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Epidemiology and Management of Fusarium Head Blight and Foliar Fungal Diseases of Wheat

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EPIDEMIOLOGY AND MANAGEMENT OF FUSARIUM HEAD BLIGHT AND FOLIAR FUNGAL DISEASES OF WHEAT

by

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Fusarium head blight (FHB) caused by *Fusarium graminearum*, the FHB-associated mycotoxin deoxynivalenol (DON), and foliar fungal diseases are significant threats to wheat production. This research 1) evaluated the effects of fungicide chemical class, application timing, and cultivar resistance on FHB and DON under field conditions; 2) evaluated the effects of field-applied fungicide chemical class, grain moisture, and time on DON under grain storage conditions; 3) evaluated the effects of field-applied fungicide chemical class and time on trichothecene-related gene (*Tri*5) expression under grain storage conditions; 4) determined the optimum *F. graminearum* spore concentration and spike bagging period following inoculation for accurately discriminating between FHB resistant and susceptible wheat cultivars under greenhouse conditions; and 5) determined the optimum timing of fungicide applications for control of foliar fungal diseases of wheat under field conditions. A triazole fungicide controlled FHB and DON more effectively than a strobilurin fungicide. A triazole applied 6 days after anthesis was as effective as an anthesis (standard timing) application, indicating a wider window of application for growers' needed flexibility. In storage, DON decreased over time in grain of a moderately resistant cultivar treated with a triazole and increased in grain of a susceptible cultivar treated with a strobilurin. During storage, DON biosynthesis *Tri*5

gene expression increased over time during storage of high grain moisture grain, a significant reduction in the relative expression of the *Tri*5 gene and a downregulation of the gene occurred in the triazole treatment whereas expression of the gene increased in the strobilurin treatment. In the greenhouse, lower concentrations of *F. graminearum* inoculum (6.25×10^3 and 1.25×10^4 spores/mL) were more efficient in discriminating between a moderately resistant and a susceptible wheat cultivar compared to the standard concentration (1×10^5 spores/mL). The optimum spike bagging period following inoculation for discrimination between a moderately resistant and a susceptible cultivar was 48 hours or 72 hours (the standard). Foliar fungicide applications in field plots at the flag leaf and boot growth stages of wheat were more effective in protecting yield than later applications. Results from this research will enhance knowledge in the epidemiology and management of FHB, DON, and foliar fungal diseases of wheat.

DEDICATION

To my beloved wife: Patricia Anaís Ballesteros-Moreta, and my son: Gabriel Abraham Bolaños-Ballesteros.

To my father, Marco Antonio Bolaños-Osorio, and my mother, Cristina Annabelle Carriel-Valdez.

To my sisters, María Belén Bolaños-Carriel and María Emilia Bolaños-Palma.

To my nieces, Emily Estefanía Salazar-Bolaños and Daniela Desiré Salazar-Bolaños.

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INTRODUCTION AND BACKGROUND

1. Importance and origin of wheat.

Wheat (*Triticum* sp.) is the most widely grown crop in the world and the staple food for 35 percent of the world's population (International Development Research Center). Wheat provides roughly 19 percent of the global dietary energy consumption (Ray et al. 2013). Wheat has vital importance for food security and food sovereignty in many countries.

The wheat crop was a driving force behind the development of human civilizations. Domestication, the process of adapting from a wild to a farm plant under human control, led to the switching from hunter-gatherer to agriculture-based populations (Gustafson et al. 2009). Humans during the Neolithic period (20,000 to 2,000 B.C.) based their diets on wild relatives of wheat such as einkorn wheat (*Triticum boeoticum*) and emmer wheat (*Triticum turgidum*) (Lev-Yadun et al. 2000).

The wheat demand by 2050 is estimated at 1,300 million metric tons (MMT) (Ray et al. 2013). Total world production for the 2016-2017 marketing year reached a record of 750 MMT (USDA - NASS). Despite the scientific and technological progress made in the crop, it is projected that there will be a shortfall in wheat production of approximately 388 MMT by 2050 (Ray et al. 2013). Worldwide, one in nine people lack sufficient food supplies and are at risk of hunger (United Nations). Wheat can play a significant role in eliminating global food insecurity.

Wheat belongs to the botanical family *Poaceae*. The genus *Triticum* can be categorized in three levels of ploidy: diploid, (2n = 2x = 14), tetraploid (2n = 4x = 28) or hexaploid (2n = 6x = 42) (Acquaah 2009). Additionally, *Triticum aestivum* has three

genomes A, B and D. Chromosomic substitutions and additions from alien DNA allowed an increase in the genetic variability of wheat (Gerlach and Dyer 1980). In 2018, an annotated and ordered reference genome was made public as the International Wheat Genome Sequencing Consortium (IWGSC) RefSeq v1.0 (Appels et al. 2018).

There are two main species of wheat cultivated for food which are bread wheat, *Triticum aestivum* (hexaploid), and pasta wheat, *Triticum durum* (tetraploid), and both species came from domesticated tetraploids. The earliest record of using a tetraploid wheat involved *Triticum dicoccoides* around 17,000 B. C. (Kislev 1992; Nevo et al. 2013). However, the origin of cultivated wheat can be traced back millions of years through speciation and later polyploidization events of its diploid relatives *Triticum urartu* (AA genome) and *Aegilops speltoides* (BB genome) for durum wheat, with the addition of *Aegilops tauschii* (DD genome) for bread wheat (Gill et al. 1991). The paleohistory of wheat evolution remains a matter of controversy and debate (El Baidouri et al. 2016).

The center of origin of wheat is distributed around the territory where modern-day Israel, Lebanon, Syria, Iraq, and Iran exist. Wheat domestication is thought to have occurred in a narrow area between the rivers Euphrates and Tigris in the southern Levant (Jordan valley) (Kislev 1992; Lev-Yadun et al. 2000).

2. Morphology of wheat and scales of growth.

Wheat is a self-pollinated crop with grass-like morphology. It is mainly produced for the caryopsis or kernel, which consists of an endosperm surrounding the embryo and coated with several layers of bran (Jackson and Williams 2016; Reed 2006). Wheat crop development is measured in growth stages. The most used scales to describe wheat development are Feekes (Fk) (Large 1954) and Zadoks growth stages (GS) (Zadoks et al. 1974).

The root development begins with the primary root penetrating the soil which provides nutrients and water to the plantule (GS 9). In a second phase (Fk 1, GS 10), secondary roots are developed, the first leaf emerges through the coleoptile, and lateral ramification or tillering begins and will continue until heading (Haun 1973; Large 1954; Zadoks et al. 1974).

The vegetative growth of wheat consists of several events of branching, beginning when the fourth leaf emerges. Usually, two or three tillers can fully develop to spikes. The tillering stage (Fk 3, GS 26) includes initial tiller formation, elongation, and erection of leaf sheaths. After this, the stages comprise of the extension of the stem starting when the first node of the stem is visible and until the sixth node is visible (Fk 4-7, GS 30-35).

The flag leaf is slightly visible at Fk 8 (GS 37), and the ligule of the flag leaf is visible at Fk 9 (GS 39). The stem extension stage ends at Fk 10 (GS 45) when the swollen flag leaf sheath, within the stem, is pushed as the stem has elongated and the tip of the flag leaf is visible—this stage is known as boot stage (Large 1954; Simmonds et al. 1985; Waldren and Flowerday 1979).

Heading goes from Fk 10.1 (GS 49) to Fk 10.5 (GS 59). The emergence of the inflorescence can be divided according to the proportion of the spike that is visible (¼, ½ or ¾) (Haun 1973; Large 1954; Simmonds et al. 1985; Zadoks et al. 1974).

Anthesis, or the blooming stage, corresponds to Fk 10.51 (GS 60). It is characterized by the extrusion of the anthers in cultivars which possess this trait (Simmonds et al. 1985). Anthesis in wheat cannot be understood as a fixed point in time (Paul et al. 2018). Flowers of wheat are self-pollinated. When anthesis is complete, the grain goes into milk development. At stage 10.54, the kernel begins to fill with the photosynthetic products of the flag leaf (Simmonds et al. 1985). During Fk 10.54, 11.1, and 11.2 (dough development) dry matter begins to increase in the kernel (soft dough stage, Fk 11.2). The ripening of wheat and other small grain cereals begins at the stage Fk 11.3 or hard kernel stage. When the kernel hardens and is difficult to dent with the thumbnail, the grain has reached the Fk 11.4 stage and is ready for harvest (Haun 1973; Large 1954; Simmonds et al. 1985; Waldren and Flowerday 1979; Zadoks et al. 1974).

3. Major diseases affecting wheat production.

Although this dissertation focuses on Fusarium head blight (FHB) and foliar diseases, this subchapter provides a brief description of the major diseases of wheat to emphasize their importance. Diseases affecting wheat can be divided based on the part of the plant where symptoms or signs are first observed. Several wheat diseases, especially those affecting leaves, are more distinguishable when the plant has reached vegetative growth (Fk 3-5, GS 26-31) than in later growth stages. Young leaves present symptoms such as yellowing, mosaics, black spots, yellow halos around brown patches, oval spots, or tan spots. These symptoms allow a straightforward identification of certain diseases and control measures can be taken according to the severity, incidence, and risk of epidemics. In mature stages, diseases affecting stems, spikelets, or florets can be distinguished from other problems due to their specificity to the part of the plant affected.

Five of the most damaging viruses in wheat belong to the group of single-strand positive-sense RNA viruses (ssRNA+) (Suzuki et al. 2015). *Barley yellow dwarf virus* (BYDV) is the type species virus in the *Luteoviridae*. Viruses associated with yellowing and dwarfing in the *Luteoviridae* are the most widespread group of cereal viruses worldwide (Figueira et al. 1997). BYDV is the most serious virus disease in winter wheat in the southeastern U.S. (Weisz et al. 2005). The virion moves in the phloem and generates symptoms of leaf discoloration that turn yellow or purple (Wegulo et al. 2015a) and leaf tip necrosis (Ibrahim and Shah 2015). The transmission of the virus is mechanical and spread through aphid vectors.

Soil-borne wheat mosaic virus (SBWMV) is the type species of the genus *Furovirus* in the *Virgaviridae* (SSRNA+ viruses) (ICTV 2015), and it was the first virus reported in wheat (McKinney 1923). The protist *Polymyxa graminis* transmits the virion. Symptoms

in young leaves appear as mottled and parallel streaks (Wegulo et al. 2015a); other symptoms are mosaics, stunting, and witches' broom. The virus can be controlled through resistance breeding (Verchot et al. 2001).

Wheat streak mosaic virus (WSMV) is the type species of the genus *Tritimovirus* in the *Potyviridae* with monopartite, linear, ssRNA+. WSMV infects all known species of wheat and has a broad host range in the *Poaceae*. WSMV is transmitted by the eriophyid wheat curl mite, *Aceria tosichella*. It is a serious pathogen of wheat in the Great Plains of the United States (French and Stenger 2003). The first symptoms associated with the viral infection are yellowing, parallel and discontinuous streaks, leaf rolling and trapping (Wegulo et al. 2015a).

High Plains disease is associated with the *High Plains wheat mosaic virus* (HPWMoV), which is also described as *Wheat mosaic virus* (WMoV) (Oliveira-Hofman et al. 2015). It was first reported in wheat fields of Kansas and rapidly spread to Texas, Nebraska, Colorado, Idaho and Utah (Seifers et al. 2009). HPWMoV belongs to the genus *Emaravirus*, and is transmitted by the wheat curl mite. In the Great Plains of the United States, yield loss in severely affected wheat fields can reach 100% (Oliveira-Hofman et al. 2015).

Triticum mosaic virus (TriMV) is the type member of the genus *Poacevirus* in the *Potyviridae* of ssRNA+ viruses (Tatineni et al. 2009). It is transmitted by the wheat curl mite which also transmits WSMV and HPWMoV (Byamukama et al. 2013). TriMV was first discovered in 2006 in Kansas (Seifers et al. 2008). TriMV causes symptoms similar to those of WSMV, and both viruses have been found co-infecting wheat. TriMV can cause significant yield losses, especially when it co-infects wheat with WSMV or HPWMoV (Byamukama et al. 2014).

Wheat spindle streak mosaic virus (WSSMV; genus *Bymovirus*, *Potyviridae*), is taxonomically related to other important viruses affecting small grains such as barley mild mosaic virus, oat mosaic virus, rice necrosis virus and wheat yellow mosaic virus (ICTV 2015). WSSMV has a non-enveloped, flexuous, filamentous, ssRNA+ genome, which serves as viral mRNA. *Polymyxa graminis* transmits the virion, and the main symptoms associated with the viral infection are yellow-green mottling, dashes, and streaks (Wegulo et al. 2015a).

The most prevalent bacterial disease of wheat is bacterial streak or black chaff, caused by *Xanthomonas campestris* pv. *translucens* (Bamberg 1936; Duveiller et al. 1992). The bacterium is widespread through the mid-west of the United States, and affects wheat, rye, triticale, and barley. Black chaff is more prevalent and severe under warm and humid conditions. Symptoms associated with the disease are inter-veinal longitudinal streaks, water-soaking lesions, and darkening of the glumes (Kandel et al. 2014; Wegulo et al. 2015b). It can be easily differentiated from other foliar diseases by the presence of a bacterial stream coming out of the vascular bundles under the light microscope.

Crown and root rot of wheat is caused by fungi in the genus *Fusarium* such as *F. graminearum, F. culmorum*, and *Fusarium pseudograminearum* (Cook 1981), and *Cochliobolus sativus*. Crown and root rot is detected in wheat fields as brown patches of dead plants. Wheat plants affected by crown and root rot have smaller heads and spindly internodes (Wegulo et al. 2015a).

Rusts are important pathogens of wheat and are known as yield-robbers. The fungi of the order Uredinales include more than 1700 species of obligate parasites of plants (Gwynne-Vaughan 1922). There are three types of rusts affecting wheat: stripe, leaf, and

stem rusts. Stripe rust, *Puccinia striiformis* f.sp. *tritici*, produces small yellow-orange round pustules (Wegulo et al. 2015b). The pustules form longitudinal stripes which are visible on the leaves. Leaf rust, *Puccinia triticina* (synonym *Puccinia recondita*), is also known as brown rust due to the presence of slightly-round orange or brown pustules (Wegulo et al. 2015b) that form a random pattern on the leaf surface. Stem or black rust, *Puccinia graminis* f.sp. *tritici*, is less prevalent in wheat fields in the United States due to the efforts conducted to eradicate barberry (the alternate host) during the early 20th century. By 1928, the Great Plains of North America were barberry-free (Roelfs and Groth 1980). Stem rust pustules are oval in shape, orange-red in color, and occur on leaves and stems (Wegulo et al. 2015b). The "Ug99" stem rust race group constitutes a major threat to wheat production worldwide (Singh et al. 2011). In 2005, the Borlaug Global Rust Initiative was launched because of the danger Ug99 posed. Nowadays, most of the bread wheat varieties planted are susceptible to Ug99 (Singh et al. 2011).

Landraces with new sources of resistance are mapped as the disease progresses. There are approximately 50 stem rust-resistance (Sr) genes reported in the Borlaug Global Rust Initiative, and the list grows every day (Singh et al. 2015). However, in 2016 there were 13 variants of the Ug99 race group detected in eastern and southern Africa (Babiker et al. 2016) and it is spreading. The control of rusts in wheat is difficult and relies on the use of resistant cultivars. However, the dependency on a few major genes for resistance has triggered mutations in the pathogens which have now overcome host resistance. New breeding efforts are trying to introduce several resistance genes into one plant, a process known as gene pyramiding (Ayliffe et al. 2008).

Powdery mildew of wheat caused by *Blumeria graminis* f. sp. *tritici* is distributed worldwide. The fungus forms abundant hyaline mycelia on the surface of the leaves, and

infections usually occur at the beginning of spring. The fungus reduces grain yield and quality (Morgounov et al. 2012). Triadimenol was very effective as a seed treatment in controlling the disease (Leath and Bowen 1989). Wheat seed treatment fungicides are based on carboxamides, dithiocarbamates, phthalimides, acylalanines, and triazoles alone or in mixtures (Wegulo 2014), and can be effective in controlling powdery mildew.

Pyrenophora tritici-repentis causes tan spot of wheat. The fungus is predominantly present in the canopy of young leaves under warm and wet conditions at the beginning of spring. Tan spot can cause losses of up to 50% (Shaber and Bockus 1988). Other foliar diseases that can reduce yield are Septoria leaf blotch (*Zymoseptoria tritici*) and glume blotch (*Parastagonospora nodorum*). These fungi are necrotrophs, which develop fruiting structures in necrotic areas of the affected leaves (Wegulo et al. 2015a).

Spot blotch (*Bipolaris sorokiniana*) causes significant yield losses (up to 40%) worldwide under warm-humid conditions and is the most destructive wheat fungal disease in warmer areas (Acharya et al. 2011; Singh et al. 2016). The pathogen is seed transmitted; however, the primary source of epidemics are conidia overwintering in crop residues. *B. sorokiniana* forms clumps of thick-walled conidia (Acharya et al. 2011; Duckzek et al. 2009; Gupta et al. 2018). An integrated approach is required to control spot blotch and consists of crop rotations, seed treatments, biological control, foliar fungicides and the planting of resistant varieties (Acharya et al. 2011).

Loose smut, caused by *Ustilago tritici*, is a common disease in the Great Plains of the United States. Although loose smut does not affect the quality of the grain, yield loss can exceed 40%; and some countries, such as Pakistan contaminated grain is not accepted. The best treatment against the disease is to use seed treatment fungicides at the time of planting (Wegulo 2017).

Common and karnal bunts of wheat kernels are associated with fungi in the genus *Tilletia* (Bonde et al. 1997; Pascoe et al. 2005; Wegulo et al. 2015b, Wright 2003). *T. indica* is a quarantine organism and the causal agent of karnal bunt. Infection levels of karnal bunt above 3% make grain unacceptable for human consumption (Wright 2003). Common bunt (*Tilletia tritici*) is widespread in the United States, where infections of over 25% can be expected during epidemics (Mathre 2000). Common bunt gives a fishy odor to the grain (Bonde et al. 1997).

4. Fusarium head blight of wheat (FHB).

FHB or scab is one of the most important diseases of wheat worldwide. In the United States, *Fusarium graminearum* is the main causal agent of FHB (McMullen et al. 1997b; O'Donnell et al. 2000). In 1838, *Fusarium graminearum* was first described by Lewis Schwein, and the fungus was illustrated as the asexual (anamorph) phase of the teleomorph *Gibberella zeae.*

Some FHB epidemics have been relevant in the history of the United States and Nebraska. In 1898, Bessey reported wheat scab in several localities in Nebraska. In 1904, some Russian and Hungarian varieties of winter wheat showed severe injury from scab, which at that time was considered a new fungal disease in the region (Lyon and Keyser 1905). Between 1919 -1920, in the United States, wheat grain losses were above 2 MMT (Atanasoff 1920; McMullen et al. 1997a), and for Nebraska, reports mentioned severe damage of the crop with 25 - 50% yield losses in eastern counties (Fromme 1920).

In 1947, Chiu (1950) reported severe blight of wheat heads with percentages of *Fusarium*-damaged kernels around 5% in eastern Nebraska counties. In the 1980's, FHB occurred sporadically, although it should be noted that in 1982 there was an epidemic of severe consequences for the US grain industry with losses of about 2.72 MMT (McMullen et al. 1997a). Major epidemics from 1991 to 1996 across the United States totaled 1.3 billion dollars in direct losses (McMullen et al. 1997a; McMullen et al. 2012). Frequency and severity of FHB epidemics have increased over the last 10 years. In Nebraska, major epidemics occurred in 2007, 2008 (Wegulo et al. 2011), and 2015 (Bolanos-Carriel et al. 2015).

In Nebraska, substantial advances have been made towards the development of new cultivars with high levels of resistance to FHB (Baenziger et al. 2008; Eckard et al.

2015), better management practices (Wegulo et al. 2015c), and knowledge of chemotype and aggressiveness of *Fusarium graminearum* strains associated with the disease (Hernandez-Nopsa et al. 2014; Panthi et al. 2014). Progress in the management of the disease and development of FHB-tolerant cultivars can be linked to notable economic benefits for farmers.

FHB is highly influenced by environmental conditions particularly during and after anthesis. *Fusarium graminearum* produces ascospores and macroconidia which are formed in perithecia and sporodochia, respectively. Ascospores are the main source of inoculum for epidemics (Osborne and Stein 2007). Ascospores are released and transported by wind and infect flower parts. Warm temperatures and high humidity are favorable conditions for complete blighting of heads in 2 to 4 days after infection (Fernando et al. 1997). Ascospores have an optimum of 25-28^{o}C for formation and 20^{o}C to 30^{o}C for infection (McMullen et al. 2012).

Perithecia and sporodochia are fruiting structures of the fungus which overwinter in crop debris. The relationship between crop debris and FHB epidemics has been well documented (Dill-Macky and Jones 2000; Sturz and Johnston 1985).

Minimum soil temperatures for perithecia production are 6^{o}C to 10^{o}C with an optimum in the range 15^{o}C to 20^{o}C (Gilbert et al. 2008; Pereira et al. 2004). High relative humidity and soil moisture content are favorable for perithecia formation; therefore, humid weather during August and September favor FHB epidemics in the following growing season.

In the spring, ascospores and macroconidia are released from the fruiting bodies. The optimum conditions for spore production are a wet substrate and high temperature. The optimum temperatures for production of ascospores are 29^{o}C and 32^{o}C for *F.*

graminearum and *F. culmorum*, respectively. In *F. graminearum*, inhibition of spores occurs when temperatures reach 36ºC or greater (Osborne and Stein 2007).

Discharge of ascospores is triggered by temperatures between 20ºC and 30ºC and high relative humidity (80–92%). Rainfall events before and during anthesis assure the presence of inoculum for FHB epidemics.

Ascospores and macroconidia land on the wheat head during the flowering stage and infection occurs. Under wet and rainy conditions, the propagules are dispersed via water splash or by wind and then infect internal flower parts, glumes, lemma, and palea. Rain splash is considered a major means of pathogen dispersal (Schmale and Bergstrom 2003).

Infections are favored when the relative humidity is higher than 80% and there is wind and rain. If the temperature is between 10ºC to 30ºC and relative humidity is higher than 90% during 4 to 6 hours at the flowering stage, these conditions are perfect for infections.

Penetration of the fungus is favored by low temperature and high relative humidity. The optimal infection occurs when the temperature is around 20ºC and relative humidity is around 100% (Beyer et al. 2006; Osborne and Stein 2007).

After infection, during wet conditions with temperatures around 25ºC to 30ºC, complete blighting of heads occurs, which explains why symptoms appear suddenly in wheat fields under these conditions. The main symptom in the field is the sudden presence of bleached spikelets. During FHB epidemics, pink to orange spore masses are evident on wheat spikes.

An important factor in the interaction of wheat and *F. graminearum* is the formation of choline and betaine by wheat during anthesis. These two components have been reported as growth stimulants for *Fusarium graminearum* (Strange et al. 1972). Hyphal

orientation is essential for successful infection (Brand and Gow 2009). Penetration by *F. graminearum* is directed towards anthers, pollen, and ovaries of wheat (Buerstmayr and Buerstmayr 2015). The hyphal growth of *F. graminearum* exhibits affinity to the above mentioned floral organs or to wheat germ (Strange et al.1974). Under experimental conditions, the growth of *F. graminearum* conidia after germination was directed to the ovary of the floret (Blumke et al. 2014). Choline and betaine play a major role in fungal attraction (Strange and Smith 1971, 1978; Strange et al. 1972). These compounds, when supplied exogenously, can be used as a source of carbon by *F. graminearum* (Strange and Smith 1978; Markham et al. 1993). Choline concentration increased hyphal extension rate and inhibited branching frequency (Robson et al. 1995; Weibe et al. 1992). Accumulation of choline and betaine in wheat anthers has been considered a susceptibility factor for *F. graminearum* (Strange et al. 1972). Hyphal chemotropism towards nutrients is a generally accepted phenomenon, but the underlying mechanisms are largely unknown (Turrà et al. 2015).

In the spikelet, *Fusarium*-damaged kernels (FDK) are the product of an *F. graminearum* infection and colonization of the head tissue. FDK appear as white, chalky collapsed grains. Other names for FDK are shriveled kernels, scabby seeds or tombstones. Associated with high severity of FHB, there is an elevated concentration of mycotoxins, especially DON, which are harmful to humans and animals.

F. graminearum isolates are divided according to their chemotype. There are three chemotypes in *F. graminearum*: 3-ADON (DON and 3-acetyl-DON producers), 15-ADON (DON and 15 acetyl-DON producers), and nivalenol (NIV) (NIV and 4 acetyl-NIV producers) (Ward et al. 2002). In the NIV chemotype, the calonectrin 4-oxygenase

(*Tri*13 gene), and the trichothecene 4-O-acetyltransferase (*Tri*7 gene) are functional and allow the conversion of the trichodiene (product of the enzyme trichodiene synthase *Tri*5) to NIV; therefore, in the NIV chemotype of *F. graminearum* the final outcome of the pathway is NIV instead of DON. Although the three chemotypes belong to the same species, their genetic differences and geographical distribution separate them into different genetic populations (Ward et al. 2002).

5. Integrated management of Fusarium head blight.

Integrated management is the most effective strategy for controlling FHB and DON (Wegulo et al. 2015c). The sporadic nature of FHB epidemics makes its control a challenge. A good forecasting system, choosing a tolerant or moderately-resistant cultivar, cultural practices such as residue management and tillage, and timely application of fungicides are the major components of an integrated management system for FHB (Wegulo et al 2015c).

Cultivar resistance is the most cost-effective option in this disease management strategy (Wegulo et al. 2015c). In monocyclic diseases such as FHB, control is made by either reducing the amount of inoculum or by reducing the efficacy of inoculum (Madden et al. 2007). Considering that spores of *F. graminearum* move via wind and rain splash, a virulent pathogen will always be present; therefore, moderately resistant cultivars will only have an effect on the infection efficiency. Total cultivar resistance to FHB does not exist. Moderate-resistance needs to be coupled with the application of fungicides to control the infection.

New systemic or locally-systemic fungicides have given farmers a valuable tool to fight the disease; however, the correct selection of the fungicide chemical class and application timing play a major role on their efficacy. Fungicide treatments that target FHB should be applied during or a few days after anthesis.

Tillage removes a large quantity of *F. graminearum* inoculum. Reducing the initial population of the fungus not only delays disease progression, but also limits the production of DON (Beyer et al. 2006). Blandino et al. (2012) found that direct sowing with a susceptible cultivar, without fungicide application showed a 97% higher DON

incidence than plowing and using a moderately resistant cultivar with a triazole application at heading.

Crop rotation is critical because the most important source of inoculum for FHB epidemics are ascospores released from fruiting bodies overwintering in crop debris especially corn and wheat stubble. Continuous wheat or wheat after corn are not a recommended rotation scheme if one wants to avoid accumulation of FHB inoculum in the soil (Osborne and Stein 2007; McMullen et al. 1997b). Survival of *F. graminearum* occurs mainly in crop stubble but not in soil (Leslie et al. 1990). Using a legume crop after wheat or corn can increase the C/N ratio accelerating the decomposition of wheat or corn stubble and could reduce the survival and the initial population of *F. graminearum*.

6. Fungicides for controlling foliar diseases, FHB and DON in wheat

Fungicides play a major role in controlling foliar fungal diseases and FHB, and in the prevention of pre- and post-harvest DON contamination in wheat grain. Unfortunately, relatively few fungicides have shown good efficacy against FHB (McMullen et al. 1997b; Mesterházy et al. 2011). Currently, triazoles are the most effective fungicides against FHB (Wegulo et al. 2015c).

Demethylation inhibitors (DMI), particularly the triazoles, inhibit the C14 demethylase in the ergosterol biosynthetic pathway (Myung and Klittich 2015). Ergosterol is a target for many antifungal compounds and the major component of the fungal cell membrane. The function of ergosterol is the bioregulation, fluidity, asymmetry, and integrity of the cell membrane. Ergosterol also plays a hormone-like role in fungal cells, because it stimulates fungal growth (Kathiravan et al. 2012). Triazoles have a direct inhibitory effect on hyphal formation (Ha and White 1999).

The effect of the triazole tebuconazole (Folicur) on infection by *Fusarium culmorum*, the main causal agent of FHB in Europe, was studied by Zange et al. (2005). Post-infection wheat heads were treated with Folicur and the fungicide effect was observed using Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM). Immediately after the treatment, morphological changes such as excessive branching, the formation of bulb-like structures in the tip of the germ tube, severe inhibition of fungal hyphae growth, and the absence of mycelium network were observed in the growth of the fungus on the surface of wheat spikes treated with the fungicide. TEM micrographs of the treated head showed thickening of the cell walls of the fungus, accumulation of vacuoles, and abnormal formation of inclusion bodies.

According to the Fungicide Resistance Action Committee (FRAC), triazoles have a medium risk for fungicide resistance; however, due to the extensive use of this chemistry, resistance has become increasingly important over the years (Becher et al. 2010). Strobilurin fungicides are beta methoxyacrylate compounds that target respiration in various fungi (Nason et al. 2007). Strobilurin A was isolated from *Strobilurus tenacellus,* and biotechnologically generated strobilurins were introduced to the market in 1996 (Ma and Michailides 2005). Strobilurins interfere with energy production by binding the cytochrome *b* of the cytochrome bc_1 complex and blocking the electron transport chain in fungi (Kathiravan et al. 2012).

Strobilurins have shown a broad spectrum of action against several fungi (powdery mildew, rusts, *Septoria*) as well as growth enhancement of plants. In plants, there are several physiological responses to strobilurin fungicides that are linked with growth-promoting effects. Changes in transpiration rates and hormonal balance and increase in nitrate reductase activity are part of the physiological changes caused by the strobilurins (Barlett et al. 2002; Tedford 2009). These changes represent a direct effect on enhancing CO_2 assimilation and water use efficiency, delaying senescence, all resulting in improved yields (Reddy 2012; Tedford 2009).

In wheat, transpiration rates are reduced as well as stomatal conductance (mmol m^{-2} s^{-1}), the net rate of photosynthesis, and intercellular carbon dioxide concentration in strobilurin treated plants versus controls (Nason et al. 2007); in essence, it results in better water use efficiency (Reddy 2012; Tedford 2009).

Strobilurin fungicides block ethylene production. Strobilurins inhibit the path from s-adenosyl-l-methionine to ethylene acting as an ACC synthase (Grossmann and Retzlaff 1997), affecting senescence directly. Azoxystrobin prolongs the time of green leaves on

wheat in a range of 8.2 to 11.2 days compared with the non-azoxystrobin treated control (Reddy 2012). Strobilurins reduce reactive oxygen species such as NO, O_2^-, H_2O_2 in plants and enhance the production of anti-oxidative enzymes such as superoxide dismutase, catalase, peroxidase, ascorbate peroxidase and glutathione reductase which could be related with the stay-green effect (Wu and Von Tiedemann 2001).

Tebuconazole alone, mixtures of tebuconazole and other fungicides, and the strobilurin azoxystrobin have shown optimal control of stem rust in wheat (Loughman et al. 2005; Wanyera et al. 2009). However, strobilurins play a different role in different wheat diseases. While strobilurin fungicides can reduce the incidence and severity of FHB and are effective in controlling foliar diseases, DON production has shown higher levels in strobilurin-treated plots than in the non-fungicide treated check plots (Amarasinghe et al. 2013).

Application of fungicides, especially demethylation inhibitors, is a part of the strategy to manage FHB (Wegulo et al. 2015c) and must be timed to protect the head; however, two applications (to control foliar and head diseases) are not economical (Wegulo et al. 2012). Although the importance of fungicides for controlling FHB and DON have been highlighted, many reviews and meta-analysis reports indicate incomplete control and differences based on cultivar. (Blandino et al. 2006, 2012; Mesterházy 2003, 2014; Paul et al. 2008; Pirgozliev et al. 2002).

The chemical control of FHB is based on the use of triazoles. Triazole dependency selects for the development of resistance in *F. graminearum* towards this chemical class. In 2014, tebuconazole-resistant *F. graminearum* was isolated for the first time in the Americas (Spolti et al. 2014). Therefore, there is a need to develop new fungicide chemistries for the control of this disease. Antifungal aminoglycosides such as K20

produced by bacterial actinomycetes (Takemoto et al. 2018) have been field tested for the control of *F. graminearum*. K20 fungicide has shown a synergistic activity in the control of *F. graminearum* when used together with triazoles (Takemoto et al. 2018). The use of two different chemistries of synergistic action with different target sites would reduce the risk of loss of efficacy of the triazoles against *F. graminearum*.

7. Cultivar resistance and techniques to evaluate breeding lines for FHB.

Genetic resistance has the potential to provide an effective control of FHB (Wegulo et al. 2015c). It is an ecologically and economically efficient strategy for management of the disease. Improvement of cultivar resistance has become a major wheat-breeding objective worldwide (Bai and Shaner 2004). However, breeding against FHB is challenging due to the quantitative nature of resistance, and technical difficulties when screening cultivars in inoculated experiments.

