Epidemiology and Variability of Disease and Deoxynivalenol in Fusarium Head Blight of Wheat in Ohio

A Thesis

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By

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ABSTRACT

Fusarium head blight (FHB) is an economically devastating disease of wheat. The pathogen, *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* (Schwein.) Petch), produces a trichothecene mycotoxin, deoxynivalenol (DON), which accumulates in grain. DON impedes protein synthesis by binding to eukaryote ribosomes, and consequently, poses a food safety risk for humans and livestock. The United States Food and Drug Administration has issued a 2 ppm advisory limit for DON in unprocessed grain, and a 1 ppm limit for milled products intended for human consumption. DON easily exceeds these limits for many *F. graminearum* infections of wheat, particularly in wet, humid years when disease is severe. Although FHB has been studied for years, several aspects of the epidemiology of the disease remain unclear. In this study, the effect of host resistance, growth stage at infection, and inoculum density, as well as the interactions of these factors, were evaluated on several disease measures (FHB index [IND], FHB incidence [INC], and Fusarium-damaged kernels [FDK]) and DON content of grain under different environmental conditions. A second study, overlaid on the first, evaluated the same effects on DON content and *F. graminearum* colonization of wheat spikes displaying no visual symptoms of
FHB. The experimental design was a randomized complete block with a split-split-split plot treatment structure, with planting date (PD) as the whole plot, wheat cultivar (CV) as the sub-plot, infection time (IT) as the sub-sub plot, and inoculum density (ID) as the sub-sub-sub plot. The incorporation of PD and cultivars with different maturation rates prolonged the window of anthesis across the experiment, so that the effects of the factors and interactions on measures of disease and DON could be evaluated under a range of environmental conditions. Main effects of CV, IT, and ID were significant in both years for DON and visual measures of disease. The greatest DON levels for the two years were seen when temperatures were cool (<20°C) at anthesis. In general DON and disease were greatest for the earliest (anthesis) infection time and increased with increasing levels of inoculum. Infections that occur at post-anthesis growth stages are sub-optimal for FHB development, yet interactions with high inoculum densities, and favorable weather conditions resulted in compensations in disease and toxin concentration. DON exceeded the acceptable limit of 2 ppm for late infections in symptomatic and asymptomatic infections and in susceptible and more resistant cultivars. These results have implications for FHB management. Since DON can be unacceptably high in grain from late season and asymptomatic infections, care should be taken to plant cultivars with high levels of resistance to toxin accumulation. In addition, it may be worthwhile to apply fungicides at post-anthesis growth stages when environmental conditions are conducive for *F. graminearum* infections. More research needs to be done to identify specific conditions that warrant additional fungicide applications. A final study evaluated
in-field disease and DON with an emphasis on variability. DON in grain is highly variable at any given level of visual symptoms on external wheat tissues. As a result, accurate in-field toxin quantification is difficult. Variability of disease and DON was evaluated for plots of six winter wheat cultivars with different levels of resistance to FHB. DON and disease index were quantified for 30 clusters of 20 spikes in each plot. Seven nonlinear mixed models were generated for DON prediction based on the level of observed disease for clusters and plots means. The best performing models contained a non-constant error term that incorporated the variance of DON into the residual. Future work will focus on the development of a dynamic sampling-protocol for DON for which determination of sample size will incorporate the level of in-field disease and variability in DON, in an effort to increase the accuracy of in-field DON quantification.
Dedicated to:

My family

I would not have made it here without you

and I certainly would not have made it through

without your support.
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CHAPTER 1: INTRODUCTION

Fusarium head blight (FHB) is a fungal disease that affects wheat and other small grains in all grain-growing regions throughout the world (67, 105). This disease causes yield reduction and grain contaminated with mycotoxins, which, if ingested in sufficient quantities are dangerous for human and animals, (63, 78, 90). In excess of twenty species of Fusarium and Microdochium are known causal agents of FHB (although Microdochium does not produce mycotoxins) (19, 67). European cereals are typically affected by several species simultaneously, including F. graminearum, F. culmorum, F. avenaceum, and F. poae, but in the United States and the rest of North America, F. graminearum Schwabe (teleomorph Gibberella zeae (Schwein.) Petch) (58) is the primary causal agent of FHB (8, 121). Among the FHB-causing Fusaria, this species is the most aggressive producer of deoxynivalenol (DON) (120); a toxin that is not the most toxic of the FHB-associated mycotoxins, but is usually found in higher concentrations than other trichothecenes (11, 90). This review will concentrate on the impact, biology, epidemiology, and management of FHB in the United States and will focus on F. graminearum in wheat, though barley and corn infections are also serious concerns for growers in North America.
COST AND CONSEQUENCES OF FHB

Since first being described in the United States in the early 1890s, FHB has consistently had serious social and economic impacts in all major wheat growing regions. Early reports (1890-1891) recorded wheat yield losses ranging from 10 to 80% throughout Indiana and Ohio (3). Stack (105) reviewed the occurrence of FHB in the United States from the initial reports through the 1980s and reported that widespread epidemics occurred in 1919, 1928, 1932, 1935, 1950, 1975, and 1981-1986. FHB development is heavily dependent upon environmental conditions, so even though spores are consistently present and available for infection, significant epidemics are not reported annually (59). In recent decades, the occurrence and severity of costly FHB epidemics have increased (29, 58), prompting the United States Department of Agriculture to call FHB “the worst plant disease to hit the United States since the stem rust epidemics of the 1950s” (118).

The most costly FHB epidemic occurred in 1993 in the Northern Great Plains. In that year, heavy rainfall coincided with the flowering stage of wheat development (anthesis), creating conditions ideal for *F. graminearum* infection (117) and FHB development. The resulting grain yield and quality losses were estimated at $1 billion (58). Yield losses due to FHB continued to be severe in the Great Plains region through 1998 (117). However, direct reduction in grain yield and quality were not the only components of economic loss associate with the FHB epidemics of the 1990s. In an economic study, Nganje et al. (65)
examined how the 1993 FHB epidemic affected the processing of harvested wheat grain and concluded that for every $1 lost by growers there was an additional $2 loss somewhere else in the wheat industry. Some of these losses are suffered by millers who, in order to fulfill contracts, may need to transport grain long distances when there is little grain available in their region, or available grain is contaminated with high levels of DON (21).

FHB threatens not only commodity production and food safety, but affects the way of life of rural communities. However, the magnitude of the social and economic impacts of FHB is difficult to quantify. Based on the 1993 epidemic in the Upper Great Plains, Windels (117) summarized a review of these impacts on the rural communities in North Dakota as follows:

“Downtown businesses are seeing fewer purchases and are cautious about extending credit. Some small towns are losing most of their businesses. One example is Michigan, ND, population 430. This spring, it lost an implement dealership, a car dealership, and a café—and dozens of people lost their jobs. Joe Lamb, who owned these businesses notes: “There were no customers due to the agricultural economy. It’s been bad for awhile. A lot of these small towns are going to dry up”."

Since the early FHB outbreaks, sporadic epidemics have also occurred in grain-growing regions outside of the Upper Great Plains. For instance, in 1996, Ohio lost an estimated 40% of its wheat yield due to FHB (54). In 2003, an estimated $13.6 million was lost by growers in the Southeastern United States (21). In 2009, Kentucky saw widespread FHB development, with severity as high
as 70 to 80% in the southwestern part of the state and between 15 and 30% for much of the rest of the state (Hershman, personal communication).

FHB and it associated toxins have also affected grain marketing and commercialization policies in the US and Europe. For instance, in 1928, a year of severe FHB outbreaks, grain shipped from the US reportedly caused livestock feeding problems in the United Kingdom and Germany (105). The US is one of the world’s leading exporters of cereals, exporting an estimated 50% of the total volume of wheat produced in 1994 (1). Problems associated with the movement of contaminated grain from one country to another have led to changes in policies governing grain commercialization. The European Union (EU) and the US Food and Drug Administration (FDA) have established strict limits on the allowable levels of DON in grain destined for human and animal consumption. The EU has established a maximal daily intake of DON at 0.5 ug/kg of body weight. The FDA advisory limit for DON in final grain products intended for human consumption is 1 ug/g of product.

**DISEASE CYCLE AND SYMPTOMATOLOGY**

*Fusarium graminearum* (teleomorph *Gibberella zeae*) produces ascospores and macroconidia, both of which are considered epidemiologically important (34, 91, 99). The fungus overwinters as perithecia or mycelia on corn and small grain residue, but can also survive between growing seasons on other plant species (76, 101). Ascospores and macroconidia are produced in the spring and summer, passively or actively liberated from fungal reproductive structures,
and disseminated by wind and rain (68, 109). Ascospores may also ooze out of perithecia and, along with sporodochia-borne macrocondia, be splash-dispersed within fields from residue to wheat spikes (68). Perithecia forcibly expel ascospores, which, under the right conditions, can be carried by wind into the planetary boundary layer (50 m – 1 km above ground) of the atmosphere for long-distance dispersal (57, 111).

Wheat spikes are most susceptible to infection at anthesis, and most infections occur when extruded anthers are colonized by hyphae from germinating spores (2, 82, 86). The anthers provide the fungus with an avenue for entry into the internal tissue of the spikelet (82, 86). Additional infections can occur by hyphal penetration of stomata, but direct tissue penetration has not been observed (44, 82, 86).

Symptoms begin to develop within a few days to a few weeks of infection, depending on environmental conditions (4, 16). Water-soaked lesions appear on affected spikelets, quickly followed by spikelet necrosis, which is manifested in the field as premature bleaching and senescence (16). Adjacent spikelets may become necrotic as the fungus spreads within the spike, or spikelets above the initial point of infection may become necrotic if the fungus penetrates and kills the rachis, impeding the movement of water and nutrients through vascular tissues (16, 85). Grains from severely infected spikes are shriveled and lightweight and are usually referred to as scabby or tombstone kernels (58). These kernels are chalky white or pinkish in color due to abundant fungal colonization, and often remain attached to the palea and lemma during harvest (2, 9, 16). Towards the
end of the season, especially if conditions are wet and humid, glumes may become covered with sporodochia and perithecia, giving the bleached spikelets an orangish tinge speckled with blackish dots.

PATHOGEN BIOLOGY

*F. graminearum* is adapted to a wide range of temperature and moisture conditions, but thrives as a pathogen in wet, humid regions (121). This fungus requires extended periods of high moisture (relative humidity >80% or rainfall) for successful infection, so it is a less successful pathogen under dry conditions (119, 121). Relative to moisture, temperature seems to be less of a limiting factor for FHB development. Optimum temperatures for infection of wheat spikes are between 22 and 29°C (2, 88, 120). However, extended periods of continuous moisture often compensate for temperature, increasing disease development at both optimal and suboptimal temperatures (2, 85, 88, 120). For example, at 25°C FHB incidence increased from 18% to 77% when the period of wetness was extended from 36 to 48 h (2).

Spore production, discharge, and germination are all affected by environmental conditions. Tschanz et al. (112) determined that exposure to ultraviolet light stimulated perithecia production by *G. zeae* under laboratory conditions, with the most effective wavelengths being between 300 nm and 320 nm. Perithecia production increases when temperatures are between 15.3°C and 28.5°C, with no production above 32.5°C (112). For ascospore and macroconidia production, the optimum temperature ranges are from 25 to 28°C and 28 to 32°C,
respectively. Macroconidia production decreases at temperatures below 16°C and above 36°C (112).

Ascospores are expelled from perithecia when turgor pressure forces asci from the ostiole (111). Trail et al. (111) reported that ascospore discharge occurred at a wide range of relative humidity (RH) treatments, but maximum discharge required high moisture conditions (either 100% RH or simulated rainfall). Correspondingly, Ayers et al. (5) and Sutton (109) reported that the greatest discharge of ascospores under field conditions occurred between 9 pm (2100 h) and 8 am (800 h); the hours of the day with the highest RH. Trail et al. (111) observed that ascospore discharge was greater when perithecia were exposed to light than to complete darkness, and estimated that under dark, humid conditions it took 1-2 h longer for perithecia to expel all asci. Discharge did not occur at temperatures above 26°C and increased as temperature fell to 16.6°C (112).

Beyer and Verreet (13) reported that ascospores successfully germinated survived for more than 20 days after expulsion from perithecia, but lost viability instantaneously if exposed to 0% RH. Germination decreases as temperatures increase above 15°C, irrespective of the relative humidity (37). Macroconidial germination occurs under conditions similar to those suitable for ascospore germination. At 20°C and 100% RH, germination of conidia occurred within 2-6 h, and decreased as RH decreased, with no germination observed when spores were incubated at 20°C and RH ≤ 80% for 18 h or when water potential was
below -6.0 MPa for 8 h (12, 108). Macroconidia lost viability when incubated at 0% RH for 6 h, 30% RH for 23 h, or 72 h at 53% RH (12).

Optimum growth of *F. graminearum* mycelia occurs at 25°C, with growth continuing at a maximum temperature of 37°C (10, 14).

**DEOXYNIVALENOL**

DON is toxic to eukaryotic cells because it inhibits protein synthesis by binding to ribosomal peptidyl transferase (DON toxicity is reviewed by (87)). Messenger RNA gets stuck in the ribosome and cannot be dislodged (87). Trichothecene-producing fungi avoid this toxicity because they possess altered ribosomal protein that is insensitive to trichothecenes (87). Deoxynivalenol is also known colloquially as vomitoxin because one of the primary reactions to DON ingestion is vomiting (58). Other signs of toxicosis include feed refusal and associated weight loss, immune system suppression, and reproductive problems (reviewed in: (87, 90).

The role of deoxynivalenol in infection and FHB development has been widely investigated. DON is produced as early as 36 h after inoculation, but may not be present at quantifiable levels until up to 4 days after infection (44, 93). To examine the role of this toxin in pathogenesis, Proctor et al. (84) engineered a non-DON-producing mutant of *F. graminearum* by knocking out the *Tri5* gene and compared disease development between this *Tri5* mutant and its *Tri5*+ wild type. Removing the *Tri5* gene, which is required for the production of DON and other trichothecene toxins, did not eliminate pathogenicity, but reduced the
severity of FHB in wheat and rye (28, 84). This suggests that DON may not be required for initial infection, but apparently allows the fungus to spread within the wheat spike after infection. Nicholson et al. (66) observed that the ability to colonize wheat grains was diminished in non-trichothece producing mutants of *F. graminearum* relative to the trichothece producing wild type.

Environmental factors are known to impact the production of DON. *F. graminearum* infection and DON production is enhanced by wetness (48). Lipps et al. (54) reported that in 2004, although FHB intensity was relatively low (13.1% average incidence), the amount of DON detected in the grain exceeded 2 ppm, which is the advisory limit established by the United States Food and Drug Administration for DON contamination of grain. The authors attributed this to the unusually high amount of precipitation that fell during kernel development in that year. Cowger et al. (20) examined the effect of post-anthesis moisture on FHB development and DON contamination of grain by mist-irrigating different field plots for 0, 10, 20, and 30 days after anthesis. They reported a significant increase in DON between the 0 and 10 day mist treatments and again between the 10 and 20 day treatments. Conducting a similar study, in which a greater total volume of moisture was applied to post-anthesis spikes than in the previously described study, Culler et al. (22) observed that grain DON content was lower in the treatment that received mist from anthesis to harvest than in the treatment misted between anthesis and disease assessment. Since DON is a water soluble toxin, the authors hypothesized that DON may have leached out of wheat tissue
in the high moisture treatment. Lemmens et al. (51) also observed a higher concentration of DON in non-mist-irrigated than in mist irrigated plots.

There is some evidence that DON production is affected by exposure of the pathogen to stressful conditions. Schmidt-Heydt et al. (98) observed that there were two separate peaks for trichothecene production in *F. culmorum*. The greatest trichothecene production occurred when the fungus was grown at optimal temperature, but a second peak occurred when grown at a sub-optimal temperature of 20°C, indicating that trichothecene production may, in part, be stress-induced. In addition to environmental conditions toxin production can be triggered by fungal competition. When Xu et al. (120) inoculated wheat with multiple species of FHB-causing organisms the amount of DON was significantly more than that detected when wheat was inoculated with a single species.

ASSOCIATION BETWEEN FHB AND DON

Many attempts have been made to characterize the relationship between visual symptoms of FHB and DON in grain. In the literature, contradictory claims exist for a positive relationship between FHB symptoms and DON, a negative relationship, or the absence of a relationship. Paul et al. (73) conducted a meta-analytic synthesis of the association between visual symptoms of FHB and DON using data from 163 studies and concluded that overall there were significant positive correlations between all measures of visual symptoms and DON. However, even though these associations exist, there are several reasons why the degree of FHB symptoms may not always be a very useful predictor of DON
contamination. First, there is a temporal lag between DON production and symptom expression. DON is detectable soon after infection, but it takes up to several weeks later for symptoms to appear, meaning that DON accumulates in seemingly healthy tissue (104). Second, the spatial separation between grain where toxin is quantified and chaff tissue where symptoms develop means that visual symptoms do not accurately reflect the degree of grain infection, and, consequently, DON production. Third, DON is water soluble, so even if infection is isolated to a single spikelet, DON may still be translocated to uninfected parts of the wheat spike (93, 102). In a point-inoculation study with *F. graminearum*, Savard et al. (93) quantified DON contamination in kernels from spikelets above and below the point of inoculation and found that DON accumulated at high levels below the point of infection, but the toxin did not travel as readily to upper portions of the spike.

Several studies have determined that DON is more closely associated with the amount of fungal biomass in grain and the percentage of Fusarium-damaged kernels than with measures of visual symptoms such as incidence and severity (31, 72, 89). Both of these measures provide more direct estimates of grain colonization than disease assessments done prior to harvest. Lemmens et al. (50) observed that visual symptoms of disease did reflect DON contamination in grain fairly well at low levels of infection, but the association broke down at high levels of infection. Paul et al. (72) also showed that the strength of relationship between FHB and DON depended on mean FHB and DON levels. Disease measures may underestimate the degree of infection and DON
accumulation under conditions not favorable for the development of visual symptoms. Unexpectedly high levels of DON have been reported in wheat spikes and wheat kernels displaying little or no visual symptoms of FHB (38, 48, 102). Conversely, visual disease measures may also overestimate the degree of infection and resulting DON accumulation. *F. graminearum* can spread to the spike rachis, blocking the flow of nutrients and water to spikelets beyond the point of infection (86, 93). The affected spikelets senesce and appear diseased even though there is no fungal colonization (93).

