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Effect Of Post-Anthesis Fungicide Applications To Manage Fusarium Head Blight In Winter Wheat

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EFFECT OF POST-ANTHESIS FUNGICIDE APPLICATIONS TO MANAGE
FUSARIUM HEAD BLIGHT IN WINTER WHEAT

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ABSTRACT

Noveroske, Anna. M.S., Purdue University, December 2014. Effect of Post-Anthesis Fungicide Applications to Manage Fusarium Head Blight in Winter Wheat. Major Professor: Kiersten A. Wise.

Fusarium head blight (FHB) of wheat, caused by the fungus *Fusarium graminearum,* is currently considered one of the most economically important diseases on wheat in the North Central United States. The fungus causes light-weight "tombstone" grains to form and produces the mycotoxin deoxynivalenol (DON), reducing the yield and quality of the grain. Currently, farmers rely heavily on the sterol demethylase Inhibitor (DMI) triazole fungicide Prosaro (Bayer CropScience) to protect their crop from this disease. The optimal fungicide application timing is traditionally believed to be early anthesis – Feekes Growth Stage (FGS) 10.5.1. However, environmental conditions and uneven flowering across a field at this growth stage can hinder precise fungicide application.

Field trials were conducted at the Agronomy Center for Research and Education in West Lafayette, IN in the 2012-2013 and 2013-2014 growing seasons to determine the impact of post-anthesis fungicide timing in conjunction with initial infection by *F. graminearum* and subsequent development of FHB and DON. Treatments consisted of single applications of Prosaro at 475 mL/ha applied at Feekes Growth Stage 10.5.1 (anthesis), and anthesis + 1, 3, 5, 7, 9, and 11 days. In 2013 all plots were inoculated with

macroconidia of *F. graminearum* and non-treated inoculated plots served as controls. In 2014, an additional treatment was included that did not receive inocula or fungicide. Disease index was assessed ten days after the final treatment. DON and yield were evaluated post-harvest.

Results indicate that fungicide applications made up to 11 days post-anthesis may be useful in reducing FHB and DON when conditions are favorable for disease development. Fungicide application had a significant effect on DON ($P < 0.0001$) in both 2013 and 2014. Mean DON values were numerically lower at every application time in both years compared to the non-fungicide treated control. These results indicate that fungicide application after anthesis may be useful in reducing FHB and mycotoxin levels in wheat.

CHAPTER 1: INTRODUCTION

1.1. Introduction

Common bread wheat (*Triticum aestivum* L. spp. *aestivum*) is an important cereal crop worldwide. It is currently the second largest source of calories for humans (after rice) and is the number one source of protein (CIMMYT et al., 2012). In 2010, over 650 million metric tons of wheat were produced worldwide, and it is estimated that the demand for wheat will continue to rise in response to the increase in global population (FAOSTAT 2011; USDA 2012a). Wheat is currently a staple cereal crop for approximately 40% of the world's population and in some countries it makes up 35 to 60% of the population's regular caloric intake (Bockus et al., 2010; CIMMYT et al., 2012).

1.2. History of *Triticum aestivum*

Although the history of wheat is somewhat ambiguous, it is thought to have evolved in a series of ploidy changes from wild and cultivated wheat ancestors to form the hexaploid species *T. aestivum* cultivated today (Bockus et al., 2010). The earliest wild ancestors of wheat likely originated in the river valleys of the Fertile Crescent (Dondlinger, 2012). The first cultivated wheat varieties are believed to be the diploid einkorn wheat (*T. monoccum*) and the tetraploid emmer wheat (*T. turgidum spp. Dicoccum*), which spread into Greece by 6,000 B.C. and into England by 3,000 B.C. (Curtis, R. et al., 2013; Dondlinger, 2012; Evans, L. T. et al., 1981). Archaeological

evidence points to hexaploid wheats having evolved under cultivation from the tetraploid emmer and one of two wild grass species from the *Aegilops* genus (*A. speltoides* and *A. tauschii*). Hexaploid wheats (6n = 42) are only found in domesticated forms and constitute the vast majority of wheat produced and consumed worldwide (Bockus et al., 2010; Zohary et al., 2012).

Approximately 20% of the cultivated land around the world is designated for wheat production (Bockus et al., 2010). *Triticum aestivum* is primarily cultivated in temperate regions where yearly temperatures fall between 3 and 32°C, averaging around 25°C. These conditions occur from 27° to 40° latitude in the southern hemisphere and 30° to 60° in the northern hemisphere. Wheat is also grown at increased elevations near the equator. There have also been reports of wheat being grown above the Arctic Circle (Bockus et al., 2010).

The species *T. aestivum* L. consists of five subspecies, that, until recently, were each considered distinct species. The subspecies are *T. aestivum* L. ssp.: *spelta* (L) Thell., *macha* (Dek. & Men.) MK, *compactum* (Host) MK, *sphaerocuccum* (Percival) MK, and *aestivum*. *Triticum aetivum* L. ssp. *aestivum* is the most widely grown wheat species in the world today, making up approximately 95% of the wheat harvested worldwide. The remaining percentage is from durum wheat (the tetraploid *T. turgidum* L. ssp. *durum* (Shrank) Thell) (Bockus et al., 2010; Zohary et al., 2012).

Within the common bread wheat subspecies, *T. aestivum* L. ssp. *aestivum*, there are four main grain classes separated by the firmness and color of their kernels and the season in which they are planted. In the United States, hard red winter (HRW) wheat is primarily grown in the southern and central portions of the Great Plains. Hard red spring

(HRS) wheat is grown in the northern Great Plains where harsher winters prevent a pre-winter planting. White wheat is typically grown in the Pacific Northwestern states, Michigan, and New York; soft red winter (SRW) wheat is grown almost exclusively in the eastern and southern states. Durum wheat is also grown in the U.S., but is primarily confined to Arizona, southern California, Montana, and North Dakota (Curtis et al., 2013; USDA-NASS 2012).

1.1.2. Growth habits

Wheat progresses through a series of well-defined growth stages as it matures. Two scales have been developed that define the various stages, Feekes and Zadoks, but the most commonly used scale is Feekes. The Feekes scale divides growth into eleven primary stages with several subdivisions (Figure 1.1; Herbek and Lee, 2009). The first stage, Feekes Growth Stage (FGS) 1 is known as spiking. This is the stage at which the first shoot emerges from the ground after planting. FGS 2 describes the stage at which tillers begin to form. A tiller is a secondary (axillary) shoot that emerges from the primary (or main) shoot and may or may not develop a wheat head by the time of maturity (Camberato et al., 2013). These are also called secondary tillers. On average, two or three of the secondary tillers will mature enough to produce a viable wheat head, thereby contributing to grain yield (Herbek and Lee, 2009). Tillers will continue to form into FGS 3.

After FGS 3, winter wheat enters a period of vernalization. Vernalization is a process required by winter wheat to produce reproductive structures. It involves exposure to cold temperatures for a given length of time. In winter wheat, approximately six weeks of exposure to temperatures between 3 and 4.4º C is sufficient, although if temperatures

drop lower than 3℃, the length of required exposure time may be reduced (Herbek and Lee, 2009). During vernalization, the winter wheat will not produce any new growth. Spring wheat does not require vernalization and moves directly from FGS 3 to FGS 4: leaf sheath elongation. Winter wheat moves into FGS 4 as it comes out of vernalization. FGS 4 to 7 occur as the wheat grows more erect, puts out more leaves (and possibly tillers, although these rarely contribute to yield), and forms the first two nodes. At FGS 8-9, the flag leaf, the leaf responsible for photosynthesizing 75% of the sugars for grain fill, emerges from the sheath and the ligule is formed. At this point, the kernel embryos are developing within the sheath and beginning to form heads within the tillers. FGS 10.0 is called the boot stage. At this stage, the wheat head has moved up the sheath and is positioned in between the topmost two nodes. As the head begins to emerge from the sheath, the wheat moves through FGS 10.0, 10.1, 10.2, 10.3, and 10.4 and at 10.5, the head is fully emerged. The next stage, FGS 10.5.1, is the stage at which anthers begin to protrude from the head and pollinate the kernels. Pollination is said to be complete at 10.5.3 (Camberato et al., 2013). The period of anthesis within a field is said to take 3 to 10 days depending on environmental factors (Curtis et al., 2002).

The last stage of wheat development is FGS 11 which is divided into four parts: milk development (early medium and late), dough development (early, soft, and hard), hard ripened kernel, and ripe for harvest. From 11.3-11.4 (hard ripened kernel to ripe for harvest), the grain does not increase in size or maturity, but undergoes drying. Harvest typically occurs once the gain moisture level falls below 15% (Herbek and Lee, 2009).
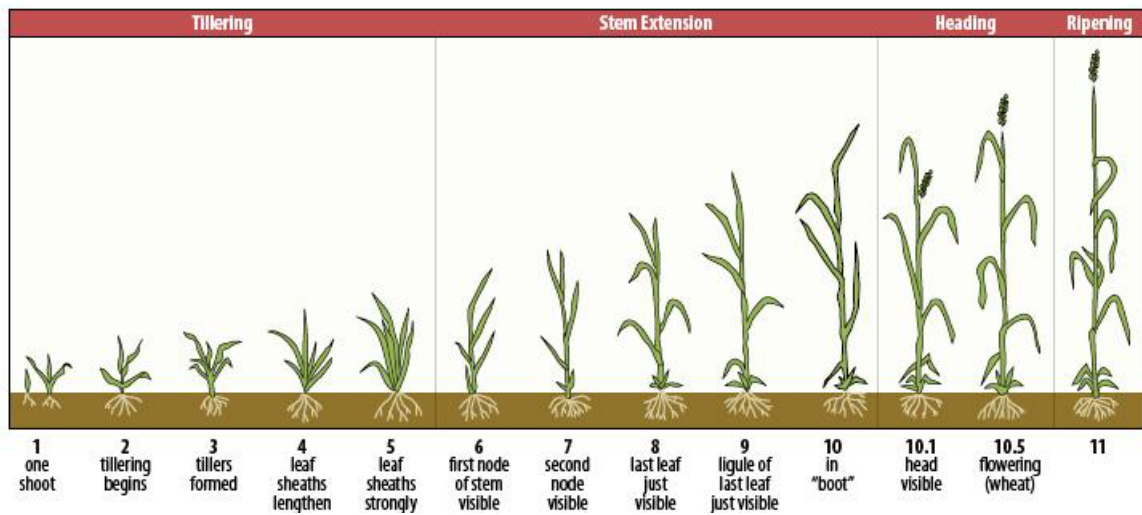
Figure 1.1. Feekes Growth Stages of wheat (image from Herbek and Lee, 2009)

1.1.3. Economic importance

1.1.3.1. United States

Triticum aestivum ssp. *aestivum* has been an important food staple in the U.S. since its introduction by Europeans traveling to the "New World". As settlers moved westward, wheat moved with them as a crop, arriving in eastern Ohio by 1850, and the east central boarder of Iowa between 1880 and 1900 (Dondlinger, 2012). Wheat is now grown in over 80% of the states in the U.S. The only states that did not report a wheat harvest in 2011-2012 were Alaska, Connecticut, Hawaii, Maine, Massachusetts, New Hampshire, Rhode Island, and Vermont (USDA-NASS, 2012).

According to the Food and Agriculture Organization (FAO), wheat is the third largest crop grown in the U.S. based on both quantity and economic value (FAOSTAT, 2011), behind maize and soybeans. Despite the large quantity of wheat grown in the U.S., production has declined since its peak in 1981, in part due to declining profitability of the crop. However the U.S. remains the third largest producer of wheat in the world after

China and India, harvesting approximately 49 million metric tons of wheat (approximately 7.5% of the global wheat production) in 2012 (USDA 2012b). Wheat is also an important export crop for the U.S. Approximately half of the wheat harvest in the U.S. is exported annually, making the U.S. the leading wheat exporter in the world (U.S. Wheat Associates, 2012).

1.1.3.2. Midwest

In Indiana, approximately 3.2% of cropland was planted with wheat in 2012, covering 390 thousand acres. In Illinois, approximately 2.8% of the cropland was used for wheat, and in Ohio, the percentage of cropland planted with wheat was nearly 8.3% (USDA-NASS, 2012). As a central crop both to Indiana and the U.S. economy as a whole, it is important that wheat production remains economical for years to come. This is a challenge when losses occur and when the costs of trying to prevent such losses rise without a guaranteed increase in yield. There are many different reasons for crop losses, including biotic and abiotic factors. Together, these factors are estimated to cause between 25 and 30% crop loss annually (Bockus et al., 2010). Abiotic factors include heat stress, drought stress, over-salination of soil, and hard frost damage, among other factors (Curtis et al., 2013). Biotic factors include insect damage and a damage from a myriad of plant pathogenic organisms that can cause disease. Pathogens of wheat include bacteria, viruses, nematodes and fungi. Currently, the pathogen of greatest concern in the U.S. is *Fusarium graminearum* Schwabe, (telemorph *Gibberella zeae* (Schwein.) Petch), the fungus that causes the disease Fusarium head blight of wheat (FHB) (Bockus et al., 2010).

1.2. Fusarium Head Blight of Wheat

Figure 1.2. Wheat heads with Fusarium head blight

1.2.1. Introduction

Fusarium head blight (FHB) is currently the most important plant disease affecting wheat in the U.S., and is becoming increasingly problematic worldwide. The disease has been reported in every country in which cereal crops are grown, and it is of particular concern in the midwestern and southern U.S. where weather conditions, combined with cultural production practices, often provide the ideal environmental conditions for proliferation of the causal pathogen (Bockus et al., 2010; McMullen et al., 1997). Although there are a variety of *Fusarium* species that cause FHB, the predominating species in the U.S. is *Fusarium graminearum* Schwabe (telemorph *Gibberella zeae* (Schwein.) Petch) (Bockus et al., 2010; Goswami and Kistler, 2004; McMullen et al., 1997).

F. graminearum initially infects wheat heads during anthesis in the spring. Disease symptoms soon become apparent as the fungus makes its way up the wheat head, causing light colored, light-weight "tombstone" grains to form in place of healthy kernels (Figure 1.2). These light-weight grains cause a decrease in overall yields (Sutton 1982).

Another problem associated with FHB is the accumulation of mycotoxins in the plant. In the U.S., *F. graminearum* commonly produces the toxic compounds deoxynivalenol (DON, commonly referred to as "vomitoxin") and nivalenol (NIV). These toxins greatly reduce the quality of grain harvested, and their levels are regulated in grain for sale or consumption are regulated by the FDA (O'Donnell et al., 2000; U.S. Food and Drug Administration, 2013).

Currently, there are no management methods that completely suppress FHB development in wheat. Several moderately resistant cultivars have been developed, but none are able to completely control FHB. Cultural practices, such as rotating between non-host crops, reduce the severity of FHB, but due to *F. graminearum*'s wide host range, which includes corn, rice, barley, soybeans and other grasses, finding a profitable non-host can be difficult (Díaz Arias et al., 2013; Dill-Macky and Jones, 2000; Goswami and Kistler, 2004; Pioli, 2004). The advent of conservation tillage has also contributed to FHB severity in recent years by allowing infested crop stubble to remain on the field surface and serve as a source of inoculum for the wheat crop in the following season (Dill-Macky and Jones, 2000).

Several commercially available fungicides have promise for FHB suppression when combined with other disease mitigation techniques through integrated pest management (IPM). The most effective of these fungicides are within the sterol biosynthesis inhibitors (SBI), demethylation inhibitors (DMI) triazole class of fungicides (FRAC group G1) (FRAC, 2011; Mesterházy et al., 2003; Mesterházy et al., 2011). Although these fungicides may suppress disease, they are an additional input cost for farmers and have been shown to have adverse environmental impacts (Knight et al.,

1997). Additionally, continued reliance on a single class of fungicides over a long period of time in the same geographic area can result in the development of fungicide resistance within the pathogen.

1.2.2. *Fusarium graminearum*

1.2.2.1. Taxonomy

According to Goswami and Kistler (2004), the taxonomy of the most common causal agent of FHB of wheat in North America is as follows:

<u>Superkingdom</u>: Eukaryotea

  <u>Kingdom</u>: Fungi

    <u>Phylum</u>: Ascomycota

      <u>Subphylum</u>: Perzizomycotina

        <u>Class</u>: Sordariomycetidae

          <u>Subclass</u>: Hypocreomycetidae

            <u>Order</u>: Hypocreales

              <u>Family</u>: Nectriaceae

                <u>Genus</u>: *Gibberella* (anamorph *Fusarium*)

                  <u>Species</u>: *zeae* (Schweinitz) Petch. (anamorph *graminearum* Schwabe)

1.2.2.1. Biological Properties

Fusarium graminearum is a homothallic, ascomycete fungus that infects a myriad of cereal crops. It is the predominating causal agent of FHB of wheat in the warmer wheat growing regions of the world, including North America, central Europe, and Australia. *Fusarium graminearum* has also been found to cause FHB in China, India,

Japan, and Yugoslavia, among others (Perry et al., 1995). Although primarily studied as a single species, studies have suggested that *F. graminearum* is actually a clade of nine organisms, likely having resulted from allopatric speciation over time. *F. graminearum* is also divided into four different chemotypes based on mycotoxin production (O'Donnell et al., 2000; O'Donnell et al., 2004).

Fusarium graminearum is a facultative parasite that over seasons on crop stubble, surviving saprophytically or forming chlamydospores. Studies show that it can survive on tissue of many hosts including corn, wheat, barley, oats, rice, and soybean (Bai and Shaner, 2004; Bockus et al., 2010; Parry et al., 1995; Trail et al., 2003). These over seasoning structures give rise to the primary inocula in the spring.

As temperatures increase in the spring, primary inocula are released in the form of macroconidia. Dark colored, flask-shaped perithecia [*Gibberella zeae* (Schweinitz) Petch.] also develop, producing unitunicate asci. These asci typically contain eight ascospores which are forcefully ejected from the perithecia and serve as another major source of primary inocula (Bockus et al., 2010; Dufalt et al., 2006; Schumann and D'Arcy, 2010; Trail et al., 2005). Ascospores typically have three septa and are between 3 and 5 μm long (Sutton, 1982). Perithecia have been shown to develop rapidly at high moisture levels and moderate temperatures (20º to 24ºC). At ideal temperature and moisture conditions, perithecia can fully develop from mycelia in the course of 10 days. At more extreme temperatures both above and below this range, development slows dramatically (Dufalt, 2006). Ascospores released from perithecia require approximately 50% relative humidity for germination (Beyer et al., 2005), and they are often released in the evenings when relative humidity is highest (Gilbert and Fernando, 2004).