Wheat has a large and complex genome which poses difficulty in breeding (Tucker et al. 2017). Host resistance to FHB is conditioned by oligogenic to polygenic inheritance. Quantitative trait loci (QTLs) involved in FHB resistance have been identified on every wheat chromosome (Eckard 2015).

In the United States, breeding efforts against FHB began in 1929 with Christensen testing 350 wheat varieties and lines for response to “Fusarial head blight”. After nine years of experiments, Christensen concluded that all plants became infected to a greater or lesser degree.

Schroeder and Christensen (1963) proposed two types of resistance to FHB in wheat. Resistance to initial infection (type I resistance) includes defense reactions such as the activation of enzymes degrading the fungal cell wall or pathogenesis-related proteins. Type I resistance is estimated by spraying a spore suspension over flowering spikes and counting diseased spikelets. The QTLs *Fhb*4 and *Fhb*5 confer type 1 resistance (Kosaka et al. 2015).

Resistance to spread of blight symptoms within a spike (type II resistance) is associated with the movement of the pathogen from one infected spikelet to another via the rachis. This type of resistance is estimated by delivering conidia into a single floret

of a spike and counting the blighted spikelets after a period of time. QTLs mapped for type II resistance are *Fhb*1, *Fhb*2 and *Fhb*3 (Kosaka et al. 2015).

Mesterhazy (1995) proposed five types or components of resistance to FHB in wheat. Additionally to type I and II, he proposed type III or kernel size and number retention; which is assessed by observing the damage to the kernels, kernel number reduction, kernel weight, test weight, or visual estimates of *Fusarium*-damaged kernels; type IV resistance, or yield tolerance, which is assessed by measuring grain yield of naturally or artificially inoculated spikes or plots and comparing the data with spikes or plots without symptoms; and type V resistance, which is the resistance to accumulation of DON or ability to decompose DON, assessed by measuring DON concentration at a given level of FHB.

Major breeding efforts have been focused on the introgression of genes from cultivars showing native resistance to FHB (from adapted cultivars and breeding lines) (Clark et al 2016). Additionally, cultivars derived from Sumai 3 have shown unique genes for resistance to FHB (Kolb et al. 2001). The nature of cultivar resistance to FHB is horizontal, that is, many genes are involved (Mesterhazy et al. 1999).

Cultivars having the *Fhb*1 QTL can conjugate DON to a less toxic glucoside (D3G). This mechanism plays an important role in wheat resistance to FHB. Transgenic wheat expressing an UDP-glucosyltransferase for DON detoxification had significantly lower DON compared to controls. (Li et al. 2015) The resistance provided by Sumai 3 to DON accumulation is related to detoxification as well.

In 2006, Husker Genetics released Overland (NE01643), a semi-dwarf hard red winter wheat cultivar, moderately-resistant to FHB (Baenziger et al. 2008). In this

cultivar, five chromosomes have resistance alleles (1A, 1B, 3A, 4A and 6A) including known QTLs such as *Fhb*1, *Fhb*5, and Rht-B1 (Eckard et al. 2015).

Cultivars of wheat have shown contradictory effects for FHB severity and DON accumulation. Hernandez-Nopsa et al. (2014) found that a good yielding cultivar with moderate resistance against FHB had high concentrations of DON. In contrast, a susceptible cultivar had lower levels of DON than the moderately resistant cultivar.

Due to the sporadic nature of FHB epidemics, the evaluation of resistant varieties in the field is a big challenge; therefore, it is necessary to develop good and precise inoculation methods in order to replicate resistance results consistently. Actual methods used for screening often result in high experimental error and inconsistent ranking of genotypes (Kumar et al. 2015). Additionally, the relationship between DON and visual symptoms is not always clear, but in years of epidemics, this relationship is highly correlated.

Marker assisted selection (MAS) can be defined as the use of molecular markers to help in or substitute for phenotypic screening (Collard et al. 2008). Molecular markers are powerful tools to evaluate breeding lines. These markers are linked to the alleles or quantitative trait loci (QTL) of interest. QTL refers to a region of the genome that evidences an effect on a character. Genes and QTL are not synonymous terms since a QTL does not necessarily represent a single gene (Acquaah 2009). During the introgression of a QTL for resistance against FHB, it is necessary to monitor the behavior of the genetic material into the new line. Genomic selection uses all the genomic information to make predictions of genotypes (Lorenz et al. 2012). Genomic selection has proven to be more accurate than classical MAS (Hayes and Goddard 2001; Hefner 2010; Lorenz et al. 2012). Genomic selection allowed a map-based cloning of the *Fhb*1 QTL

from the Chinese wheat cultivar Sumai 3 (Rawat et al. 2016), which was a significant milestone in developing durable breeding strategies for resistance against FHB.

8. Storage of wheat grain.

Grain storage is an ancient practice implemented since early civilization, approximately 7,000 years ago (Reed 1992). Although stored grains are easily maintained in semi-arid conditions, this situation is less predictable with climate change. There are also significant costs associated with maintaining farm storage under both dry and cold conditions. Storage of food products is more critical under tropical conditions, where high relative humidity and temperature play a major role in spoilage. A common practice among wheat growers is to store grain in farm bins and silos. Storage time is driven by economic factors. Properly stored wheat grain should be cleaned and dried before storage. One of the major constraints during storage is the risk of losing grain quality; therefore, good management practices during storage of wheat grain are fundamental.

Wheat grain, after harvesting, enters a period of dormancy; at this stage, there is a reduction in respiration rates and physiological processes within the grain (Reed 2006). However, grain is a living organism susceptible to attack by pathogens, especially under deficient storage conditions. Loses during storage are mainly due to infestation by insects, damage caused by birds and rodents, and microbial growth—especially by molds (Sauer 1992).

Temperature and moisture (grain moisture content and environmental relative humidity) are the two physical factors most directly related to grain spoilage (Magan et al. 2004). During storage, grain with high moisture content can increase overall relative humidity and moisture in dry grain until equilibrium between dry grain and moistened grain is attained; additionally, dormancy of wheat seeds is broken when grain is moistened. The major problem with excess moisture is the formation of hot spots by respiration of storage microbes which can potentially cause bin burn (Fleurat-Lessard

2017). Broken kernels are more difficult to aerate as well as more susceptible to colonization by insects and molds (Sauer 1992).

Culture methods, such as direct plating of kernels in semi-selective media, are of little value when making accurate estimations of spoilage potential. Assessing grain infection visually can provide a rapid diagnostic of the deteriorating situation in grain; however, this evaluation can underestimate the concentration of compounds such as mycotoxins in healthy-looking grain. More precise estimations can be obtained by evaluating ergosterol concentrations or using molecular techniques to quantify molds and transcriptionally active genes of the mycotoxin production pathways.

9. Molds and mycotoxins in wheat grain.

Grain molds contribute to deterioration and rapid loss of quality. Mycotoxins associated with mold growth have enormous influence on grain quality loss. Molds can become a problem during grain storage, especially in silos that do not provide sufficient protection against external relative humidity or direct inflow of water, either by capillary exchange from the ground to the grain or by infiltration of rainwater in permeable silos.

During storage, *Penicillium*, *Aspergillus*, and *Fusarium* are the most prevalent mycotoxin producing molds (Wilson and Abramson 1992). Mold contamination of grain cannot be eliminated, but the biomass of fungi entering storage can be greatly diminished through good post-harvest practices (Reed 2006).

Mycotoxins are compounds produced by the secondary metabolism of molds. Although mycotoxins are not essential for growth, these compounds can play a major role in pathogenicity as well as in the colonization of an ecological niche by the mold which produces the mycotoxin. Worldwide, it is estimated that around 1 billion metric tons of food and food products are lost due to mycotoxin contamination every year (Schmale and Munkvold 2009).

Mycotoxin contamination can occur before, during, and after harvest, and it is rarely associated with only one phase (Wilson and Abramson 1992). Additionally, mycotoxin accumulation is an additive process which begins in the field and continues during and after harvest.

During storage, grain moisture content and temperature have a direct effect on spoilage, self-heating, grain respiration rates, and increased mycotoxin contamination (Magan et al. 2014; Reed 2006).

Total mold present, infection, and respiration rates are parameters used to evaluate grain deterioration during storage. Under conducive conditions, mold spores germinate and the hyphae extend and colonize the grain from the outside to the inside. Postharvest management, such as cleaning and drying of grain, assures a reduction of fungal growth rates as well as lower contamination of mycotoxins during storage.

In North America, the principal toxin accumulated in wheat grains is deoxynivalenol (DON) or vomitoxin, a member of a group of related mycotoxins, the trichothecenes. Grain elevators test for DON and will establish a discount in grain prices if the level of contamination surpasses acceptable limits. In the United States, the acceptable limit in grain is 2 μg/g at the elevators. The U.S. Food and Drug Administration (FDA) limits DON to 1 μg/g in food products and 10 μg/g in grain for feed purposes (Henry 2006). DON contamination is suspected to have caused a number of important health problems, such as mycotoxicosis in China associated with consumption of moldy wheat from 1961 to 1988; deoxynivalenol toxicosis in India with human food poisonings reported in 1987; Kashin-Beck disease in Russia (Miller and Trenholm 1994), and common dermatoxicosis among field workers on wheat fields (Reed 2006). In humans, when contaminated grain is ingested, it produces a toxic syndrome with symptoms such as diarrhea, nausea, vomiting, abdominal pain, headaches, and dizziness. In animals, symptoms of mycotoxin intoxication are vomiting, feed refusal, weakness and emaciation (Wegulo 2012).

Enzymatic activity has important consequences on DON. Feed additives can enzymatically de-epoxidize DON (Karlovsky 2011). Increases of 118% and 189% in DON have been registered in doughnuts during the baking step due to enzymatic conversions and not associated with fungal growth (Miller and Trenholm 1994). DON is highly stable and resistant to heat. Baking and brewing do not reduce DON; therefore,

DON produced in the field can be found at the end of the food chain, on the table of consumers.

Other important mycotoxins have been found in wheat, namely nivalenol (NIV) and zearalenone (ZEA). Although these toxins are less prevalent in wheat grain, their toxicity is higher compared to DON (Desjardins 2006). NIV causes liver damage, failure of the immune system, and toxicity to the reproductive system (Calori-Domingues et al. 2016). ZEA is an estrogenic mycotoxin and is linked with abortions in livestock, and immune and endocrine system morbidity (Zinedine et al. 2007).

10. qRT-PCR for gene expression analysis.

Quantitative reverse transcript polymerase chain reaction (qRT-PCR) is a molecular technique used to quantify RNA in real-time. Quantitative techniques such as qRT-PCR and q-PCR are highly sensitive and robust, and are applied in pathogen detection (Lievens et al. 2006), quantification of gene expression and microarray validation (Hallen-Adams et al. 2011), to identify single nucleotide polymorphisms (SNP) for genotyping (Yeh et al. 2004), for DNA damage measurements (Furda et al. 2014), and GMO detection (Vaitilingom et al. 1999).

qRT-PCR consists of several steps beginning with RNA extraction and subsequent amplification of its complementary DNA strand (cDNA). The messenger RNA (mRNA), or target, is a transient molecule, which acts as an intermediary between the DNA and the protein. A gene of interest may be expressed or not under given conditions or in a given tissue (Glazer and Nikaido 2007) and qRT-PCR is useful for quantifying the variation in expression of the gene.

The amount of mRNA per gene of interest varies depending on cellular factors (RNA half-life, type of tissue, cell age, etc.), post-transcriptional or post-translational control of gene expression (mRNA regulation, promoters, enhancers, etc.), or external factors (heat, stress, moisture, pH, organic components) (Weaver 2012). Additionally, there are genes for which mRNA is always present and in high quantities (housekeeping genes); inversely, there are genes that have only one mRNA molecule per cell. The abundance of mRNA is reflected in the number of times a reverse-transcribed cDNA appears in the pool of transcriptionally active genes (Baker et al. 2003; Weaver 2012).

qRT-PCR is advantageous as it is based on mRNA of transcriptionally active organisms while utilizing cDNA, which is more stable than the mRNA (transient

molecule and temperature-sensitive) (Weaver 2012). qRT-PCR is more reliable than quantitative end-point PCR methods, because there is no linear relationship between the starting copy number of a gene and the final yield of the amplified product (Green and Sambrook 2014).

In qRT-PCR, the PCR product is monitored cycle-by-cycle by combining thermal cycling, fluorescence detection, and application of a specific software. qRT-PCR uses different chemistries to report fluorescence at the moment a desired PCR amplicon is detected. The two most common methods for monitoring the change in amounts of double stranded PCR product are SYBR green and TaqMan (Green and Sambrook 2014). Fluorescence detection by SYBR green chemistry, also known as the intercalation method, consists of the binding of double stranded DNA with the dye at each cycle of amplification. Fluorescence is emitted and then detected during the amplification.

The main output of the qRT-PCR assay is the Ct (cycle threshold) value, which is a measure of the hybridization kinetics (Livak et al. 1995). Defining the threshold is an important step towards the correct and reliable analysis of qRT-PCR data. The threshold must be defined above the linear phase of the reaction and must exclude the background fluorescence (Green and Sambrook 2014). Several analytical methods consider the efficiency of a reaction; however, modifying the efficiency of the reaction per sample has been shown to yield highly variable results (Ruijter et al. 2009). Ct values are associated with the exponential growth of the PCR product and are turned into a usable value for ANOVA (VanGilder et al. 2008). Schmittgen and Livak (2008) proposed the report of individual quantitative real-time PCR Ct values as 2^{-Ct}.

Analyzing relative gene expression has been proposed to avoid bias due to intrinsic biological variation in the Ct values of replicates belonging to the same

condition/treatment. Gene expression can be relative to: i) a housekeeping gene (for example glyceraldehyde 3-phosphate dehydrogenase, *GAPDH*), ii) an external synthetic RNA, or iii) a reference index (average of several controls) (Pfaffl 2004). The most commonly-used method is the normalization of the gene expression through the ratio of the Ct of the housekeeping gene to the Ct of the gene of interest in a sample. Cts are used for the calculation of the $2^{-\Delta\Delta Ct}$ (Schmittgen and Livak 2008) where ΔCt is variation between the gene of interest and the reference gene and ΔΔCt is the variation between the ΔCt of the treatment and the ΔCt of a reference or calibrator.

11. *Tri5* gene expression in *F. graminearum* and its relationship with DON in grain.

The gene *Tri5* encodes the trichodiene synthase enzyme (Desjardins 2006) which is a key enzyme for the production of all trichothecene mycotoxins (Niessen et al. 2004). Trichodiene synthase catalyzes the isomerization and cyclization of farnesyl pyrophosphate to trichodiene, which is the first step in the trichothecene production pathway (Desjardins 2006). qRT-PCR has been used to study gene expression of *Tri5* (Doohan et al. 1999; Hallen-Adams et al. 2011; Han et al. 2018; Mudge et al. 2006).

Tri5 is a gene involved in secondary metabolism; therefore, the fungus can live without this gene. Furthermore, DON is not essential for stem colonization by *F. graminearum* or *F. pseudograminearum* in crown rot disease (Mudge et al. 2006). However, the ability of *F. graminearum* to colonize wheat is severely affected when the *Tri5* gene is disrupted (Proctor et al. 1995). DON appears to give *F. graminearum* an advantage under growth-limiting or stress conditions (Audenaert et al. 2013; Doohan et al. 1999; Glazer and Nikaido 2007; Jansen et al. 2005; Miller et al. 1983). The regulation of fungal secondary metabolites is complex. It is hypothesized that the fungus recognizes the host environment and induces genes for DON biosynthesis (Mudge et al. 2006).

In wheat, the expression of the *F. graminearum Tri5* gene is related to the inhibition of plant defense responses such as thickening of the cell wall during the colonization process (Mudge et al. 2006; Jansen et al. 2005). *Tri5* detection and expression in wheat is representative of an actively growing fungus. DON produced at the end of the biosynthetic pathway that begins with *Tri5* inhibits protein synthesis allowing movement of the fungus from cell-to-cell (Brown et al. 2012).

qRT-PCR can measure the abundance of transcripts of the *Tri5* gene *in planta* (Mudge et al. 2006). Hallen-Adams et al. (2011) detected significant differences in the expression pattern of DON biosynthetic genes. *F. graminearum in vitro* showed lower expression of DON biosynthetic genes when compared to growth *in planta* (Hallen-Adams et al. 2011). *Tri5* has been detected and is highly up-regulated at 7 days post inoculation in wheat heads (Mudge et al. 2006); after 72 hours post inoculation in barley (Hallen-Adams et al. 2011); and between cell division and cell differentiation stages in susceptible wheat cultivars (Chetouhi et al. 2016). In addition, in wheat, Brown et al. (2011) detected a high level of *Tri5* gene expression during initial asymptomatic infection. Infection by *F. graminearum* in susceptible wheat can be divided into three phases: an initial phase where DON and *Tri5* gene expression are correlated, an intermediate phase characterized by a rapid fungal growth and colonization of the head, and a third phase where *F. graminearum* develops independently of *Tri5* gene expression (Chetouhi et al. 2016). However, *Tri5* expression can be detected in senescent host tissue of an FHB-susceptible cultivar (Hallen-Adams et al. 2011).

High and positive correlations have been found between *Fusarium graminearum* biomass and DON accumulation (Horevaj et al. 2011; Kumar et al. 2015; Nicolaisen et al. 2009; Pirgozliev et al. 2008; Zhang et al. 2009). However, few gene expression studies have found positive relationships between *Tri5* expression and DON (Brown et al. 2012; Lee et al. 2014). Also, in some studies it has been shown that DON and *Tri5* expression are not correlated, especially in specific events of the incubation period. Hallen-Adams et al. (2011) found that DON levels were high and the ratio of gene expression *Tri5*/*GAPDH* was low, near or at the infection front. In the same study, the authors found that DON concentration increased later in the infection process. Early induction of *Tri*

genes in asymptomatic tissue has been detected in several studies of *Tri* gene expression (Hallen-Adams et al. 2011; Mudge et al. 2006; Chetouhi et al. 2016; Brown et al. 2011). Lee et al. (2014) hypothesized that *Tri* transcript accumulation is necessary before initiating DON or NIV biosynthesis. If DON biosynthesis is dependent on an initial *Tri* accumulation, then correlations between DON and gene expression of members of the *Tri* cluster are unlikely, as mRNA is transient and may have degraded by the time of detectable DON accumulation.

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CHAPTER II

EFFECTS OF CULTIVAR RESISTANCE, FUNGICIDE CHEMICAL CLASS, AND FUNGICIDE APPLICATION TIMING ON FUSARIUM HEAD BLIGHT IN WINTER WHEAT

1. Abstract.

Fusarium head blight (FHB), caused mainly by *Fusarium graminearum*, can cause devastating economic losses in small grain cereal crops. Management of FHB is by a combination of strategies and tactics including cultivar resistance, fungicide application at anthesis, and cultural practices. This study evaluated, under field conditions, the effects of cultivar resistance, fungicide chemical class (triazole versus strobilurin), fungicide application timing, and environment on FHB and its associated mycotoxin deoxynivalenol (DON). The moderately resistant hard red winter wheat cultivar Overland consistently had lower levels of FHB-index, *Fusarium*-damaged kernels (FDK), and DON, and higher yield compared to the susceptible hard red winter wheat cultivar Overley. Under the most FHB-favorable conditions (irrigation and wet growing season) and no fungicide application, FHB-index, FDK, DON, and yield in Overland were 39%, 55%, 46 µg/g, and 1819 kg/ha, respectively, compared to 80%, 79%, 78 µg/g, and 1122 kg/ha, respectively in Overley. The most effective fungicide treatment in reducing FHB, FDK, and DON and increasing yield was Prosaro (prothioconazole + tebuconazole) applied at anthesis. Application of Prosaro 6 days post anthesis (DPA) achieved a slightly lower but comparable efficacy to that achieved by the anthesis application. Application of Prosaro 12 DPA was least effective. The strobilurin fungicide Headline (pyraclostrobin) controlled FHB, FDK, and DON, but was less effective than

Prosaro. Under the most FHB-favorable conditions (irrigation and wet growing season) in the susceptible cultivar Overley, control relative to the untreated check achieved by Prosaro applied at anthesis, 6DPA, and 12 DPA was 47%, 30%, and 10%, respectively for FHB-index; 48%, 37%, and 13% for FDK; and 76%, 78%, and 52% for DON. Under the same conditions in the same cultivar, control relative to the untreated check achieved by Headline applied at anthesis, 6DPA, and 12 DPA was 23%, 9%, and -8%, respectively for FHB-index; 24%, 14%, and 5% for FDK; and 47%, 49%, and 39% for DON. In both cultivars, FHB-index, FDK, and DON were higher under irrigated compared to rainfed conditions and in a wet compared to a relatively dry growing season. The results from this study assert the benefits of using cultivar resistance as a management strategy for FHB and DON. Based on the results, triazole fungicides are recommended over strobilurins for control of FHB and DON, and the window of fungicide application can be extended by up to approximately one week after anthesis without significant loss of efficacy.

2. Introduction.

Fusarium head blight (FHB) or scab is a devastating disease of wheat and other small grain cereal crops. In the United States, the main causal agent of FHB is *Fusarium graminearum* (Dill-Macky 2010). FHB causes economic losses not only in yield and grain volume weight reduction, but also in discounts at the elevator due to accumulation of mycotoxins such as deoxynivalenol (DON, vomitoxin). In Nebraska, recent major outbreaks occurred in 2007, 2008, and 2015 (McMullen et al. 2012; Bolanos-Carriel et al. 2015). Although considerable research efforts have been undertaken to develop effective management strategies and tactics for FHB, the disease continues to be a major challenge

for growers. The acreage of wheat and barley have declined drastically due to, among other factors, FHB epidemics (McMullen et al. 2012).

Fusarium graminearum synthesizes mycotoxins as a byproduct of its secondary metabolism, especially under stress situations (Glazer and Nikaido 2007). Important mycotoxins produced by *F. graminearum* include the trichothecenes DON and nivalenol (NIV), as well as zearalenone (ZEA) (Desjardins 2006). Isolates are divided into chemotypes based on whether they produce NIV or one of the acetylated forms of DON (Liang et al. 2014; Ward et al. 2008). The main acetylated forms of DON are 3-ADON and 15-ADON (Greenhalgh et al. 1986).

Fungicide application is one of the management tactics for FHB. Fungicide chemical class and optimal application timing are critical for effective management of the disease. Several fungicides have been proposed to control FHB and DON; however, few of them have shown good efficacy. Efficacy of triazole-based fungicides in controlling FHB and DON has been demonstrated in field trials (Paul et al. 2008). Fungicide application is timed at anthesis because the most damaging infections occur on wheat spikes during that growth stage or thereafter (Andersen 1948). The narrow window of fungicide application at anthesis presents challenges to the grower who may be unable to apply at that time due to various reasons including unfavorable weather (wind, rainfall), scheduling with commercial applicators, or unavailability due to personal commitments. A wider application window can provide needed flexibility to the grower. However, it is not known how long after anthesis a fungicide can be applied and still provide acceptable efficacy. This knowledge gap can be filled by comparing the efficacy of a suitable fungicide applied at various time intervals starting at anthesis.

In the field, it has previously been demonstrated that strobilurin fungicides (quinone outside inhibitors (Q_oI)) significantly increase the levels of DON in wheat grain (Amarasinghe et al. 2013; Ellner 2005; Madden et al. 2014; Pirgozliev et al. 2002; Simpson et al. 2001). However, this phenomenon has not been demonstrated under Nebraska conditions.

Genetic resistance is the most effective and economical strategy for managing FHB. In 2006, the winter wheat cultivar Overland (NE01643) with moderate resistance to FHB and adaptation to rainfed conditions of Nebraska, South Dakota, and the northern Great Plains was released by the Nebraska wheat breeding program (Baenziger et al. 2008). Winter wheat cultivar Overley was released in Kansas in 2003 (Fritz et al. 2004) for its high yields, but it is susceptible to FHB. Knowledge of the reaction of moderately resistant and susceptible wheat cultivars to triazole and strobilurin fungicides will be useful in developing more effective management strategies for FHB. The objectives of this study were to evaluate the effects of 1) cultivar resistance, 2) fungicide chemical class, 3) fungicide application timing, and 4) environment on FHB, *Fusarium*-damaged kernels (FDK), DON, thousand kernel weight (TKW), and yield in a moderately resistant and a susceptible winter wheat cultivar under Nebraska field conditions.

3. Materials and methods.

Field experiments were conducted under rainfed (one experiment) and irrigated (one experiment) conditions during each of the 2015 and 2016 wheat growing seasons at the Eastern Nebraska Research and Extension Center (ENREC), formerly the Agricultural Research and Development Center (ARDC)) l near Mead, Nebraska (41.2286° N, 96.4892° W). Two hard red winter wheat cultivars, Overley (susceptible to FHB) and Overland (moderately resistant to FHB), were planted in the fall of 2014 and 2015. Plots

measured 1.22 m x 6.10 m in 2015 and 1.22 m x 4.57 m in 2016. Plots were maintained according to standard agricultural practices.

Weather data (Table 1) at the experimental sites were recorded with Watchdog® portable weather stations (Spectrum Technologies, Thayer Court, IL) placed in the alleys of plots. In the irrigated experiments, impact sprinklers (model 30H, Rain Bird, Azusa, CA) delivered approximately 0.6 inches / day of water to the plots for 5 minutes at 15-minute intervals from 10 a.m to 5 p.m. every day for the period May to June.

The experimental design was split plot in randomized complete blocks with four replications, with cultivars as the main plots and fungicide treatments as the subplots. Treatments consisted of a factorial arrangement of three fungicide treatments (triazole, strobilurin, and check) by three application timing (anthesis, 6 days post-anthesis (DPA), and 12 DPA). The fungicides used were tebuconazole + prothioconazole (triazole, Prosaro) at a rate of 0.47 L/ha and pyraclostrobin (strobilurin, Headline) at a rate of 0.66 L/ha.

Plots were inoculated by spreading *F. graminearum*-colonized maize kernels at a rate of 1.08 g/m^2 or approximately 300 kernels/m^2 weekly for three weeks beginning in late April. At anthesis and 6 and 12 DPA, fungicides were applied to the spikes using a CO_2-powered backpack sprayer equipped with four Tee-jet 800-LVS nozzles (Tee-Jet Technologies, Dillsburg, PA) spaced 30.5 cm apart and set at 241 kPa (35 psi). A volume of 150 L/ha of spray mixture was delivered to the plots. Nonionic surfactant (NIS 90-10, Precision laboratories, Waukegan, IL) was added to the spray mixture at 0.125% v/v. At 24 h after fungicide treatments, plots were inoculated by spraying the spikes with a suspension of *F. graminearum* spores using a back-pack sprayer. Spores

were harvested from potato dextrose agar (PDA) culture plates on which five Nebraska *F. graminearum* isolates were grown for three to five weeks at room temperature. The spores from the different isolates were mixed in sterile distilled water and the concentration was adjusted to 100, 000 spores/mL.

FHB-index (percentage of symptomatic spikelets on all spikes sampled), was assessed on 100 spikes in each plot (20 spikes in each of five randomly selected spots in each plot) at 16 and 21 days after fungicide application. At maturity, plots were harvested with a small plot combine (Wintersteiger, Dimmelstrasse, Austria) which recorded yield. The percentage of FDK was visually determined in subsamples of the harvested grain. Gas chromatography with electron capture detection (Tacke and Casper 1996) was used to measure DON in subsamples of grain that were previously ground to flour. DON measurement was done at the North Dakota Veterinary Diagnostic Laboratory.

Fungicide efficacy for FHB-index, FDK and DON was determined with the following formula:

$$E = \left[\frac{(C - F)}{C}\right] \times 100$$

where E is the efficacy, C is the check value, and F is the fungicide treatment value

Fungicide efficacy for yield was determined using the following the formula:

$$E = \left[\frac{(F - C)}{F}\right] \times 100$$

Data were analyzed with SAS software version 9.4 (SAS Inc, Cary, NC) using generalized linear mixed models. Based on heterogeneous error variances determined by

the F-ratio test (Gomez and Gomez 1984), data from each experiment were analyzed separately. Fisher's least significant difference (LSD) test at P = 0.05 was used to compare pairs of treatment means. In this paper, when comparing treatment means, use of "significant", "significantly", "differed", "did not differ", "higher", "lower", "increased", or "reduced" is in reference to the LSD at P = 0.05. F-values for treatment effects and their interactions were considered significant at $P \leq 0.05$. To determine the effect of irrigated or non-irrigated environment on FHB, FDK, DON, and yield, year was considered a replication and means of these variables in each cultivar were averaged over fungicide treatments. Means were averaged over fungicide treatments and irrigation environments to determine the effect of year-specific environment on the same variables.

4. Results.

4.1 2015 Growing season, rainfed experiment. Under rainfed conditions, the effect of cultivar on all measured variables (FHB-index, FDK, DON, TKW, and yield) was significant ($P \leq 0.0139$). The effect of fungicide chemical class (Prosaro = triazole versus Headline = strobilurin) was significant ($P \leq 0.0320$) for all variables except FHB-index and yield. The effect of fungicide application timing was similarly significant ($P \leq 0.0024$) for all variables except FHB-index. The orthogonal comparisons "fungicide application at anthesis versus application at 6 and 12 DPA" were significant ($P \leq 0.0119$) for all measured variables whereas the orthogonal comparisons "chemical class x fungicide application timing versus the check" were significant ($P < 0.0001$) for all variables except FHB-index and DON (Table 2).

Overall, the FHB-index was lower in cv. Overland (here after referred to as Overland) than in cv. Overley (here after referred to as Overley) (Fig. 1). In Overley, the untreated

check plots had the highest FHB-index, which did not significantly differ (P = 0.05) from that in Headline- or Prosaro-treated plots at 12 DPA. In Overland, FHB-index did not differ among any treatments including the untreated check (Table 3). Overley had much higher FDK than Overland (Fig. 2). In both cultivars, plots treated at anthesis and 6 DPA with both fungicides had lower FDK than plots treated at 12 DPA and the untreated check plots. Within each cultivar, differences in FDK between the untreated check plots and Headline-treated plots at 12 DPA were not significant (Table 3). DON was higher in Overley than in Overland (Fig. 3) and ranged from 33 to 46 µg/g in the fungicide treated plots of Overley compared to 64 µg/g in the untreated check plots (Table 3). There were no significant differences between Prosaro-treated Overley plots sprayed at anthesis, 6 DPA, and 12 DPA versus Headline-treated plots sprayed at anthesis (Table 3). In Overland, DON concentrations in fungicide treated plots ranged from 6 to 14 µg/g compared to 16 µg/g in the untreated check (Table 3). DON in Headline-treated plots did not differ from that in untreated check plots regardless of application timing (anthesis, 6 DPA, and 12 DPA) (Table 3). TKW was higher in Overland than in Overley and lowest in Overley untreated check and Headline-treated plots sprayed at 12 DPA (Table 3). The highest TKW was from Overland plots treated with either fungicide at anthesis. Yield was higher in Overland than in Overley in all fungicide treatments including the untreated check (Fig. 4). The highest yield was from Overland plots treated with Headline at anthesis and the lowest yield was from Overley untreated check plots (Table 3).

4.2 2015 Growing season, irrigated experiment. Under irrigated conditions, differences between cultivars and among fungicide treatments were more evident, with the effects of cultivar, fungicide chemical class and fungicide application timing on all

variables measured (FHB-index, FDK, DON, TKW, and yield) significant ($P \leq 0.0283$) except the effects of fungicide chemical class on TKW and yield. The orthogonal comparisons "fungicide application at anthesis versus application at 6 and 12 DPA" were significant ($P \leq 0.0231$) for all measured variables except yield whereas the orthogonal comparisons "chemical class x fungicide application timing versus the untreated check" were significant ($P \leq 0.0097$) for all variables except DON (Table 2). FHB-index was approximately twice as high in Overley as in Overland (Fig. 1). In Overley, Headline-treated plots at 12 DPA showed the highest FHB-index. This value did not significantly differ from the values in Headline-treated plots at 6 DPA, Prosaro–treated plots at 12 DPA, and the untreated check plots. In Overland, there were no differences in FHB-index among all treatments (Table 4). Overall, Overley had higher FDK than Overland (Fig. 2). In Overley, untreated check plots and Headline-treated plots sprayed at 12 DPA showed the highest levels of FDK. There were no significant differences in the levels of FDK between the Prosaro-treated plots sprayed at anthesis and at 6 DPA, as well as in the Headline-treated plots sprayed at anthesis and 6 DPA. In Overland, the highest FDK levels were from Headline-treated plots sprayed at 6 and 12 DPA and in the untreated check and the lowest levels were from Prosaro-treated plots sprayed at anthesis and 6 DPA (Table 4). DON was higher in Overley compared to Overland (Fig. 3). In Overley, it ranged from 17 to 48 µg/g in fungicide treated plots compared to 78 µg/g in the untreated check. It was lowest in Prosaro-treated plots sprayed at anthesis and 6 DPA. DON in these two treatments was similar but significantly lower than in all Headline treatments and the Prosaro treatment at 12 DPA. In Overland, DON in fungicide treated plots ranged from 8 µg/g in the Prosaro anthesis treatment to 37 µg/g in the Headline anthesis treatment compared to 46 µg/g in the untreated check (Table 4). TKW was

generally higher in Overland than in Overley. The lightest kernels in both cultivars were from the untreated check plots and Headline-treated plots sprayed at 12 DPA. Yield was significantly higher in Overland than in Overley only in the untreated check plots. Although it was higher in Overland than in Overley in Prosaro- and Headline-treated plots, these differences in yield between the two cultivars were not significant (Fig. 4). The highest yield was from Overland plots treated with Prosaro at anthesis. Differences in yield among all other fungicide application timing in both cultivars and for both fungicides were mostly non-significant (Table 4).

