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FUSARIUM HEAD BLIGHT: WINTER WHEAT CULTIVAR RESPONSES AND CHARACTERIZATION OF PATHOGEN ISOLATES

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FUSARIUM HEAD BLIGHT: WINTER WHEAT CULTIVAR RESPONSES AND CHARACTERIZATION OF PATHOGEN ISOLATES

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FUSARIUM HEAD BLIGHT: WINTER WHEAT CULTIVAR RESPONSES AND CHARACTERIZATION OF PATHOGEN ISOLATES

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Fusarium head blight (FHB) is a destructive disease of wheat (*Triticum aestivum* L). FHB reduces yield and grain quality and causes accumulation of the mycotoxin deoxynivalenol (DON) in grain. Cultivar resistance is one of the most effective management strategies for FHB. Experiments were conducted to 1) identify winter wheat cultivars with resistance to FHB and DON accumulation, 2) determine the effect of winter wheat cultivar on the relationship between FHB and DON concentration, and 3) identify the major species of *Fusarium* causing FHB in Nebraska and characterize its isolates. Differences ($P \leq 0.05$) were detected among cultivars in FHB index, *Fusarium*-damaged kernels, DON, and yield. The cultivars Alliance, Harry, Hondo, Infinity CL, and Overland were moderately resistant to FHB and DON accumulation. Harry was resistant to FHB but susceptible to DON accumulation. Overley, Jagalene, Wesley, and 2137 were susceptible to FHB and DON accumulation. The relationship between FHB severity and DON concentration was linear and positive regardless of cultivar. However, regression slopes indicated that this relationship was cultivar dependent. Forty of 41 isolates of *Fusarium* obtained from infected winter wheat kernels in grain collected from

elevators and fields in Nebraska were identified by polymerase chain reaction (PCR) morphological characteristics as *Fusarium graminearum* Schwabe (Teleomorph: *Gibberella zeae* (Schwein.) Petch). Seventeen selected isolates differed ($P \leq 0.05$) in the number and size of perithecial units (single perithecium or clusters of perithecia) produced in culture and seven selected isolates differed ($P \leq 0.05$) in aggressiveness on wheat spikes and detached leaves. Based on aggressiveness on wheat spikes, the seven isolates were grouped into three categories: 1) highly aggressive (isolates 103, 110, and 119), 2) moderately aggressive (isolates 91 and 98), and 3) weakly aggressive (isolates 90 and 97).

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TABLE OF CONTENTS

CHAPTER II

EVALUATION OF WINTER WHEAT CULTIVARS FOR RESISTANCE TO FUSARIUM HEAD BLIGHT AND DEOXYNIVALENOL ACCUMULATION

CHAPTER III

EFFECT OF CULTIVAR ON THE RELATIONSHIP BETWEEN FUSARIUM HEAD BLIGHT SEVERITY AND DEOXYNIVALENOL CONCENTRATION IN WINTER WHEAT

CHAPTER IV

CHARACTERIZATION OF NEBRASKA ISOLATES OF *Fusarium graminearum* FROM WINTER WHEAT.

TABLES OF FIGURES AND TABLES

CHAPTER II

CHAPTER III.

CHAPTER I

INTRODUCTION AND BACKGROUND

1. WHEAT.

Farming was the revolution that provided food surpluses for the development of humanity. Domestication of crops occurred around 10,500 years before present (bp) for wheat, 9,000-8,000 years bp for maize, and 8,000 years bp for rice (4). Wheat, maize and rice support the world food supply, providing 44% of total edible dry matter and 40% of food consumed in developing countries. Wheat plays an important role in the world. It is grown in more than 70 countries on 5 continents (20) and is the most widely grown crop in the world (2). In 2008, the production of wheat worldwide was 683,406,527 MT. The production of wheat in the U.S. during the same year was 68,016,100 MT (FAOSTAT 2010, Verified November 17, 2010 in http://faostat.fao.org/site/339/default.aspx).

The two most important commercial wheat types are durum wheat (*Triticum durum* L. $2n = 4x = 28$) and common wheat (*Triticum aestivum* L. $2n = 6x = 42$). Based on its growth habits, wheat can be divided into winter wheat sown primarily in the fall, requiring vernalization to flower, and tolerant of freezing temperatures; facultative wheat, sown during winter months in mild climates, requiring or not requiring vernalization to flower, and intolerant of long periods of freezing temperatures; and spring wheat, sown mainly in the spring and summer months (2).

All winter wheat grown in North America is common wheat and can be divided into four groups: Eastern and Southeastern soft wheats, Southern Great Plains wheats, Northern Great Plains wheats, and Pacific Northwest wheats (2). Each of them has different agroecological adaptations and different end-use properties. In the Great Plains of North America, wheat production is mostly winter wheat. Given the size of this area (southern Texas to South Dakota, and from the Missouri river valley to the Rocky mountains, the region is divided into two broad gene pools: the Southern Great Plains, including Texas, Oklahoma, Kansas, and Colorado; and the Northern Great Plains comprising Nebraska, South Dakota, North Dakota, Wyoming, and Montana. U.S. winter wheat production in 2008 was 50,836,848 MT (NASS, Agricultural statistics 2009 http://www.nass.usda.gov/Publications/Ag_Statistics/ verified November 17, 2010).

2. FUSARIUM HEAD BLIGHT OF WHEAT

The Great Plains region generally is semiarid, and irrigation is commonly used for high value crops such as corn (2). Wheat in this region is mostly grown under dryland environments; however, irrigated wheat is also grown. Several diseases cause yield loss and reduce grain quality in wheat. They include leaf spots (e.g. tan spot, Septoria tritici blotch), rusts (leaf rust, steam rust, and stripe rust), and Fusarium head blight (FHB).

Fusarium head blight, caused by *Fusarium graminearum* Schwabe (Teleomorph: *Gibberella zeae* (Schwein.) Petch)), is a destructive disease of wheat *(Triticum aestivum* L.) and has a significant economical impact in the United States and other parts of the

world. *F. graminearum* causes damage not only in wheat but also in other cereal crops such as oat (*Avena sativa* L.), barley (*Hordeum vulgare* L.), wild rice (*Zizania palustris* L.), maize (*Zea mays* L.), and sorghum (*Sorghum bicolor* (L.) Moench). It can also cause disease in *Acacia* (Mill.), *Eucalyptus*, and carnation (*Dianthus caryophyllus* L.) (32, 54). FHB results in yield loss and poor grain quality, low kernel weight, reduced seed germination, seedling blight, and poor stands (18, 34, 43). FHB causes premature bleaching of spikelets on the wheat spike (57). Bleached spikelets are sterile or contain shriveled and/or discolored kernels, commonly referred as *Fusarium*-damaged kernels (FDK). In addition, *F. graminearum* produces the toxin deoxynivalenol (DON), which accumulates in grain (34, 43).

3. EPIDEMICS OF FUSARIUM HEAD BLIGHT IN THE U.S.

Since the mid 1990s, FHB has increased its importance as a wheat disease with severe epidemics in the U.S. and especially in the central Great Plains (2). The disease has caused significant yield, quality, and economic losses (34). In the U.S., epidemics of significant importance occurred in 1917 causing grain losses amounting to 288,000 metric tons (MT). In 1919, a new epidemic occurred causing losses totaling 2.18 million MT. During the period between 1928 and 1937, large yield losses were also recorded. In 1982, losses amounting to 2.72 million MT were caused by FHB and 4.78 million MT were lost in 1993 in North Dakota, South Dakota, Minnesota, and Manitoba. The

epidemics that occurred in 1998-2000 caused losses in the U.S. estimated at 1.3 million MT (34, 52).

Since the early 1990s, FHB outbreaks have become more frequent in the Great Plains region and other wheat growing areas in the United States (19), causing significant economic losses in hard winter wheat in the states of Kansas, Nebraska, and South Dakota. FHB has occurred yearly to varying levels of severity and prevalence since 2007, with the worst years occurring in 2007 and 2008.

Different factors have contributed to the increase in FHB epidemics. They include more frequent precipitation during spring and summer, the use of cultivars with high susceptibility to FHB, and an increase in the area under corn cultivation which, together with reduced or no tillage practices has favored development of epidemics.

4. CAUSAL AGENT OF FHB

F. graminearum, a homothallic fungus, is the most prominent causal agent of FHB in the United States, Canada, and Europe (34, 58, 25). Nevertheless, other *Fusarium* species including *F. culmorum* (W. G. Smith) Sacc.1892., *F. poae* (Peck) Wollenw. 1913., *F. pseudograminearum* O'Donnell & T. Aoki, 1999., *F. avenaceum* (Fr.:Fr.) Sacc, 1886., *Microdochium nivale* (Fr.) Samuels & I.C. Hallett 1983., and *M. majus* (Wollenw.) Glynn & S.G. Edwards 2005., may also be associated with FHB in wheat and other small grains (59, 43, 54). The distribution of these pathogens varies within a region and is influenced by the weather and climate. Several or all these

pathogens can be found simultaneously in wheat spikes (59). *F. graminearum* occurs worldwide (32). It has been documented in North America, Europe, South America, and Asia. Other species causing FHB have also been found in Europe, Asia, and Australia (1, 21, 22, 23, 59).

5. LIFE CYCLE OF *Fusarium graminearum*

F. graminearum overwinters as mycelium on residues of cereal crops such as corn, wheat, and barley (59). Perithecia (sexual fruiting structures) form on the crop residue in the spring and, when mature, release ascospores. Natural infection occurs when ascospores land on spikelets during anthesis, where they germinate and enter the tissues using natural openings in the lemma, glume, and palea or through the anther (54, 59, 43). After entering the plant, *F. graminearum* can grow initially intercellularly, continuing intracellularly and rapidly colonizing the tissue (12). Symptoms at this stage include water soaking and bleaching of the tissues affected. This premature bleaching of infected spikes can be present in a few spikelets or in most or all of the spikelets on the spike, a typical symptom of FHB (54).

Expression of genes for production of DON starts almost immediately after infection, allowing *F. graminearum* to spread into the rachis from florets in wheat (26). Colonization of developing kernels is accompanied by DON accumulation resulting in shriveled, undersized grain referred to as *Fusarium*-damaged kernels (FDK) (34, 54). If infected grain is used as seed for the next crop, damping off and seedling blights occur.

6. DISEASE ASSESSMENT

FHB researchers commonly use four measures to quantify the disease (44, 45, 46): incidence, defined as the proportion of diseased spikes in a sample; severity, defined as the proportion of diseased spikelets per spike; index, defined as the product of incidence and severity; and FDK, defined as the proportion of visually scabby kernels in a sample of harvested grain.

7. RESISTANCE TO FHB

Resistance of wheat to FHB is a character of highly complex inheritance (35). Introducing and maintaining traits for FHB resistance is a difficult task. Mesterházy (37) described five types of physiological resistance: Type I or resistance to the initial infection (51), Type II or resistance to spreading through the spike (51), Type III or resistance to kernel infection (36, 39), type IV or tolerance to infection (36, 39), and type V or resistance to DON accumulation. Different breeding programs are developing cultivars with resistance to FHB. In addition to the traditional breeding programs, transgenic wheat with resistance to FHB is also being tested (2).

8. MYCOTOXINS AND *F. graminearum* INFECTION

Different *Fusarium* species have been described as producers of toxic secondary metabolites that affect human and animal health. Among these, trichothecenes have been identified as an important type (50). Trichothecenes are classified as macrocylic and nonmacrocylic, depending on the presence of a macrocylic ester or an ester-ether bridge between C-4 and C-15 (5). Nonmacrocylic trichothecenes are divided into type A and type B. Type B contains the mycotoxins fusarenon-x, nivalenol, and DON. Trichothecenes are extremely potent inhibitors of eukaryotic proteins synthesis. Therefore, they are toxic to both animals and plants (33). *F. graminearum* produces several mycotoxins, including nivalenol, DON and its derivatives, zearalenone, fusarin C, and aurofusarin (33, 5, 54). The primary economic and health consequence of FHB is due to DON contamination even with its relatively low acute toxicity (45). Chemotypes 3-acetyldeoxynivalenol (chemotype 3-ADON) and 15- acetyldeoxynivalenol (chemotype 15-ADON) have been described in *F. graminearum* DON producers, and chemotype NIV in 4-acetylnivalenol producers (59).

DON is a potent protein biosynthesis inhibitor affecting the digestive system and major organ function in humans and animals. When ingested in sufficient doses, it causes nausea, vomiting, and diarrhea. Farm animals fed with contaminated grain have weight loss and food refusal (5) and for this reason DON is also called vomitoxin or food refusal factor. It is a virulence factor in wheat, causing tissue necrosis (47, 17). DON is the only mycotoxin shown to be a virulence factor (33, 54).

Tolerance limits of DON in the U.S. are 1, 10, 5, and 5 ppm, respectively, in finished wheat products, grain and grain byproducts destined for ruminating beef and feedlot cattle older than 4 months and chickens (not exceeding 50 % of the total diet), grain and grain byproducts destined for swine (not exceeding 20 % of their diet), and grain and grain byproducts for other animals (not exceeding 40 % of their diet) (18). In the upper Midwestern region of the United States, DON levels frequently exceed this limit (54). In addition to the health consequences, wheat grain with DON concentrations exceeding the minimum limits allowed may be rejected or devalued at grain intake points (14).

9. RELATIONSHIP BETWEEN FUSARIUM HEAD BLIGHT INTENSITY AND DEOXYNIVALENOL

Different studies have shown that DON accumulation might be related to the level of FHB damage. However, different degrees of association between FHB intensity and DON accumulation in harvested grain have been reported in the literature. Paul et al. (45, 46) using meta-analysis observed that overall there were significant, positive relationships between all commonly used measures of FHB intensity and DON. It was also shown that levels of DON might vary considerably from epidemic to epidemic as a function of precipitation. Lacey et al. (29) showed that DON contamination was highest after inoculating spikes at about mid anthesis; nevertheless small amounts of DON were produced without visible symptoms of FHB.

Previous studies have shown that FHB-susceptible wheat cultivars generally accumulate more DON than resistant cultivars. In North Carolina, Cowger et al. (14) showed that when post-anthesis mist was applied or not applied to *F. graminearum*-inoculated soft red winter wheat, the susceptible cultivar USG 3592 accumulated more DON than moderately resistant cultivars. In Hungary, Mesterházy et al. (38, 39) observed more DON accumulation in susceptible than in resistant winter wheat cultivars. In Germany, Koch et al. (28) similarly observed that a highly susceptible winter wheat cultivar accumulated more DON than a moderately resistant cultivar. In field and greenhouse studies, DON concentration in grain and wheat spikes was explained with a linear regression where the concentration continued to increase with additional inoculum (53).

Lemmens et al. (31) found that environmental conditions had an impact on both disease development and DON accumulation. Extended periods of free moisture with relative humidity (RH) greater than 90% and moderate temperatures (15 to 30 °C) are known to be required for successful infection before or during anthesis (53, 16, 58). It has been demonstrated (16, 58) that environmental conditions prior to anthesis, often 7 to 14 days, are known to affect sporulation and subsequently FHB severity. Hooker et al. (25) showed that infection is mainly dependent on the combination of rainfall, the duration of canopy wetness, and temperature conditions relative to the stage of wheat development.

Although the relationship between DON and FHB has been shown to be generally linear and positive (31, 44, 45), it has not been determined whether the strength of the relationship varies among cultivars in winter wheat.

10. EVALUATION OF WINTER WHEAT CULTIVARS FOR RESISTANCE TO FUSARIUM HEAD BLIGHT

Due to the sporadic nature of FHB, there is lack of information on hard winter wheat cultivar resistance to FHB and DON accumulation in the central Great Plains of the U.S. In adjacent regions with higher rainfall (the northern Great Plains spring wheat region and the eastern soft wheat region) considerably more is known about the disease and cultivar resistance. However, many recently released hard winter wheat cultivars in the central Great Plains have not been tested in the field under high FHB intensity.

Knowledge of the reaction to FHB and DON accumulation of hard winter wheat cultivars under field conditions will enable growers to make informed decisions regarding the choice of cultivars to plant and the level of risk they have during the sporadic occurrences of the disease. Therefore, there is a need to identify hard winter wheat cultivars with resistance to FHB and DON accumulation in the central Great Plains. Such cultivars can be used as germplasm in small grain breeding programs for future improvement and their use by growers can significantly reduce the risk of growing wheat and the losses associated with FHB and DON. In addition, the food processing industries will benefit immensely from a consistent supply of high quality grain with little or no DON when such cultivars are grown in years with high FHB intensity.

Few studies have reported the evaluation of hard winter wheat cultivars for resistance to FHB in the U.S. Ransom and McMullen (48) reported that hard winter wheat cultivars differed significantly in FHB, DON concentration, and FDK. In cultivar by fungicide interaction studies, Wegulo et al. (56) demonstrated differences among a

limited number of hard winter wheat cultivars in their reaction to FHB and DON accumulation. Bai et al. (3) demonstrated significant differences among 116 wheat genotypes in their reaction to FHB. They showed that DON concentration was significantly correlated with FHB. Cowger et al. (14) demonstrated that there were significant differences among eight soft red winter wheat cultivars in FHB incidence, FHB severity, FDK, and DON.

In Europe, Mesterházy et al. (38) found that winter wheat cultivars differed in yield loss, severity, FDK, and DON. FHB-resistant cultivars had less FDK, severity, DON, and yield loss compared with susceptible cultivars. DON, severity, FDK, and yield loss were significantly correlated. In a separate study, Mesterházy et al. (39) found that DON decreased with decreasing FHB severity. Severity was best correlated with yield loss and least correlated with DON. The most resistant cultivars had no or very low DON. Lehoczki-Krsjak et al. (30) found that some winter wheat genotypes with medium FHB severity had low FDK and DON concentration. However, there were some genotypes with relatively low FHB severity but high levels of DON.

Brennan et al. (8) tested commercially grown European wheat cultivars against a range of *Fusarium* species. They found non-significant differences among cultivars in DON production. DON content was generally correlated with FHB parameters (incidence, severity, and FDK).

11. IDENTIFICATION OF *FUSARIUM* SPECIES CAUSING FHB

Leslie and Summerell (32) stated that three different species concepts (morphological, biological, and phylogenetic) contribute to the construction of what species is in the genus *Fusarium*. Each of these concepts has techniques that can be used to describe a species in daily work. The morphological concept is based on the idea that a morphological 'type' or individual represents the entire species. The biological concept is commonly based on a description given by Mayr and stated by Leslie and Summerell (32): '…species as group of populations that actually or potentially inbreed with each other'. The phylogenetic concept defines species as the smallest phylogenetic subgroup (clade) of individuals or of a population that share a fixed suite of diagnostic characters. Nowadays, this concept usually uses molecular markers.