Macroconidia develop from haploid mycelia and, depending on the temperature at which they form, tend to be 3 to 7 septate (Andersen, 1948; Sutton, 1982). The conidia range from ~2.5 to 5μm long, depending on the number of septations, and they form an elongated, canoe-like shape, being smaller at either end than they are in the middle and generally having a slight curve to them. They also have a distinctive foot cell (Sutton, 1982).

The primary inoculum of *F. graminearum* is disseminated mainly by wind and splashing water, although ascospores are also ejected from perithecia and some birds and insects may function as vectors (Ferenando et al., 1997; Paul, 2004; Sutton, 1982; Trail et al., 2002). Once the spores reach wheat heads, they germinate and begin extending mycelia into the surrounding plant tissues. *Fusarium graminearum* enters the wheat spikelets primarily via the anthers, and colonization is favored when infection occurs prior to the anthers shedding pollen. *Fusarium graminearum* can also infect wheat heads via wounds (Sutton, 1982). Since the period of greatest susceptibility (anthesis) is only 10 to 20 days long, FHB is primarily considered a monocyclic disease, but some debate exists on this point due to the potential for spores to infect the secondary tillers (Fernando et al., 1997; McMullen et al., 2008; Willyerd et al., 2012).

Germination, the process whereby spores begin producing hyphae, is favored by extended periods (>24 hours) of high moisture and moderately warm temperatures (between 25° and 30°C) around the time of primary wheat head infection. These conditions allow a shorter incubation period between the initial infection and the development of FHB symptoms (Andersen, 1948). Macroconidia require a relative

humidity of at least 80% at 20ºC in order to germinate (Beyer et al., 2005). In favorable conditions, spores can germinate within three hours (Andersen, 1948).

Once hyphae begin to grow, *F. graminearum* will colonize the rachis, spike, grain, other flower parts, and subsequent spikelets (Brown et al., 2010). Hyphae have also been shown to colonize wheat glumes, entering through the stomata since the epidermis is too formidable a barrier for direct penetration (Pritsch et al., 2000). Although hyphal infection routes include both the apoplast and vascular tissue, spread of *F. graminearum* from one spikelet to another only occurs via the plant cortex (Brown et al., 2010).

Currently, *F. graminearum* is thought to function like a biotroph in the earlier stages of infection, feeding off extracellular exudates. As cell death occurs in the host plant, the pathogen shifts toward the necotrophic end of the spectrum, obtaining nutrients from dead host cells (Brown et al., 2010; Jansen et al., 2005). Host cells have been shown to empty of their contents just prior to colonization by *F. graminearum*, and no specialized feeding structures were observed (Brown et al., 2010).

Mycelia of *F. graminearum* is haploid and ranges in color from white to a deep salmon-pink. Macroconidia form from the hyphal tips of the mycelia, and if environmental factors are favorable, they can form within 72 hours of initial wheat head infection (Sutton, 1982; Andersen, 1948). These new spores can be carried by the wind to infect other susceptible host plants, including flowering wheat heads, maize, barley, and rice. They can also form over seasoning chlamydospores that serve as primary inocula for the next cycle of infection (Schumann and D'Arcy, 2010; Sutton, 1982).

1.2.2.3. Mycotoxins

Fusarium graminearum produces various mycotoxins during the infection process. Mycotoxins are defined as naturally produced fungal secondary metabolites that have demonstrated toxicity toward humans and/or animals upon their consumption. They are non-living byproducts of infection and are believed to contribute to pathogen aggressiveness (Sinha and Bhatnagar, 1998; Wagacha and Muthomi, 2007). *Fusarium graminearum* is known to produce three different types of mycotoxins: Deoxynivalenol and its derivatives (DON, also called "vomitoxin"), zearelenone (ZON), and nivalenol (NIV). DON and NIV are trichothocenes and are known to inhibit protein biosynthesis in eukaryotes, while ZON is an estrogenic mycotoxin (O'Donnell et al., 2000). The derivatives of DON that *F. graminearum* is capable of producing are 3-Acetyl-deoxynivalenol (3AcDON) and 15-Acetyl-deoxynivalenol (15AcDON) (Sinha and Bhatnagar, 1998). *Fusarium graminearum* is grouped into four chemotypes based on the mycotoxin that it is able to produce. Each chemotype has the potential to produce ZON. The most prevalent chemotype, and the one of greatest economic concern in the U.S., produces DON (Ichinoe et al., 1983; O'Donnell et al., 2000). Levels of DON have been shown to positively correlate to the number of Fusarium damaged kernels (FDK) in a field, and to a high relative humidity (Beyer et al., 2005; Paul et al., 2005; Sinha and Bhatnagar, 1998).

DON is considered a virulence factor of *F. graminearum* and functions by suppressing the plant's defense responses at the front of advancing hyphae (Brown et al., 2011; Jansen et al., 2005). In the absence of DON, wheat plants develop thick cell walls in the rachis node as a defense against the pathogen's spread. DON acts by inhibiting

protein synthesis, thereby preventing the formation of this thick, hardened wall (Ueno, 1969). This allows the pathogen to colonize the rachis node and advance into subsequent spikelets. However, DON does not function as a virulence factor in the colonization of fruit coat tissue. When wheat spikelets were inoculated with a *F. graminearum* mutant isolate (one lacking the ability to synthesize trichothecenes), colonization of the coat tissue was not affected (Jansen et al., 2005).

DON poses a significant danger for human and animal health, and the U.S. Food and Drug Administration (FDA) has developed advisory levels to regulate their presence in various wheat products. Currently, the FDA advises that DON levels not exceed 1 ppm in finished wheat products created for human consumption, 5 ppm for wheat products to be consumed by swine and other animals, and 10 ppm for brewers' grains, and grain for chickens and ruminating animals at least four months of age (U.S. Food and Drug Administration, 2013). Although integrated pest management (IPM) measures attempt to prevent DON from exceeding these levels, in years of moderate disease pressure, it is estimated that the DON advisory levels are exceeded with an infection of less than one fourth of a field (Ichinoe et al., 1983). Such losses can cause great economic impact for wheat farmers and thus require reliable disease mitigation tactics.

1.2.3. Disease cycle

Fusarium head blight in wheat, otherwise known as Fusarium head scab, is characterized by white, light-weight, scabby kernels and high yield losses. It is considered a re-emerging disease and is of economic importance in wheat growing regions on the world (McMullen et al., 1997; Yin et al., 2009). In 1993, the economic loss in the United States due to FHB was estimated at US $1 billion, and in 1994 it was

estimated at US $500 million (Curtis et al., 2013). The disease can be caused by a variety

of different *Fusarium* species, including *F. avenaceum, F.culmorum,* and *F. poae*, but the

primary causal agent in the U.S. and Canada is *F. graminearum* (Parry, 1995).

Infection begins when spores of *F. graminearum* land on young wheat heads.

Wheat heads are most susceptible near anthesis, Feekes Growth Stage (FGS) 10.5.1. Prior

to extruding anthers, wheat heads are not susceptible to FHB. The susceptibility period

lasts through FGS 11.2 (soft dough stage) (Andersen, 1948; Large, 1954). After initial

infection, disease will progress as the fungus moves throughout the spikelet, making its

way into rachis node, and eventually into to the rachis ear via hyphal growth (Brown et

al., 2010). Symptoms are typically seen within five days of initial infection, but the

precise latent period (the period of time between infection and symptom development) is

dependent upon climate conditions (Andersen, 1948). Symptoms include premature

bleaching of the spikelets and light-weight, scabby kernels (also called "tombstone"

grains, (Bockus et al., 2010; Sutton, 1982). Pigmented mycelia are often visible on the

infected heads, giving them a salmon-pink coloration. During harvest, many of the

infected grains are often lost due to their light weight, reducing the overall yield (Bockus

et al., 2010).

1.2.4. History of Fusarium head blight in the United States

The first occurrence of the disease FHB was noted in England as early as 1884, at

which point the disease was attributed to *Fusisporum culmorum*. This same pathogen was

recorded as the cause of FHB in the United States when it was first noted in 1890 in

Ohio. The first record of FHB caused by *F. graminearum* in the U.S. was made in the

1920s, at which time *F. graminearum* was already considered the primary causal agent of

the disease (Parry et al., 1995). Between 1928 and 1937, field surveys documented large

yield losses resulting from FHB infections. Then, in 1980 and 1982, FHB reached

epidemic levels in at least eight different states, including southern Illinois and Indiana. It

was estimated that the epidemic of 1982 caused a 4% decrease in the amount of wheat

produced that year in the U.S. (McMullen et al., 1997). It was not until the 1990s, when a

series of epidemic years devastated many wheat growing regions in the U.S. and Canada,

that FHB received its designation as one of the most economically important wheat

diseases in the U.S (Figure 1.3). The increased attention to FHB and the growing need for

better management techniques eventually led to the creation of the U.S. Wheat and

Barley Scab Initiative (USWBSI) in 1997 that now funds many research programs

focused on fighting this disease (Bockus et al., 2010; McMullen et al., 1997; McMullen

et al., 2012).
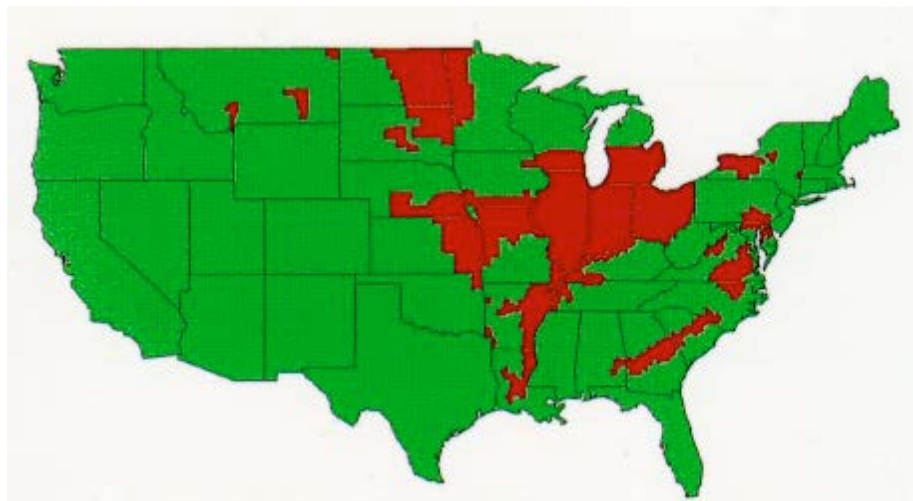
Figure 1.3. Major outbreaks of Fusarium head blight (highlighted in red) on wheat and
barley reported by scientists in the United States from 1991 to 1996 via a questionnaire.
Image from McMullen et al. (1997).

1.2.5. Disease management

Due to the widespread damage caused by FHB, various management techniques have been implemented in an effort to mitigate damage. Together, these techniques are referred to as integrated pest management (IPM). Current IPM for FHB incorporates crop rotation, planting moderately resistant wheat cultivars, burying infected crop residues, and the use of various fungicides (Bockus et al., 2010; Mesterházy et al., 2003; Paul et al., 2008).

The first management goal has been to limit the amount of primary inoculum present in a wheat field. FHB increases in wheat planted in consecutive years or following corn. Therefore, rotating to a non-host crop can decrease the initial field inocula. (Dill-Macky and Jones, 2000; Teich and Hamilton, 1985). Another means of managing the initial inoculum is the use of tillage to bury infected debris under the soil, such as moldboard plowing, thus inhibiting the initial spread of *F. graminearum* spores. However, these tillage practices are not in line with current conservation practices that promote no-till farming for soil and water conservation. Since Indiana, Illinois, and Ohio are among the states that practice the most no-till corn farming in the U.S., these states rely primarily on crop rotation to reduce initial inoculum (Dill-Macky and Jones, 2000; McMullen et al., 1997; Teich and Hamilton, 1985).

The optimal disease management method is to plant cultivars that are completely resistant to the disease, but no such wheat cultivar currently exists for FHB. Therefore, common cultural practices now incorporate the use of moderately resistant wheat varieties as a component of IPM (Willyerd et al., 2011). Wheat breeders believe that gains in FHB resistance are primarily due to physiological (active) resistance as

compared to morphological (passive) resistance (Rudd et al., 2000). Resistance toward

FHB is inherited quantitatively (Bockus et al., 2010). Mesterházy (1995) described five

different types of FHB resistance in wheat (as described in Table 1.1): the most resistant

variety currently available is the Chinese spring wheat cultivar Sumai 3, which exhibits

type II resistance. This cultivar has been used as a parent in U.S. breeding efforts for

spring and winter wheat varieties. While breeding efforts continue to develop FHB

resistant wheat cultivars that combine different types of resistance, challenges include the

limited quantity of effective resistance genes, and the complexity of incorporating

resistant genes into *T. asiaticum*'s hexaploid genome (Mesterházy, 1995; Rudd et al.,

2000; Willyerd, 2011). Currently, FHB is commonly measured in terms of FHB Index, a

measurement of the mean percent disease per head across a field. It therefore assesses the

combined type I and type II resistance using the formula: Index = (% severity) x (disease

incidence out of 100 heads) (Stack and McMullen, 2011).

Table 1.1: Types of Fusarium head blight resistance in wheat cultivars, as described by
Mesterhazy (1995).

Type	Description
I	Resistance to initial infection
II	Resistance to spread within the infected tissue
III	Resistance of the kernel to infection
IV	Yield tolerance to pathogen
V	Ability to decompose/not accumulate mycotoxin produced by pathogen

Due to the lack of resistant cultivars, fungicides are often applied to help suppress

FHB. A number of effective fungicides historically existed including benomyl and

carbendazime (FRAC group B1, methyl benzimidizole carbamate class (MBC) fungicides), fludioxonil (FRAC group E2, a Phenylpyrrole (PP) fungicide), and pyraclostrobin and azoxystrobin (FRAC group C3, quinone-outside inhibitor fungicides (QoI)) (FRAC Mode of Action of Fungicides, 2011; Jones, 2000; Chen, 2012). QoI fungicides, commonly referred to as strobilurin fungicides, have been successfully utilized in the past, but can potentially increase mycotoxin levels when applied after boot stage and are not currently labeled for FHB control in the U.S. (Bradley et al., 2011). Studies suggest that the most effective fungicides for controlling both disease and DON levels are in the sterol demethylation inhibitor (SBI-DMI) triazole class of fungicides (Mesterházy et al., 2003; Jones, 2000). In a multivariate analysis of over 100 uniform fungicide trials (UFT), Paul et al. (2008) determined that the most effective DMI triazole fungicides for controlling FHB were tebuconazole+prothioconazole, followed by prothioconazole and metconazole. Metconazole was shown to be the most effective for reducing DON levels, followed by prothioconazole and tebuconazole+prothioconazole (Paul et al., 2008). The greatest disease reduction obtained was 52%, and the greatest reduction of DON was 40% compared to a non-treated control.

Due to the timing of initial infection and penetration of *F. graminearum* into wheat heads, fungicides are typically applied at anthesis (Paul et al., 2008; Willyerd et al., 2012). However, some studies indicate that the most effective application timing for disease control may not fully align with the timing for optimal DON level control, and that the effect of application timing may further vary among fungicides (Chen 2012; Yoshida et al., 2012). Yoshida et al. (2012) determined that the most effective time to apply fungicide to control DON is around 20 days after anthesis (approximately FGS

11.2). In this same study, FHB was significantly reduced by fungicide application at anthesis compared to untreated plots, but not by fungicide treatments at 10, 20, or 30 days after anthesis. Del Ponte et al. (2007) saw a similar trend in DON, finding that levels were highest when wheat was inoculated with *F. graminearum*, up to the hard dough stage (FGS 11.3). However, a recent study found that FHB Index was reduced more by post-anthesis fungicide application than by anthesis fungicide applications and that both index and DON were reduced in fungicide treated plots compared to untreated plots regardless of the application time (from anthesis to anthesis + 6 days) (D'Angelo et al., 2014).

IPM practices for FHB in wheat have demonstrated greater reduction in FHB than single management strategies alone (Mesterházy et al., 2003). In the U.S., up to 76% FHB control and 71% DON reduction have been attained by combining fungicides with moderately resistant wheat cultivars. Cultivar resistance to FHB and fungicide use have shown an additive effect on reducing FHB index and DON levels (Willyerd et al., 2011). Willyerd et al. (2011) also found that combining a moderately resistant cultivar with a fungicide treatment was the most stable treatment combination across environments for control of index and DON across 37 environments in the wheat growing regions of the U.S..

Several harvesting strategies have also been used to separate out light-weight, infected kernels after infection has occurred. These strategies include increasing combine fan speed and increasing the combine shutter opening in an attempt to separate out the Fusarium damaged kernels with the chaff (Saldago et al., 2011, Saldago et al., 2014). Although these studies demonstrate reduction in Fusarium damaged kernels (FDK) and

DON, Paul (2008), points out that in years where disease pressure is high, even IPM techniques will likely not be able to reduce the DON content in wheat to an acceptable level. Cultivar development and testing is a slow process, meaning that fungicides will likely remain an integral part of IPM for FHB for many years to come. As such, it is important to conduct additional research on optimal fungicide application timing to refine application recommendations for the greatest economic benefit.

1.3. Fungicides

1.3.1. Introduction

Fungicides are chemicals that inhibit the growth and proliferation of fungi. They typically fall into one of two categories: protectant (contact) fungicides or systemic (penetrant) fungicides. Protectant fungicides are applied to the surface of plants and protect the plant tissue against fungal penetration. Systemic fungicides are capable of moving or spreading within the plant by penetrating the plant surface and moving through either the plant tissue or xylem, depending on the fungicide. They tend to have a more site-specific mode of action, typically only interfering with one or two essential fungal enzymes. Several systemic fungicides also exhibit curative properties (Schumann and D'Arcy, 2010).

Fungicides are classified by their mode of action. Currently there are fourteen recognized mode of action groups. These mode of action groups are divided into subgroups and categorized based on their target site of action and chemical group name. The fungicides most commonly used to inhibit *F. graminearum* in wheat are all in the sterol biosynthesis in membranes FRAC group (G), sub-group G1: SBI class 1: DMI, triazole chemistry fungicide group. (FRAC, 2011).

Fungicides are useful, and often necessary in order to prevent major crop losses that would make farming less profitable. However, a variety of concerns surround fungicide use. One concern is that fungicide contaminated crop runoff will cause damage to the environment. Also, there are potential health implications of ingesting residual amounts of fungicides during the consumption of treated crops. The U.S. Environmental Protection Agency (EPA) is responsible for addressing fungicide toxicity issues, and each fungicide is required to undergo toxicity testing prior to receiving registration and licensure from the EPA (Schumann and D'Arcy, 2010; US EPA, 2013).