4.3 2016 Growing season, rainfed experiment. The effect of cultivar was significant ($P \leq 0.0339$) for all measured variables except yield. The effect of fungicide chemical class was significant ($P = 0.0141$) only for FHB-index whereas the effect of fungicide application timing was significant ($P \leq 0.0136$) for FHB-index, DON, and yield. The orthogonal comparisons "fungicide application at anthesis versus application at 6 and 12 DPA" were significant ($P \leq 0.0380$) for all measured variables except DON whereas the orthogonal comparisons "chemical class x fungicide application timing versus the check" were significant ($P < 0.0001$) for FHB-index and yield (Table 2).

FHB intensity was lower in 2016 compared to 2015. As in 2015, FHB-index was higher in Overley than in Overland (Fig. 1). In Overley, it ranged from 14% in Prosaro-treated plots sprayed at anthesis to 33% in Headline-treated plots sprayed at 12 DPA and 38% in untreated check plots. Both fungicides were most effective in reducing FHB when applied at anthesis followed by 6 DPA and least effective when applied at 12 DPA. In Overland, FHB-index ranged from 3% in plots treated with Prosaro at anthesis to 14% in the untreated check. All fungicide treatments (Prosaro and Headline) timed at anthesis,

6 DPA, and 12 DPA did not significantly differ in FHB-index, but had index values that were significantly lower than that in the untreated check (Table 3). FDK was low (4.2-11 %) but generally higher in Overley than in Overland (Fig. 2). Differences in FDK among fungicide treatments were not significant in both cultivars (Table 3). DON was similarly low (0.4-1.4 µg/g in Overley; 0.1-0.2 µg/g in Overland, Table 3) but higher in Overley than in Overland (Fig. 3). TKW was higher in Overley than in Overland (Fig. 4). In Overley, it ranged from 27 g in the untreated check to 33 g in Headline plots sprayed at anthesis and 35 g in Prosaro-treated plots sprayed at 6 DPA. In Overland, TKW did not differ among treatments and ranged from 23 g in the untreated check to 25 g in Headline plots sprayed at anthesis (Table 3). Overland yielded higher in the untreated check, Prosaro, and Headline treatments; however, these differences were not significant (Fig. 4). Yield ranged from 2208 kg/ha (Headline applied at 12 DPA) to 4061 kg/ha (Headline applied at anthesis). Later application timing of both fungicides (12 DPA) resulted in lower yield than earlier application timing (anthesis and 6 DPA) (Table 3).

4.4 2016 Growing season, irrigated experiment. The effect of cultivar was significant ($P \leq 0.0039$) for FHB-index, DON, and TKW. The effect of fungicide chemical class was significant ($P \leq 0.0310$) only for FHB-index and FDK whereas the effect of fungicide application timing was significant ($P \leq 0.0530$) for all measured variables except DON. The orthogonal comparisons “fungicide application at anthesis versus application at 6 and 12 DPA” were significant ($P \leq 0.0280$) for all measured variables except DON and TKW whereas the orthogonal comparisons “chemical class x fungicide application timing versus the check” were significant ($P \leq 0.0236$) for all variables except DON (Table 2). FHB-index was higher in Overley compared to Overland (Fig. 1). In Overley it ranged from 23% in the Headline anthesis treatment to

67% in the Headline treatment applied 12 DPA and 74% in the untreated check. In Overland it ranged from 13% to 37% in the Headline treatment applied 12 DPA and 38% in the untreated check. In both cultivars and for both fungicides, the anthesis treatments were the most effective and the treatments applied 12 DPA were the least effective in reducing FHB-index. FDK was higher in Overley than in Overland in the untreated check and Headline treatments but not in the Prosaro treatment (Fig. 2). In Overley, FDK was highest in the untreated check (39%) and Headline treatment applied at 12 DPA (43%) and lowest in Prosaro anthesis treatment (22%). In Overland, FDK was similarly highest in the untreated check (26%) and Headline treatment applied at 12 DPA (27%) and lowest in Prosaro anthesis treatment (14%) (Table 4). DON was generally low but significantly higher in Overley than in Overland (Fig. 3). In Overley, it ranged from 2.6 µg/g in the Prosaro treatment applied at 6 DPA to 4.2 µg/g in the anthesis Headline treatment. DON was very low in Overland, ranging from 0.6 to 1.2 µg/g with no significant differences among all treatments (Table 4). TKW was greater in Overley than in Overland (Fig. 4). In Overley, it highest in the Headline anthesis treatment (30 g) and lowest in the Headline treatment applied 12 DPA (25 g) and the untreated check (26 g) with the values in the latter two treatments significantly lower than in all other treatments (Table 4). Yield was not significantly different among cultivars and fungicide treatments (Fig. 5) and ranged from 1865 kg/ha in the Overley untreated check to 3492 kg/ha Prosaro anthesis treatment in Overley (Table 4).

4.5 Effect of fungicide application timing on FHB-index, FDK, DON, TKW, and yield. In the 2015 rainfed experiment, fungicide application timing (0, 6 or 12 DPA) generally did not have an effect on FHB-index in either Overley or Overland. In the irrigated experiment, however, FHB-index in Overley was higher when Headline was

applied 6 and 12 DPA compared to the anthesis application. In Overland in the irrigated experiment, fungicide application timing had no effect on FHB-index. In the 2016 rainfed experiment, FHB-index was significantly higher in Overley treated with Headline 12 DPA compared to the anthesis and 6 DPA applications. In Overland in the same experiment, fungicide application timing had no effect on FHB-index. In the 2016 irrigated experiment, FHB-index increased with each delay in fungicide application in both cultivars and for both fungicides, and this trend was most pronounced in Overley treated with Headline (Fig. 6).

In the 2015 rainfed experiment, FDK was significantly higher in Overley treated at 12 DPA with both Headline and Prosaro compared to the anthesis and 6 DPA applications. In Overland, there was no effect of fungicide application timing on FDK. In the 2015 irrigated experiment in Overley, FDK was higher in both the Headline and Prosaro treatments applied 12 DPA compared to the anthesis treatments. In Overland, FDK was similar in the 6 and 12 DPA Headline treatments, but higher in these two treatments than the anthesis Headline treatment. In the Prosaro treatments in Overland, FDK significantly increased with each delay in fungicide application. In the 2016 rainfed experiment, there was no effect of fungicide application timing on FDK in both cultivars and for either fungicide. In the irrigated experiment, FDK was higher in Overley treated with Headline at 12 DPA compared to the anthesis and 6 DPA treatments whereas in Overland, FDK was higher in the Prosaro treatments applied at 6 and 12 DPA compared to the anthesis treatment (Fig. 7).

A significant effect of fungicide application timing on DON in the 2015 rainfed experiment was observed only in Overley treated with Headline where the 12 DPA

treatment resulted in more DON than the anthesis and 6 DPA treatments. In the 2015 irrigated experiment, there was no effect of fungicide application timing on DON in either cultivar. Similarly, there was no effect of fungicide application timing on DON in either cultivar in both the rainfed and irrigated experiments in 2016 (Fig. 8).

An effect of fungicide application timing on TKW was observed only in 2015. In the rainfed experiment, TKW was lower when both fungicides were applied to both cultivars at 12 DPA compared to the anthesis applications. In the rainfed experiment, TKW was lower in Overley treated with both fungicides at 12 DPA compared to the anthesis and 6 DPA treatments (Fig. 9). Yield was significantly reduced with each delay in fungicide application in both experiments in 2015 and in the rainfed experiment in 2016 (Fig. 10).

4.6 Fungicide efficacy. Overall, Prosaro applied at anthesis achieved the highest efficacy in reducing FHB-index, FDK, and DON, as well as increasing yield. Among the Headline treatments, the anthesis application also achieved the highest efficacy, but this efficacy was in general lower than that achieved by Prosaro applied at anthesis. In both Overley and Overland and for both fungicides, efficacy declined with each delay in fungicide application, with applications at 12 DPA having the lowest efficacy. Efficacy in reducing DON was much higher for Prosaro than for Headline (Table 5).

4.7 Effect of environment on FHB-index, FDK, DON, and yield. Averaged over fungicide treatments and years, FHB-index and FDK were significantly higher in the irrigated compared to the rainfed environment in both cultivars. DON did not differ between the irrigated and rainfed environments in Overley, but was higher in the irrigated compared to the rainfed environment in Overland. Yield did not differ between the

irrigated and rainfed environments in Overley, but was lower in the irrigated compared to the rainfed environment in Overland (Fig. 11). Averaged over fungicide treatments and irrigation environments, FHB-index, FDK, and DON were higher and yield was lower in 2015 when there was above average rainfall that favored high FHB intensity compared to 2016 when a dryer environment was unfavorable to disease development (Fig. 12).

5. Discussion

Integration of cultivar resistance with other management strategies is an effective and recommended approach to managing FHB in wheat and other small grain cereal crops. In this study, Overland, a moderately resistant winter wheat cultivar, consistently had lower FHB-index, FDK, and DON and yielded higher than the susceptible Overley. In a previous study which evaluated 363 U.S. winter wheat accessions (Jin et al. 2014), Overland was among the accessions that displayed low levels of FHB and DON, which is in agreement with the results obtained in this study. Previous research has similarly shown moderately resistant cultivars to have lower FHB-index, FDK, DON and higher yield than susceptible cultivars. Wegulo et al. (2011) demonstrated in the field that under high FHB intensity and no fungicide application, the moderately resistant winter wheat cultivars Roane and Truman had lower FHB severity, FDK, and DON and higher yield than susceptible cultivars Overley and Tomahawk. In a field study by Amarasinghe et al. (2013), the moderately resistant spring wheat cultivar Glenn similarly had lower FHB severity, FDK, and DON and yielded higher than the susceptible cultivar Roblin. Willyerd et al. (2012) analyzed data from more than 40 field trials conducted in 12 U.S. states and found that the wheat cultivars classified as moderately resistant, fungicide-untreated had lower FHB-index and DON compared to the cultivars classified as susceptible or moderately susceptible, fungicide-untreated.

Triazoles and strobilurins are the two classes of fungicides most widely used to control foliar and spike diseases of wheat. In this study, the triazole fungicide Prosaro was more effective in reducing FHB, FDK, and DON and increasing TKW and yield than the strobilurin fungicide Headline. This result is consistent with results from previous studies that compared the efficacy of triazoles and strobilurins in controlling FHB and DON. Pirgozliev et al. (2002) found the strobilurin azoxystrobin to be less effective in reducing FHB and DON than the triazole metconazole. In a meta-analysis of 292 uniform fungicide field trials conducted in 17 U.S. states from 1995 to 2013, Paul et al. (2018) found that triazoles applied to wheat at anthesis were more effective in reducing FHB and DON than strobilurins.

In addition to the inferior efficacy of strobilurins in reducing FHB and DON, this class of fungicides has been demonstrated in several studies to elevate DON. Ellner (2005) reported that in 85% of the plots in 23 field trials, strobilurins applied before anthesis (Zadoks growth stages 33, 49, and 55; Zadoks et al. 1974) increased DON by up to 65% compared to the untreated check plots. Simpson et al. (2001) and Mesterházy et al. (2003) similarly reported increases in DON in wheat grain from field plots treated with the strobilurin azoxystrobin. In this study, the field-applied strobilurin did not increase DON over the untreated check. However, when grain from the 2015 growing season was cleaned to remove FDK so it could be used in a separate study, DON in grain from strobilurin-treated plots was higher than DON from untreated check plots (Bolanos-Carriel et al. 2016).

The optimum fungicide application timing to control FHB and DON in the field is at anthesis because most infections occur during this growth stage or shortly thereafter

(Andersen 1948). However, the short window during anthesis presents challenges to the grower who may not be able to spray a fungicide during that time due to various reasons including unfavorable weather, scheduling with commercial applicators, or unavailability due to personal commitments. Therefore, there have been efforts to determine whether post-anthesis fungicide applications can be effective in controlling FHB and DON. In this study, the effect of fungicide application timing was most apparent in the triazole (Prosaro) treatments applied to the susceptible cultivar Overley in the irrigated environments, which favored high levels of FHB. In these treatments, FHB-index, DON, and FDK were similar between the anthesis and 6 DPA applications, but generally lower in the anthesis application. However, FHB-index, FDK, and DON the levels were significantly higher in the 12 DPA application compared to the two earlier applications. These results suggest that fungicide application can be delayed by about one week and still achieve effective control of FHB and DON.

These results are similar to those obtained by D'Angelo et al. (2014) who found that FHB-index, FDK, and DON levels in post-anthesis applications of up to 6 days were generally not significantly different from those in earlier applications, although the earlier applications resulted in higher percent control compared to the untreated check. Meta-analysis of data from 19 years of fungicide field trials (Paul et al. 2018) showed that pre-anthesis (at heading) applications of the triazole fungicides Caramba (metconazole) and Prosaro were much less efficacious in controlling FHB and DON than applications at anthesis or 5 to 7 days later. The anthesis applications resulted in better percent control of FHB-index compared to applications 5 to 7 days later. However, the difference in percent control between the two timing was not large (6% and 10% for Caramba and Prosaro, respectively). For DON there was no difference in percent control between the

anthesis and the late (5 to 7 days) applications (3% and 1% for Caramba and Prosaro, respectively). These results are similar to those obtained in this study and similarly suggest that a triazole application about one week post anthesis can control FHB and DON with comparable efficacy to that achieved with anthesis applications.

The meta-analysis by Paul et al. (2008) discussed in the preceding paragraph and the results from this study indicate that applications of triazole fungicides to wheat before anthesis or too long past anthesis (12 days in this study) are not recommended because their efficacy in reducing FHB and DON is too low. In this study, efficacy of Prosaro in reducing FHB-index, FDK, and DON was highest when it was applied at anthesis, generally decreased with each 6-day delay in application, and was lowest when the fungicide was applied 12 DPA. This suggests, as stated above, that the best window for triazole fungicide application to wheat for control of FHB and DON is anthesis to approximately one week later.

Environment played a significant role in the development of FHB. FHB and DON developed to higher levels in the irrigated compared to the rainfed experiments in both years, and in 2015 when there was above average rainfall before and during anthesis compared to 2016 when there was much less precipitation. This was expected since FHB is favored by moisture before and during anthesis, as demonstrated in previous studies (Cowger et al. 2009; Hernandez Nopsa et al. 2012; Kriss et al. 2012)

This study demonstrated the value of cultivar resistance as a management strategy for FHB and DON. The moderately resistant cultivar Overland consistently had lower levels of FHB, FDK, and DON in FHB-favorable field environments compared to the susceptible cultivar Overley. The study confirmed, for the first time, that under Nebraska

field conditions, a strobilurin fungicide was less effective in reducing FHB, FDK, and DON compared to a triazole fungicide. In addition, the study showed that anthesis applications of a triazole fungicide were most effective in reducing FHB, FDK, and DON, but applications at 6 DPA had comparable efficacy, indicating that growers have a wider window during which they can apply a triazole fungicide to control FHB and DON during disease-favorable growing seasons.

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Table 1. Weather conditions at the experimental plots near Mead, Nebraska during the 2015 and 2016 growing seasons.

Environment		Temperature ºC						Precipitation (mm)[a]		Relative Humidity (%)	
		2015			2016			2015	2016	2015	2016
		Min.	Max.	Avg.	Min.	Max.	Avg.				
Rainfed	May	0.72	31.2	16.0	0.94	30.7	15.6	193	186	66.4	60
	June	11.2	35.8	22.5	10.2	38.4	25.9	104	68	71.3	60
Irrigated	May	1.8	33.6	15.9	0.83	31.0	15.8	234[a]	251[a]	64.4	60.6
	June	10.9	36.0	22.4	10.0	37.3	25.1	599[a]	572[a]	69.7	63.7

[a] Precipitation includes rainfall and irrigation water

N/A = not applicable as the values of DON in the dryland experiment for the growing season 2016 were below of the limit of quantification.

Conservative T Grouping for cult*trt Least Squares Means (α =0.05)

Negative values are means for treatment lower than the means for the non-fungicide sprayed check plots

Same letters in the same column are not different (LSD α=0.05)

Table 2. P > F values from analysis of variance of data from experiments conducted near Mead, Nebraska to determine the effects of cultivar resistance, fungicide chemical class, and fungicide application timing on Fusarium head blight (FHB) index, *Fusarium*-damaged kernels (FDK), deoxynivalenol (DON), thousand kernel weight (TKW), and yield in two winter wheat cultivars under rainfed and irrigated conditions during the 2015 and 2016 growing seasons

Effect	Index		FDK		DON		TKW		Yield	
	2015	2016	2015	2016	2015	2016	2015	2016	2015	2016
					Rainfed					
Cultivar (C)	0.0058	0.0037	0.0019	0.0328	0.0006	0.0339	<.0001	0.0020	0.0139	0.1074
Fungicide (F)	0.3315	0.0141	0.0082	0.1425	0.0171	0.3698	0.0320	0.7424	0.3987	0.8286
Timing (T)	0.2984	<.0001	<.0001	0.4883	0.0024	0.0136	<.0001	0.1571	<.0001	<.0001
A vs. 6 and 12	0.0119	<.0001	<.0001	0.0263	<.0001	0.4056	<.0001	<.0001	0.0003	0.0380
Fact vs. Add[a]	0.6060	<.0001	<.0001	0.2998	0.0678	0.8870	<.0001	0.5438	<.0001	<.0001
F*T	0.8828	0.5244	0.4342	0.7483	0.7735	0.8517	0.5619	0.0797	0.0085	0.6182
C*F	0.3211	0.0835	0.1675	0.1425	0.3903	0.6663	0.7579	0.7396	0.5262	0.7055
C*T	0.0923	0.0054	0.0068	0.0393	0.3066	0.0032	0.0121	0.2156	0.0098	0.0809
C*M*F	0.7368	0.6236	0.9559	0.7724	0.5104	0.8677	0.0349	0.0086	0.0256	0.1192
					Irrigated					
Cultivar (C)	<0.0001	<0.0001	0.0019	0.0789	0.0283	<0.0001	0.0090	0.0039	0.1217	0.1974
Fungicide (F)	0.0071	<0.0001	<.0001	0.0310	<.0001	0.0862	0.8095	0.5999	0.2174	0.6536
Timing (T)	0.0191	<0.0001	<.0001	0.0002	0.0009	0.1890	0.0037	0.0006	<.0001	0.0530
A vs. 6 and 12	0.0231	<.0001	<.0001	0.0020	<.0001	0.1452	0.0017	0.9766	0.2983	0.0280
Fact. vs. Add.	0.0097	<.0001	<.0001	0.0009	0.7536	0.6442	0.0062	<.0001	0.0001	0.0236
F*T	0.4024	0.0387	0.0347	0.0425	0.0320	0.6232	0.2534	0.0149	0.1680	0.6514
C*F	0.1587	0.2126	0.2441	0.0108	0.8106	0.1635	0.1698	0.1900	0.1506	0.4549
C*T	0.0250	0.0035	0.3060	0.2398	0.1330	0.0881	0.1021	0.4278	0.2866	0.5575
C*M*F	0.8673	0.0428	0.8744	0.4335	0.2236	0.4615	0.2590	0.6976	0.6187	0.8388

[a]Additional contrasts (Add) were fungicide application at anthesis versus 6 and 12 days post anthesis (A vs. 6 and 12 DPA) and factorial (Fact) (chemical class x fungicide application timing) versus additional (non-sprayed check)

Table 3. Means of Fusarium Head blight index (FHB-index), *Fusarium*-damaged kernels (FDK), deoxynivalenol (DON), thousand kernel weight (TKW), and yield from experiments conducted near Mead, Nebraska to determine the effects of cultivar resistance, fungicide chemical class, and fungicide application timing on the variables in two winter wheat cultivars under rainfed conditions during the 2015 and 2016 growing seasons

Cultivar	Fungicide Treatment	FHB-index (%)		FDK (%)		DON (µg/g)		TKW (g)		Yield (kg/ha)[5]	
		2015	2016	2015	2016	2015	2016	2015	2016	2015	2016
Overley	Check	59.5 a[a]	38.4 a	77.5 a	11.0 a	63.8 a	1.2 ab	16.5 f	26.5 e-g	677 j	2731 ef
	Prosaro A	40.5 bc	13.5 fg	43.2 c	6.2 bc	34.9 cd	0.8 b-d	23.9 b	31.2 b-d	1484 e-h	3542 a-d
	Prosaro 6 DPA	26.9 cd	20.9 de	43.8 c	6.0 bc	33.0 d	0.4 ed	22.1 cd	34.8 a	1284 g-i	3160 c-e
	Prosaro 12 DPA	48.2 ab	29.1 bc	61.0 b	7.2 a-c	37.8 cd	1.4 a	22.1 cd	30.3 cd	1114 h-j	2775 ef
	Headline A	43.8 b	18.9 ef	48.0 c	8.8 ab	37.8 cd	1.1 a-c	21.7 d	33.2 ab	1468 e-h	4061 a
	Headline 6 DPA	39.6 bc	26.6 cd	53.0 bc	7.8 a-c	40.2 bc	0.6 c-e	18.4 e	31.3 b-d	1428 g-i	2756 ef
	Headline 12 DPA	50.2 ab	33.4 ab	74.0 a	10.2 a	45.6 b	1.4 a	18.0 ef	31.8 b-d	933 ij	2208 f
Overland	Check	13.5 de	13.8 ef	29.2 d	7.2 a-c	16.3 e	0.1 e	23.9 bc	22.9 h	1942 c-e	3113 c-e
	Prosaro A	6.5 e	2.6 h	11.5 f	5.8 bc	6.2 f	0.1 e	28.2 a	23.8 gh	2531 b	3958 ab
	Prosaro 6 DPA	11.8 de	5.9 gh	16.5 ef	7.8 a-c	10.5 ef	0.1 e	27.3 a	23.3 h	2159 b-d	3590 a-d
	Prosaro 12 DPA	7.4 de	9.6 fg	17.8 ef	4.2 c	9.6 ef	0.1 e	25.4 b	24.2 gh	2340 bc	3077 de
	Headline A	6.2 e	4.5 gh	11.5 f	4.2 c	10.7 ef	0.1 e	28.4 a	24.6 f-h	3088 a	3745 a-c
	Headline 6 DPA	10.5 e	8.6 f-h	20.2 d-f	8.2 ab	11.3 ef	0.2 e	25.0 b	24.3 f-g	1874 d-f	3636 a-d
	Headline 12 DPA	8.6 de	7.2 gh	23.0 de	5.2 bc	14.0 e	0.1 e	24.9 b	23.2 h	1694 e-g	3313 b-d

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

Table 4. Means of Fusarium Head blight index (FHB-index), *Fusarium*-damaged kernels (FDK), deoxynivalenol (DON), thousand kernel weight (TKW), and yield from experiments conducted near Mead, Nebraska to determine the effects of cultivar resistance, fungicide chemical class, and fungicide application timing on the variables in two winter wheat cultivars under irrigated conditions during the 2015 and 2016 growing seasons

Cultivar	Fungicide Treatment	Index (%)		FDK (%)		DON (µg/g)		TKW (g)		Yield (kg/ha)[5]	
		2015	2016	2015	2016	2015	2016	2015	2016	2015	2016
Overley	Check	80.3 ab[a]	73.5 a	79.2 a	39.0 a	78.1 a	4.1 ab	19.8 ef	25.8 b-d	1122 cd	1865 c
	Prosaro A	42.2 de	28.1 e-h	41.5 g-h	21.5 b-d	19.0 de	2.9 cd	23.7 a-d	27.9 ab	1656 b-d	3492 a
	Prosaro 6 DPA	56.1 cd	34.9 d-f	50.2 e-g	22.5 c-d	16.8 de	2.6 d	25.1 ab	27.5 a-c	1272 b-d	2605 a-c
	Prosaro 12 DPA	72.3 a-c	51.3 b	68.8 bc	25.0 bc	37.4 bc	3.7 a-c	22.2 b-f	28.0 ab	988 d	2928 ab
	Headline A	62.0 b-c	23.0 gh	60.5 cd	26.8 b	41.4 bc	4.2 a	24.4 a-c	29.6 a	1525 b-d	3044 ab
	Headline 6 DPA	72.7 a-c	45.3 bc	68.2 bc	26.2 bc	40.2 bc	3.1 b-d	20.6 d-f	27.6 ab	1324 b-d	2798 a-c
	Headline 12 DPA	86.4 a	67.2 a	75.0 ab	43.2 a	48.0 b	4.1 ab	19.6 f	25.1 c-e	1147 b-d	2376 bc
Overland	Check	38.6 d-f	37.7 c-d	54.8 d-f	25.5 cd	46.1 g	1.1 e	21.5 c-f	21.2 fg	1819 b-d	2332 bc
	Prosaro A	19.2 f	13.1 h	22.0 i	13.8 d	7.8 e	0.6 e	24.6 a-c	20.1 f-h	2914 a	2817 a-c
	Prosaro 6 DPA	22.7 ef	18.9 gh	34.2 i	25.2 b	11.2 e	1.1 e	22.9 b-e	17.6 i	2050 bc	2662 a-c
	Prosaro 12 DPA	28.4 ef	31.0 d-f	45.8 fg	24.2 bc	18.3 de	0.8 e	24.8 ab	18.7 g-i	1481 b-d	2166 bc
	Headline A	35.4 ef	19.6 gh	42.8 g-h	15.2 cd	37.2 b-e	0.7 e	26.5 a	21.9 ef	2079 b	2945 ab
	Headline 6 DPA	25.1 ef	33.4 de	58.2 c-e	18.5 b-d	18.2 de	0.9 e	24.5 ab	19.1 g-i	1864 b-d	2823 a-c
	Headline 12 DPA	26.9 ef	37.4 cd	58.0 c-e	27.0 b	29.1 cd	1.2 e	23.0 b-e	18.1 hi	1491 b-d	2079 bc

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

Table 5. Fungicide efficacy (%) in experiments conducted near Mead, Nebraska to determine the effects of cultivar resistance, fungicide chemical class, and fungicide application timing on the variables in two winter wheat cultivars under irrigated conditions during the 2015 and 2016 growing seasons

		Rainfed								Irrigated							
Cultivar	Treatment	FHB-Index		FDK		DON		Yield		FHB-Index		FDK		DON		Yield	
		2015	2016	2015	2016	2015	2016	2015	2016	2015	2016	2015	2016	2015	2016	2015	2016
Overley	Prosaro A	41.9 ab	65.4 ab	43.5 ab	42.8 a	44.4 ab	28.7 ab	41.9 a	20.2 ab	50.5 a	62.2 ab	47.1 b	43.4 a	75.2 a	23.2 ab	28.3 ab	46.6 a
	Prosaro 6 DPA	44.2 ab	47.2 b-d	43.8 ab	44.9 a	48.2 ab	67.4 a	42.9 a	11.1 a-c	30.4 a	52.0 bc	36.0 bc	39.4 bc	77.6 a	27.8 ab	5.8 ab	7.2 ab
	Prosaro 12 DPA	33.6 ab	27.1 b-d	21.6 cd	34.0 a	37.2 a-c	-16.1 b	32.2 ab	-8.4 cd	10.1 ab	30.2 de	12.2 d-f	33.3 ab	45.5 a-c	10.4 ab	-18.3 b	35.6 a
	Headline A	30.1 ab	52.0 b-d	38.1 bc	22.2 a	39.3 ab	-4.1 b	48.9 a	31.6 a	23.9 ab	68.5 a	22.8 cd	28.1 ab	43.7 a-c	-5.7 ab	25.5 ab	32.0 a
	Headline 6 DPA	16.5 ab	31.2 b-d	32.7 bc	28.1 a	37.5 a-c	59.5 a	50.2 a	-7.0 b-d	10.1 ab	38.0 cd	11.0 d-f	33.3 ab	49.7 a-c	10.4 ab	14.3 ab	28.0 ab
	Headline 12 DPA	6.5 b	12.4 d	3.6 d	5.9 a	27.1 bc	-40.6 b	2.1 ab	-29.8 d	-8.9 b	8.0 ef	4.5 d-f	-19.6 c	34.3 bc	-2.2 ab	1.3 ab	9.6 ab
Overland	Prosaro A	53.0 a	76.8 a	59.7 a	23.4 a	61.2 a	N/A	19.4 ab	21.2 ab	44.3 a	65.0 a	59.0 a	47.3 a	82.1 a	35.9 a	39.1 a	16.9 ab
	Prosaro 6 DPA	14.0 ab	48.0 b-d	42.6 a-c	-4.7 a	29.5 bc	N/A	8.2 ab	12.2 a-c	38.1 a	50.2 bc	36.5 bc	1.5 bc	73.9 a	-8.8 ab	-6.1 ab	11.1 ab
	Prosaro 12 DPA	49.4 ab	11.9 d	37.0 bc	37.4 a	24.4 bc	N/A	16.3 ab	-2.4 b-d	7.8 ab	17.0 ef	15.7 de	2.3 bc	62.0 ab	21.9 ab	-27.2 b	-12.0 b
	Headline A	53.2 a	62.3 a-c	60.2 a	39.6 a	30.9 bc	N/A	35.6 a	16.4 ab	4.0 ab	47.0 c	18.9 c-e	39.6 ab	14.1 c	35.9 ab	12.7 ab	20.3 ab
	Headline 6 DPA	21.8 ab	18.3 cd	31.1 bc	34.0 a	35.7 a-c	N/A	-4.8 b	12.3 a-c	32.5 a	11.0 ef	-7.7 ef	28.7 ab	57.5 a-c	21.8 ab	25.8 ab	8.8 ab
	Headline 12 DPA	37.8 ab	44.2 cd	17.6 cd	5.8 a	10.6 c	N/A	-17.0 b	5.8 a-d	25.0 a	-2.0 f	-9.1 f	-5.6 bc	34.3 bc	-14.1 b	-22.8 b	-15.6 b

[a]Means followed by the same letter within a column are not significantly different according the least significant difference (LSD) test at P = 0.05)

Figure captions

Fig. 1. Fusarium head blight (FHB) index averaged over years and fungicide application timing treatments in experiments conducted near Mead, Nebraska to determine the effects of cultivar resistance, fungicide chemical class, and fungicide application timing in hard red winter wheat cultivars Overland (moderately resistant) and Overley (susceptible) under rainfed and irrigated conditions during the 2015 and 2016 growing seasons.

Fig. 2. Fusarium-damaged kernels (FDK) averaged over years and fungicide application timing treatments in experiments conducted near Mead, Nebraska to determine the effects of cultivar resistance, fungicide chemical class, and fungicide application timing in hard red winter wheat cultivars Overland (moderately resistant) and Overley (susceptible) under rainfed and irrigated conditions during the 2015 and 2016 growing seasons.

Fig. 3. Deoxynivalenol (DON) averaged over years and fungicide application timing treatments in experiments conducted near Mead, Nebraska to determine the effects of cultivar resistance, fungicide chemical class, and fungicide application timing on the variable in in hard red winter wheat cultivars Overland (moderately resistant) and Overley (susceptible) under rainfed and irrigated conditions during the 2015 and 2016 growing seasons.

Fig. 4. Thousand kernel weight (TKW) index averaged over years and fungicide application timing treatments in experiments conducted near Mead, Nebraska to determine the effects of cultivar resistance, fungicide chemical class, and fungicide application timing in hard red winter wheat cultivars Overland (moderately resistant) and

Overley (susceptible) under rainfed and irrigated conditions during the 2015 and 2016 growing seasons.

Fig. 5. Yield averaged over years and fungicide application timing treatments in experiments conducted near Mead, Nebraska to determine the effects of cultivar resistance, fungicide chemical class, and fungicide application timing in hard red winter wheat cultivars Overland (moderately resistant) and Overley (susceptible) under rainfed and irrigated conditions during the 2015 and 2016 growing seasons.

Fig. 6. Fusarium head blight (FHB) index in hard red winter wheat cultivars Overland (moderately resistant) and Overley (susceptible) treated with the triazole fungicide Prosaro (prothioconazole + tebuconazole) and the strobilurin fungicide Headline (pyraclostrobin) at anthesis, 6 days post anthesis (DPA), and 12 DPA in field experiments conducted near Mead, Nebraska under rainfed and irrigated conditions during the 2015 and 2016 growing seasons.