According to Leslie and Summerell (32), the greatest potential for characterization of species in fungi is the combined use of biological and phylogenetic species concepts. Species identification using molecular and morphological characteristics simultaneously can give more confidence than identification using either method alone. Molecular methods for identification of fungal species causing FHB have been used broadly (40), sometimes without total success in species-specific identification. Some techniques used are amplification from the internal transcribed regions (ITS) of nuclear ribosomal DNA, duplex PCR, and amplification from sequences of specific genes like the galactose oxidase gene (42, 41, 27). De Biazio et al. (15) have proposed a new method that amplifies a fragment of 435 bp of an internal region of the gene GO.

Characterization and identification of the causal agent of FHB is crucial for disease management and for basic research. It is important to know if isolates of the species differ in characteristics such as pathogenicity (ability to cause disease), aggressiveness (rate of disease progression), and DON production. Perithecia production has been linked to pathogenicity (17). Characterization of isolates of a *Fusarium* species causing FHB can be done using different techniques such as quantification of perithecia production, measurement of aggressiveness on wheat spikes and detached leaves, and determination and quantification of the trichothecene chemotype produced by the isolate.

Under laboratory conditions, perithecia can be produced both in vivo and in vitro, (6, 24, 55). Detached leaf assays have been used to evaluate wheat lines and commercial cultivars for resistance to FHB (9, 10). This technique can also be utilized in the evaluation of the aggressiveness of isolates of a given species of *Fusarium* causing FHB. Pathogen aggressiveness can be measured as lesion size at a given time following inoculation or as area under the disease progress curve AUDPC (1, 11, 7, 13, 49).

The research reported in this dissertation was conducted to 1) identify winter wheat cultivars with resistance to FHB and DON, 2) determine the effect of winter wheat cultivar on the relationship between FHB and DON, and 3) identify the major species of *Fusarium* causing FHB in Nebraska and characterize its isolates.

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CHAPTER II

EVALUATION OF WINTER WHEAT CULTIVARS FOR RESISTANCE TO FUSARIUM HEAD BLIGHT AND DEOXYNIVALENOL ACCUMULATION

1. INTRODUCTION

Fusarium head blight (FHB), caused by *Fusarium graminearum* Schwabe (sexual stage *Gibberella zeae* (Schwein.) Petch is an economically important disease of wheat and other small grain cereals. Losses from FHB are due to yield reduction, presence of *Fusarium*-damaged kernels (FDK), and production by *F. graminearum* of the mycotoxin deoxynivalenol (DON) which accumulates in grain. Major epidemics of FHB with varying intensity (incidence, severity, or index = incidence x severity) have recently occurred in the Great Plains of the United States, causing significant economic losses in hard winter wheat in several states including Kansas, Nebraska, and South Dakota (personal observation). Various strategies are used to manage FHB, including the use of resistant cultivars, crop rotation, residue management, and fungicide application (20,24).

Due to the sporadic nature of FHB, there is lack of information on hard winter wheat cultivar resistance to FHB and DON accumulation in the central Great Plains of the U.S. In adjacent regions with higher rainfall (the northern Great Plains spring wheat region and the eastern soft wheat region) considerably more is known about the disease and cultivar resistance. However, many recently released hard winter wheat cultivars in

the central Great Plains have not been tested in the field under high FHB intensity. Moreover, published ratings (Table 1) of older cultivars from state trials and breeder nurseries have been inconsistent for some cultivars. Knowledge of the reaction to FHB and DON accumulation of hard winter wheat cultivars under field conditions will enable growers to make informed decisions regarding the choice of cultivars to plant and the level of risk they have during the sporadic occurrences of the disease. Therefore, there is a need to identify hard winter wheat cultivars with resistance to FHB and DON accumulation in the central Great Plains. Such cultivars can be used as germplasm in small grain breeding programs for future improvement and their use by growers can significantly reduce the risk of growing wheat and the losses associated with FHB and DON. In addition, the food processing industries will benefit immensely from a consistent supply of high quality grain with little or no DON when such cultivars are grown in years with high FHB intensity.

Few studies have reported the evaluation of hard winter wheat cultivars for resistance to FHB in the U.S. Ransom and McMullen (26) reported that hard winter wheat cultivars differed significantly in FHB, DON concentration (hereafter referred to as DON), and FDK. In cultivar by fungicide interaction studies, Wegulo et al. (32) demonstrated differences among a limited number of hard winter wheat cultivars in their reaction to FHB and DON accumulation.

In Europe, Mesterházy et al. (21) found that winter wheat cultivars differed in yield loss, severity, FDK, and DON. FHB-resistant cultivars had less FDK, severity, DON, and yield loss compared with susceptible cultivars. DON, severity, FDK, and yield loss were significantly correlated. In a separate study, Mesterházy et al. (22) found

that DON decreased with decreasing FHB severity. Severity was best correlated with yield loss and least correlated with DON. The most resistant cultivars had no or very low DON. Lehoczki-Krsjak et al. (19) found that some winter wheat genotypes with medium FHB severity had low FDK and DON concentration. However, there were some genotypes with relatively low FHB severity but high levels of DON.

Due to the limited information regarding the reaction to FHB and DON of hard winter wheat cultivars currently grown in the central Great Plains, there is a need to evaluate these cultivars for resistance to the disease and the mycotoxin. The objectives of this study were to i) evaluate hard winter wheat cultivars for resistance to FHB and DON accumulation, ii) assess relationships among FHB intensity, DON, and FDK from the cultivar evaluations in objective 1, and iii) compare DON in grain from symptomatic spikes to DON in grain bulked from plots in the field experiments in objective 1.

2. MATERIALS AND METHODS

In 2008-2010, a total of 22 hard red winter wheat cultivars (Table 1) were evaluated for resistance to FHB and DON in field and greenhouse experiments. Cultivars were chosen to represent those commonly grown in Nebraska and neighboring states.

2.1. 2008 field trial. In fall 2007, a field at the University of Nebraska Agricultural Research and Development Center (ARDC) near Mead, NE was planted on October 26 with 12 hard red winter wheat cultivars following corn (*Zea mays*). The cultivars were

2137, Alliance, Goodstreak, Harry, Hondo, Infinity CL, Jagalene, Karl 92, Millennium, Overley, Wahoo, and Wesley. Plot size was 0.6 m by 1.4 m. Seed was planted at a rate of 101 kg/ha and standard agronomic practices for wheat production were followed. In spring 2008, plots were artificially inoculated with a spore suspension of *F. graminearum* at 1 x 10^5 spores/ml at mid-anthesis (Zadoks growth stage 65) (34) using a backpack sprayer. To obtain the spore suspension, an isolate of *F. graminearum* obtained from a Nebraska wheat field was grown on potato dextrose agar (PDA) plates on a laboratory bench for three weeks.

Sterile distilled water (5 ml) was added to each plate and a rubber policeman was used to dislodge spores. The spore suspension was filtered through two layers of cheesecloth into a beaker and the concentration was adjusted to 1 x 10^5 spores/ml with distilled water. There also was natural inoculum in the field. Mid anthesis was considered to be the day on which 50% of the spikes of a given cultivar had extruded anthers. Thus, cultivars were inoculated on different dates ranging from 2 June to 4 June. Cultivars were arranged in a randomized complete block design with four replications. A second inoculation was made on individual spikes using a hand-held bottle sprayer. Approximately 2 ml of the same spore suspension used to inoculate the plots was applied to each of 20 spikes per plot with a hand-held bottle sprayer and each spike was covered with a transparent plastic bag for 24 hours following inoculation. Inoculated spikes were tagged for identification during disease assessment and harvesting.

Disease incidence (percentage of diseased spikes) and severity (percentage of diseased spikelets on a spike) were determined 21 days after inoculation on 10 spikes in each of 10 arbitrarily selected locations in each plot and used to calculate index using the

formula: index (%) = [incidence (%) x severity (%)]/100. Hand harvesting of tagged spikes in each plot was done when grain moisture content dropped below 15%. The rest of the plot was harvested with a small plot combine to estimate grain yield and obtain grain samples for FDK and DON analysis.

2.2. 2009 field trial. In fall 2008, a commercial field near Paxton, NE was planted on 11 October with 20 hard red winter wheat cultivars following soybean (*Glycine max*). The cultivars were 2137, Alliance, Art, Bill Brown, Bond CL, Camelot, Goodstreak, Harry, Hatcher, Hawken, Infinity CL, Jagalene, Mace, Millennium, Overland, Overley, Postrock, Settler CL, Wahoo, and Wesley. Plot size was 2.1 m by 1.3 m. Seed was planted at a rate of 122 kg/ha and standard agronomic practices for wheat production were followed. Plots were not inoculated but there was natural inoculum in the field. Cultivars were arranged in a randomized complete block design with three replications. Two disease assessments were made. In the first assessment on 2 July, disease incidence and severity were determined on 10 spikes in each of 10 arbitrarily selected locations in each plot and used to calculate index using the formula stated above. The second assessment was similarly done on 9 July. Twenty symptomatic spikes were tagged in each plot and hand-harvested when grain moisture content dropped below 15%. The rest of the plot was harvested on 20 July with a small plot combine to estimate grain yield and obtain grain samples for FDK and DON analysis.

2.3. Greenhouse trial. In 2010, 13 hard winter wheat cultivars were planted in 15-cm-diameter pots on 22 February, one seed per pot, after 7 weeks of vernalization at 4^{o}C.

Twelve of the cultivars were the same as those in the 2008 field trial (above). The thirteenth cultivar was Overland. The soil mixture consisted of 1 part clay loam soil, 1/2 part sand, 1/2 part vermiculite, and 1 part Canadian sphagnum peat moss. Cultivars were arranged in a randomized complete block design with four replications and were grown on a greenhouse bench. The pots were fertilized with 20:20:20 NPK injected daily at a rate of 250 ppm during regular watering. Temperature ranged from 20ºC (night) to 26ºC (day). To induce flowering, the days were extended by artificial light from 5 p.m. to 10 p.m. Five to seven spikes were artificially inoculated with a spore suspension of an isolate of *Fusarium graminearum* at 1 x 10^5 spores/ml at mid-anthesis (Zadoks growth stage 65) (34) using a hand-held bottle sprayer. The isolate was obtained from an infected kernel in grain supplied by an elevator in south central Nebraska in 2007.

Spores of the isolate were stored in 15% glycerol at -80^{o}C until needed for experiments. The surface of the frozen glycerol was scraped with a sterile spatula and the particles were sprinkled on potato dextrose agar (PDA) plates which were then incubated at 25^{o}C in 12 h light and 12 h dark. After 7 days, 10-mm-diameter mycelial plugs from actively growing edges of the isolate were transferred, mycelial face down, onto fresh PDA plates which were then incubated at 25^{o}C in 12 h light and 12 h dark for 3 weeks. A spore suspension was made as described above. Approximately 1 ml of the spore suspension was applied to each spike with a hand-held bottle sprayer and the spike was then covered with a transparent plastic bag for 7 days following inoculation. Disease severity on spikes was assessed 10 and 21 days after inoculation. Spikes were harvested when grain moisture content dropped below 15%. The experiment was conducted twice.

2.4. FDK and DON analysis. The percentage of *Fusarium*-damaged kernels (FDK) in grain from field trials was determined at the USDA ARS Center for Grain and Animal Health Research, Engineering and Wind Erosion Research Unit, Manhattan, KS using a single-kernel near-infrared (SKNIR) system. Accuracy of the SKNIR system in measuring FDK has been validated (33). FDK from the greenhouse trial was estimated visually by dividing the number of shriveled and/or chalky white kernels by the total number of kernels in a sample and multiplying by 100. Ten-gram grain samples were ground to flour (Laboratory Construction CO. Kansas City, MO. Model 256) and sent to the North Dakota Veterinary Diagnostic Laboratory, North Dakota State University, Fargo, ND for DON analysis using gas chromatography with electron capture detection (GC/ECD) (31). In the field trials, DON concentration was determined in grain bulked from each plot (DONplot) and in grain from tagged spikes (DONtag).

2.5. Data analysis. The general linear models (GLM) procedure of SAS version 9.1 (SAS Institute, Cary, NC) was used to analyze data. To show the effects of environment and the interaction between environment and cultivar and to compare average DON between environments, ten cultivars common to both locations (Mead in 2008 and Paxton in 2010) were used in analysis of variance. A combined analysis of the two runs of the greenhouse trial was done based on homogeneity of error variance (14) between the two runs. Fisher's least significant difference (LSD, $P = 0.05$) test was used to compare pairs of cultivar means. Cultivars were ranked for resistance to FHB, FDK, and DON accumulation (the more resistant, the lower the ranking) and for yield (the greater the yield, the lower the ranking). Cultivar means were used in correlation analysis (14,30) to

determine the relationship between FHB index or severity and DON and between FHB index or severity and FDK in each trial. Data for all cultivars in a trial (12 cultivars at Mead, 20 cultivars at Paxton, and 13 cultivars in the greenhouse) were used in correlation analysis. In the greenhouse trial, grain samples from the cultivar Wahoo were insufficient for DON analysis; therefore, 12 cultivars were used in the FHB severity/DON correlation. Effects of environment, cultivar, and environment by cultivar interaction were considered significant at $P \leq 0.05$.

3. RESULTS

3.1. Index, DON, FDK, and yield from field trials. The effect of environment was significant for yield, FDK, and DONplot (Table 2). Environment by cultivar interaction was significant for index, yield, DONplot, and DONtag. At Mead, the most susceptible cultivars to FHB were Overley, Jagalene, and Wesley and the most resistant were Harry, Hondo, and Goodstreak (Table 3). High concentrations of DON occurred in Overley, Jagalene, and Wesley, which were the cultivars that also had the highest susceptibility to FHB. However, Harry, which was among the cultivars with the lowest susceptibility, had DON levels similar to those in the FHB-susceptible cultivars. The cultivars with the highest FDK were Wahoo, Harry, and Jagalene. Yield was very low due to late planting in fall 2007 and high FHB and foliar disease intensity in 2008. Jagalene and Karl 92 had the lowest and highest yield, respectively (Table 3).

At Paxton, Overley and Jagalene were the most susceptible cultivars to FHB based on the second disease assessment whereas the least susceptible cultivars were Goodstreak, Wahoo, Millennium, and Overland (Table 4). DON in grain bulked from plots (DONplot) was highest in Postrock followed by Overley and lowest in Overland and Goodstreak. DON in grain from symptomatic spikes (DONtag) was highest in Hatcher and 2137 and lowest in Art. Harry and Overley had the highest and lowest FDK, respectively. Bond CL and Mace were the highest and lowest yielding cultivars, respectively (Table 4).

3.2. Severity, FDK, and DON from the greenhouse trial. Severity, not index, was measured in the greenhouse trial due to the limited number of spikes. Hondo, Harry, and Overland were the most resistant cultivars based on the first disease assessment at 10 days post-inoculation (dpi) whereas the most susceptible cultivars were Overley and Jagalene (Table 5). At 21 dpi, Hondo, Harry, and Overland were still the most resistant cultivars. FDK was lowest in Hondo and highest in Karl 92 whereas DON was lowest in Hondo and highest in Jagalene (Table 5).

3.3. Comparison of DON from symptomatic spikes to DON bulked from plots. Tagged spikes in both field trials had FHB severity ranging from 80% to 100%. A comparison of DON concentration in these spikes (DONtag) with DON in grain bulked from plots (DONplot) showed a higher concentration of DONtag than DONplot at Mead ($P < 0.0001$) and Paxton ($P < 0.0001$) (Fig 1). Averaged over all cultivars in a location, DONtag concentration was 1.7 and 7.3 times the concentration of DONplot at Mead and

Paxton, respectively. Averaged over all cultivars in a location, DONtag did not differ ($P = 0.5828$) between Mead (9.3 ppm) and Paxton (9.9 ppm); however, average DONplot at Mead (6.1 ppm was higher ($P < 0.0001$) than average DONplot at Paxton (1.2 ppm) (Fig 2).

3.4. Cultivar rankings. Except for the most susceptible and most resistant cultivars, cultivar rankings (the more resistant, the lower the ranking) generally fluctuated from trial to trial and from variable to variable within a trial. At Mead, Harry and Hondo were the most resistant and Overley and Jagalene were the most susceptible to FHB (Table 6). 2137 and Alliance were the most resistant to FDK whereas Wahoo and Harry were the most susceptible. Karl 92 and Hondo were the most resistant to DONplot whereas Harry and Overley were the most susceptible. Karl 92 and Jagalene were the best and worst yielding cultivars, respectively (the higher the yield, the lower the ranking, Table 6).

At Paxton, Goodstreak was the most resistant and Overley and Jagalene the most susceptible to FHB (Table 7). Overley and Harry were the most resistant and most susceptible, respectively, to FDK. Overland and Postrock were the most resistant and most susceptible, respectively, to DONplot. Art and Hatcher were the most resistant and most susceptible, respectively, to DONtag. The best and worst yielding cultivars were Bond CL and Mace, respectively (Table 7).

In the greenhouse trial, Hondo and Harry were the most resistant and Jagalene and Overley the most susceptible to FHB (Table 8). Hondo and Wahoo were the most resistant to FDK whereas Karl 92 and Alliance were the most susceptible. 2137 and

Hondo were the most resistant to DON whereas Jagalene and Wesley were the most susceptible.

3.5. Correlation between FHB index and FDK and between FHB index and DON. In both field trials, index and FDK were not correlated (r = -0.01, P = 0.9725 at Mead and r = -0.19, P = 0.4229 at Paxton). At Mead, correlation between index and DON was positive (Fig. 3). It was significant for DONtag (r = 0.75, P = 0.0050), but not for DONplot (r = 0.48, P = 0.118). At Paxton, correlation between index and DON was positive and significant for DONplot (r = 0.62, P = 0.0037 – first disease assessment, data not shown; r = 0.49, P = 0.0275 – second disease assessment, Fig. 3). Correlation between index and DONtag was non-significant (r = -0.20, P = 0.3877, Fig. 3).

3.6. Correlation between FHB severity and FDK and between FHB severity and DON. In the greenhouse, correlation between severity and FDK was positive and significant at 10 dpi (r = 0.67, P = 0.0124) and 21 dpi (r = 0.61, P = 0.0261). Correlation between severity and DON was positive and significant at 10 dpi (r = 0.65, P = 0.0233); it was positive but non-significant at 21 dpi (r = 0.49, P = 0.1029, Fig. 4). These results show that in the greenhouse, correlation between severity and DON and between severity and FDK was stronger at 10 dpi than at 21 dpi.

4. DISCUSSION

Few studies have been done in the U.S. to evaluate hard winter wheat cultivars for resistance to FHB. This study provides information on the reaction to FHB of hard winter wheat cultivars, including newly released ones, in the central Great Plains. Cultivars differed in their reaction to FHB in all three trials. The cultivars Overley, Jagalene, and Wesley were consistently the most susceptible to FHB (index or severity) whereas the cultivars Harry, Hondo, and Overland were among those with low FHB intensity in field and greenhouse evaluations. Based on the results of this study, cultivars whose reaction to FHB was previously unknown (Table 1) were classified as moderately resistant (Alliance and Infinity CL), moderately susceptible (Mace), and susceptible (Bill Brown and Bond CL).