Further studies are needed to investigate factors affecting the relationship between FHB and DON and to determine what causes disparities in the relationship between these two variables. In particular, little is understood about asymptomatic infections and DON accumulation. The absence of visual symptoms may be a result of infections that occur too late in the growing season for symptoms to develop before physiological maturity. Additionally, weather conditions may delay fungal growth and/or symptom development even when infections occur at anthesis. Differences in types of resistance among cultivars (see Management and Control of FHB) may also lead to disparity in the FHB/DON relationship.

**SAMPLING FOR DON**

Testing for DON has now become a routine part of the commercialization of wheat grain. Based largely on ELISA-based tests, grain with DON contamination exceeding critical limits is either downgraded in price or rejected
entirely at grain elevators. However, the exact level of grain contamination is
difficult to quantify accurately, partly because of the high variability in toxin
content among kernels of a given grain lot (115). This variability is due in part to
the fact that infection and FHB development is highly variable in wheat fields.

Wheat is most susceptible to infection by *F. graminearum* at anthesis
(Feeke’s 10.5.1) (49), and as such FHB develops best when wet, humid
conditions coincide with this growth stage. For a given cultivar, anthesis is highly
variable among plants within the same field and even among spikelets on the
same spike. Consequently, plants in a single field may reach anthesis under a
wide range of weather conditions over several days, resulting in high in-field
variability in infection, disease development, and DON production. However,
asynchronous crop development is not the only reason for high variability in FHB
and DON in wheat field. Variations in in-field microclimate and spore deposition
and abundance also contribute to variably infection among plants. Studying the
spatial pattern of deposition of viable spores of *G. zeae* in wheat fields between
heading and early grain fill, Schmale et al. (97) reported that more than 90% of
the spore deposition events were random, and the number of deposited spores
varied from one location in the field to another

Considerable research effort has gone into evaluating DON variability in
harvested grain and how sampling techniques can minimize such variability (92,
115). However, in-field FHB and DON variability is still poorly understood.
Champeil et al. (18) found that taking 10 samples, each containing 100 wheat
spikes, returned less variable DON results than sampling in quadrats. The total
sample size was larger for the 10-sample procedure than for the quadrat procedure and the authors suggest that the sample size instead of the procedure itself may have accounted for the reduction in variability. Xu et al. (122) examined within-in field DON variability by sampling from multiple quadrats in fields from four European countries. DON ranged from 17 to 880 ppb. The field with the highest level of DON also had quadrats with no detectable DON. The authors suggested that this variability resulted from disease aggregation or microclimate differences, and urged that when sampling for DON, adequately large samples be taken from a composite of groups of spikes from throughout the field. Del Ponte et al. (27) examined the spatial pattern of FHB symptoms in New York wheat fields and concluded that FHB is randomly distributed, but that aggregation can occur when infected corn residue is present. Hart and Schabenberger (40) sampled in-field for DON and recommended that a total of 20 randomly distributed samples, each comprised of 20-25 wheat spikes, be used for DON testing. Champeil et al. (18) suggested that samples be distributed evenly throughout fields for DON assessment.

Accurate quantification of DON and disease is crucial for research, disease management, and grain marketing. Screening for resistance and evaluation of chemical and other management strategies are all dependent on adequate sampling, which is critical for accurate disease and toxin quantification. In breeding nurseries, if DON or FHB symptoms are inaccurately quantified, cultivars or lines may be misclassified, which can lead to the abandonment of lines that are resistance or the selection of those that not less resistant. Similarly,
when evaluating fungicides, DON and FHB need to be accurately quantified in order to determine the true efficacy of the product being tested, and ultimately, recommend the most effective fungicide. Accurate FHB and DON quantification is also important for developing and validating prediction models and for modeling the relationship between these two measures of disease.

**MANAGEMENT AND CONTROL OF FHB**

Management options for FHB are limited. Planting resistant wheat cultivars is the most effective way to prevent disease, and because it does not require any additional financial inputs, it is also the most economical (15, 43, 124). Worldwide, the most widely used source of FHB resistance is from the Asian cultivar Sumai 3, which was bred from two moderately susceptible parents (6). Cultivars possessing resistance may lack traits that are desirable for agricultural production. Combining resistance and high yield potential has proven to be a difficult task (6, 104).

Currently there are five putative types of resistance to FHB that have been proposed by Schroeder and Christensen (100) and Mesterházy (61) including, i) resistance to infection, ii) resistance to spread, iii) resistance to kernel infection, iv) tolerance to infection, and v) DON decomposition. Assessing visual measures of disease (Type I and II resistance) is less expensive and less time consuming than assessing DON (Type V resistance) or quantifying fungal biomass (Type III resistance), and can be accomplished in-field prior to grain harvest. As a result, Type I and II are the resistance types that are most commonly selected for in
breeding programs. Type II resistance has been characterized as the most common and stable form of FHB resistance available (43, 124). In many cases, fungal growth inhibition may have the added benefit of minimizing DON production (124), leading to high levels of Type III resistance in cultivars with Type II resistance. The standard screening procedure for Type II resistance involves injecting infectious spores into the central spikelet of a wheat spike and then determining how many of the surrounding spikelets also become visibly diseased. In general, in susceptible cultivars, infection spreads rapidly, often resulting in premature bleaching of the entire spike, while symptoms in more resistant cultivars are localized around the point of infection (7). This procedure allows many new lines of wheat to be tested quickly and inexpensively for Type II resistance, but it does not account for the disparity among visual symptoms on chaff material, fungal colonization, and DON contamination in grain, and the effects environmental conditions have on disease development.

Most *F. graminearum* infections occur during anthesis, making this the most critical period for chemical control. Anthesis typically begins with the spikelets in the center of the spike and then spreads to the upper and lower spikelets (49). Depending on weather conditions, it can take several days for a single wheat field to complete anthesis, since different spikes within the field may reach anthesis at different times. Consequently, adequate timing of protectant fungicide application is difficult. Many fungicides, particularly the triazoles are moderately effective against FHB and DON (70, 71, 126). In certain cases, the efficacy of these products is not enough to warrant the additional expense of
fungicide application (125). There is also evidence that some fungicides, belonging to the Strobilurin group of compounds, may trigger an increase in DON production in infected spikes (126).

The occurrence of FHB epidemics has increased over the last several decades (58). This has been attributed to widespread adoption of conservation tillage and no-till cropping practices (29, 58, 58). These practices are intended to protect against top-soil erosion by retaining crop residue from previous growing seasons, but in the case of *F. graminearum*, retention of infected residue also increases inoculum availability. Currently, little quantitative information is available on the direct relationship between inoculum abundance and the severity of FHB and DON production. However, it is clear that increasing spore density could increase infection and toxin content of grain. Burying infected residue promotes decomposition and minimizes spore production (45, 77). However, even if a grower chooses conventional tillage, it may not be effective at controlling FHB because *F. graminearum* spores can be transported long distances by wind (57, 97).

The intent of the research presented in this thesis was to address some of the unanswered biological and epidemiological questions about FHB and its associated toxin. The overall goals were i) to determine the effect of multiple factors including cultivar resistance, environmental conditions, infection timing, and inoculum density on FHB development and DON content of grain and ii) quantify infield FHB and DON variability and determine the effect of variability on the relationship between FHB and DON. This thesis is organized into four
chapters, this introductory chapter, plus three additional chapters. The specific objectives for chapter II were to i) quantify the effects of inoculum density, cultivar resistance, and timing of infection on FHB development and DON accumulation under field conditions ii) examine how inoculum density interacts with cultivar resistance, infection timing, and weather conditions to impact FHB and DON and iii) identify and characterize factors that result in DON accumulation in excess of the acceptable limit when conditions are sub-optimal for disease development. For Chapter III, the objective was to establish the basis for the development of models to estimate the probability of DON exceeding critical thresholds in seeming healthy grain by i) quantifying the effects of infection timing, host resistance, inoculum density, environmental conditions and their interactions on \textit{F. graminearum} biomass and DON accumulation in grain from asymptomatic wheat spikes, ii) evaluating the association between DON and fungal biomass in asymptomatic grain as influenced by cultivar resistance, inoculum density, and environmental conditions, and iii) identifying situations in which asymptomatic \textit{F. graminearum} infections result in toxin contamination of grain in excess of 2 ppm. For Chapter IV, the objective was to establish the basis for the development of a sampling protocol for in-field quantification of DON by i) quantifying the variability in FHB and DON in inoculated plots ii) determine the relationship between variance and mean for FHB and DON; and iii) determining the effect of variability on the relationship between FHB and DON.
CHAPTER 2: FUSARIIUM HEAD BLIGHT DEVELOPMENT AND DEOXYNIVALENOL ACCUMULATION IN WHEAT AS INFLUENCED BY INFECTION TIMING, CULTIVAR RESISTANCE, AND INOCULUM DENSITY

INTRODUCTION

Fusarium head blight (FHB) is an important disease of wheat and other small grains that has profound economic consequences for the wheat and barley industries in the United States and other grain-growing regions of the world. As early as 1890, Arthur reported yield losses from FHB that reached 80% in Madison County, Ohio (3). More recently, losses due to FHB in the United States have been estimated at $3 billion for the period between 1990 and 2002, with $1 billion of these losses being attributed to the 1993 epidemic in the northern Great Plains (21, 58). As a result, the United States Department of Agriculture has classified FHB as “the worst plant disease to hit the United States since the stem rust epidemics of the 1950s” (118). Primary yield losses result from spikelet and whole spike necrosis which leads to the development of light-weight grain (67, 81). Additional FHB-related losses are attributable to grain contamination with the mycotoxin deoxynivalenol (DON). DON in grain poses a serious food safety risk for humans and livestock because it binds to eukaryotic ribosomes and inhibits the elongation and termination steps of protein synthesis (87, 113).
In North America, the main causal agent of FHB is *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* (Schwein.) Petch) (58). Epidemics of FHB are favored by host susceptibility, as well as environmental conditions. *F. graminearum* does not directly penetrate the tissue of the wheat spike, but enters through stomates or by colonizing anthers (82, 86). Hence, the most susceptible period for wheat infection by *F. graminearum* is at anthesis (Feeke’s 10.5.1) (49).

Favorable environmental conditions for infection include prolonged wetness (precipitation or high relative humidity ≥ 80%) and temperatures between 15°C and 30°C (2, 13, 24). If relative humidity is low, ascospores rapidly lose viability and will not germinate (13, 37), and if the water potential is too low (≤ -6.0 MPa for macroconidia) for at least eight hours, germination will not occur (108).

The timing of anthesis is highly variable among wheat cultivars, among plants of the same cultivar, and even among spikelets of the same wheat spike. Consequently, depending on the weather, a wheat crop may reach its most susceptible period for *F. graminearum* infection over several days, during which time plants may be exposed to a range of environmental conditions. There is also evidence suggesting that infections after anthesis can occur and these infections may result in DON contamination above acceptable limits for grain commercialization (25, 38, 48, 75, 123).

Wheat cultivars vary in their resistance to FHB and DON, but no cultivar is immune to this disease-toxin complex. Although cultivar resistance alone can explain much of the variability in DON contamination of grain, this relationship is affected by environmental conditions (96). Studying the effects of post-infection
moisture on disease symptoms and DON, Culler et al. (22) found that under high inoculum density, cultivars typically considered resistant to FHB developed grain with unexpectedly high levels of DON. These results suggest that under conditions of high inoculum density and favorable weather, even the most resistant cultivars may become infected, leading to DON accumulation above critical threshold levels.

It is well known that the availability and abundance of inoculum is important for the development of plant diseases (56). In the epidemiology of FHB, circumstantial evidence supports the role of inoculum density in disease development and DON accumulation. For instance, recent increases in FHB epidemics have been attributed, in part, to the widespread implementation of reduced and no-till cropping systems, which retain more crop residue on the soil surface from one growing season to the next than more traditional cropping practices (29, 58, 95). *F. graminearum* overwinters as a saprophyte on infected corn and wheat residue, so increasing the amount of retained residue is equivalent to increasing the amount of available inoculum for the next growing season (36, 45, 58, 125). However, some field studies found that tillage practices to reduce surface residue did not always affect the incidence of FHB (62, 96). This was probably because spores may have been introduced into the test fields from sources other than the in-field residue. Maldonado-Ramirez et al. (57) reported that ascospores of *G. zeae* were consistently found in the planetary boundary layer of the atmosphere (50 m to 1 km above the earth surface), and consequently, can travel long distances, perhaps playing a vital role in regional
epidemics. Schaafsma et al. (95) found that the number of spores detected in in-field traps was not a good predictor of FHB and DON in grain. Unless condition are favorable for spore dissemination and deposition, spores on residue and in the air may not reach the infection court, resulting in weak or the lack of associations among the amount of surface residue, spores in the air, and FHB.

Paul et al. (69) recently used polynomial distributed lag regression to model the relationship between daily *F. graminearum* spore density on wheat spikes and ambient weather. They reported significant linear relationships between the log of daily spore density and ambient relative humidity, temperature, and rainfall. However, quantitative information on associations among inoculum density on wheat spikes, FHB development, and DON accumulation under field conditions is still lacking. In a survey of colony forming units (CFU) of *F. graminearum* washed from wheat spikes, Francl et al. (36) found that in locations where FHB epidemics occurred, there were more CFUs of *F. graminearum* on spikes than in areas where epidemics did not develop, suggesting an association between spike inoculum density and FHB epidemics. In a more direct study of the relationship between spike inoculum density and FHB/DON, Stein et al. (107) demonstrated that under controlled conditions, both DON and FHB (incidence and severity) were affected by the number of spores per wheat spike. Similarly, in other greenhouse studies, Andersen (2) and Stack and McMullen (106) observed an increase of disease with an increase in the amount of inoculum applied to spikes.
It is clear that FHB development and DON accumulation are influenced by complex interactions among cultivar characteristics (timing and duration of anthesis and resistance), inoculum density, and weather conditions. Additional research into the effect of in-field inoculum density is necessary to understand the dynamics of FHB. It is unclear how inoculum density interacts with other factors such as, timing of infection, weather conditions and host resistance to impact disease development and toxin contamination. The objectives of this study were to i) quantify the effects of inoculum density, cultivar resistance, and timing of infection on FHB development and DON accumulation under field conditions ii) examine how inoculum density interacts with cultivar resistance, infection timing, and weather conditions to impact FHB and DON and iii) identify and characterize factors that result in DON accumulation in excess of the acceptable limit when conditions are sub-optimal for disease development.

**MATERIALS AND METHODS**

**Experimental design and treatment layout.** Field experiments were conducted during the consecutive growing seasons of 2007-2008 and 2008-2009 at the Ohio Agricultural Research and Development Center, Wooster, Ohio. The study was designed as a randomized complete block, with three replicate blocks and a split-split-split plot treatment structure. Wheat planting date was the whole plot (early and late), and cultivar (Cooper, Hopewell, Truman), timing of inoculation (anthesis; one, two, and three weeks post-anthesis), and inoculum density (0, 10, 20, and 30 x 10^4 spores/ml) were the sub-plot, sub-sub plot, and
sub-sub-plots, respectively. In both years, plots were planted into fields previously planted with oats using a Kincaid wheat drill, calibrated to plant at an approximate seeding rate of 400 seeds/m². There were seven rows per plot, with 19 cm spacing between rows.

The planting date factor (PD) consisted of two levels, a normal (PD1) and a late planting (PD2). In 2007, PD1 was on October 8 and in 2008 on September 23. PD2 was 11 days after the first in 2007 and 14 days after in 2008. At each planting date, 34 m-long strips of three locally adapted soft red winter wheat cultivars, with different levels of resistance to FHB based on visual symptoms (Cooper, susceptible [S]; Hopewell, moderately susceptible [MS]; Truman, moderately resistant [MR]), were planted as the sub-plot. The purpose of using two planting dates was to increase the staggering of anthesis and later growth stages throughout the growing season, and consequently, increase the chance of inoculation/infection of the different cultivars occurring under a range of environmental conditions.

Each strip of each cultivar was divided into four 8.5 m-long sub-sub-plots for subsequent inoculation at four different growth stages, anthesis (Feeke’s 10.5.1), 1 week, 2 weeks, and 3 weeks after anthesis. The late inoculation times roughly corresponded to the milky ripe (Feeke’s 11.1), soft dough (Feeke’s 11.2), and hard dough (Feeke’s 11.3) stages of kernel development. Throughout this paper, abbreviations GS1-GS4 refer to the Feeke’s growth stages, 10.5.1, 11.1, 11.2, and 11.3, respectively. Each sub-sub-plot was further divided into four 1.5 m plots (sub-sub-sub-plots) to which four inoculum density treatments, 0, 10, 20,
and $30 \times 10^4$ spores/ml, were randomly assigned. The first inoculum density was applied as a water control (C) and served as a measure of natural infection. The remaining levels were chosen based on previous research (69) to represent spike inoculum densities within the range commonly observed under natural conditions, and will be referenced to here as the low (L), medium (M), and high (H) inoculum density treatments.

**Inoculation and data collection.** Inoculum consisted of a mixture of spores (macroconidia and ascospores) from ten *F. graminearum* isolates collected from diseased spikes in wheat fields in Ohio. Isolations were done on streptomycin-amended Komada media (47), which is selective for *Fusarium*. Putative *F. graminearum* colonies were transferred to potato dextrose agar (PDA) (64) and carnation leaf agar (35) for species identification based on colony and spore morphology (52, 64, 110). Ascospores were produced on carrot agar (46) and macroconidia on mung bean agar (both supplemented with 0.3 g/L streptomycin sulfate) (33). Carrot agar plates were seeded with plugs from cultures stored on PDA and incubated under ultraviolet and white light for 12 h per day. Five days after seeding, mycelia were scrapped from the plate with a sterile metal spatula. This reduced the growth of aerial mycelia and subsequent macroconidia production, and stimulated the production of ascospores. Separate mung bean agar plates were seeded with spore suspensions from the 10 isolates and grown under a 12-h photoperiod. After approximately one week of incubation, plates were flooded with sterile water and a glass rod was used to suspend spores. The resulting suspension was filtered through several layers of
sterile cheesecloth to remove fragments of mycelia and pieces of perithecia. Concentrated stock suspensions of both ascospores and macroconidia were stored at -20°C until inoculation.

Approximately 12 hours before being used for inoculation, stock suspensions were thawed at room temperature (22 - 25°C), and after thawing, stored at 4°C for all subsequent inoculations. The concentrations of these suspensions were determined by counting spores on a hemacytometer, and the desired L, M, and H inoculum densities were adjusted by adding sterile water to aliquots of the stock suspension. A 1:1 suspension of ascospores and macroconidia was prepared by mixing equal volumes of suspension of the two spore types. Inoculum was applied to sub-sub-sub plots beginning at anthesis (GS1) followed by inoculations at GS2, GS3, and GS4. Crop development was more uniform in 2008 than in 2009. In 2008, GS1 inoculations began on June 2 and GS4 inoculations ended on June 27; whereas, in 2009, the first set of GS1 inoculations was done on May 22 and the last set of GS4 inoculations on June 25. At the time of inoculation, Tween 20 was added to the inoculum at a rate of 0.01% (v/v). Applications were done using a backpack sprayer (R&D Sprayers, Opelousas LA) calibrated to deliver approximately 100 ml/m from three nozzles at 0.21 MPa. Each sub-sub-sub plot received approximately 150 ml of the inoculum suspension.