Another concern is the possibility of fungal populations developing resistance to a specific fungicide mode of action. In response to concerns about resistance, a committee of scientists and agrochemical company representatives, called the Fungicide Resistance Action Committee (FRAC), was formed in 1981. FRAC is incorporated in the Global Crop Protection Federation (GCPF) and seeks to develop guidelines for fungicide use that will prevent, or at least slow, the development of fungicide resistance (FRAC, 2005).

1.3.2. DMI triazoles

1.3.2.1. Mode of action

DMI triazoles are members of the G1 class of fungicides as designated by FRAC. This group of fungicides is characterized by their ability to inhibit sterol biosynthesis in fungal membranes by inhibiting C14-demethylase (FRAC 2011). They are officially called DMI-fungicides, or sterol biosynthesis inhibitors (SBI): class I, within FRAC code 3. The DMI triazoles are a subset of this larger group, categorized based on their chemical structure. Other types of chemical structures that fall into the G1 fungicides include pyridines, imidazoles, and piperazines (FRAC, 2011). Their fungicidal properties

come from their ability to inhibit cytochrome P450 sterol 14α-demethylase, preventing the enzymatic demethylation of C-14 in lanosterol. This is a precursor step in the biosynthesis of ergosterol from lanosterol in the smooth endoplasmic reticulum of fungal cells (Köller, 1992; Schnabel and Jones, 2001; Siegel, 1981). As a result, free fatty acid and sterol ergosterol precursors build up in the fungal cells, causing abnormal growth patterns and growth inhibition. The lack of ergosterol also contributes to these effects since it is an important component of the fungal cell membrane, and serves an analogous role in fungal cell membranes to cholesterol in eukaryotic cell membranes (Köller, 1992; Siegel, 1981). The effect of DMI triazole fungicides on spore germination is still unclear. While some studies claim that spore germination is not inhibited by these fungicides, others have found that several fungicides within this group are capable of inhibiting spore germination (Klix et al., 2007; Siegel, 1981).

The DMI triazole fungicides are partially systemic, broad range, site-specific fungicides (Kuck, 1986; Siegel, 1981). Partially systemic refers to their ability to penetrate a plant and move within its tissue without being able to enter the xylem and move throughout the entirety of the plant. The first DMI triazole introduced was triadimefon (trade name Bayleton®) by Bayer in 1973 (Kuck, 1981; Morton and Staub, 2008). Since that time, many other fungicides with the same mode of action have been developed, the most recent being prothioconazole in 2004 by Bayer (trade name Proline). The newer fungicides have demonstrated better fungicidal activity than their precursors, as well as lower levels of environmental toxicity (Klix et al., 2007). The DMI triazoles have been used worldwide for control of a broad range of diseases including Fusarium head blight in wheat (Jørgensen and Olsen, 2007; Mesterházy, 2003; Waard et al., 1986;

Yin et. al., 2008). Currently the most widely used fungicides for suppressing this disease

are metconazole, prothioconazole, and tebuconazole + prothioconazole, trade names

Caramba (BASF Corporation), Proline (Bayer CropScience), and Prosaro (Bayer

CropScience) respectively (Mesterházy et al., 2003; Paul et al., 2008).

<center>1.3.2.2. Fungicide use in the U.S.</center>

Tebuconazole was first introduced in 1986 (Russel, 2005) but did not become

registered for use on FHB in wheat in the U.S. until the spring of 2008. However, due to

the threat of a FHB epidemic, it was granted a Section 18 Crisis Exemption in 1997 under

the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) in North Dakota. In

subsequent years it was granted a Section 18 label in six other states for at least one year

between 1998 and 2008, when it was officially registered for use (McMullen et al., 2012).

Tebuconazole has been shown to decrease Fusarium damaged kernels (FDK) and reduce

DON levels (Mesterházy et al., 2011). In the United States, a multivariate meta-analysis

performed by Paul et. al. (2008) using over one hundred uniform fungicide trials that

spanned 14 states and 11 years, determined that tebuconazole decreased FHB index by

40% and DON levels by 23% (Paul et al., 2008). While a reduction, these levels still may

not be acceptable.

The newest DMI triazole developed to date is prothioconazole. It was first

introduced in 2002 by Bayer CropScience, but was not labeled for use on wheat in the

U.S. until late 2006, and its use on wheat was limited until 2008. Most recently,

prothioconazole has been used in combination with tebuconazole under the trade name

Prosaro 421 SC (Bayer CropScience). This product was first registered for use on wheat

in the U.S. in the spring of 2008. In the multivariate meta-analysis conducted by Paul et

al. (2008), prothioconazole was shown to decrease FHB Index and DON levels by 48% and 43% respectively. Prothioconazole + tebuconazole decreased FHB Index and DON levels by 52% and 42% respectively, demonstrating the best FDK control of the fungicides tested (Paul et al. 2008). Prothioconazole has demonstrated highly systemic properties and it is believed to have the best control benefit for environmental cost of all the DMIs triazoles currently labeled for use on wheat (Klix et al., 2007; Russel, 2005).

1.3.3. Application timing

As with all fungicides, proper application timing and technique is essential for DMI triazole fungicides to be effective in suppressing FHB and DON accumulation. In order for fungicides to function in a preventative manner, the fungicide must be on the plant prior to initial fungal infection. Infection by *F. graminearum* occurs primarily at anthesis (FGS 10.1-10.5) between the flowering and soft dough stages. Therefore, spraying directly prior to initial flowering is thought to be the most effective timing for fungal suppression (Homdork et al., 2000; Sutton, 1982). However, the uneven heading of wheat spikelets across a field, inhibit fungicide application at the optimal timing for the entire field. Late infection due to extended periods of favorable weather for disease development (rainy periods) can also cause serious problems. This is particularly true in winter wheat because these varieties tend to have longer grain fill periods than spring wheat (Paul et al., 2008). Studies continue to attempt to better understand the fungicide application timing window to achieve suppression of FHB and DON (Bradley et al., 2011).

Additionally, the optimal fungicide application timing for suppressing FHB is not necessarily the same as the optimal timing for the suppression of DON. Several studies

suggest that fungicide application at anthesis is the most effective timing for FHB, but *F. graminearum* infections occurring as late as 20 days after anthesis (DAA) can still cause significant levels of mycotoxin accumulation (Yoshida et al., 2012; Yoshida and Nakajima, 2010). For this reason, it has been suggested that multiple fungicide applications may be necessary, although cost prohibitive. More research is needed to determine the precise timing at which fungicide application provides the most profitable return, both by increasing yield and limiting DON levels.

1.3.4. Resistance to fungicides

Fungicide resistance is defined as the reduced sensitivity of a fungal population toward a particular fungicidal chemical. It often occurs after prolonged use of a particular fungicide mode of action to combat a specific fungal pathogen, and it poses significant financial problems for both growers and chemical manufacturers. The Fungicide Resistance Action Committee (FRAC) records the various pathogens that have developed resistance toward specific fungicide groups and classes. FRAC has also evaluated the risk factors associated with each class of fungicides and categorized them into low, medium, or high risk of fungal resistance development (FRAC, 2005b). Factors used to evaluate the risk include the number of sprays typically used per season, the fungicide mode of action, and the likelihood of virulent mutants to develop based upon in-vitro mutagenesis studies. Other factors include whether or not other fungicide modes of action are also used to manage a given pathogen, and the number generations a fungal population goes through in a given season (Brent and Hollomon, 2007b).

Fungicide resistance is broken down into two major categories: qualitative resistance and quantitative resistance. Qualitative resistance, also referred to as 'discrete',

'discontinuous', or 'single-step' resistance, occurs when resistance is conferred by a single mutation. This type of resistance tends to develop toward fungicides that act on a single target site in the fungus. When a mutation occurs to the target gene, the fungus directly changes from susceptible to resistant. For this reason, qualitative resistance is said to be bimodal, meaning that any given fungal isolate will be either susceptible or resistant, with few to no intermediate levels of susceptibility detectable in a given population (Georgopoulos, 1988). When a mutation confers resistance without diminishing the pathogen's ability to infect its host, it will survive to pass its resistance on to the next generation. With continued selective pressure from fungicide applications, the fungal population will shift from susceptible to resistant. Examples of this include the benzimidazole fungicides and the QoI fungicides (Brent and Hollomon, 2007b, Quello et al., 2010). Qualitative resistance is difficult to detect in low levels, and can accumulate very quickly. For this reason, fungicides that fall under this pattern of resistance development typically have a higher risk of practical or field resistance (i.e. loss of disease control with a given fungicide class in the field).

Quantitative resistance, also referred to as 'multi-site', 'continuous', 'directional', or 'progressive' resistance, occurs when multiple mutations, each conferring small amounts of resistance, build up in a pathogen population over time (Brent and Hollomon, 2007b). This type of resistance is typically associated with broad spectrum fungicides because susceptibility is not determined by a single site of action. Quantitative resistance tends to progress in a continuous manner, slowly shifting toward reduced sensitivity over time. For this reason, there will likely be a wide range of fungal sensitivity levels existent within a population at any given time. Screening for sensitivity shifts tends to be easier

than with qualitative resistance because there will be a detectable shift in sensitivity before an entire population makes a jump to complete or practical resistance. Qualitative resistance is conveyed by the synergistic relationship between multiple mutations that convey small degrees of resistance (Georgopoulos, 1988). As these mutations build up in a population, the mean sensitivity levels begin to shift.

The DMI triazole fungicides are said to be at medium risk of resistance according to FRAC (FRAC, 2005b). They follow a quantitative pattern of resistance, but even so, several pathogens have demonstrated resistance toward fungicides within this group, including the fungal pathogen *Venturia inaequalis*, causal organism of apple scab (Chapman et al., 2011). Since the early 1980s, fungicide resistance toward DMI, SBI class I fungicides have been reported in 34 different pathogens (FRAC, 2012b). The first record of resistance toward a DMI triazole was in barley powdery mildew (*Blumeria graminis* f.sp. *hordei*) in the early 1980s (Russel, 2005), followed closely by powdery mildew of cucumbers (*Sphaerotheca fuligenea*) (FRAC, 2012b; Russel, 2005). Four resistance mechanisms have been described for the DMI triazoles. One proposed mechanism is an increase in efflux of the fungicide via overexpression of ABC transporters (Stergiopoulos et al., 2003). The overexpression of *CYP51A1* due to the presence of transcriptional tandem repeats is also capable of conferring losses in sensitivity, because this causes an overproduction of the cytochrome P450 sterol 14α-demethylase enzyme that are inhibited by DMI fungicides (Hamamoto et al., 2000; Schnabel and Jones, 2001). Other potential mechanisms include an alteration in the target site (CYP450) and a decreased demand for ergosterol in the fungal cell membrane (Brent and Hollomon, 2007b).

Despite reports of resistance, reduction in fungicide sensitivity has not developed quickly in all pathogens (Brent and Hollomon, 2007b). Cross resistance, defined as the phenomenon whereby the mutations within a given pathogen population that have resulted in resistance toward one fungicide have, in tandem, conferred resistance to another fungicide or set of fungicides, has been reported in *F. graminearum* mutants to the various azole fungicides, but to varying degrees (Betcher et al., 2010; Brent and Hollomon, 2007a; Siegel, 1981). Klix et al. (2007) reported decreases in sensitivity of *F. graminearum* toward metconazole and tebuconazole by factors of 1.391 and 1.393 respectively within the first 10 years of introduction in Germany. A more recent study conducted on *F. graminearum* isolates from New York found a single isolate that is resistant to tebuconazole (Spolti et al., 2014). The fact that FHB is primarily a monocyclic disease will also help slow the development of DMI resistance since more time is required for one generation to supersede the previous, potentially more susceptible generation. However, since resistance potential has been sufficiently demonstrated, it is necessary to be aware of reductions in field efficacy. It is also imperative that fungicides are used properly, as superfluous sprays may contribute an unnecessary amount of selective pressure toward reduced fungal sensitivity toward current fungicides.

1.4. Study Objectives

The objective of this study is to determine the window of effective application timing of the fungicide Prosaro (Bayer CropScience LP, Research Triangle Park, North Carolina) when its application coincides with inoculum availability. Prosaro, which contains the active ingredients prothioconazole and tebuconazole, is typically applied at

anthesis (FGS 10.5.1). This is the stage currently believed to be most vulnerable to primary infection. However, anthesis is not a single day event, and *F. graminearum* will produced inoculum whenever weather conditions are favorable. Secondary tillers flower in succession after the primary tiller, and natural variation in growth stages exists across fields, making the crop susceptible past FGS 10.5.1. This means the potential for infection exists beyond the initial assessment of field anthesis. The difficulty of timing a spray for FGS 10.5.1 is compounded when rain potential is factored in, which would delay the application of a fungicide. It is, therefore, important to understand how wide the window is during which wheat is susceptible to inoculum availability and, in conjunction with this window of susceptibility, the range of time during which fungicide application effectively reduces FHB and DON.

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CHAPTER 2: EFFECT OF FUNGICIDE APPLICATION TIMING ON CONTROL OF
FUSARIUM HEAD BLIGHT

2.1. Introduction

Fusarium graminearum Schwabe [telemorph *Giberella zea* (Schweinitz) Petch] is

the primary causal agent of Fusarium head blight (FHB) of wheat, *Triticum aestivum* ssp.

aestivum, in the United States (Goswami and Kistler, 2004). This fungus infects wheat

heads during anthesis, causing salmon to white colored "tombstone" kernels to form in

lieu of healthy grain (Sutton, 1982). The fungus also produces several mycotoxins,

including deoxynivalenol (DON), which is known to inhibit protein synthesis in

eukaryotes, making it harmful to both humans and other mammals (O'Donnell et al.,

2000). Although FHB has been a problem in the U.S. since the mid 1920's, it has

recently re-emerged as a disease of great economic importance after a series of epidemics

in the mid 1990's (McMullen et al., 1997; McMullen et al., 2012). The pathogen is also

able to infect corn, another important crop in the midwestern U.S., causing the disease

Gibberella ear rot (Sutton, 1982). Currently, FHB is considered the pathogen of greatest

concern to wheat cultivation in the U.S. (Bockus et al., 2010).

Wheat is an important crop in the U.S., ranking as the third largest crop both by

quantity and economic value (FAOSTAT, 2011). The U.S. is also the world's leading

exporter of wheat, and midwestern states are the greatest contributors to the export

market (USDA, 2012; U.S. Wheat Associates, 2012; USDA-NASS, 2012).

Unfortunately, the Midwest is also at high risk for FHB outbreaks due to the combination

of weather conditions and common farming practices that occur in this area (Bockus et al., 2010; McMullen et al., 1997).

Currently no single management practice will completely suppress FHB. Typical integrated pest management (IPM) strategies for FHB include planting wheat after soybean instead of corn, using moderately resistant wheat cultivars, and applying fungicide at beginning anthesis (Mesterházy, 1995; Willyerd et al., 2011). The most effective fungicides currently labeled for use against *F. graminearum* on wheat are Prosaro (Bayer CropScience LP, Research Triangle Park, NC), composed of the demethylase inhibitor (DMI) triazole active ingredients prothioconazole and tebuconazole (FRAC, 2011), and Caramba (BASF, Research Triangle Park, NC, composed of the active ingredient metconazole (Paul et al., 2008; Wise, 2014). All of the active ingredients in these fungicides are sterol biosynthesis inhibitors (SBI) and are members of FRAC group G1: SBI class 1: DMI fungicides (FRAC, 2011). Both products are broad spectrum fungicides and are also used to control foliar diseases (Wise, 2014). The DMI triazole fungicides inhibit sterol biosynthesis in fungal membranes by inhibiting the enzyme C14-demethylase from acting on C-14 in lanosterol. This is a necessary step in the biosynthesis of ergosterol, and it leads to the buildup of fatty acids and sterol ergosterol precursors in the fungal cells, concluding in abnormal growth patterns and inhibition of fungal growth (Köller, 1992; Schnabel and Jones, 2001; Siegel, 1981). The DMI triazole fungicides are also partially systemic, meaning they can penetrate the plant and move within its tissue, but they are unable to enter the xylem (Mueller and Bradley, 2008; Siegel, 1981).

Proper application timing and techniques are essential for optimum fungicide efficacy. Current recommendations state that fungicide should be applied at early anthesis, or Feekes Growth Stage (FGS) 10.5.1, the time at which 50% of the primary tillers in a field have 50% of their anthers extruding (Large, 1954). In winter wheat there are several limitations to meeting this optimal application timing. Winter wheat produces tillers that flower in stages, beginning with the primary tiller and proceeding to secondary tillers. Because of this, the flowering period of a single plant can extend up to two weeks. Therefore, spraying fungicide at beginning anthesis will likely not coincide with beginning anthesis for the secondary tillers. Since *F. graminearum* is strongly influenced by environmental factors such as weather, initial infection can occur at any point during this two-week anthesis period, provided environmental conditions are suitable and primary inoculum is present (Sutton, 1982). Rain can also pose an obstacle to spraying at precisely FGS 10.5.1 due to the inability of spray equipment to enter a field under wet conditions. Several studies have also determined that the optimum application timing for FHB suppression and DON reduction may be different (Yoshida et al., 2012; Yoshida and Nakajima, 2010). Finally, it is important that fungicide sprays do not interfere with harvest. Every fungicide has a pre-harvest interval, dictating the amount of time that must pass between the final fungicide application and harvest. If fungicide is applied after the optimum timing and weather conditions favor a prompt harvest, it is possible that the 30 day pre-harvest interval for Prosaro will not be met.

Several studies have demonstrated that fungicide applications can reduce FHB and DON levels when applied up to 6 days past FGS 10.5.1 and that DON may be reduced by applications made up to 20 days after anthesis (DAA) (D'Angelo et al., 2014;

Hart, 1984; Yoshida et al., 2012). However, all of these studies have focused on the effect of post-anthesis fungicide applications when inoculum became available at FGS 10.5.1, not when fungicide applications coincided with inoculum availability. Post-anthesis fungicide applications may be effective because they target secondary tillers, which have been shown to lag in developmental growth stage when compared to the primary tillers. Therefore, post-anthesis applications for primary tillers would actually coincide with beginning anthesis for these secondary tillers (D'Angelo et al., 2014; Powers and Alessi, 1978). However, this relationship has yet to be quantified experimentally.

The objectives of this study were 1) to determine the impact of fungicide timing, in conjunction with initial infection by *F. graminearum*, on FHB and DON and 2) determine the range of growth stages across tillers during anthesis, and assess the growth stage at which fungicide is most effective at reducing disease incidence.

Based on previous studies, we predicted that all fungicide applications would provide a similar amount of disease control and that applications made near the end of anthesis would provide the greatest control of DON (Yoshida et al. 2012; Yoshida and Nakajima, 2010). It was also predicted that several tillers would still be entering anthesis by the end of the experiment, and we hypothesized that tillers at FGS 10.5.1 would benefit most from the fungicide application since anthesis is the time at which wheat heads are most susceptible to the pathogen (Sutton, 1982).