Fig. 7. Fusarium-damaged kernels (FDK) in hard red winter wheat cultivars Overland (moderately resistant) and Overley (susceptible) treated with the triazole fungicide Prosaro (prothioconazole + tebuconazole) and the strobilurin fungicide Headline (pyraclostrobin) at anthesis, 6 days post anthesis (DPA), and 12 DPA in field experiments conducted near Mead, Nebraska under rainfed and irrigated conditions during the 2015 and 2016 growing seasons.

Fig. 8. Deoxynivalenol (DON) concentration in hard red winter wheat cultivars Overland (moderately resistant) and Overley (susceptible) treated with the triazole

fungicide Prosaro (prothioconazole + tebuconazole) and the strobilurin fungicide Headline (pyraclostrobin) at anthesis, 6 days post anthesis (DPA), and 12 DPA in field experiments conducted near Mead, Nebraska under rainfed and irrigated conditions during the 2015 and 2016 growing seasons.

Fig. 9. Thousand kernel weight (TKW) in hard red winter wheat cultivars Overland (moderately resistant) and Overley (susceptible) treated with the triazole fungicide Prosaro (prothioconazole + tebuconazole) and the strobilurin fungicide Headline (pyraclostrobin) at anthesis, 6 days post anthesis (DPA), and 12 DPA in field experiments conducted near Mead, Nebraska under rainfed and irrigated conditions during the 2015 and 2016 growing seasons.

Fig. 10. Yield in hard red winter wheat cultivars Overland (moderately resistant) and Overley (susceptible) treated with the triazole fungicide Prosaro (prothioconazole + tebuconazole) and the strobilurin fungicide Headline (pyraclostrobin) at anthesis, 6 days post anthesis (DPA), and 12 DPA in field experiments conducted near Mead, Nebraska under rainfed and irrigated conditions during the 2015 and 2016 growing seasons.

Fig. 11. Fusarium head blight (FHB) index, *Fusarium*-damaged kernels (FDK), deoxynivalenol (DON) concentration, and yield averaged over fungicide treatments and years in hard red winter wheat cultivars Overland (moderately resistant) and Overley (susceptible). Experiments were conducted near Mead, Nebraska to determine the effects of cultivar resistance, fungicide chemical class, and fungicide application timing under rainfed and irrigated conditions during the 2015 and 2016 growing seasons.

Fig. 12. Fusarium head blight (FHB) index, *Fusarium*-damaged kernels (FDK), deoxynivalenol (DON) concentration, and yield averaged over fungicide treatments and

irrigation environments in hard red winter wheat cultivars Overland (moderately resistant) and Overley (susceptible). Experiments were conducted near Mead, Nebraska to determine the effects of cultivar resistance, fungicide chemical class, and fungicide application timing under rainfed and irrigated conditions during the 2015 (wet) and 2016 (relatively dry) growing seasons.

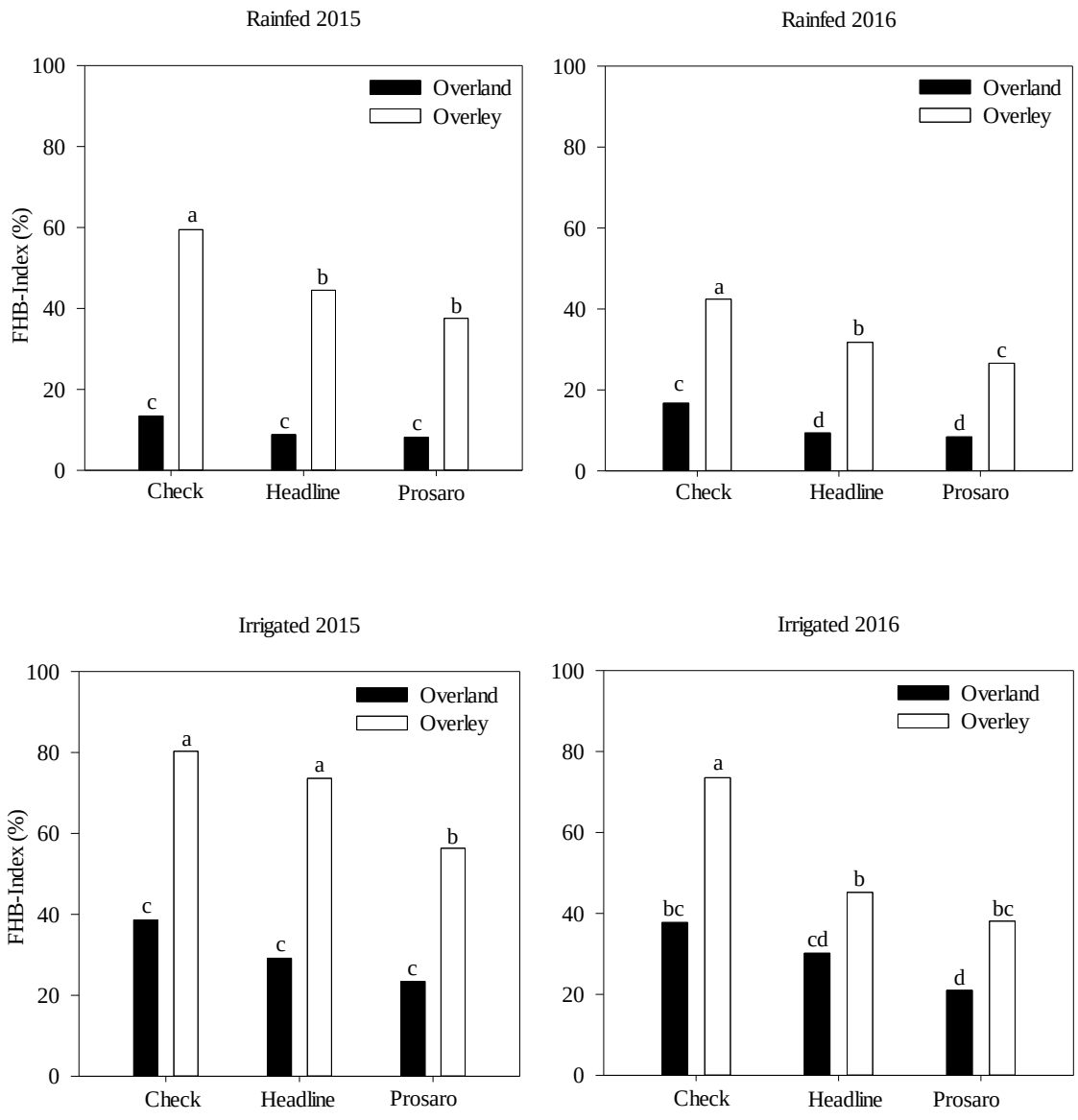
Rainfed 2015
Rainfed 2016
Irrigated 2015
Irrigated 2016
Overland
Overley
FHB-Index (%)
0
20
40
60
80
100
Check
Headline
Prosaro
a
b
b
c
c
c
a
b
c
c
d
d
a
a
b
c
c
c
a
b
bc
cd
d
bc

Fig. 1.

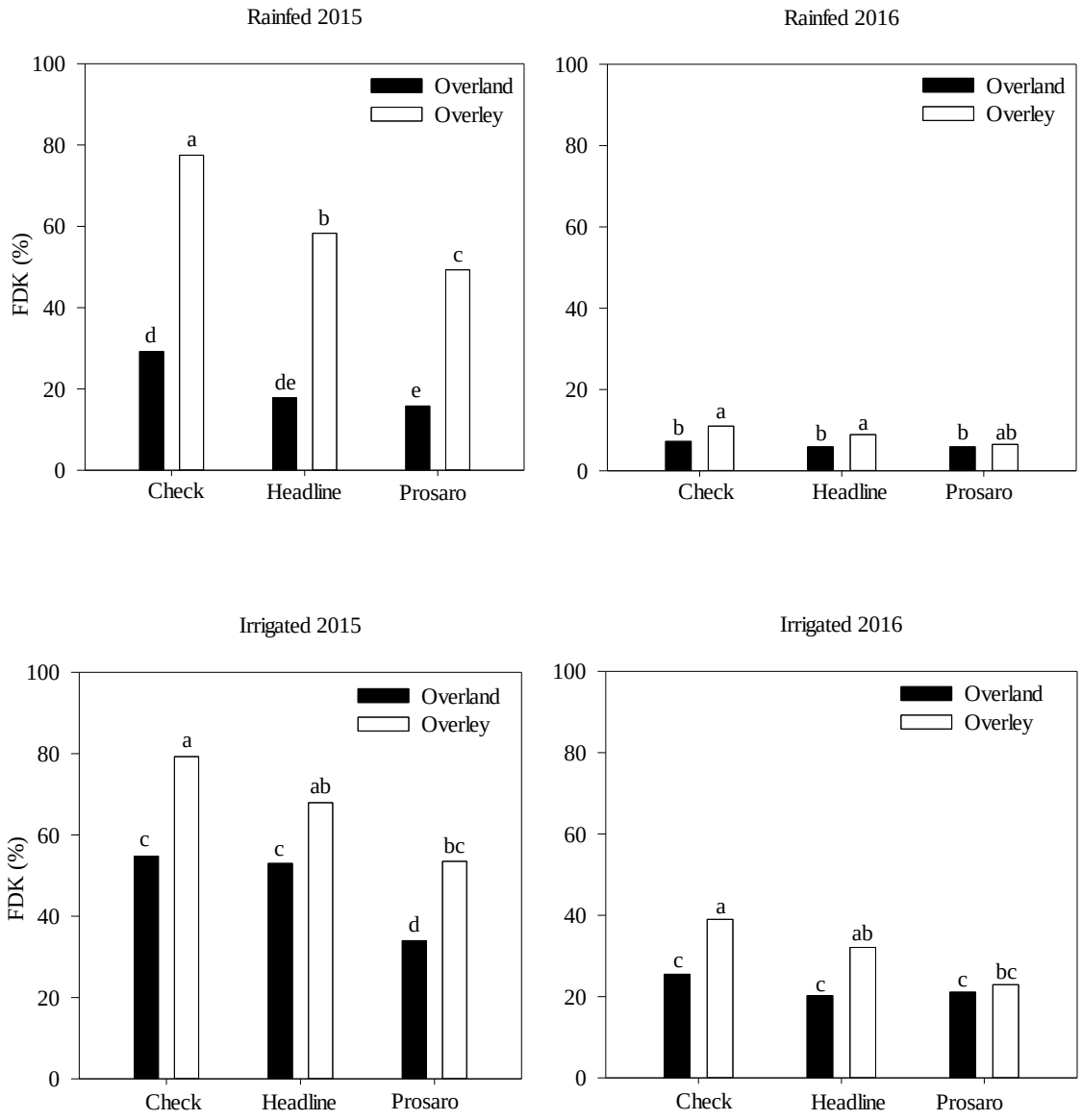
Rainfed 2015
Rainfed 2016
Irrigated 2015
Irrigated 2016
Overland
Overley
FDK (%)
Check
Headline
Prosaro
0
20
40
60
80
100
a
b
c
d
de
e
b
a
b
a
b
ab
c
a
c
ab
d
bc
c
a
c
ab
c
bc

Fig. 2.

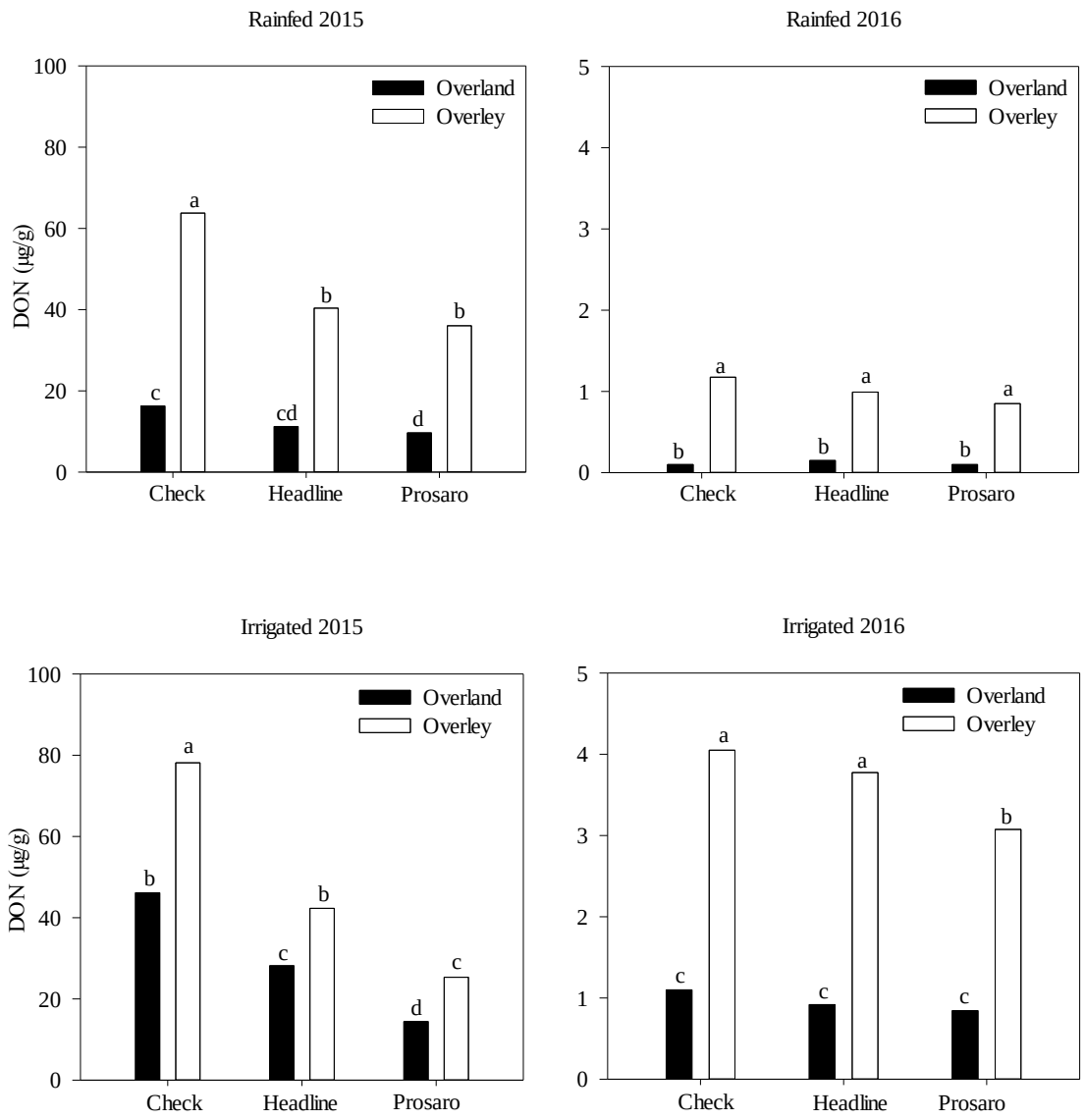
Rainfed 2015
Rainfed 2016
Irrigated 2015
Irrigated 2016
Overland
Overley
DON (µg/g)
Check
Headline
Prosaro
a
b
c
cd
d

Fig. 3.

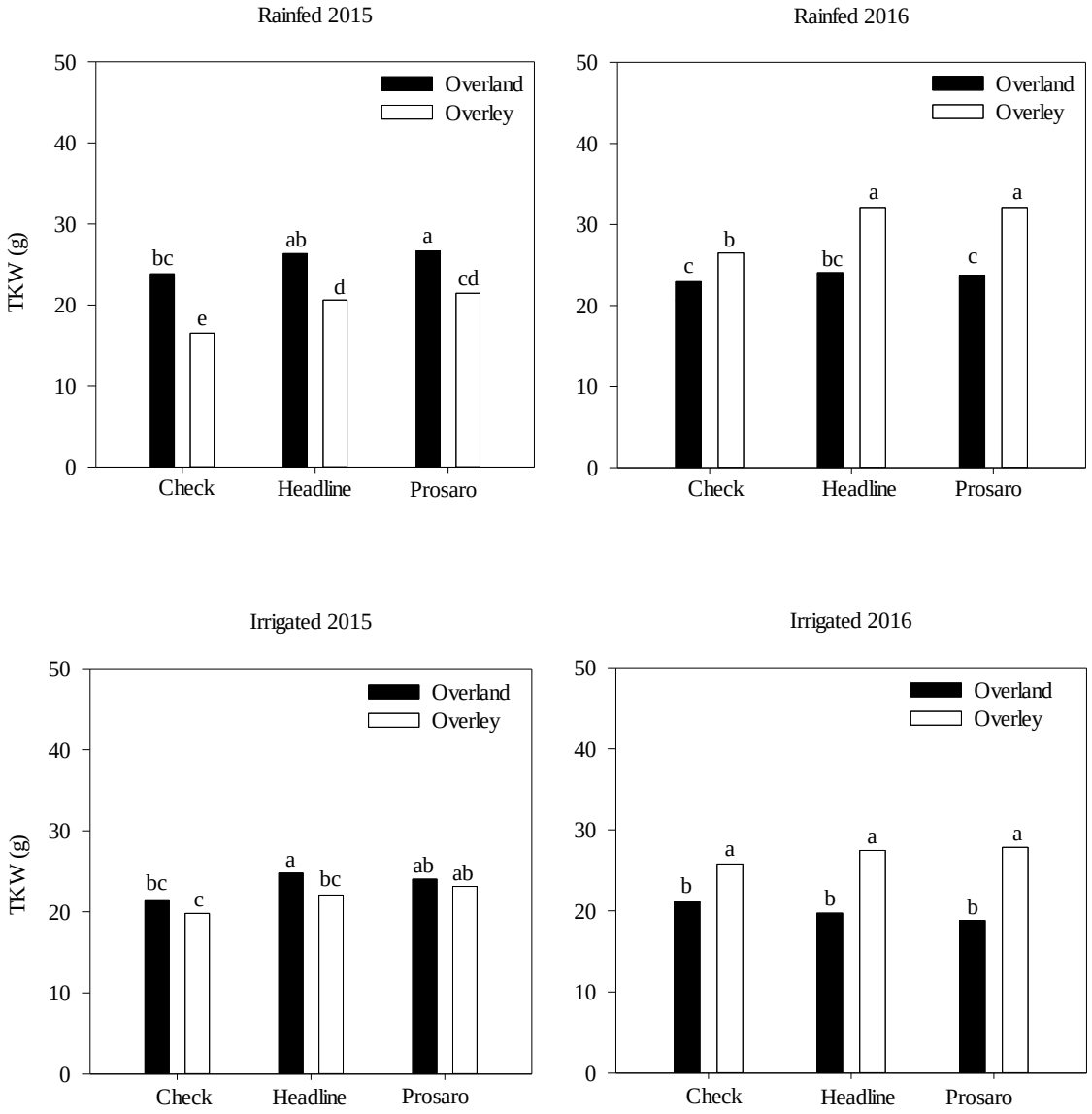
Rainfed 2015
Rainfed 2016
Irrigated 2015
Irrigated 2016
TKW (g)
Overland
Overley
Check
Headline
Prosaro
0
10
20
30
40
50
bc
e
ab
d
a
cd
c
b
bc
a
c
a
bc
c
a
bc
ab
ab
b
a
b
a
b
a

Fig. 4.

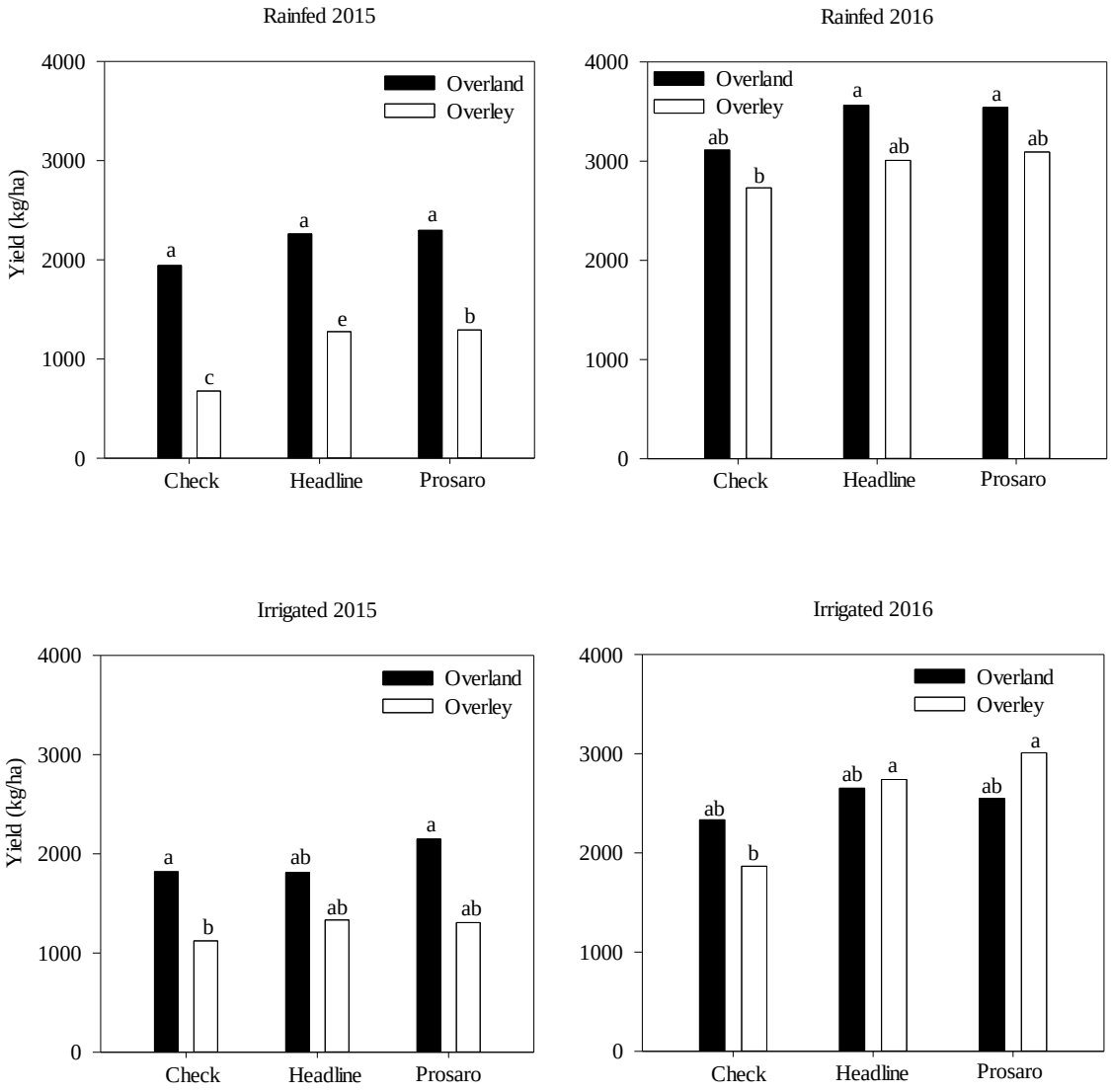
Rainfed 2015
Rainfed 2016
Irrigated 2015
Irrigated 2016
Yield (kg/ha)
Overland
Overley
Check
Headline
Prosaro
0
1000
2000
3000
4000
a
c
a
e
a
b
ab
b
a
ab
a
ab
a
b
ab
ab
a
ab
ab
b
ab
a
ab

Fig. 5.

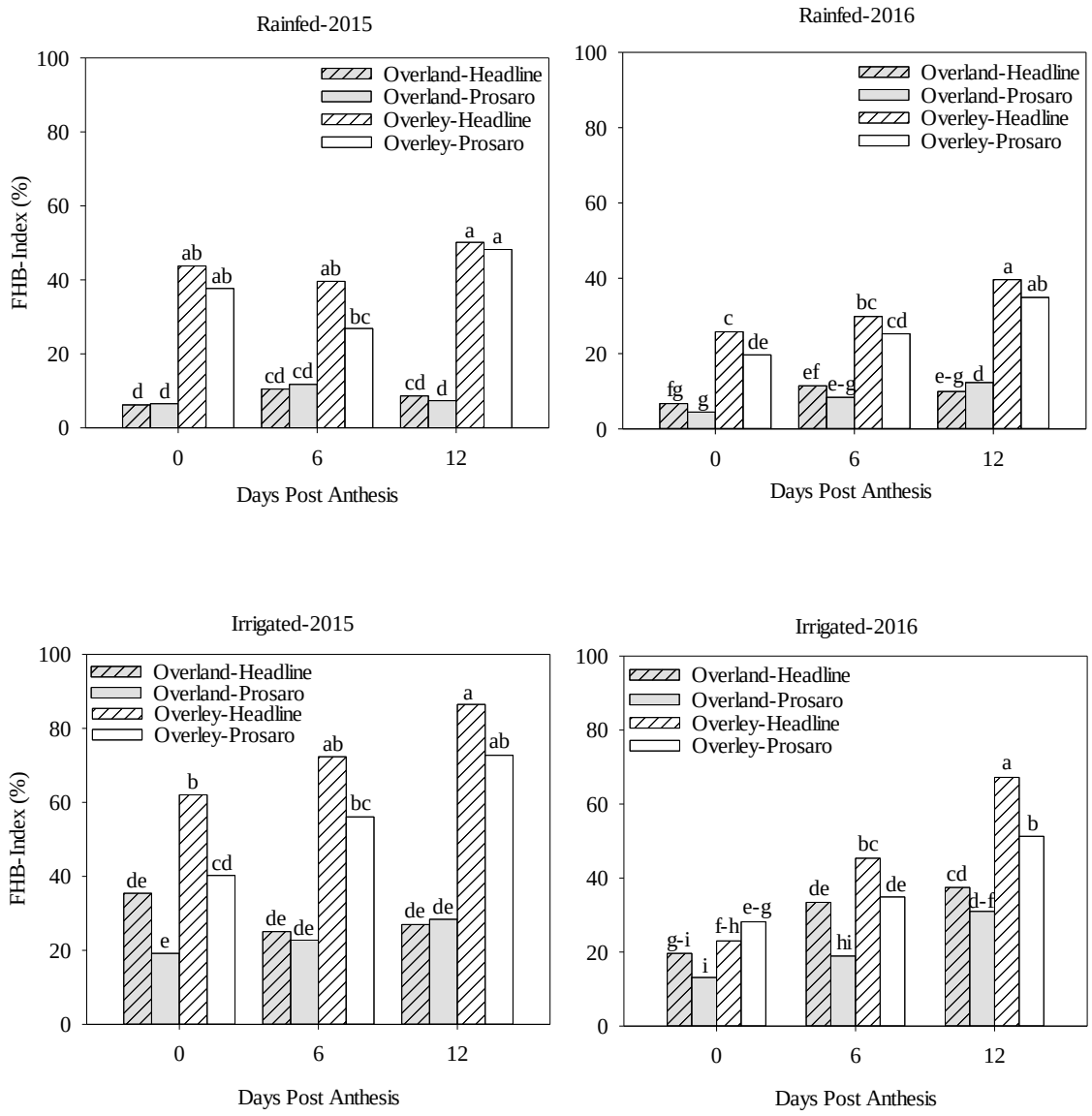
Rainfed-2015
Rainfed-2016
Irrigated-2015
Irrigated-2016
Overland-Headline
Overland-Prosaro
Overley-Headline
Overley-Prosaro
FHB-Index (%)
Days Post Anthesis
0
6
12
0
20
40
60
80
100

Fig. 6.

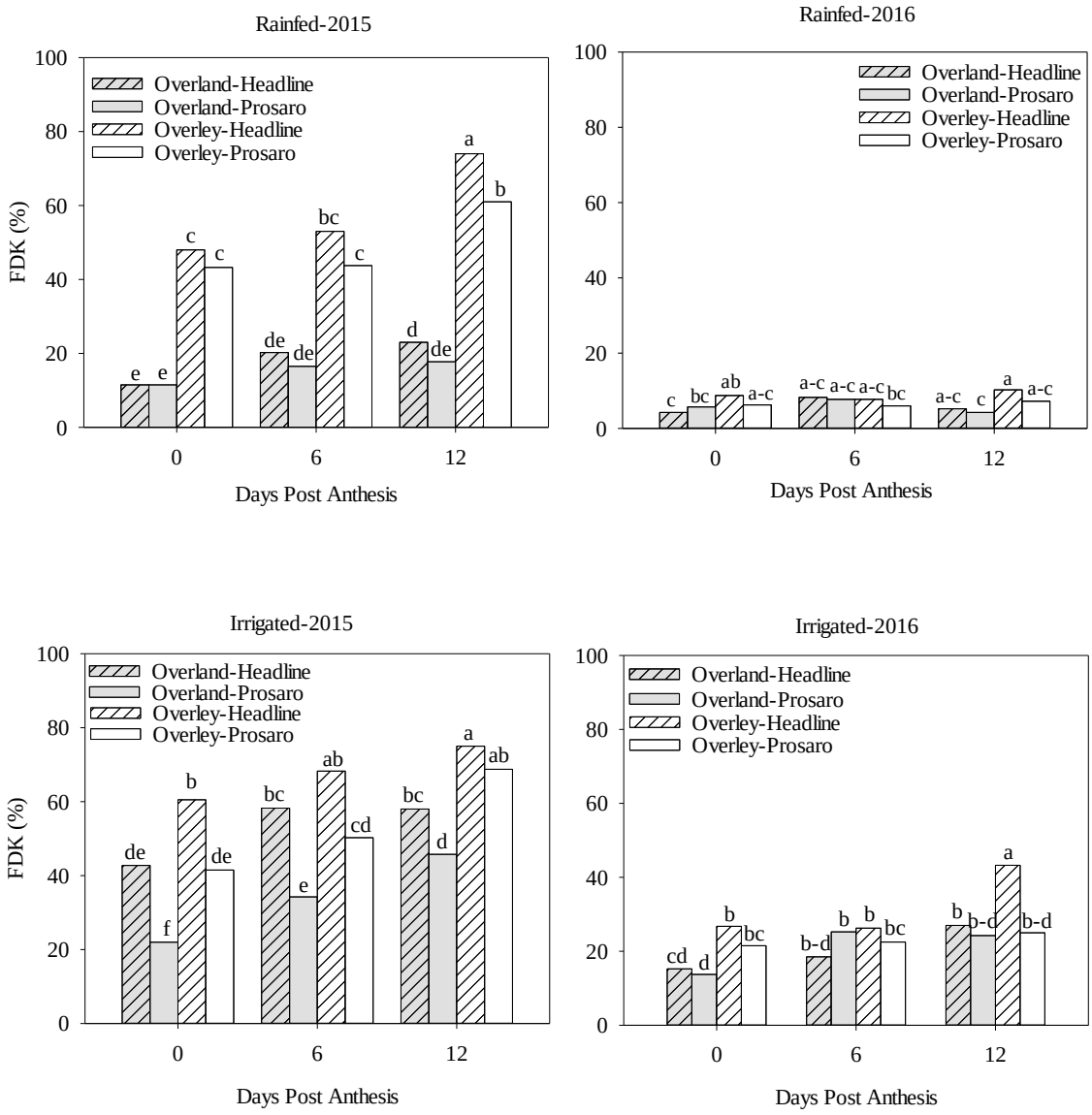
Rainfed-2015
Rainfed-2016
Irrigated-2015
Irrigated-2016
Overland-Headline
Overland-Prosaro
Overley-Headline
Overley-Prosaro
FDK (%)
Days Post Anthesis
0
6
12
20
40
60
80
100

Fig. 7.