Goodstreak which was among the cultivars with the lowest FHB index in the field has been rated as susceptible to FHB (26). The low FHB index on Goodstreak in the field in this study may have been due to a genotype by environment interaction or disease escape since it is a tall cultivar. Ransom and McMullen (26) observed that taller winter wheat cultivars tended to show less FHB severity in the field and attributed this observation to the greater distance between the inoculum source (soil surface) and spikes of taller cultivars compared to spikes of shorter cultivars.

Winter wheat cultivars evaluated in this study also differed in DON. However, differences among cultivars in DON were not consistent among trials except DONtag at the Mead field trial and DON in the greenhouse trial, in which the susceptible cultivars Overley, Jagalene, and Wesley consistently had high levels of DON. Harry with a

moderately resistant reaction to FHB had DON levels comparable to those in the susceptible cultivars Overley, Jagalene, and Wesley in both field trials, implying that cultivars with resistance to FHB are not necessarily resistant to DON accumulation. In Europe, Lehoczki-Krsjak et al. (19) and Mesterházy et al. (22) also showed that some winter wheat genotypes with low FHB intensity accumulated high levels of DON and vice versa.

A notable observation is that the levels of DON recorded in the greenhouse trial were much higher than those recorded in the field trials (152.8 ppm average DON in the greenhouse compared to 9.8 and 9.0 ppm average DONtag at Mead and Paxton, respectively). This difference in DON concentration in the greenhouse trial compared to the field trials may be due to differences in *F. graminearum* isolates and/or the fact that the environment in the greenhouse was very conducive to FHB development and DON accumulation (spikes were bagged for 7 days after inoculation) compared to the environment in the field (tagged spikes were bagged for 24 h after inoculation at Mead and tagged spikes were neither inoculated nor bagged at Paxton). In addition, final disease severity in the greenhouse was higher than in the field (data not shown).

Cultivars also differed in FDK. However, these differences were not consistent among the three trials except for Harry which consistently had high FDK levels in all three trials. The yield at Paxton was much higher than that at Mead. This was due to late planting in fall 2007 and high FHB and foliar disease intensity in 2008 at Mead which resulted in an unusually low yield. In addition, plots at Mead were not irrigated whereas those at Paxton were irrigated.

In this study, the effect of environment by cultivar interaction was significant for index, FDK, DON, and yield. Miedaner et al. (23) similarly found a highly significant genotype by environment interaction when they evaluated winter wheat, rye, and triticale for resistance to FHB and DON in six environments. As expected, DON in grain bulked from plots (DONplot) was lower than DON in grain from spikes with visible FHB symptoms (DONtag) in both field trials. A comparison of DONtag between Mead and Paxton showed that the two locations did not significantly differ in this variable (Fig. 2), implying that symptomatic spikes accumulated high levels of DON regardless of the location.

Correlation between index (or severity) and DON was generally positive. Previous studies which evaluated winter wheat cultivars for resistance to FHB and DON have similarly demonstrated a positive correlation between FHB intensity and DON (10,23). The lack of correlation between index and FDK in the field trials may have been due in part to the fact that a portion of the FDK was blown out at the back of the combine during harvest. In cultivar by fungicide interaction field experiments, Wegulo et al. (32) found a positive correlation between FHB index and FDK in all five experiments. However, this correlation was consistently weaker than the correlation between index and DON.

The results from this study indicate that in the Central Great Plains, there is moderate resistance to FHB and DON accumulation in some hard winter wheat cultivars. However, some commonly grown cultivars are highly susceptible. Growers can use these results to make informed decisions regarding the choice of cultivars to plant in order to reduce losses to FHB and DON. FHB-resistant cultivars identified in this study can be

used as germplasm in breeding programs. Evaluation of a wider range of hard winter wheat cultivars grown in the region for resistance to FHB and DON accumulation will provide more choices and increased benefits to producers and the food processing industries.

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6. TABLES

Table 1. Fusarium head blight (FHB) reaction and maturity characteristics of 22 winter wheat cultivars evaluated for resistance to FHB and DON accumulation in the field and greenhouse, 2008-2010

Cultivar	FHB reaction	Maturity	Reference
2137	Susceptible	Early	12,29
Alliance	Not available	Medium early	8
Art	Intermediate	Early	1,12
Bill Brown	Not available	Medium early	12,16
Bond CL	Not available	Medium early	17
Camelot	Moderately susceptible	Moderately late	6
Goodstreak	Susceptible	Medium early	3,26
Harry	Moderately resistant	Late	4
Hatcher	Intermediate to susceptible	Medium	12,18,27
Hawken	Moderately susceptible	Medium late	1,12,27
Hondo	Moderately resistant	Medium	11
Infinity CL	Not available	Medium	2
Jagalene	Susceptible	Early	12,26
Karl 92	Intermediate	Very early	12,28
Mace	Not available	Moderately late	15
Millennium	Moderately resistant to moderately susceptible	Medium	9,12,26,27
Overland	Intermediate	Moderately late	5,12
Overley	Susceptible	Early	12,13
Postrock	Moderately susceptible	Medium	1,12
Settler CL	Susceptible	Moderately late	27
Wahoo	Susceptible	Medium	7,27
Wesley	Moderately susceptible	Medium	12,25

Table 2. Environment and cultivar effects on Fusarium head blight (FHB) index, yield, *Fusarium*-damaged kernels (FDK), and deoxynivalenol (DON) concentration in 10 winter wheat cultivars evaluated in the field for resistance to FHB and DON accumulation at Mead (2008) and Paxton (2009)

		Mean square				
Source of variation	**df**	**Index**	**Yield**	**FDK**	**DONplot [a]**	**DONtag [b]**
Environment (E)	1	0.42	379225838.5****	2760.82****	358.19****	5.40
Reps within environment	4	546.93****	89634.6	237.93***	0.63	29.68
Cultivar (C) [c]	9	1610.37****	218947.6*	207.34****	7.20****	7.26
E x C	9	84.49*	458301.0****	68.16	4.17***	47.01**
Pooled error	36	33.91****	84914.4****	36.40****	0.89****	12.00*
Total	59					

[a]DON in grain bulked from plots.

[b]DON in grain from symptomatic spikes.

[c] Ten cultivars common in both environments were used in analysis of variance. They were 2137, Alliance, Harry, Goodstreak, Infinity CL, Jagalene, Millennium, Overley, Wahoo, and Wesley.

* $0.01 \leq P \leq 0.05$

** $0.001 \leq P \leq 0.01$

*** $0.0001 \leq P \leq 0.001$

**** $P \leq 0.0001$

Table 3. Fusarium head blight (FHB) index, deoxynivalenol (DON) concentration in grain bulked from plots (DONplot), DON concentration in grain from symptomatic spikes (DONtag), *Fusarium*-damaged kernels (FDK), and yield in twelve winter wheat cultivars evaluated for resistance to FHB and DON accumulation at Mead in 2008

Cultivar	Index (%)	DONplot (ppm)	DONtag (ppm)	FDK (%)	Yield (kg/ha)
Overley	63.5 a [a]	8.8 ab	18.6 a	25.8 cd	442 c-e
Jagalene	35.0 b	8.0 bc	17.6 a	38.0 a	395 e
Wesley	30.0 b	5.9 de	13.1 a-c	35.3 ab	475 c-e
2137	21.5 c	4.6 e-g	5.3 d	21.3 d	731 a-c
Karl 92	19.3 cd	3.7 g	7.9 b-d	23.5 d	939 a
Millennium	18.5 cd	5.6 d-f	8.0 b-d	32.8 a-c	660 a-e
Infinity CL	17.3 cd	6.7 cd	6.6 cd	23.8 cd	689 a-d
Wahoo	17.3 cd	5.7 de	6.7 cd	41.8 a	438 de
Alliance	16.8 cd	4.1 fg	6.0 cd	22.3 d	808 ab
Goodstreak	13.5 d	4.5 e-g	6.8 cd	27.0 b-d	627 b-e
Hondo	13.5 d	3.8 g	6.6 cd	22.8 d	719 a-d
Harry	13.0 d	9.9 a	14.6 ab	41.5 a	533 b-e

[a] Means with the same letter within a column are not significantly different at $P = 0.05$ according to the least significant difference test.

Table 4. Fusarium head blight (FHB) index, deoxynivalenol (DON) concentration in grain bulked from plots (DONplot), DON concentration in grain from symptomatic spikes (DONtag), *Fusarium*-damaged kernels (FDK), and yield in 20 winter wheat cultivars evaluated for resistance to FHB and DON accumulation at Paxton in 2009

Cultivar	Index 1[a] (%)	Index 2[b] (%)	DONplot (ppm)	DONtag (ppm)	FDK (%)	Yield (Kg/ha)
Overley	20.2 a[c]	59.7 a	2.9 ab	6.4 c-e	5.8 e	5701 a-g
Jagalene	13.3 b	46.0 b	1.7 bc	8.8 a-e	20.3 a-d	5948 a-e
2137	12.1 bc	31.7 c-e	1.5 bc	12.5 a	15.3 a-e	4483 h
Bond CL	11.7 bc	30.7 c-e	1.4 bc	11.0 a-c	14.7 a-e	6262 a
Wesley	11.6 bc	38.3 bc	1.6 bc	8.1 a-e	15.2 a-e	5920 a-e
Bill Brown	10.4 b-d	37.0 b-d	0.8 bc	7.8 a-e	19.5 a-d	5606 b-g
Camelot	10.4 b-d	31.7 c-e	1.3 bc	11.7 a-c	8.7 de	6042 ab
Postrock	9.1 b-e	23.0 e-i	4.3 a	8.0 a-e	12.0 b-e	5252 g
Hatcher	7.9 c-f	23.3 e-h	0.8 bc	12.6 a	18.4 a-d	5672 a-g
Hawken	7.9 c-f	25.7 e-g	1.1 bc	6.7 b-e	22.7 ab	5353 e-g
Alliance	7.6 c-f	20.0 f-j	1.0 bc	9.7 a-e	16.2 a-e	5303 fg
Settler CL	6.1 d-g	23.0 e-i	1.2 bc	5.9 c-e	21.5 a-c	5984 a-c
Harry	5.6 d-g	21.3 f-i	1.1 bc	8.9 a-e	24.1 a	5863 a-f
Millennium	5.3 e-g	14.0 ij	0.6 c	11.6 a-c	14.9 a-e	5440 c-g
Wahoo	4.7 e-g	11.0 jk	1.0 bc	10.9 a-d	14.8 a-e	5725 a-g
Overland	4.4 e-g	14.3 h-j	0.2 c	7.3 a-e	15.1 a-e	5379 d-g
Art	4.3 e-g	19.3 g-j	0.8 bc	4.7 e	20.4 a-c	5836 a-g
Infinity CL	4.2 e-g	20.7 f-i	0.6 c	12.2 ab	15.1 a-e	5963 a-d
Mace	4.0 fg	28.7 d-f	0.6 c	5.2 de	12.5 a-e	4198 h
Goodstreak	2.4 g	3.7 k	0.3 c	9.6 a-e	11.0 c-e	5420 c-g

[a] Index measured on 2 July.

[b] Index measured on 9 July.

[c] Means with the same letter within a column are not significantly different at $P = 0.05$ according to the least significant difference test.

Table 5. Fusarium head blight (FHB) severity, *Fusarium*-damaged kernels (FDK), and deoxynivalenol (DON) concentration in 13 winter wheat cultivars evaluated for resistance to FHB and DON accumulation in the greenhouse, 2010

Cultivar [a]	Severity 1[a] (%)	Severity 2[b] (%)	FDK (%)	DON (ppm)
Overley	86.5 a	93.4 ab	82.8 a-c	222.1 b
Jagalene	86.6 a	94.3 a	78.0 a-c	338.2 a
Wesley	76.0 a-c	86.4 b-d	83.3 ab	223.8 b
2137	76.4 a-c	93.1 ab	75.0 a-c	66.2 e
Millennium	78.6 ab	88.8 a-d	80.5 a-c	152.8 c
Infinity CL	79.0 ab	89.0 a-d	71.8 a-c	94.2 e
Wahoo	75.8 bc	89.3 a-d	65.3 bc	.[c]
Alliance	83.9 ab	93.3 ab	83.5 ab	147.8 cd
Goodstreak	80.5 ab	91.6 a-c	75.3 a-c	114.2 c-e
Harry	63.9 d	83.1 de	71.5 a-c	95.5 e
Karl 92	83.3 ab	93.3 ab	84.8 a	205.3 b
Overland	66.4 cd	85.4 cd	76.0 a-c	97.6 de
Hondo	58.9 d	77.3 e	63.8 c	76.1 e

[a] FHB severity 10 days post-inoculation (dpi).

[b] FHB severity 21dpi.

[c]Grain sample was insufficient for DON analysis.

Table 6. Rankings (1 = most resistant/highest yielding; 12 = least resistant/lowest yielding) of winter wheat cultivars by Fusarium head blight index, yield, *Fusarium*-damaged kernels (FDK), deoxynivalenol (DON) concentration in grain bulked from plots (DONplot), and DON concentration in grain from symptomatic spikes (DONtag) at Mead in 2008

	Mead				
Cultivar	**Index**	**Yield**	**FDK**	**DONplot**	**DONtag**
Harry	1	8	11	12	10
Hondo	2	4	3	2	3
Goodstreak	3	7	7	4	6
Alliance	4	2	2	3	2
Wahoo	5	11	12	7	5
Infinity CL	6	5	5	9	4
Millennium	7	6	8	6	8
Karl 92	8	1	4	1	7
2137	9	3	1	5	1
Wesley	10	9	9	8	9
Jagalene	11	12	10	10	11
Overley	12	10	6	11	12

Table 7. Rankings (1 = most resistant/highest yielding; 20 = least resistant/lowest yielding) of winter wheat cultivars by Fusarium head blight index, yield, *Fusarium*-damaged kernels (FDK), deoxynivalenol (DON) concentration in grain bulked from plots (DONplot), and DON concentration in grain from symptomatic spikes (DONtag) at Paxton in 2009

	Paxton					
Cultivar	**Index 1[a]**	**Index 2[b]**	**Yield**	**FDK**	**DONplot**	**DONtag**
Goodstreak	1	1	14	3	2	12
Mace	2	13	20	5	3	2
Infinity CL	3	7	4	9	4	18
Art	4	5	8	17	6	1
Overland	5	4	15	10	1	6
Wahoo	6	2	9	7	9	14
Millennium	7	3	13	8	5	16
Harry	8	8	7	20	11	11
Settler CL	9	9	3	18	13	3
Alliance	10	6	17	13	10	13
Hawken	11	12	16	19	12	5
Hatcher	12	11	11	14	8	20
Postrock	13	10	18	4	20	8
Camelot	14	15	2	2	14	17
Bill Brown	15	17	12	15	7	7
Wesley	16	18	6	11	17	9
Bond CL	17	14	1	6	15	15
2137	18	16	19	12	16	19
Jagalene	19	19	5	16	18	10
Overley	20	20	10	1	19	4

[a]Index on 2 July.

[b]Index on 9 July.

Table 8. Rankings (1 = most resistant; 13 = least resistant) of winter wheat cultivars by Fusarium head blight (FHB) severity, *Fusarium*-damaged kernels (FDK), and deoxynivalenol (DON) concentration in a greenhouse trial in 2010

Cultivar	**Severity 1**[a] **(%)**	**Severity 2**[b] **(%)**	**FDK (%)**	**DON (ppm)**
Hondo	1	1	1	2
Harry	2	2	3	4
Overland	3	3	7	5
Wahoo	4	7	2	.[c]
Wesley	5	4	11	11
2137	6	9	5	1
Millennium	7	5	9	8
Infinity	8	6	4	3
Goodstreak	9	8	6	6
Karl 92	10	10	13	9
Alliance	11	11	12	7
Overley	12	12	10	10
Jagalene	13	13	8	12

[a]Severity at 10 days post-inoculation (dpi).

[b]Severity at 21 dpi.

[c] Grain sample was insufficient for DON analysis.

7. FIGURE CAPTIONS

Fig. 1. Deoxynivalenol (DON) concentration in grain from spikes with Fusarium head blight (FHB) symptoms (DONtag) and in grain bulked from plots (DONplot) at Mead (2008) and Paxton (2009). Bars with the same letter within a location are not significantly different according to the least significant difference test at $P = 0.05$.

Fig. 2. Deoxynivalenol (DON) concentration in grain from spikes with Fusarium head blight (FHB) symptoms (DONtag) and in grain bulked from plots (DONplot) at Mead (2008) and Paxton (2009). Bars with the same letter with a DON category are not significantly different according to the least significant difference test at $P = 0.05$.

Fig. 3. Correlation between Fusarium head blight (FHB) index and deoxynivalenol concentration in grain bulked from plots (DONplot) and in grain from symptomatic spikes (DONtag). A, C: Mead, 2008; B, D: Paxton, 2009, second disease assessment.

Fig. 4. Correlation between Fusarium head blight (FHB) severity and deoxynivalenol (DON) concentration and between FHB severity and *Fusarium*-damaged kernels (FDK) in a greenhouse cultivar trial in 2010. A, C: FHB severity at 10 days post-inoculation (dpi); B, D: FHB severity at 21 dpi.

FIGURE 1.

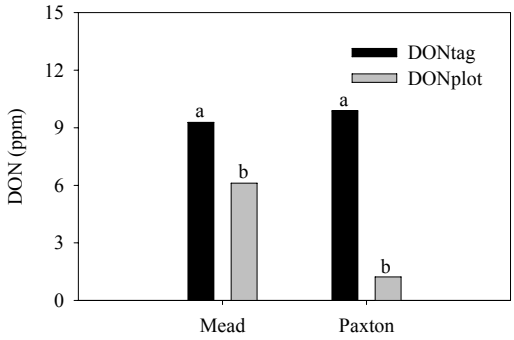


FIGURE 2.

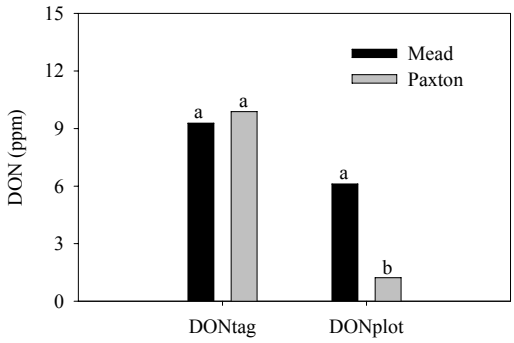


FIGURE 3.