2.2. Materials and Methods

Field studies were conducted in two field seasons, 2012-2013 and 2013-2014, at Purdue's Agronomy Center for Research and Education (ACRE) in West Lafayette, Indiana.

2.2.1. 2013 Field experiment

Plots were established on October 12, 2012 with soft red winter wheat variety P25R47, a moderately FHB susceptible wheat variety. Seed was drilled into soil that had been disked and field cultivated at a seeding rate of 5.0×10^6 seeds/ha using a Great Plains drill. The previous crop was corn. Fertilizer in the form of diammonium phosphate (DAP) was applied at 100.8 kg/ha on September 19, 2012, followed by an application of potash at 336 kg/ha on September 25 and an application of urea at 224 kg/ha on March 21, 2013. Weeds were controlled by hand prior to anthesis.

The experimental design consisted of a random complete block design with a 2 x 7 factorial arrangement of 14 plots. Factorial level one refers to the treatment type: presence or absence of fungicide given an inoculation with *F. graminearum*. Level two refers to the day of treatment application relative to the beginning of anthesis (FGS 10.5.1). Anthesis was defined as the first day that 50% of the primary tillers across the field were extruding 50% of their anthers. The application occurring at anthesis was given a designation of day 0. Applications occurring after anthesis were designated as the number of days after anthesis (DAA), with six applications occurring at 1, 3, 5, 7, 9, and 11 DAA (Table 2.1). In 2013, anthesis occurred on May 25. Each plot was designated as an experimental unit with the inoculated, no fungicide plots serving as controls within each application time. Each treatment was replicated four times within the experiment. ARM 8.5.0 (Gylling Data Management, Inc. 2012) was used to randomly assign treatments to plots within each replication.

Experimental plots were 2.1 m wide and approximately 6.1 m long with a 1.5 m wide alley between each plot. Border plots of the same size were established between

experimental plots to prevent the effects of inoculum and/or fungicide drift during treatment applications. Border plots were planted with variety INW0803 at a seeding rate of 3.4 x 10^6 seeds/ha.

2.2.2. 2014 Field experiment

Plots were established on October 15, 2013 with soft red winter wheat cultivar P25R47 at a seeding rate of 3.4 x 10^6 seeds/ha using a Great Plains Drill. The previous crop was corn. The field was disked three times prior to planting, and once the day after planting. Fertilizer, in the form of DAP (at a rate of 100.8 kg/ha) and nitrogen (at a rate of 107.3 kg/ha), was applied on September 2, 2013 and March 28, 2014 respectively. Weeds were controlled by hand prior to anthesis. Due to a harsh winter that lead to winter kill of wheat plants, the healthiest 21 plots in each replication, from a total of 30, were selected for use in the experiment prior to treatment randomization.

The experimental design in 2014 consisted of a randomized complete block design with a 3 x 7 factorial arrangement of 21 plots. Factorial level one refers to the treatment type: inoculum and fungicide (inoculum, fungicide), inoculum and no fungicide (inoculum, non-fungicide), or no inoculum and no fungicide (naturally infected, non-fungicide). The additional treatment level added in 2014 served as a means of evaluating the baseline level of disease in naturally infected plots alongside inoculated plots. Factorial level two referred to the treatment application time relative to the beginning of anthesis (FGS 10.5.1). Treatments occurred on 1, 3, 5, 8, 9, and 11 days after anthesis (DAA; Table 2.2). In 2014, anthesis occurred on May 28. Treatment applications scheduled for 7 DAA were moved to 8 DAA due to rain. ARM 9.1.5 (Gylling Data

Management, Inc. 2014) was used to randomly assign treatments to experimental plots within each replication.

Experimental plots were 2.1 m wide and approximately 6.1 m long with a 1.5 m wide alley between each plot. Border plots of the same size were established between experimental plots to prevent the effects of inoculum and/or fungicide drift during treatment applications. Border plots were planted with variety INW0803 at a seeding rate of 3.4 x 10^6 seeds/ha.

2.2.3. Tiller growth stages throughout anthesis

In 2014, an experiment to determine the effect of tiller growth stage on disease development was established within the previously described field experiment. Three wheat plants per plot were arbitrarily selected on each designated day of treatment application (Table 2.2). Care was taken to avoid wheat plants near the borders of the plots and to choose plants in several areas of the plot (northern end, middle, and southern end). The primary tiller of each plant was tagged with a piece of colored tape and its growth stage was determined according to Feekes Growth Stages. Then, counting in a clockwise direction, each additional tiller on the wheat plant was counted and growth staged. If the growth stage of any tiller was not yet at FGS 8.0 (flag leaf), the tiller was counted, but the growth stage was not recorded.

2.2.4. Inocula preparation

Macroconidia inocula of *F. graminearum* was prepared in the laboratory prior to field inoculation. A mix of isolates collected in Indiana were used each year to simulate natural disease conditions. In 2013 the isolates 09INDecaturF3S1, 09INDecaturF1S1, and 10INSWS2U112 were used. In 2014, isolates 09DecaturF3S1, 10INSWS2U112 and

13INHunt600NPH5 were used. Each isolate was screened for virulence on wheat in a greenhouse prior to being selected. Isolates were grown on full strength potato dextrose agar (PDA) amended with ampicillin at 0.05mg/mL. After approximately 1-2 weeks, a single plug of each isolate was transferred into separate Erlenmeyer flasks containing sterile mung bean broth according to the protocol described by Bai and Shaner (1996), with the following alterations: Beans were added to water (at 95 to 99°C) and left to soak for 10 minutes before the broth was divided into 200mL flasks with 100 to 150mL of broth per flask. Flasks were plugged with pieces of cotton wrapped in cheesecloth and covered with aluminum foil before autoclaving. Inoculated flasks of broth were placed on a VWR shaker plate (model 15000-1, VWR Scientific, Randor, PA) until the concentration of the macroconidia was greater than 50,000 conidia/ mL (~3 weeks). Macroconidia were enumerated using a hemacytometer. The final inocula solution was created by combining equal parts (by spore count) of broth from each isolate before being diluted to 50,000 spores/mL with water. Inocula was kept at 4.4°C until use.

2.2.5. Inocula application

In 2013, inoculum was applied to experimental plots using a handheld 1.5 m wide boom mounted with four Teejet 8002 nozzles spaced 48 cm apart, powered by compressed CO_2. The boom was calibrated to deliver 190 L/ha, at 276 kPa to a total volume of 300 mL/ plot. In 2014, the handheld boom was fitted with Teejet 8001 nozzles, with all other factors consistent with 2013 applications. Plots were inoculated in early evening on each treatment day.

2.2.6. Fungicide application

The DMI triazole fungicide Prosaro (Bayer CropScience) was the only fungicide tested in this study. It was applied at the recommended rate of 475 mL/ha (Bayer CropScience). Preference non-ionic surfactant and anti-foaming agent (AgriSolution, LLC) was included in the application at 0.125% v/v to improve fungicide coverage. Fungicide was applied using a backpack sprayer and spray boom with four Teejet 8001 nozzles spaced 48 cm apart. The boom was powered by compressed CO_2 set at 276 kPa and was held approximately 25 cm above the plot during application. Fungicides were applied in the morning on each treatment day.

2.2.7. Disease assessment

FHB incidence and index were assessed on June 14 in 2013, and on June 18 in 2014, corresponding to FGS 11.1, 10 days after the last treatment application. Assessments were made on 4 arbitrary handfuls of 25 tillers from each plot for a total of 100 tillers. FHB incidence was determined by counting the number of heads with FHB symptoms in each group of tillers. Disease index, also known as disease severity, as defined by Paul et al. (2005), was determined using a visual rating scale to assessing the percentage of total head area with FHB symptoms across the 25 tillers in each sample (Stack and McMullen, 2011). In 2013, foliar diseases such as leaf rust, (*Puccinia triticina*), Septoria leaf blotch, (*Septoria tritici*), and Stagonospora leaf blotch (*Stagonospora nodorum*) incidence and index were assessed on the flag leaves of the same 100 wheat tillers per plot using visual rating scales from Severity Pro (Iowa State University, Ames, IA). Foliar diseases were not as prevalent in 2014 and therefore were

not rated, although traces of leaf rust (*Puccinia triticina*) and glume blotch (*Stagonospora nodorum*) were noted.

In the tiller study, disease severity of individual wheat heads was visually rated 10 days after the last treatment (June 18, 2014) for each of the marked plants. Tillers were rated in a clockwise manner, beginning with the primary tiller, so that they would correspond to the order in which the tillers were initially growth staged.

2.2.8. Post-harvest assessments

The middle 1.75 m of each plot was harvested on July 9, and July 16, in 2013, and 2014 respectively, with a small plot Kincaid 8XP combine. This coincided with 36 and 31 days after the 11 DAA treatments, thereby meeting Prosaro's 30 day pre-harvest interval. Percent kernel moisture, test weight, and yield were obtained for each plot and used to calculate the adjusted yield (kg/ha). Adjusted yield (kg/ha) is used to equate a harvest weight to a market standard, taking moisture content into account. The market standard for wheat is 769 kg/m^3 at 13.5 % moisture for wheat (Hellevang 1995). In addition to yield, an arbitrary sample of approximately 2.3 kg of harvested grain was sampled from each plot. Post-harvest analyses were performed on subsamples of this grain. A Key-mat Model 946 Seed Counter (Key-mat Equipment Company Inc., St. Charles, IL), adjusted for wheat kernel size, was used to enumerate 1000 kernels that were subsequently weighed to obtain the 1000 kernel weight. Percent Fusarium damaged kernels (FDK), was visually assessed for each plot using a percentage based visual scale created by Jones and Mirocha (1999).

Grain samples were prepared for deoxynivalenol (DON) analysis by grinding approximately 4 L of kernels from each respective plot into a rough powder using a

Romer Series II Mill (Romer Labs, Inc., Union, MO). A 20 g subsample of this wheat-meal was then used for DON assay. The mill was thoroughly vacuumed out between each sample to avoid cross- contamination. DON analysis was performed using DON3 QuickTox kits (EnviroLogix, Portland, ME) catalog number AQ 204 BG in 2013 and a DON3 QuickTox kit, catalog number AQ 254 BG in 2014. The DON3 kit used in 2013 had a detection limit of 5.0ppm, while the 2014 kit had a detection limit of 12.0ppm. Analyses were performed according to the instructions provided in the kit and DON levels were obtained for each sample unit using the QuickScan (Environlogix, Portland, ME) system. In 2013, if DON levels exceeded 5.0 ppm, the sample was re-tested using another 20g subsample. Samples were diluted 2-fold after the extraction step and buffer was added according to kit instructions. The resulting DON value was doubled to attain the final measurement. In 2014, no dilutions were required.

2.2.9. Statistical analysis

Due to differences in experimental design by year, trials were analyzed separately. All data analyses were performed were performed using the PROC MIXED procedure of SAS 9.3 (SAS Institute Inc., Cary, NC). Data from all four replications per year were averaged prior to analysis. Since FHB index is a factor of both FHB incidence and FHB severity and is typically the unit used to quantify FHB, only FHB index is reported. FHB index was arcsine-square root transformed to attain homogeneity of variance. A Box-Cox regression analysis on the post-harvest data (DON, FDK, and 1000 kernel weight) indicated that a log transform was appropriate to use on the 2013 and 2014 FDK values in order to achieve homogeneity of variance.

All dependent variables (FHB index, FDK, 1000 kernel weight, DON and adjusted yield, hereafter referred to as 'yield') were subjected to analysis of variance (ANOVA) to test for significant differences between fungicide treatment levels (fungicide, control, and check) and application timing. The interaction between treatment level and application timing was also tested. Least squares means (LSM) tests were performed for significant variables using PROC MIXED with a Kenward-Roger correction for adjusting degrees of freedom and estimation of random effects. Treatment, application timing, and their interaction were treated as fixed effects and replication was treated as the random effect. A separate residual variance was estimated for each treatment level. A Tukey-Kramer adjustment was used in the comparisons of Least-squares means. Fixed effects were said to be significant if $P > 0.05$.

Pearson's correlation tests were run on the untransformed values of dependent variable as deemed appropriate based upon the results of the ANOVA.

Fungicide efficacy (inoculum, fungicide versus inoculum, no fungicide) was calculated using the formula $((Q-R)/Q) \times 100$ where Q represents the back-transformed means estimate value of the inoculum, no fungicide control, and R represents the back-transformed means estimate value for INOCULUM, FUNGICIDE treatments within a given application time (Yoshida et al. 2012). All values are recorded as back transformed mean estimates.

Tillers on separate wheat plants within a plot were grouped and replications were combined for further analysis. Histograms were generated in SAS to determine the relative frequency of tillers at each growth stage (FGS 8-11) over the course of application timings. The frequency of FHB incidence within growth stage groups was

then graphically analyzed for inoculum, no fungicide and inoculum, fungicide treated

plots, disregarding application time, to test for the effect of growth stage on susceptibility

to inoculum.

2.3. Results

The scale of replication effect relative to other random effects was non-significant

in both years of the trials, and therefore the effect of block is not included in further

discussion. Weather in 2013 was more conducive to disease than in 2014 (Figures 2.1 and

2.2). In 2013, temperatures remained in the ideal range for *F. graminearum* infection (20-

25°C) for the majority of anthesis. Temperatures did not reach 30°C during the

experiment, and there were six rain events for a total accumulation of 72.38 mm.

Although rain did occur during anthesis, 2014 was much warmer and drier than 2013.

Total precipitation reached only 6.6 mm (excluding June 5 where precipitation level

information is missing) over the course of this trial, only three days had temperatures that

fell within the ideal temperature range for infection by *F. graminearum*. Seven of the 12

days of anthesis experienced temperatures over 30°C.

2.3.1. Effect of fungicide treatment and inoculum application timing on FHB Index

In 2013, there was no significant interaction between fungicide treatment and

inoculum application time on FHB Index ($P = 0.415$; Table 2.3). Both fungicide

treatment and inoculum application time (the time *F. graminearum* inoculum became

available to wheat heads) significantly affected FHB Index ($P = 0.012$ and $P = 0.028$

respectively). Inoculum applied 9 days after anthesis (DAA) resulted in significantly less

($P = 0.034$) disease severity than inoculum applied at 0 DAA. FHB Index among all other

timings were statistically similar. Fungicide application reduced FHB index numerically

at all application timings with the exception of 11 DAA where FHB Index was higher in the inoculated, non-fungicide treated plots than the inoculated, fungicide-treated plots (Figure 2.3). Percent disease control ranged from -32% at 11 DAA to 41.8% at 3 DAA, although neither value represents a statistically significant difference within an inoculum application time (Table 2.4).

In 2014, there was a significant interaction between fungicide treatment and inoculum application timing for FHB Index ($P = 0.003$). Therefore, differences in LSMs were evaluated to determine the effect of fungicide treatment within inoculum application time and inoculum application time within fungicide treatment. At anthesis, FHB Index in inoculated, non-fungicide treated plots was significantly higher than in naturally infected, non-fungicide treated plots ($P = 0.0011$). No other significant differences were observed for FHB Index among fungicide treatment. When inoculum application time was evaluated within inoculated, non-fungicide treated plots, inoculum applied at anthesis resulted in significantly higher FHB Index than all other application timings with the exception of 3 DAA ($P < 0.05$). No significant differences in FHB Index were observed among inoculum application times for inoculated, fungicide-treated plots or for naturally infected, non-fungicide treated plots.

2.3.2. Effect of fungicide treatment and inoculum application time on deoxynivalenol

High levels of DON were observed in all plots in 2013, with mean values ranging from 3.5 to 4.5 ppm in inoculated, fungicide-treated plots and from 5.1 to 7.0 ppm in inoculated, non-fungicide treated plots. There was no significant interaction between fungicide treatment and inoculum application time on DON ($P = 0.510$).

Fungicide treatment significantly reduced DON regardless of inoculum application timing ($P < 0.0001$) up to 11 DAA. DON levels were numerically reduced at every inoculum application time in inoculated, fungicide-treated plots compared to inoculated, non-fungicide treated plots, and significantly reduced at 3 DAA ($P = 0.0003$), 7 DAA ($P = 0.0094$), and 9 DAA ($P = 0.0232$). A spike in DON levels occurred at 3 DAA in the inoculated, non-fungicide treated plots but not in the inoculated, fungicide-treated plots. This increase coincided with a heavy rain event within one day of inoculation.

In 2014, the range of mean DON levels was smaller in the inoculated, fungicide-treated plots compared to the inoculated, non-fungicide treated plots (2.1 to 4.8 ppm and 3.4 to 6.5 ppm respectively). In the naturally infected, non-fungicide treated plots, mean DON levels ranged from 3.5 to 4.3 ppm. There was a significant interaction between inoculum application time and fungicide treatment in 2014 ($P = 0.002$). Differences in LSM within inoculum application time indicated that there were significantly higher levels of DON in inoculated, non-fungicide treated plots than in the naturally infected, non-fungicide plots at anthesis ($P = 0.0013$). Also, at 5 DAA, fungicide significantly reduced DON in inoculated, fungicide treated plots by 48.3% compared to inoculated, non-fungicide treated plots ($P = 0.0122$; Table 2.5). No other statistically significant differences were observed for DON within inoculum application timings. However, DON levels were numerically reduced at every inoculum application timing in the inoculated, fungicide treated plots relative to inoculated, non-fungicide treated plots.

Within inoculated, non-fungicide treated plots, DON levels were significantly higher when inoculum was applied at anthesis compared to 11 DAA ($P = 0.0097$), but

statistically similar levels of DON were observed among all other inocula application timings. Within inoculated, fungicide-treated plots, those inoculated at anthesis developed higher levels of DON compared to those inoculated at 9 and 11 DAA (P = 0.0175 and 0.0032 respectively) and plots inoculated at 1 DAA had significantly higher DON levels than those inoculated at 11 DAA (P = 0.323). Two spikes in DON levels were observed within the inoculated, non-fungicide treated plots, one at anthesis and another at 5 DAA (Figure 2.4). Both of these spikes corresponded to rain events within one day of the plots being inoculated. This same spike was not observed in the inoculated, fungicide-treated plots.

2.3.3. Effect of fungicide treatment and inoculum application time on FDK

Fungicide treatment was the only fixed effect that significantly affected FDK in 2013 (P = 0.0002). Fungicide provided the greatest percent control of FDK at 7 DAA (63.6%) where mean FDK was reduced from 15.9% to 5.8% (Figure 2.5). However, fungicide did not significantly reduce FDK within a given inocula application time. Statistically similar levels of FDK developed when plants were inoculated from anthesis to 11 DAA.