Between Feekes growth stages 11.2 and 11.3, visual estimates of disease intensity were recorded. Incidence (INC) was quantified as the number of visually diseased spikes out of sixty spikes per sub-sub-sub plot and index (IND) as the
mean percent of necrotic tissue for those same spikes. When grain moisture fell to approximately 15%, each plot was individually harvested using a research plot combine (ALMACO SPC20, Nevada, IA), and grain was assessed for percentage Fusarium damaged kernels (FDK) with the aid of a visual rating scale (32). A sample of grain from each sub-sub-sub plot was ground using a Laboratory Mill (Model LM 3303, Perten Instruments Inc. Springfield, IL) and sent for DON analysis by gas chromatography mass-spectrometry at the U.S. Wheat and Barley Scab Initiative DON Testing Laboratory at the University of Minnesota.

Throughout each growing season, temperature, surface wetness, and relative humidity information were collected at hourly intervals between jointing (Feekes 6) and harvest using a Campbell CR10 (Campbell Scientific, Logan, UT) weather station, mounted adjacent to the plots.

**Data analysis.** Prior to data analysis, all response variables were transformed to stabilize variance. The effects of planting date (PD), cultivar (CV), infection timing (IT), inoculum density (ID) and their interactions on arcsine-transformed FHB incidence (INC), index (IND), and log-transformed DON were determined with a linear mixed model (55). In the analysis, PD, CV, IT and ID were all treated as qualitative factors and considered fixed effects in the model, whereas, block and all interactions involving block were considered random effects. Analyses were done using PROC MIXED of SAS (SAS Institute, Cary, NC). Fixed effects were evaluated with $F$ tests and random effects were evaluated with standard normal test statistics. *Estimate, lsmeans*, and *contrast* statements were used to compare treatments and treatment combinations.
RESULTS

Fusarium head blight and deoxynivalenol. In 2008, planting date, PD 1 and PD2 flowered on the same days (Table 2.1). All of the plots planted with the S cultivar reached anthesis on day 154 (Table 2.1). The MS and MR cultivars each flowered on two days, but there was no distinction between PD1 and PD2 (Table 2.1). In 2009, there was a temporal separation between anthesis for PD1 and PD2 for all cultivars (Table 2.1). On day 146, some plots from both PD1 and PD2 of the S cultivar reached anthesis, but this was the only day that there was overlapping anthesis between planting dates for any given cultivar (Table 2.1).

FHB intensity was higher in 2008 than in 2009. In the former year, mean index (IND), incidence (INC), and percent Fusarium damaged kernels (FDK) ranged from 0 to 32%, 0 to 70%, and 0 to 50%, respectively, compared to 0 to 18%, 0 to 33%, and 0 to 40%, respectively, in the latter (Fig. 2.1). However, mean DON content of harvested grain was lower in the first year of the study than in the second, ranging from not detectable (< 0.05 ppm) to 13 ppm in year 1 and 0.1 to 19.6 ppm in year 2 (Fig. 2.1).

In 2008, averaged across inoculum density, inoculation timing, and cultivar, all measures of FHB and DON were similar between planting dates 1 (PD1) and 2 (PD2), with means of 3.15 % for IND and 1.45 ppm for DON for PD1 and 2.78 % and 1.32 ppm for PD2. Among the cultivars, moderately susceptible (MS) Hopewell had the highest level of mean IND (3.88 %) and DON (3.05 ppm) followed by susceptible (S) Cooper (1.49 % for IND and 1.29 ppm for DON) and
moderately resistant (MR) Truman (0.53 % for IND and 0.77 ppm for DON). In the first year, for both planting dates and for all three cultivars, mean IND and DON were generally highest in plots inoculated at anthesis (GS1), decreasing progressively for inoculations done at later growth stages (Fig. 2.2 and Fig. 2.3). Mean DON levels, averaged across cultivars and planting dates, for GS1, GS2, GS3 and GS4 were 3.69, 1.53, 0.87, and 0.73 ppm. In general, all measures of disease and DON increased with increasing inoculum density for all cultivars, with the response to inoculum density being more pronounced for GS1 and GS2 (Figs. 2.2, 2.3, and 2.4).

Trends were very similar in 2009 (Figs. 2.1, 2.5, 2.6, 2.7), however, contrary to what was observed in 2008, DON contamination was considerably lower for PD1 than for PD2 in 2009, with means for the two planting dates being 1.38 and 2.03 ppm, respectively (Fig. 2.6). In addition, in 2008, mean DON contamination exceeded 2 ppm when the susceptible and moderately susceptible cultivars were inoculated at GS1 and approached the 2 ppm level in the moderately resistant cultivar when the highest inoculum density was applied at GS1 or in the moderately susceptible cultivar when applied at GS2 (Fig. 2.3); however, for the second planting date of 2009 DON reached or exceeded 2 ppm for all combinations of inoculum density and inoculation timing for Hopewell (Fig. 2.6). DON accumulation also exceeded 2 ppm when the moderately resistant cultivar was inoculated with medium and high inoculum densities at GS1 for planting PD1 and at GS2 for PD2 (Fig. 2.6).
Differences in mean disease and DON levels between the two years and between the two planting dates in 2009 were probably reflective of differences in weather conditions. In 2008, mean ambient temperature, relative humidity (RH), and daily rainfall for the period between Feekes GS 10 (boot stage) for the earliest cultivar and harvest was 20.2°C, 62.8 %, and 5.2 mm, respectively (Figs. 2.8-2.10). The corresponding means for 2009 were 18.9°C, 73.9 %, and 2.2 mm (Figs. 2.11-2.13). For the susceptible cultivar, mean temperature at anthesis was similar for PD1 (19.7°C) and PD2 (19.2°C) in 2009, while mean temperature was lower during anthesis in the second planting date for the moderately susceptible (20.0°C for PD1 and 16.6°C for PD2) and moderately resistant cultivars (17.8°C for PD1 and 14.2°C for PD2). Mean RH was 68.0, 74.0, and 77.3% during anthesis for S, MS, and MR respectively during PD1 of 2009, and 84.9, 72.7, and 77.7% during PD2. For S in PD1, there was no rainfall at anthesis, while 17.7 mm fell during anthesis for PD2. Total rainfall for MS at anthesis was 6.0 and 27.1 mm for PD1 and PD2, respectively, and 11.7 and 24.9 mm for MR for PD1 and PD2.

Mixed model analysis of main and interaction effects. Separate linear mixed model analyses were performed to evaluate the effects of planting date (PD) cultivar (CV), inoculation timing (IT) and inoculum density (ID) and their interactions on arcsine-transformed FHB incidence (arcINC), index (arcIND), Fusarium damages kernels (arcFDK), and log-transformed DON (lnDON). For measures of FHB intensity (arcIND, arcINC, and arcFDK), the main effects of CV, IT, and ID were statistically significant in both years, and the effect of PD
was only statistically significant for lnDON and arclNC in 2009, but not in 2008 (Table 2.2). Two-way interactions of CV x IT and IT x ID were significant for all responses in both years of this study (Table 2.2). Additionally, the interaction effects of CV x ID and CV x ID x IT on all response variables were statistically significant in 2008, but not in 2009 (Table 2.2). The effects of CV, IT, and ID depended on PD; for instance, in 2008, the effect of PD on arclND depended on ID, and in 2009 the effects of PD on lnDON, arclND, and arcFDK varied with IT and/or CV (Table 2.2). In fact, in 2009, for all responses, with the exception of arcFDK, the four-way interaction effect of PD x CV x IT x ID was statistically significant (Table 2.2).

In an effort to characterize the compensatory effects of PD, CV, IT and ID on FHB development and DON accumulation, the *lsmeans* statement was used to compare mean arclND and lnDON between specific treatment combinations, based on the significant interactions presented in Table 2.2 and the graphical summaries shown in Figures 2.2-2.7. For instance, based on the assumption, that under favorable environmental conditions, high inoculum density will compensate for sub-optimum infection timing (post-anthesis) to produce comparable levels of disease development and toxin accumulation, mean responses for a give cultivar were compared at different combinations of PD x ID x IT. If, for instance, a non-significant difference is observed between the highest inoculum density applied at GS2 (the milky ripe growth stage) and the lowest inoculum density applied at GS1, this would be suggestive of inoculum density compensating for infection occurring later then the optimum time (anthesis) for
FHB development. For FHB IND, in 2008, trends were consistent with what is generally expected for this disease, with the highest levels of intensity occurring in the susceptible cultivars inoculated with the highest spore densities at anthesis (Fig. 2.2). However, in 2009, there were several instances of inoculations at later growth stages having comparable levels of IND to inoculations done at anthesis or the moderately resistance cultivar having levels of disease similar to that of the susceptible cultivars (Fig. 2.5). For instance, for the second planting date of 2009, Truman (MR) inoculated with the two highest spore concentrations at GS1 and GS2 had comparable levels of disease to Hopewell (MS) inoculated at GS2 with the M spore concentration and Cooper (S) inoculated at GS2 with the H spore concentration (Table 2.3).

For DON, occurrences suggestive of interactions leading to compensations for suboptimum conditions for toxin accumulation were even more evident, especially for the second planting date of 2009 (Fig. 2.6). In 2008, MR inoculated with the highest spore concentration at GS1 had comparable DON contaminated to MS and S inoculated with a similar concentration at GS2 (Table 2.4). Similarly, for growth stage 2 of 2009, MR with the H and M inoculation treatments had higher or comparable DON levels to S inoculated with all inoculation treatments at GS1 and MS with the L inoculation treatment at GS2 (Table 2.4). For the moderately susceptible cultivar (Hopewell) weather condition during the second planting and natural infection lead to relative high (above 2 ppm) toxin accumulation at GS3 and GS4, for all inoculation treatments (Fig. 2.6).
DISCUSSION

The amount of available inoculum is epidemiologically important for the development of many plant diseases (56). FHB is generally thought to increase with inoculum density, but little research has focused on the relationships between inoculum density and disease measures, including DON (2, 106). Other factors known to impact FHB include host resistance, timing of infection, and environmental conditions at infection and throughout the growing season (24, 38, 48, 61). Few studies have investigated the combined effects of, or characterized compensations among, these factors on FHB development and DON accumulation. Hence, the impact of the interaction of these factors on FHB and DON remains largely unknown. For what is known, relationships are often extrapolated from studies conducted under controlled greenhouse or growth chamber conditions, with the result that knowledge of in-field effects are inadequately understood.

From the results of this study it is evident that host resistance, infection timing, and inoculum density individually have significant impacts on DON and all measures of disease (Table 2.2), and that because of the interactions among these factors, there may be compensation for sub-optimum levels of one or more factors under certain field conditions. DON and disease measures were greatest when infection occurred at GS1 and generally increased with inoculum density treatment (Figs. 2.2-2.7). These trends were anticipated from the direct and circumstantial evidence available in the literature. Stein et al. (107) demonstrated
that DON and the incidence and severity of FHB increased with applied inoculum density under controlled conditions, whereas Paulitz et al. (75) and Hart et al. (38) demonstrated that disease and DON decreased with post-anthesis infections. Additionally, the increased occurrence and severity of FHB epidemics over recent decades has often been attributed to tillage-related increases in available inoculum (29, 58, 77).

The pattern of cultivar response was somewhat unanticipated. For FHB, host resistance exists in five putative types. Resistance to infection (Type I), and resistance to fungal colonization in spikes (Type II) are evaluated based on visual symptoms on external wheat tissue, while resistance to kernel infection (Type III), tolerance to infection (Type IV), and DON decomposition (Type V) are not visible on external tissues and are not typically assessed when screening cultivars for resistance (61, 100). In this study, it was anticipated that the S cultivar would be the one with the most disease and highest level of DON, followed by MS, and then MR, because of their resistance classifications, yet this was clearly not what happened in all cases. As expected, Truman had consistently lower levels of disease and DON than Hopewell and Cooper for any given combination of growth stage, inoculation timing, and inoculum density, but moderately susceptible Hopewell had higher or comparable responses to those of susceptible Cooper. The differences between Hopewell and Cooper (in favor of Hopewell) were much greater in 2009 than in 2008, suggesting that the relative response of these two cultivars depended on the weather conditions. Disparity between the observed responses and the resistance classification of Hopewell
and Cooper may be attributed to the fact that Hopewell may have become more susceptible to FHB than Cooper between the time they was classified as MS and S respectively, and now, or that more favorable weather conditions for DON production, such as the cooler temperatures during PD2 of 2009, occurred at the time of anthesis for Hopewell than for Cooper.

Compensatory interactions were also observed, where a combination of optimal and sub-optimal conditions resulted in unacceptably high levels of DON. Most of these interactions occurred for inoculations conducted at GS2 with the H inoculum density treatment. The results of this study also suggest that these compensatory interactions are heavy influenced by environmental conditions. In 2008, because of environmental conditions, PD1 and PD2 reached anthesis nearly simultaneously; consequently there was very little environmental difference between the planting dates at inoculation. In 2009, PD1 and PD2 flowered at different times, under different environmental conditions, and the effect of PD was significant. DON and disease were much greater for PD2 than PD1. In 2008, late inoculations resulted in DON in excess of 2 ppm only when conducted at GS2 with the H inoculum treatment, but for PD2 of 2009, DON in excess of 2 ppm occurred more frequently and for a wider range of situations. DON exceeded 2 ppm for all infection times and inoculum densities for MS, implying that not only is MS more susceptible than the other cultivars, it is highly susceptible for a longer period of time under favorable environmental conditions. In 2008, DON in MR exceeded 2 ppm only under the most ideal conditions (GS1 H treatment), while in 2009, DON exceeded 2 ppm for M and H treatments at the
GS2 inoculation, but not at all for the GS1 inoculations, suggesting that environmental conditions may have a greater impact on DON in MR than growth stage at infection. This is consistent with Culler et al. (22) where an interaction between favorable environmental conditions, resistant cultivars, and high inoculum density resulted in unexpectedly high levels of DON. Although S had higher levels of disease, the response of DON and FDK when inoculated at late growth stages was more similar to the response of MR than to MS. The cultivar S did not accumulate DON in excess of 2 ppm in PD2 of 2009; this could imply that although S has an inherent susceptibility to FHB, DON contamination was comparable to that of the moderately resistant cultivar under the conditions of this study.

The accumulation of DON in grain has economic consequences when it exceeds 2 ppm, and FHB affects grain quality by reducing test weight. Collectively, FHB and DON reduce the market value of grain. Throughout this study there were many occurrences of DON exceeding the 2 ppm threshold, and although most of these occurrences were the result of infections at anthesis on the MS and S cultivars (Figs. 2.3 and 2.6), there were instances in which and DON exceeded 2 ppm under suboptimal conditions in terms of resistance and timing of infection. In particular, the results clearly showed that high inoculum density may compensate for suboptimum timing of infection and cultivar resistance, leading to comparable levels of diseases and DON between resistant and susceptible cultivars under certain weather conditions. However, with only two years of data, it is unlikely that the full range of conditions affecting the
relationships investigated herein were fully explored. Continued research is needed to better identification and quantification the influence of environmental conditions on the interaction effects of inoculum density, infection timing and resistance on the development of FHB symptoms and contamination of grain with DON. Understanding the epidemiology of FHB development and DON production will allow for the implementation of more effective FHB management programs and contribute to the accuracy of FHB and DON prediction models.
Figure 2.1:
Boxplots for FHB (A) percent disease incidence (INC), (B) percent disease index (IND) for 2008 and 2009, as well as (C) DON from combine harvested grain, and (D) percent Fusarium-damaged kernels (FDK). Each gray box represents the middle 50% of the data, and the line within the boxes indicates the median value of (A) INC, (B) IND, (C) DON, and (D) FDK for each year.
Figure 2.2:
Percent Fusarium head blight disease incidence (gray) and disease index (black) for 2008. Results are partitioned by planting date (one [PD 1] and two [PD 2]), cultivar (susceptible [S], moderately susceptible [MS], and moderately resistant [MR]), growth stage at infection (GS1-GS4), and inoculum density (water control [C], low density [L], medium density [M], high density [H]).
Figure 2.3:
Deoxynivalenol (DON) content in ppm from combine harvested grain in 2008. Results are partitioned by planting date (one [PD 1] and two [PD 2]), cultivar (susceptible [S] – medium gray bars, moderately susceptible [MS] – black bars, and moderately resistant [MR] – light gray bars), growth stage at infection (GS1-GS4), and inoculum density (water control [C], low density [L], medium density [M] and high density [H]). The dashed black reference line indicates the critical threshold of 2 ppm for DON in grain intended for human consumption.
Figure 2.4:
Percent Fusarium damaged kernels (FDK) in combine harvested grain from 2008. Results are partitioned by planting date (one [PD 1] and two [PD 2]), cultivar (susceptible [S] – medium gray bars, moderately susceptible [MS] – black bars, and moderately resistant [MR] – light gray bars), growth stage at infection (GS1-GS4), and inoculum density (water control [C], low density [L], medium density [M] and high density [H]).
Figure 2.5:
Percent Fusarium head blight disease incidence (gray) and disease index (black) for 2009. Results are partitioned by planting date (one [PD 1] and two [PD 2]), cultivar (susceptible [S] – medium gray bars, moderately susceptible [MS] – black bars, and moderately resistant [MR] – light gray bars), growth stage at infection (GS1-GS4), and inoculum density (water control [C], low density [L], medium density [M] and high density [H]).
Figure 2.6:
Deoxynivalenol (DON) content in ppm of combine harvested grain in 2009. Results are partitioned by planting date (one [PD 1] and two [PD 2]), cultivar (susceptible [S] – medium gray bars, moderately susceptible [MS] – black bars, and moderately resistant [MR] – light gray bars), growth stage at infection (GS1-GS4), and inoculum density (water control [C], low density [L], medium density [M] and high density [H]). The dashed black line indicates the critical 2 ppm threshold for DON in grain intended for human consumption.
Figure 2.7:
Percent Fusarium damaged kernels (FDK) in combine harvested grain from 2009. Results are partitioned by planting date (one [PD 1] and two [PD 2]), cultivar (susceptible [S] – medium gray bars, moderately susceptible [MS] – black bars, and moderately resistant [MR] – light gray bars), growth stage at infection (GS1-GS4), and inoculum density (water control [C], low density [L], medium density [M] and high density [H]).
Figure 2.8:
Average temperature (C) for each day of the year (DOY) from boot stage (Feeke’s growth stage 10) on day 147 (May 27) to wheat harvest on day 191 (July 10) in 2008. Arrows indicate anthesis dates for S (dark gray), MS (white), MR (light gray) cultivars. Both planting dates were represented at all anthesis times. Mean temperature for DOY 147-191 is indicated by the dashed line.
Figure 2.9:
Average daily relative humidity (RH) for each day of the year (DOY) from boot stage (Feeke's growth stage 10) on day 147 (May 27) to wheat harvest on day 191 (July 10) in 2008. Arrows indicate anthesis dates for S (dark gray), MS (white), MR (light gray) cultivars. Both planting dates were represented at all anthesis times. Overall mean relative humidity for DOY 147-191 is indicated by the dashed line.
Figure 2.10:
Total daily rainfall in mm for each day of the year (DOY) from boot stage (Feeke’s growth stage 10) on day 147 (May 27) to wheat harvest on day 191 (July 10) in 2008. Arrows indicate anthesis dates for S (dark gray), MS (white), MR (light gray) cultivars. Both planting dates were represented at all anthesis times.
Figure 2.11:
Average temperature (C) for each day of the year (DOY) from boot stage (Feeke’s growth stage 10) on day 137 (May 17) to wheat harvest on day 196 (July 15) in 2009. Arrows indicate anthesis dates for S (dark gray), MS (white), MR (light gray) cultivars. Days when planting date 1 reached anthesis are indicated by arrows with thin outlines, while the thick black outlined arrows indicate days when planting date 2 flowered. For S there was overlap between the planting dates on day 146. Overall mean temperature for DOY 137-196 is indicated by the dashed black line.
Figure 2.12:
Average daily relative humidity (RH) for each day of the year (DOY) from boot stage (Feeke’s growth stage 10) on day 137 (May 17) to wheat harvest on day 196 (July 15) in 2009. Arrows indicate anthesis dates for S (dark gray), MS (white), MR (light gray) cultivars. Days when planting date 1 reached anthesis are indicated by arrows with thin outlines, while the thick black outlined arrows indicate days when planting date 2 flowered. For S there was overlap between the planting dates on day 146. Overall mean relative humidity for DOY 137-196 is indicated by the dashed black line.
Figure 2.13:
Total daily rainfall in mm for each day of the year (DOY) from boot stage (Feeke’s growth stage 10) on day 137 (May 17) to wheat harvest on day 196 (July 15) in 2009. Arrows indicate anthesis dates for S (dark gray), MS (white), MR (light gray) cultivars. Days when planting date 1 reached anthesis are indicated by arrows with thin outlines, while the thick black outlined arrows indicate days when planting date 2 flowered. For S there was overlap between the planting dates on day 146.
Table 2.1: Day of the year (DOY) when sub-sub-sub plots reached anthesis for planting dates 1 and 2 for the susceptible (S), moderately susceptible (MS), and moderately resistant (MR) cultivars in 2008 and 2009.