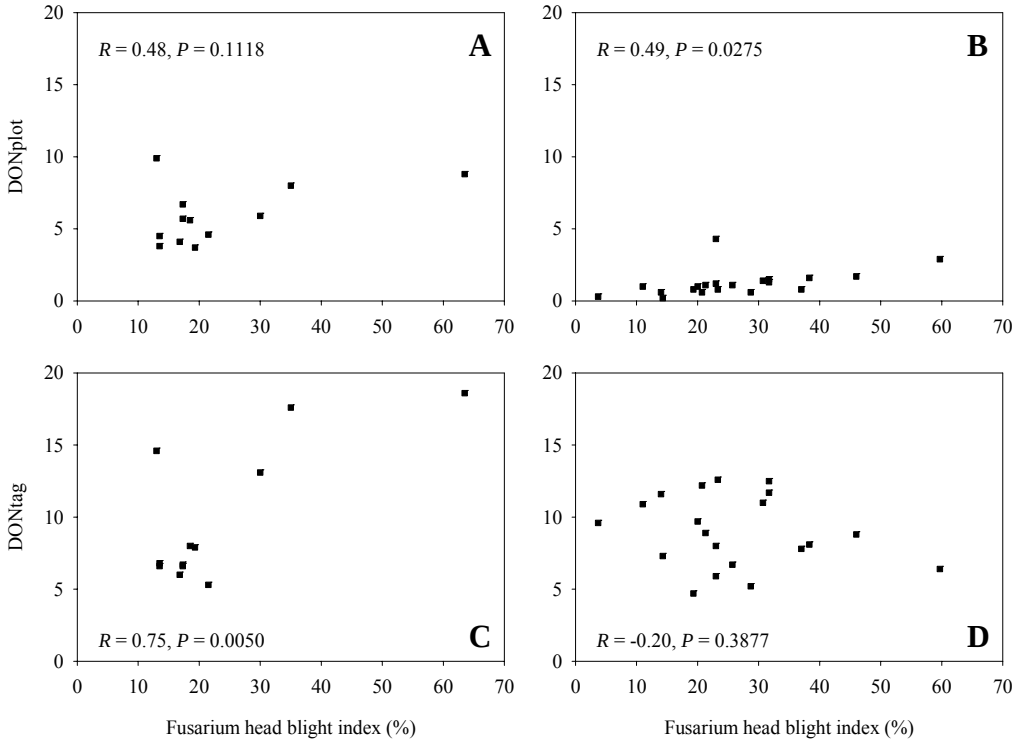
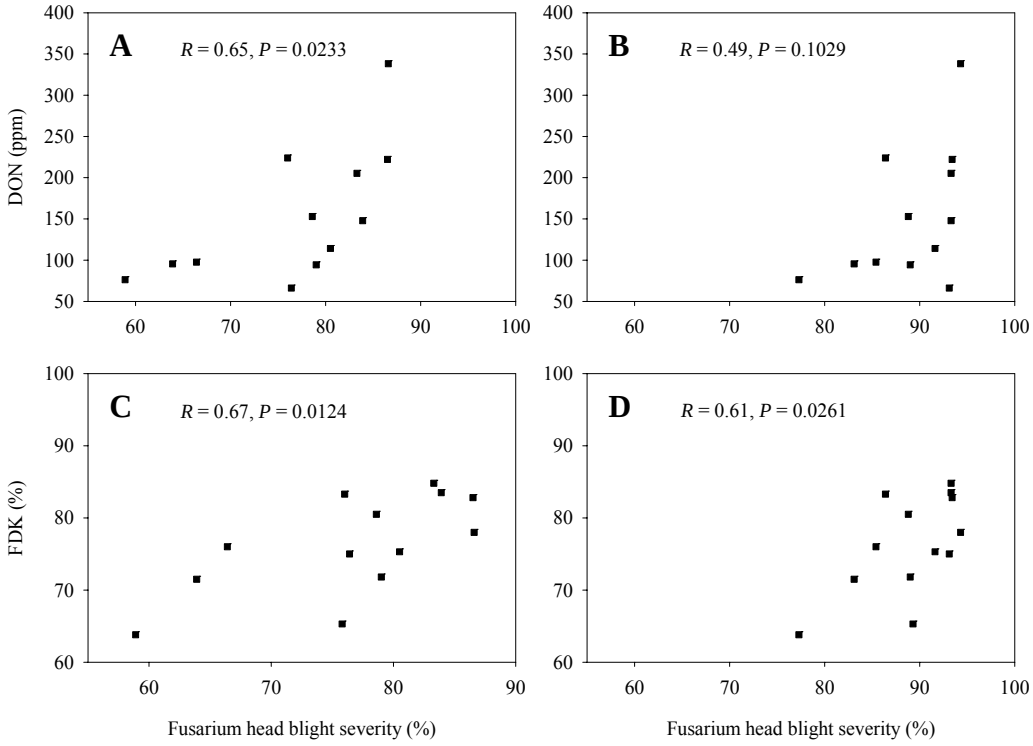

A
R = 0.48, P = 0.1118
B
R = 0.49, P = 0.0275
C
R = 0.75, P = 0.0050
D
R = -0.20, P = 0.3877
DONplot
DONtag
Fusarium head blight index (%)
Fusarium head blight index (%)

FIGURE 4.


A
R = 0.65, P = 0.0233
B
R = 0.49, P = 0.1029
C
R = 0.67, P = 0.0124
D
R = 0.61, P = 0.0261
DON (ppm)
FDK (%)
Fusarium head blight severity (%)
Fusarium head blight severity (%)

CHAPTER III

EFFECT OF CULTIVAR ON THE RELATIONSHIP BETWEEN FUSARIUM HEAD BLIGHT SEVERITY AND DEOXYNIVALENOL CONCENTRATION IN WINTER WHEAT

1. INTRODUCTION

Fusarium head blight (FHB), caused mainly by *Fusarium graminearum* (sexual stage, *Gibberella zeae*) is an economically important disease of wheat and other small grain cereals in the United States and other parts of the world. FHB results in yield loss and poor grain quality (6,14,17). In addition, *F. graminearum* produces the toxin deoxynivalenol (DON) which accumulates in grain (14,17). Since 2007, major epidemics of FHB have occurred in the Central Great Plains, causing significant economic losses in hard winter wheat in the states of Kansas, Nebraska, and South Dakota. Research is needed to develop effective management strategies for FHB and DON in this region. Specifically, there is lack of information on the relationship between FHB intensity and DON concentration in winter wheat cultivars grown in the Central Great Plains.

Although this relationship has been shown to be generally linear and positive (13,18), it has not been determined whether the strength of the relationship varies among cultivars in winter wheat. Information on the cultivar dependence of this relationship can enable wheat producers to make informed decisions regarding choice of cultivars and

control strategies. In addition, such information can be useful to researchers who wish to model this relationship.

Previous studies have shown that FHB-susceptible wheat cultivars generally accumulate more DON than resistant cultivars. In North Carolina, Cowger et al. (4) showed that when post-anthesis mist was applied or not applied to *F. graminearum*-inoculated soft red winter wheat, the susceptible cultivar USG 3592 accumulated more DON than moderately resistant cultivars. In Hungary, Mesterhazy et al. (15,16) observed more DON accumulation in susceptible than in resistant winter wheat cultivars. In Germany, Koch et al. (11) similarly observed that a highly susceptible winter wheat cultivar accumulated more DON than a moderately resistant cultivar. In the current study we report the accumulation of high concentrations of DON in a winter wheat cultivar with a moderately resistant FHB phenotype. The objectives of the study were to 1) assess differences in resistance/susceptibility to FHB and DON among three winter wheat cultivars commonly grown in the Central Great Plains, 2) model the relationship between FHB severity and DON concentration in the three winter wheat cultivars in objective 1 (above), and 3) determine the effect of winter wheat cultivar on the relationship between FHB severity and DON concentration. Preliminary results have been published (9).

2. MATERIALS AND METHODS

2.1. Weather. Weather data were obtained from the Mead station of the Automated Weather Data Network near Mead, NE.

2.2. Cultivars. Two winter wheat cultivars were planted on 5 October 2006 (Harry) and 9 October 2006 (2137) following soybean (*Glycine max*) harvest, and three winter wheat cultivars (Harry, 2137 and Jagalene) were planted on 27 October 2007 and 3 October 2008 following corn (*Zea mays*) harvest at the University of Nebraska Agricultural Research and Development Center near Mead, NE. Plot size was 3.1 m by 9.2 m in 2006, 2.0 m by 9.2 m in 2007, and 1.5 m by 3.3 m in 2008. Harry is a late maturing hard red winter wheat cultivar (1) with a moderately resistant FHB phenotype (3,27). Jagalene and 2137 are hard red winter wheat cultivars (10,21) susceptible to FHB (3,5)

2.3. Planting and inoculation. Seed was planted with a small plot drill at a seeding rate of 72 kg/ha in 2006, 101 kg/ha in 2007, and 80 kg/ha in 2008. Standard agronomic practices for wheat production were followed. Field plots were artificially inoculated with a spore suspension of *F. graminearum* at 1 x 10^5 spores/ml at mid-anthesis (Zadoks growth stage 65; 28) using a backpack sprayer. There also was heavy natural inoculum in the field. Mid anthesis was considered to be the day on which 50% of the heads of a given cultivar had extruded anthers. Thus, cultivars were inoculated on different dates within a season ranging from 28 May to 4 June. Cultivars were arranged in a randomized complete block design with three replications.

2.4. Disease assessment and harvesting. Two separate disease assessments were made 21 days after inoculation in all three years. In the first assessment, twenty spikes were tagged representing each of the following FHB severity categories in each plot: 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50% severity in 2007 and 2009. Two more severity

categories (70% and 90%) were added in the 2008 experiment. Tagging facilitated the identification, separation and hand harvesting of each spike in each disease severity category. In the second assessment, disease incidence and severity were determined on 20 spikes in each of 30 arbitrarily selected locations in each plot and used to calculate index using the formula: index (%) = [incidence (%) x severity (%)]/100. Hand harvesting of tagged spikes was done when grain moisture content dropped below 15%. The rest of the wheat was harvested with small plot combine.

2.5. FDK and DON analysis. The percentage of *Fusarium*-damaged kernels (FDK) in grain from each plot was determined at the USDA ARS Grain Marketing and Production Research Center, Engineering Research Unit, Manhattan, KS using a single-kernel near-infrared (SKNIR) system (7,26). Samples were then ground to flour (Laboratory Construction CO. Kansas City, MO. Model 256), and sent to the North Dakota Veterinary Diagnostic Laboratory, North Dakota State University, Fargo ND for DON analysis using gas chromatography with electron capture detection (GC/ECD) (24). DON concentration was determined in grain bulked from each plot (DON_{plot}) and in grain from tagged spikes in each FHB severity category (DON_{cat}).

2.6. Data analysis. The general linear models (GLM) procedure of SAS version 9.1 (SAS Institute, Cary, NC) was used to analyze data. To test for differences in regression slopes (FHB severity regressed on DON concentration) between cultivars, a split plot design was used to analyze data, with cultivar as the main plot and FHB severity category as the subplot. The severity category sums of squares were partitioned into linear and

quadratic components. Regression slopes were significantly different between cultivars if the interaction between cultivar and the linear component of the partitioned sums of squares was significant at $P \leq 0.05$.

A randomized complete block design was used to analyze plot data (index, yield, FDK, DON). To determine the effects of year and year x cultivar interaction on measured variables, only the two cultivars that were tested in all three years (Harry and 2137) were used in analysis of variance. Fisher's least significant difference test (LSD, $P = 0.05$) (8,22) was used to compare pairs of cultivar means. Regression analysis (8,22) was used to model the relationship between FHB severity (independent variable) and DON (dependent variable) in each cultivar. Effects of cultivar and year x cultivar interaction were considered significant at $P \leq 0.05$.

3. RESULTS

3.1. Weather. There was above average rainfall in May 2007 (16.0 cm total) followed by a relatively dry June (4.0 cm) (Table 1). Average relative humidity (RH) was 68.1% and 70.2% in May and June, respectively. Average temperature was 18.7^{o}C and 21.8^{o}C in May and June, respectively. In 2008, there was above average rainfall during both May (13.7 cm) and June (23.4 cm). Average relative humidity (RH) was 65.6% and 70.0% in May and June, respectively. Average temperature was 15.3^{o}C and 21.9^{o}C in May and June, respectively. In 2009, May was relatively dry (2.5 cm of rain). Rain started in late May and continued through June, with a moderate amount of 10.7 cm total

rainfall for the month of June. Maximum RH was > 90% in May and June in all three years except May 2009. Therefore, weather conditions during the three week period before wheat flowering and through the flowering period in late May to early June favored FHB infections in 2007 and 2008, but not in 2009.

3.2. Differences among cultivars in yield and FHB parameters. The effect of cultivar was significant ($P \leq 0.05$) for index, average DON from severity categories (DON_{catavg}), and total DON from severity categories (DON_{cattot}) (Table 2). The effect of year was significant for all measured variables whereas year by cultivar interaction was significant for index, DON measured in grain bulked from plots (DON_{plot}), DON_{catavg}, and DON_{cattot} (Table 2). In 2007 there were significant differences between cultivars in FHB index ($P = 0.0074$) and average DON in grain from severity categories (DON_{catavg}) ($P = 0.0146$). 2137 had a higher FHB index (45.7%) than Harry (16.7%) whereas Harry had higher DON_{catavg} (2.9 ppm) than 2137 (0.9 ppm). The yield in 2137 (3958 kg ha^{-1}) was higher than that in Harry (2708 kg ha^{-1}); however, this difference was not significant ($P = 0.0758$). The two cultivars similarly did not significantly differ in FDK ($P = 0.3316$), and DON bulked from plots (DON_{plot}, $P = 0.3727$) (Table 3).

In 2008, there were significant differences among cultivars in FHB index ($P = 0.0132$) and FDK ($P = 0.0036$). Jagalene had the highest FHB index (29.3%) followed by 2137 (19.7%) and Harry (12.0%). However, index did not differ between Harry and 2137. Harry had the highest FDK (64%) followed by Jagalene (55.3%) and 2137 (46.0%). Due to late planting followed by very low soil temperatures in the fall of 2007, the yield in 2008 was very low and did not differ among cultivars, ranging from 208 kg

ha^{-1} (Jagalene) to 625 kg ha^{-1} (2137). Cultivars similarly did not differ in DON_{plot} (P = 0.1240) and DON_{catavg} (P = 0.0655) (Table 3).

In 2009, there were significant differences among cultivars in yield (P = 0.0240). The yield in Harry (2352 kg ha^{-1}) and 2137 (2338 kg ha^{-1}) was similar and significantly higher than the yield in Jagalene (1102 kg ha^{-1}). Cultivars did not differ in FHB index (P = 0.1031), FDK (P = 0.9842), DON_{plot} (P = 0.3739), and DON_{catavg} (P = 0.1591) (Table 3).

In 2007, significant differences among cultivars in DON from different FHB severity categories (DON_{cat}) were found in five out of eleven categories: 10% (P = 0.0173), 25% (P = 0.0023), 30% (P = 0.0101), 45% (P = 0.0326), and 50% (P = 0.0497). An analysis of total DON accumulated in all categories (DON_{cattot}) showed that there were significant differences among cultivars in the amount of DON, with Harry having more DON_{cattot} than 2137 (P = 0.0146) (Table 4).

In 2008, regardless of significant differences at P = 0.05, Harry again accumulated more DON_{cat} than 2137 in all 13 categories. There was a significant difference in three categories: 10% (P = 0.0095), 15% (P = 0.0072), and 20% (P = 0.0186). Harry accumulated more DON_{cat} than Jagalene in only two out of thirteen categories: 10% (P = 0.0095) and 15% (P = 0.0072). Harry accumulated more DON_{cattot} (418.2 ppm) than 2137 (241.0 ppm), but had a DON_{cattot} amount similar to that in Jagalene (378.0 ppm) (Table 4).

In 2009, regardless of significant differences at P = 0.05, Harry accumulated more DON_{cat} than 2137 in ten out of eleven categories. Significant differences were found in

two categories: 15% ($P = 0.0250$), and 45% ($P = 0.0231$). DON_{cattot} did not significantly differ among cultivars ($P = 0.1591$) (Table 4).

3.3. Relationship between FHB severity and DON concentration. There was a positive linear relationship between FHB severity and DON from grain in each severity category (DON_{cat}) in all three years regardless of cultivar ($0.41 \leq R^2 \leq 0.84$, Figs. 1-3). In 2007, this relationship was stronger for Harry ($R^2 = 0.55$) than 2137 ($R^2 = 0.50$) (Fig.1). A test of differences in regression slopes (DON concentration regressed on FHB severity category) showed significant differences between the two cultivars ($P = 0.0008$, Table 5). In 2008, the relationship was strongest for Jagalene ($R^2 = 0.84$) followed by 2137 ($R^2 = 0.75$), and Harry ($R^2 = 0.41$) (Fig. 2). A test for differences in regression slopes showed that there were highly significant differences among cultivars ($P < 0.0001$, Table 5). In 2009, the relationship was strongest for Jagalene ($R^2 = 0.83$), followed by Harry ($R^2 = 0.60$), and 2137 ($R^2 = 0.54$) (Fig. 3). A test for differences in regression slopes showed that unlike the previous two years, there were no differences among cultivars ($P = 0.8976$, Table 5). The slope and intercept of the regression line for each cultivar varied from year to year. Harry had the highest intercept in all three years (Figs. 1-3) indicating that in years of both low and high infection, Harry was consistently a high DON accumulator.

4. DISCUSSION

This study has demonstrated a positive linear relationship between DON accumulation and FHB severity (Figs. 1-3). This suggests that DON accumulation in grain increases with increasing FHB severity. This relationship was found to be consistent during all three years of this study. This result is consistent with previous findings. Paul et al. (18) used meta-analysis to analyze 163 studies for associations between FHB intensity and DON concentration. They found more than 65% of all correlation coefficients to be > 0.50. However, they also found negative correlations, with correlation coefficients (*r*) ranging from -0.58 to 0.99. In the current study, all associations between FHB severity and DON were linear and positive. This may be due to the fact that in the current study, spikes with increasing FHB severity categories were systematically identified and FHB severity in these categories was correlated to DON concentration in individual cultivars, whereas in the study of Paul et al. (18), *r* values were obtained from many different studies by correlating several FHB intensity parameters (incidence, severity, index, and *Fusarium*-damaged kernels (FDK)) with DON concentration. In controlled environment studies, Stein et al. (29) similarly found a positive linear relationship between inoculum concentration and DON concentration in inoculated spikes of spring wheat (disease incidence and severity increased with increasing inoculum concentration).