In 2014, fungicide treatment had a significant effect on FDK (P = 0.0002; Figure 2.6). Differences in LSM demonstrated that this effect is primarily due to a significant difference between inoculated, non-fungicide treated and inoculated, fungicide-treated plots, and between inoculated, non-fungicide treated and naturally infected, non-fungicide treated plots (P = 0.0007 and 0.001 respectively). Inoculum application timing did not have a significant effect on FDK.

2.3.4. Effect of fungicide treatment and inoculum application time on 1000 kernel weight

Neither the interaction between inoculum application time and fungicide treatment nor inoculum application time was significant on 1000 kernel weight in 2013 ($P > 0.05$). Fungicide treatment had a significant effect ($P < 0.0004$) on 1000 kernel weight. Seed weight in inoculum, fungicide plots was numerically increased at every inoculum application time compared to inoculated, non-fungicide treated plots.

In 2014, a significant interaction between inoculum application time and fungicide treatment was observed ($P = 0.019$). However, this interaction is largely explained by the significant difference between inoculated, non-fungicide treated plots and both inoculated, fungicide-treated and naturally infected, non-fungicide plots at beginning anthesis (0 DAA; $P = 0.022$ and 0.064 respectively). Across all inoculum application timings, 1000 kernel weight of inoculated, fungicide-treated plots was significantly different from inoculated, non-fungicide treated plots ($P < 0.0001$). Additionally, 1000 kernel weight of inoculated, fungicide-treated plots was significantly different from naturally infected, non-fungicide treated plots ($P = 0.0004$), and 1000 kernel weight of inoculated, non-fungicide treated plots was significantly different from that of naturally infected, non-fungicide treated plots ($P = 0.0106$). At every inoculum application time, fungicide application numerically increased the 1000 kernel weight compared to the inoculated, non-fungicide treated plots.

2.3.5. Effect of fungicide treatment and inoculum application time on yield

No significant interaction was observed between fungicide and inoculum application timing for yield in 2013 or in 2014. In both years, only fungicide treatment had a significant effect on yield ($P < 0.0001$ and 0.0003 respectively). Inoculum

application did not have a significant effect on yield in either year, and statistically similar yields were observed when plots were inoculated from anthesis to 11 DAA. In 2013, fungicide application significantly increased yield at 1, 3, and 5 DAA compared to yield in inoculated, non-fungicide treated plots treated on those same days. In 2014, yield was numerically increased in inoculated, fungicide-treated plots at every application time in both years compared to both inoculated, non-fungicide treated plots, and to naturally infected, non-fungicide treated plots. The highest mean yield was found in fungicide treated plots in 5 DAA in both years (6,109.8 kg ha^{-1} and 5,838.0 kg ha^{-1} respectively). Foliar disease was only measured in 2013. Fungicide treatment had a significant effect on foliar index levels ($P < 0.0001$; data not shown). There was no significant interaction between *F. graminearum* inoculation application time and fungicide treatment on foliar disease severity, nor was inoculum application time significant at $P < 0.05$.

2.3.6. Correlations

In 2013, DON was not significantly associated with either FHB Index or FDK in inoculated, non-fungicide treated plots across all inoculum application timings (Table 2.6). However, in inoculated, fungicide-treated plots, DON was positively associated with FHB Index at $P = 0.0165$. However, the *r*-value was relatively weak at 0.4492. In 2014, DON was positively associated with both FHB Index and FDK ($P = 0.0032$ and 0.0003 respectively; Table 2.7). The association between FDK and DON was stronger than the association between FHB Index and DON ($r = 0.6338$ versus 0.5267). In inoculated, fungicide-treated plots, no association was observed between FHB Index and DON, but FDK and DON were positively associated at $P = 0.0021$.

2.3.7. Range of tiller growth stages and susceptibility to FHB throughout anthesis

On the first designated day of anthesis (FGS 10.5.1), in 2014, 15.97% of the tillers across all plots were still at FGS 8.0. Only 26.05% of the tillers had reached beginning anthesis (FGS 10.5.1; Figure 2.7). By 3 DAA, 60.17% of plot tillers had reached or were past FGS 10.5.1. At the last application time (11 DAA), 66.15% of tillers had completed anthesis and entered FGS 11. However, 5.39% of tillers had still not yet reached FGS 10.5.1, and 3.08% were at FGS 10.5.1. Tillers were observed to be undergoing anthesis (FGS 10.5.1-10.5.3) within any given plot up to 11 days past the initial designation of FGS 10.5.1 within the field.

To assess at what growth stage fungicide was most effective at reducing FHB, the frequency of wheat heads that developed FHB (reported as FHB incidence) were compared (Figure 2.8). Tillers were grouped by fungicide treatment and only those that received inoculum were evaluated. The greatest FHB incidence was observed on tillers that were inoculated at FGS 10.5 and did not receive a fungicide treatment (37%, n = 30). The greatest difference in FHB incidence between inoculated, non-fungicide treated tillers and inoculated, fungicide treated tillers occurred at FGS 10.5. Tillers receiving a fungicide treatment (inoculum and fungicide) at all growth stages except FGS 8, 10.4, and 10.5.3 had numerically lower FHB incidence than inoculated, non-fungicide treated tillers. Overall, tillers inoculated just prior to, or during, anthesis had higher FHB incidence than tillers inoculated before the wheat head had fully emerged from the sheath, regardless of fungicide treatment.

2.4. Discussion

The results of this study confirm that winter wheat is susceptible to infection by *F. graminearum* from beginning anthesis (10.5.1) up to 11 DAA. Results also demonstrate that post-anthesis applications of Prosaro can reduce FHB Index, DON, and FDK, and increase yield similarly to fungicide applications at beginning anthesis when inocula is available to infect the plant. These results are consistent with D'Angelo et al. (2014) who found that fungicide applications up to 6 days post-anthesis consistently reduced DON and FDK levels when inoculum was applied at anthesis. This study indicates that post-anthesis fungicides applications are efficacious for yet an additional 5 days beyond what was has previously been described. It is also consistent with work conducted by Del Ponte et al (2007) who found that wheat could incur FHB and develop DON when inoculated as late as FGS 11.3 (hard dough). Current recommendations for fungicide application for suppression of FHB in the Midwest are to apply fungicide when 50% of the primarily tillers are at beginning anthesis in order to protect as many high yielding wheat heads as possible from infection by *F. graminearum*. This is based on research that demonstrated that wheat is most susceptible to infection by *F. graminearum* from FGS 10.5.1 through FGS 11.2 (Andersen 1948). Our results indicate that the recommendation to apply fungicides precisely at beginning of anthesis could be modified, particularly when it coincides with initial inoculum becoming available to the wheat plant. This means that growers, who often have a difficult time spraying wheat at beginning anthesis due to uneven flowering across a field and heavy rain events near flowering, may have more flexibility in timing an effective fungicide spray than originally believed.

This is also the first study to formally evaluate the range of growth stages present within a winter wheat field during anthesis and further elucidates how the timeframe of initial infection by *F. graminearum* can vary within a plant and field given the restricted growth stages of susceptibility for an individual head. Our data supports the hypothesis that there is a relatively wide timeframe of both fungicide efficacy and susceptibility to infection in winter wheat heads near anthesis due to uneven growth stages across a field and among tillers on a single plant. Secondary tillers have the potential to contribute greatly to yield (between 40 and 62%) and therefore it is desirable to have plants with strong tiller development (Powers and Alessi, 1978). However, in spring wheat and in barley, secondary tillers have been shown to be equally susceptible to infection by *F. graminearum* as primary tillers (McCallum and Tekauz, 2002). In a study conducted in North Dakota on spring wheat, the growth stage of secondary tillers tended to lag several days behind that of the main tiller (Powers and Alessi, 1978). This is consistent with our findings in winter wheat. Several tillers in this study had not yet reached FGS 10.5.1 up to 11 DAA, demonstrating that anthesis can last at least 11 days. However, this timeframe is likely dependent upon other factors that contribute to tiller development such as weather, cultivar, fertilizer applications, and planting density (D'Angelo, 2014; Powers and Alessi, 1978). When the growth stages of both primary and secondary tillers were evaluated, approximately 25% of the tillers were at or beyond FGS 10.5.1 at the time that 50% of the primary tillers were visually determined to be at anthesis (FGS 10.5.1). It was not until 3 DAA that over 50% of the tillers in an experimental plot reached or were past FGS 10.5.1.

Tillers that were between FGS 10.5 and FGS 11 at the time of inoculation were most susceptible to infection by *F. graminearum* according to final FHB incidence estimates, which is consistent with previous research (Andersen 1948; Sutton 1982). Several tillers inoculated prior to FGS 10.5 ultimately developed disease, but this is likely attributed to natural infection once the tillers reached anthesis. Tillers that were inoculated at FGS 10.5 and did not receive a fungicide treatment had the highest percent FHB incidence relative to inoculated non-fungicide treated tillers at all other growth stages.

Since FGS 10.5.1 is determined based on the time at which half of the primary tillers across a given area are at beginning anthesis, but does not explicitly examine secondary tillers, the protection offered by fungicide application post-anthesis in our study was likely provided to secondary tillers. Post-anthesis applications likely also had some post-infection activity on primary tillers that were infected during anthesis. Although fungicide application was able to reduce DON levels in our experiment, mean DON levels in all treatments across both years exceeded 2 ppm. This is the level at which growers typically begin to experience price dockages when selling grain. DON levels exceeded 2 ppm even when weather conditions did not favor disease development and wheat was treated with fungicide up to 11 DAA. In the U.S., DON levels in finished grain products are regulated by the Food and Drug Administration (FDA) because the mycotoxin is toxic to human and animal consumption (U.S. Food and Drug Administration, 2013). Therefore, it is important for both the safety of humans and animals, and the economy of wheat farming, that we continue to find ways to improve DON control. A study conducted by Yoshida et al. (2012) in Japan demonstrated that

DON could be reduced by applying thiophanate-methyl at 20 DAA, but this application timing would not be practical in winter wheat due to the relatively short period of time between anthesis and harvest (~30-40 days). Several studies have evaluated the effect of moderately resistant cultivars on DON levels, but no cultivars have yet been developed that are completely resistant to DON accumulation (Bai et al., 2001; Saldago et al., 2014). Therefore, additional strategies besides fungicide and cultivar use are needed to reduce DON levels in years with high levels of *F. graminearum* inocula.

Since complete control of DON is not yet possible, several strategies are employed to predict the impact of FHB on grain quality and attempt to determine final DON levels prior to sale or use of grain. Two visual estimators of FHB severity used are FHB Index and FDK grain quality assessment. In this study, several positive correlations were found between FHB Index, FDK, and DON, but significant associations were not consistent between years. These results are similar to those from a meta-analysis conducted by Paul and Madden (2005) who found that associations between disease variables were higher in years with lower disease intensity. This may be due in part to the differences in timing between DON production and disease symptoms (Hart et al., 1984). DON can continue to accumulate up to 45 days after anthesis in wheat tissue, while FHB symptoms take approximately 5 days to develop and can often not be distinguished from natural senescence due to ripening after approximately 25 DAA.

Since FHB Index was rated 21 DAA in this experiment, it is not surprising that FHB Index is not consistently associated with final DON levels (Andersen, 1948; Cowger and Arellano, 2012). Our results further serve to demonstrate that FHB Index and FDK are not consistently useful estimators of DON levels, and quantitative methods such as

immunostrip DON quantification kits, high performance liquid chromatography (HPLC), or an enzyme-linked immunosorbant assay (ELISA) should be used to assess DON prior to sale of grain.

In both years of this study, DON levels increased in non-fungicide treated plots that were inoculated on the evening prior to a rain event. DON functions as a virulence factor in the colonization of wheat by *F. graminearum*, allowing hyphae to colonize the rachis node and move from spikelet to spikelet (Brown et al., 2011; Jansen et al., 2005). DON has been shown to be most active at the infection front and transcription of DON biosynthesis genes have been detected within 24 hours of initial infection (Hallen-Adams et al., 2011). DON levels have also been shown to increase in wheat tissue up to 45 DAA and to be positively influenced by increased levels of moisture during grain fill up to 30 DAA (Cowger and Arellano, 2013). In a series of models developed by Hooker et al. (2002) to predict DON levels in spring wheat in Ontario, Canada, rain events where precipitation exceeded 3 mm, 3 to 6 DAA was one of the most important predictors for final DON accumulation, and it was the most important predictor 7 to 10 DAA. In this study, plots treated with fungicide did not exhibit increases in DON levels, suggesting that the fungicide may reduce the impact of moisture on DON levels. Since DON is synthesized primarily at hyphal tips, and DMI triazole fungicides function by preventing the normal growth of hyphae through disruption of the ergosterol biosynthetic pathway (Köller, 1992; Siegel, 1981), it is possible that the fungicide disrupts the advancing fungal hyphae and prevents an upsurge in DON production after a rain event.

In this study, fungicide was applied approximately 8 hours prior to inoculation. Therefore, it was primarily investigating the protective effect of

prothioconazole+tebuconazole on infection by *F. graminearum*. DMI triazole fungicides

have been shown to have both pre- and post-infection activity, meaning they function

both to protect the plant from initial infection and to hinder further colonization of plant

tissue after infection begins (Andersen et al., 2014; Ivic, 2010; Mueller and Bradley,

2008; Szkolnik, 1981). Post-infection activity has demonstrated useful activity for 1 to 5

days, and is less likely to be adversely affected by rain events than the protective effect

once the fungicide has been absorbed into the plant tissue (Andersen et al., 2014; Ivic,

2010). The dual action of DMI fungicides is particularly important in extending the time

of fungicide efficacy after a spray due to the fact that infection can occur from FGS

10.5.1 (early anthesis) through FGS 11.2 (soft dough) and the optimal spray time for

managing FHB Index differs from that of DON (Brown, 2010; Yoshida and Nakajima,

2010; Yoshida et al., 2012). Therefore, although this study primarily investigated the

protective effect of fungicide, differences in FHB Index between inoculated, fungicide

treated plots compared to inoculated, non-fungicide treated plots indicate that fungicide

likely also had post-infection activity on wheat heads that had been naturally infected

from 1 to 5 days prior. Additionally, in both years, FHB Index and DON were

numerically decreased, and yield numerically increased, in plots where fungicide was

applied 3 to 7 DAA compared to plots where fungicide was applied at anthesis. This may

indicate that the fungicide was exhibiting some post-infection activity on natural

infection that had occurred at anthesis, as well as protecting the wheat heads that were

susceptible at the time inoculum was applied.

In summary, we found that new options exist for winter wheat growers with

respect to timing fungicide applications to manage FHB and DON. We also demonstrated

the importance of predicting when environmental conditions are conducive for *F. graminearum* spore production and infection near anthesis. With a wide timeframe of fungicide efficacy and wheat head susceptibility, an accurate forecasting system could help growers optimize their fungicide application. Lastly, we discovered that additional methods are still needed to reduce DON levels in wheat. However, further research is necessary to determine if these results are consistent across wheat varieties, fungicides, and environments. Research is also needed to evaluate the contribution of secondary tiller infection to DON levels and to assess whether alternative methods of assessing FHB index levels would lead to stronger associations between FHB Index and DON.

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2.6. Tables and Figures

Table 2.1. Description of application times and treatment factors applied to winter wheat near anthesis for the 2013 field experiment at the Agronomy Center for Research and Education, West Lafayette, IN

Growth Stage	Treatment	Treatment Factors	
		Inoculum[a]	Fungicide[b]
Anthesis	Fungicide	Yes	Prosaro
	Control	Yes	None
Anthesis + 1 day	Fungicide	Yes	Prosaro
	Control	Yes	None
Anthesis + 3 days	Fungicide	Yes	Prosaro
	Control	Yes	None
Anthesis+ 5 days	Fungicide	Yes	Prosaro
	Control	Yes	None
Anthesis + 7 days	Fungicide	Yes	Prosaro
	Control	Yes	None
Anthesis + 9 days	Fungicide	Yes	Prosaro
	Control	Yes	None
Anthesis + 11 days	Fungicide	Yes	Prosaro
	Control	Yes	None

[a.] Inoculum was applied at a total volume of 300mL/plot at 50,000 *Fusarium graminearum* macroconidia/mL.

[b.] Prosaro was applied at the recommended field rate of 475 mL/ha with 0.125% v/v of Preference (AgriSolutions) non-ionic surfactant.