<table>
<thead>
<tr>
<th>Year</th>
<th>S&lt;sup&gt;b&lt;/sup&gt;</th>
<th>MS&lt;sup&gt;c&lt;/sup&gt;</th>
<th>MR&lt;sup&gt;c&lt;/sup&gt;</th>
<th>S&lt;sup&gt;c&lt;/sup&gt;</th>
<th>MS&lt;sup&gt;c&lt;/sup&gt;</th>
<th>MR&lt;sup&gt;c&lt;/sup&gt;</th>
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<td>158</td>
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<td>158</td>
</tr>
<tr>
<td>2009</td>
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<td>148</td>
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<td>148</td>
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<td></td>
<td></td>
<td></td>
<td>152</td>
<td>155</td>
</tr>
</tbody>
</table>

<sup>a</sup> Day of the year (DOY) designation. In 2008, DOY 154 was June 3 and DOY 158 was June 7. In 2009, DOY 142 was May 22 and DOY 155 was June 4.

<sup>b</sup> In 2008, the planting dates (PD) reached anthesis simultaneously, while in 2009 there was no overlap in planting date flowering except for the susceptible (S) cultivar.

<sup>c</sup> The three cultivars, susceptible (S), moderately susceptible (MS), and moderately resistant (MR), do not mature at the same rate and so reached anthesis at different times.
Table 2.2: Main and interaction effects of planting date (PD), cultivar (CV), infection time (IT), and inoculum density (ID) on deoxynivalenol (DON) in grain, Fusarium head blight index (IND) and incidence (INC), and Fusarium-damaged kernels (FDK) in 2008 and 2009.

| Effects | 2008 | | | | 2009 | | | |
|---------|------|------|------|------|------|------|------|------|------|
|         | DON<sup>a</sup> | IND<sup>b</sup> | INC<sup>b</sup> | FDK<sup>b</sup> | DON<sup>a</sup> | IND<sup>b</sup> | INC<sup>b</sup> | FDK<sup>b</sup> |
| PD      | F   | p   | F   | p   | F   | p   | F   | p   | F   | p   |
| CV      | 57.02 <.0001 | 14.12 0.0024 | 12.46 0.0035 | 30.03 0.0002 | 104.44 <.0001 | 45.07 <.0001 | 49.82 <.0001 | 163.12 <.0001 |
| PD*CV   | 0.3 0.7461 | 0.17 0.8442 | 0.31 0.7423 | 0.64 0.5513 | 5.42 0.0324 | 0.05 0.949 | 1.32 0.2707 | 4.23 0.0558 |
| IT      | 151.59 <.0001 | 87.29 <.0001 | 90.96 <.0001 | 66.54 <.0001 | 48.4 <.0001 | 62.24 <.0001 | 43.1 <.0001 | 29.23 <.0001 |
| PD*IT   | 0.76 0.5241 | 0.52 0.6742 | 0.72 0.5491 | 0.48 0.6985 | 5.07 0.005 | 1.99 0.1328 | 1.11 0.3459 | 1.60 0.2052 |
| CV*IT   | 12.84 <.0001 | 11.1 <.0001 | 12.31 <.0001 | 12.79 <.0001 | 4.91 0.0009 | 14.17 <.0001 | 4.51 0.0003 | 5.7 0.0003 |
| PD*CV*IT | 0.92 0.49 | 0.79 0.5805 | 0.36 0.9017 | 0.84 0.5501 | 1.24 0.3082 | 2.47 0.042 | 2.15 0.0501 | 2.61 0.0334 |
| ID      | 83.38 <.0001 | 19.29 <.0001 | 19.35 <.0001 | 14.47 <.0001 | 11.04 <.0001 | 7.5 0.0001 | 8.55 <.0001 | 6.76 0.0003 |
| PD*ID   | 0.16 0.9248 | 3.61 0.0149 | 1.2 0.3113 | 0.93 0.4298 | 1.32 0.2714 | 0.9 0.4428 | 1.33 0.266 | 4.36 0.0057 |
| CV*ID   | 4.29 0.0005 | 2.64 0.0187 | 2.95 0.0095 | 3.25 0.005 | 1.32 0.2514 | 0.82 0.5527 | 1.41 0.2113 | 1.34 0.2416 |
| PD*CV*ID | 0.21 0.9737 | 0.5 0.807 | 0.92 0.4833 | 1.3 0.2618 | 1.14 0.3424 | 1.65 0.1388 | 1.31 0.2548 | 0.68 0.6697 |
| IT*ID   | 47.78 <.0001 | 16.94 <.0001 | 16.43 <.0001 | 15.96 <.0001 | 4.72 <.0001 | 3.53 0.0006 | 4 0.0001 | 4.03 0.0001 |
| PD*IT*ID | 0.89 0.535 | 3.02 0.0025 | 1.7 0.0943 | 1.22 0.2863 | 0.86 0.5582 | 1.61 0.1179 | 1.05 0.4034 | 1.41 0.1905 |
| CV*IT*ID | 3.34 <.0001 | 1.99 0.0137 | 2.56 0.0011 | 2.89 0.0002 | 1.54 0.0849 | 1.19 0.2745 | 1.21 0.2531 | 1.08 0.3792 |
| PD*CV*IT*ID | 1.12 0.3393 | 0.58 0.906 | 0.54 0.9347 | 0.97 0.5013 | 1.83 0.0273 | 1.71 0.0442 | 1.88 0.0195 | 0.95 0.5214 |

<sup>a</sup> Deoxynivalenol (DON) content in grain was log-transformed prior to mixed model analysis to normalize the data and stabilize variance.

<sup>b</sup> Fusarium head blight index (IND) and incidence (INC), and Fusarium-damaged kernel data was transformed with an angular transformation prior to mixed model analysis to stabilize variance and normalize the data.

<sup>c</sup> All effects were calculated for transformed data using mixed model analysis. Planting date (PD), cultivar (CV), infection time (IT), and inoculum density (ID) were designated as fixed factors in the analysis.
Table 2.3: Differences of least square means from mixed model analysis for index of Fusarium head blight for selected interactions of planting date (PD), cultivar (CV), infection time (IT) and inoculum density (ID).

<table>
<thead>
<tr>
<th>Year</th>
<th>PD&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CV&lt;sup&gt;c&lt;/sup&gt;</th>
<th>IT&lt;sup&gt;d&lt;/sup&gt;</th>
<th>ID&lt;sup&gt;e&lt;/sup&gt;</th>
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<th>ID&lt;sup&gt;e&lt;/sup&gt;</th>
<th>T-value&lt;sup&gt;f&lt;/sup&gt;</th>
<th>P-value</th>
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<tbody>
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<td>PD2</td>
<td>MR</td>
<td>GS1</td>
<td>H</td>
<td>PD2</td>
<td>MS</td>
<td>GS2</td>
<td>M</td>
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<td>0.2460</td>
</tr>
<tr>
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<td>GS1</td>
<td>M</td>
<td>PD2</td>
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<td>GS2</td>
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<td>PD2</td>
<td>MS</td>
<td>GS2</td>
<td>M</td>
<td>-1.52</td>
<td>0.1307</td>
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<td>M</td>
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<td>GS2</td>
<td>H</td>
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<td>0.6249</td>
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</tbody>
</table>

<sup>a</sup> The 1 and 2 designate to the first and second interactions for which means are being compared.

<sup>b</sup> PD1 is the first planting date, and PD2 is the second planting date.

<sup>c</sup> MR refers to the moderately resistant (Truman) cultivar, while MS and S refer to the moderately susceptible (Hopewell), and susceptible (Cooper) cultivars, respectively.

<sup>d</sup> Inoculum was applied at four stages of kernel development, including anthesis (GS1), milky ripe (GS2), soft dough (GS2), and hard dough (GS4).

<sup>e</sup> The letters L, M, and H refer to the low, medium, and high density spore concentrations.

<sup>f</sup> Prior to mixed model analysis, Fusarium head blight disease index was transformed with an angular transformation to normalize the data and stabilize variance. The t-value is on the transformed scale.
Table 2.4: Differences of least square means from mixed model analysis for deoxynivalenol (DON) content of grain for selected interactions of cultivar (CV), infection time (IT) and inoculum density (ID).

<table>
<thead>
<tr>
<th>Year</th>
<th>CV</th>
<th>IT</th>
<th>ID</th>
<th>CV</th>
<th>IT</th>
<th>ID</th>
<th>T-value</th>
<th>P-value</th>
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</thead>
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<td>MS</td>
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<td>GS2</td>
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<td>L</td>
<td>1.16</td>
<td>0.2469</td>
</tr>
</tbody>
</table>

Note: The 1 and 2 designate to the first and second interactions for which means are being compared.

MR refers to the moderately resistant (Truman) cultivar, while MS and S refer to the moderately susceptible (Hopewell), and susceptible (Cooper) cultivars, respectively.

Inoculum was applied at four stages of kernel development, including anthesis (GS1), milky ripe (GS2), soft dough (GS2), and hard dough (GS4).

The letters L, M, and H refer to the low, medium, and high density spore concentrations.

Prior to mixed model analysis, deoxynivalenol (DON) content in grain was transformed with a log transformation to normalize the data and stabilize variance. The t-value is on the transformed scale.
CHAPTER 3: FACTORS INFLUENCING POST-ANTHESIS INFECTION AND DEOXYNIVALENOL ACCUMULATION IN WHEAT GRAIN HARVESTED FROM ASYMPTOMATIC SPIKES

INTRODUCTION

Fusarium head blight (FHB) is a fungal disease that limits the production of wheat and other small grain in all major grain-growing regions of the world. In North America, the primary causal agent of FHB is *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* (Schwein.) Petch) (58). Yield reduction results from fungal invasion of wheat florets, leading to production of shriveled, low-weight kernels (67, 81). Reduced kernel weight negatively impacts grain quality, resulting in a devaluation of the crop (58, 117). Additional quality losses result from grain contamination with mycotoxins produced by the infecting fungus. The toxin of primary concern, deoxynivalenol (DON), impedes protein synthesis in eukaryote cells and poses a serious food safety risk to humans and livestock, even at low levels (80, 87, 113). DON in excess of 2 ppm increases the processing costs for millers and others in the wheat industry who must produce a final product with 1 ppm or less DON to meet the advisory limit established by the US Food and Drug Administration for products intended for human consumption.
FHB development is strongly dependent on environmental conditions (24, 96). Moisture is necessary for the germination of ascospores and macroconidia, and when periods of prolonged rainfall or high relative humidity coincide with the most susceptible stage of wheat development, anthesis (Feeke’s 10.5.1) (49), severe infections occur. These infections typically lead to high levels of grain contamination with DON (2, 13, 24, 37, 108). In the development of FHB, DON is considered a virulence factor (28, 60, 83, 84), with the level of disease severity being proportional to the toxin producing ability of the fungus (60, 83). As such, there is, in general, a significant positive relationship between FHB development (visual symptoms) and DON accumulation (72, 73, 74). However, this relationship is highly variable. In some instances, relatively high levels of DON may accumulate in grain harvested from spikes with little or no visible symptoms of FHB (17, 54, 102) or, conversely, disproportionately low levels of DON may accumulate when the levels of visual symptoms are high (17, 103). Under certain weather conditions, there may be greater or lesser spread of the toxin and fungus within the wheat spike than that suggested by the level of visual symptoms. If the vascular tissue within the rachis is killed at the point of infection, spikelets beyond that point, and beyond the point of fungal spread, may become bleached and necrotic. On the other hand, spikelets beyond the point of infection may become colonized without visual symptom manifestation. Consequently, visual estimates of disease, which is a measure of damage to the chaff tissue, may not reflect the true extent of fungal spread and toxin accumulation within the
inner tissues of the spike, leading to disparity between visual symptoms, colonization, and DON accumulation.

Several explanations have been given for disparities in the association between FHB and DON, including post-anthesis infection (38, 48, 123), weather conditions (21) and differences in cultivar resistance to FHB and DON. For instance, Lipps et al. (54) reports that in 2004 DON contamination of grain was much higher than that expected based on visual symptoms and attributed this to the unusually high amount of post-anthesis rainfall that occurred in that year. Developing DON prediction models in Canada, Hooker et al. (41) identified rainfall in excess of 5 mm 4-7 days prior to heading or in excess of 3 mm between 3 and 10 days after heading to be risk factors for DON. Cowger et al. (20) and Culler et al. (22) conducted field experiments under overhead mist irrigation to determine the effects of post-anthesis moisture on FHB development and DON accumulation. In Cowger et al. (20), misting wheat for 10 days after anthesis significantly increased DON over the non-mist-irrigated control, and misting for 20 days significantly increased DON over the 10 day misting treatment. In contrast, Culler et al. (22) found that DON contamination was lower in plots that received a mist treatment from anthesis to harvest than in plots misted for a shorter period, from anthesis to disease assessment. Cowger et al. (20) attributed the discrepancy between the findings of these two studies to the higher total volume of water applied during misting in the study by Culler et al. (22) than in their study. Culler et al. (22) suggested that the observed decrease in
DON in response to extended periods of high moisture may have been due to this water-soluble toxin being leached from the wheat spikes.

Although wheat is most susceptible to *F. graminearum* infection at anthesis, later infections at the milk and soft and hard dough stages of kernel development have been reported (2, 38, 39, 48, 75). In some cases, these late-season infections have resulted in DON contamination in excess of 2 ppm (38, 39). It is unclear how environmental conditions interact with other factors such as cultivar resistance and inoculum density to affect disease development, fungal colonization of grain and DON accumulation when infections occur after anthesis. DON production usually begins shortly after infection (10) and the toxin spreads through spike tissue ahead of fungal growth, meaning that DON may begin to accumulate in grain well before colonization and visual symptom development (44, 104). Under warm, wet conditions at anthesis, the spread of DON within the spike is quickly followed by FHB symptom development (44). However, when conditions are less conducive for disease development or infections occur after anthesis, visual symptoms may take longer to appear (2) or remain completely unapparent, potentially leading to disparity between visual symptoms and DON. In addition, there is evidence suggesting that stresses, such as sub-optimal growth conditions (e.g. < or > 20°C) or competition with other *Fusaria* may lead to increases in toxin production (42, 98, 120). Since late infections are known to occur, and may occur under sub-optimal conditions, it is possible that at harvest spikes are infected but asymptomatic and grain from these spikes may contain unacceptably high levels of DON. Also contributing to the relatively high levels of
DON in seemingly disease-free spikes is the fact that cultivars differ in their DON response at a given level of disease (53). Willyerd (116) observed that under a give set of temperature conditions, initial DON levels were significantly higher in grain from a moderately resistant cultivar (based on visual symptoms) than a susceptible cultivar following point inoculation of both cultivars with an equal suspension of *F. graminearum* spores.

Further research is needed to better characterize factors affecting DON accumulation in healthy-looking grain. This phenomenon is of concern to producers and researchers alike, since it has implications for disease management and grain marketing decision-making, DON forecasting, cultivar resistance screening, and evaluation of management strategies for FHB and DON, all of which are usually based on infections at anthesis and the assumption that visual disease levels are indicative of actual DON contamination. The goal of this study was to establish the basis for the development of models to estimate the probability of DON exceeding critical thresholds in seeming healthy grain. Towards this goal, there were three objectives: i) quantify the effects of infection timing, host resistance, inoculum density, ambient environmental conditions and their interactions on *F. graminearum* biomass and DON accumulation in grain from asymptomatic wheat spikes; ii) evaluate the association between DON and fungal biomass in asymptomatic grain as influenced by cultivar resistance, inoculum density, and environmental conditions; and iii) identify situations in which asymptomatic *F. graminearum* infections result in toxin contamination of grain in excess of 2 ppm.
MATERIALS AND METHODS

Establishment of field plots. Field experiments were conducted during the 2007-2008 and 2008-2009 wheat growing seasons at the Ohio Agricultural Research and Development Center, Wooster, Ohio. The experimental design was a randomized complete block, with three replicate blocks and a split-split-split plot treatment structure. Wheat planting date was the whole plot, and cultivar, timing of inoculation, and inoculum density were the sub-plot, sub-sub plot, and sub-sub-sub plots, respectively. Whole plots were established by planting plots of each cultivar at two planting dates (normal and late). In 2007, the first planting was done on October 8 and in 2008 on September 23. The second planting was done 11 days after the first in 2007 and 14 days after in 2008. In both years, wheat was planted after oats using a Kincaid wheat drill, calibrated to plant at a seeding rate of 400 seeds/m². There were seven rows per plot, with 19-cm spacing between rows.