This study demonstrated differences among winter wheat cultivars in the relationship between FHB severity and DON concentration. The slope and intercept of the regression line (DON regressed on FHB severity) for each cultivar varied from year to year, implying that environment can influence the amount of DON that accumulates

per unit of FHB severity, as evidenced by the significant effect of year and the significant interaction between year and cultivar (Table 2). Harry consistently had the highest intercept, implying that Harry, with a moderately resistant FHB phenotype, accumulated more DON than the susceptible 2137. Paul et al. (26) reported differences in the slope and intercept of the regression line between FHB intensity and DON concentration between wheat types (spring versus winter wheat). In contrast, this study reports differences in these parameters in individual cultivars within a wheat type (winter wheat). These differences imply that winter wheat cultivars differ in the amount of DON they accumulate per unit of FHB severity.

Harry had a significantly lower FHB index (disease assessment in plots) than 2137 in 2007 and 2008 when disease intensity was high. This result is consistent with the results of separate studies conducted concurrently with this study (25). In 2009, FHB intensity was very low compared to 2007 and 2008. Hence, cultivars did not differ in FHB index. The higher (but non-significant) FHB index in Harry in 2009 (Table 3) was due to the timing of rain and flowering. It was dry during the three weeks in May preceding flowering of 2137 and Jagalene, as well as during flowering of these two cultivars (Table 1). However, rainfall coincided with flowering of the late maturing Harry in late May to early June, hence more FHB infections occurred in Harry compared to 2137 and Jagalene.

The fact that Harry, a moderately resistant cultivar, accumulated more DON than the susceptible 2137 and accumulated DON amounts comparable to Jagalene, also a susceptible cultivar, suggests that cultivars with resistance to FHB are not necessarily resistant to DON accumulation. Results from this study are in agreement with the results

of Lemmens et al. (13), who also found genotypic differences in DON accumulation when ten winter wheat genotypes were inoculated with a DON-producing *F. culmorum* strain and kept under mist-irrigated or non-irrigated conditions. Cowger et al. (4) similarly demonstrated differences in DON accumulation among winter wheat cultivars in a misted field nursery. However, in the study of Cowger et al. (4), moderately resistant cultivars accumulated less DON than susceptible cultivars whereas in this study the moderately resistant Harry accumulated more DON than the susceptible 2137. The reason for the greater accumulation of DON in the moderately resistant Harry compared to the susceptible 2137 is not known and warrants investigation.

Harry and 2137 did not differ in yield in all three years of this study. However, Jagalene had lower yield than Harry and 2137 in the two years (2008 and 2009) it was tested. This was due to the greater susceptibility of Jagalene not only to FHB, but to several foliar diseases including leaf rust (*Puccinia triticina*), tan spot (*Pyrenophora tritici-repentis*), and Septoria tritici blotch (*Septoria tritici*) that occurred during the growing season in all three years. The very low yield in 2008 compared to 2007 and 2009 was due mainly to late planting followed by very low soil temperatures in the fall of 2007. FDK did not differ among cultivars in 2007 and 2009. However, in 2008, differences were significant, with Harry having the highest FDK and 2137 the lowest. Separate studies conducted concurrently with this study also showed inconsistency in differences in FDK among the three cultivars (25).

In all three years of this study, cultivars did not differ in DON measured in grain bulked from plots, whereas there were differences among cultivars in DON measured in grain from symptomatic spikes in the different FHB severity categories (Tables 3 and 4).

In addition, in 2009 when FHB intensity was very low, cultivars did not differ in DON regardless of whether it was measured in grain bulked from plots or from symptomatic spikes. These results suggest that differences among winter wheat cultivars in DON accumulation were more discernible if DON was measured in grain from symptomatic spikes and in years when FHB intensity was high compared to grain bulked from plots and years when FHB intensity was low.

Asymptomatic spikes (spikes that were in category zero of FHB severity) accumulated DON in all three cultivars. This is evidence of symptomless infections of wheat spikes by *F. gramineaum*. Lacey et al. (12) reported accumulation of small amounts of DON and isolation of *F. culmorum* in the absence of head blight symptoms in winter wheat. In this study, however, significant amounts of DON (up to 23.7 ppm, Table 4) were detected in spikes that apparently had no visible symptoms. It is possible that symptoms in the 0% FHB category developed after tagging of heads, but they were not noticeable due to the natural turning of spike color as the wheat crop matured.

This study has demonstrated a positive linear relationship between FHB severity and DON concentration in winter wheat cultivars 2137, Harry, and Jagalene under Nebraska conditions. This relationship was shown to be cultivar dependent, implying that some cultivars can accumulate more DON per unit of FHB severity than others. Therefore, wheat producers should consider resistance to both FHB and DON when selecting cultivars. The cultivar Harry with a moderately resistant FHB phenotype accumulated DON amounts greater than those in the susceptible 2137 and similar to those in the susceptible Jagalene. More research is needed to determine why Harry with a moderately resistant FHB phenotype is susceptible to DON accumulation. This study

also determined that differences in DON accumulation among winter wheat cultivars were more discernible in years with high disease intensity and in grain from symptomatic spikes compared to years with low disease intensity and grain bulked from plots. Small grain breeders and other researchers can use this information to more accurately determine differences in DON concentration among cultivars and breeding lines.

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6. TABLES

Table 1. Weather parameters in May and June at Mead, NE, 2007-2009

	Total rain (cm)	Average RH (%)	RH Range (%)	Average Min Temp (^{o}C)	Average Max Temp (^{o}C)	Average Temp (^{o}C)
2007						
May	16.0	68.1	46.3-91.1	12.4	25.0	18.7
June	4.0	70.2	43.4-90.6	15.4	28.1	21.8
2008						
May	13.7	65.6	35.5-93.8	8.4	22.2	15.3
June	23.4	70.0	48.0-90.9	15.4	28.4	21.9
2009						
May	2.5	58.4	37.7-85.8	9.5	24.5	17.0
June	10.7	75.5	40.1-94.8	15.2	27.1	21.1

Table 2. Year and cultivar effects on Fusarium head blight (FHB) index, yield, *Fusarium*-damaged kernels (FDK), and deoxynivalenol (DON) concentration in two winter wheat cultivars (Harry, and 2137), 2007-2009

Source of variation	d.f.	Mean square					
		Index	Yield	FDK	DON_{plot}[a]	DON_{catavg}[b]	DON_{cattot}[c]
Year (Y)	2	1159.35****	2526.91****	2321.45*	172.99****	932.12****	165593.69****
Reps (Year)	6	19.14	33.93	100.90	5.20	8.85	1470.77
Cultivar (C)	1	467.16**	174.22	327.68	4.10	175.47**	27043.63**
Y x C	2	469.16***	147.19	116.18	3.47*	60.89*	11041.03*
Pooled error	6	14.22	32.07	348.11	1.45	6.04	1012.34
Total	17						

[a]DON measured in grain bulked from plots.

[b]DON averaged across FHB severity categories (11 categories in 2007 and 2009, 13 categories in 2008).

[c]Total DON in all FHB severity categories.

* $0.01 \leq P \leq 0.05$

** $0.001 \leq P \leq 0.01$

*** $0.0001 \leq P \leq 0.001$

**** $P \leq 0.0001$

Table 3. Fusarium head blight (FHB) index, yield, *Fusarium*-damaged kernels (FDK), deoxynivalenol concentration in grain harvested from plots (DON_{plot}), and average deoxynivalenol concentration from grain harvested from tagged heads in increasing FHB severity categories (DON_{catavg}) in three winter wheat cultivars, 2007-2009

Year	Cultivar	Index[a] (%)	Yield (kg/ha)	FDK (%)	DON_{plot} (ppm)	DON_{catavg} (ppm)
2007	Harry	16.7[b]	2707.8[a]	19.7[a]	1.1[a]	2.9[a]
	2137	45.7[a]	3957.5[a]	12.7[a]	1.5[a]	0.9[b]
2008	Harry	12.0[b]	544.2[ab]	64.0[a]	11.9[a]	32.2[a]
	2137	19.7[b]	624.9[a]	46.0[c]	9.3[a]	18.6[b]
	Jagalene	29.3[a]	208.3[b]	55.3[b]	12.5[a]	29.1[ab]
2009	Harry	6.5[a]	2351.7[a]	30.4[a]	1.7[a]	8.0[a]
	2137	0.4[a]	2338.2[a]	29.8[a]	1.0[a]	4.8[a]
	Jagalene	1.3[a]	1101.9[b]	27.2[a]	0.8[a]	4.3[a]

[a] Means within a column with in a year followed by the same letter are not significantly different according to Fisher's least significant difference test at $P = 0.05$.

Table 4. Deoxynivalenol (DON) concentration by Fusarium head blight severity category in three winter wheat cultivars in field plots, 2007-2009

Year	Cultivar	FHB severity categories (%)													
		0	5	10	15	20	25	30	35	40	45	50	70	90	DON_{cattot}
2007	Harry	0.4[a]	0.4[a]	1.7[a]	3.0[a]	3.1[a]	4.7[a]	4.5[a]	2.6[a]	2.4[a]	4.3[a]	4.4[a]	---	---	31.5[a]
	2137	0[a]	0[a]	0.2[b]	1.2[a]	1.6[a]	0.9[b]	1.1[b]	1.7[a]	1.0[a]	0.9[b]	1.6[b]	---	---	10.3[b]
2008	Harry	23.7[a]	31.4[a]	32.5[a]	32.1[a]	31.5[a]	35.2[a]	29.2[a]	31.6[a]	33.4[a]	30.0[a]	36.6[a]	33.5[a]	37.4[ab]	418.2[a]
	2137	12.9[a]	13.3[a]	12.9[b]	12.4[c]	10.8[b]	17.1[b]	18.6[a]	23.1[a]	21.0[a]	23.2[a]	25.2[a]	27.0[a]	24.5[b]	241.9[b]
	Jagalene	17.5[a]	24.9[a]	21.6[b]	22.8[b]	24.4[a]	23.6[ab]	25.1[a]	28.9[a]	30.8[a]	35.2[a]	33.0[a]	33.7[a]	56.6[a]	378.0[ab]
2009	Harry	3.9[a]	6.1[a]	5.1[a]	9.2[a]	6.9[a]	8.6[a]	6.3[a]	12.2[a]	8.7[a]	10.2[a]	10.2[a]	---	---	87.5[a]
	2137	2.1[ab]	1.8[ab]	2.8[a]	4.2[b]	5.2[a]	4.6[a]	7.0[a]	7.1[a]	6.6[a]	3.0[b]	8.1[a]	---	---	52.4[a]
	Jagalene	0.4[b]	0.9[b]	3.6[a]	3.8[b]	3.7[a]	4.9[a]	5.7[a]	6.4[a]	5.0[a]	5.8[b]	6.9[a]	---	---	47.0[a]

[a] Means within a column followed by the same letter are not significantly different according to Fisher's least significant difference test at $P = 0.05$.

Table 5. Analysis of variance to test for differences in regression slopes (DON concentration regressed on FHB severity categories) in three field-grown winter wheat cultivars, 2007-2009

Source of variation	d.f.			Mean square		
	2007	2008	2009	2007	2008	2009
Rep[a]	2	2	2	2.71*	81.22	33.89*
Cultv[b]	1	2	2	61.48****	1969.73****	131.70****
Error (a)	2	4	4	0.92	338.92****	43.69**
Severity category[c]						
Linear (S)[d]	(1)	(1)	(1)	37.73****	3125.19****	298.71****
Quadratic (S*S)[e]	(1)	(1)	(1)	9.89***	0.43	24.40
Residual (Cat)	(8)	(10)	(8)	1.68*	29.96	5.56
(S-Linear)*Cultv[f]	1	2	2	7.72***	414.32****	0.95
(S-quadratic)*Cultv	1	2	2	1.38	131.55*	0.24
Cultv*Cat	8	20	16	1.67*	29.96	5.56
Error (b)	40	72	60	0.59	27.57	8.74
Total	65	116	98			

[a] Replications.

[b] Winter wheat cultivars: Harry and 2137 (2007); Harry, 2137, and Jagalene (2008 and 2009).

[c] FHB severity categories in wheat spikes (2007 and 2009 categories were 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50%. 2008 additionally had 70 and 90%).

[d]Test for linear model.

[e]Test for quadratic model.

[f] Test for differences among slopes.

* $0.01 \leq P \leq 0.05$

** $0.001 \leq P \leq 0.01$

*** $0.0001 \leq P \leq 0.001$

**** $P \leq 0.0001$

7. FIGURE CAPTIONS

Fig. 1. Linear regression of DON on FHB severity in winter wheat cultivars Harry and 2137 in field experiments conducted in 2007.

Fig. 2. Linear regression of DON on FHB severity in winter wheat cultivars Harry, 2137, and Jagalene in field experiments conducted in 2008.

Fig. 3. Linear regression of DON on FHB severity in winter wheat cultivars Harry, 2137, and Jagalene in field experiments conducted in 2009.

FIGURE 1.

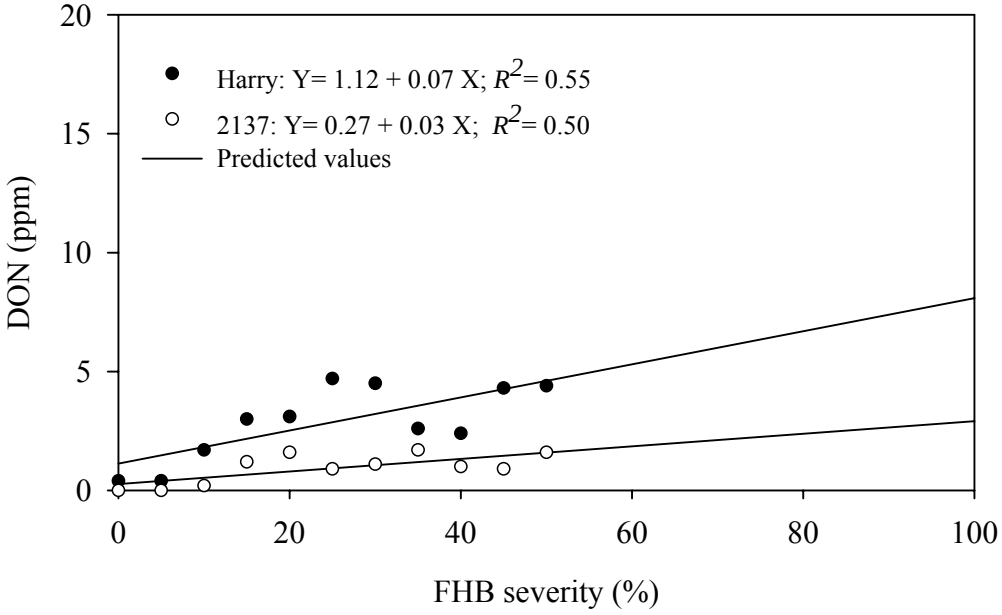

Harry: Y= 1.12 + 0.07 X; R^2= 0.55
2137: Y= 0.27 + 0.03 X; R^2= 0.50
Predicted values
DON (ppm)
FHB severity (%)
0
5
10
15
20
0
20
40
60
80
100

FIGURE 2.

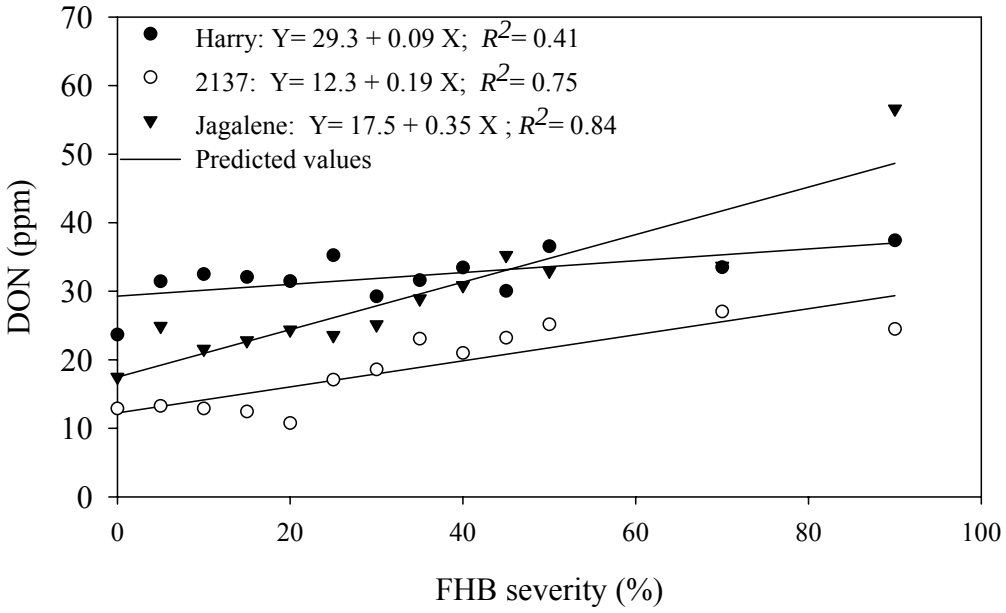
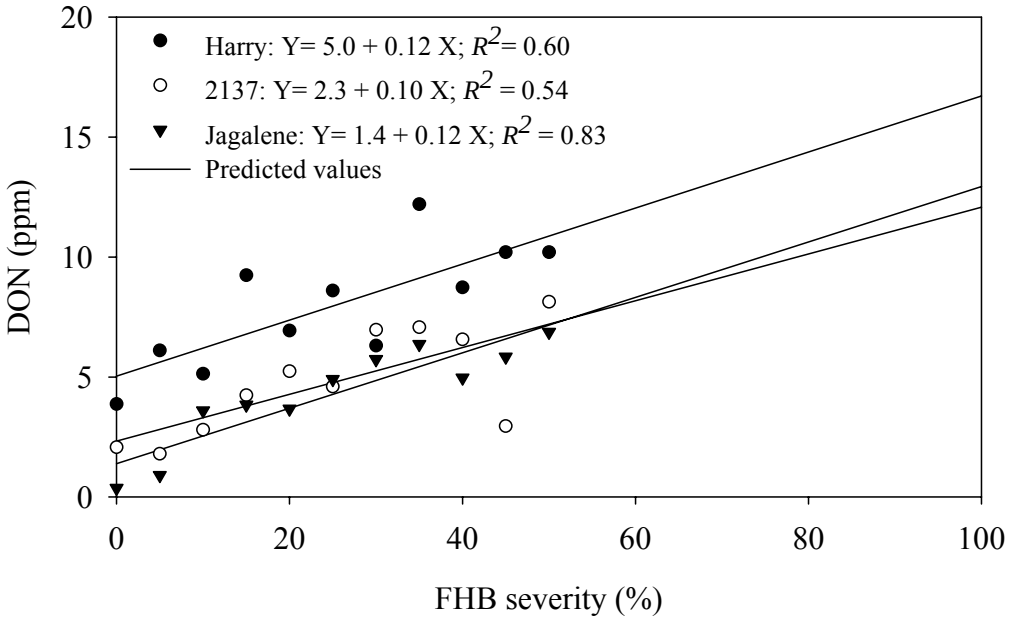

Harry: Y= 29.3 + 0.09 X; R^2= 0.41
2137: Y= 12.3 + 0.19 X; R^2= 0.75
Jagalene: Y= 17.5 + 0.35 X ; R^2= 0.84
Predicted values
DON (ppm)
FHB severity (%)
0
10
20
30
40
50
60
70
0
20
40
60
80
100

FIGURE 3.


