder. DON was quantified using competitive direct ELISA as described above.
2.5. Data analysis.

The general linear models (GLM) procedure of SAS version 9.1 (SAS Institute, Inc., Cary, NC) was used to analyze data. In the experiment to determine DON production by 16 isolates grown in vitro, each of the three runs of the experiment was considered a replication. A combined analysis of spore concentration data from the two replicate experiments was done based on homogeneity of error variance determined from the F-test (15). The least significant difference test at $P = 0.05$ was used to compare pairs of treatment means (15). Linear regression analysis (15) was used to evaluate the relationships between i) disease variables (severity and AUDPC) and DON concentration in grain in the aggressiveness experiments, ii) AUDPC and DON concentration in vitro, iii) AUDPC and spore concentration determined in vitro, and iv) DON concentration in grain and DON concentration in vitro for the eight *F. graminearum* isolates used in the aggressiveness experiments.

3. RESULTS

3.1. Preliminary screening of *F. graminearum* isolates for DON production.

All seventy seven isolates produced DON, but in variable amounts (see Appendix IV), ranging from 0.1 ppm to 23.8 ppm. Based on this variation in DON production, isolates were selected for further experimentation in vitro (comparison of DON production among 16 isolates) and in the greenhouse (evaluation of aggressiveness among eight isolates).
3.2. *In vitro* DON production.

DON concentration determined *in vitro* varied widely among the 16 *F. graminearum* isolates tested, with five isolates producing low amounts, four isolates producing intermediate amounts, and 7 isolates producing high amounts. Average DON concentration ranged from 0.6 ppm for isolate NE210 to 18.8 ppm for isolate NE161 (Fig. 3).

3.3. Culture appearance and spore production.

Visual observation of cultures of 16 selected isolates on PDA revealed variation in the density and color of mycelia. Mycelia were sparse in some isolates and very dense in others. Mycelial color varied from white to yellow to pale orange (Fig. 4). The isolates formed pigments ranging in color from dark red to bright red to yellow (Fig. 5). There was wide variation among the isolates in spore production which ranged from hardly any spores for isolates NE102, NE160, NE167, and NE168 to abundant for isolates NE90 and NE172 (Fig. 6).

3.4. Aggressiveness on wheat spikes.

There were significant differences in disease severity among the eight *F. graminearum* isolates tested for aggressiveness on wheat spikes of cv. Wheaton in the greenhouse. These differences were observed in both experiments and on all the five days on which disease severity was measured. From all isolates, disease severity increased with time up to 100 and 99% in experiments 1 and 2, respectively, for
isolate NE110 (Tables 1 and 2). Similarly, there were significant differences in AUDPC among the isolates. In experiment 1, AUDPC values ranged from 329 percent days for isolate NE90 to 1355 percent days for isolate NE110 (Table 1). In experiment 2, AUDPC values ranged from 136 percent days for isolate NE121 to 1202 percent days for isolate NE110 (Table 2). Based on the results from both experiments, the three most aggressive isolates were NE110, NE168, and NE201 whereas the three least aggressive isolates were NE121, NE90, and NE210 (Tables 1 and 2). In both experiments, DON concentration in grain differed significantly among isolates and was highest for isolates NE110, NE168, and NE201 and lowest for isolates NE121, NE90, and NE210. Therefore, the more aggressive isolates produced higher concentrations of DON whereas the less aggressive isolates produced lower concentrations of DON (Tables 1 and 2).

3.5. Relationship between disease variables and DON concentration in grain.

When DON concentration in grain for the eight isolates tested for aggressiveness on wheat spikes was regressed on disease severity in each of the two experiments, there was a strong, significant linear relationship between the two variables on each day disease severity was measured. Similarly, there was a strong, significant linear relationship between DON and AUDPC (Tables 3 and 4).
3.6. Relationship between DON concentration *in vitro* and AUDPC.

There was a strong, significant linear relationship between AUDPC and DON concentration measured *in vitro* for the eight isolates tested for aggressiveness on wheat spikes. This result was similar in both experiments 1 and 2 (Fig. 7). However, regression of AUDPC on spore concentration determined *in vitro* for the eight isolates did not reveal a significant relationship between the two variables in both experiments (data not shown).