Table 2.2. Description of application times and treatment factors applied to winter wheat near anthesis for the 2014 field experiment at the Agronomy Center for Research and Education, West Lafayette, IN

Growth Stage	Treatment	Treatment Component	
		Inoculum[a]	Fungicide[b]
Anthesis	Check	None	None
	Control	Yes	None
	Fungicide	Yes	Prosaro
Anthesis + 1 day	Check	None	None
	Control	Yes	None
	Fungicide	Yes	Prosaro
Anthesis + 3 days	Check	None	None
	Control	Yes	None
	Fungicide	Yes	Prosaro
Anthesis + 5 days	Check	None	None
	Control	Yes	None
	Fungicide	Yes	Prosaro
Anthesis + 8 days	Check	None	None
	Control	Yes	None
	Fungicide	Yes	Prosaro
Anthesis + 9 days	Check	None	None
	Control	Yes	None
	Fungicide	Yes	Prosaro
Anthesis + 11 days	Check	None	None
	Control	Yes	None
	Fungicide	Yes	Prosaro

[a.] Inoculum was applied at a total volume of 300mL/plot at 50,000 *Fusarium graminearum* macroconidia/mL.
[b.] Prosaro was applied at the recommended field rate of 475 mL/ha with 0.125% v/v of Preference (AgriSolutions) non-ionic surfactant.
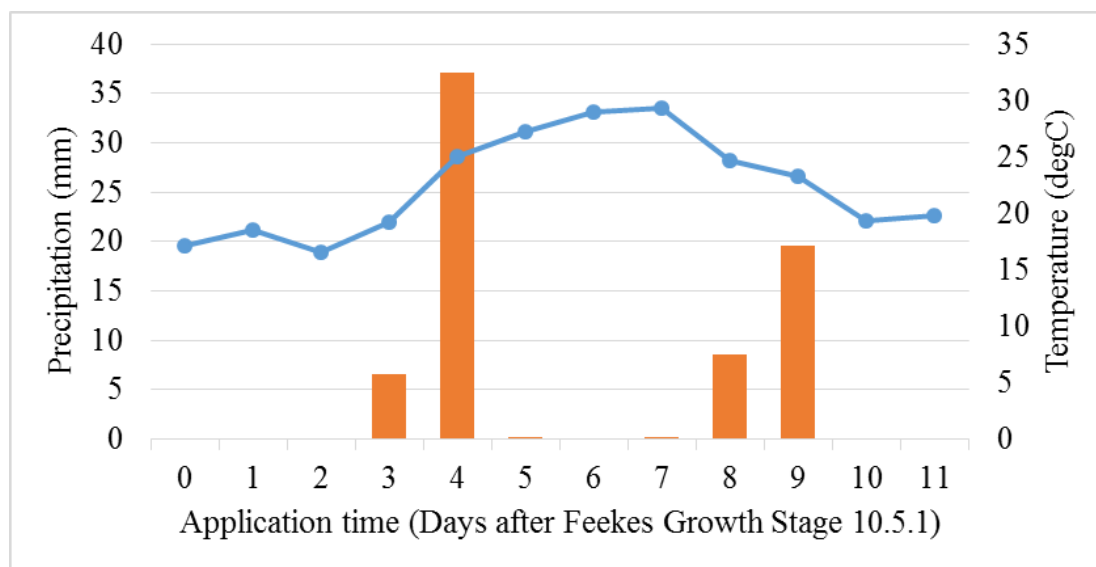
Figure 2.1. Daily maximum temperatures (solid line) and precipitation (bars) during the course of the experiment in 2013. Inoculum application time zero (0) corresponds to May 24, 2013. Weather data was retrieved from the Agronomy Center for Research and Education (ACRE) and Indiana State Climate Office, iClimate.org.
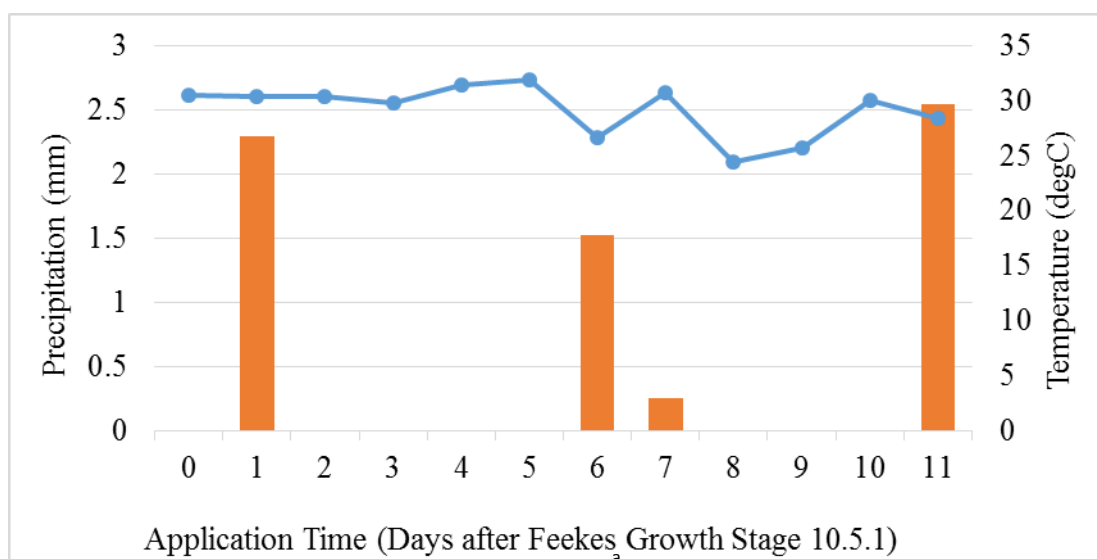

Figure 2.2. Daily maximum temperatures (solid line) and precipitation (bars) during the course of the experiment in 2014. Inoculum application time zero (0) corresponds to May 28, 2014. Weather data retrieved from the Agronomy Center for Research and Education (ACRE) and Indiana State Climate Office, iClimate.org.
[a.] On Application day 8, daily high temperature was retrieved from accuweather.com and precipitation data is missing.

Table 2.3. Results from the two-way analysis of variance on Fusarium head blight Index (FHB Index), deoxynivalenol (DON), yield, Fusarium damaged kernels (FDK), and 1000 kernel weight from the 2013 (A) and 2014 (B) field experiments treating inoculum application time and fungicide treatment as main effects

A)

2013	FHB Index[a]			DON[b]			Yield[c]			FDK[d,f]			1000 Kernel Weight[e]		
	df	*F* value	*P* value	df	*F* value	*P* value	df	*F* value	*P* value	df	*F* value	*P* value	df	*F* value	*P* value
Inoculum timing	6, 34.7	3.26	**0.012**[g]	6, 30.3	0.90	0.510	6, 34.8	1.20	0.329	6, 58.2	1.99	0.082	6, 54.2	0.94	0.476
Fungicide	1, 34.7	5.26	**0.028**	1, 30.3	76.93	**<0.0001**	1, 34.8	67.70	**<0.0001**	2, 37.7	10.93	**0.0002**	2, 39.6	20.79	**<0.0001**
Inoculum timing* Fungicide	6, 34.7	1.04	0.415	6, 30.3	2.15	0.077	6, 34.8	1.14	0.362	12, 56	0.78	0.668	12, 46.5	2.34	0.019*

B)

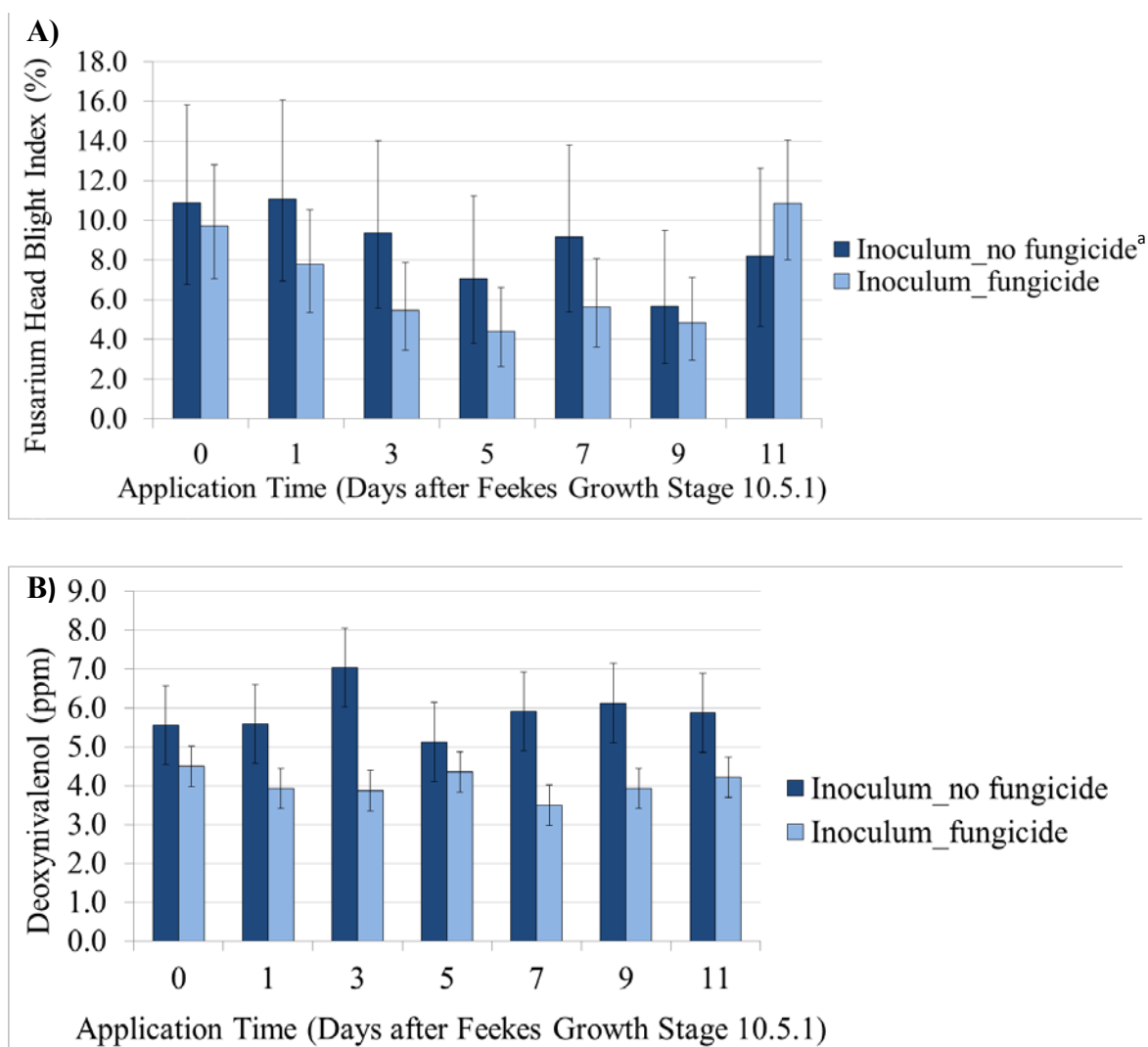| 2014	FHB Index			DON			Yield			FDK			1000 Kernel Weight		
	df	*F* value	*P* value	df	*F* value	*P* value	df	*F* value	*P* value	df	*F* value	*P* value	df	*F* value	*P* value
Inoculum timing	6, 50.4	2.83	**0.019**	6, 52.2	7.34	**<0.0001**	6, 59.9	0.72	0.631	6, 58.2	1.99	0.082	6, 54.2	0.94	0.476
Fungicide	2, 36.4	1.77	0.185	2, 36.3	17.90	**<0.0001**	2, 39.3	10.19	**0.0003**	2, 37.7	10.93	**0.0002**	2, 39.6	20.79	**<0.0001**
Inoculum timing* Fungicide	12, 44	3.04	**0.003**	12, 44.4	3.24	**0.002**	12, 47	0.90	0.557	12, 56	0.78	0.668	12, 46.5	2.34	**0.019**

Table 2.3.

Figure 2.3. Effect of inoculum application time and fungicide treatment on **A)** Fusarium head blight (FHB) Index and **B)** deoxynivalenol (DON, as measured in parts per million) in 2013. Error bars represent the upper and lower limits of the standard error of the mean values based on least squares means estimations.

[a.] Inoculum was applied at a total volume of 300mL/plot at 50,000 *Fusarium graminearum* macroconidia/mL and Prosaro was applied at 475 mL/ha with 0.125% v/v of a non-ionic surfactant.

Table 2.4. Percent reduction of Fusarium head blight Index (FHB Index), deoxynivalenol (DON), and Fusarium damaged kernels (FDK) means in inoculated, fungicide treated plots compared to inoculated, non-fungicide treated plots within each inoculum application time in 2013.

Application Time	Percent Control (%)[a]		
	FHB Index[b]	DON (ppm)[c]	FDK (%)[d]
Anthesis[e]	10.6	19.6	51.2
Anthesis + 1 day	29.9	30.4	9.4
Anthesis + 3 days	41.8	44.3	29.8
Anthesis + 5 days	37.8	13.7	61.4
Anthesis + 7 days	38.6	40.7	63.6
Anthesis + 9 days	14.7	36.1	24.9
Anthesis + 11 days	-32.4	28.8	40.1

[a] Percent control was calculated using the formula $((Q-R)/Q) \times 100$ where Q represents the back-transformed means estimate value of the inoculated, no-fungicide control, and R represents the back-transformed means estimate value for inoculated, fungicide treatments within a given application time (Yoshida et al. 2012).
[b] FHB Index was calculated from visual disease ratings taken 21 days after anthesis.
[c] DON, as measured in parts per million (ppm) was quantified from a post-harvest grain sample.
[d] FDK, the percent of kernels damaged by *F. graminearum*, was visually estimated post-harvest from a 40 mL grain sample from each experimental plot.
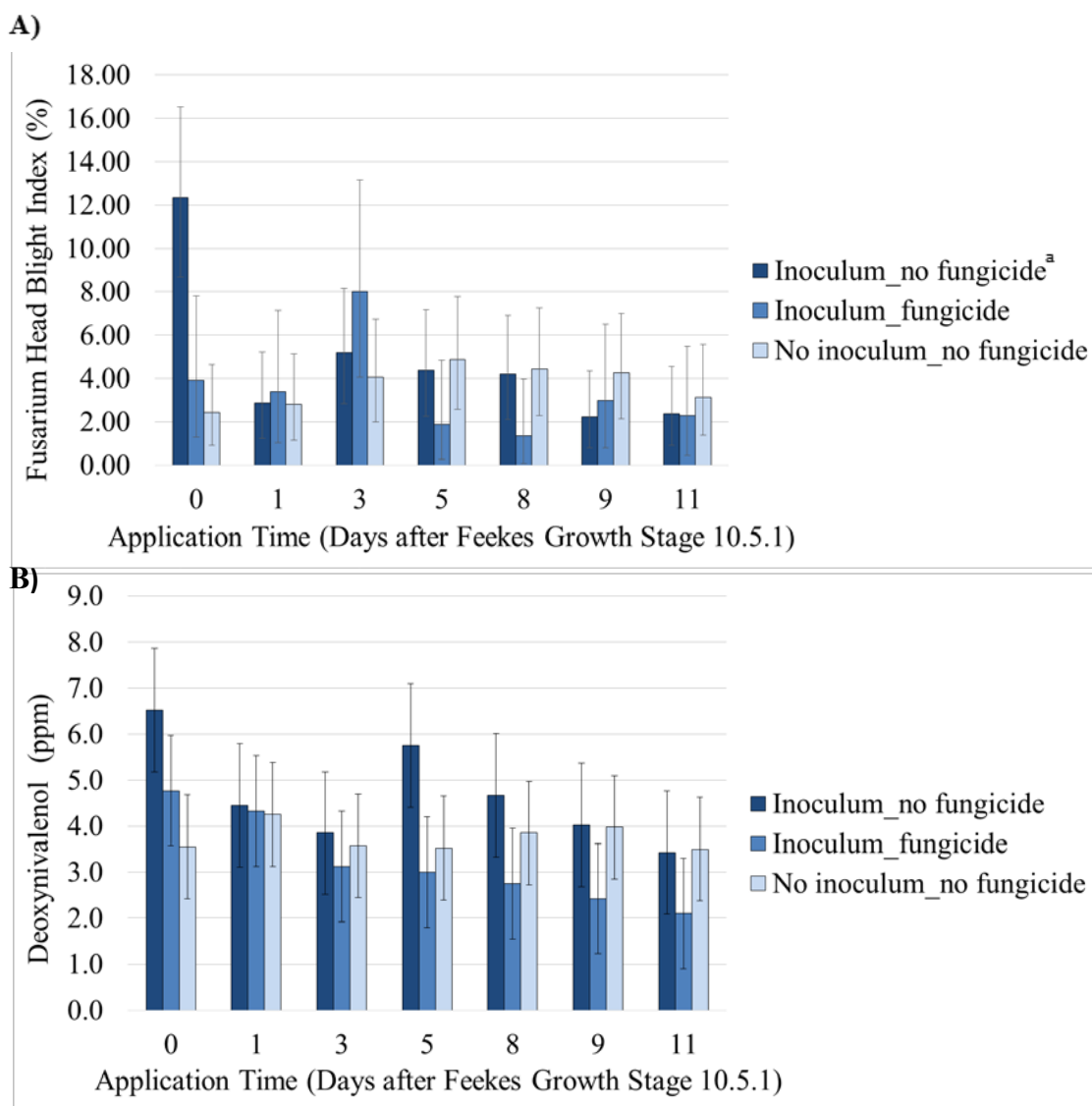[e] Anthesis refers to the Feekes Growth Stage 10.5.1

Figure 2.4. Effect of inoculum application time and fungicide treatment on **A)** Fusarium head blight (FHB) Index and **B)** deoxynivalenol (DON, as measured in parts per million) in 2014. Error bars represent the upper and lower limits of the standard error of the mean values based on least squares means estimations.

[a.] Inoculum was applied at a total volume of 300mL/plot at 50,000 *Fusarium graminearum* macroconidia/mL and Prosaro was applied at 475 mL/ha with 0.125% v/v of a non-ionic surfactant.

Table 2.5. Percent reduction of Fusarium head blight Index (FHB Index), deoxynivalenol (DON), and Fusarium damaged kernels (FDK) means in inoculated, fungicide treated plots compared to inoculated, non-fungicide treated plots within each inoculum application time in 2014.