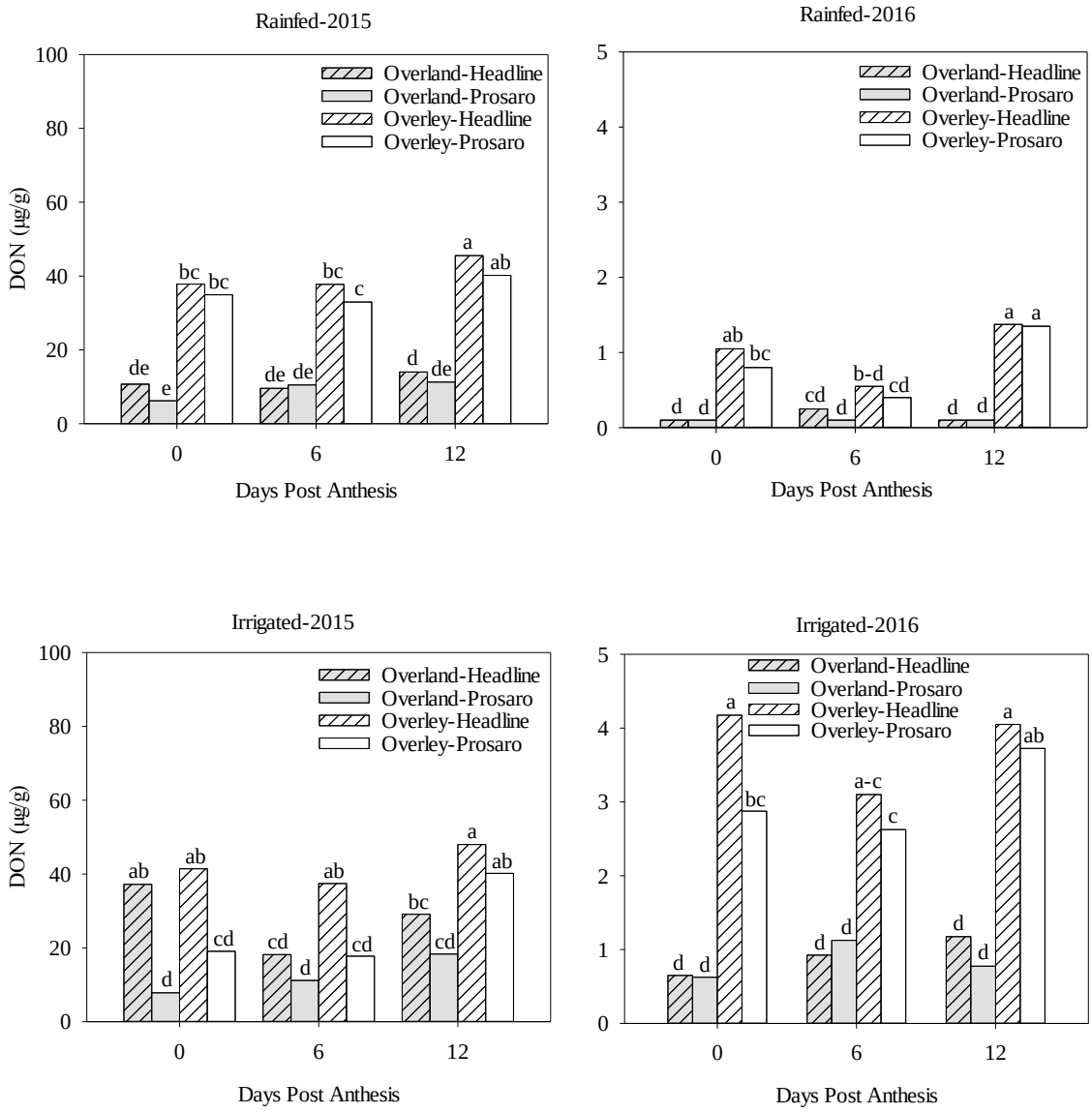
Rainfed-2015
Rainfed-2016
Irrigated-2015
Irrigated-2016
Overland-Headline
Overland-Prosaro
Overley-Headline
Overley-Prosaro
DON (µg/g)
Days Post Anthesis
0
6
12

Fig. 8.

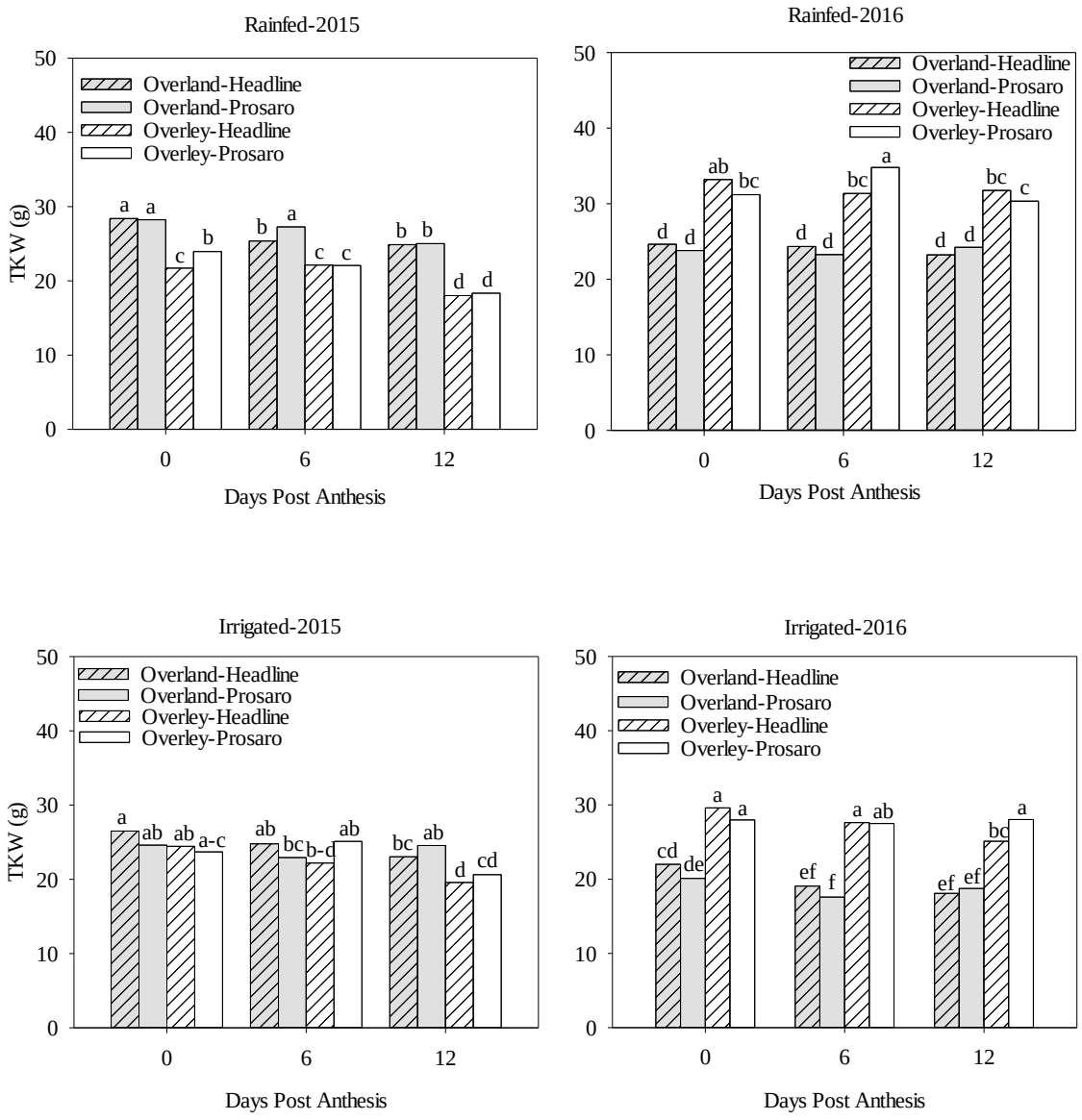
Rainfed-2015
Rainfed-2016
Irrigated-2015
Irrigated-2016
Overland-Headline
Overland-Prosaro
Overley-Headline
Overley-Prosaro
TKW (g)
Days Post Anthesis
0
6
12
10
20
30
40
50

Fig. 9.

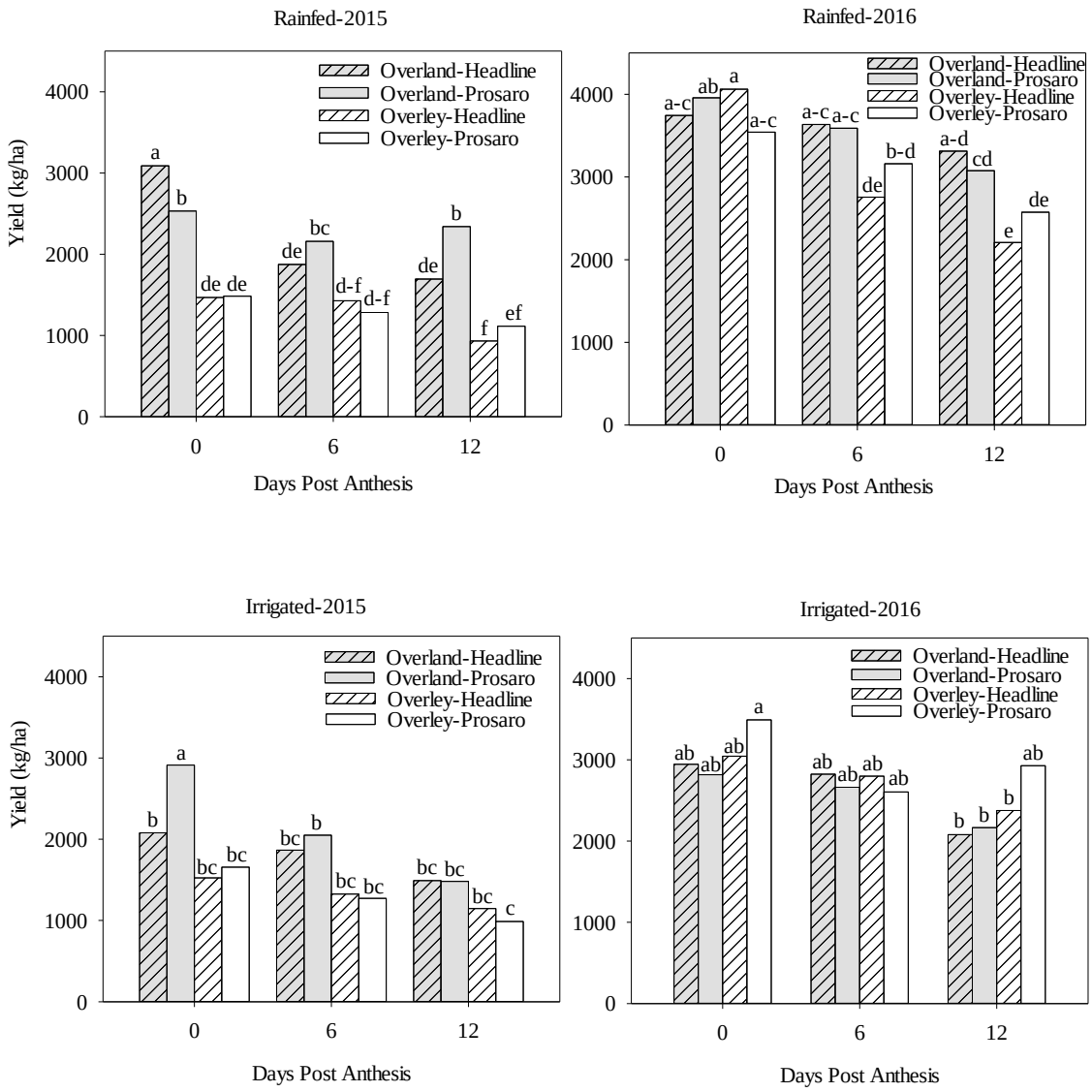
Rainfed-2015
Rainfed-2016
Irrigated-2015
Irrigated-2016
Overland-Headline
Overland-Prosaro
Overley-Headline
Overley-Prosaro
Yield (kg/ha)
Days Post Anthesis
0
1000
2000
3000
4000
0
6
12

Fig. 10.

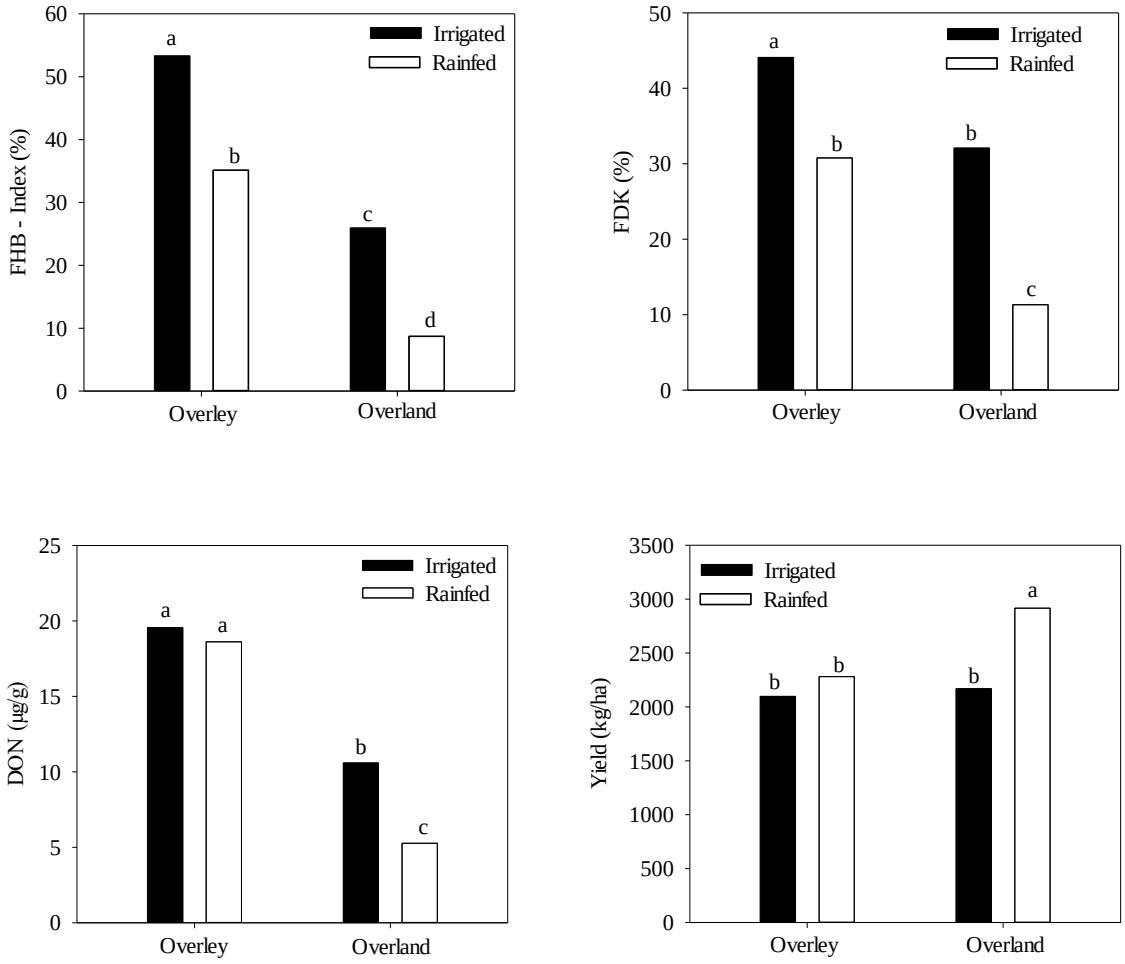
FHB - Index (%)
60
50
40
30
20
10
0
a
b
c
d
Irrigated
Rainfed
Overley
Overland
FDK (%)
50
40
30
20
10
0
a
b
b
c
Irrigated
Rainfed
Overley
Overland
DON (µg/g)
25
20
15
10
5
0
a
a
b
c
Irrigated
Rainfed
Overley
Overland
Yield (kg/ha)
3500
3000
2500
2000
1500
1000
500
0
b
b
b
a
Irrigated
Rainfed
Overley
Overland

Fig. 11.

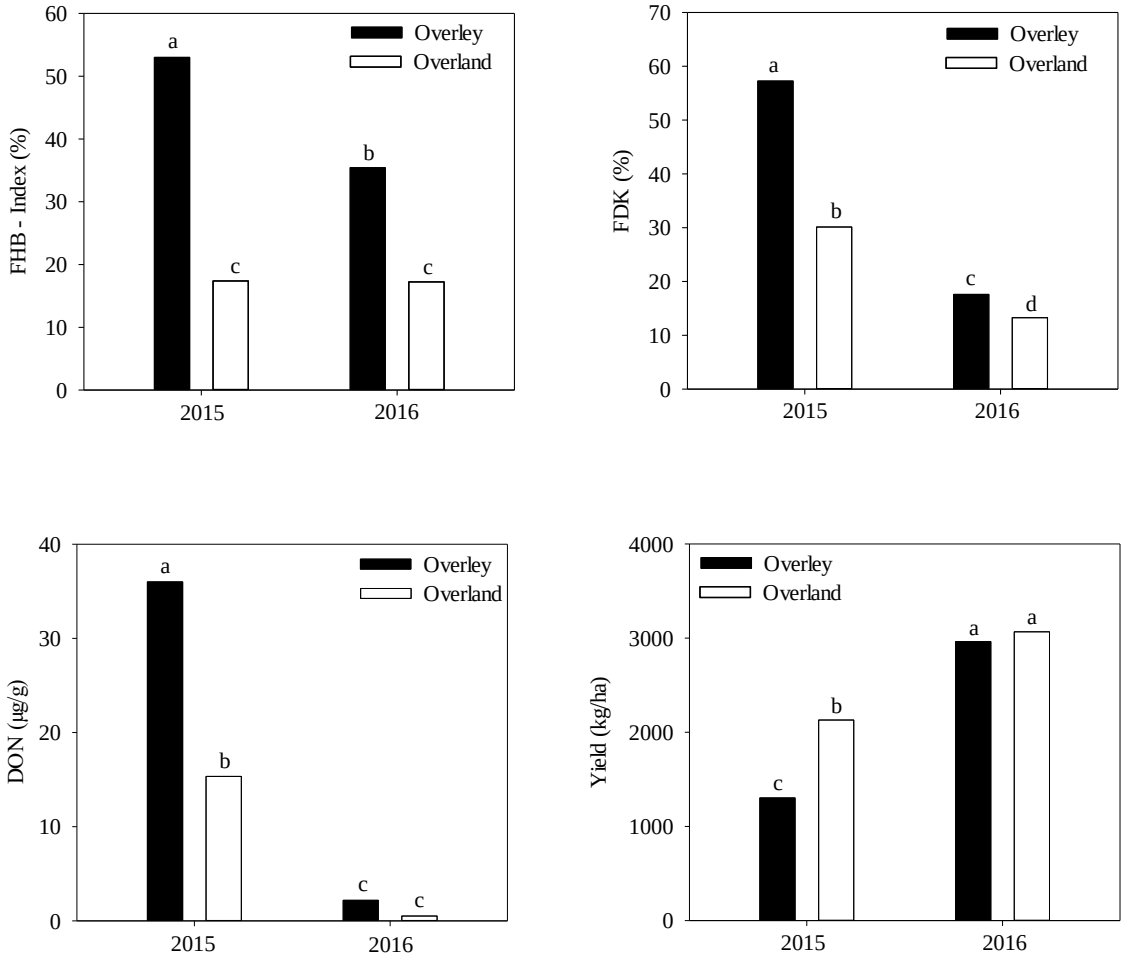
FHB - Index (%)
60
50
40
30
20
10
0
a
c
b
c
2015
2016
Overley
Overland
FDK (%)
70
60
50
40
30
20
10
0
a
b
c
d
2015
2016
Overley
Overland
DON (µg/g)
40
30
20
10
0
a
b
c
c
2015
2016
Overley
Overland
Yield (kg/ha)
4000
3000
2000
1000
0
c
b
a
a
2015
2016
Overley
Overland

Fig. 12.

CHAPTER III

EFFECTS OF FIELD-APPLIED FUNGICIDES, MOISTURE, AND TIME ON DEOXYNIVALENOL DURING POSTHARVEST STORAGE OF WINTER WHEAT GRAIN

1. Abstract.

Fusarium head blight (FHB), caused mainly by *Fusarium graminearum*, causes major losses in wheat. Triazole fungicides have been shown to be more effective than strobilurins in controlling FHB and the associated mycotoxin deoxynivalenol (DON) when applied at anthesis. The effects of field-applied fungicides on DON during grain storage have not been investigated. DON concentration was monitored during 120 days of grain storage in darkness at 10ºC, 40% RH, and 10%, 16%, or 20% grain moisture following harvest of winter wheat cultivars Overland (moderately resistant) and Overley (susceptible) to determine the effects on DON of a triazole (Prosaro) and a strobilurin (Headline) applied at anthesis. In cv. Overland, DON decreased significantly (P = 0.05) from 3.6 to 3.0 and 2.7 to 2.2 µg/g in the untreated check and Prosaro treatments, respectively, whereas in the Headline treatment, there was a non-significant decrease from 4.4 to 4.1 µg/g. In cv. Overley, DON increased significantly from 3.1 to 3.6 µg/g and 2.9 to 3.5 µg/g in the untreated check and Headline treatments, respectively, but remained the same at 2.2 µg/g in the Prosaro treatment. DON did not differ between 16% (3.2 µg/g) and 20% (3.1 µg/g) grain moisture, but was significantly lower (2.7 µg/g) at 10% moisture. These results indicate that the effects on DON of fungicides applied at anthesis in the field can extend through the grain storage period. Triazoles are

recommended over strobilurins and grain should be stored at the lowest possible moisture maintained throughout the storage period.

2. Introduction.

Fusarium head blight (FHB) is a devastating disease of wheat and other small grain cereals that results in major economic losses worldwide. It is caused mainly by *Fusarium graminearum*, but other species of *Fusarium* are known to be causal agents. Symptoms are manifested as premature whitening or bleaching of one or more spikelets on the spike, which results in partial or entire bleaching of the spike. Bleached spikelets are sterile or contain shriveled kernels that appear chalky white or pink, referred to as *Fusarium*-damaged kernels (FDK), scabby kernels, or "tombstones". In addition to poor grain quality, infection by this pathogen also results in contamination of grain with the mycotoxin deoxynivalenol (DON) which is harmful to human and animal health (McMullen et al. 2012). In humans, ingestion of DON-contaminated grain results in food poisoning symptoms including diarrhea, nausea, vomiting, abdominal pain, headache, and dizziness (Desjardins 2006). In animals, symptoms include vomiting, feed refusal, weakness, and emaciation (Pestka 2007).

Management strategies and tactics for FHB include cultural practices such as crop rotation and tillage to reduce residue-borne inoculum, the use of genetic resistance, and fungicide application timed at anthesis (Parry et al. 1995; Wegulo et al. 2015). Following an FHB-favorable growing season, grain quality losses and mycotoxin concentration in grain can be reduced during harvest. Because FDK are lighter than healthy kernels, they can be blown away by adjusting the combine's fan speed and shutter opening (Salgado et al. 2011, 2014). To further reduce DON contamination in grain, post-harvest practices

can be undertaken including the use of sieves and specific gravity tables to clean grain by removing the lighter FDK (Dexter and Nowicki 2003).

Even after pre-storage measures to remove FDK, grain from an FHB-favorable growing season will still contain DON at the time it is stored. Suboptimal storage conditions including higher than ideal moisture and temperature can lead to an increase of DON during storage (Birzele et al. 2000). High grain moisture content, measured as water activity (a_w), is favorable to fungal growth and mycotoxin formation in grain during storage (Magan et al. 2003; Magan et al. 2014). Water activity is the ratio of the partial pressure of water vapor in the grain to the saturation vapor pressure of pure water under the same environment. It is numerically equivalent to equilibrium relative humidity (ERH) expressed as a decimal and is the major environmental factor, along with other factors including temperature, that influences stability or spoilage of stored food or grain (Pitt and Hocking 2009). Control of a_w is critical to reducing the growth of fungi and their metabolic activities during food or grain storage (Comerio et al. 1999; Schwabe and Kramer 1995). The optimal conditions for *in vitro* growth of *F. graminearum* are 25^{o}C and a_w = 0.85 (Brennan et al. 2003; Hope et al. 2005).

In the field, FHB and DON are controlled by applying a fungicide to the wheat heads at anthesis (Wegulo et al. 2011, 2015). Demethylation inhibitor (DMI) fungicides, also known as triazoles, slow fungal growth by inhibiting the biosynthesis of sterols which are essential in the maintenance of cell membrane integrity (Chen et al. 2014; Hewitt 1998). Strobilurins are quinone outside inhibitors (Bartlett et al. 2002; Myung 2015; Nason et al. 2007) which interfere with energy production in fungi. In the wheat-FHB pathosystem, strobilurin fungicides applied at anthesis have been correlated with increased DON levels in grain (Amarasinghe et al. 2013; Ellner 2005; Madden et al. 2014). However, this

increase in DON does not always occur (Pirgozliev et al. 2002). The effects of field-applied fungicides on DON during wheat grain storage have not been investigated. The objectives of this study were to i) compare the effects a triazole and a strobilurin fungicide applied at anthesis in the field on DON concentration during wheat grain storage, and ii) determine the effect of wheat grain moisture and time on DON concentration during storage.

3. Materials and Methods

Grain of winter wheat cv. Overland (moderately resistant to FHB, Baenziger et al. 2008) from a 2015 rain-fed field trial and winter wheat cv. Overley (susceptible to FHB, Fritz et al. 2004) from a 2016 irrigated field trial was used in postharvest storage experiments. In both field trials which were conducted at the Eastern Nebraska Research and Extension Center (ENREC) near Mead, Nebraska (41.2286° N, 96.4892°W), the triazole Prosaro® (prothioconazole + tebuconazole, 0.475 L/ha) and the strobilurin Headline® (pyraclostrobin, 0.658 L/ha) were applied at label rates during anthesis (mid-spring) or not applied (untreated check) to the spikes with a CO_2-powered backpack sprayer equipped with four tee-jet nozzles (TeeJet Technologies, Dillsburg, PA) spaced 30.5 cm apart on a boom and set at a pressure of 241 kPa. Plots (1.2 m x 6.1 m) were inoculated 24 h after fungicide application by spraying a spore suspension of *F. graminearum* (100,000 spores/mL) on the spikes. This was in addition to *F. graminearum*-colonized corn kernels that had been spread on the soil surface of the plots in early spring at the rate of 67 kernels/m^2. Because of the high FHB intensity in 2015, FDK levels were very high and therefore grain of cv. Overland from the rain-fed trial was used after cleaning with a modular fractionating aspirator (Carter Day International, Inc., Minneapolis, MN, USA) to remove FDK. In contrast, FHB intensity in 2016 was very

low and therefore grain of cv. Overley from the irrigated trial was used without removing FDK.

Before initiation of the postharvest grain storage experiments, grain samples were stored in paper bags at room temperature for 264 days (cv. Overland from the 2015 growing season, experiment 1) or 79 days (cv. Overley from the 2016 growing season, experiment 2). At the time the experiments were initiated, moisture content had dropped from 15% (a_w 0.53) at the time of harvest to 8% (a_w 0.26) in grain from the 2015 growing season, and from 15% to 11% (a_w 0.40) in grain from the 2016 growing season.
To calculate the amount of water needed to hydrate the grain to a desired moisture content (16% and 20%), two hydration curves were generated based on preliminary experiments. For grain from the 2015 growing season, the equation used was $Y = 2.11 + 0.12X$, $r^2 = 0.70$; and for grain from the 2016 growing season, the equation used was $Y = 6.47X - 3.85$, $r^2 = 0.96$; where X is total milliliters of sterile distilled water (SDW) to be added to the grain and Y is the difference between the initial moisture content of the grain and the desired moisture content.

Tempering was achieved by evenly spreading a 300 g sample of non-sterile grain from each field plot on a plastic tray (50 cm long x 30 cm wide x 3 cm high) and sprinkling evenly with SDW using a heavy-duty manual sprayer (Rubbermaid, Wooster, OH). After tempering, grain samples were separated and homogenized manually and transferred to a sterile Microbox® hermetically-sealed micro-propagation container of dimensions 15 cm long x 15 cm wide x 20 cm high ($SacO_2$, Veldeken, Belgium). Microbox's containers have a filter in the lid for gas exchange which blocks the entrance of external organisms or spores (Birzele et al. 2000). Therefore, these containers prevent external contamination of the grain's environment. Water activity (a_w) was determined

using a Pawkit® water activity meter (Decagon Devices, Pullman, WA). Grain moisture content (%) was determined using a grain moisture seed tester (Dickey Jhon Corp., Auburn, IL), model GAC 500-XT. After 14 days of tempering, grain moisture and a_w were determined, and SDW was added again if the initial tempering was not enough to adjust to 16% (a_w 0.60) or 20% (a_w 0.75) grain moisture. In experiment 2 (Overley grain from the 2016 irrigated trial), non-tempered grain at a moisture content 10% (a_w 0.40) was added as a third moisture treatment.

After initial tempering, grain samples in Microbox® containers were stored in a seed cooler (Bally Case & Cooler, Inc., Bally, PA) under dark conditions at 10^{o}C and 40% RH and monitored for changes in moisture content and water activity at monthly intervals. RH and temperature inside the containers were monitored using Watchdog® sensors model 1400 (Spectrum Technologies, Thayer Court, IL). Sampling was conducted at 0, 30, 60, 90, and 120 days after tempering. From each of the micro propagation containers, at the specific storage time, a random sample of 20 mL of grain volume was taken and milled using a cyclone sample laboratory mill (UDY Corporation, Fort Collins, CO). Deoxynivalenol and its acetylated derivatives 3-ADON and 15-ADON were quantified in an Agilent 6890/5975 system using gas chromatography-mass spectrometry (GC-MS). Each experiment was designed as a split-split plot with four replications and was repeated once. The whole plot, subplot, and sub-subplot consisted of fungicide treatments, grain moisture treatments, and grain storage time, respectively.

Statistical analysis for each experiment was carried out using SAS software version 9.4. In this study, there were two different experiments (Experiment 1 and Experiment 2) with two runs (experiment repeated once) in each experiment.

Based on homogeneity of error variance using F-ratio test (Gomez and Gomez 1983), the two runs of the same experiment were combined and analyzed using generalized mixed models.

Experimental units were arranged in a split-split plot design (SSPD) with containers serving as samples from the whole plot unit (fungicide treatment by reps), split-plot was grain moisture, and split-split-plot was storage time.

First, data were analyzed as repeated measures split-plot-in-time assuming compound symmetry, using PROC GLM to test all the elements of the SSPD. Random effects of the model were: reps (blocks), reps by fungicide treatment, runs, the effect of fungicide within runs by reps, fungicide within grain moisture by reps, fungicide within time by reps, as well as fungicide within time by moisture by reps, and the variation among containers within reps, fungicide, and moisture combinations. Second, PROC GLIMMIX was used to obtain the standard errors throughout. Here, the statement: random slash residual, type equals compound symmetry, subject equals (reps, runs, moisture, fungicide) within container, was added to analyze the data with the compound symmetry covariance structure. LS-means were estimated in PROC GLIMMIX (SAS Inc, Cary, NC), and the Fisher - least significant difference test (Fisher – LSD, α=0.05) was used to determine differences between main effects and interactions among factors. In this manuscript, highly significant differences were considered if the $P \leq 0.01$, and significant differences were considered if the $P \leq 0.05$.

Second and third order polynomial models were tested using PROC GLIMMIX. The model can be explained as:

$$\mu i = \beta 0 + \beta 1\, x + \beta 2 x 2 + \beta 3 x 3 + \beta 4 x 4$$

where μi is DON concentration (μg/g), x is the quantitative factor storage time, β0 is the intercept, and β1, β2, β3, β4, are the linear, quadratic, cubic and quartic coefficients relating storage time and DON, respectively. Type I or sequential sum of squares was used to find the response curve that best fit the data.

4. Results

4.1 Effects of fungicide treatments and grain storage time on DON. The effects of fungicide and storage time treatments on DON were highly significant in both experiments. Two-way or three-way interactions between fungicide, moisture, and time treatments were not significant at P = 0.05 (Table 1), indicating that differences in DON among fungicide treatments were not significantly affected by moisture levels and vice versa. Therefore, comparison of fungicide treatment means was done by averaging over moisture treatments and time, and comparison of moisture treatments was done by averaging over fungicide treatments and time (Gomez and Gomez 1984). When grain of the moderately resistant cv. Overland from the 2015 growing season was cleaned to remove FDK, the Headline® treatment had more DON than the untreated check and Prosaro® treatments (Fig. 1, 0 DAT). In comparably clean grain of the susceptible cv. Overley from the 2016 growing season, the Headline® treatment had a DON level similar to that in the untreated check but higher than that in the Prosaro® treatment (Fig. 2, 0 DAT). In cleaned grain of the moderately resistant cv. Overland, DON declined significantly over 120 days of storage in the untreated check and Prosaro® treatments whereas in the Headline® treatment, there was a slight but non-significant decline (Fig. 1). In comparably clean grain of the susceptible cv. Overley, DON increased over 120 days of storage in the untreated check and Headline® treatments, but not in the Prosaro® treatment (Fig. 2).

4.2 Effect of moisture treatments on DON. The effect of grain moisture at 16% or 20% on DON was non-significant in cv. Overland (Table 1). However, in cv. Overley, a significant effect (P = 0.0168) was observed, with DON similar at 16% and 20% moisture but higher at these moisture levels than at 10% moisture (Table 1, Fig. 3).

4.3 Variation in DON over grain storage time. In cv. Overland, DON concentration declined during the first 30 days by an average (over the two moisture levels) of 21, 34, and 40% in the Headline®, untreated check, and Prosaro® treatments, respectively. Over the next 30 days, DON levels increased by an average of 19, 27, and 51% in the Headline®, check, and Prosaro® treatments, respectively. Thereafter, DON levels in all treatments stabilized with slight fluctuations. During the 120 days of storage, DON decreased over time in all three treatments (Figs. 1 and 4). In cv. Overley, DON concentration in the Headline treatment increased by an average (over the three moisture levels) of 31% over the first 30 days, decreased by 20% over the following 60 days, then increased by 15% over the last 30 days. In the untreated check, DON concentration increased by 25% during the first 60 days, decreased by 21% during the following 30 days, and increased by 20% during the last 30 days. In the Prosaro treatment, DON concentration increased by 17% over the first 30 days, decreased by 23% over the following 60 days, and increased by 12% over the last 30 days. Over the 120 days of storage time, DON significantly increased in the Headline and untreated check but remained the same in the Prosaro treatment (Figs. 2 and 5). The trends in DON concentration over time in moisture treatments averaged over fungicide treatments were similar to those in fungicide treatments averaged over moisture treatments (Fig. 6).