At each planting date, 34-m-long strips of three soft red winter wheat cultivars, with different levels of resistance to FHB and locally adapted to growing conditions in Ohio, were planted as the sub-plot factor. Based on visual symptoms, these cultivars, Cooper, Hopewell, and Truman, are considered susceptible, moderately susceptible, and moderately resistant, respectively. In addition the cultivars differed in maturity, with Cooper being the earliest and Truman the latest maturing cultivars. Each strip of each cultivar was divided into four 8.5-m-long sub-sub plots for subsequent inoculation at four different growth
stages, anthesis (Feeke’s 10.5.1), 1 week, 2 weeks, and 3 weeks after anthesis. The late inoculation times roughly corresponded to the milky ripe (Feeke’s 11.1), soft dough (Feeke’s 11.2), and hard dough (Feeke’s 11.3) stages of kernel development. Throughout this paper, abbreviations GS1, GS2, GS3 and GS4 will be used to refer to Feekes growth’s stages 10.5.1, 11.1, 11.1 and 11.3. Each sub-sub plot was further divided into four 1.5-ft-long plots (sub-sub-sub plots) to which four inoculum density treatments, 0, 10, 20, and $30 \times 10^4$ spores/ml, were randomly assigned. The first inoculum density was applied as a water control, while the others were chosen based on previous research (69) to represent spike inoculum densities within the range commonly observed under field conditions.

Combinations of different planting dates, inoculation timings, and cultivars with different maturities were used with the hope of spreading out anthesis and later growth stages over several days or weeks during the growing season, and consequently, increasing the chance of having inoculation/infection coincide with a range of environmental conditions.

**Inoculation.** Inoculum was prepared using a mixture of macroconidia and ascospores from ten *F. graminearum* isolates collected from diseased spikes in wheat fields in Ohio. Isolations were done on streptomycin-amended Komada media (47), which is selective for *Fusarium*, and putative *F. graminearum* colonies were sub-cultured onto potato dextrose agar (PDA) (64) and carnation leaf agar (35) for species identification based on colony and spore morphology (52, 64, 110). Ascospores were produced on carrot agar supplemented with streptomycin sulfate (0.3 g/L), as described by Klittich and Leslie (46). Agar plugs
were transferred from cultures stored on PDA to carrot agar and plates were incubated under ultraviolet and white light for 12 h each day. Five days after seeding, the cultures were scrapped to remove aerial mycelia and minimize subsequent macroconidia production, while also stimulating the production of perithecia. After an additional week, plates were flooded with sterile water and perithecia and ascospores were suspended by gently rubbing the surface of the culture with a glass rod. The resulting suspension was filtered through several layers of sterile cheesecloth to remove fragments of mycelia and pieces of perithecia. Macroconidia were produced on mung bean agar (supplemented with 0.3g/L streptomycin sulfate) (33). Plates were seeded with a mixture of spores from the ten isolates and grown under a 12-h photoperiod. After one week, plates were flooded with sterile water and a glass rod was used to liberate spores. Concentrated stock suspensions of both ascospores and macroconidia were stored at -20°C until inoculation.

Stock spore suspensions were thawed at room temperature (22 - 25°C) for approximate 12 hours prior to inoculation. After thawing, the suspensions were stored at 4°C for the duration of the experiment. Spore concentrations were determined using a hemacytometer, and the final concentrations adjusted to 10, 20, and 30 x 10^4 spores/ml with sterile water. Equal volumes of ascospore and macroconidial suspensions of a given concentration were mixed and applied to sub-sub-plots, beginning at anthesis followed by three subsequent inoculations to the appropriate plots at weekly intervals. Crop development was more uniform in 2008 than in 2009. In 2008, inoculations began on June 2 at the
start of anthesis and ended on June 27, whereas, in 2009, the first set of inoculations was done at anthesis on May 22 and the last set on June 25. A backpack sprayer (R&D Sprayers, Opelousas LA) containing the appropriate spore concentration plus 0.01% (v/v) Tween 20 was used to apply inoculum. Each sub-sub-sub plot received a total of approximately 150 ml of the inoculum suspension, applied at 0.21 MPa via three nozzles at a combined rate of 100 ml/m.

**Fungal biomass and deoxynivalenol analysis.** Prior to physiological maturity (Feeke’s 11.4), thirty asymptomatic spikes were marked with colored tape at arbitrary locations within each sub-sub-sub plot and later hand harvested when grain moisture fell to approximately 15%. Grain was separated from chaff material with a tabletop thresher and ground using a Laboratory Mill (Model LM 3303, Perten Instruments Inc. Springfield, IL). Sub-samples of ground grain from each plot were analyzed for deoxynivalenol (DON) and assayed for fungal biomass.

DON analysis was done at the US Wheat and Barley Scab Initiative testing laboratory at the University of Minnesota by way of gas chromatography mass-spectrometry (GC/MS). DON was extracted by shaking a sample of ground grain in a mixture of acetonitrile and water (18:16 v/v) for an hour. The extract was then passed through a column packed with C18 and aluminum oxide (1:3) and an aliquot of the filtrate was evaporated to dryness under nitrogen and derivatized by the silylating reagent (TMSI/TMCS 100:1, Pierce Chemical Co., IL) for GC/MS analysis (Shimadzu GCMS-QP2010, Shimadzu Corporation, Kyoto, Japan; J & W
DB-5MS capillary column, 0.25 µm film thickness, 0.25 mm i.d., and 30 m). A temperature program was used for GC/MS analysis with initial column temperature of 150°C for 1 min, followed by an increase to 280°C at a rate of 30°C/min and a 5-min hold at 280°C. The injection temperature was kept at 300°C, and column flow rate was 1 ml/min. Selected ion monitoring (SIM) was applied to detect the characteristic ions of DON with fragment ion (m/z value) of 235.10 as target ion and 259.10 and 422.10 as reference ions (Y. Dong, personal communication).

A multiplex quantitative real-time polymerase chain-reaction (qRT-PCR) assay was used to quantify fungal and wheat DNA in the grain samples. The advantages of this technique are that i) throughput is high, allowing rapid processing and accurate quantification of many samples, ii) wheat and fungal DNA can be quantified simultaneously and do not require separate assays, and iii) in contrast to quantifying ergosterol, qRT-PCR specifically quantifies the target DNA, which in this case is Tri5, a requisite gene for trichothecene production.

DNA was extracted from ground grain samples using a modified protocol for the DNeasy 96 Plant Kit (Qiagen; Valencia, CA). Grain samples (0.1 g) were suspended in 400 µl of the lysis buffer with 2 µl of RNAse A (100mg/ml), vortexed and incubated at 65°C for 10 min in a waterbath. Samples were incubated on ice for 5 min after the addition of 130 µl of precipitation buffer, and then centrifuged at 14,000 rpm for 5 min. The liquid from each sample was transferred into a filter column on the provided 96-well DNeasy Plate. The remaining steps of the extraction were conducted according to the protocol provided by the manufacturer. Total DNA was quantified using a Nanodrop 2000.
(ThermoScientific; Wilmington, DE), and then diluted to 5 ng/µL with sterile water. Extractions and dilutions were stored at -20 ºC.

For pure reference culture standards, *F. graminearum* was grown on PDA from a single macroconidium and then transferred to potato dextrose broth. DNA was extracted from vacuum filtered fungal tissue and from healthy wheat grain according to the protocols of Doyle and Doyle (30) and Cullings et al. (23). A standard curve was generated from seven serial dilutions of wheat (ranging from 128 pg/µl to 5 ng/µl) and *F. graminearum* (ranging from 3.2 pg/µl to 32 ng/µl) genomic DNA with the addition of a sterile deionized water negative control.

Reactions were prepared for a 96-well format with the standard curve and samples present in triplicate on optical microtiter plates (Bio-Rad; Hercules, CA). Each assay well contained 10 µL PerfeCTa Multiplex qPCR Supermix (Quanta Biosciences; Gaithersburg, MD), 1 µL each of 10 µM primers for amplification of wheat and *Tri5* of *F. graminearum* (Table 3.1)(114), 1 µL of 5 µM fluorescent probes for detection of wheat and *Fusarium* amplification (Table 3.1), 1.5 µL sterile water, and 2.5 µL of target DNA. Plates were covered with optical plate seals (Bio-Rad; Hercules, CA), and assays were conducted using an iCycler. The thermocycler protocol begins with probe activation at 95ºC for 7 min, followed by 45 cycles of melting at 95ºC for 15 s and annealing at 60ºC for 1 min. Reactions were then cooled to 55ºC to stop probe activity and held at 70ºC until removal. The data analysis window was set to 25% of the length of the cycle.

**Data analysis:** Prior to data analysis, fungal biomass and DON data were log-transformed to stabilize variance. The effects of planting date (PD), cultivar
(CV), inoculation timing (IT), inoculum density (ID) and their interactions on log-transformed biomass and DON were determined with a linear mixed model (55). In the analysis, PD, CV, IT and ID were all treated as qualitative factors and considered fixed effects in the model, whereas, block and all interactions involving block were considered random effects. Analyses were done using PROC MIXED of SAS (SAS Institute, Cary, NC). Fixed effects were evaluated with F tests and random effects were evaluated with standard normal test statistics. *Estimate*, *lsmeans* and *contrast* statements were used to compare treatments. The analysis was then expanded, using log-transformed fungal biomass as a continuous covariate in the analysis of PD, CV, IT and ID effects on log DON in grain from asymptomatic spikes. Separate models were fitted for each CV*IT treatment combination and *estimate* and *contrast* statements were again used to compare models parameters and log DON at specific levels of log biomass across CV*IT treatment combinations.

**RESULTS**

**Deoxynivalenol and *F. graminearum* biomass.** In 2008, planting dates 1 (PD1) and 2 (PD2) flowered on the same days (Table 3.2). All of the plots planted with the S cultivar reached anthesis on day 154 (Table 3.2). The MS and MR cultivars flowered on two days, but there was no distinction between PD1 and PD2 (Table 3.2). In 2009 there was a temporal separation between anthesis for PD1 and PD2 for all cultivars (Table 3.2). On day 146, some plots from PD1 and PD2 of the S cultivar reached anthesis, but this was the only day that there
was an overlap in anthesis between planting dates for any given cultivar (Table 3.2).

In 2008, planting date (PD) 1 and PD2 flowered on the same days (Table 3.3). All of the plots planted with the S cultivar reached anthesis on day 154 (Table 3.3). The MS and MR cultivars flowered on two days, but there was no distinction between PD1 and PD2 (Table 3.3). In 2009 there was a temporal separation between anthesis for PD1 and PD2 for all cultivars (Table 3.3). On day 146, some plots from PD1 and PD2 of the S cultivar reached anthesis, but this was the only day that there was overlap between planting dates flowering for any given cultivar (Table 3.3).

Mean DON (ppm) and *F. graminearum* biomass (mg *F. graminearum* DNA/g total DNA [mg/g]) across all factor levels were greater in 2008 (0.91 ppm and 6.64 mg/g) than in 2009 (0.85 ppm and 5.77 mg/g) (Fig. 3.1A). However the range in DON, from not detectable (< 0.05 ppm) to 8.2 ppm, was greater in 2009 than in 2008, when the highest detected value was 5.2 ppm (Fig. 3.1A). The range and mean of DON for post-anthesis (GS2-GS4) inoculations was greater the second year (8.1 ppm; 0.73 ppm) than the first year (4.2 ppm; 0.52 ppm) (Fig. 3.1B). Fungal biomass ranged from 0.23 mg/g to 96.60 mg/g in 2008 and from 0.42 to 30.07 mg/g in 2009 (Fig. 3.1C).

DON contamination was similar between planting dates in 2008, with mean values of 0.85 ppm for PD1 and 0.98 for PD2. Fungal biomass was not quantified for PD2 in 2008. Among cultivars, the highest concentrations of DON (1.43 ppm) and fungal biomass (14.21 mg/g) were found in Hopewell, the
moderately susceptible (MS) cultivar, followed by the susceptible (S) cultivar Cooper (0.91 ppm DON and 3.53 mg/g fungal biomass), and the moderately resistant (MR) cultivar Truman (0.41 ppm DON and 2.17 mg/g fungal biomass). The mean concentration of DON was greatest for infections at anthesis (2.06 ppm), and decreased greatly for the post-anthesis infections (0.73 ppm for GS2, 0.50 ppm for GS3, and 0.36 ppm for GS4) (Figs. 3.2-3.3). The overall trend was for toxin concentration to increase with inoculum density, although for the latest infections (GS3 and GS4), and particularly for Truman, patterns were not clear (Figs. 3.2-3.3). For Truman there was very little difference in fungal biomass among growth stages and inoculum densities (Figs. 3.4-3.5). In contrast, fungal biomass was much greater for Cooper treated with the H inoculum density, and for Hopewell treated with the M and H inoculum densities at anthesis than for post-anthesis infections (Figs. 3.4-3.5).

Trends between 2008 and the first planting date of 2009 were similar for DON, although there was less overall DON (0.45 ppm) for PD1 of 2009 (Fig. 3.2-3.3). In 2008, DON approached or exceeded 2 ppm when the S and MS cultivars were inoculated at anthesis with the L, M, and H inoculum densities and when MS was inoculated at GS2 with the H inoculum density (Fig. 3.2). For both PDs of 2009, however, DON above 2 ppm was only observed for the MS cultivar (Fig. 3.3). In PD1, DON exceeded 2 ppm when inoculated at anthesis with the H inoculum density (2.40 ppm), while for PD2 the M and H inoculum applications at anthesis (4.47 and 4.53 ppm), as well as all inoculum applications at GS2 (L: 4.53 ppm, M: 3.33 ppm, H: 4.83 ppm) resulted in unacceptably high DON (Fig. 3.3).
3.3). Even the water control treatment at GS2 had high DON (2.40 ppm), indicating that DON was high even for natural infections (Fig. 3.3). Consistent with DON, the highest levels of *F. graminearum* biomass were observed for the MS cultivar at both planting dates (PD1: 5.89 mg/g, PD2: 9.32 mg/g), with the highest values observed for inoculations at GS2 in PD2 (16.53 mg/g) (Fig. 3.5). Overall patterns in relation to inoculum density treatment and infection timing were not especially evident in this year (Fig. 3.5).

For FHB, the relationship between visual measures of disease and DON is highly variable, so that predictions of DON from disease are not always accurate (72). Since fungal biomass directly quantifies infection levels in grain, it is potentially a more accurate predictor of DON, particularly in asymptomatic infections. Figure 3.6 displays the relationship between fungal biomass and DON for all infection times and specifically for post-anthesis infections. There was a positive relationship between fungal biomass and DON for each cultivar, although as fungal biomass increases there was also an increase in the variability of DON. Variability in DON and fungal biomass between years and planting dates, as well as in the relationship between DON and biomass is probably reflective of differences in weather conditions. In 2008, mean ambient temperature, relative humidity (RH), and daily rainfall for the period between Feekes GS 10 (boot stage) for the earliest cultivar and harvest was 20.2°C, 62.8 %, and 5.2 mm, respectively (Figs. 3.7-3.9). The corresponding means for 2009 were 18.9°C, 73.9 %, and 2.2 mm (Figs. 3.10-3.12). For the susceptible cultivar, mean temperature was similar for the period of anthesis for PD1 (19.7°C) and
PD2 (19.2°C) in 2009, while mean temperature was lower during anthesis in the second planting date for the moderately susceptible (20.0°C for PD1 and 16.6°C for PD2) and moderately resistant cultivars (17.8°C for PD1 and 14.2°C for PD2). Mean RH was 68.0, 74.0, and 77.3% during anthesis for S, MS, and MR respectively during PD1 of 2009, and 84.9, 72.7, and 77.7% during PD2. For S in PD1, there was no rainfall at anthesis, while 17.7 mm fell during anthesis for PD2. Total rainfall for MS at anthesis was 6.0 mm and 27.1 mm for PD1 and PD2 and was 11.7 mm and 24.9 mm for MR in PD1 and PD2.

Mixed model analysis of main and interaction effects. Separate linear mixed model analyses were performed to evaluate the effects of planting date (PD) cultivar (CV), inoculation timing (IT) and inoculum density (ID) and their interactions on log-transformed DON (lnDON) and fungal biomass (lnbiomass). The main effects of CV, IT, and ID were significant in both years for DON, while the effect of PD was only significant in 2009 (Table 3.3). For fungal biomass, the effects of CV, IT, and ID depended on other factors (Table 3.3). Two-way interactions of CV x IT and IT x ID were significant for DON in both years (Table 3.3). Additionally, the interaction of CV x ID was significant in 2008, but not in 2009, while in 2009, the effect of PD x CV was also significant (Table 3.3). For fungal biomass, the only two-way interaction that had a significant effect was CV x IT in 2009, though the effect of CV x ID was marginally significant (p = 0.054) in the same year (Table 3.3). Three-way interactions did not have a significant effect on DON except for PD x CV x IT in 2009, though there was a marginal effect of the interaction of CV x IT x ID in both years (Table 3.3). Three- and four-
way interactions were non-significant for fungal biomass, and four-way interactions were also non-significant for DON in both years (Table 3.3).