3.7. Relationship between DON concentration *in vitro* and in grain.

When DON concentration in grain was regressed on DON concentration *in vitro* for the eight *F. graminearum* isolates used in the aggressiveness experiments, there was a strong, significant linear relationship between the two variables in both experiments (Fig. 8). Eighty six and 81% of the variation in DON concentration in grain was explained by variation in DON concentration *in vitro* for experiments 1 and 2, respectively.
4. DISCUSSION

Screening of *F. graminearum* isolates for DON production *in vitro* showed a wide variation in the amount of DON produced by the isolates, ranging from 0.1 ppm to 23.8 ppm in the preliminary screen of 77 isolates and from 0.63 ppm to 18.8 ppm in 16 isolates selected for further screening. A similar variation (0.0-11.2 ppm) was observed in DON produced in grain of FHB-susceptible spring wheat cv. Wheaton inoculated on the spikes with eight selected isolates. These results are in agreement with previously published results. Goswami and Kistler (16) found large variation in the ability of 31 strains belonging to the *F. graminearum* species complex to produce DON and other trichothecenes in wheat spikelets or rice florets. This variation in DON production was evident among seven of the 31 *F. graminearum* strains tested. The investigators observed that the variation appeared to be strain-specific rather than species-specific. Other studies (14, 23, 33, 36) have similarly found wide variation in DON production among *F. graminearum* isolates.

The colors (white to orange to yellow) of mycelial cultures of 16 selected isolates of *F. graminearum* grown on PDA were consistent with those reported in the literature (18). The majority of the isolates produced a red pigment as expected (18). However, a yellow pigment was also produced by some of the isolates. It was not determined whether production of the yellow pigment was isolate-specific. It is known that the red pigment produced by *F. graminearum* is pH-sensitive and changes to yellow when the pH drops (18). Sporulation on PDA among the 16 isolates varied widely, with some isolates sporulating poorly and others sporulating abundantly. Tunali et al. (35) similarly found significant within-species differences in spore production among isolates of *F. graminearum, F. culmorum*, and *F.*
pseudograminearum. In this study, macroconidia were the most abundant spore type observed. Isolates identified as abundant spore producers if possess useful features such as virulence and toxin production can be used for inoculum production for greenhouse or field studies involving inoculation of wheat spikes.

Aggressiveness (measured as FHB severity or AUDPC) differed significantly among eight *F. graminearum* isolates inoculated onto wheat spikes of FHB-susceptible cultivar Wheaton in the greenhouse. Differences in aggressiveness among isolates may due to genetic recombination, mutation, or selection. In *F. graminearum*, there is large genetic variation within a given population, even in samples collected from a small area within a field (21). Miedaner et al. (23) analyzed the aggressiveness on young winter rye plants of populations of *F. graminearum* and *F. culmorum* collected from natural field epidemics of FHB and rye foot rot. They found high genotypic variance in the two species and a high degree of diversity of aggressiveness within single field populations of either species.

In this study, the most aggressive isolates on wheat spikes of cv. Wheaton were high DON producers whereas the least aggressive isolates were low DON producers as determined from DON measurement in grain. This was evidenced by the strong, positive linear relationship between disease intensity (severity or AUDPC) and DON with coefficients of determination ($R^2$) ranging from 0.59 to 0.88 in experiment 1 and 0.79 to 0.95 in experiment 2. This observation is consistent with previously published results from other studies. Tunali et al. (35) found a significant, positive relationship (correlation coefficient, $r = 0.71$) between FHB severity and DON in grain when wheat plants were inoculated with seven isolates of *F. graminearum* under greenhouse conditions. Similarly, Mesterhazy (22) reported that
the aggressiveness of *F. graminearum* and *F. culmorum* was correlated with their DON and nivalenol-producing capacity. Desjardins et al. (7) demonstrated in field tests that trichothecene-nonproducing mutants of *F. graminearum* caused less disease on wheat spikes than trichothecene-producing parental and revertant strains.