Harry: Y= 5.0 + 0.12 X; R^2= 0.60
2137: Y= 2.3 + 0.10 X; R^2 = 0.54
Jagalene: Y= 1.4 + 0.12 X; R^2 = 0.83
Predicted values
DON (ppm)
FHB severity (%)
0
5
10
15
20
0
20
40
60
80
100

CHAPTER IV

CHARACTERIZATION OF NEBRASKA ISOLATES OF *Fusarium graminearum* FROM WINTER WHEAT.

1. INTRODUCTION

Fusarium head blight (FHB) caused by *Fusarium graminearum* Schwabe (Teleomorph: *Gibberella zeae* (Schwein.) Petch)) is a destructive disease and has an important economical impact not only in wheat but also in other small grains. Losses to FHB result from yield reduction, presence of *Fusarium*-damaged kernels (FDK), and accumulation of the mycotoxin deoxynivalenol (DON) in grain. In the central Great Plains, FHB epidemics have occurred sporadically due to a variable climate. However, since the early 1990s, FHB outbreaks have become more frequent in this region and other wheat growing areas in the United States (12).

In Nebraska, FHB has occurred yearly to varying levels of severity and prevalence since 2007, with the worst epidemics in over 20 years occurring in 2007 and 2008. In addition to *F. graminearum*, several other species of *Fusarium* are causal agents of FHB, including *F. culmorum* (Wm. G. Smith) Sacc. and *F. avenaceum* (Fr.:Fr.) Sacc. It is known that the most important causal agent of FHB in the U.S. is *F. graminearum* (14). However, this has not been confirmed in Nebraska in recent years. Knowledge of the major species of *Fusarium* causing FHB in Nebraska will be useful to researchers and in devising management strategies for the disease. Traditional diagnostic methods for

identification of *F. graminearum* are based on morphological characteristics, but this procedure can be time consuming and may not be accurate in distinguishing between similar species (9).

Species identification using molecular and morphological techniques simultaneously can give more confidence than identification using either method alone. Different molecular techniques have been used to identify *F. graminearum* but without total success in species-specific identification (25). Niessen and Vogel (24) described a duplex PCR method for identification of *F. graminearum* using a set of primers designed to detect a galactose oxidase-producing *Gibberella zeae* strain. A fragment of 900 bp was amplified using this method. Based on this technique, Knoll et al. (19) described an identification method using DNA detection with test stripes. However, the authors of this set of primers reported that this method failed to identify one *F. graminearum* strain that was a galactose oxidase producer (9). A PCR method for detection of *F. culmorum*, *F. graminearum* and *F. avenaceum* was published by Schilling et al. (28). This method used internal transcribed regions (ITS) of nuclear ribosomal DNA but they were not polymorphic enough to make a clear distinction among the species analyzed. It is known today how variable the ITS can be. A new protocol for *F. graminearum* identification was developed using the internal 3' coding region of the *gao*A gene. The set of primers designed for this purpose consisted of GOFW 5'-ACCTCTGTTGTTCTTCCAGACGG-3' and GORV 5' -CTGGTCAGTATTAACCGTGTGTG- 3'. The amplification product of this set is a fragment of 435 bp of an internal region of the gene (9). This test showed specificity for *F. graminearum* with as low concentrations as 4.0 ng of DNA.

Other methods have been used for *F. graminearum* identification without isolation and morphological identification of the fungus (21, 7). In the Netherlands, Waalwijk et al. (33) used a set of 18 primers to identify *F. avenaceum*, *F. culmorum*, *F. graminearum*, *F. poae*, *F. proliferatum*, *Microdochium nivale* var. *nivale*, and *M. nivale* var. *majus*. Once the species of *Fusarium* causing FHB in a region or state is identified, it is important to know if isolates of the species differ in characteristics such as pathogenicity (ability to cause disease) aggressiveness (rate of disease progression), and DON production.

Perithecia production has been linked to pathogenicity. Urban et al. (32) identified the *MAP1* gene in *F. graminearum* and showed that *MAP1* mutants were non-pathogenic and lost the ability to produce perithecia, hence establishing a link between pathogenicity and perithecia production. Some studies have shown a positive correlation between DON production and aggressiveness in the *F. graminearum*-wheat pathosystem (11). However, other studies have failed to demonstrate a positive correlation. Gilbert et al. (15) reported a significant variation in the levels of mycotoxin production in 16 Canadian isolates of *F. graminearum*. Knowledge about the isolate characteristics of the major *Fusarium* species causing FHB in Nebraska can be used to develop management strategies for the disease.

Several techniques can be used to characterize isolates of a *Fusarium* species causing FHB. They include quantification of perithecia production, measurement of aggressiveness on wheat spikes and detached leaves, and determination and quantification of the trichothecene chemotype produced by the isolate (23, 14). Under laboratory conditions, perithecia can be produced both in vivo (18) and in vitro (30).

Carrot agar has been the medium of choice for in vitro production of perithecia in several studies (3, 30, 16, 27, 13, 31). Browne et al. (5, 6) used detached leaf assays to evaluate wheat lines and commercial cultivars for resistance to FHB. This technique can also be utilized in the evaluation of the aggressiveness of isolates of a given species of *Fusarium* causing FHB. Advantages of using a detached leaf assay instead of wheat spikes for this purpose include the evaluation of a large number of isolates within a short period of time and savings in time and resources since leaves are more plenty than spikes and the requirements of vernalization and growing plants to the spike stage are eliminated. Pathogen aggressiveness can be measured as lesion size at a given time following inoculation or as area under the disease progress curve AUDPC (1, 8, 4, 10, 29).

Due to the recent FHB epidemics in Nebraska and the need to develop management strategies for the disease, laboratory and greenhouse experiments were conducted in 2009-2010 to 1) use Polymerase Chain Reaction and morphological characteristics to identify the major species of *Fusarium* causing FHB in the state, and 2) quantify perithecia production and the aggressiveness of selected isolates of the species of *Fusarium* identified in objective 1.

2. MATERIALS AND METHODS

2.1. Isolation of *F. graminearum* isolates. Samples of wheat kernels from elevators and fields in south central and southeastern Nebraska were collected during the growing

season in 2007 and 2008. *Fusarium*-damaged kernels were disinfected using 1% sodium hypochlorite for 1 min, rinsed with double distilled sterile water (ddwater), and disinfected again with 70% ethanol for 1 min, followed by a second rinse with ddwater for 1 min. After disinfection, two *Fusarium*-damaged kernels (FDK) per Petri plate were incubated at 25°C in 12 h light and 12 h dark on Nash & Snyder peptone PCNB medium (NS) (22) for 5 to 7 days in a low temperature illuminated incubator, model 818, (Thermo Electron Corporation Waltham, MA). Ten-millimeter-diameter mycelial plugs from the actively growing edges of the NS plates were transferred to potato dextrose agar (PDA) plates. After 4 days, approximately 1cm^2 of a mycelial plug was placed into an Eppendorf tube containing 1 ml of ddwater. The mycelia were disrupted with a sterile needle and homogenized with a vortex machine. One hundred microliters of this suspension was used to make serial dilutions (1:10, 1:100, and 1:1000). Three hundred microliters from each dilution were spread and incubated on 2% water agar (WA) plates for 12 - 48 h under the same incubation conditions described above. A single conidium was isolated from each of these plates and placed on NS plates. After 72 h of incubation, transfer to PDA plates was done as described above. Mycelia and spores from the PDA plates were kept in vials at -80°C in a 15% glycerol suspension until needed for experiments. A total of 41 pure culture, single conidium isolates from infected kernels were obtained. Seventeen isolates were from samples collected in 2007 (NE90 to NE110), and 24 isolates were from samples collected in 2008 (NE111 to NE165).

2.2. Molecular identification of *F. graminearum* isolates. Protocols for morphological (32) and molecular (9) characterization for *F. graminearum* were used.

Isolates were grown on carnation leaf agar (CLA) and PDA for their morphological identification. A DNA extraction of each isolate was done using mycelia grown in 25 ml of potato dextrose broth (PDB) in a 125 ml glass flask on a rotation shaker (New Brunswick Scientific Co. Inc. Edison, NJ) at 100 rpm for 48 - 72 h at 25°C, in a 12 h light/dark cycle. Approximately 300 µl of mycelial suspension were used for DNA isolation. A variation of the protocol for isolation of genomic DNA using MPBio GeneClean Spin Kit was used as follows. In Eppendorf tubes 500 µl of DNA extraction buffer (20 ml 1M Tris-HCl (pH 8.5), 8.33 ml 3M NaCl, 5 ml 0.5M EDTA, 5 ml 10% SDS, and 61.67 ml ddwater) was added to the suspension followed by incubation at 65ºC for 20 min. Mycelia were periodically macerated with a small plastic pestle every 5 min. Five hundred microliters of phenol-chloroform (1:1) were added and the Eppendorf tubes were vortexed. Samples were centrifuged at 14,000 rpm for 5 min. While centrifuging, the glass milk solution of MPBio GeneClean Spin Kit was vigorously shaken. Four hundred microliters of the glass milk solution were added to the spin filter and 300 µl of the aqueous phase (top phase) were placed into the solution. Tubes were inverted to mix and incubated for 5 min at room temperature. Samples were centrifuged at top speed for 1 min and flow-through was decanted. Five hundred microliters of the GeneClean wash solution were added. Samples were centrifuged for 1 min and the flow-through was decanted. This step was repeated once. After the second wash step, centrifugation of tubes at top speed was done for 2 min. Filters were transferred to a catch tube. Twenty-five microliters of elution buffer were added to elute the DNA. The samples were then centrifuged for 1 min. The DNA was recovered in the water at the bottom of the tube.

DNA obtained from all isolates was tested for the suitability of PCR amplification using primers ITS4 and ITS5 targeting the ITS 5.8S rRNA region (34). A specific PCR amplification was conducted to identify *F. graminearum* using primers GOFW (5`-ACCTCTGTTGTTCTTCCAGACGG-3`) and GORV (5` -CTGGTCAGTATTAACCGTGTGTG-5`) (9). The amplification was performed in a DNA Engine Peltier thermal cycler, single block model, 60V alpha unit (BioRad Hercules, CA). The reaction was made in PCR tubes according to de Biazio et al. (9): 50 µL of final reaction volume, PCR buffer (Invitrogen Carlsbad, CA) 1X, 1.5 mM $MgCl_2$, 0.2 mM dNTPs mix (Invitrogen), 25 pmol GOFW, 25 pmol GORV, and 20-400 ng of DNA, and 1.5 U of platinum Taq DNA polymerase (Invitrogen). The PCR reaction for GOFW and GORV consisted of 25 cycles of 1 min and 30 s at 94ºC, 1 min and 30 s at 55ºC, and 2 min at 72ºC. Amplification with ITS4 and ITS5 primers differed from amplification using GOFW and GORV in the annealing temperature (50ºC). For both reactions, an initial heating was done at 94ºC for 5 min and a final extension time of 72ºC for 10 min was applied. A positive control (DNA from *F. graminearum,* strain PH-1) was used for amplification and a negative control (no DNA) was also used. Ten microliters of PCR product were analyzed in 1% agarose gel containing 0.25 µg/ml of ethidium bromide in 1X TBE buffer at 80 V. Molecular weight markers (100 bp DNA ladder, Invitrogen) were used to determine the weight of the PCR products. PCR products were visualized and photographed using a Molecular imager Chemi-doc, serial # 765100922 (BioRad).

2.3. Perithecia production. Seventeen Nebraska isolates of *F. graminearum* collected in 2007 were tested for perithecia production. Additionally, a wild type (PH-1) was used as a control. Carrot agar (CA) was the medium used for this fertility study (22). The protocol used is a modification of the protocol of Pasquali and Kistler (27). In a 9-cm-diameter Petri plate, 20 ml of CA was poured. One-centimeter-diameter PDA plugs from the actively growing edge of each isolate were transferred onto CA plates. Each isolate was incubated at 25ºC in 12 h light and 12 h dark. After 96 hours of incubation, 1 ml of 2.5% Tween 60 was applied to each plate and mycelia were homogenized for 30 s with an L-shaped cell spreader (Fisher Scientific Waltham, MA). Plates were incubated as previously described. After 10 days, perithecial units were counted and the percentage of perithecia-covered area in each Petri plate was estimated visually. A perithecial unit consisted of a single perithecium or a cluster of perithecia. A randomized complete block design with 4 replications was used and the experiment was conducted twice.

2.4. Pathogenicity and aggressiveness on wheat spikes. An experiment was conducted to determine pathogenicity and quantify the aggressiveness of seven isolates of *F. graminearum* on the spikes of two soft winter wheat cultivars, Coker 9835 and VA03W-433. Based on field evaluations, Cocker is FHB-susceptible and VA03W-433 is FHB-resistant (Carl Griffey, personal communication). Seed of the two cultivars was planted in 15-cm-diameter pots. The soil mix consisted of 1 part clay loam soil, 1/2 part sand, 1/2 part vermiculite and 1 part Canadian sphagnum peat moss. Seed was planted on 3 March 2010 at a rate of one seed per pot after 7 weeks of vernalization at 4ºC. The pots were placed on a greenhouse bench and fertilized daily. Fertilizer consisted of

20:20:20 NPK injected daily at a rate of 250 ppm during regular watering. Temperature ranged from 20ºC (night) to 26ºC (day). To induce flowering, days were extended by artificial light from 5 p.m. to 10 p.m. The experimental design was a split plot randomized complete block with three replications. Cultivar was the main plot and isolate was the subplot. At mid-anthesis, two spikes per pot were each inoculated with 0.5 ml of a spore suspension of one of the seven isolates of *F. graminearum* at 1 x 10^5 spores/ml (Zadoks growth stage 65) (36) using a hand-held bottle sprayer. Following inoculation, each spike was covered with a transparent plastic bag for 72 h.

The spore suspension was obtained from isolates grown on PDA for up to 3 weeks in 9-cm-diameter Petri plates in a low temperature illuminated incubator set at 25ºC and a 12 h light/dark cycle. Five milliliters of ddwater 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 and kept in a 50 ml Falcon tube at 4ºC until needed for inoculation. Inoculation was done within 6 h of inoculum preparation. The isolates used were NE90, NE91, NE97, NE98, NE103, NE110, and NE119. A volume of 0.5 ml of spore suspension was applied to each spike and the spike was then covered with a transparent plastic bag for 72 h.

Inoculated spikes were tagged with colored tape for identification during harvesting. Disease severity (%) 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. Area under the disease progress curve (AUDPC) was calculated using the formula:

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

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.

2.5. Pathogenicity and aggressiveness on detached leaves. An experiment was conducted to evaluate the aggressiveness of seven isolates of *F. graminearum* by measuring mycelial growth and necrotic area on the leaves of two soft red winter wheat cultivars, Coker 9835 and VAO3W-433. A split plot experimental design randomized complete block with cultivar as the main plot and isolate as the subplot was used. A portion of the flag leaf measuring approximately 14 cm from the leaf apex was cut with a sterile surgical blade, surface disinfected with 70% ethanol for 30 s, and rinsed with sterile ddwater. Each leaf portion was cut into two pieces. A 3-mm-diameter wound was created with a sterile circular metallic bar on the adaxial surface at the basal part of each leaf piece. The two leaf pieces were then placed on a 9-cm-diameter WA plate with the abaxial side in contact with the agar. A 1-cm-diameter PDA mycelial plug of each *F. graminearum* isolate was placed on the wound with the mycelial side of the plug in contact with the wound. Plates were incubated for 12 days at 25°C in 12 h light and 12 h

dark. The percentage of the leaf surface covered with mycelia was visually estimated daily for 4 days and the values for the two leaf pieces were averaged.

Area under the mycelia growth curve (AUMGC) was calculated according to the formula:

$$\text{AUMGC: } \sum_{i=1}^{n-1} [(0.5)\,(S_i + S_{i+1})]\,(t_{i+1} - t_i)$$

Where S_i is the percentage of the leaf area covered by mycelia 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. The percentage of necrotic area on the two leaf pieces was visually estimated 12 days after inoculation and the values for the two leaf pieces were averaged.

2.6. Fitting models for analyzing disease progress on FHB severity data on wheat spikes. To further characterize the *F. graminearum* isolates, three models for temporal analysis of disease progress were fit to FHB severity data for the three most aggressive *F. graminearum* isolates (NE103, NE110, and NE119) and two soft red winter wheat cultivars (Coker 9835 and VA03W-433). The logistic, monomolecular, and Gompertz models were selected based on the shape of disease progress curves (10). The general linear models procedure of SAS version 9.1 (SAS Institute, Cary, NC) was used to evaluate the goodness-of-fit to the complete set of disease progress data for each isolate. The shape of the rate curve (FHB severity/day plotted against time (days)) and fit statistics (higher coefficient of determination and lower mean square error) were used to determine the model that best fit the disease progress data for each isolate.

2.7. Data analysis. The general linear models (GLM) procedure of SAS version 9.1 was used to analyze data. The least significant difference test at P = 0.05 (17) was used to compare pairs of treatment means. Spearman's coefficients of rank correlation were used as a measure of repeatability between replicate experiments (correspondence between isolate rankings in aggressiveness on spikes and detached leaves). Pearson correlation coefficients were used to determine if measurements on detached leaves (necrotic and mycelial area) could be used to predict disease severity on spikes.

3. RESULTS

3.1. Characterization of *F. graminearum* isolates. Polymerase chain reaction analysis using primers ITS4 and ITS5 was tested to verify the quality of amplification of DNA from 41 Nebraska isolates of *F. graminearum*. A DNA fragment of approximately 550 bp was obtained, as reported by White et al. (34), indicating that the DNA obtained had optimal conditions for amplification. Amplification with the specific set of primers GOFW and GORV showed that 40 of the 41 isolates were *F. graminearum* (Fig. 1). Isolate NE145 did not amplify (Table 1). All 41 isolates produced perithecia and macroconidia characteristic of *F. graminearum*, including isolate NE145 which did not amplify with *F. graminearum*-specific primers. Sporulation (production of macroconidia and ascospores) was abundant in all isolates except NE160 and NE161 (Table 1).