In order to characterize the compensatory effects of PD, CV, IT and ID on DON accumulation and *F. graminearum* colonization in grain of asymptomatic spikes, the *lsmeans* statement was used to compare mean lnDON and lnbiomass between specific treatment combinations, based on the significant interactions presented in Table 3.3 and the graphical summaries shown in Figures 3.2-3.5. For example, in PD2 of 2009 when the moderately resistant (MR) cultivar was inoculated at GS2 with the M inoculum density treatment, mean lnDON was not significantly different from lnDON in the susceptible (S) cultivar inoculated at anthesis with the H inoculum density treatment (Table 3.4). Indicating that, for DON production, certain weather conditions (in this case low-temperature), can compensate for late infection time, low inoculum density, and host resistance. Many similar compensatory interactions can be observed for PD2 of 2009, such as the comparison between MS x GS1 x L and MR x GS4 x M, where DON accumulation is non-significantly different (Table 3.4). Trends in DON in 2008 were more consistent with expectations, with highest mean levels occurring at anthesis inoculations, and an increase in DON with an increase in inoculum density treatment, although similar levels of DON were observed for PD1 between the S and MS cultivars when inoculated respectively at GS1 with the L inoculum density treatment and at GS2 with the H inoculum density treatment (Table 3.4).
For fungal biomass, particularly in 2009, trends and, consequently, compensatory interactions, were difficult to distinguish (Fig. 3.5). In 2008, fungal biomass was similar when both the S and MS cultivars were inoculated with the H density treatment at anthesis (GS1) and at the milky ripe growth stage (GS2) (Table 3.5). The range and mean for fungal biomass were both less in 2009 than in 2008, and there was a lot of similarity between levels of fungal colonization at different growth stages; for example, in PD2, fungal biomass was similar between the MR and MS cultivars when both were treated with the L inoculum density even though MR was inoculated at hard dough (GS4) and MS at anthesis (GS1) (Table 3.5).

DISCUSSION

Unacceptably high deoxynivalenol content in grain can be very costly for wheat growers and others in the wheat industry that process contaminated grain. FHB management is based on minimizing infection and toxin contamination of grain by planting cultivars with high levels of resistance and sometimes applying fungicides at anthesis, when wheat is most vulnerable to infection. Asymptomatic infections are particularly troubling because DON is present in apparently healthy wheat grain and because little is understood about the circumstances that encourage DON production without accompanying symptom expression, although post-anthesis infections and environmental conditions (specifically high moisture levels after infection) have been suggested as possible explanations (21, 123)
The results of this study demonstrate that host resistance, growth stage at infection, and inoculum density individually have significant effects DON in grain from wheat spikes that are asymptomatic for FHB, and suggest that under certain environmental conditions, may have significant effects on fungal biomass. Several studies have indicated that DON production is, in part, a result of a fungal response to stressful growing conditions (84, 98, 120). It was observed that the concentration of toxin was generally greater for higher inoculum density treatments, even when the level of *F. graminearum* colonization was similar among treatments (see MR in PD1 of 2008; Fig. 3.4). Work by Rossi et al. (89) indicates that DON production does not increase linearly with the level of *F. graminearum* colonization. Additionally, sub-optimal environmental conditions for *F. graminearum* growth, such as temperatures below 20°C, as seen in PD2 of 2009, appear to encourage DON production, and the effect can compensate for other sub-optimal conditions such as post-anthesis infection or host resistance. Asymptomatic infections may be more common under these conditions which are generally less favorable for symptom development.

The moderately resistant cultivar, Truman, was the only cultivar for which DON did not exceed 2 ppm in asymptomatic infections in any of the conditions evaluated in this study. This was not surprising because of Truman’s high resistance classification. It was unexpected that Hopewell, the moderately susceptible cultivar, and not Cooper, which is classified as susceptible, consistently had the largest concentration of toxin. Further, it was unanticipated that Truman would have greater mean DON than Cooper in PD2 of 2009. For all
year and planting date combinations, *F. graminearum* biomass was greater for the MS cultivar than for the other cultivars. Except for PD2 of 2009 there was no significant difference between fungal biomass in the S and MR cultivars. The relationship between external visual symptoms, on which cultivar resistance classifications are based, and kernel infection is not well understood and this may be why the high toxin and *F. graminearum* biomass levels are high in a cultivar classified as moderately susceptible. The susceptible cultivar Cooper is the earliest flowering cultivar. For Cooper, in 2009, anthesis and all subsequent inoculation times occurred when temperatures were favorable for fungal growth, while for Truman, the latest flowering cultivar, inoculations during PD2 of 2009 coincided with cool temperatures. This interaction of environmental conditions and host resistance may explain the unanticipated responses of the MR and S cultivars in 2009.

Compensatory interactions were observed, where a combination of optimal and sub-optimal conditions resulted in unacceptably high levels of DON, but only for the MS cultivar. In 2008, all such interactions occurred when inoculated at GS2 with the H inoculum density treatment, and for both planting dates 1 and 2, the level of DON only exceeded 2 ppm slightly. For PD 1 of 2009, no post-anthesis infections resulted in DON above 2 ppm, while in PD2, DON exceeded 2 ppm for all inoculum applications at GS2. MS infected at GS2 with the H inoculum density treatment resulted in the most DON (4.83 ppm) of any combination of factors for 2009, and was similar to the highest concentrations observed in 2008 (5.00 ppm and 4.57 ppm). This indicates that environmental
conditions have a significant impact on DON accumulation even when infection time is sub-optimal, and that when favorable conditions interact with a host that is susceptible to toxin accumulation, asymptomatic infections can result in DON above the acceptable limit.

Severe DON-related losses can occur for post-anthesis infections even if symptoms are absent. Since the DON response, particularly at GS2, was cultivar related, special emphasis should be placed on planting low-DON accumulating cultivars. Unacceptably high DON levels may also be preventable by treating wheat with fungicides to prevent *F. graminearum* infection throughout the growing season, not just at anthesis when wheat is most susceptible to infection. However, additional fungicide applications are not the ideal form of control for late season infection and DON production because of the additional expense, product label restrictions, and because there is evidence that some fungicides, particularly Strobilurins-based fungicides, may increase the production of DON in pre-existing infections (126). Fungal biomass is generally considered a more accurate predictor of DON. The results of this study demonstrate that DON can be influenced by CV, IT, and ID without significant effect on fungal biomass. This indicates that it is important to identify situations when DON production is enhanced for both symptomatic and asymptomatic infections for incorporation into DON prediction models, because a particular level of infection can result in a range of DON levels.

During the two years of this study, FHB symptoms were mild and DON in excess of 2 ppm was not observed for MR or for S inoculated at GS2 or later.
growth stages; however, it is possible that DON can exceed the levels observed in this study when environmental conditions are more favorable for *F. graminearum* infection and FHB development, especially if inoculum density is high. In spite of the relatively low levels of DON for most late infection timings, there was clearly an increase in DON with inoculum density at these growth stages. Future research should focus on the influence of weather conditions, particularly moisture, on DON accumulation in grain from asymptomatic spikes.
Figure 3.1:
Boxplots for deoxynivalenol (DON) in grain from spikes asymptomatic for FHB inoculated at (A) all infection times (GS1-GS4), (B) post-anthesis infections, and for (C) Fusarium graminearum biomass in 2008 and 2009. Each gray box represents the middle 50% of the data, and the line within the boxes indicates the median value of (A-B) DON, and (C) F. graminearum biomass for each year.
Deoxynivalenol (DON) in ppm from grain harvested from wheat spikes asymptomatic for Fusarium head blight in 2008. Results are partitioned by planting date (one [PD 1] and two [PD 2]), by cultivar (susceptible [S] – medium gray bars, moderately susceptible [MS] – black bars, and moderately resistant [MR] – light gray bars), growth stage at infection (GS1-GS4), and inoculum density (water control [C], low density [L], medium density [M] and high density [H]). The dashed black line indicates the 2 ppm critical threshold for DON in grain intended for human consumption.
Figure 3.3:
Deoxynivalenol (DON) in ppm from grain harvested from wheat spikes asymptomatic for Fusarium head blight in 2009. Results are split partitioned by planting date (one [PD 1] and two [PD 2]), by cultivar (susceptible [S] – medium gray bars, moderately susceptible [MS] – black bars, and moderately resistant [MR] – light gray bars), growth stage at infection (GS1-GS4), and inoculum density (water control [C], low density [L], medium density [M] and high density [H]). The dashed black line indicates the 2 ppm critical threshold for DON in grain intended for human consumption.
Figure 3.4:
Fungal biomass (mg *F. graminearum* DNA/ g total DNA) in grain harvested from wheat spikes asymptomatic for Fusarium head blight in 2008. Results are partitioned by cultivar (susceptible [S] – medium gray bars, moderately susceptible [MS] – black bars, and moderately resistant [MR] – light gray bars), growth stage at infection (GS1-GS4), and inoculum density (water control [C], low density [L], medium density [M] and high density [H]). Biomass was only quantified for the first planting date (PD 1) in 2008.
Figure 3.5:
Fungal biomass (mg *F. graminearum* DNA/g total DNA) in grain harvested from wheat spikes asymptomatic for Fusarium head blight in 2009. Results are partitioned by planting date (one [PD1] and two [PD2]), cultivar (susceptible [S] – medium gray bars, moderately susceptible [MS] – black bars, and moderately resistant [MR] – light gray bars), growth stage at infection (GS1-GS4), and inoculum density (water control [C], low density [L], medium density [M] and high density [H]).
Figure 3.6:
The relationship between fungal biomass (mg *F. graminearum* DNA/g total DNA) and deoxynivalenol (DON) in grain harvested from wheat spikes displaying no symptoms of Fusarium head blight is split by cultivar (susceptible [S], moderately susceptible [MS], and moderately resistant [MR]) for (A) all infection times, and for (B) post-anthesis infections. Note the x-axis scale differences between the two panels.
Figure 3.7:
Average temperature (C) for each day of the year (DOY) from boot stage (Feeke’s growth stage 10) on day 147 (May 27) to wheat harvest on day 191 (July 10) in 2008. Arrows indicate anthesis dates for S (dark gray), MS (white), MR (light gray) cultivars. Both planting dates were represented at all anthesis times. Mean temperature for DOY 147-191 is indicated by the dashed line.
Figure 3.8:
Average daily relative humidity (RH) for each day of the year (DOY) from boot stage (Feeke’s growth stage 10) on day 147 (May 27) to wheat harvest on day 191 (July 10) in 2008. Arrows indicate anthesis dates for S (dark gray), MS (white), MR (light gray) cultivars. Both planting dates were represented at all anthesis times. Overall mean relative humidity for DOY 147-191 is indicated by the dashed line.
Figure 3.9:
Total daily rainfall in mm for each day of the year (DOY) from boot stage (Feeke’s growth stage 10) on day 147 (May 27) to wheat harvest on day 191 (July 10) in 2008. Arrows indicate anthesis dates for S (dark gray), MS (white), MR (light gray) cultivars. Both planting dates were represented at all anthesis times.
Figure 3.10:
Average temperature (C) for each day of the year (DOY) from boot stage (Feeke's growth stage 10) on day 137 (May 17) to wheat harvest on day 196 (July 15) in 2009. Arrows indicate anthesis dates for S (dark gray), MS (white), MR (light gray) cultivars. Days when planting date 1 reached anthesis are indicated by arrows with thin outlines, while the thick black outlined arrows indicate days when planting date 2 flowered. For S there was overlap between the planting dates on day 146. Overall mean temperature for DOY 137-196 is indicated by the dashed black line.
Figure 3.11:
Average daily relative humidity (RH) for each day of the year (DOY) from boot stage (Feeke’s growth stage 10) on day 137 (May 17) to wheat harvest on day 196 (July 15) in 2009. Arrows indicate anthesis dates for S (dark gray), MS (white), MR (light gray) cultivars. Days when planting date 1 reached anthesis are indicated by arrows with thin outlines, while the thick black outlined arrows indicate days when planting date 2 flowered. For S there was overlap between the planting dates on day 146. Overall mean relative humidity for DOY 137-196 is indicated by the dashed black line.
Figure 3.12:
Total daily rainfall in mm for each day of the year (DOY) from boot stage (Feeke’s growth stage 10) on day 137 (May 17) to wheat harvest on day 196 (July 15) in 2009. Arrows indicate anthesis dates for S (dark gray), MS (white), MR (light gray) cultivars. Days when planting date 1 reached anthesis are indicated by arrows with thin outlines, while the thick black outlined arrows indicate days when planting date 2 flowered. For S there was overlap between the planting dates on day 146.
**Table 3.1:** Probes and Primers used in quantitative real-time polymerase chain reaction assays

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGB_F(^a)</td>
<td>GGCGCTTCTCGTGAACACA</td>
</tr>
<tr>
<td>MGB_R(^a)</td>
<td>TGGCTAAACAGCAGCAATGC</td>
</tr>
<tr>
<td>MGB_Probe(^a)</td>
<td>FAM-AGATATGTCTCTTTCAAGTCT-TAMRA</td>
</tr>
<tr>
<td>Pin447L2(^b)</td>
<td>TGGCGGAGGAGGTTGGTTC</td>
</tr>
<tr>
<td>Pin447R2(^b)</td>
<td>CGACATTGTGGTGCTATCTGG</td>
</tr>
<tr>
<td>Pin484Probe(^b)</td>
<td>HEX-AAATGGTGGAAGGGCGGGCTG-TAMRA</td>
</tr>
</tbody>
</table>

\(^a\) Minor Groove Binder (MGB) ligands from Waalwijk et al. (114)

\(^b\) Pin primers and probe developed by Mary J. Guttieri
Table 3.2: Day of the year (DOY) when sub-sub-sub plots reached anthesis for planting dates 1 and 2 for the susceptible (S), moderately susceptible (MS), and moderately resistant (MR) cultivars in 2008 and 2009.

<table>
<thead>
<tr>
<th>Year</th>
<th>S</th>
<th>MS</th>
<th>MR</th>
<th>PD1</th>
<th>S</th>
<th>MS</th>
<th>MR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>c</td>
<td>c</td>
<td>c</td>
<td>b</td>
<td>c</td>
<td>c</td>
<td>c</td>
</tr>
<tr>
<td>2008</td>
<td>154</td>
<td>154</td>
<td>156</td>
<td>154</td>
<td>154</td>
<td>156</td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>142</td>
<td>144</td>
<td>148</td>
<td>146</td>
<td>148</td>
<td>151</td>
<td></td>
</tr>
<tr>
<td></td>
<td>143</td>
<td>146</td>
<td>149</td>
<td>147</td>
<td>149</td>
<td>152</td>
<td></td>
</tr>
<tr>
<td></td>
<td>144</td>
<td>147</td>
<td>150</td>
<td>148</td>
<td>150</td>
<td>153</td>
<td></td>
</tr>
<tr>
<td></td>
<td>145</td>
<td></td>
<td></td>
<td>149</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>146</td>
<td></td>
<td></td>
<td>152</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **PD1** and **PD2** denote planting dates 1 and 2, respectively.
- **S**, **MS**, and **MR** represent the susceptible, moderately susceptible, and moderately resistant cultivars, respectively.

- **DOY**

- **DOY** designation. In 2008, DOY 154 was June 3 and DOY 158 was June 7. In 2009, DOY 142 was May 22 and DOY 155 was June 4.
- In 2008, the planting dates (PD) reached anthesis simultaneously, while in 2009 there was no overlap in planting date flowering except for the susceptible (S) cultivar.
- The three cultivars, susceptible (S), moderately susceptible (MS), and moderately resistant (MR), do not mature at the same rate and so reached anthesis at different times.
Table 3.3: Main and interaction effects of planting date (PD), cultivar (CV), infection time (IT), and inoculum density (ID) on deoxynivalenol (DON) in grain, and fungal biomass in grain from wheat spikes asymptomatic for Fusarium head blight in 2008 and 2009.

<table>
<thead>
<tr>
<th>Fixed Effects</th>
<th>2008</th>
<th></th>
<th>2009</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DON(^a)</td>
<td>Fungal Biomass(^a)</td>
<td>DON(^a)</td>
<td>Fungal Biomass(^a)</td>
</tr>
<tr>
<td></td>
<td>F value</td>
<td>P value</td>
<td>F value</td>
<td>P value</td>
</tr>
<tr>
<td>PD</td>
<td>2.9</td>
<td>0.2306</td>
<td>n/a</td>
<td>240.09</td>
</tr>
<tr>
<td>CV</td>
<td>93.61</td>
<td>&lt;.0001</td>
<td>10.94</td>
<td>0.0239</td>
</tr>
<tr>
<td>PD*CV</td>
<td>1.59</td>
<td>0.262</td>
<td>n/a</td>
<td>13.33</td>
</tr>
<tr>
<td>IT</td>
<td>111.83</td>
<td>&lt;.0001</td>
<td>2.9</td>
<td>0.0637</td>
</tr>
<tr>
<td>PD*IT</td>
<td>0.77</td>
<td>0.5197</td>
<td>n/a</td>
<td>1.7</td>
</tr>
<tr>
<td>CV*IT</td>
<td>12.93</td>
<td>&lt;.0001</td>
<td>1</td>
<td>0.4527</td>
</tr>
<tr>
<td>PD<em>CV</em>IT</td>
<td>1.35</td>
<td>0.2613</td>
<td>n/a</td>
<td>2.58</td>
</tr>
<tr>
<td>ID</td>
<td>58.1</td>
<td>&lt;.0001</td>
<td>1.94</td>
<td>0.1304</td>
</tr>
<tr>
<td>PD*ID</td>
<td>0.41</td>
<td>0.7471</td>
<td>n/a</td>
<td>1.48</td>
</tr>
<tr>
<td>CV*ID</td>
<td>3.12</td>
<td>0.0066</td>
<td>0.43</td>
<td>0.858</td>
</tr>
<tr>
<td>PD<em>CV</em>ID</td>
<td>1.52</td>
<td>0.1744</td>
<td>n/a</td>
<td>1.69</td>
</tr>
<tr>
<td>IT*ID</td>
<td>21.8</td>
<td>&lt;.0001</td>
<td>1.47</td>
<td>0.1748</td>
</tr>
<tr>
<td>PD<em>IT</em>ID</td>
<td>0.63</td>
<td>0.7706</td>
<td>n/a</td>
<td>0.89</td>
</tr>
<tr>
<td>CV<em>IT</em>ID</td>
<td>1.62</td>
<td>0.0612</td>
<td>0.81</td>
<td>0.6872</td>
</tr>
<tr>
<td>PD<em>CV</em>IT*ID</td>
<td>0.56</td>
<td>0.9199</td>
<td>n/a</td>
<td>1.34</td>
</tr>
</tbody>
</table>

\(^a\) Deoxynivalenol (DON) content and fungal biomass in grain were log-transformed prior to mixed model analysis to normalize the data and stabilize variance.

\(^b\) All effects were calculated for transformed data using mixed model analysis. Planting date (PD), cultivar (CV), infection time (IT), and inoculum density (ID) were designated as fixed factors in the analysis.
Table 3.4: Differences of least square means from mixed model analysis for deoxynivalenol (DON) in grain from asymptomatic infections for selected interactions of planting date (PD), cultivar (CV), infection time (IT) and inoculum density (ID).