In this study, DON measured *in vitro* for eight *F. graminearum* isolates was strongly correlated with aggressiveness of the same isolates on wheat spikes of cv. Wheaton in the greenhouse. It was also strongly correlated with DON measured in grain, although the concentration of DON in grain was lower than that *in vitro*. These results indicate that DON measured in vitro can be a reliable predictor of *F. graminearum* isolate aggressiveness as well as DON production in planta. Therefore, *in vitro* measurement of DON, which can be done in a relatively short period, can be used to select *F. graminearum* isolates for use in greenhouse and field experiments.

This study demonstrated that *F. graminearum* isolates differ in DON production *in vitro* and in planta and that high DON producers were more aggressive than low DON producers. The existence of variability in aggressiveness among isolates suggests that breeders should use mixtures of isolates to screen for FHB resistance in wheat lines. Information on the relative levels of aggressiveness and DON production capacity of *F. graminearum* isolates in a given location can be used to select isolates for use in greenhouse and field experiments. In addition, the information can be used by small grain producers to make decisions pertaining to deployment of FHB management strategies.
REFERENCES


33. Puri, K.D., and Zhong, S. 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.


Table 1. Deoxynivalenol (DON) concentration, Fusarium head blight (FHB) severity, and area under the disease progress curve (AUDPC) from greenhouse experiment 1 conducted to compare the aggressiveness, on wheat spikes of FHB-susceptible cultivar Wheaton, of eight selected *Fusarium graminearum* isolates collected from Nebraska grain elevators and winter wheat fields affected by Fusarium head blight in 2007-2010.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>DON(^a)</th>
<th>5d(^b)</th>
<th>7d(^b)</th>
<th>10d(^b)</th>
<th>14d(^b)</th>
<th>21d(^b)</th>
<th>AUDPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE201</td>
<td>9.30(^a)</td>
<td>11.97(^c)</td>
<td>50.96(^a)</td>
<td>61.20(^b)</td>
<td>79.48(^{abc})</td>
<td>97.62(^a)</td>
<td>1162.40(^a)</td>
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<td>NE168</td>
<td>9.13(^a)</td>
<td>27.35(^a)</td>
<td>59.96(^a)</td>
<td>75.96(^{ab})</td>
<td>84.52(^{ab})</td>
<td>96.22(^a)</td>
<td>1313.10(^a)</td>
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<td>15.30(^b)</td>
<td>60.92(^{a})</td>
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<td>89.67(^a)</td>
<td>100.00(^a)</td>
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<tr>
<td>NE157</td>
<td>6.10(^{ab})</td>
<td>22.34(^{ab})</td>
<td>54.06(^a)</td>
<td>56.92(^{b})</td>
<td>62.39(^{bc})</td>
<td>82.37(^{ab})</td>
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<td>8.31(^{cd})</td>
<td>40.13(^{ab})</td>
<td>63.24(^{b})</td>
<td>80.8(^{ab})</td>
<td>95.77(^a)</td>
<td>1130.40(^a)</td>
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<td>NE210</td>
<td>3.50(^{bc})</td>
<td>2.51(^d)</td>
<td>16.08(^{c})</td>
<td>34.14(^{c})</td>
<td>55.59(^c)</td>
<td>83.07(^{ab})</td>
<td>765.00(^{bc})</td>
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<td>NE90</td>
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<td>1.78(^d)</td>
<td>4.31(^c)</td>
<td>10.35(^{d})</td>
<td>14.79(^{d})</td>
<td>55.63(^b)</td>
<td>329.30(^d)</td>
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<tr>
<td>NE121</td>
<td>0.30(^c)</td>
<td>3.83(^{d})</td>
<td>18.84(^{bc})</td>
<td>14.26(^{cd})</td>
<td>24.57(^d)</td>
<td>75.00(^{ab})</td>
<td>508.10(^{cd})</td>
</tr>
</tbody>
</table>

\(^a\)Means followed by the same letter within a column are not significantly different according to the least significant difference test at \(P = 0.05\).