5. Discussion

Previous research has demonstrated the superior efficacy of triazole fungicides in controlling FHB and DON in wheat when applied in the field at anthesis compared to the inferior efficacy of strobilurin fungicides and their tendency to elevate DON in grain when applied in the field before or during anthesis (Blandino and Reyneri, 2009; Ellner, 2005; Oldenburg et al. 2001). However, the effects of field-applied triazole and strobilurin fungicides on DON concentration during storage have not been investigated. This study was designed to fill this knowledge gap.

In both experiments at the beginning of grain storage, the higher or similar DON concentration in the field-applied strobilurin (Headline) treatment compared to the untreated check and the lower DON concentration in the field-applied triazole (Prosaro) treatment compared to the strobilurin and untreated check (Figs. 1 and 2) are consistent with previous reports (Blandino and Reyneri, 2009; Ellner, 2005; Oldenburg et al. 2001). It was remarkable to observe in the moderately resistant cv. Overland a decrease in DON over 120 days of grain storage in the triazole and untreated check but not in the strobilurin treatment. In contrast, in the susceptible cv. Overley, DON increased over 120 days of grain storage in the strobilurin and untreated check but did not increase in the triazole treatment. Although the two storage experiments were conducted at different times and the grain of each cultivar was from different growing seasons, the most notable difference is that cv. Overland is moderately resistant to FHB (Baenziger et al. 2008) whereas cv. Overley is susceptible (Fritz et al. 2004). Therefore, the decrease in DON during storage of grain of cv. Overland and the increase in the mycotoxin during storage of grain of cv. Overley was likely due to the difference in genetic resistance to FHB and DON between the two cultivars. Further research is needed to validate this observation.

The lower DON concentration at 10% compared to 16% or 20% grain moisture during storage observed in cv. Overley is consistent with results from previous research by other investigators. Ramirez et al. (2006) showed that the growth rate of and DON production by two isolates of *F. graminearum* on irradiated wheat grain increased as water activity (a_w), a measure of grain moisture, increased. Birzele et al. (2000) found DON concentration in stored wheat grain at 20% moisture to be approximately three times the concentration at 17% moisture by the 5^{th} and 6^{th} week of storage. In contrast, in this study DON concentration at 16% moisture did not differ from that at 20% moisture over a period of 17 weeks (120 days) of grain storage. This difference in the results between the two studies is likely due to the temperature at which grain was stored. In the study by Birzele et al. (2000), grain was stored at 20^{o}C whereas in this study grain was stored at 10^{o}C. In this study, however, DON concentration at 10% moisture was lower than that at 16% and 20% moisture, which is in agreement with the results of Birzele et al. (2000) that demonstrate higher DON production in *Fusarium*-contaminated wheat grain stored at higher compared to lower grain moisture. Comerio et al. (1999) and Hope et al. (2005) similarly showed that more DON was produced by *F. graminearum* in stored grain at higher than at lower a_w values.

In both experiments, the reasons for the fluctuation in DON concentration over time (Figs. 4-6) are not known, but may be related to biochemical processes in grain that may be influenced by a range of factors including environmental conditions (moisture, temperature), the amount of fungal biomass present in the grain, the level of expression of trichothecene biosynthesis genes (Hallen-Adams et al. 2011) or, as shown in this study, the level of resistance of the wheat cultivar. Kolmanicˇ et al. (2010) and Zhang et al. (2016) found DON to decrease over time in stored wheat flour and wheat grain,

respectively. However, Zhang et al. (2016) also observed an increase in DON in stored wheat flour. Similarly, in the study by Birzele et al. (2000) in which DON was measured weekly for six weeks in winter wheat grain stored at 20^{o}C and 17% or 20% moisture content, DON increased over time at both moisture levels, but the increase was much greater at the 20% moisture level by the fifth week, indicating that grain moisture content played a significant role in the synthesis of DON during grain storage.

This research has demonstrated that the reduction in DON observed in the field from applying a triazole fungicide (Prosaro) at anthesis can be extended through the period of grain storage. A strobilurin fungicide (Headline) applied at anthesis in the field was ineffective in reducing DON in grain of the moderately resistant cv. Overland stored in the dark at 10^{o}C for 120 days in storage. In contrast, DON in grain of the same cultivar treated at anthesis in the field with a triazole fungicide (Prosaro) declined in storage under the same conditions and period. In the susceptible cv. Overley treated and stored similarly, DON increased during storage in the strobilurin and untreated check, but in the triazole treatment, it was lower at the beginning of storage than in the strobilurin and untreated check and did not increase over time. More DON was produced in storage in grain of cv. Overley tempered to 16% or 20% moisture compared to non-tempered grain at 10% moisture, indicating the importance of proper drying of grain before storage. Based on these results, triazole but not strobilurin fungicides are recommended for control of FHB and DON, and grain should be stored at the lowest moisture content possible to prevent or limit DON production during storage.

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Table 1. Analysis of variance from experiments conducted to evaluate the effects of field-applied fungicides on deoxynivalenol (DON) during storage of grain of two winter wheat cultivars

	Experiment 1 (cv. Overland)		Experiment 2 (cv. Overley)	
Source of variation	d.f.	P > F	d.f.	P > F
Replicated experiment (runs)[2]	1	0.8294	1	0.2424
Fungicide Treatments (F)	2	<.0001	2	0.0124
Reps	3	0.0035	3	0.0414
F * Reps[3]	6	0.0011	6	0.0947
Runs*Reps(F)	11	0.0049	11	0.0788
Grain moisture (M)	1	0.8777	2	0.0168
Linear	-	-	1	0.0001
Quadratic	-	-	1	0.4619
F * M	2	0.0058	4	0.8778
Reps * M(F)	9	0.1045	18	0.1695
Container (Reps*Runs*F*M)	12	0.0063	24	0.0044
Storage time (T)	4	<.0001	4	<.0001
Linear	1	0.1729	1	0.4623
Quadratic	1	<.0001	1	0.0779
Cubic	1	<.0001	1	<.0001
Quartic	1	<.0001	1	0.0822
F x T	8	0.4360	8	0.0620
M x T	4	0.2061	8	0.2542
F x M x T	8	0.1537	16	0.5218
Reps * T(F)	36	0.0153	36	0.2045
Reps*M*T(F)	36	0.5447	72	0.4295
Residual	96		143	

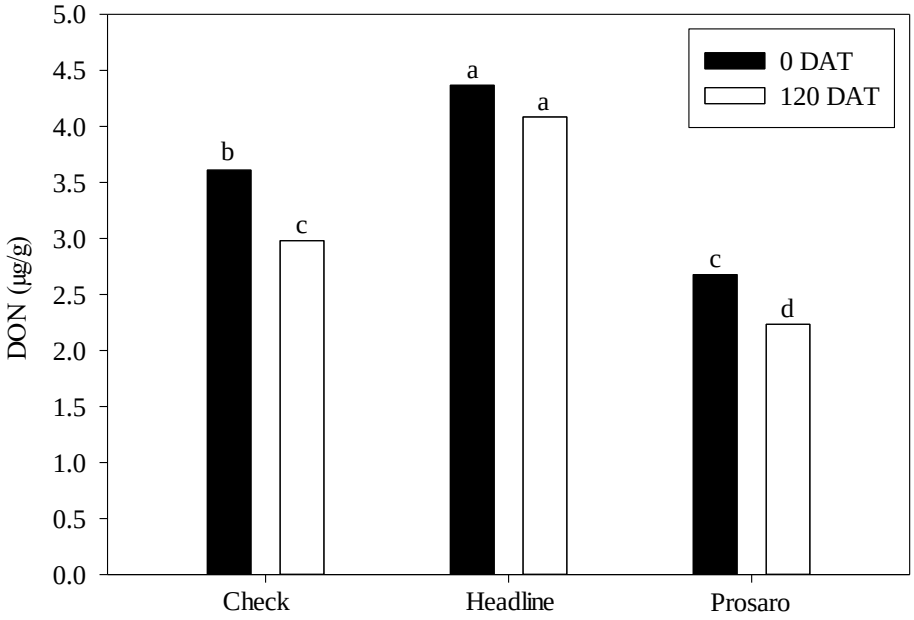

Fig. 1. DON concentration in storage averaged over moisture and time at 0 days after tempering (DAT) compared to 120 DAT in grain of winter wheat cv. Overland not treated (check) or treated at anthesis with the strobilurin fungicide Headline and the triazole fungicide Prosaro during the 2015 growing season. Means with the same letter are not significantly different according to the least significant difference (LSD) test at P = 0.05.

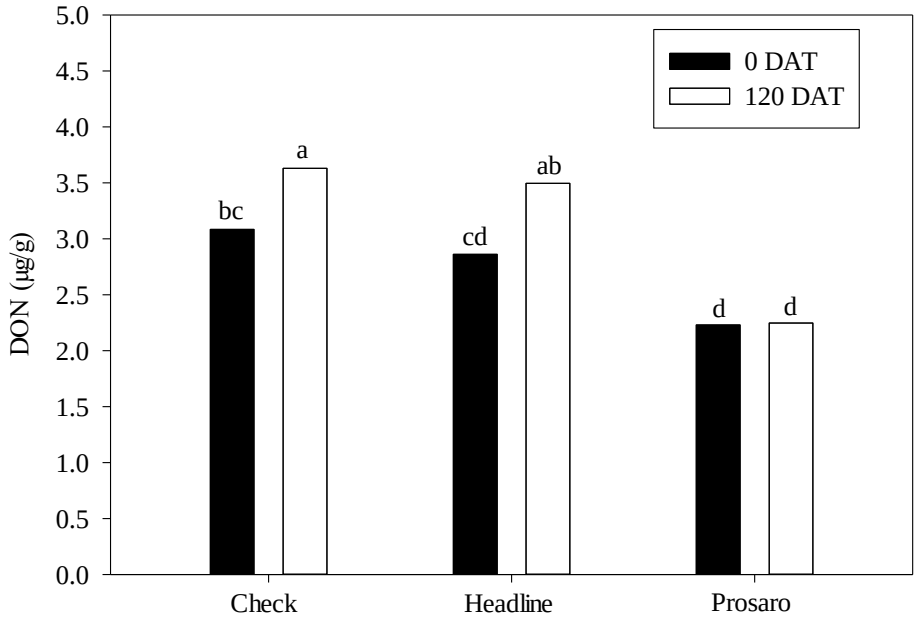

Fig. 2. DON concentration in storage averaged over moisture and time at 0 days after tempering (DAT) compared to 120 DAT in grain of winter wheat cv. Overley not treated (check) or treated at anthesis with the strobilurin fungicide Headline and the triazole fungicide Prosaro during the 2016 growing season. Means with the same letter are not significantly different according to the least significant difference (LSD) test at P = 0.05.

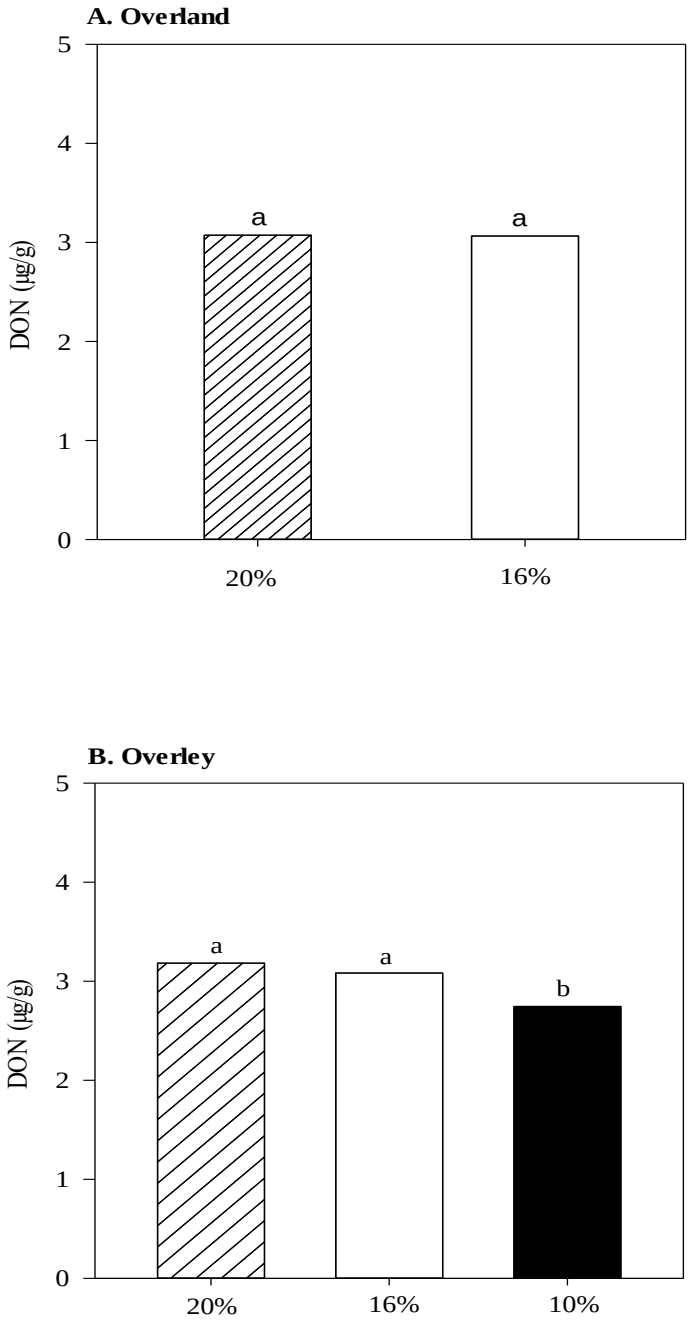

Fig. 3. Effects of moisture on DON concentration in storage averaged over fungicide treatments and time (120 days) in grain of winter wheat cultivars Overland and Overley.

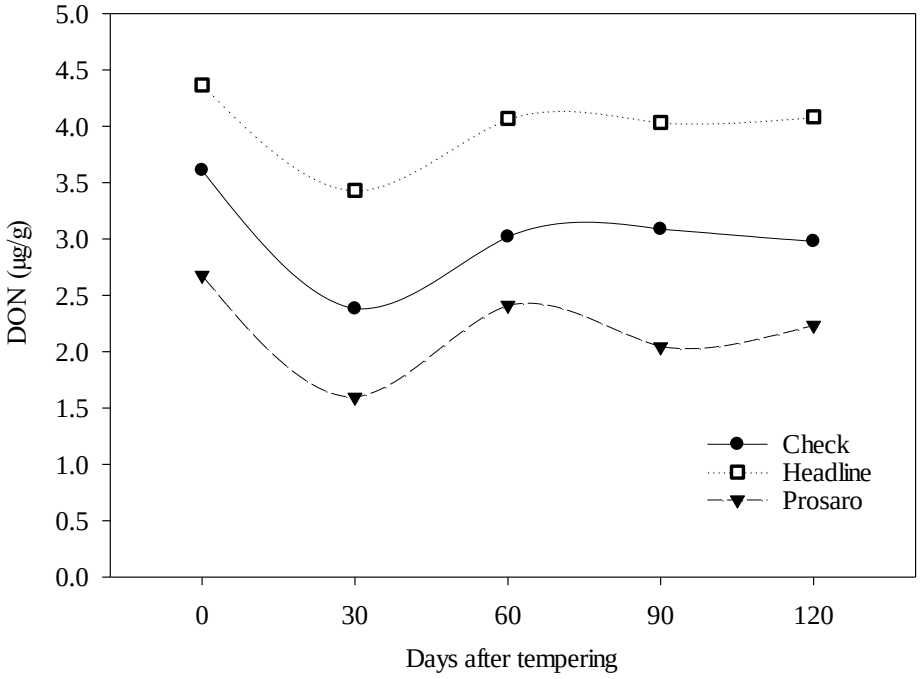

Fig. 4. DON concentration in storage averaged over moisture treatments in grain of winter wheat cv. Overland not treated (check) or treated at anthesis with the strobilurin fungicide Headline and the triazole fungicide Prosaro during the 2015 growing season.

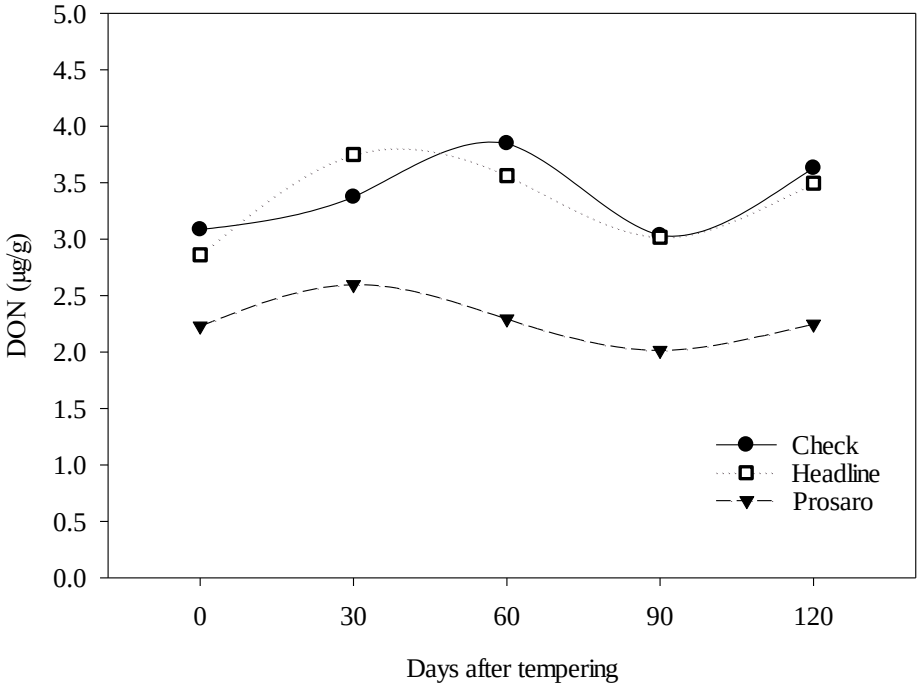

Fig. 5. DON concentration in storage averaged over moisture treatments in grain of winter wheat cv. Overley not treated (check) or treated at anthesis with the strobilurin fungicide Headline and the triazole fungicide Prosaro during the 2016 growing season.

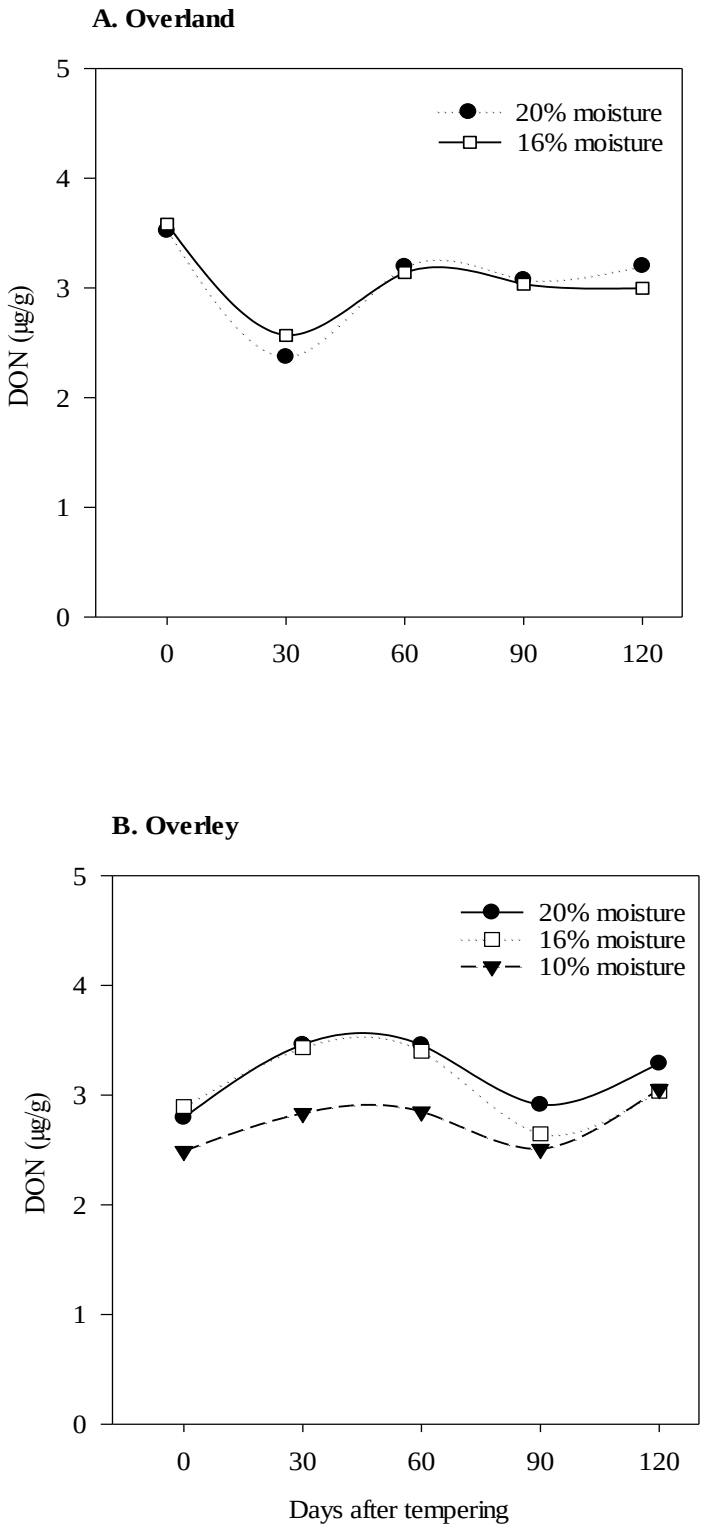

Fig 6. Effects of moisture on DON concentration in storage averaged over fungicide treatments in grain of winter wheat cultivars Overland and Overley.

CHAPTER IV

Tri5 GENE EXPRESSION ANALYSIS DURING POSTHARVEST STORAGE OF WHEAT GRAIN FROM FIELD PLOTS TREATED WITH A TRIAZOLE AND A STROBILURIN FUNGICIDE

1. Abstract

Fusarium head blight (FHB) and the trichothecene mycotoxin deoxynivalenol (DON) have profound negative impact on the wheat industry, worldwide. In the United States, FHB is mainly associated with *Fusarium graminearum*. The purpose of this study was to evaluate expression of the trichodiene synthase gene (*Tri*5) of *F. graminearum* in hard-red winter wheat susceptible or moderately resistant to FHB from field plots treated with a triazole and a strobilurin fungicide or left untreated during storage. Aliquots of infected wheat collected from field plots were put in storage and periodically sampled to determine *Tri*5 gene expression using quantitative reverse transcript PCR (qRT-PCR). Results showed consistent detection of the *GAPDH* (housekeeping gene), indicative of metabolically active fungi, and significantly high ($\chi 2 : P < 0.0001$) detection of *Tri*5 in the FHB-susceptible compared to the FHB-moderately resistant cultivar. *Tri*5 gene expression and DON were not or were minimally correlated which conveys that the DON prediction was not accurate. The strobilurin fungicide did not significantly reduce *Tri*5 gene expression compared with untreated wheat, while only in the triazole treatment, a significant reduction in the relative expression of the *Tri*5 was detected after 120 days, as well as a downregulation of *Tri*5 from 60 to 120 days of storage in the FHB-susceptible cultivar. In wheat grain from strobilurin-treated plots, the expression of *Tri*5 went up

from 0 to 30 days after tempering the grain in grain from Overland -2015 dryland as well as in grain from Overley- 2016 irrigated. Genetic expression of *Tri*5 that is necessary for DON production can increase during storage of high-moisture grain. *Fusarium* fungi can persist in wheat kernels for several months post-harvest and may actively produce toxin during this period.

2. Introduction

Fusarium head blight (FHB) is a major threat for wheat production worldwide. In the United States, FHB is mainly associated with *Fusarium graminearum*. FHB causes economic losses due to reduction in yield and accumulation of mycotoxins such as deoxynivalenol (DON). Mycotoxins are considered a global food security issue especially in low-income countries and places with deficient management of cereal grain during postharvest storage (Haubruge et al. 2003).

DON is one (of many) trichothecene mycotoxins, a group of related sesquiterpenoid compounds, produced by a wide range of Sordariomycetes. DON plays a function as a virulence factor, and it is essential for pathogen movement from florets to rachis in wheat heads (Jansen et al. 2005). The trichodiene synthase gene (*Tri*5) catalyzes the isomerization and cyclization of farnesyl pyrophosphate to trichodiene. This is the initial step in the DON production pathway (Hohn and Beremand 1989; Desjardins 2006). In the FHB-susceptible spring wheat cultivar Wheaton, strains of *F. graminearum* with a disrupted *Tri*5 gene showed reduced virulence and slow development of FHB symptoms (Proctor et al. 1995). Additionally, expression of the *Tri*5 gene during wheat infection is related to the inhibition of plant defense responses such as thickening of the cell wall

during the colonization process (Mudge et al. 2006; Jansen et al. 2005). Conjugation of DON to a less toxic glucoside (deoxynivalenol-3-glucoside; D3G) plays an important role in wheat resistance to FHB. Transgenic wheat expressing an UDP-glucosyltransferase for DON detoxification had significantly lower FHB compared to controls (Li et al. 2015).

DON inhibits protein synthesis allowing movement of the fungus from cell-to-cell (Brown et al. 2012). Quantitative reverse transcript PCR (qRT-PCR) can be used to measure the abundance of transcripts of the *Tri*5 gene *in planta* (Mudge et al. 2006) and thereby predict DON concentrations. Hallen-Adams et al. (2011) detected significant differences in the pattern of expression of DON biosynthetic genes during a wheat infection time course. *Tri*5 has been shown to be highly up-regulated at 7 days after inoculation of wheat heads (Mudge et al. 2006), 72 hours after inoculation (Hallen-Adams et al. 2011), and between cell division and cell differentiation stages in susceptible wheat (Chetouhi et al. 2016), while Brown et al. (2012) detected a peak in *Tri*5 gene expression during initial asymptomatic infection.

In the wheat-*F. graminearum* pathosystem, FHB and DON are controlled not only by using moderately-resistant cultivars, but also by applying fungicides. However, the selection of the fungicide chemical class and the fungicide application timing are critical for effective management. In the field, strobilurin fungicides can increase DON levels in wheat (Blandino and Reyneri 2009; Edwards et al. 2001; Ellner, 2005; Madden et al. 2014; Mesterhazy et al. 2003; Pirgozliev et al. 2002; Simpson et al. 2001). The mechanism producing this increase is not known. In contrast, triazole fungicides are

effective at controlling FHB and DON (Amarasinghe et al. 2013; Edwards et al. 2001; Mesterhazy et al. 2003; Pirgozliev et al. 2002; Wegulo 2012; Wegulo et al. 2015).

Grain mold pathogens can be divided into pre-harvest or field, and post-harvest or storage, according to their prevalence in different phases during development in the grain ecosystem. FHB-associated pathogens are considered as field molds; however, poor drying and cleaning practices can lead to postharvest mycotoxin accumulation (Aldred and Magan 2004). During storage, one of the critical factors influencing grain quality is moisture content. High grain moisture content is conductive to mycotoxin accumulation (Comerio et al. 1999; Hope et al. 2005). The dynamic of DON during postharvest storage of *F. graminearum*-infected winter wheat after different field applied fungicide treatments is unknown. The use of qPCR in wheat treated with fungicides provides a method to discriminate among fungicide efficacies which is not apparent in visual disease assessments (Doohan et al. 1999).

Pre-harvest and post-harvest management strategies heavily impact FHB pathogen structure and population in the field and during storage. The abundance of FHB pathogens in grain are well correlated with DON (Demeke et al. 2010; Horevaj et al. 2011). The correlation between DNA from trichothecene-producing *Fusarium* species and DON in harvested grain has been studied using competitive PCR to determine the efficacy of fungicides applied at anthesis in winter wheat (Edwards et al. 2001). *Tri*5 DNA and DON were at high levels and were positively correlated in inoculated field trials. Additionally, the *Tri*5-PCR assay showed that metconazole and tebuconazole (triazole fungicides) were highly effective in controlling trichothecene-producing

Fusarium, and that the highest concentration of *Tri*5 DNA (pg/ng of total DNA) was obtained in grain from azoxystrobin (a strobilurin)-treated plots.

Seed grain provides a good reservoir for FHB pathogens. High spore loads of *F. graminearum* and DON concentrations can be readily detected in grain harvested from wheat fields in an FHB epidemic year. Grain inoculated with *F. culmorum* at anthesis showed 16 times higher concentration (pg/ng of total DNA) of *Fusarium* DNA than non-inoculated seed lots (Glynn et al. 2007). The *Tri*5 gene has been used in qRT-PCR to evaluate the relative transcript abundance at different points of kernel colonization. The expression of *Tri*5 never ceased during the whole process of kernel colonization (Hallen-Adams et al. 2011). Furthermore, *Tri*5 gene expression and mycelial *in vitro* growth rate of *F. graminearum* were independent and the expression of *Tri*5 remained constant irrespective of the solute stress and incubation temperature (Marin et al. 2010).

This study aimed to evaluate the expression of the *Tri*5 gene in wheat with high grain moisture content during postharvest storage using qRT-PCR, as well as to determine the effect of postharvest storage time on *Tri*5 gene expression in winter wheat from field plots treated at anthesis with Headline (pyraclostrobin; a strobilurin) and Prosaro (prothioconazole + tebuconazole; triazoles). Information on the effects of field applied fungicide chemical class on DON production in storage will enable growers to make informed decisions on the choice of fungicide to apply to control FHB and DON. Also, information on DON accumulation in storage will be useful in setting the optimum grain storage conditions following harvest.

Knowing the relationship between the expression of DON biosynthesis genes (e.g. Tri5) and DON concentration during storage will enable researchers to accurately predict DON accumulation in storage. Understanding the conditions for transcriptional changes of the *Tri*5 gene may give clues behind the response of high DON in strobilurin-treated field trials, and the dynamics of *Tri*5 and DON during postharvest storage.

3. Materials and Methods

3.1 Field conditions. During the growing seasons 2015 and 2016, field trials were conducted at the Eastern Nebraska Research and Extension Center (ENREC), formerly the Agricultural Research and Development Center (ARDC) near Mead, Nebraska (41.2286° N, 96.4892° W). Hard red winter wheat (HRWW) cultivars Overley, which has shown susceptibility to FHB and DON accumulation in grain (Peiris et al. 2016); and Overland, which has shown moderate resistance to FHB and DON accumulation in grain (Baenziger et al. 2008; Jin et al. 2013; Nopsa et al. 2014), were sown under dryland and irrigated conditions. During the third week of April, in both 2015 and 2016, field plots were inoculated by spreading corn kernels colonized with *Fusarium graminearum* (67 kernels/m^2) on the soil where the wheat plants were growing.

A triazole-based fungicide (prothioconazole and tebuconazole) available commercially as Prosaro® (Bayer Ag life science, Kaiser-Wilhelm-Allee, Leverkusen, Germany) was applied at a rate of 0.467 L/ha to the wheat heads. A strobilurin-based fungicide (pyraclostrobin) available commercially as Headline® (BASF Corporation, Davis Drive, Research Triangle Park, NC) was applied at a rate of 0.657 L/ha to the wheat heads. Both fungicides were applied in the field plots at anthesis (A), when 30 to

40 percent of anthers were extruded. Fungicides were sprayed using a CO_2 propelled-backpack sprayer coupled with four tee-jet nozzles (TeeJet Technologies, Dillsburg, PA, USA) at a rate of 150 L/ha, and at a spray pressure of 241 kPa (35 psi). A nonionic surfactant (Induce, Helena Agri-Enterprises, Collierville, TN, USA) was added to the mixture at a rate of 0.125% vol./vol.

During anthesis, and 24 hours after applying the fungicide treatments, a second inoculation was carried out by spraying a spore suspension of *F. graminearum* (1×10^5 spores/mL) at a rate of 27 mL/m^2 on the wheat heads.

Wheat heads were harvested when grain moisture content dropped below 15% using a small combine for trial plots (Wintersteiger, Ried im Innkreis, Austria). Grain samples from each trial unit were threshed twice using a single-head thresher (Precision Machine, Lincoln, NE, USA).

3.2 Postharvest storage trials. Two postharvest storage trials were conducted with grain of the cultivar Overland from the 2015 growing season planted under dryland conditions (trial 1), and grain of the cultivar Overley, from the 2016 growing season planted under irrigated conditions (trial 2). Postharvest storage trials were conducted in duplicate at the wheat pathology laboratory and greenhouses at the University of Nebraska- Lincoln. Each experiment had three biological reps from each fungicide treatment by postharvest storage time combination.