<table>
<thead>
<tr>
<th>Year</th>
<th>PD</th>
<th>CV</th>
<th>IT</th>
<th>ID</th>
<th>PD</th>
<th>CV</th>
<th>IT</th>
<th>ID</th>
<th>T-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2008</td>
<td>PD1</td>
<td>MS</td>
<td>GS2</td>
<td>H</td>
<td>PD1</td>
<td>S</td>
<td>GS1</td>
<td>L</td>
<td>0.08</td>
<td>0.9389</td>
</tr>
<tr>
<td>2009</td>
<td>PD2</td>
<td>MR</td>
<td>GS2</td>
<td>M</td>
<td>PD1</td>
<td>S</td>
<td>GS1</td>
<td>H</td>
<td>-1.89</td>
<td>0.0610</td>
</tr>
<tr>
<td>2009</td>
<td>PD2</td>
<td>MR</td>
<td>GS4</td>
<td>M</td>
<td>PD1</td>
<td>MS</td>
<td>GS1</td>
<td>L</td>
<td>0.16</td>
<td>0.8748</td>
</tr>
</tbody>
</table>

* The 1 and 2 designate to the first and second interactions for which means are being compared.

* PD1 is the first planting date, and PD2 is the second planting date.

* MR refers to the moderately resistant (Truman) cultivar, while MS and S refer to the moderately susceptible (Hopewell), and susceptible (Cooper) cultivars, respectively.

* Inoculum was applied at four stages of kernel development, including anthesis (GS1), milky ripe (GS2), soft dough (GS2), and hard dough (GS4).

* The letters L, M, and H refer to the low, medium, and high density spore concentrations.

* Prior to mixed model analysis, deoxynivalenol (DON) content in grain was transformed with a log transformation to normalize the data and stabilize variance. The t-value is on the transformed scale.
Table 3.5: Differences of least square means from mixed model analysis for *Fusarium graminearum* biomass in grain from asymptomatic FHB infections for selected interactions of planting date (PD), cultivar (CV), infection time (IT) and inoculum density (ID).

<table>
<thead>
<tr>
<th>Year</th>
<th>PD&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CV&lt;sup&gt;c&lt;/sup&gt;</th>
<th>IT&lt;sup&gt;d&lt;/sup&gt;</th>
<th>ID&lt;sup&gt;e&lt;/sup&gt;</th>
<th>PD&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CV&lt;sup&gt;c&lt;/sup&gt;</th>
<th>IT&lt;sup&gt;d&lt;/sup&gt;</th>
<th>ID&lt;sup&gt;e&lt;/sup&gt;</th>
<th>T-value&lt;sup&gt;f&lt;/sup&gt;</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2008</td>
<td>MS</td>
<td>GS2</td>
<td>H</td>
<td></td>
<td>S</td>
<td>GS1</td>
<td>H</td>
<td></td>
<td>-0.393</td>
<td>0.6966</td>
</tr>
<tr>
<td>2009</td>
<td>PD2</td>
<td>MR</td>
<td>GS4</td>
<td>L</td>
<td>PD2</td>
<td>MS</td>
<td>GS1</td>
<td>L</td>
<td>-1.42</td>
<td>0.1574</td>
</tr>
</tbody>
</table>

<sup>a</sup> The 1 and 2 designate to the first and second interactions for which means are being compared.

<sup>b</sup> PD1 is the first planting date, and PD2 is the second planting date. Fungal biomass was not quantified for PD2 in 2008, so all interactions in 2008 refer are from PD1.

<sup>c</sup> MR refers to the moderately resistant (Truman) cultivar, while MS and S refer to the moderately susceptible (Hopewell), and susceptible (Cooper) cultivars, respectively.

<sup>d</sup> Inoculum was applied at four stages of kernel development, including anthesis (GS1), milky ripe (GS2), soft dough (GS2), and hard dough (GS4).

<sup>e</sup> The letters L, M, and H refer to the low, medium, and high density spore concentrations.

<sup>f</sup> Prior to mixed model analysis, *F. graminearum* biomass was transformed with a log transformation to normalize the data and stabilize variance. The t-value is on the transformed scale.
CHAPTER 4: QUANTIFICATION OF WITHIN-Plot VARIABILITY IN FUSARIUM HEAD BLIGHT DEVELOPMENT AND DEOXYNIVALENOL ACCUMULATION IN WHEAT GRAIN

INTRODUCTION

Deoxynivalenol (DON) is a mycotoxin found in grain of wheat and other cereal crops infected by Fusarium-head-blight-(FHB)-causing *Fusarium* species. In the United States the principal pathogen is *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* (Schwein.) Petch) (58). DON inhibits eukaryotic protein synthesis, and consequently, poses a serious food safety risk for humans and livestock (87, 113). To minimize the risk of DON entering the food chain, the United States, Food and Drug Administration has issued DON advisory limits that range from 2-10 ng/g for unprocessed grain intended for human and animal consumption. Testing for DON has now become a routine part of the commercialization of wheat grain. Based largely on ELISA-based tests, grain with DON contamination exceeding critical limits is either downgraded in price or rejected entirely at grain elevators. However, the exact level of grain contamination is difficult to quantify accurately, partly because of the high variability in toxin content among kernels of a given grain lot (115). This variability
is due in part to the fact that infection and FHB development is highly variable in wheat fields.

Wheat is most susceptible to infection by *F. graminearum* at anthesis (Feeke’s 10.5.1) (49), and FHB develops best when wet, humid conditions coincide with this growth stage. For a given cultivar, anthesis is variable among plants within the same field and even among spikelets on the same spike. Consequently, plants in a single field may reach anthesis under a range of weather conditions over several days, resulting in high in-field variability in infection, disease development, and DON production. However, asynchronous crop development is not the only reason for high variability in FHB and DON in wheat field. Variations in in-field microclimate as well as in spore deposition and abundance also contribute to variable infection among plants. Studying the spatial pattern of deposition of viable spores of *G. zeae* in wheat fields between heading and early grain fill, Schmale et al. (97) reported that more than 90% of the spore deposition events were random, and the number of deposited spores varied from one location in the field to another.

Considerable research effort has gone into evaluating DON variability in harvested grain and how sampling techniques can minimize such variability (92, 115). However, in-field FHB and DON variability is still poorly understood. This variability can directly affect the observed relationship between DON and index, and account for differences in results among studies. Champeil et al. (18) found that taking 10 samples, each containing 100 wheat spikes, returned less variable DON results than sampling in quadrats. The total sample size was larger for the
10-sample procedure than for the quadrat procedure and the authors suggested that the sample size instead of the procedure itself may have accounted for the reduction in variability. The researchers sampled DON in grain from fields in four European countries, and they found that the quadrat with the highest DON concentration came from a field that also had quadrats with no detectable DON (122). The authors suggested, in contrast to the results of Schmale et al. (97), that this variability resulted from disease aggregation or microclimate differences, and urged that when sampling for DON, adequately large samples be taken from a composite of groups of spikes from throughout the field. Del Ponte et al. (27) examined the spatial pattern of FHB symptoms in New York wheat fields and concluded that FHB is randomly distributed at the field scale, but that aggregation can occur when infected corn residue is present. Hart and Schabenberger (40) sampled in-field for DON and recommended that a total of 20 randomly distributed samples, each comprised of 20-25 wheat spikes, be collected for DON testing, while Champeil et al. (18) suggested that samples should be distributed evenly throughout fields.

Accurate quantification of DON and disease is crucial for research, disease management, and grain marketing. Screening for resistance and evaluation of chemical control and other management strategies are all dependent on adequate sampling, which is critical for accurate disease and toxin quantification. In breeding nurseries, if DON or FHB symptoms are inaccurately quantified, cultivars or lines may be misclassified, which can lead to the abandonment of lines that are resistance or the selection of those that not
resistant. Similarly, when evaluating fungicides, DON and FHB need to be accurately quantified in order to determine the true efficacy of the product being tested, and ultimately, recommend the most effective fungicide. The development and validation of statistical prediction models requires accurate DON and FHB symptom quantification.

The objective of this study was to establish the basis for the development of a sampling protocol for in-field quantification of DON by i) quantifying the variability in FHB and DON in inoculated plots, ii) determining the relationship between variance and mean for FHB and DON, and iii) determining the effect of variability on the relationship between FHB and DON at two hierarchical scales. Accurate DON quantification could lead to more effective management of FHB.

**MATERIALS AND METHODS**

**Plot establishment and inoculation.** Field experiments were conducted during the 2007-2008 and 2008-2009 growing seasons at the Ohio Agricultural Research and Development Center, Wooster, Ohio. The experimental design was a randomized complete block, with three replicate blocks of six soft-red winter wheat cultivars (for a total of 18 plots/year). Plots were planted on October 1, 2007 and on September 24, 2008. In both years, plots were 3 m x 6 m and were planted following oats at a seeding rate of 392 seeds/m². The six wheat cultivars chosen for the study are adapted to growing conditions in Ohio and vary in their FHB resistance classification. Based on visual symptoms, Truman and McCormick are considered moderately resistant (MR), Hopewell and AGI 101
moderately susceptible (MS), and Cooper and Pioneer 25R37 susceptible (S). In addition the cultivars varied in maturity, with Cooper being the earliest and Truman the latest maturing cultivars. Cultivars with different levels of resistance and maturity were selected in an attempt to generate plots with a range of FHB levels.

**Inoculation.** All plots were inoculated with a 1:1 mixture of macroconidia and ascospores from ten *F. graminearum* isolates collected from diseased spikes in wheat fields in Ohio. Isolations were done on streptomycin-amended Komada selective media (47) and putative *F. graminearum* colonies were sub-cultured onto potato dextrose agar (PDA) (64) and carnation leaf agar (35) for species identification based on colony and spore morphology (52, 64, 110). Ascospores and macroconidia were produced on streptomycin sulfate-amended carrot and mung bean agar, respectively, and stock suspensions of both spore types were stored at -20°C until used for inoculation.

The spore concentration of the stock suspension was determined by counting spores on a hemacytometer and the final concentration was adjusted to 50,000 spores/ml with sterile water. Equal volumes of ascospores and macroconidial suspension were mixed for inoculation. Plots were inoculated when approximately 50% of the spikes had freshly extruded anthers (Feekes 10.5.1). In 2008 inoculations were conducted between May 29 and June 4, and in 2009 between May 25 and May 28, depending on the maturity of the cultivar. Tween 20 was added to the spore suspension at a rate of 0.01% v/v and a
backpack sprayer (R&D Sprayers, Opelousas LA) was used for inoculation. Each plot received approximately of 1.2 L of the inoculum in 2008 and 1 L in 2009.

**Sampling and disease quantification.** Between Feekes’ growth stages 11.2 and 11.3, thirty clusters of twenty spikes were tagged and bound with twine in each plot. In each cluster, FHB incidence (INC) was visually estimated as the proportion of diseased spikes (number of spikes with nonzero severity divided by the total number of spikes [20] sampled) and index (IND, field or plot severity) as the average proportion of diseased spikelets per spike (sum of the proportion of diseased spikelets per spike divided by the total number of spikes [20] sampled, including those with zero severity). In 2009, because of the low overall levels of FHB in all plots, plots planted with moderately resistant cultivars (Truman and McCormick) were not assessed. Instead, three additional plots each of susceptible cultivar Cooper and moderately susceptible cultivar Hopewell from an adjacent experiment were used. These plots were of the same dimension as the original plots, but were inoculated 25,000 spores/ml. Moderate levels of FHB developed in these plots, adding to the range index and incidence across the 18 plots. These additional “higher inoculum” treatments were considered as additional fixed-effect levels in the analysis.

At physiological maturity, when grain moisture content fell to approximately 15%, clusters were hand-harvested from each plot, labeled with plot and cluster number, and placed into separate bags. Each cluster was threshed separately with a tabletop thresher, ground using a Laboratory Mill (Model LM 3303, Perten Instruments Inc. Springfield, IL), and a sub-sample of
ground grain was sent DON quantification by gas chromatography/mass-spectrometry to the U.S. Wheat and Barley Scab Initiative (USWBSI) DON Testing Laboratory at the University of Minnesota.

**Data analysis.** There were 30 INC-IND-DON clusters from each of 17 plots in 2008 and 18 plots in 2009. In this investigation, each plot is considered a sampling unit and each INC-IND-DON cluster is considered an observation, giving a total of 510 and 540 observations in years 1 and 2, respectively. Initial evaluation of graphs of the data (Fig. 4.1) showed that variances for IND and DON were skewed and increased with mean IND and DON, respectively, suggesting a power-law relationship (56). In the first step of the analysis, separate means and variances were estimated for each sampling unit. Details of, and justifications for, such a relationship are provided in Chapter 9 of Madden et al. (56). Means and variances for DON and IND were log-transformed prior to regression analysis.

The regression models were as follows:

\[ \log_{10}(S_{DON}^2)_j = \log_{10}(a) + b\log_{10}(DON)_j \]  \hspace{1cm} (1)

\[ \log_{10}(S_{IND}^2)_j = \log_{10}(a) + b\log_{10}(IND[1-IND])_j \]  \hspace{1cm} (2)

where \( S_{DON}^2 \) and \( S_{IND}^2 \) are the respective plot variances of DON and IND, \( \overline{DON} \) and \( \overline{IND} \) are the means for DON (ppm) and proportion IND, and \( a \) and \( b \) are intercept and slope regression parameters. The subscript \( j \) is an index for the \( j \)-th plot (defined as each unique combination of year x cultivar x block).

Separate regression analyses were performed for each year using PROC REG in SAS (SAS Institute, Cary, NC). T-tests were used to evaluate whether
intercepts and slopes were different between years, and to evaluate the equality of b to the specified constants (such as 1).

The relationship between IND and DON was modeled using non-linear mixed models (56). Models were fitted to the data from individual clusters and plot means. The basic model was of the form:

$$DON_i = \beta_0 + \beta_0 IND_i^\phi + \varepsilon_i$$  \hspace{1cm} (3)

where $\varepsilon$ is the residual, (with an assumed normal distribution and variance $\sigma^2$), $[\varepsilon_i \sim N(0, \sigma^2)]$, $\beta_0$, $\beta_1$, and $\phi$ are parameters, and $i$ is the index for the individual cluster. $\beta_0$ is the expected level of DON when IND is 0, and $\beta_1$ is the rate at which DON increases with increasing IND, and the exponent $\phi$ is a parameter controlling the curvature to the relation. When $\phi = 1$, a linear straight line model results. When $0 < \phi < 1$, the model curve is concave. Equations for each of the models are presented in Table 4.1, and all models were fitted using PROC NLMIXED of SAS. The first three models, A-C, were fitted to all observations, a combined total of 1049 for both years of the study ($i = 1, \ldots, 1049$).

For model A, the residual $\varepsilon_i$ accounts for the between-cluster variability, whether the clusters are from the same or different plots. Thus, this model does not account for the hierarchical nature of the data. Models B and C do account for the hierarchy through the inclusion of a between-plot variability term ($\mu_j$) and explicit consideration of $\varepsilon$ as a between-cluster-within-plot variability term ($\varepsilon_{ij}$) (see table 4.1). Model C is an expansion of B, by making the within-plot variance
be a function of the level of DON (Table 4.2). This is a natural extension of the power-law results for the variation in DON (equation 1).

The remaining models, D-G, are based on the plot means of IND and DON (i.e., the average over all clusters in each plot). IND_\text{j} and DON_\text{j} are the means for plot \text{j} (for the simplicity of notation, overbars are not used). For these data, there is only one level to the hierarchy (plot), and thus only one random effect for between-plot variability (\varepsilon_\text{j}). For model D, it was assumed that \varepsilon_\text{j} had a constant variance, and for model E, it was assumed that \varepsilon_\text{j} had a variance proportional to the mean DON (Table 4.1). Models F and G made the same variability assumptions as did D and E, respectively, but restricted \Phi to be equal to 1.

In addition to \beta_0, \beta_1, and \phi, the NLMIXED procedure was used to estimate the variance terms (\sigma^2_\varepsilon and, where appropriate, \sigma^2_\mu), or the \text{a} and \text{b} parameters for the \sigma^2_\varepsilon model components. Maximum likelihood was used for all model fitting. Goodness of the fit was evaluated using the corrected Akaike information criterion (AICC) statistic.

In all analyses, the cultivar or inoculum density was utilized only to obtain different levels of IND and/or DON, and was not explicitly evaluated for effect on the response variable.

**RESULTS**

**DON and IND means and variances.** In 2008, DON in clusters ranged from 0.16 to 32.7 ppm, and plot means from 1.14 to 17.00 ppm (Fig. 4.1A-B). In
2009, DON in clusters ranged from below the detectable limit (< 0.05 ppm) to 47.9 ppm, with mean DON per plot ranging from 2.90 to 18.55 ppm (Fig. 4.1C-D). For FHB index, mean plot and cluster values ranged from 0.9 to 31.6% and from 0.0 to 64.0%, respectively, in 2008 (Fig. 4.2A-B). The corresponding ranges for 2009 were between 1.5 to 16.0% for plots and 0 to 36.0% for clusters (Fig. 4.2 C-D).

The range of values for IND variances were higher in 2008 than in 2009, ranging from 1.43 to 140.40 and from 4.70 to 62.02 in the years 1 and 2, respectively. For DON, variances ranged from 0.50 to 39.05 in 2008 and from 3.30 to 107.91 in 2009. In both years, plots with the highest mean IND and DON had correspondingly high variances.

**Plot mean and variance relationship.** IND and DON variances increased as the plot mean of IND and DON increased, respectively (Fig. 4.3). The results from the regression analysis of the relationship between log-transformed means and variances for IND and DON (equations 1 and 2) showed that there were significant positive linear relationships between the plot means and variances on a log scale for both IND and DON (Fig. 4.4). Based on the t-test, regression coefficients were not significantly different between the two years, with slopes for IND of 1.32 and 1.09 for 2008 and 2009, respectively. For DON, slopes for the two years were 1.41 and 1.62, respectively (Table 4.2). This suggested that the form of the functional relationship between transformed means and variances for both IND and DON was consistent across years (environmental conditions). Moreover the estimates of $b$ were significantly
greater than 0 for all situations. This indicates that the variation depended on the mean. Among other things, this also indicates that a transformation of the observations is needed to stabilize variances (56, 79) for analyses that require a constant variance.

**Relationship between DON and IND.** Parameter and error variance estimates, along with standard errors and AICC values for goodness of fit, are presented in Table 4.3 for each model. Also included in this table are the results from t-tests for each model that tested whether $\phi$ was significantly less than 1. For the models A-C fitted using IND and DON data from individual clusters ($n = 1049$), model A had the highest AICC value, and model C, the lowest. Model A can be rejected outright because it does not account for the hierarchy of the data structure. Thus, model C was the best fitting of these three. Because model C was based on unequal error variances, this result is consistent with the power-law results for variances (independent of the functional relationship between DON and IND).