3.2. Perithecia production. Fifteen of the 17 isolates of *F. graminearum* collected in 2007 produced perithecia on carrot agar. In experiment 1, the F-value for number of perithecial units (1 unit = a single perithecium or a cluster of perithecia) was highly significant ($P < 0.0001$), indicating that isolates differed in the number of perithecial units they produced (Table 2). In experiment 2, the F-value for number of perithecial units was significant at the 10% level ($P = 0.0667$). The F-value for the percentage of the Petri plate surface covered with perithecial units was highly significant in both experiments ($P \leq 0.0002$). Isolates NE98 and NE108 produced no or very few perithecia (Fig. 2, Table 2). Isolate NE92 produced the largest number of perithecial units which, along with those of isolate NE110, also covered the largest area on the Petri plate (Table 2). Isolates differed in the size of perithecial units they produced (Fig. 2). However, this difference was not consistent for some isolates which produced both small and large perithecial units.

3.3. Pathogenicity and aggressiveness on wheat spikes. All seven isolates of *F. graminearum* were pathogenic on wheat spikes of both cultivars Coker 9835 and VA03W-433. The effect of cultivar was significant only for final severity on spikes in experiment 1 (Table 3). However, the effect of isolate was significant for all variables in both experiments except necrotic area on leaves in experiment 1. The effect of cultivar by isolate interaction was significant only for AUMGC in experiment 1. Based on the lack of a significant effect of cultivar and cultivar by isolate interaction for most of the variables in both experiments, isolate data (averaged over all cultivars) are presented. Cultivar by isolate interaction data (Fig. 3) are presented to demonstrate differences in the

rate of disease progression on spikes in the FHB-susceptible Coker 9835 versus the FHB-resistant VA03W-433.

Based on final FHB severity and AUDPC on spikes, the most aggressive isolates in both experiments were NE103, NE110, and NE119 whereas the least aggressive isolates were NE90 and NE97 (Table 4). Isolates NE91 and NE98 were intermediate in their aggressiveness. This characterization of aggressiveness of *F. graminearum* isolates was similar on the susceptible and the resistant cultivar. Although cultivars did not differ ($P > 0.05$) in final FHB severity and AUDPC on spikes (data not shown), the rate of disease progression for the three most aggressive isolates (NE103, NE110, and NE119) during the first 5 days after inoculation was higher in Coker 9835 than in VA03W-433 (Figs. 3 and 4). This observation was consistent in both experiments. Based on Spearman's coefficients of rank correlation, the two experiments were repeatable (r_s = 0.82, $P = 0.0234$ for final FHB severity; $r_s = 0.89$, $P = 0.0068$ for AUDPC).

3.4. Pathogenicity and aggressiveness on detached leaves. All seven isolates were pathogenic on detached leaves (Table 4). In experiment 1, isolates NE90, NE103, and NE110 were the most aggressive in producing mycelia on leaves whereas isolate 91 was the least aggressive. Isolates NE98 and NE119 were the most aggressive in causing necrosis on leaves whereas isolate NE90 was the least aggressive (Table 4). In experiment 2, isolates NE90 and NE110 were the most aggressive in producing mycelia on leaves whereas isolate 98 was the least aggressive. Isolate 98 was the most aggressive in causing necrosis on leaves whereas isolates 103 and 110 were the least aggressive (Table 4). Spearman's coefficients of rank correlation showed that the two experiments

were not as repeatable (r_s = 0.69, P = 0.0856 for final mycelial area; r_s = 0.57, P = 0.1802 for AUMGC) as the spike experiments.

3.5. Correlation between measurements on detached leaves and measurements on spikes. Pearson correlation coefficients showed that measurements on detached leaves (mycelial area, necrotic area, and AUMGC) were not significantly correlated with measurements on spikes (FHB severity and AUDPC), indicating that the detached leaf assays could not be used to reliably to predict FHB severity on spikes of live plants (-0.21 $\leq r \leq$ 0.33; 0.4668 $\leq P \leq$ 0.9826 for experiment 1 and (-0.62 $\leq r \leq$ 0.29; 0.1357 $\leq P \leq$ 0.8679 for experiment 2).

3.6. Fitting models for analyzing disease progress on FHB severity data on wheat spikes. The model that best fit the disease progress data was largely influenced by wheat cultivar. In cultivar Coker (FHB-susceptible), the monomolecular model best fit disease progress data for all three isolates in both experiments (Tables 5 and 6, Fig. 4). In cultivar VA03W-433 (FHB-resistant), the logistic model best fit disease progress data for isolate NE103 whereas the Gompertz model best fit disease progress data for isolate NE119 (Tables 5 and 6, Fig. 4). The monomolecular model best fit disease progress data for isolate NE110 in experiment 1. However, in experiment 2, none of the models had a good fit for disease progress data for this isolate (Tables 5 and 6, Fig. 4).

4. DISCUSSION

Using molecular and morphological identification techniques, this study has confirmed that the major species of *Fusarium* causing FHB in Nebraska is *F. graminearum*. This finding is in accordance with published reports on the distribution of *F. graminearum* worldwide and in North America (2, 26, 35). Because *F. graminearum* also causes ear and stalk rots in corn and wheat is often grown in rotation with corn in Nebraska, this confirmation reinforces the need for growers to adopt crop rotation schemes that avoid planting wheat following corn, especially where a reduced or no-till system is practiced to conserve soil and moisture.

With the exception of *F. graminearum* isolates NE98 and NE108 which barely produced any perithecia in vitro, all 40 isolates confirmed to be *F. graminearum* produced perithecia, but to varying degrees of abundance. It has been shown that in some isolates of *F. graminearum* pathogenicity is linked to perithecia production (32). In this study, however, the isolates NE98 and NE108 which barely produced any perithecia were pathogenic on both wheat spikes and leaves. The possibility that these two isolates may have the ability to produce perithecia in vivo was not tested in this study.

Seven selected *F. graminearum* isolates differed significantly in their aggressiveness on wheat spikes (FHB severity), indicating that in Nebraska, there may be populations of the pathogen with different levels of fitness. Therefore, the level of FHB severity in a given field may depend on the level of aggressiveness of the predominant *F. graminearum* isolate in that field or localized region. The seven isolates tested for aggressiveness in this study were grouped into highly aggressive (isolates NE103,

NE110, and NE119), moderately aggressive (isolates NE91 and NE98) and weakly aggressive (isolates NE90 and NE97). This grouping was the same regardless of cultivar resistance. Knopf and Miedaner (20) spray-inoculated binary *F. graminearum* mixtures and single isolates on spring wheat plots. As in this study, they found that aggressiveness of the isolates varied significantly and the effect of host resistance was independent of aggressiveness.

The seven isolates evaluated for aggressiveness on wheat spikes in this study were also evaluated for aggressiveness on detached leaves (mycelia production and necrosis) of the same wheat cultivars. The isolates again differed significantly in their aggressiveness on detached leaves. However, aggressiveness data from detached leaf assays were not correlated with aggressiveness data from wheat spikes, implying that detached leaf assays were not reliable predictors of disease progression on spikes. In addition, detached leaf assays were not as repeatable as in vivo spike assays. This observation may be due to the fact that in nature, *F. graminearum* colonizes spikes and not leaves of wheat plants.

Analysis of final disease severity and AUDPC on spikes showed that the two cultivars used did not differ in either variable. This was surprising because the cultivar Coker 9835 is susceptible to FHB whereas VA03W-433 is resistant (C. Greffey, personal communication). The lack of differences in final disease severity and AUDPC between the two cultivars may have been due to high disease intensity in the greenhouse compared to field conditions. Susceptibility of Coker and resistance of VA03W-433 were observed in the rate of disease progression during the first five days following inoculation. The rate of disease progression during this period was higher in Coker compared to VA03W-

433 (Figs. 3 and 4). This finding has implications in the management of FHB in these two cultivars. If this observation was true under field conditions, the timing of fungicide application to suppress FHB would be more critical for Coker than VA03W-433.

Analysis of disease progress on spikes of the susceptible Coker and the resistant VA03W-433 inoculated with the three most aggressive *F. graminearum* isolates revealed a significant effect of cultivar resistance/susceptibility on disease progression. This type of analysis and the results from it have not been reported before in the wheat-*F. graminearum* pathosystem. Disease progression on spikes of the susceptible cultivar was best described by the monomolecular model (10) regardless of *F. graminearum* isolate. On the other hand, disease progression on spikes of the resistant cultivar was best described by the logistic model for isolate NE103 and the Gompertz model for isolates NE110 and NE119. In monomolecular disease progression, the rate of disease development is initially high and declines over time. Therefore, significant damage can be done to a crop during the period immediately following infection. Susceptible cultivars would benefit from early fungicide application at early flowering. In contrast, in logistic and Gompertz disease progression, the rate of disease development is low initially, rises to a maximum an then declines. The results from this study indicate that under the conditions in which the experiments were carried out in the greenhouse, resistance of VA03W-433 was manifested by slowing down disease progression on spikes during the 5-day period following inoculation.

In summary, this study has confirmed that the major species of *Fusarium* causing FHB in Nebraska is *F. graminearum*. Selected isolates of the pathogen from infected wheat grain collected from elevators and fields differed in perithecia production and

aggressiveness on wheat spikes and detached leaves. Aggressiveness data from detached leaf assays were not correlated with aggressiveness data on wheat spikes. Disease progression on spikes of a susceptible soft red winter wheat cultivar was best described by the monomolecular model regardless of *F. graminearum* isolate. Disease progression on spikes of a resistant soft red winter wheat cultivar was best described by the logistic or the Gompertz model depending on the isolate of *F. graminearum*. The results from the study have important implications for the management of FHB in the two cultivars studied, including the timing of a fungicide application to suppress the disease.

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6. TABLES

Table 1. Molecular and morphological characterization of Nebraska isolates of *Fusarium graminearum* from infected wheat kernels

Isolate	PCR primers ITS4[a] & ITS5[b]	PCR primers GOFW[c] & GORV[d]	Perithecia production	Macroconidia production	Sporulation (PDA[e] & CLA)[f]	*F. graminearum* identification
NE90	+	+	+	+	Abundant	+
NE91	+	+	+	+	Abundant	+
NE92	+	+	+	+	Abundant	+
NE93	+	+	+	+	Abundant	+
NE96	+	+	+	+	Abundant	+
NE97	+	+	+	+	Abundant	+
NE98	+	+	+	+	Abundant	+
NE99	+	+	+	+	Abundant	+
NE100	+	+	+	+	Abundant	+
NE101	+	+	+	+	Abundant	+
NE102	+	+	+	+	Abundant	+
NE103	+	+	+	+	Abundant	+
NE105	+	+	+	+	Abundant	+
NE107	+	+	+	+	Abundant	+
NE108	+	+	+	+	Abundant	+
NE109	+	+	+	+	Abundant	+
NE110	+	+	+	+	Abundant	+
NE111	+	+	+	+	Abundant	+
NE112	+	+	+	+	Abundant	+
NE115	+	+	+	+	Abundant	+
NE119	+	+	+	+	Abundant	+
NE121	+	+	+	+	Abundant	+

NE123	+	+	+	+	Abundant	+
NE124	+	+	+	+	Abundant	+
NE125	+	+	+	+	Abundant	+
NE129	+	+	+	+	Abundant	+
NE133	+	+	+	+	Abundant	+
NE134	+	+	+	+	Abundant	+
NE143	+	+	+	+	Abundant	+
NE145	+	-	+	+	Abundant	-
NE146	+	+	+	+	Abundant	+
NE148	+	+	+	+	Abundant	+
NE151	+	+	+	+	Abundant	+
NE156	+	+	+	+	Abundant	+
NE157	+	+	+	+	Abundant	+
NE158	+	+	+	+	Abundant	+
NE160	+	+	+	+	Scarce	+
NE161	+	+	+	+	Scarce	+
NE162	+	+	+	+	Abundant	+
NE164	+	+	+	+	Abundant	+
NE165	+	+	+	+	Abundant	+

[a] ITS4: Primer targeting ITS 5.8 S rRNA region as described by White et al 5'-TCCTCCGCTTATTGATATGC-3'.

[b] ITS5: Primer targeting ITS 5.8 S rRNA region as described by White et al 5'-GGAAGTAAAAGTCGTAACAAGG-3'.

[c] GOFW : *F. graminearum* specific primer forward 5'-ACCTCTGTTGTTCTTCCAGACGG- 3'.

[d] GORV: *F. graminearum* specific primer reverse 5' -CTGGTCAGTATTAACCGTGTGTG- 3'.

[e] PDA: Potato dextrose agar medium

[f] CLA: Carnation leaf agar medium

Table 2. Number of perithecia and perithecia covered area of 17 Nebraska isolates of *Fusarium graminearum*

Isolate	Experiment 1		Experiment 2	
	Perithecial units	Perithecia-covered area (%) [z]	Perithecial units	Perithecia-covered area (%)[z]
NE90	11.3 e-i	8.8 c-e	18.3 b	11.5 c-g
NE91	5.8 f-i	1.6 ef	81.3 ab	14.3 b-f
NE92	53.5 a	18.0 ab	129.5 a	28.8 a
NE93	21.8 b-e	20.0 ab	26.0 b	15.8 a-e
NE96	26.5 bc	19.8 ab	11.5 b	6.5 d-g
NE97	8.3 f-i	4.0 ef	13.0 b	6.0 d-g
NE98	0.0 i	0.0 f	0.5 b	0.1 g
NE99	21.0 b-e	8.5 c-e	5.5 b	1.1 fg
NE100	0.8 hi	0.4 f	0.3 b	0.4 g
NE101	16.5 c-f	13.4 b-d	13.8 b	4.8 e-g
NE102	11.0 e-i	6.1 d-f	6.5 b	6.3 d-g
NE103	12.8 d-h	7.0 d-f	18.5 b	2.0 fg
NE105	13.8 d-g	13.0 b-d	16.8 b	11.3 c-g
NE107	24.0 b-d	15.8 a-c	24.3 b	19.8 a-c
NE108	0.0 i	0.0 f	0.5 b	0.1 g
NE109	4.0 g-i	1.9 ef	25.3 b	8.5 c-g
NE110	32.8 b	23.3 a	47.8 ab	27.0 ab
PH-1	14.3 d-g	4.9 ef	132.8 a	18.0 a-d

[a] Means within a column followed by the same letter are not significantly different according to Fisher's least significant difference test at $P = 0.05$.

[z] Percent of the surface area of a 9-cm-diameter Petri plate covered by perithecia.

Table 3. Effects of cultivar and *Fusarium graminearum* isolates on mycelial area on leaves, area under the mycelia growth curve (AUMGC) on leaves, necrotic area on leaves, spike severity, and area under the disease progress curve (AUDPC) on spikes

Source of variation	**df**	**Mean square**				
Experiment 1		**Mycelial area on leaves**	**AUMGC**	**Necrotic area on leaves**	**Final spike severity**	**AUDPC**
Rep	2	148.66	831.14	23.80	25.81	153.78
Cultivar (C)	1	418.01	49.83	201.52	1920.38*	57980.01*
Error (a)	2	812.65**	3110.66*	372.18	45.17	3145.46
Isolate (I)	6	1011.71***	4226.98**	692.09	7987.91****	1930583.98****
C x I	6	230.85	2579.96*	321.04	166.89	27885.97
Error (b)	24	130.65	888.51	348.74	138.05	43716.63
Total	41					
Experiment 2						
Rep	2	36.76	163.67	173.68	68.76	34735.48
Cultivar (C)	1	7.29	455.07	841.52	1065.05	1351.50
Error (a)	2	501.04*	1636.35	465.18	532.30	67543.95
Isolate (I)	6	898.56****	3801.64**	3371.46****	7065.43****	1583632.73****
C x I	6	180.90	790.11	421.91	730.97	147824.50
Error (b)	24	2766.07	688.27	426.71	566.91	110809.13
Total	41					

* $0.01 \leq P \leq 0.05$

** $0.001 \leq P \leq 0.01$

*** $0.0001 \leq P \leq 0.001$

**** $P \leq 0.0001$

Table 4. Mycelial area and area under the mycelia growth curve (AUMGC) on leaves, perithecia number and perithecia-covered area, necrotic area on leaves, spike severity and area under the disease progress curve (AUDPC) on spikes of Nebraska isolates of *Fusarium graminearum*

Isolate	FHB severity on spikes 21 dpi[a] (%)	AUDPC (% days)	Mycelial area on leaves 4 dpi	AUMGC (% days)	Necrotic area on leaves 12 dpi (%)
Experiment 1					
NE90	15.0 d[b]	183.2 d	86.7 a	173.9 a	21.3 b
NE91	77.2 b	1033.1 b	53.3 c	110.2 c	33.7 ab
NE97	16.7 d	199.5 d	71.3 b	148.5 ab	36.4 ab
NE98	47.0 c	536.5 c	68.3 b	125.8 bc	53.3 a
NE103	91.6 a	1240.4 ab	86.3 a	168.7 a	35.5 ab
NE110	97.7 a	1467.5 a	87.9 a	164.7 a	35.0 ab
NE119	95.6 a	1484.6 a	67.5 b	115.8 bc	49.8 a
Experiment 2					
NE90	26.2 cd	259.7 c	78.3 a	152.2 a	51.1 b-d
NE91	54.4 bc	666.4 b	58.3 b	102.3 dc	63.3 bc
NE97	9.2 d	135.3 c	58.3 b	117.1 bc	68.8 b
NE98	56.2 b	716.2 b	45.0 c	74.7 d	94.3 a
NE103	96.8 a	1289.8 a	70.8 ab	108.3 bc	31.0 d
NE110	82.2 ab	1265.5 a	77.9 a	138.9 ab	27.9 d
NE119	98.0 a	1409.4 a	70.8 ab	119.4 bc	38.8 cd

[a]Days post-inoculation

[b]Means within a column followed by the same letter are not significantly different according to Fisher's least significant difference test at $P = 0.05$.

Table 5. Linear regression statistics used to evaluate the goodness-of-fit of three growth models to Fusarium head blight progress data from two soft red winter wheat cultivars and three *Fusarium graminearum* isolates in an a greenhouse study, 2010, experiment 1.