Application Time	Percent Control (%)[a.]		
	FHB Index [b.]	DON (ppm) [c.]	FDK (%) [d.]
Anthesis[e.]	68.3	26.2	43.6
Anthesis + 1 days	-17.2	4.4	20.7
Anthesis + 3 days	-53.8	20.5	33.7
Anthesis + 5 days	56.8	48.3	53.1
Anthesis + 8 days	69.0	40.4	64.7
Anthesis + 9 days	-36.4	40.0	69.3
Anthesis + 11 days	4.2	38.2	66.9

[a.] Percent control was calculated using the formula $((Q-R)/Q) \times 100$ where Q represents the back-transformed means estimate value of the inoculated, no-fungicide control, and R represents the back-transformed means estimate value for inoculated, fungicide treatments within a given application time (Yoshida et al. 2012).
[b.] FHB Index was calculated from visual disease ratings taken 21 days after anthesis.
[c.] DON, as measured in parts per million (ppm) was quantified from a post-harvest grain sample.
[d.] FDK, the percent of kernels damaged by *F. graminearum*, was visually estimated post-harvest from a 40 mL grain sample from each experimental plot.
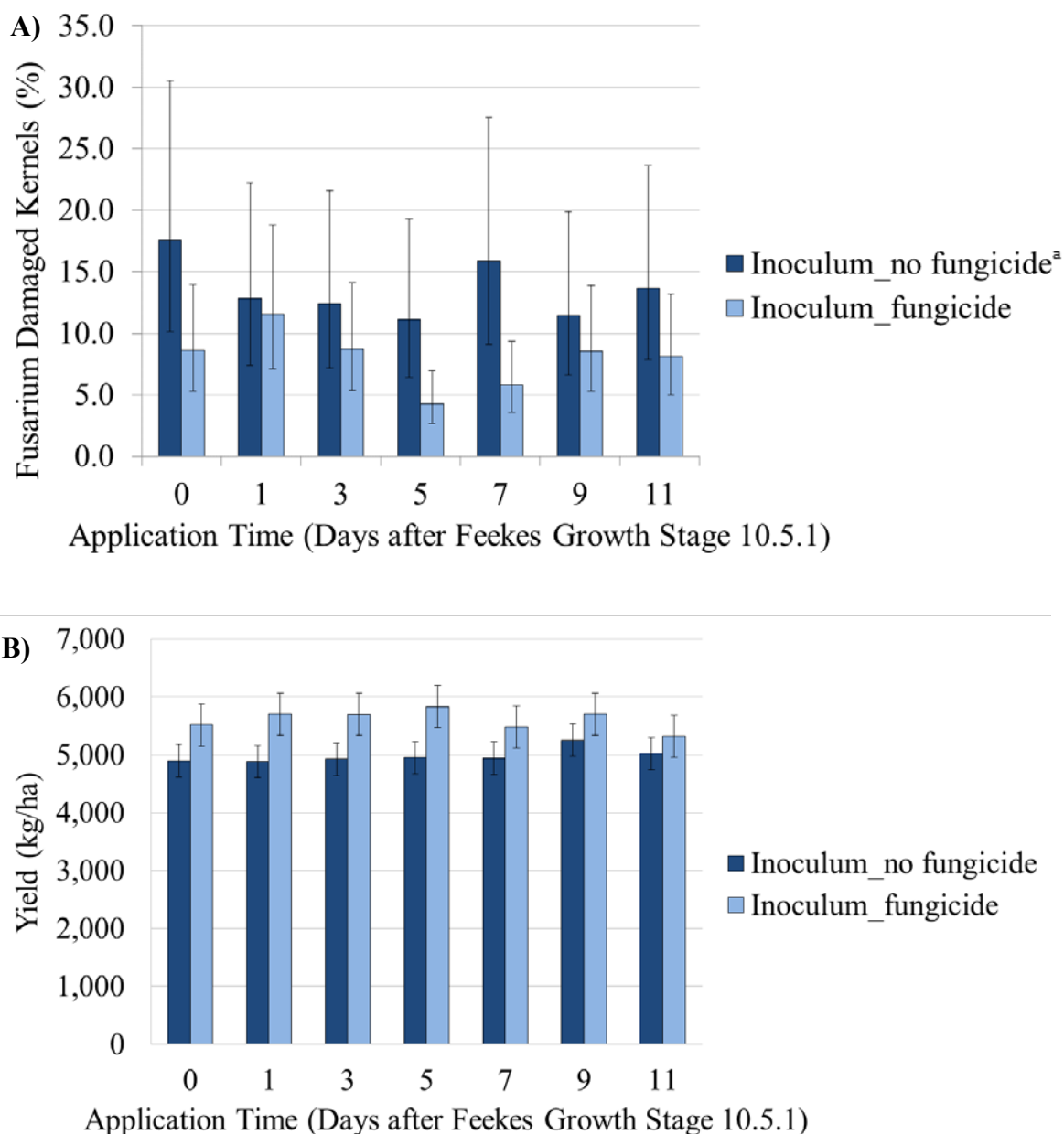[e.] Anthesis refers to the Feekes Growth Stage 10.5.1

Figure 2.5. Effect of inoculum application time and fungicide treatment on **A)** Fusarium damaged kernels (FDK), reported as % visually damaged kernels, and **B)** yield, adjusted for moisture at 13.5%, in 2013. Error bars represent the upper and lower limits of the standard error of the mean values based on least squares means estimations.
[a.] Inoculum was applied at a total volume of 300mL/plot at 50,000 *Fusarium graminearum* macroconidia/mL and Prosaro was applied at 475 mL/ha with 0.125% v/v of a non-ionic surfactant.
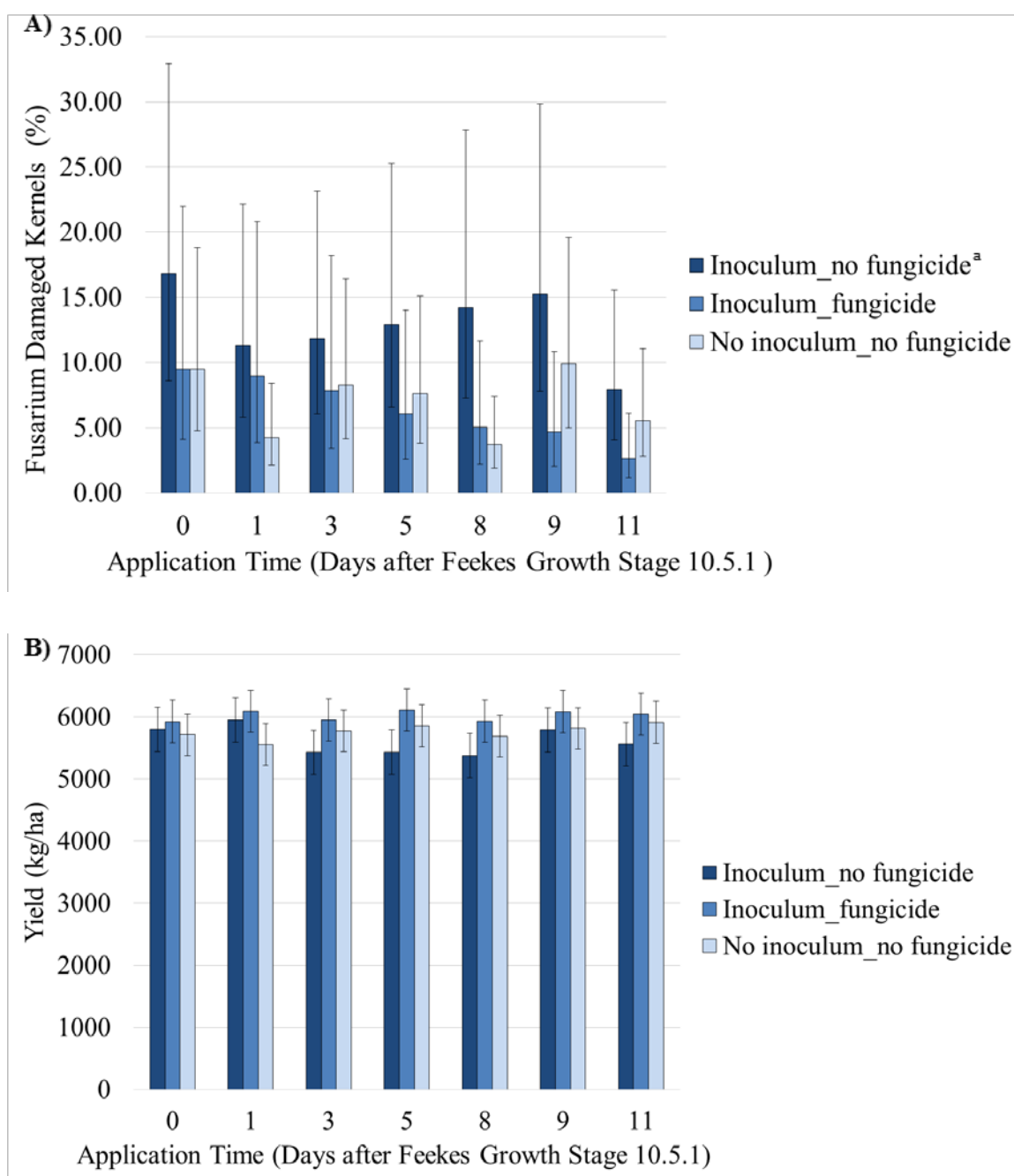
Figure 2.6. Effect of inoculum application time and fungicide treatment on **A)** Fusarium damaged kernels (FDK), reported as % visually damaged kernels, and **B)** yield, adjusted for moisture at 13.5%, in 2014. Error bars represent the upper and lower limits of the standard error of the mean values based on least squares means estimations.
[a.] Inoculum was applied at a total volume of 300mL/plot at 50,000 *Fusarium graminearum* macroconidia/mL and Prosaro was applied at 475 mL/ha with 0.125% v/v of a non-ionic surfactant.

Table 2.6. Pearson's correlation tests for associations between Fusarium head blight (FHB) Index, deoxynivaleol (DON), Fusarium damaged kernels (FDK), 1000 kernel weight (1000 KW), and yield within fungicide treatments from 2013.

	Inoculum, No Fungicide		Inoculum, Fungicide	
Relationship	*r*	*P*-value	*r*	*P*-value
Index[a] – DON[b]	-0.20344	0.2991	0.44916	**0.0165[f]**
Index – FDK[c]	0.28557	0.1407	0.34194	0.0749
Index – 1000 KW[d]	-0.27770	0.1525	0.15420	0.4334
Index – Yield[e]	-0.49377	**0.0076**	-0.31966	0.0973
DON – FDK	-0.07809	0.6929	0.27269	0.1603
DON – 1000 KW	-0.26056	0.1805	0.08055	0.6837
DON – Yield	0.15744	0.4237	0.01573	0.9367
FDK – 1000 KW	0.07568	0.7019	-0.08256	0.6762
FDK – Yield	-0.31586	0.1015	0.03613	0.8552
Yield – 1000 KW	0.42391	**0.0246**	0.21219	0.2784

[a.] FHB Index was calculated from visual disease ratings taken 21 days after anthesis.

[b.] DON as measured in parts per million (ppm) quantified from a post-harvest grain sample.

[c.] FDK, the percent of kernels damaged by *F. graminearum*, was visually estimated post-harvest from a 40 mL grain sample from each experimental plot.

[d.] One thousand kernel weight (1000 kernel weight) as measured in grams (g) was determined by enumerating 1000 kernels and weighing them.

[e.] Yield (kg/ha) was adjusted for moisture content (13.5%) prior to analysis.

[f.] Bolded *P*-value indicates significance at $\alpha = 0.05$.

Table 2.7. Pearson's correlation tests for associations between Fusarium head blight (FHB) Index, deoxynivaleol (DON), Fusarium damaged kernels (FDK), 1000 kernel weight (1000 KW), and yield within fungicide treatments from 2014.

Relationship	Inoculum, No Fungicide		Inoculum, Fungicide		Natural Infection, No Fungicide	
	r	P-value	r	P-value	r	P-value
Index[a] – DON[b]	0.53669	**0.0032[a]**	-0.14298	0.4679	-0.17512	0.3728
Index – FDK[c]	0.19652	0.3162	-0.03227	0.8705	-0.26507	0.1728
Index – 1000 KW[d]	-0.36021	0.0597	0.12550	0.5245	0.17203	0.3814
Index – Yield[e]	0.11037	0.5761	0.05384	0.7856	0.03127	0.8745
DON – FDK	0.63380	**0.0003**	0.55565	**0.0021**	-0.05363	0.7864
DON – 1000 KW	-0.45563	**0.0148**	-0.43058	**0.0222**	0.20888	0.2861
DON – Yield	-0.10082	0.6097	-0.39759	**0.0362**	-0.46894	**0.0118**
FDK – 1000 KW	-0.35534	0.0635	-0.38193	**0.0449**	0.01452	0.9415
FDK – Yield	-0.14129	0.4733	-0.34001	0.0767	-0.00746	0.9699
Yield – 1000 KW	-0.05823	0.7685	0.04088	0.8364	-0.10471	0.5959

[a.] FHB Index was calculated from visual disease ratings taken 21 days after anthesis.

[b.] DON as measured in parts per million (ppm) quantified from a post-harvest grain sample.

[c.] FDK, the percent of kernels damaged by *F. graminearum*, was visually estimated post-harvest from a 40 mL grain sample from each experimental plot.

[d.] One thousand kernel weight (1000 kernel weight) as measured in grams (g) was determined by enumerating 1000 kernels and weighing them.

[e.] Yield (kg/ha) was adjusted for moisture content (13.5%) prior to analysis.
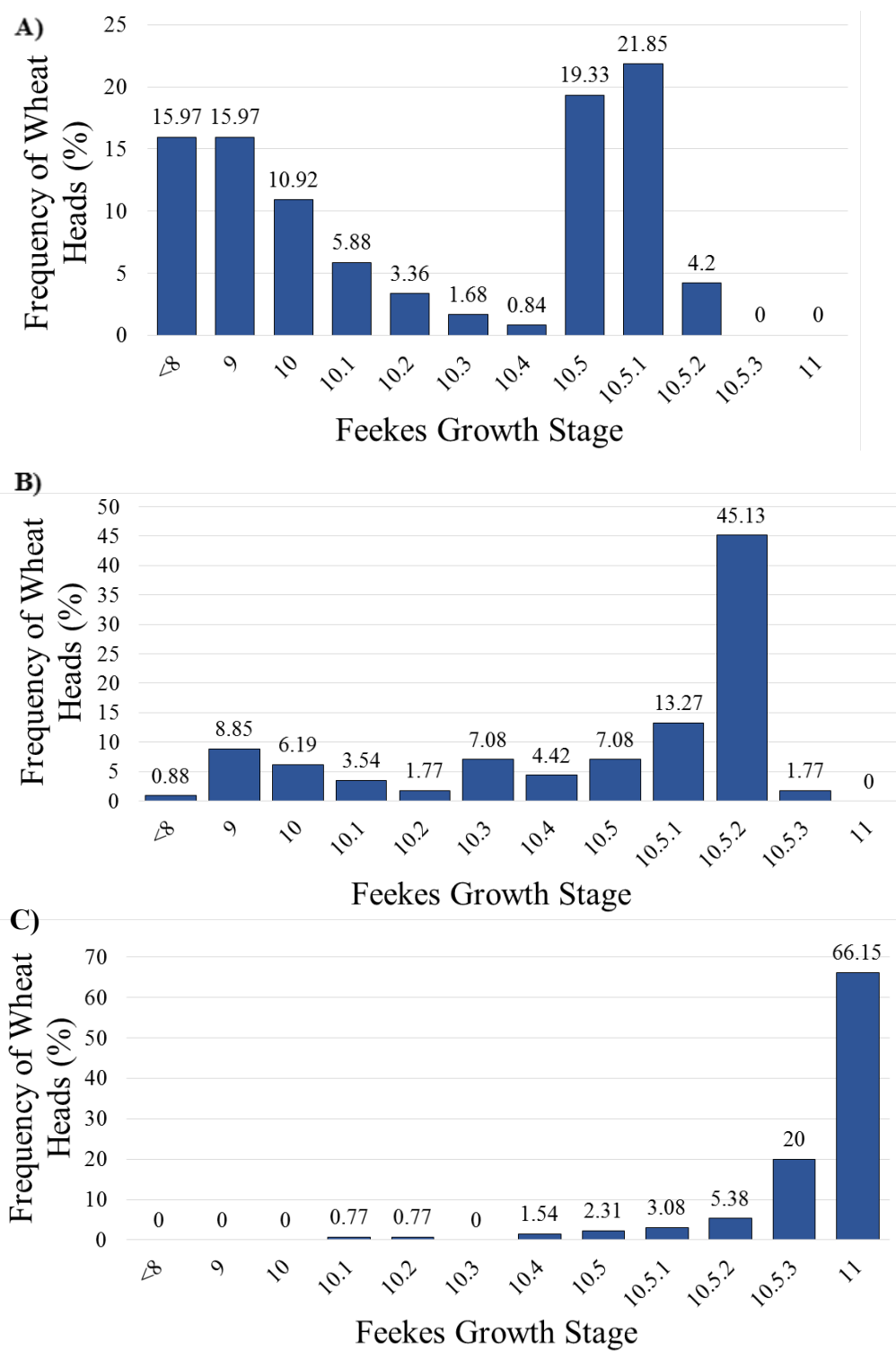
[f.] Bolded *P*-value indicates significance at α= 0.05.

Figure 2.7. Relative frequency (%) of tiller growth stages combined across all fungicide treatments at inoculum application times **A)** 0 days after anthesis (n = 119), **B)** 3 days after anthesis (n = 113) and **C)** 11 days after anthesis (n =130) in 2014.
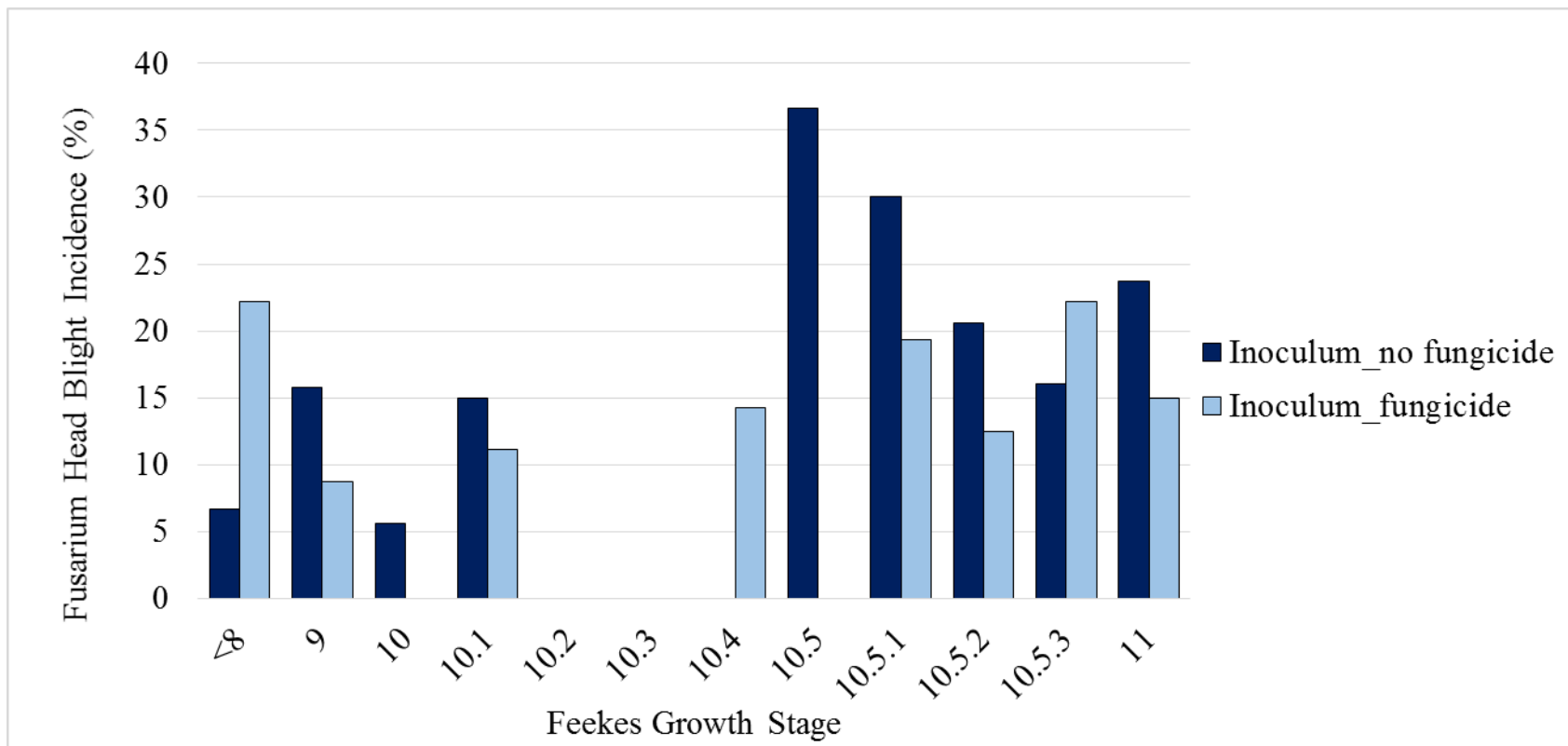
Figure 2.8. Frequency of tillers that developed Fusarium head blight after receiving an inoculation treatment at the growth stage indicated on the x-axis. Data is separated by inoculated, fungicide-treated, and inoculated, non-fungicide treated plots.