\(^b\)FHB severity measured 5, 7, 10, 14, and 21 days after inoculation.
Table 2. Deoxynivalenol (DON) concentration, Fusarium head blight (FHB) severity, and area under the disease progress curve (AUDPC) from greenhouse experiment 2 conducted to compare the aggressiveness, on wheat spikes of FHB-susceptible cultivar Wheaton, of eight selected *Fusarium graminearum* isolates collected from Nebraska grain elevators and winter wheat fields affected by Fusarium head blight in 2007-2010.

<table>
<thead>
<tr>
<th>Isolate name</th>
<th>DON&lt;sup&gt;a&lt;/sup&gt;</th>
<th>5d&lt;sup&gt;b&lt;/sup&gt;</th>
<th>7d&lt;sup&gt;b&lt;/sup&gt;</th>
<th>10d&lt;sup&gt;b&lt;/sup&gt;</th>
<th>14d&lt;sup&gt;b&lt;/sup&gt;</th>
<th>21d&lt;sup&gt;b&lt;/sup&gt;</th>
<th>AUDPC</th>
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<tr>
<td>NE201</td>
<td>11.20&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>17.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45.85&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>57.42&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>70.44&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>90.56&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1080.60&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>NE168</td>
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<td>46.97&lt;sup&gt;ab&lt;/sup&gt;</td>
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<td>135.70&lt;sup&gt;f&lt;/sup&gt;</td>
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<sup>a</sup>Means followed by the same letter within a column are not significantly different according to the least significant difference test at *P* = 0.05.

<sup>b</sup>FHB severity measured 5, 7, 10, 14, and 21 days after inoculation.
Table 3. Statistics from the regression of deoxynivalenol (DON) concentration measured in grain on Fusarium head blight (FHB) severity and area under the disease progress curve in greenhouse experiment 1 conducted to compare the aggressiveness, on wheat spikes of FHB-susceptible cultivar Wheaton, of eight selected *Fusarium graminearum* isolates collected from wheat fields and grain elevators in Nebraska in 2007-2010.

<table>
<thead>
<tr>
<th>Independent variable</th>
<th>Intercept</th>
<th>Slope</th>
<th>Standard error</th>
<th>R-square</th>
<th>MSE</th>
<th>p-value</th>
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<td>0.82</td>
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<tr>
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<sup>a</sup>FHB severity measured 5, 7, 10, 14, and 21 days after inoculation.

<sup>b</sup>Area under disease progress curve
Table 4. Statistics from the regression of deoxynivalenol (DON) concentration measured in grain on Fusarium head blight (FHB) severity and area under the disease progress curve in greenhouse experiment 2 conducted to compare the aggressiveness, on wheat pikes of FHB-susceptible cultivar Wheaton, of eight selected *Fusarium graminearum* isolates collected from Nebraska grain elevators and winter wheat fields affected by Fusarium head blight in 2007-2010

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<sup>a</sup>FHB severity measured 5, 7, 10, 14, and 21 days after inoculation.