For the 2015 growing season (trial 1), due to high levels of infection, Fusarium-damaged kernels (FDK) were removed from the mass of grain using a fractionating aspirator (Carter Day International, Inc., Minneapolis, MN, USA). The aspirator removed light kernels (FDK) by rotating a wire mesh which allowed the classification and

separation of grain with < 2% of FDK. Grain samples were passed through the scalperator three times. The mass of grain from each trial plot used for the postharvest storage trial 2 (2016 growing season, Overley- irrigated) consisted of a mixture of apparently healthy kernels and FDK (i.e., FDK were not separated).

Non-sterile grain (300 g), free of impurities, was placed on a plastic tray (50 cm x 30 cm x 3 cm height) and sprinkled evenly over the tray's surface. Grain samples were tempered with sterile distilled water using a heavy-duty manual sprayer (Rubbermaid, Wooster, OH). After tempering, grain samples were separated and homogenized manually and transferred to a sterile Microbox® hermetically-sealed micropropagation container of dimensions 15 cm x 15 cm x 20 cm height ($SacO_2$, Veldeken, Belgium). Microbox containers have a filter in the lid for gas exchange which blocks the entrance of external organisms or spores. Water activity (a_w) was determined using a Pawkit® water activity meter (Decagon Devices, Pullman, WA, USA). Grain moisture content (%) was determined using a grain moisture seed tester (Dickey John Corp., Auburn, IL, USA), model GAC 500-XT. After 14 days of tempering, grain moisture and a_w were determined. Sterile distilled water was added to reach 20% grain moisture. Grain samples were monitored for changes in the percentage of moisture content and a_w at monthly intervals. Relative humidity and temperature inside the containers were monitored using Watchdog® sensor model 1400 (Spectrum Technologies, Thayer Court, IL, USA). Samples were stored in a seed cooler (Bally Case & Cooler, Inc., Bally, PA, USA) in the dark at 10^{o}C and 40% environmental RH.

3.3 Deoxynivalenol determination. From each of the micro-propagation containers at the specified storage time, a random sample of grain was taken and milled using a

cyclone sample laboratory mill (UDY Corporation, Fort Collins, CO, USA). Deoxynivalenol (DON) quantification was performed in an Agilent 6890/5975 system at the Plant Pathology, Physiology, and Weed Science Department of the Virginia Polytechnic Institute and State University (Blacksburg, VA, USA) using gas chromatography-mass spectrometry (GC - MS).

3.4 RNA extraction and purification. Molecular studies were conducted in the laboratories of the Food Innovation Complex at the University of Nebraska-Lincoln. Samples of grain from cultivars Overland (trial 1) and Overley (trial 2), were freeze-dried and stored at -80^{o}C until RNA extraction. RNA extraction was performed using the hot phenol-chloroform and lithium chloride precipitation method according to the specifications of Goswami et al. 2006 with modifications. Briefly, freeze-dried grain samples were ground in a mortar and mixed with a heated (80^{o}C) mixture 1:1 of extraction buffer (Tris-LiCl-EDTA-SDS) and phenol. The extract was transferred into 30 mL tubes, and a half volume of chloroform was added to the mixture. The mixture was centrifuged for 30 minutes at 2500 x *g*. An aqueous layer formed at the top of the tube was transferred to a new tube and one third volume of 8M LiCl was added. The tube was incubated on ice for at least 2 hours. Centrifugation was performed for 5 minutes at 12000 x *g* and 4^{o}C. The pellet formed at the bottom of the tube was washed with 3 mL of 2M LiCl and 3 mL of 70% ethanol. The supernatant was removed and re-suspended in 2 mL ultra-pure DEPC-treated water, followed by the addition of 200 μL of 3M NaOAc, and 5.5 mL of 95% ethanol. The suspension was kept at -80^{o}C for 15 minutes, and then centrifuged for five minutes at 12000 x *g*. The pellet at the bottom of the tube was

washed with 3 mL of 70% ethanol. Centrifugation and washing were repeated twice. The pellet was dissolved in 100 µL ultra-pure DEPC-treated water, and transferred to a 1.5 mL Eppendorf tube. RNA was quantified using an Eppendorf Bio-Photometer plus (Eppendorf North America, Hauppauge, NY, USA). For DNase treatment, a mixture of 2 µL (20 U) of Thermo scientific DNase I (Life technologies, Carlsbad, CA, USA), 4 µL of 10X incubation buffer, and 88 µL of nucleic acids was incubated at 37^{o}C for 15 minutes, and then incubated further after adding 0.2 M EDTA at 75^{o}C for 10 minutes. The sample was purified with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. After RNA purification using the Qiagen kit, the concentration and absorbance at 260/280 nm were taken with the BioPhotometer.

3.5 cDNA assay. Complementary strand DNA (cDNA) was prepared using the Thermo-Fisher Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Life technologies, Carlsbad, CA, USA). RNA concentration was adjusted to 1 µg using molecular biology grade water. On ice, 4 µL of 5X reaction mix and 2 µL of reverse transcriptase were mixed together with 14 µL of the RNA- water suspension for a total of 20 µL volume reaction. cDNA reaction was conducted in a T-100^{TM} thermal cycler (BIO-RAD, Hercules, CA, USA) and the amplification protocol consisted of an initial cycle at 25^{o}C for 10 minutes, followed by incubation at 50^{o}C for 15 minutes, and final cycle at 85^{o}C for 5 minutes.

3.6 Quantitative reverse transcript PCR. Quantitative reverse transcript PCR was conducted in an Eppendorf MasterCycler RealPlex (Eppendorf North America, Hauppauge, NY, USA) using SYBR Green I chemistry. Data acquisition and

visualization was carried out by the MasterCycler ep RealPlex software. Primer pairs *Tri*5-F (5'-TCT ATG GCC CAA GGA CCT GTT TGA- 3') and *Tri*5-R (5'- TGA CCC AAA CCA TCC AGT TCT CCA -3'), and *Gapdh*–F (5'- CTA CAT GCT CAA GTA CGA CTC TTC C – 3') and *Gapdh* –R (5'- GCC GGT CTC GGA CCA CTT G – 3'), amplifying *Tri5* and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), respectively (Hallen-Adams et al. 2011) were used in a paired qRT-PCR assay. The *GAPDH* (housekeeping gene) was amplified to normalize the expression of the *Tri*5 gene. qRT-PCR assays were conducted in a 96-well PCR plate (half of the plate to amplify the *GAPDH* gene, and half for the *Tri*5 gene). A preliminary assay was conducted using cDNA samples from wheat grain to determine the adequate primer concentration (0.5, 1, 1.5, 2 and 2.5 µL/reaction) in the qRT-PCR. Final volume of qRT-PCR reactions contained: 1 µL of cDNA product, 2.5 µL of each *Tri*5 primer (forward and reverse) or 1.5 µL of each *GAPDH* primer, 12.5 µL of the Thermo Fisher Maxima SYBR Green qPCR Master Mix (Life technologies, Carlsbad, CA), and water to 25 µL.

Amplification consisted of an initial denaturation step at 95ºC for 2 minutes, followed by 40 cycles which consisted in denaturation at 95ºC for 15 seconds, annealing at 55ºC for 15 seconds, and extension at 68ºC for 20 seconds. Final holding temperature was 4ºC.

3.7 Data analysis. Threshold values from each qRT-PCR assay were adjusted manually. Cycle threshold (C_t) values were archived for *Tri*5 and *GAPDH*. Individual C_t values of the target *Tri*5 gene were compared with that of the housekeeping gene *GAPDH*, and the relative ratio of expression was calculated (cycle threshold [C_t] ratio). Normalization of the *Tri*5 gene expression was done based on the C*t* value of the fungal *GAPDH* from each corresponding assay. Relative gene expression was calculated using

the $2^{-\Delta\Delta Ct}$ (Schmittgen and Livak 2008) using the expression of the untreated check plots as calibrators.

Statistical analysis was carried out using SAS software version 9.4 (SAS Inc, Cary, NC) for the ratio of *Tri*5 gene expression relative to *GAPDH.* In each postharvest storage trial, both experiments were analyzed using generalized linear mixed models (PROC GLIMMIX). The Fisher-least significant difference (Fisher-LSD, α=0.05) was used to determine differences among LS-means through the main effects of the factors (fungicide treatments at anthesis and postharvest storage time) and the interaction fungicide treatments by storage time. The F-ratio test (Gomez and Gomez 1984) was used to determine homogeneity of error of variances. Based on these results, experiments were combined and LS-means, standard errors (SE) and standard deviations (SD) were used for graphics.

Pearson correlation coefficients among the ratio *GAPDH*/*Tri*5 versus DON, and C_t*Tri*5 versus DON were requested using PROC CORR in SAS, using LS-means by fungicide treatment and postharvest storage time. LS-means were compared separately by trials. Chi-square (χ^2) test was used to determine if the threshold at which *Tri*5 gene was detected differed by cultivar (Overley versus Overland). Logit test on the binary response distribution was conducted using PROC GLIMMIX. The binary variable consisted in the detection or absence of the gene of interest and/or reference gene. The results of the type II test of fixed effects were used to determine if there was an effect of fungicide treatments, postharvest storage time or the interaction fungicide treatment by storage time on the detection of the *Tri*5 gene.

4. Results

In Overland (moderately resistant to FHB; trial 1), *Tri*5 was detected in 74 % of the total reactions conducted. In Overley (susceptible to FHB and DON accumulation; trial 2), *Tri*5 was detected in 96% of the reactions. Pearson's Chi-square test showed a significant difference ($p<0.0001$) in the frequency of reactions at which *Tri*5 gene was detected in Overley versus Overland (Table 3). Descriptive statistics by trial in each combination of fungicide treatment by postharvest storage time are presented in Table 1. Overall, the coefficients of variation were low in both trials with 12.8% and 8.2% for C_t*Tri*5 and 13.8% and 12.7% for the ratio of *Tri*5 expression relative to *GAPDH* in trials 1 and 2, respectively. In most cases the standard deviation in the C_t*GAPDH* was higher than in the C_t*Tri*5 (Table 1). The housekeeping gene *GAPDH* showed high relative expression (Table 1) denoted as lower C_t values than *Tri*5 in both trials (Table 1).

The effect of the interaction fungicide treatments by storage time was consistently detected as significant on *Tri*5 gene expression relative to *GAPDH* (Ratio C_t*GAPDH*/C_t*Tri*5) (Table 2). Overall, there was an increase in relative *Tri5* gene expression during storage (Figure 1). LSD-Fisher over the total reactions at which *Tri*5 was detected in both trials (653) showed a significant difference in *Tri*5 gene expression at 120 days of storage (Ratio C_t*GAPDH*/C_t*Tri*5 = 0.79) compared to the expression registered at 0 days (0.75) and 30 days of storage (0.76). The trend over postharvest storage time is summarized in Figure 1 for both trials. In Overland grain from triazole-treated plots, the C_t ratio of *GAPDH*/*Tri*5 increased from 60 days to 120 days of postharvest storage (Figure 1). However, *Tri*5 expression in these combinations of fungicide and storage time were not significant compared to strobilurin-treated grain at 30 days and untreated checks at 60 and 120 days of storage (Fisher-LSD; $\alpha = 0.05$).

Although, a significant increase of *Tri*5 gene expression was detected in Overland grain from strobilurin-treated plots from 60 to 120 days of storage, the detection of the gene of interest was significantly lower ($p = 0.0154$) in the grain from triazole-treated (*Tri*5 detected in 66% of samples/reac in trial 1) than in the strobilurin-treated (81% *Tri*5 detection) and untreated check plots (77% *Tri*5 detection) according to the Logit test of the binary response distribution.

In Overley, *Tri*5 gene expression levels were higher in the grain from strobilurin-treated plots (Ratio C_t*GAPDH*/C_t*Tri*5 = 0.79) compared to the grain that came from triazole treated plots (0.73) and untreated check plots (0.75). The strobilurin fungicide did not significantly reduced *Tri*5 gene expression compared with the untreated grain (Figure 1).

At the first evaluation after tempering the grain (30 days of storage), grain that came from strobilurin-treated plots showed a consistent increase in *Tri*5 gene expression over that from untreated check plots (Figure 2). A 2.6-fold and 4.4-fold change in gene expression was detected in Overland (trial 1) and Overley (trial 2), respectively. In both trials, from 30 to 120 days of storage, fold changes in gene expression were variable without a clear trend and dependent of the expression of the reference sample (untreated check) instead of a clear fungicide effect (Figure 2). In grain from Overland (trial 1), Tri5 was downregulated from 0 to 30 days of storage (Figure 2).

Pearson's correlation coefficients were not significant among DON concentration and either the Ct ratio *GAPDH*/*Tri*5 or DON-C_t*Tri*5, for either cultivar. (Figure 3).

5. Discussion

The population of toxigenic fungi in stored grain depends largely on the field and storage conditions, as well as harvest process (Magan et al. 2014). Under poor storage conditions the risk of mycotoxin contamination increases due to the growth of *F. graminearum* in the mass of grain. In this study, qRT-PCR was conducted to determine changes in gene expression of the *Tri*5 gene under postharvest storage conditions in grain that came from field plots treated with triazole or strobilurin fungicides at anthesis, or left untreated.

Detection of *Tri*5 gene was higher in the post-harvest trial using Overley grain (FHB-susceptible) than in the Overland grain (moderately resistant). Spread of *Fusarium* through the wheat head is promoted by DON, and higher *Tri*5 expression (and thus higher DON) in the susceptible cultivar would lead to higher levels of infection and spread of the fungus within plant. (Bai et al. 2001; Jansen 2005; Jiao et al. 2008; Kumar et al. 2015). Our results showed significantly higher (χ^2; $p < 0.0001$) levels of *Tri*5 gene detection in the FHB-susceptible compared to the FHB-moderately resistant cultivar. Jansen et al. (2005) reported that the progress of infection in an FHB-moderately resistant barley (*Hordeum vulgare*) cultivar was slower than in FHB-susceptible cultivars. Also, Hallen-Adams et al. (2011) found that spring wheat carrying the *Fhb*1 allele for FHB resistance showed minimal detection of *Tri*5 and significant deviation in *Tri*5 gene expression compared with a FHB-susceptible cultivar.

Levels of expression of the *Tri*5 gene by *F. graminearum* may be related to the activation of genes of resistance or susceptibility by the host. Gene expression is highly variable between an FHB-susceptible and an FHB-moderately resistant cultivar. In a microarray study conducted by Bernardo et al. (2007), as many as 86% of the wheat

genes differentially expressed in response to *F. graminearum* came from the FHB-susceptible cultivar (Clark), and 14% of the genes came from the FHB-moderately resistant cultivar (Ning 7840). The activation of susceptibility genes in the FHB-susceptible cultivar could trigger a synergistic expression of *Tri*5 in *F. graminearum*, and therefore increase of *Tri*5 detection in the FHB-susceptible than in the FHB-moderately resistant cultivar.

Fungicide treatments not only control the disease in the field but also result in higher quality grain that is less affected by pathogens which could potentially increase mycotoxin levels during storage. Fungicides applied to control FHB pathogens reduce the amount of trichothecene-producing *Fusarium* present in grain and, indirectly, DON concentrations (Pirgozliev et al. 2002). DON biosynthesis *Tri*5 gene expression was significantly reduced in the relative expression of the *Tri*5 gene and a downregulation of the gene occurred in the triazole treatment from 0 to 30 days of storage in Overland grain, whereas expression of the gene increased in the strobilurin treatment from 0 to 30 days in both trials using Overland and Overley grain. In the FHB-susceptible cultivar, the *Tri*5 gene expression levels were lower in grain that came from triazole-treated plots than either non-fungicide treated check or strobilurin-treated plots. Furthermore, only in grain from triazole-treated plots was a significant reduction in the relative expression of the *Tri*5 detected, while in the grain from the non-fungicide sprayed check plots and strobilurin-treated plots an increase in the relative expression of the *Tri*5 was detected at the end of the postharvest trial. Therefore, the population of *F. graminearum* actively producing DON was apparently diminished by the action of the triazole fungicide. Conversely, in grain from strobilurin-treated plots, the expression of *Tri*5 went up after

tempering the grain (30 days) in both trials (Figure 1). Gene expression decreased in wheat treated with a triazole fungicide. Also, *Tri*5 was significantly downregulated from 60 to 120 days of storage. However, DON was still being produced in stored grain 120 days after harvest. These findings emphasize the value of triazole fungicide usage (and the fact that treating wheat for foliar diseases with strobilurins will not protect against head blight).

Storage conditions, especially grain moisture, had a significant impact on DON. Our study demonstrates that the genetic expression of the *Tri*5 (necessary for the production of DON) can increase postharvest due to the presence of a transcriptionally-active mass of fungi under conditions of high grain moisture content. In grain of both cultivars, the high and consistent expression of the housekeeping gene *GAPDH* indicated the presence of living postharvest fungi (Table 1). Therefore, there is potential for metabolically active fungi to grow and produce toxin during several months of storage. Transcriptional activity of the *Tri*5 gene from *F. graminearum* was detectable in grain with high grain moisture content (20%, $a_w = 0.75$) after 120 days of postharvest storage after tempering the grain. Hallen-Adams et al. (2011) detected *Tri*5 activity in senescent tissue of a susceptible cultivar. The authors suggested the ability of the fungus to resume DON biosynthesis in dried infected grain. This study corroborates that suggestion, demonstrating increases of transcriptional activity of *F. graminearum-Tri*5 from 0 to 120 days after tempering the grain.

*Tri*5 gene expression was not a good predictor of deoxynivalenol grain concentration, as DON was not correlated with *Tri*5 relative expression. Similarly, Bernaldez et al. (2017) found that the expression of *afl*R gene in *Aspergillus flavus* was not a good

indicator of aflatoxin B1 production. These results are also in agreement with Hallen-Adams et al. (2011) who observed inconsistent correlation of *Tri*5 expression relative to fungal *GAPDH* and DON concentration. Regulation of the trichothecene production is complex, with unknown positive and negative factors affecting the expression of the toxin gene *Tri*5 (Hallen-Adams et al. 2011) and other genes in the *Tri* cluster (Jiao et al. 2008; Schmidt-Heydt et al. 2011). Early induction of *Tri* genes in asymptotic tissue has been detected in several studies (Brown et al. 2012; Chetouhi et al. 2016; Hallen Adams et al. 2011; Mudge et al. 2006). Lee et al. (2014) hypothesized that *Tri* transcript accumulation is necessary before initiating DON biosynthesis. *Tri* gene expression has been shown to be maximal during symptomless infection. If DON biosynthesis is dependent on an initial *Tri* accumulation, then direct temporal correlations between DON and gene expression of members of the *Tri* cluster are unlikely.

In conclusion, DON can be present during postharvest storage even if *F. graminearum* is not actively transcribing *Tri*5. Cultivar resistance to FHB seems to affect both detection and expression of the *Tri*5 gene. If conditions are favorable for mold growth, *F. graminearum* can express *Tri*5 and therefore produce higher accumulation of DON in grain storage. Field management practices, such as cultivar resistance and fungicide chemical class and application timing have an impact on the quality of stored grain, and therefore should not be underestimated.

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Table 1. Summary statistics on the evaluation of the effect of fungicide treatments applied at anthesis and postharvest storage time on gene detection (C_t*GAPDH* and C_t*Tri*5) in grain from the FHB – moderately resistant cultivar Overland (dryland – growing season 2015; trial 1) and FHB-susceptible cultivar Overley (irrigated – growing season 2016; trial 2) averaged over two experiments.

Fungicide treatment	Storage time	Variable	Total reactions		Mean		Standard Deviation		Minimum		Maximum	
			Overland	Overley	Overland	Overley	Overland	Overley	Overland	Overley	Overland	Overley
Check	0	C_t*GAPDH*	21	21	24.34	23.82	4.03	3.56	18.65	18.65	33.91	30.71
	0	C_t*Tri5*	20	20	32.63	32.28	2.68	2.23	29.14	29.14	39.37	36.34
	30	C_t*GAPDH*	22	22	22.25	22.00	1.93	1.56	18.57	18.57	27.55	25.53
	30	C_t*Tri5*	21	21	31.08	31.08	1.91	1.91	28.19	28.19	35.33	35.33
	60	C_t*GAPDH*	20	20	25.34	25.34	3.43	3.43	20.53	20.53	34.61	34.61
	60	C_t*Tri5*	20	20	32.9	32.90	3.09	3.09	27.99	27.99	39.71	39.71
	90	C_t*GAPDH*	21	21	25	25.00	2.99	2.99	21.7	21.70	33.26	33.26
	90	C_t*Tri5*	21	21	31.95	31.95	1.55	1.55	28.54	28.54	34.92	34.92
	120	C_t*GAPDH*	20	20	24.36	24.36	3.7	3.70	19.53	19.53	35.25	35.25
	120	C_t*Tri5*	21	21	30.51	30.57	1.52	1.53	27.19	27.19	32.8	32.80
Strobilurin	0	C_t*GAPDH*	29	29	22.65	22.44	2.8	2.60	18.22	18.22	31.11	31.11
	0	C_t*Tri5*	29	29	30.27	30.34	3.03	3.07	24.95	24.95	36.14	36.14
	30	C_t*GAPDH*	29	29	24.06	24.03	3.95	4.02	18.88	18.88	34.27	34.27
	30	C_t*Tri5*	29	29	30.31	30.36	1.84	1.86	26.77	26.77	33.9	33.90
	60	C_t*GAPDH*	27	27	24.32	23.98	3.25	2.79	18.77	18.77	33.09	29.64
	60	C_t*Tri5*	27	27	31.61	31.56	2.7	2.75	26.7	26.70	36.29	36.29
	90	C_t*GAPDH*	28	28	24.35	23.93	3.22	2.96	18.82	18.82	32.19	29.76
	90	C_t*Tri5*	26	26	31.75	31.61	2.56	2.51	27.23	27.23	36.6	36.60
	120	C_t*GAPDH*	29	29	23.44	23.23	4.73	4.58	17.8	17.80	34.79	34.79
	120	C_t*Tri5*	28	28	30.99	30.68	3.54	3.18	24.92	24.92	39.47	37.08
Triazole	0	C_t*GAPDH*	25	25	24.1	24.15	2.46	2.47	20.74	20.74	29.61	29.61
	0	C_t*Tri5*	22	22	31.64	31.62	3.27	3.23	27.29	27.29	38.2	37.96
	30	C_t*GAPDH*	25	25	25.05	24.71	2.49	2.28	21.34	21.34	29.45	28.54
	30	C_t*Tri5*	23	23	33.1	33.10	2.25	2.25	29.49	29.49	37.59	37.59
	60	C_t*GAPDH*	24	24	23.18	23.27	2.43	2.52	19.51	19.51	31.67	31.67
	60	C_t*Tri5*	22	22	32.13	32.13	2.04	2.04	27.56	27.56	35.61	35.61
	90	C_t*GAPDH*	25	25	24.45	24.45	3	3.00	19.87	19.87	31.43	31.43
	90	C_t*Tri5*	25	25	32.45	32.45	2.92	2.92	27.74	27.74	38.87	38.87
	120	C_t*GAPDH*	25	25	23.43	23.43	2.88	2.88	20.19	20.19	31.6	31.60
	120	C_t*Tri5*	25	25	30.93	30.93	2.56	2.56	26.99	26.99	36.5	36.50

Table 2. ANOVA summary on the evaluation of Fungicide treatments applied at anthesis and storage time on the *Tri5* gene expression relative to *GAPDH* (Ratio Ct*GAPDH*/Ct*Tri5*) in grain from the FHB-moderately resistant cultivar Overland (Dryland – growing season 2015; trial 1) and FHB-susceptible cultivar Overley (Irrigated – growing season 2016; trial 2).

	2015-Overland Dryland (*Trial 1*)						2016-Overley Irrigated (*Trial 2*)					
	Experiment[a] *1*			*Experiment 2*			*Experiment 1*			*Experiment 2*		
Source	**df**[b]	**F value**	**P-value**	**Df**	**F value**	**P-value**	**df**	**F value**	**P-value**	**df**	**F value**	**P-value**
Fungicide Treatment (F)	2	0.29	0.7658	2	0.72	0.5271	2	1.09	0.3949	2	0.53	0.6119
Storage Time (T)	4	1.11	0.3529	4	2.55	0.0421	4	0.12	0.9742	4	1.92	0.1097
*F*T*	8	1.90	0.0653	8	2.45	0.0163	8	2.46	0.0155	8	2.28	0.0246
Residual	130			140			167			156		

[a] Each experiment had three independent biological reps

[b] The number of degrees of freedom was dependent of the total reactions at which *Tri5* and *GAPDH* were detected

Table 3. Count frequency of qRT-PCR reactions at which *Tri5* gene was or was not detected in two winter wheat cultivars with different reaction to FHB and DON, Overland (moderately-resistant – trial 1) and Overley (susceptible – trial 2).

***Tri5* gene**	**Overland-2015 Dryland**		**Overley-**		**Total**
	O[a]	**E[b]**	**O**	**E**	
Detected	300	337.82	353	315.18	653
Non-detected	103	65.18	23	60.82	126
Total[c]	403		376		779
χ^2; P<0.0001					

[a] O = Observed values represent the total counts of reactions at which Tri5 was or was not detected

[b] E = Expected counts under the independence hypothesis (*Ha* = Detection of *Tri5* is dependent of the cultivar)

[c] Totals are the sum of reactions conducted in two independent experiments at each trial.

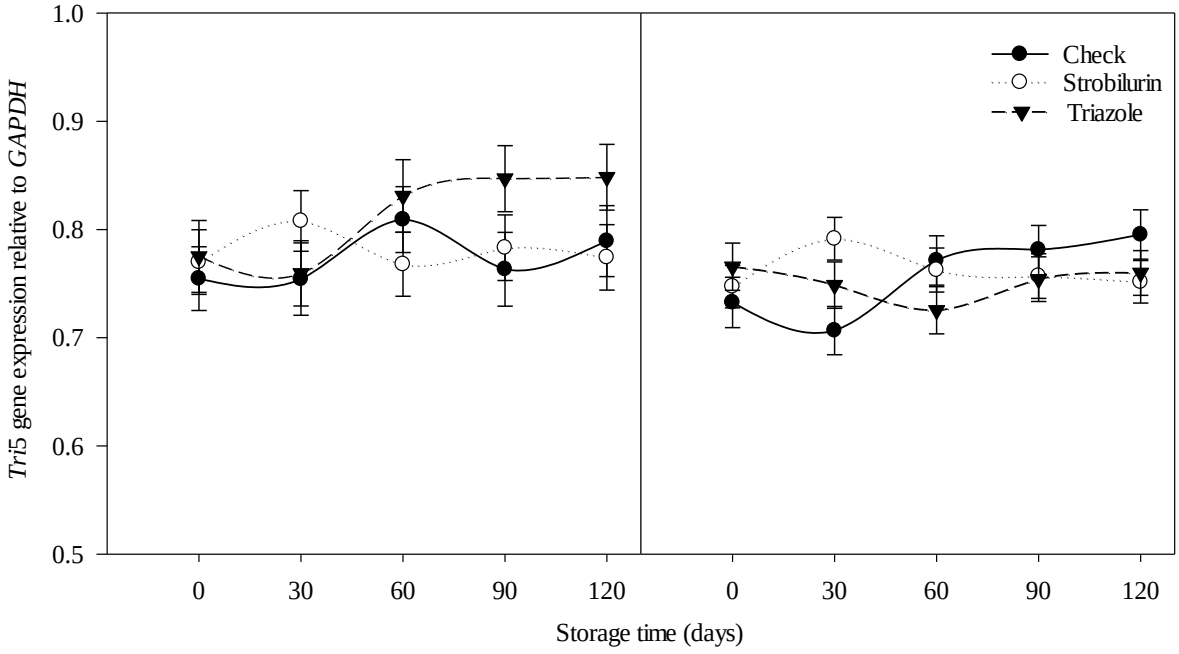

Fig. 1. Effect of postharvest storage time on *Tri5*-gene expression relative to *GAPDH* (Ratio C_t*GAPDH*/C_t*Tri5*) in grain that came from triazole- and strobilurin-treated plots at anthesis, and non-fungicide sprayed plots in the (A) 2015 growing season in the cultivar Overland (moderately resistant to FHB and DON accumulation) – Dryland (trial1), and the (B) 2016 growing season in the cultivar Overley (susceptible to FHB and DON accumulation) –Irrigated (trial2). LS-means came from two independent experiments at each trial.

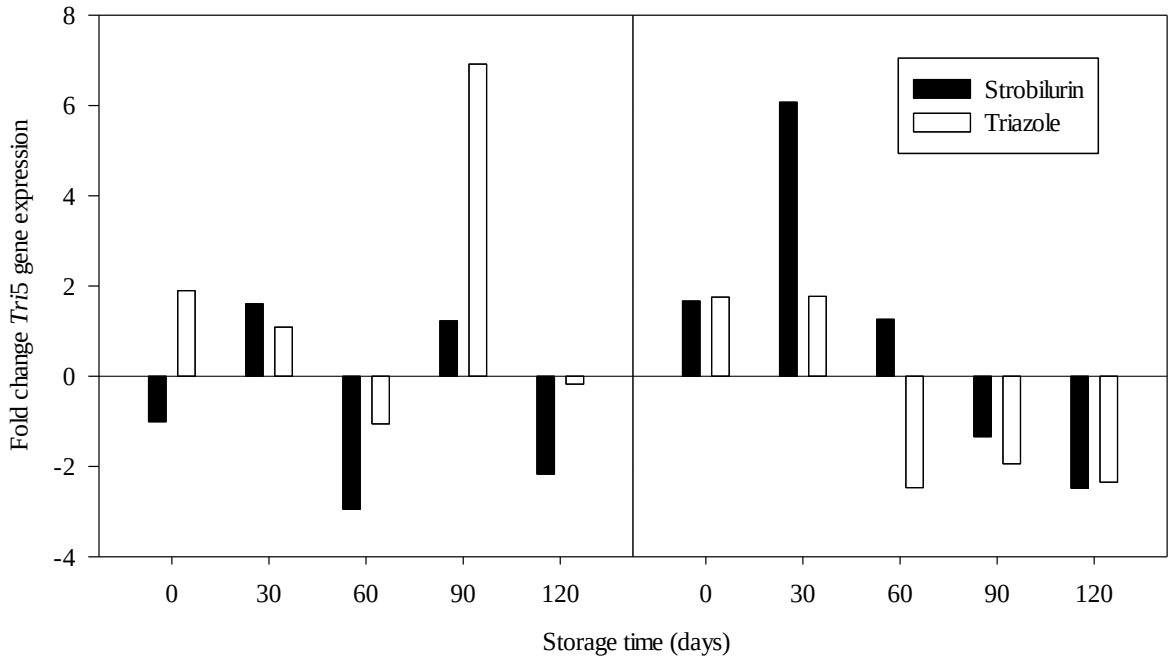

Fig. 2. Fold change in *Tri5* gene expression relative to the GAPDH in wheat grain from Triazole- and Strobilurin-treated plots of the cultivars (A) Overland (moderately-resistant to FHB) – 2015 Dryland (Trial 1) and Overley (susceptible to FHB) – 2016 Irrigated (Trial 2).Non-fungicide treated plots were used as calibrators. Data for $2^{-\Delta\Delta Ct}$ calculation correspond to LS-means averaged over two independent experiments at each Trial.

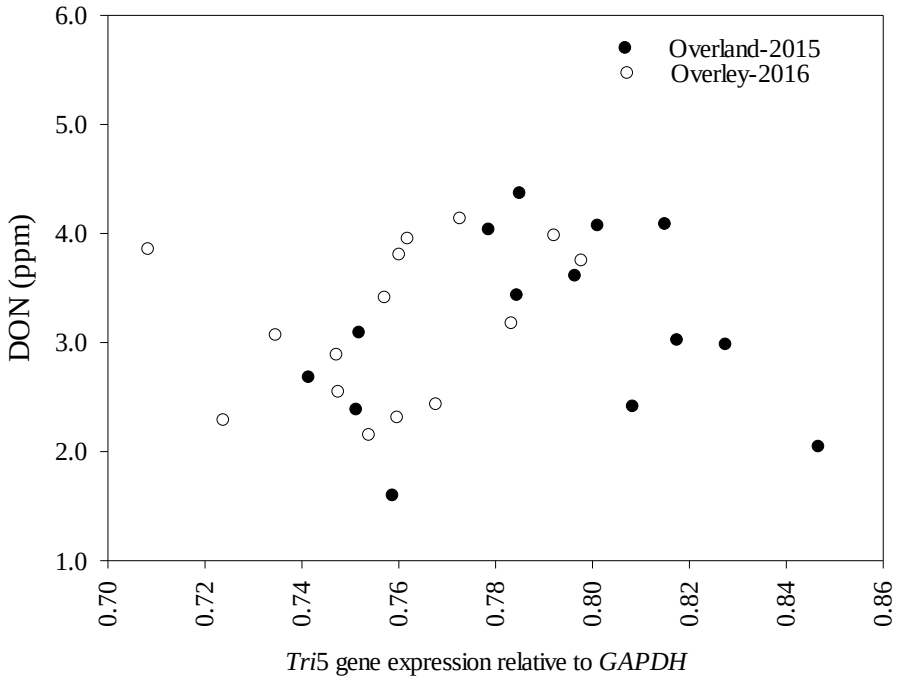

Fig. 3. Dependence of the amount of DON and the *Tri5* relative gene expression to *GAPDH* in grain of the cultivars Overland (moderately resistant to FHB and DON accumulation) 2015 – Dryland (Trial 1), and Overley (susceptible to FHB) 2016 - Irrigated (Trial 2) for the combinations of fungicide treatments by postharvest storage time (n = 15). LS-means came from two independent experiments at each Trial.