APPENDIX

APPENDIX

Table A.1. Least squares means estimations for Fusarium head blight Index (FHB Index), deoxynivalenol (DON), Fusarium damaged kernels (FDK), 1000 kernel weight (1000 KW) and yield by inoculum application time and fungicide treatment for the 2013 experiment at the Agronomy Center for Research and Education (ACRE), West Lafayette, IN

Inoculum application time[a]	Treatment [b]	FHB Index (%)[c]	DON (ppm) [d]	FDK (%)[e]	1000 KW (g) [f]	Yield (kg/ha) [g]
Anthesis[h]	Prosaro	9.7 a[i]	4.5	8.6	39	5500
	None	11	5.6	18	37	4900
Anthesis + 1 day	Prosaro	7.8 ab	3.9	12	38	5700
	None	11	5.6	13	36	4890
Anthesis + 3 days	Prosaro	5.4 ab	3.9	8.7	38	5700
	None	9.4	7.0	12	37	4900
Anthesis + 5 days	Prosaro	4.4 ab	4.4	4.3	39	5800
	None	7.1	5.1	11	37	5000
Anthesis + 7 days	Prosaro	5.6 ab	3.5	5.8	39	5500
	None	9.2	5.9	16	39	4900
Anthesis + 9 days	Prosaro	4.8 b	3.9	8.6	39	5700
	None	5.7	6.1	11	38	5300
Anthesis + 11 days	Prosaro	11 ab	4.2	8.2	38	5300
	None	8.2	5.9	14	36	5000

Table A.1.

[a.] Inoculum was applied at a total volume of 300mL/plot at 50,000 *Fusarium graminearum* macroconidia/mL.

[b.] Prosaro was applied at the recommended field rate of 475 mL/ha with 0.125% v/v of a non-ionic surfactant.

[c.] FHB Index was calculated from visual disease ratings taken 21 days after anthesis.

[d.] DON as measured in parts per million (ppm) quantified from a post-harvest grain sample.

[e.] FDK, the percent of kernels damaged by *F. graminearum*, was visually estimated post-harvest from a 40 mL grain sample from each experimental plot.

[f.] One thousand kernel weight (1000 kernel weight) as measured in grams (g) was determined by enumerating 1000 kernels and weighing them.

[g.] Yield (kg/ha) was adjusted for moisture content (13.5%) prior to analysis.

[h.] Anthesis corresponds to Feekes growth stage 10.5.1

[i.] Within a column, values followed by the same letter designate that the mean within that inoculum application time is not significantly from different from the mean value within another inoculum application time based on the least squared means. If no letters are present, no significant differences exist across inoculum application time means.

Table A.2. Least squares means estimations for Fusarium head blight Index (FHB Index), deoxynivalenol (DON), Fusarium damaged kernels (FDK), 1000 kernel weight (1000 KW) and yield by inoculum application time and fungicide treatment for the 2014 experiment at the Agronomy Center for Research and Education (ACRE), West Lafayette, IN

Inoculum Application time[a]	Treatment[b]	FHB Index (%)[c]	DON (ppm)[d]	FDK (%)[e]	1000 K.W. (g)[f]	Yield (kg/ha)[g]
Anthesis[h]	Inoculum, fungicide	3.9	4.8 a[i]	9.5 a	39	5900
	Inoculum, no fungicide	12	6.5	17	35	5800
	No inoculum, no fungicide	2.4	3.6	9.5	38	5700
Anthesis + 1 day	Inoculum, fungicide	3.4	4.3 ac	9.0 ab	38	6100
	Inoculum, no fungicide	2.9	4.5	11	36	5900
	No inoculum, no fungicide	2.8	4.3	4.2	37	5600
Anthesis + 3 days	Inoculum, fungicide	8.0	3.1 bc	7.8 ab	39	6000
	Inoculum, no fungicide	5.2	3.9	12	37	5400
	No inoculum, no fungicide	4.0	3.6	8.3	37	5800
Anthesis + 5 days	Inoculum, fungicide	1.9	3.0 ac	6.1 ab	39	6100
	Inoculum, no fungicide	4.4	5.8	13	36	5400
	No inoculum, no fungicide	4.9	3.5	7.6	38	5900
Anthesis + 8 days	Inoculum, fungicide	1.3	2.8 bc	5.0 ab	39	5900
	Inoculum, no fungicide	4.2	4.7	14	37	5400
	No inoculum, no fungicide	4.4	3.9	3.7	38	5700
Anthesis + 9 days	Inoculum, fungicide	3.0	2.4 bc	4.7 ab	38	6100
	Inoculum, no fungicide	2.2	4.0	15.2	37	5800
	No inoculum, no fungicide	4.2	4.0	9.9	37	5800
Anthesis + 11 days	Inoculum, fungicide	2.3	2.1 b	2.6 ab	39	6000
	Inoculum, no fungicide	2.4	3.4	8.0	38	5600
	No inoculum, no fungicide	3.1	3.5	5.6	37	5900

Table A.2.

a. Inoculum was applied at a total volume of 300mL/plot at 50,000 *Fusarium graminearum* macroconidia/mL.

b. Prosaro was applied at the recommended field rate of 475 mL/ha with 0.125% v/v of a non-ionic surfactant.

c. FHB Index was calculated from visual disease ratings taken 21 days after anthesis.

d. DON as measured in parts per million (ppm) quantified from a post-harvest grain sample.

e. FDK, the percent of kernels damaged by *F. graminearum*, was visually estimated post-harvest from a 40 mL grain sample from each experimental plot.

f. One thousand kernel weight (1000 kernel weight) as measured in grams (g) was determined by enumerating 1000 kernels and weighing them.

g. Yield (kg/ha) was adjusted for moisture content (13.5%) prior to analysis.

h. Anthesis corresponds to Feekes growth stage 10.5.1

i. Within a column, values followed by the same letter designate that the mean within that inoculum application time is not significantly from different from the mean value within another inoculum application time based on the least squared means. If no letters are present, no significant differences exist across inoculum application time means.
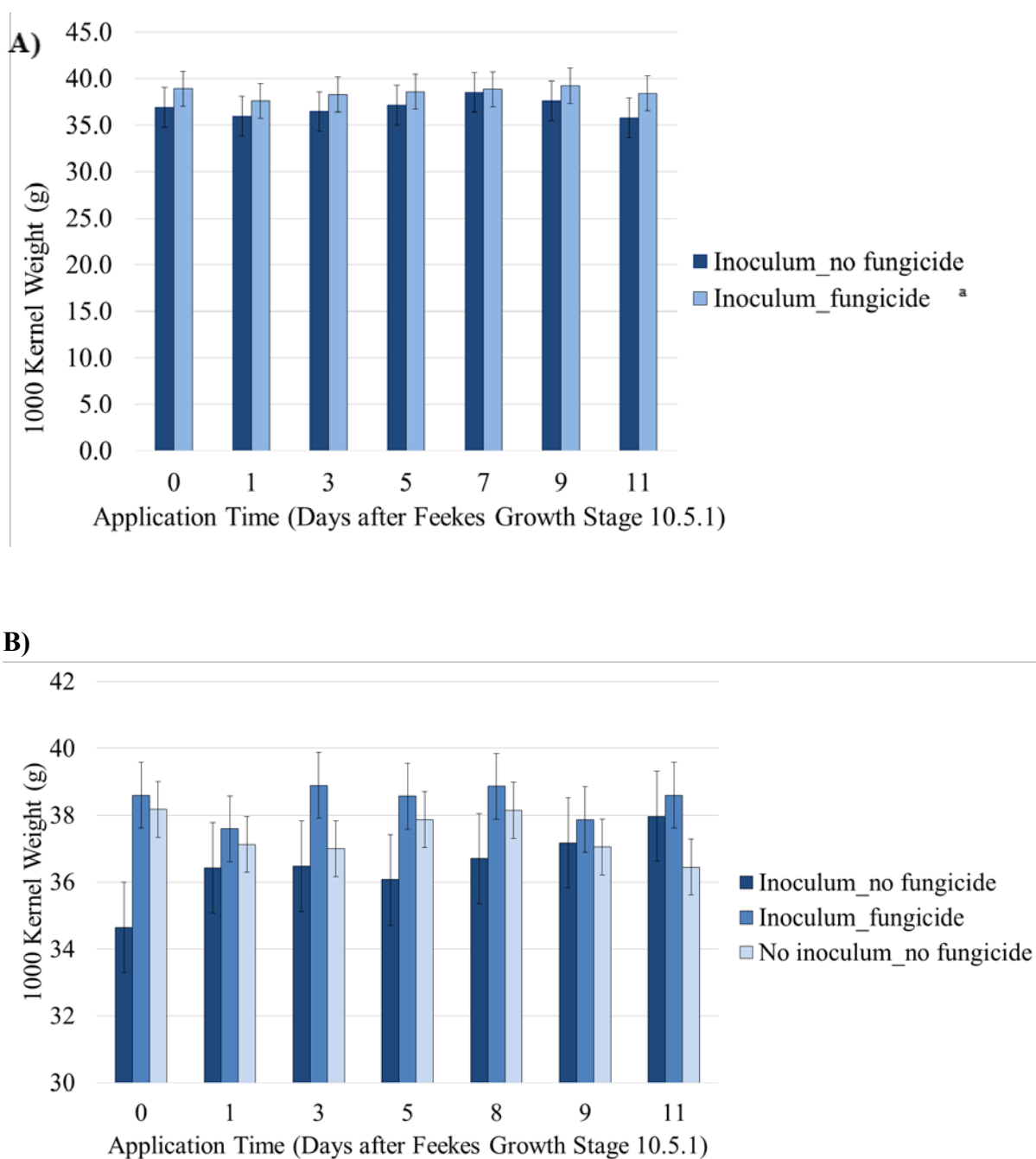
Figure A.1. Effect of inoculum application time and fungicide treatment on 1000 kernel weight in **A)** 2013 and **B)** 2014. Error bars represent the upper and lower limits of the standard error of the mean values based on least squares means estimations.

[a.] Inoculum was applied at a total volume of 300mL/plot at 50,000 *Fusarium graminearum* macroconidia/mL and Prosaro was applied at 475 mL/ha with 0.125% v/v of a non-ionic surfactant.
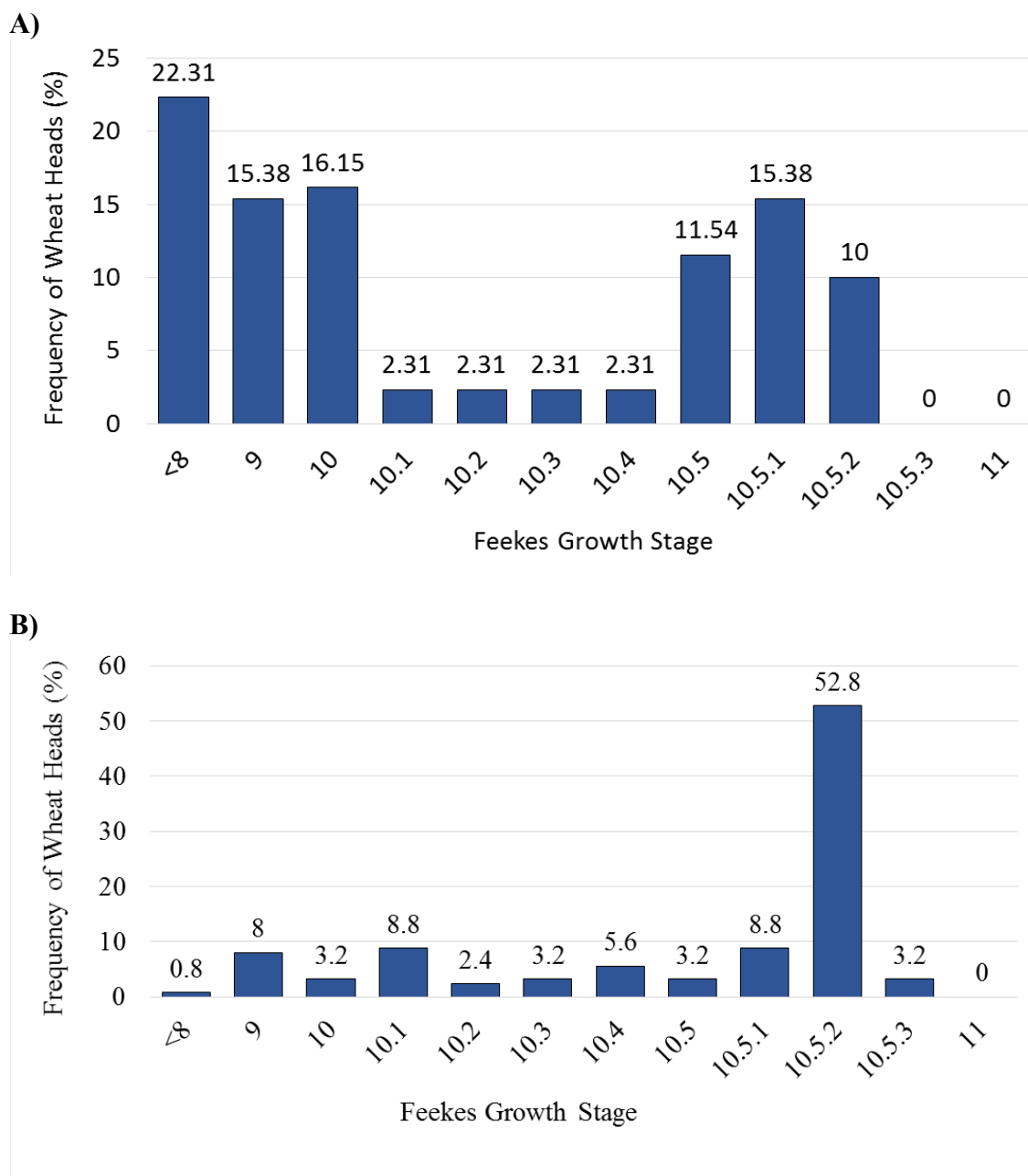
A)

B)

Figure A.2. Relative frequency (%) of tiller growth stages combined across all fungicide treatments at inoculum application times **A)** 1 day after anthesis (n = 130), and **B)** 5 days after anthesis (n = 125), combined across all fungicide treatments in 2014.
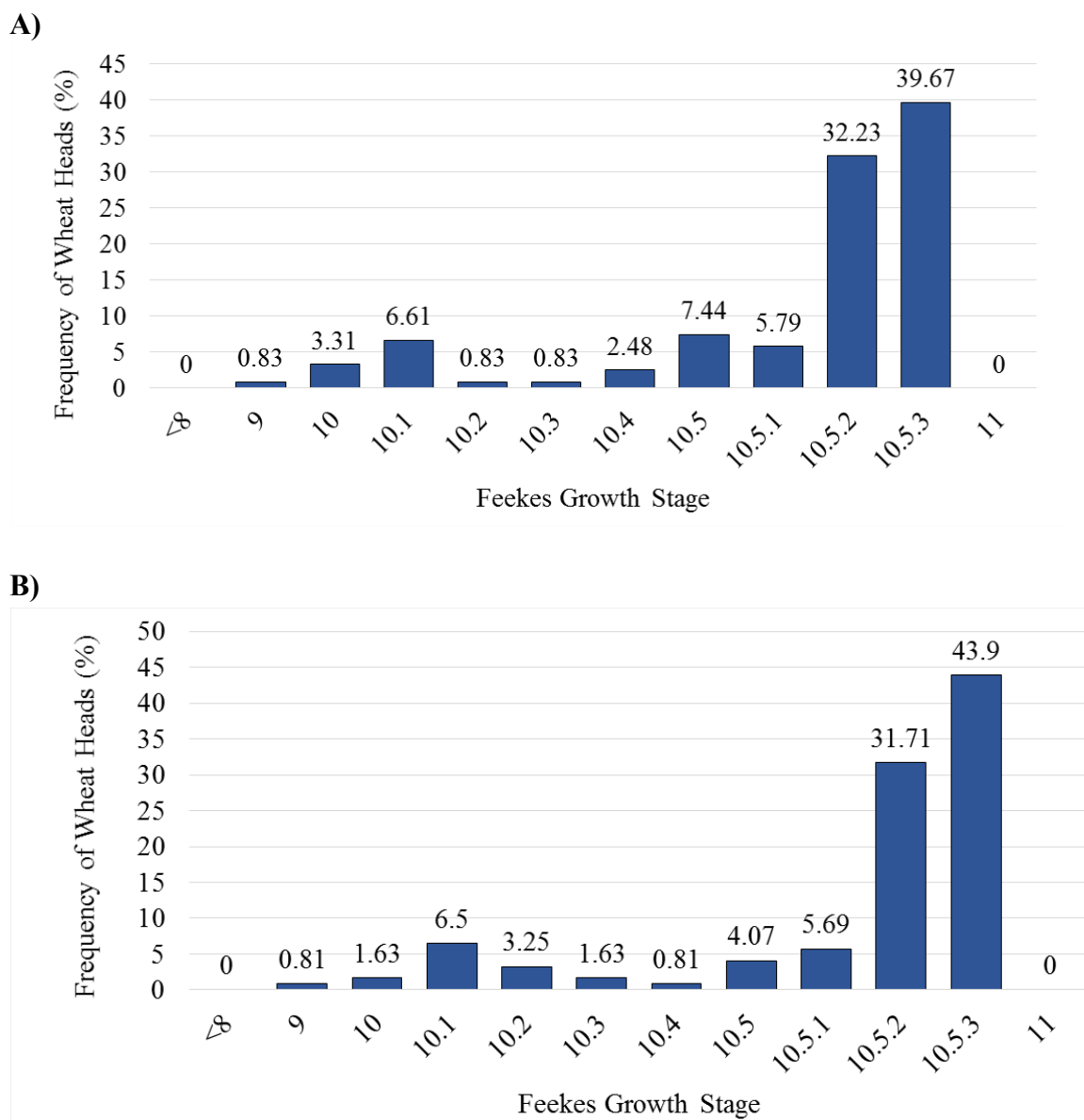
A)

B)

Figure A.3. Relative frequency (%) of tiller growth stages combined across all fungicide treatments at inoculum application times **A)** 8 days after anthesis (n = 121) and **B)** 9 day after anthesis (n = 123), combined across all fungicide treatments in 2014.