The estimate of $\phi$ was significantly less than 1 for models A-C. This reflects the concave relationship found for the cluster observations (Fig. 4.5). The estimate of $\beta_0$ is the estimated expected value of DON when $\text{IND}^\phi$ is 0 (which is also when $\text{IND} = 0$). Based on the results of model C, the expected DON when IND was 0 was 2.0 ppm.

For the modeling results based on plot means (D, E), the estimate of $\phi$ was not significantly different from 1 (Table 4.3). This suggests that a straight-line model may be appropriate for plot-level data. In fact, the best fitting model was
model G, ($\phi = 1$), based on AICC. This model also accounted for unequal residual variances. Thus, results showed that curvature to the relationship at one scale in a hierarchy was reduced when observations were aggregated at a higher level (Fig. 4.5). Based on the intercept term ($\beta_0$) of model G, the estimated expected DON when IND equaled 0 was 2.8 ppm. This number is similar to the results with the cluster data.

A comparison of model fits shows further aspects of data aggregation (Fig. 4.6). The predicted value of DON (which is also the estimated expected DON) at a given IND was lower for aggregated data than for the original cluster observations, for values of IND up to about 0.22. There were few data points above 0.25. This figure also shows that the model fits were very similar for linear models F and G (without and with unequal variances), and between models B and C (also without and with unequal variances).

**DISCUSSION**

Many attempts have been made to evaluate in-field variability in FHB and DON and to characterize the relationship between visual symptoms of FHB and DON in harvested grain (122). However, to our knowledge, this was the first study specifically designed to evaluate the influence of mean IND and DON on the in-field variability of these two measures for this disease and to incorporate the mean/variance relationship into models for the relationship between IND and DON. Based on power-law regression models (56), there were significant, positive relationships between (log-transformed) means and variances for both
IND and DON. The relationships were consistent across the 2 years of this study, with no significant differences between slopes and intercepts between 2008 and 2009, two years with considerable differences in weather conditions.

In the literature, contradictory claims exist for a positive relationship between FHB symptoms and DON, a negative relationship, or the absence of a relationship. Paul et al. (73) conducted a meta-analytic synthesis of the association between visual symptoms of FHB and DON using data from 163 studies and concluded that overall there were significant positive correlations between all measures of visual symptoms and DON. However, in a much more limited data set, Lemmens et al. (50) observed that visual symptoms of disease did reflect DON contamination in grain fairly well at low levels of infection, but the association broke down at high levels of infection. Paul et al. (72) also showed that as mean FHB and DON levels increased in field studies, the height of the DON:IND line (regression intercept) also increased. In this study it was observed that DON was highly variable and that this variability increased with FHB index (Fig. 4.3), especially for data at the cluster-within-plot scale. This mean-dependent variability makes accurate DON quantification and prediction difficult. Despite this, FHB remains a useful predictor for DON because of the relative ease and low cost of measurement, even though there remains considerable uncertainty in model predictions.

FHB disease measurements and DON content in grain are known to be greatly influenced by environmental conditions (24, 26, 94). Data from the 2 years of this study were initially treated separately in terms of variance/mean
relationships, but comparison of the estimated regression parameters demonstrated that the slope was not significantly different between the 2 years for the transformed data (Fig. 4.4). This suggests that the relationship between means and variances of IND and DON is consistent under a range of conditions. FHB index (field plot severity) did not exceed 36% in 2008 or 16% in 2009. Hence, it is unclear how higher levels of IND and DON will affect the relationship between means and variances for both measure of FHB. However, as disease intensity increases and approaches 100%, one would expect that there will be less variation among clusters and, as such, the variance will likely decrease at very high mean index levels. The chosen variability model takes the later situation into account (equation 2), so that declining variances are directly handled within the modeling framework (56). The relationship between means and variance should continue to be examined especially in years when environmental conditions favor higher levels of disease.

The hierarchical structure of the experimental design allows for examination of the relationship between IND and DON for individual clusters and also for plot means. It would be inappropriate to judge the model fit between the models constructed with cluster data (models A-C) and the models constructed from plot mean data (models D-G), because of the differences in data structure (i.e., degree of aggregation). For each group of models, AICC was lowered when non-constant variance based on the estimate of DON was incorporated into the between-plot residual term (Tables 4.1 and 4.3). Therefore, utilization of the form of variance/mean relationship for DON (equation 1) in the DON:IND model
resulted in a better fit to the data. For the models based on mean plot data, AICC was reduced when $\phi$ was held constant at 1. For models D and E, $\phi$ was not held constant, but the parameter was not significantly different from 1. Thus, for aggregated data, a linear (straight-line) relationship was found to be appropriate.

For modeling results based on individual cluster data (models A-C), $\phi$ was significantly less than 1, and these model predictions displayed considerable concave curvature in the DON:IND relation. Thus, the degree of consolidation (“aggregation”) of data into means affects the realized slop of the DON:IND relationship. Different degree of data consolidation in different studies could be partly responsible for heterogeneity in results among separate studies. Interestingly, it can be shown that if there is high variability at a lower scale in a hierarchy, and also a concave (and positive) relationship between two variable, (DON and IND), then two things will happen under many circumstances (56): (1) the expected DON at a given IND will be lower for the mean data; and (2) there will be less curvature in the relationship for the mean data. Both of these predictions were realized with these data, at least up to an IND of around 25%. Given that there were few data points above 25%, results in this latter index range would not reliable. Additional work is needed to see if these results are found under a wide range of disease/DON data.
Figure 4.1:
Boxplots for deoxynivalenol (DON) measurements for cluster observations in each plot for (A) 2008 and (C) 2009, with corresponding histograms for (B) 2008 and (D) 2009. The plots with large DON variance (large gray boxes) also have the highest median (indicated by the middle line in each gray box) value for DON.
Figure 4.2:
Boxplots for FHB disease index (IND) measurements for cluster observations in each plot for (A) 2008 and (C) 2009, with corresponding histograms for (B) 2008 and (D) 2009. The plots with large IND variance (large gray boxes) also have the highest median (indicated by the middle line in each gray box) value for IND.
Figure 4.3:
Scatterplots showing the relationship between plot means and plot variances for (A) Fusarium head blight index (IND), and (B) deoxynivalenol (DON) content in grain. Each dot represents the results from 30 cluster observations within a single plot.
Figure 4.4:
Transformed plot means and variances with regression lines for deoxynivalenol (DON) in (A) 2008, in (B) 2009, and for transformed mean Fusarium head blight index (IND) and IND variances (IND var) for (C) 2008 and (D) 2009. Regression equations are included on the individual panels.
Figure 4.5:
The nonlinear relationship between the proportion Fusarium head blight index (IND) and predicted deoxynivalenol (DON) in ppm is displayed for (A) model B, and (B) model which were generated from the cluster observations (n = 1049), and (C) model D, (D) model E, (E) model F, and (F) model G which were generated from mean plot data (n = 35). The exponent parameter $\phi$ is held constant at 1 for models F and G, so that the models relationship is linear.
Figure 4.6:
The relationship between proportion Fusarium head blight index (IND) and predicted values of deoxynivalenol (DON) in ppm is shown for models B-G overlain on one graph for comparison. Models B and C were generated from all cluster observations (n = 1049) and models D-G were generated from plot mean data (n = 35). Models C, E, and G possess random effects with non-constant variance based on the estimate of DON. For models F and G, $\phi$ is held constant at 1, equating the models to linear models.
Table 4.1: Equations and random effect distribution for the relationship between deoxynivalenol (DON) and Fusarium head blight index (IND) for models using cluster observations (models A-C) and mean plot data (models D-G).

<table>
<thead>
<tr>
<th>Model</th>
<th>Equation</th>
<th>Random effect distributions</th>
</tr>
</thead>
<tbody>
<tr>
<td>A&lt;sup&gt;a&lt;/sup&gt;</td>
<td>DON&lt;sub&gt;i&lt;/sub&gt; = β₀ + β₁IND&lt;sub&gt;i&lt;/sub&gt; + ε&lt;sub&gt;i&lt;/sub&gt;</td>
<td>ε&lt;sub&gt;i&lt;/sub&gt; ~ N(0, σ&lt;sub&gt;ε&lt;/sub&gt;²)</td>
</tr>
<tr>
<td>B&lt;sup&gt;a&lt;/sup&gt;</td>
<td>DON&lt;sub&gt;ij&lt;/sub&gt; = β₀ + β₁IND&lt;sub&gt;ij&lt;/sub&gt; + μ&lt;sub&gt;j&lt;/sub&gt; + ε&lt;sub&gt;ij&lt;/sub&gt;</td>
<td>μ&lt;sub&gt;j&lt;/sub&gt; ~ N(0, σ&lt;sub&gt;μ&lt;/sub&gt;²), ε&lt;sub&gt;ij&lt;/sub&gt; ~ N(0, σ&lt;sub&gt;ε&lt;/sub&gt;²)</td>
</tr>
<tr>
<td>C&lt;sup&gt;a&lt;/sup&gt;</td>
<td>DON&lt;sub&gt;ij&lt;/sub&gt; = β₀ + β₁IND&lt;sub&gt;ij&lt;/sub&gt; + μ&lt;sub&gt;j&lt;/sub&gt; + ε&lt;sub&gt;ij&lt;/sub&gt;</td>
<td>ε&lt;sub&gt;ij&lt;/sub&gt; ~ N(0, σ&lt;sub&gt;ε&lt;/sub&gt;²), where σ&lt;sub&gt;ε&lt;/sub&gt;² = aDON&lt;sub&gt;ij&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>D&lt;sup&gt;b&lt;/sup&gt;</td>
<td>DON&lt;sub&gt;j&lt;/sub&gt; = β₀ + β₁IND&lt;sub&gt;j&lt;/sub&gt; + ε&lt;sub&gt;j&lt;/sub&gt;</td>
<td>ε&lt;sub&gt;j&lt;/sub&gt; ~ N(0, σ&lt;sub&gt;ε&lt;/sub&gt;²)</td>
</tr>
<tr>
<td>E&lt;sup&gt;b&lt;/sup&gt;</td>
<td>DON&lt;sub&gt;j&lt;/sub&gt; = β₀ + β₁IND&lt;sub&gt;j&lt;/sub&gt; + ε&lt;sub&gt;j&lt;/sub&gt;</td>
<td>ε&lt;sub&gt;j&lt;/sub&gt; ~ N(0, σ&lt;sub&gt;ε&lt;/sub&gt;²), where σ&lt;sub&gt;ε&lt;/sub&gt;² = aDON&lt;sub&gt;j&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>F&lt;sup&gt;b&lt;/sup&gt;</td>
<td>DON&lt;sub&gt;j&lt;/sub&gt; = β₀ + β₁IND&lt;sub&gt;j&lt;/sub&gt; + ε&lt;sub&gt;j&lt;/sub&gt;</td>
<td>ε&lt;sub&gt;j&lt;/sub&gt; ~ N(0, σ&lt;sub&gt;ε&lt;/sub&gt;²)</td>
</tr>
<tr>
<td>G&lt;sup&gt;b&lt;/sup&gt;</td>
<td>DON&lt;sub&gt;j&lt;/sub&gt; = β₀ + β₁IND&lt;sub&gt;j&lt;/sub&gt; + ε&lt;sub&gt;j&lt;/sub&gt;</td>
<td>ε&lt;sub&gt;j&lt;/sub&gt; ~ N(0, σ&lt;sub&gt;ε&lt;/sub&gt;²), where σ&lt;sub&gt;ε&lt;/sub&gt;² = aDON&lt;sub&gt;j&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Models A-C use all cluster observations (n = 1049). The subscript i is an index for cluster, while the subscript j is an index for plot. Model A does not account for the hierarchy of the data and only contains the cluster index. Random effects are normally distributed in models A and B, but the variance of ε<sub>ij</sub> is dependent on the estimate of DON in model C. β₀, β₁, and φ are model parameters.

<sup>b</sup> Models D-G use plot mean data (n = 35). β₀, β₁, and φ are model parameters. The subscript j is an index for plot. Models D and F have normally distributed random effects, while the random effects in models E and G have non-constant variance dependent on the estimate of DON. For models F and G the exponent parameter φ is held constant at 1, equating the models to linear models.
Table 4.2: Slope parameter estimates and \( t \)-tests between years for regression of transformed IND and DON plot means and variances.

<table>
<thead>
<tr>
<th></th>
<th>IND(^a)</th>
<th>T-test(^b)</th>
<th>DON(^a)</th>
<th>T-test(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Year</td>
<td>( \hat{b} )</td>
<td>SE</td>
<td>T-value</td>
<td>P-value</td>
</tr>
<tr>
<td>2008</td>
<td>1.3266</td>
<td>0.667</td>
<td>0.6172</td>
<td>0.7292</td>
</tr>
<tr>
<td>2009</td>
<td>1.0975</td>
<td>0.1184</td>
<td>1.6203</td>
<td>0.2099</td>
</tr>
</tbody>
</table>

\(^a\) Plot means and variances for Fusarium head blight index (IND) and deoxynivalenol (DON) in grain were transformed using the power-law relationship prior to regression. The value of the slope parameter estimate (\( \hat{b} \)) is on the transformed scale.

\(^b\) A \( t \)-test was performed between the slope parameter estimates (\( \hat{b} \)) in 2008 and 2009 for both Fusarium head blight index (IND) and deoxynivalenol (DON) to evaluate if the slopes were significantly different between the two years of the study.
Table 4.3: Parameter estimates, $\phi$ evaluation, variance estimates, goodness of fit tests for all models for the DON:IND relationship.

<table>
<thead>
<tr>
<th>Model</th>
<th>$\beta_0$</th>
<th>$\beta_1$</th>
<th>$\phi$</th>
<th>T</th>
<th>P</th>
<th>$a$</th>
<th>$b$</th>
<th>Clustering variance</th>
<th>Between Plot variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.872</td>
<td>29.316</td>
<td>0.692</td>
<td>-</td>
<td>&lt;0.0001</td>
<td>19.148</td>
<td>(0.836)</td>
<td>6081.9</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>2.226</td>
<td>29.822</td>
<td>0.726</td>
<td>4.329</td>
<td>&lt;0.0001</td>
<td>3.774</td>
<td>(0.339)</td>
<td>17.366 (1.10)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>2.022</td>
<td>28.695</td>
<td>0.691</td>
<td>-</td>
<td>&lt;0.0001</td>
<td>5.189 (0.759)</td>
<td>0.213 (0.063)</td>
<td>1.924 (0.136)</td>
<td>5621.9</td>
</tr>
<tr>
<td>D</td>
<td>2.744 (1.861)</td>
<td>39.285</td>
<td>0.952</td>
<td>-</td>
<td>0.4514</td>
<td>8.921</td>
<td>(2.133)</td>
<td>185.3</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>1.398 (2.273)</td>
<td>30.092</td>
<td>0.690</td>
<td>0.123</td>
<td>1.2869</td>
<td>0.670</td>
<td>(0.761)</td>
<td>1.26 (0.563)</td>
<td>183.3</td>
</tr>
<tr>
<td>F</td>
<td>2.940 (0.824)</td>
<td>41.278 (5.45)</td>
<td>1</td>
<td>0.1869</td>
<td>8.925 (2.134)</td>
<td>0.776 (0.907)</td>
<td>1.202 (0.582)</td>
<td>181.4</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>2.772 (0.631)</td>
<td>42.770 (6.115)</td>
<td>1</td>
<td>0.1869</td>
<td>8.925 (2.134)</td>
<td>0.776 (0.907)</td>
<td>1.202 (0.582)</td>
<td>181.4</td>
<td></td>
</tr>
</tbody>
</table>

$^b$ Models A-C were generated using all cluster observations ($n = 1049$), whereas models D-G were generated using mean plot data ($n = 35$).

$^c$ $\beta_0$, $\beta_1$, and $\phi$ are parameters of the non-linear models. $\beta_0$ is the intercept, $\beta_1$ is the slope, and the exponent parameter $\phi$ controls the curvature of the line. In models F and G $\phi$ is held constant at 1, so the models become the equivalent of linear models. The numbers in the parentheses are the standard errors of each parameter estimate.

$^d$ This $t$-test evaluates whether the exponent parameter $\phi$ is significantly different from 1.

$^e$ Cluster variances are not included for models D-G because models were generated using mean plot data and not cluster observations. The numbers in parentheses are the standard errors of the estimated variances.

$^g$ The corrected Akaike information criterion (AICC) was used to judge the goodness of fit of each model. Models A-C and models D-G were compared separately because of the differences in datasets used for model generation. A lower AICC value indicates a better model fit.


44. Kang Z., and Buchenauer H. 2002. Studies on the infection process of Fusarium culmorum in wheat spikes: Degradation of host cell wall


APPENDIX A: CULTURE MEDIA RECIPES

Komada media: Prior to autoclaving, dissolve 2 g L-asparagine, 0.5 g MgSO$_4$·7H2O, 0.5 g KCl, 20 g D-galactose, 1 g K$_2$HPO$_4$, 5 mg Fe(EDTA), and 20 g agar in 1 L of water. Autoclave the solutions for 30 min. After cooling to 45°C, add 1 g pentachloronitrobenzene, 0.5 g oxgall, 1 g Na$_2$B$_4$O$_7$·10H$_2$O, and 0.3 g streptomycin sulfate and aliquot in Petri plates immediately.

Potato Dextrose Agar: Mix 24 g PDA powder into 1 L H$_2$O and autoclave for 30 min. After removal allow the liquid to cool to 45°C and add 0.3 g streptomycin sulfate. Immediately aliquot into Petri plates.

Carnation Leaf Agar: Combine 20 g agar with 1 L water and autoclave for 30 minutes. Add 0.3 g streptomycin to liquid media when it has cooled to 45°C. Immediately aliquot the liquid into Petri plates. When the media has solidified, place 3-5 short (~1-5 cm) pieces of sterile carnations leaves onto the surface using appropriate aseptic technique.

Carrot Agar: Peel and cut 400 g of carrots into 2 cm wide pieces and add to 400 mL of water. Autoclave the carrots and water for 30 min. Allow to cool completely, preferably overnight, before blending. Blend the carrot mixture until smooth, then add 20 g agar and blend until agar is well mixed with the carrots.
Return the mixture to a flask and bring the total volume to 1 L using water. Autoclave the mixture for an additional 30 min. When the mixture has cooled to 45°C add 0.3 g streptomycin sulfate and immediately aliquot into Petri plates.

**Mung Bean Agar:** Forty grams of mung beans were added to boiling water and heated for 23 min until the beans cracked open. Filter the liquid through cheesecloth, and discard the solids. Bring the liquid to a volume of 1 L with water before autoclaving for 30 min. When the liquid has cooled to 45°C, add 0.3 g streptomycin sulfate and aliquot into Petri plates.