<sup>b</sup>Area under disease progress curve
**Figure 3.** Deoxynivalenol (DON) concentration measured *in vitro* for 16 selected *Fusarium graminearum* isolates collected from wheat fields and grain elevators in Nebraska in 2007-2010. For each isolate, the error bar represents the standard error of the mean.
Figure 4. Appearance of mycelial cultures (front of Petri plate) of 16 selected *Fusarium graminearum* isolates collected from wheat fields and grain elevators in Nebraska in 2007-2010.
Figure 5. Appearance of mycelial cultures (back of Petri plate) of 16 selected *Fusarium graminearum* isolates collected from wheat fields and grain elevators in Nebraska in 2007-2010.
Figure 6. Spore concentration for 16 selected *Fusarium graminearum* isolates collected from wheat fields and grain elevators in Nebraska in 2007-2010. For each isolate, the error bar represents the standard error of the mean.
Figure 7. Regression of area under the disease progress curve (AUDPC) on deoxynivalenol (DON) concentration measured in vitro for eight selected *Fusarium graminearum* isolates collected from wheat fields and grain elevators in Nebraska in 2007-2010 and tested for aggressiveness on wheat spikes of *Fusarium* head blight-susceptible cultivar Wheaton in the greenhouse.
**Figure 8.** Regression of deoxynivalenol (DON) concentration measured in grain on DON concentration measured *in vitro* for eight selected *Fusarium graminearum* isolates collected from wheat fields and grain elevators in Nebraska in 2007-2010 and tested for aggressiveness on wheat spikes of Fusarium head blight-susceptible cultivar Wheaton in the greenhouse.
Appendix I: Agarose gel showing DNA band of 550bp-570bp using ITS primers (ITS1 and ITSF) amplifying Nebraska isolates of *Fusarium* species from year 2009. Lane M represents 100bp DNA ladder, lanes 1 to 20 represents (NE166, NE167, NE168, NE169, NE170, NE171, NE172, NE173, NE174, NE175, NE176, NE179, NE180, NE182, NE183, NE184, NE185, NE186, NE187 and NE188), lane N represents negative control, water.
Appendix II: Agarose gel showing DNA band of 550bp-570bp using ITS primers (ITS1 and ITSF) amplifying Nebraska isolates of *Fusarium* species from year 2009 (two isolates) and 2010. Lane M represents 100bp DNA ladder, lanes 1 to 22 represents (NE189, NE190, NE191, NE192, NE193, NE194, NE195, NE196, NE197, NE198, NE199, NE200, NE201, NE202, NE203, NE204, NE205, NE206, NE207, NE208, NE209 and 210), lane N represents negative control, water.
Appendix III: Identification of *Fusarium* species by sequence homology to GENE BANK using BLAST.

**NE166**

gb|HQ832817.1| Gibberella zeae isolate LH184 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=570 Score = 906 bits (1004), Expect = 0.0

**NE167**

gb|JQ412111.1| Gibberella zeae strain 535-10 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=528 Score = 899 bits (996), Expect = 0.0

gb|HQ832817.1| Gibberella zeae isolate LH184 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=570 Score = 915 bits (1014), Expect = 0.0

**NE168**

gb|JQ412111.1| Gibberella zeae strain 535-10 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=528 Score = 901 bits (998), Expect = 0.0

**NE169**

gb|JX534351.1| Fusarium culmorum strain F167 18S ribosomal RNA, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA, partial sequence Length=509 Score = 901 bits (998), Expect = 0.0

**NE170**

gb|HQ832817.1| Gibberella zeae isolate LH184 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=570 Score = 895 bits (992), Expect = 0.0

**NE171**

gb|HQ832817.1| Gibberella zeae isolate LH184 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=570 Score = 917 bits (1016), Expect = 0.0

**NE172**

gb|JQ412111.1| Gibberella zeae strain 535-10 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=528 Score = 904 bits (1002), Expect = 0.0

**NE173**

gb|HQ832817.1| Gibberella zeae isolate LH184 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=570 Score = 906 bits (1004), Expect = 0.0

**NE174**

gb|HQ832817.1| Gibberella zeae isolate LH184 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=570 Score = 921 bits (1020), Expect = 0.0

**NE175**

gb|HQ333193.1| Gibberella zeae isolate SMCD F15 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=557 Score = 935 bits (1036), Expect = 0.0
Gibberella zeae isolate LH184 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=570 Score = 922 bits (1022), Expect = 0.0

Gibberella zeae isolate LH184 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=570 Score = 913 bits (1012), Expect = 0.0

Gibberella zeae isolate LH184 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=570 Score = 919 bits (1018), Expect = 0.0

Gibberella zeae isolate LH184 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=570 Score = 919 bits (1018), Expect = 0.0

Gibberella zeae isolate LH184 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=570 Score = 919 bits (1018), Expect = 0.0

Gibberella zeae strain 535-10 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=528 Score = 904 bits (1002), Expect = 0.0

Gibberella zeae genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence, strain: NBRC 9462 Length=2265 Score = 915 bits (1014), Expect = 0.0