Cultivar Isolate Model	R^2	MSE	Intercept	Std. Dev of Int.	Slope	Std. Dev. of Slope
Coker						
Isolate NE103						
Logistic	0.15	0.80	0.00	0.54	0.07	0.05
Monomolecular	0.12	0.40	0.76	0.38	0.04	0.03
Gompertz	0.14	0.56	0.40	0.46	0.05	0.04
Isolate NE110						
Logistic	0.66	0.38	-0.03	0.36	0.14	0.03
Monomolecular	0.68	0.25	0.48	0.29	0.12	0.02
Gompertz	0.67	0.31	0.23	0.32	0.13	0.03
Isolate NE119						
Logistic	0.44	0.45	0.58	0.39	0.10	0.03
Monomolecular	0.45	0.31	0.96	0.32	0.08	0.03
Gompertz	0.44	0.37	0.78	0.35	0.09	0.03
VA03W-433						
Isolate NE103						
Logistic	0.75	1.21	-3.14	0.79	0.38	0.07
Monomolecular	0.82	0.42	-1.35	0.43	0.28	0.04
Gompertz	0.79	0.67	-2.12	0.59	0.32	0.05
Isolate NE110						
Logistic	0.83	0.63	-1.92	0.53	0.35	0.05
Monomolecular	0.82	0.47	-0.94	0.46	0.29	0.04
Gompertz	0.83	0.53	-1.40	0.48	0.32	0.04
Isolate NE119						
Logistic	0.82	0.34	-1.19	0.39	0.25	0.04
Monomolecular	0.89	0.11	-0.25	0.22	0.19	0.02
Gompertz	0.86	0.19	-0.68	0.29	0.22	0.03

Table 6. Linear regression statistics used to evaluate the goodness-of-fit of three growth models to Fusarium head blight progress data from two soft red winter wheat cultivars and three *Fusarium graminearum* isolates in an a greenhouse study, 2010, experiment 2.

Cultivar Isolate Model	R^2	MSE	Intercept	Std. Dev of Int.	Slope	Std. Dev. of Slope
Coker						
Isolate NE103						
Logistic	0.54	0.41	-0.27	0.39	0.12	0.03
Monomolecular	0.57	0.21	0.41	0.28	0.09	0.02
Gompertz	0.56	0.30	0.09	0.33	0.11	0.03
Isolate NE110						
Logistic	0.78	0.27	-0.02	0.30	0.16	0.02
Monomolecular	0.77	0.24	0.37	0.28	0.15	0.02
Gompertz	0.78	0.25	0.18	0.29	0.15	0.02
Isolate NE119						
Logistic	0.80	0.17	0.11	0.24	0.14	0.02
Monomolecular	0.81	0.13	0.52	0.21	0.12	0.02
Gompertz	0.81	0.15	0.32	0.22	0.13	0.02
VA03W-433						
Isolate NE103						
Logistic	0.90	0.56	-3.046	0.45	0.40	0.04
Monomolecular	0.91	0.24	-1.36	0.30	0.27	0.02
Gompertz	0.92	0.30	-2.26	0.34	0.32	0.03
Isolate NE110						
Logistic	0.04	9.69	0.66	2.24	-0.13	0.20
Monomolecular	0.03	1.25	0.56	0.74	0.04	0.07
Gompertz	0.00	3.14	0.66	1.28	-0.02	0.11
Isolate NE119						
Logistic	0.84	0.67	-2.90	0.54	0.38	0.05
Monomolecular	0.85	0.34	-1.23	0.39	0.28	0.04
Gompertz	0.86	0.42	-1.96	0.43	0.32	0.04

7. FIGURE CAPTIONS

Fig. 1. Agarose gel resulting from a specific polymerase chain reaction to identify Nebraska isolates of *Fusarium graminearum* using primers GOFW and GORV. M: Molecular weight markers 100bp ladder, lane 1: isolate NE90, lane 2: isolate NE91, lane 3: isolate NE92, lane 4: isolate NE93, lane 5: isolate NE97, lane 6: isolate NE98, lane 7: isolate NE99, lane 8: isolate NE100, lane 9: isolate NE101, lane 10: isolate NE102, lane 11: isolate NE103, lane 12: isolate NE105, lane 13: isolate NE107, lane 14: isolate NE108, lane 15: isolate NE110, lane 16: isolate NE111, lane 17: isolate NE112, lane 18: isolate NE115, lane 19: isolate NE119, lane 20: isolate NE123, lane 21: isolate NE125, lane 22: isolate NE133, lane 24: blank.

Fig. 2. Perithecial units formed by *Fusarium graminearum* isolates NE98 (A), NE91 (B), NE99 (C), and NE93 (D) on carrot agar. Note the absence of perithecial units (1 unit = a single perithecium or a cluster of perithecia) and/or the different sizes of perithecial units.

Fig. 3. Disease (Fusarium head blight) progress curves of seven Nebraska isolates of *Fusarium graminearum* on spikes in two soft red winter wheat cultivars, Coker 9835 and VA03W-433.

Fig. 4. Rate of disease progression of the three most aggressive *Fusarium graminearum* isolates on wheat spikes of two soft red winter wheat cultivars, Coker 9835 and VA03W-433. A: isolate NE103, experiment 1; B: isolate NE103, experiment 2; C: isolate NE110, experiment 1; D: isolate NE110, experiment 2; E: isolate NE119, experiment 1; F: isolate NE119, experiment 2.

8. FIGURES

FIGURE 1.

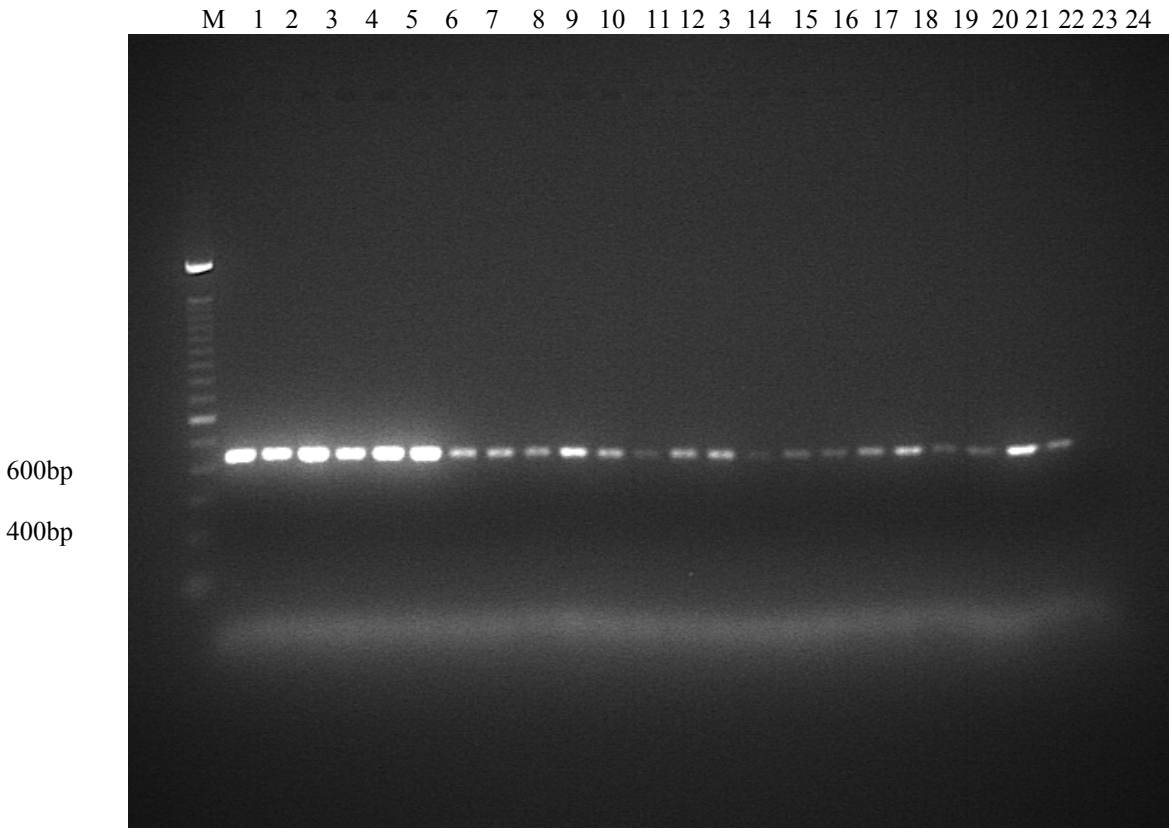

FIGURE 2

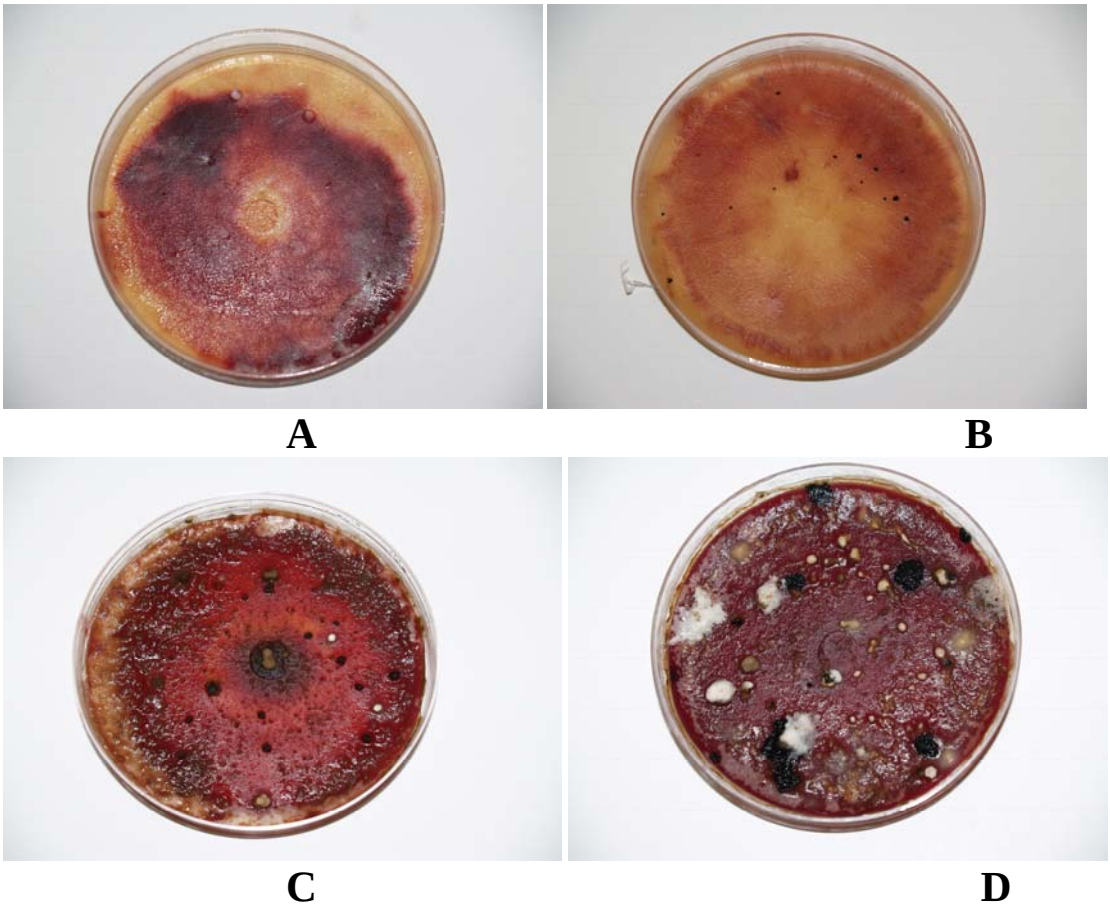

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FIGURE 3

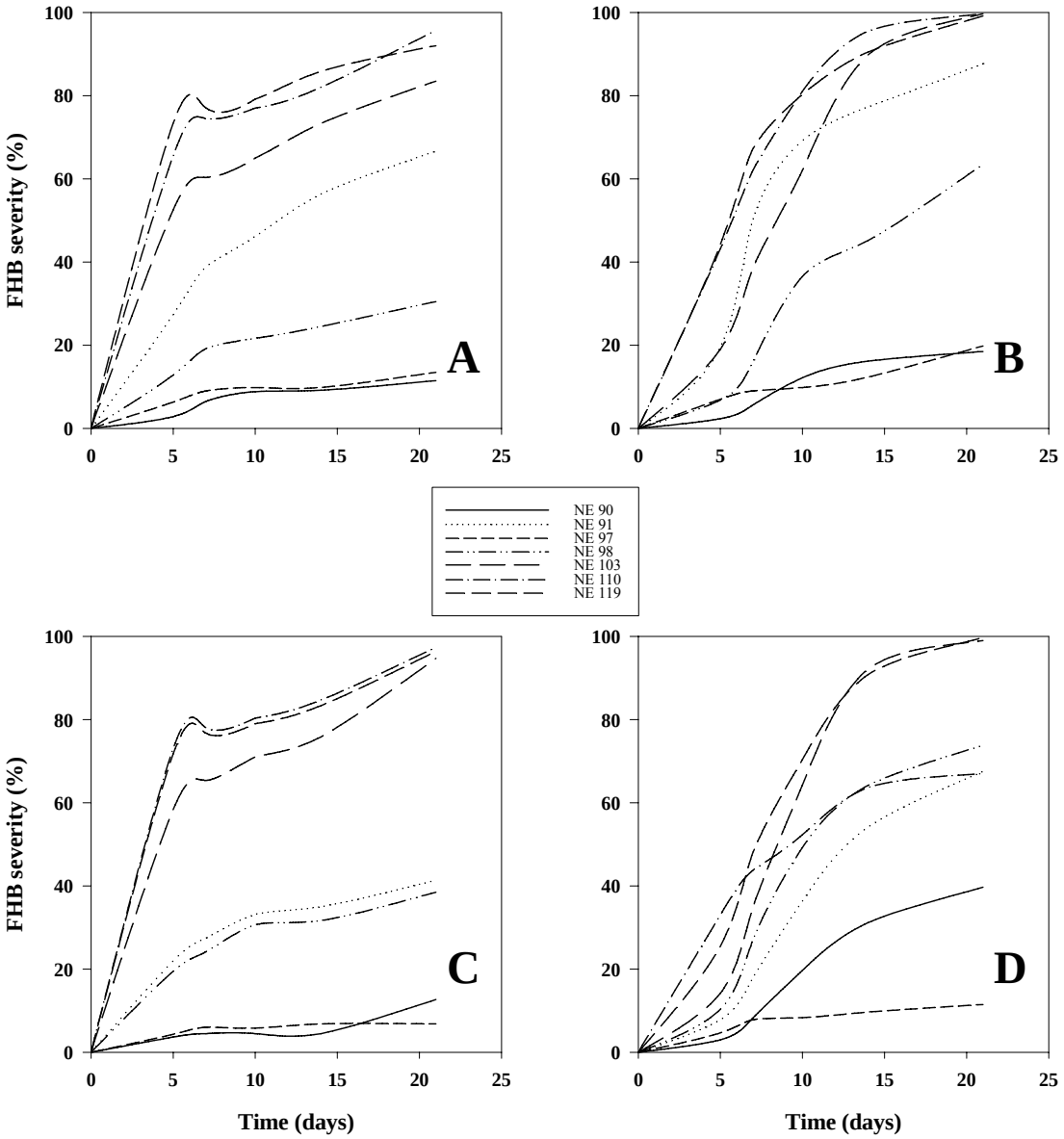
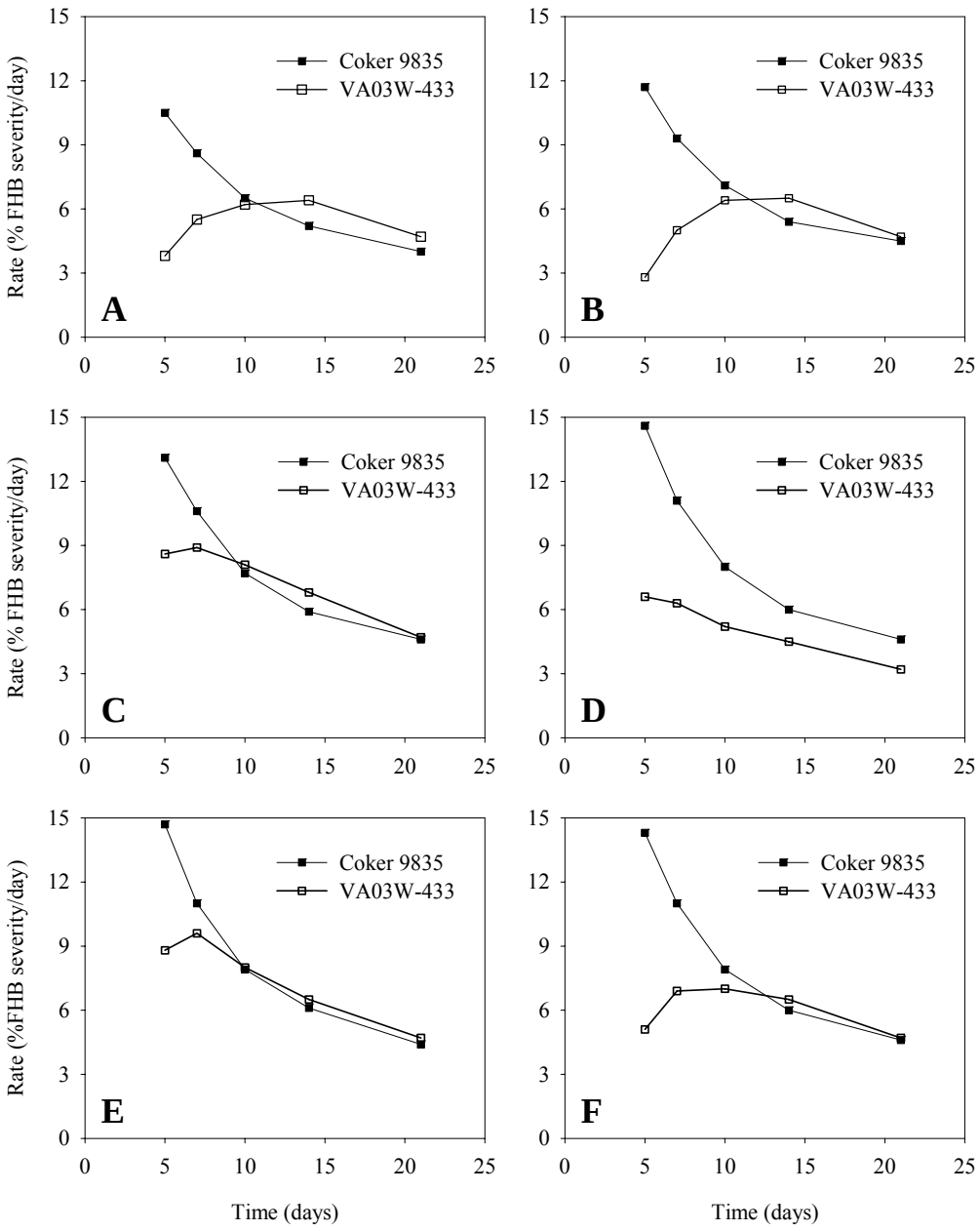

FHB severity (%)
Time (days)
A
B
C
D
NE 90
NE 91
NE 97
NE 98
NE 103
NE 110
NE 119

FIGURE 4


Rate (% FHB severity/day)
Rate (%FHB severity/day)
Time (days)
Coker 9835
VA03W-433
A
B
C
D
E
F
0
3
6
9
12
15
0
5
10
15
20
25