Gibberella zeae isolate LH184 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=570 Score = 897 bits (994), Expect = 4e-141

Gibberella zeae strain 535-10 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=528 Score = 884 bits (980), Expect = 0.0
NE191
gb|JQ412111.1| Gibberella zeae strain 535-10 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=528 Score = 865 bits (958), Expect = 0.0

NE192
gb|JQ412111.1| Gibberella zeae strain 535-10 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=528 Score = 899 bits (996), Expect = 0.0

NE193
gb|JQ412111.1| Gibberella zeae strain 535-10 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=528 Score = 895 bits (992), Expect = 0.0

NE194
gb|JQ412111.1| Gibberella zeae strain 535-10 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=528 Score = 897 bits (994), Expect = 0.0

NE195
gb|JQ412111.1| Gibberella zeae strain 535-10 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=528 Score = 895 bits (992), Expect = 0.0

NE196
gb|JQ412111.1| Gibberella zeae strain 535-10 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=528 Score = 890 bits (986), Expect = 0.0

NE197
gb|JQ412111.1| Gibberella zeae strain 535-10 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=528 Score = 897 bits (994), Expect = 0.0

NE198
gb|JX534351.1| Fusarium culmorum strain F167 18S ribosomal RNA, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA, partial sequence Length=509 Score = 868 bits (962), Expect = 0.0

GB|JQ412111.1| Gibberella zeae strain 535-10 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=528 Score = 868 bits (962), Expect = 0.0

NE199
gb|JX534351.1| Fusarium culmorum strain F167 18S ribosomal RNA, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA, partial sequence Length=509 Score = 895 bits (992), Expect = 0.0

GB|JQ412111.1| Gibberella zeae strain 535-10 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=528 Score = 895 bits (992), Expect = 0.0
NE200  
**gb|JX534351.1**  
Fusarium culmorum strain F167 18S ribosomal RNA, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA, partial sequence Length=509 Score = 883 bits (978), Expect = 0.0

NE201  
**gb|JQ412111.1**  
Gibberella zeae strain 535-10 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=528 Score = 901 bits (998), Expect = 0.0

NE202  
**gb|JQ412111.1**  
Gibberella zeae strain 535-10 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=528 Score = 888 bits (984), Expect = 0.0

NE203  
**gb|JX534351.1**  
Fusarium culmorum strain F167 18S ribosomal RNA, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA, partial sequence Length=509 Score = 883 bits (978), Expect = 0.0

**gb|JQ412111.1**  
Gibberella zeae strain 535-10 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=528 Score = 883 bits (978), Expect = 0.0

NE204  
**gb|JQ412111.1**  
Gibberella zeae strain 535-10 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=528 Score = 875 bits (970), Expect = 0.0

NE205  
**gb|JX534351.1**  
Fusarium culmorum strain F167 18S ribosomal RNA, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA, partial sequence Length=509 Score = 863 bits (956), Expect = 0.0

**gb|JQ412111.1**  
Gibberella zeae strain 535-10 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=528 Score = 863 bits (956), Expect = 0.0

NE206  
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Fusarium culmorum strain F167 18S ribosomal RNA, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA, partial sequence Length=509 Score = 874 bits (968), Expect = 0.0

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Gibberella zeae strain 535-10 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=528 Score = 874 bits (968), Expect = 0.0

NE207  
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Fusarium culmorum strain F167 18S ribosomal RNA, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA, partial sequence Length=509 Score = 881 bits (976), Expect = 0.0

**gb|JQ412111.1**  
Gibberella zeae strain 535-10 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=528 Score = 881 bits (976), Expect = 0.0
NE208

GenBank|JQ412111.1| Gibberella zeae strain 535-10 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=528 Score = 897 bits (994), Expect = 0.0

NE209

GenBank|JX534351.1| Fusarium culmorum strain F167 18S ribosomal RNA, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA, partial sequence Length=509 Score = 888 bits (984), Expect = 0.0

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Appendix IV: DON mycotoxin screening results of Isolates from year (2007-2010)