CHAPTER V

DETERMINING THE OPTIMUM INOCULUM CONCENTRATION AND SPIKE BAGGING PERIOD FOR DISCRIMINATING BETWEEN FHB-SUSCEPTIBLE AND -RESISTANT WHEAT CULTIVARS UNDER GREENHOUSE CONDITIONS

1. Abstract

Fusarium head blight (FHB), caused mainly by *Fusarium graminearum*, results in devastating economic losses in small grain cereal crops. In regions where FHB occurs frequently, breeding for resistance to the disease is a priority in small grain breeding programs. Screening lines or cultivars for resistance to FHB under controlled conditions is necessary due to the sporadic nature of the disease under field conditions. However, screening for resistance is challenging due to the quantitative nature of resistance to FHB and the variability in aggressiveness of pathogen isolates. Too high or too low inoculum concentration or too much humidity can lead to inaccurate results. Greenhouse experiments were conducted to determine the optimum inoculum concentration and spike bagging period following inoculation for discriminating between a susceptible and a moderately resistant spring wheat cultivar. The cultivars used were Samson (susceptible) and Glenn (moderately resistant). In one experiment, spikes were inoculated at anthesis with the standard spore concentration of 1×10^5 *F. graminearum* spores/mL and 1/2, 1/4, 1/8, and 1/16 of the standard concentration. In a second experiment, spikes were inoculated at anthesis with the standard spore concentration of 1×10^5 *F. graminearum* spores/mL and covered with Ziplock® bags for 12, 24, 36, 48, or 72 hours. In both experiments, FHB severity was visually assessed seven times at 3-day intervals following

inoculation. The percentage of *Fusarium*-damaged kernels (FDK) and deoxynivalenol (DON) concentration were determined after harvest. FHB severity was the best variable for discriminating between the two cultivars. FHB severity results showed that 1/16 and 1/8 of the standard spore concentration discriminated between the two cultivars whereas higher concentrations did not. The best discrimination between the two cultivars was achieved by bagging spikes for 48 or 72 hours following inoculation. The results from this study indicate that for screening wheat cultivars for resistance to FHB under greenhouse conditions, lower concentrations of *F. graminearum* spores (6.25×10^3 or 1.25×10^4 spores/mL) are better than higher concentrations, and the optimum spike bagging period following inoculation is 48 to 72 hours.

2. Introduction

Fusarium head blight (FHB) is an economically important disease of wheat and other small grain cereals. The main causal agent of FHB in North America is *Fusarium graminearum* (O'Donnell et al. 2000; Hernandez-Nopsa et al. 2014). The disease is characterized by sudden bleaching of wheat spikes, and by the sporadic nature of its epidemics (McMullen et al. 1997). In Nebraska, recent epidemics of FHB in wheat occurred in 2007, 2008, and 2015 (McMullen et al. 2012; Bolanos-Carriel et al. 2015; Wegulo et al. 2011).

Genetic resistance provides effective control of the disease, and the use of moderately resistant cultivars is the most cost-effective management strategy (Wegulo et al. 2015). Major sources of resistance to FHB are found in cultivars that have native resistance such

as Sumai 3, Glenn, and Overland (Anderson et al. 2001; Baenziger et al. 2008; Mergoum et al. 2006, 2007; Schweiger et al. 2016; Waldron et al. 1999; Zhou et al. 2003). Sumai 3 has been extensively studied to identify quantitative trait loci (QTL) conferring FHB resistance. Overland is adapted to rainfed conditions of the Great Plains of the United States and has moderate resistance to FHB. Glenn is a hard red spring wheat cultivar that has Sumai 3 FHB resistance.

Schroeder and Christensen (1963) proposed two types of FHB resistance in wheat: type I and II. Type I, or resistance to initial infection, includes defense reactions such as activation of enzymes that degrade the fungal cell wall or pathogenesis-related (PR) proteins (Walter et al. 2010). Type I resistance is estimated by spraying a spore suspension over flowering spikes and counting diseased spikelets (Miedaner et al. 2003). QTLs *Fhb*4 and *Fhb*5 confer type I resistance (Buerstmayr et al. 2003). Type II, or resistance to spread of the pathogen within a spike, is associated with movement of the pathogen from one infected spikelet to another via the rachis. This type of resistance is estimated by delivering conidia into a single floret of a spike and counting the blighted spikelets after a period of time. *Fhb1*, *Fhb2*, and *Fhb3* are QTLs mapped for type II resistance (Buerstmayr et al. 2003).

Mesterhazy (1995) proposed five types or components of resistance to FHB in wheat. In addition to type I and II described above, he proposed type III or kernel size and number retention, type IV or yield tolerance, and Type V or resistance to accumulation of mycotoxins.

Host resistance to FHB is complex and is conditioned by several genes providing small effects. Genes for FHB resistance have been identified on every wheat chromosome (Eckard et al. 2015). Breeding against FHB is challenging due to the quantitative nature of resistance and the technical difficulties encountered when screening cultivars in inoculated experiments.

Despite the importance of breeding for resistance against FHB, actual methods used for screening often result in high experimental error or inconsistent ranking of genotypes (Kumar et al. 2015). FHB reaction and DON accumulation differ among cultivars and environmental conditions (McMullen et al. 2012; Wilcoxson et al. 1992). Inoculation methods and techniques to enhance infection are critical in differentiating the severity of FHB among lines or cultivars that the breeder seeks to develop. Among the most commonly used methods for evaluating FHB resistance are needle and spray inoculation. Needle inoculation involves the use of a thin syringe to infiltrate a water suspension of *F. graminearum* into a healthy spikelet of wheat (Mesterhazy 2014). Significant and consistent differences in FHB area under the disease progress curve (AUDPC) have been detected in greenhouse trials using the technique of floral injection of spores (Bai and Shaner 1996). However, this method is inefficient in estimating DON and yield.

Practical experience has shown that the standard spore concentration (1×10^5 spores/mL) and the standard spike bagging period following inoculation (72 hours) may be too high and too long to accurately discriminate between wheat lines or cultivars. The objectives of this study were to determine 1) the optimum spore concentration and 2) the

optimum spike bagging period following inoculation for discriminating between an FHB susceptible and a moderately resistant spring wheat cultivar.

3. Materials and Methods

3.1 Plant material and greenhouse conditions. Spring wheat cultivars: Glenn (moderately resistant to FHB, Mergoum et al. 2006) and Samson (susceptible to FHB, Ransom et al. 2010; Westbred 2016), were grown in the greenhouse in 15-cm-diameter clay pots. Substrate consisted of a mixture of sphagnum peat moss (Premier Horticulture Inc., Quakertown, PA), black clay-loam soil, vermiculite (Palmetto Vermiculite Company, Woodruff, SC), and sand in the proportion 1:1:0.5:0.5, respectively. The substrate was sterilized using a Lindig-150 soil pasteurizer (Lindig Manufacturing Corp., St. Paul, MN). Pots were drip irrigated every day. Fertilization was made in conjunction with irrigation using a 20-20-20 N-P-K liquid fertilizer injected at a rate of 250 µg/g. In the first set of experiments, daily photoperiod was extended by five hours of light (5 pm to 10 pm) using Lumigrow Pro 325 LED Grow Lights (Lumigrow Inc., Emeryville, CA). In the second set of replicate (repeated) experiments, photoperiod was extended using 400-watts incandescent Day Brite lighting (Emerson Electric Co., St. Louis, MO). There was a total of twelve plants in each pot.

3.2 Isolates and strains of *F. graminearum*. Isolates used in the experiments were obtained from wheat spike samples collected from Nebraska wheat fields during the 2015 and 2016 growing seasons. Isolates were obtained and characterized by Valverde-Bogantes (2017). Wheat kernels were surface sterilized with 70% ethanol for 1 minute,

washed with distilled sterilized water, and dried on sterile paper towels in a laminar flow cabinet. Kernels were placed on Fusarium selective medium (KH_2PO_4 1 g; $MgSO_4 \cdot 7H_2O$ 0.5 g; peptone 15 g; agar 20 g; PCNB 1 g; distilled water 1000 mL) (Nash and Snyder 1962) contained in 9-cm-diameter Petri plates which were then incubated at room temperature for 5 to 7 days.

Colonies showing morphological characteristics of *Fusarium* were transferred to PDA on which they grew for 7 days. Sterile distilled water was added to each plate and mycelia and spores were dislodged using an L shaped plastic rod followed by filtering through four layers of sterile cheesecloth. The spore suspension was serially diluted and 300 µL were spread onto PDA in 9-cm-diameter plates. After 24 h of incubation at room temperature, single spore isolates were obtained and maintained on PDA.

3.3 . **Preparation of inoculum.** Seven *F. graminearum* isolates were transferred onto PDA plates and incubated in a Thermo Scientific™ Precision™ Low Temperature Incubator (Thermo Fisher Scientific Inc., Waltham, MA) at 25ºC with a 12 hour light-dark period. Under these conditions, all isolates grew and covered the whole surface of the plate after 10 to 14 days. After 21 days of incubation, culture plates were stored at 4ºC until needed for inoculation. To prepare spore suspensions, culture plates were removed from storage and incubated on a shelf for 12 hours at room temperature. Sterile distilled water was added to each plate and mycelia and spores were dislodged with an L-shaped plastic rod followed by filtration through four layers of cheesecloth. Spore concentration was determined using INCYTO™ C-Chip™ disposable hemocytometers (INCYTO, Korea).

3.4 Inoculation of spikes. Wheat spikes were inoculated on different days when they reached full anthesis (GS 65). The spore suspension was prepared in the morning and inoculation was performed in the afternoon and night of the same day. Each wheat spike was numerically identified using Fisherbrand™ colored label tape (Thermo Fisher Scientific Inc., Waltham, MA) and inoculated with approximately 1 mL of spore suspension using 8-oz super-mist manual sprayers (Sprayco, Livonia, MI).

3.5 Experimental layout. Each experiment was repeated once. Treatments were arranged in a split plot design in randomized complete blocks with four replications. Main plots were the cultivars and subplots were spore concentration or spike bagging period treatments. In the inoculum concentration experiment, spore concentrations were the standard ($1x10^5$ spores/mL), one half ($5x10^4$ spores/mL), one quarter ($2.5x10^4$ spores/mL), one eighth ($1.25x10^4$ spores/mL), and one-sixteenth ($6.25x10^3$ spores/mL) of the standard. Wheat spikes were covered with a 7.5 x 13 cm Ziplock® bag for 72 hours after inoculation. In the spike bagging period experiments, wheat spikes were inoculated using a spore suspension ($1x10^5$ spores/mL) prepared using a mixture of the seven *F. graminearum* isolates described above. Inoculated spikes were covered with Ziplock® bags for 12, 24, 36, 48, or 72 hours.

3.6 Disease assessment and grain processing. Following inoculation, disease severity (percentage of symptomatic spikelets on the spikes) was visually assessed seven times at 3-day intervals on 12 to 30 spikes in each pot. After 40 to 56 days following inoculation, spikes were harvested by hand and threshed individually using a single spike thresher (Precision Machine, Lincoln, NE). The grain from each pot was kept separately,

and the total number of kernels was counted using a seed counter (Agriculex, Guelph, Ontario, Canada). A sample of 100 kernels was used to determine the percentage of *Fusarium*-damaged kernels (FDK). Grain samples were ground to flour using a cyclone sample laboratory mill (UDY Corporation, Fort Collins, CO). Samples were sent to the North Dakota Veterinary Diagnostic Laboratory for DON analysis using gas-chromatography with electron capture detection (GC-ECD) (Tacke and Casper 1996).

3.7 Data analysis. Data were analyzed with SAS software version 9.4 using generalized linear mixed models PROC GLIMMIX. Based on the F-test for homogeneity of variances (Gomez and Gomez 1984), a combined analysis of the two replicated experiments of each experiment was conducted. Greenhouse room, cultivars, inoculum concentration, and spike bagging period were considered fixed effects. Block, block within greenhouse room, and cultivar by block within greenhouse room were considered random effects.

4. Results

4.1 Inoculum concentration experiments. In the experiment conducted to determine the optimum spore concentration for discriminating between cultivar resistance levels, the effect of cultivar was significant for all measured variables (FHB severity, FDK and DON; Table 1). The susceptible cultivar Samson had higher FHB severity, FDK, and DON than the moderately resistant cultivar Glenn. Neither the cultivar by greenhouse room interaction nor the third-order interaction greenhouse room by cultivar by spore concentration was significant. However, the interaction spore concentration by cultivar

was significant for FDK and FHB severity at 21 days post inoculation (dpi) (Table 1, Figure 5). The effect of spore concentration was significant for FHB severity and DON (Table 1). FHB severity was significantly higher in the spikes inoculated with 1 x 10^5 and 5 x 10^4 spores/mL in the susceptible cultivar Samson (Fisher-LSD $\alpha = 0.05$) (Table 2).

A change in magnitude of the response to inoculum concentration by cultivar was detected at 21 dpi. Concentrations 6.25 x 10^3 and 1.25 x 10^4 spores/mL allowed clear rank separation between the moderately resistant and the susceptible cultivar (Figures 2 and 5). In Glenn, the lowest DON was detected in grain from spikes inoculated with *F. graminearum* at 6.25 x 10^3 spores/mL (25.8 µg/g) (Table 2). However, this value was not significantly different compared to DON in the rest of the inoculum concentrations, with the exception of DON in spikes inoculated with 5 x 10^4 spores/mL, which was a very high concentration (64.1 µg/g). In Samson, DON in grain from spikes inoculated with 5 x 10^4 spores/mL had 95.3 µg/g (Table 2). The high levels of variability detected in these experiments did not allow the separation of this value from the treatments 1 x 10^5 spores/mL (82.6 µg/g) and 2.5 x 10^4 spores/mL (81.3 µg/g).

The effect of greenhouse room was highly significant for FHB severity and DON (Table 1). It is noteworthy that DON in grain from the room with LED lights (85.9 µg/g) was 2.4 times higher than that in the room with incandescent lights (35.5 µg/g) (Table 2). Temperature in the room with LED lights was 24.2^{o}C compared 23.1^{o}C in the room with incandescent lights.

Linear regression analysis was conducted where the orthogonal polynomials showed a significant linear effect (Tables 1 and 2). Spore concentrations were highly and positively related to FHB severity ($3.1 \leq \beta_1 \leq 14.5$, $0.31 \leq R^2 \leq 0.94$) (Table 4). The strongest relationship between FHB severity and incremental concentrations of inoculum was at 3 dpi ($R^2 = 0.94$, $P < 0.0001$), followed by 6 dpi ($R^2 = 0.59$, $P = 0.0092$), and 18 dpi ($R^2 = 0.50$, $P = 0.0220$).

4.2 Spike bagging time experiments. Highly significant effects of cultivar and spike bagging period following inoculation of spikes with *F. graminearum* were detected for FHB severity, FDK, and DON. In the spike bagging experiment, a significant difference between cultivars in FHB severity was detected at 3 dpi in the 36 h and 48 h bagging treatments. This difference was remarkably increased at 9 dpi in the 48 h and 72 h bagging treatments (Table 1, Figures 6 and 7).

FDK did not differ among bagging treatments in Samson. FDK in the 72 h bagging treatment in Glenn was similar to that in the 12 h and 72 h bagging treatments in Samson (Table 2). For DON, 12 h of bagging did not separate the moderately resistant cultivar Glenn from the susceptible cultivar Samson (Table 2). Likewise, no significant differences between the cultivars were detected following 72 hours of bagging. However, there was a difference of 8 µg/g DON between Samson (49.8 µg/g) and Glenn (41.8 µg/g) in the 72 h bagging treatment.

The greenhouse room effect was highly significant for DON and FDK (Table 2). Contrary to the results in the spore concentration experiment, in the bagging period

experiment higher values of DON and FDK were recorded in the room with incandescent lights than in the room with LED lights. This was unexpected given that temperature in the room with LED lights was 1^{o}C higher than that in the room with incandescent lights. DON was linearly and positively related to spike bagging treatments ($R^2 = 0.60$) (Table 4).

Correlations among variables evaluated in the spike bagging experiment are summarized in Table 5. FDK and DON were highly and positively correlated in the susceptible Samson ($R = 0.89$; $n = 10$; $P = 0.0006$) and the moderately resistant Glenn ($R = 0.78$; $n = 10$; $P = 0.0075$), as well as when means of both cultivars were analyzed together ($R = 0.81$; $n = 20$; $P < 0.0001$).

5. Discussion

Screening cultivars for FHB resistance was conducted using the two spring wheat cultivars Samson (susceptible) and Glenn (moderately resistant). These cultivars were chosen as they represent two different genetic backgrounds. The standard spore concentration used in FHB studies is 1×10^5 spores/mL, and the standard spike bagging period following inoculation is 72 hours. Practical experience indicates that both standards (concentration and spike bagging period) may be too high and too long, respectively, for accurate discrimination between moderately resistant and susceptible wheat lines or cultivars. This study demonstrates that 6.25×10^3 spores/mL (1/16 of the standard) and 1.25×10^4 spores/mL (1/8 of the standard) are sufficient to discriminate

between cultivars, and that the spike bagging time following inoculation of wheat spikes needed to differentiate among cultivars should be 48 to 72 hours.

A significant difference in DON between cultivars was detected in spikes inoculated with 6.25 x 10^3 spores/mL. The average was 25.8 µg/g for Glenn compared with 60.2 µg/g for Samson. A high concentration of spores resulted in very high FHB severity, and as a result differences between cultivars were masked. A high inoculum concentration will result in too much disease, which will lower the accuracy of discriminating between a resistant and a susceptible cultivar. Similarly, a low inoculum concentration will result in too little disease that will not be useful in differentiating between a resistant and a susceptible cultivar. Therefore, insufficient or excessive inoculum concentration can lead to inaccurate results in screening and can increase type II error (failure to discriminate cultivars by not rejecting the null hypothesis). Although a significant difference between the susceptible and moderately resistant cultivar was detected at 9 dpi in the 12 h and 36 h bagging treatments, a better discrimination and separation of cultivars was detected in the 48 h and 72 h bagging treatments.

In a similar study, different inoculum concentrations were used to characterize the development of FHB and DON in SD3845 (susceptible line) (Stein et al. 2009). It was found that FHB incidence and severity and inoculum concentration followed a negative exponential function with an asymptote at approximately 5000 conidia/spike and no further effect of inoculum concentration after adding more inoculum. In addition, DON showed a positive linear relationship with significant increases of the toxin up to 2.5 x

10^4 conidia/spike. Our results contrast with this finding as no linear relationship was detected between DON and inoculum concentration in the susceptible cultivar Samson and the moderately resistant cultivar Glenn. High levels of variability in these experiments did not allow the separation of treatments which showed differences in means for DON of 32 µg/g. This was likely because most of the susceptible plants showed symptoms but also some spikelets may have escaped infection, resulting in a large coefficient of variation (59%) of the mean for the susceptible cultivar (mean 76 µg/g; n = 40; standard deviation 46 µg/g). Inaccurate evaluation of resistance may be due in part to escape of infection in susceptible lines due to external influences such as moisture, opening of florets and other non-direct variables (Bai and Shaner 1996).

In the spore concentration experiment, significantly higher FHB severity and DON were observed in the LED-lit room compared to the room with incandescent lights. This may have been due in part to the optimal spike bagging period of 48 or 72 hours and the higher room temperature in the LED-lit room. A significant reduction in FDK and DON was observed in the LED-lit room compared to the room with incandescent lights. The reason why there was significantly less FDK and DON in this room is unknown. A hypothesis is that the blue-purple light supplemented in the LED-lit room could have caused death of actively growing *F. graminearum* hyphae when spikes were exposed by removal of bags in the treatments with shorter bagging periods (12 h and 24 h). Trzaska et al. (2017) demonstrated that blue light at 405 nm triggered a high production of ROS in living cells and subsequent death of actively growing hyphae. In *Fusarium* spp., blue

light was highly effective in inhibiting fungal growth of germinated spores leading to production of immature hyphae (Trzaska et al. 2017). Lumigrow Pro 325 LED Grow Lights produce two main peaks at 660 and 440 nm. Blue LED light at 440 nm effectively inactivates *Candida albicans* when irradiated over planktonic cells (Dovigo et al. 2011). LED lights are a relatively new technology in agriculture. Exposing microorganisms to different wavelengths can be useful in disease management in greenhouse crops.

Screening cultivars for FHB resistance is a difficult task even when comparing lines that are clearly different in QTLs for resistance. Inoculation techniques can have important consequences in the advance of a breeding program targeting resistance to FHB. Methodology, availability of inoculum, and control of the environment are critical factors to consider in phenotyping FHB resistance. In addition, variation in FHB screening can be a result of the genotype x environment interaction (Geddes et al. 2008).

Screening for FHB resistance represents an investment in time and resources. Optimization of techniques will prevent removal of good lines in the early stages of the breeding program. The results from this study indicate that spore concentration and spike bagging time following inoculation are critical in accurate discrimination between resistant and susceptible cultivars. The results also indicate that differences in environmental conditions such as temperature and type of incident light can have a significant effect on the results obtained when screening cultivars for resistance to FHB.

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Table 1. Effects of cultivar, spore concentration, and spike bagging period following inoculation on FHB severity in greenhouse experiments.

Source	gl	Inoculum concentration							Spike bagging						
		3 dpi	6 dpi	9 dpi	12 dpi	15 dpi	18 dpi	21 dpi	3 dpi	6 dpi	9 dpi	12 dpi	15 dpi	18 dpi	21 dpi
		P > F													
Greenhouse room[a]	1	0.0004	<.0001	0.0055	0.0153	0.1754	0.8689	0.1360	0.0016	0.0121	0.0336	0.0340	0.0981	0.0476	0.0961
GR (Rep)	6	0.9720	0.9956	0.6058	0.4838	0.2053	0.1492	0.0318	0.7937	0.8404	0.8051	0.7125	0.2715	0.3847	0.2619
Cultivar (CV)[b]	1	1.0000	0.2920	0.0120	0.0036	0.0019	0.0157	<.0001	0.0123	0.0183	0.0097	0.0076	0.0012	0.0003	0.0004
CV * GR	1	0.8364	0.0218	0.1316	0.0798	0.1373	0.3004	0.7371	0.6699	0.7354	0.2940	0.4154	0.4524	0.6093	0.1589
CV * Rep(GR)	6	0.0779	0.0049	0.0524	0.0591	0.2460	0.1486	0.9518	0.0316	0.0086	0.0405	0.1666	0.6609	0.8267	0.9139
Treatment (T)[c]	4	<.0001	0.0036	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
linear	1	<.0001	0.0245	0.0061	0.0009	0.0003	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
quadratic	1	0.3819	0.5947	0.0500	0.0329	0.0169	0.0336	0.0114	0.2848	0.5714	0.3103	0.5653	0.7396	0.8469	0.2241
cubic	1	0.8260	0.8322	0.5760	0.3802	0.3796	0.6091	0.8841	0.0579	0.1826	0.1894	0.0373	0.0479	0.1198	0.5527
quartic	1	0.5149	0.8078	0.8456	0.8815	0.8510	0.7767	0.8036	0.8981	0.5891	0.5629	0.4808	0.4006	0.5178	0.7439
T * GR	4	0.5872	0.7219	0.5609	0.5198	0.1373	0.3124	0.6327	0.8758	0.7747	0.5358	0.4855	0.4455	0.7184	0.7657
T * CV	4	0.9752	0.2608	0.5446	0.1917	0.4241	0.8035	0.0064	0.0316	0.1070	0.0330	0.2242	0.4083	0.1184	0.5844
T * CV * GR	4	0.1101	0.2382	0.7410	0.2769	0.4112	0.5804	0.2972	0.9796	0.6229	0.4937	0.8832	0.7580	0.2962	0.2369

[a] Sets of replicate (repeated) experiments were conducted in separate greenhouse rooms for a total of four independent experiments (two replicates for spore concentration, and two for spike bagging period).
[b] Cultivars used in this study were Glenn (FHB moderately-resistant) and Samson (FHB-susceptible).
[c] Treatments for the spore concentration experiments were 10^5, $5x10^4$, $2.5x10^4$, $1.25x10^4$ and $6.25x10^3$ spores/mL; and for spike bagging time were 12, 24, 36, 48, and 72 hours post inoculation.

Table 2. Effects of cultivars, spore concentration, and spike bagging period on Fusarium-damaged kernels (FDK) and deoxynivalenol (DON) under greenhouse experimental conditions.

Effect	d.f.	Inoculum concentration		Spike bagging	
		FDK	DON	FDK	DON
		P > F			
Greenhouse room (GR)[a]	1	0.2297	0.0005	<0.001	0.0278
(GR)rep	6	0.4326	0.4852	0.5877	0.7465
Cultivar (CV)[b]	1	0.0020	0.0062	0.0004	0.0250
CV * GR	1	0.8433	0.1217	0.8821	0.6500
CV * Rep(GR)	6	0.0126	0.4832	0.3003	0.6179
Treatment (T)[c]	4	0.0892	0.0481	0.0236	0.0008
Linear	1	-	0.2483	0.1253	0.0010
quadratic	1	-	0.1439	0.3078	0.8966
Cubic	1	-	0.7027	0.6379	0.2975
quartic	1	-	0.1626	0.5570	0.5211
T * GR	4	0.0168	0.2576	0.1329	0.1692
T * CV	4	0.0480	0.3170	0.3145	0.7265
T * CV * GR	4	0.1705	0.5384	0.5386	0.4447

[a] Sets of replicate (repeated) experiments were conducted in a separate greenhouse rooms for a total of four independent experiments (two replicates for spore concentration, and two for spike bagging period).

[b] Cultivars used in this study were Glenn (FHB moderately-resistant) and Samson (FHB-susceptible).

[c] Treatments for the spore concentration experiments were 10^5, $5x10^4$, $2.5x10^4$, $1.25x10^4$ and $6.25x10^3$ spores/mL; and for spike bagging time were 12, 24, 36, 48, and 72 hours post inoculation.

Table 3. LS-means for Fusarium-damaged kernels (FDK) and deoxynivalenol (DON) in spring wheat cultivars Samson (FHB-susceptible) and Glenn (moderately resistant) cultivars used to determine the effects of inoculum concentration (spores/mL) and spike bagging period following inoculation under greenhouse conditions

Cultivar	Inoculum concentration				Spike bagging period		
	spores mL	Fraction standard[a]	*FDK %*	*DON (µg/g)*	hours spike bagged	*FDK %*	*DON (µg/g)*
Samson	$1x10^5$	1	67.4 a	82.6 ab	12	39.3 a-c	20.4 d
	$5x10^4$	1/2	63.4 ab	95.3 a	24	43.7 a	40.1 ab
	$2.5x10^4$	1/4	57.1 bc	58.5 b-d	36	47.9 a	38.6 a-c
	$1.25x10^4$	1/8	57.9 a-c	81.3 ab	48	46.2 a	43.2 ab
	$6.25x10^3$	1/16	57.0 bc	60.2 bc	72	41.9 ab	49.8 a
Glenn	$1x10^5$	1	34.9 e	31.1 cd	12	20.2 e	20.2 d
	$5x10^4$	1/2	50.2 cd	64.1 a-c	24	26.8 de	29.8 b-d
	$2.5x10^4$	1/4	41.4 de	58.0 b-d	36	32.5 cd	23.6 cd
	$1.25x10^4$	1/8	39.5 e	50.5 b-d	48	27.6 de	32.1 b-d
	$6.25x10^3$	1/16	40.8 e	25.8 d	72	34.9 b-d	41.8 ab
Greenhouse room effect							
	A2-LED		53.8	85.9		24.7	20.3
	B2-INC		48.2	35.5		47.6	47.3
ANOVA factor P ≤			NS	0.0001		0.0001	0.0001

Table 4. Linear regression analysis between spore concentration, spike bagging time and FHB-severity evaluated at 3, 6, 9, 12, 15, 18, and 21 days post inoculation.

		Linear regression parameters				
	Variable	Intercept	Slope	R^2	F - value	P > F
Spore concentration	FHB-SEV^{3dpi}	2.92	3.10	0.94	134.11	<.0001
	FHB-SEV^{6dpi}	16.99	5.68	0.59	11.65	0.0092
	FHB-SEV^{9dpi}	35.40	11.96	0.32	3.78	0.0878
	FHB-SEV^{12dpi}	52.08	14.44	0.31	3.63	0.0934
	FHB-SEV^{15dpi}	67.88	14.52	0.32	3.75	0.0889
	FHB-SEV^{18dpi}	79.18	14.43	0.50	8.04	0.0220
	FHB-SEV^{21dpi}	89.77	8.95	0.33	3.94	0.0823
Spike bagging	FHB-SEV^{3dpi}	0.98	0.15	0.78	27.61	0.0008
	FHB-SEV^{6dpi}	10.89	0.28	0.65	15.29	0.0045
	FHB-SEV^{9dpi}	25.01	0.33	0.59	11.36	0.0098
	FHB-SEV^{12dpi}	41.88	0.31	0.57	10.57	0.0118
	FHB-SEV^{15dpi}	57.26	0.31	0.60	11.97	0.0086
	FHB-SEV^{18dpi}	72.37	0.26	0.57	10.77	0.0112
	FHB-SEV^{21dpi}	84.73	0.18	0.61	12.43	0.0078

Table 5. Correlation coefficients for Fusarium head blight (FHB) severity, *Fusarium*-damaged kernels (FDK), and deoxynivalenol (DON) concentration from experiments conducted to determine the effect of inoculum concentration and spike bagging period following inoculation on the efficiency of discrimination between a susceptible and a moderately resistant cultivar.

	Inoculum concentration						Spike bagging period					
	susceptible N = 10		moderately resistant N = 10		combined N = 20		susceptible N = 10		moderately resistant N = 10		combined N = 20	
	R	Prob > |r|	R	Prob > |r|	R	Prob > |r|	R	Prob > |r|	R	Prob > |r|	R	Prob > |r|
FHB-sev 3 dpi/ FDK	0.6577	0.0387	0.2261	0.5299	0.2614	0.2657	-0.2456	0.494	-0.131	0.7183	-0.0392	0.8697
FHB-sev 3 dpi/ DON	0.796	0.0059	0.4193	0.2278	0.5545	0.0112	0.0459	0.8998	0.0242	0.9471	0.0917	0.7006
FHB-sev 6 dpi/ FDK	0.4814	0.1589	0.3105	0.3826	0.3717	0.1065	-0.2621	0.4644	-0.1517	0.6757	0.0536	0.8225
FHB-sev 6 dpi/ DON	0.8856	0.0007	0.4458	0.1966	0.75	0.0001	0.0544	0.8814	0.0307	0.9329	0.1432	0.5471
FHB-sev 9 dpi/ FDK	0.5955	0.0693	0.4132	0.2352	0.6266	0.0031	-0.2312	0.5204	0.1336	0.713	0.1683	0.4782
FHB-sev 9 dpi/ DON	0.9298	<.0001	0.6273	0.0522	0.867	<.0001	0.0797	0.8267	0.1775	0.6237	0.2076	0.3798
FHB-sev 12 dpi/ FDK	0.6195	0.0561	0.3381	0.3393	0.6817	0.0009	-0.2692	0.452	0.04744	0.8965	0.1367	0.5655
FHB-sev 12 dpi/ DON	0.8383	0.0024	0.5449	0.1033	0.8	<.0001	0.0176	0.9615	0.0578	0.874	0.1409	0.5534
FHB-sev 15 dpi/ FDK	0.7412	0.0142	0.3421	0.3333	0.7614	<.0001	-0.1955	0.5884	0.0582	0.8732	0.1759	0.4582
FHB-sev 15 dpi/ DON	0.7245	0.0178	0.316	0.3737	0.6813	0.0009	0.1138	0.7543	0.0388	0.9152	0.1865	0.4312
FHB-sev 18 dpi/ FDK	0.8178	0.0038	0.1035	0.7761	0.6621	0.0015	-0.0631	0.8624	-0.1172	0.747	0.1897	0.4231
FHB-sev 18 dpi/ DON	0.5388	0.1081	0.01671	0.9635	0.4631	0.0398	0.2583	0.4712	-0.0899	0.8048	0.2326	0.3237
FHB-sev 21 dpi/ FDK	0.2596	0.4688	0.1981	0.5832	0.5571	0.0107	0.0912	0.802	-0.037	0.9191	0.2624	0.2638
FHB-sev 21 dpi/ DON	-0.0934	0.7974	-0.0153	0.9665	0.2093	0.3759	0.4108	0.2383	-0.054	0.8822	0.2991	0.2001
FDK/DON	0.5995	0.0857	0.697	0.0251	0.6739	0.0011	0.8905	0.0006	0.7824	0.0075	0.8189	<.0001

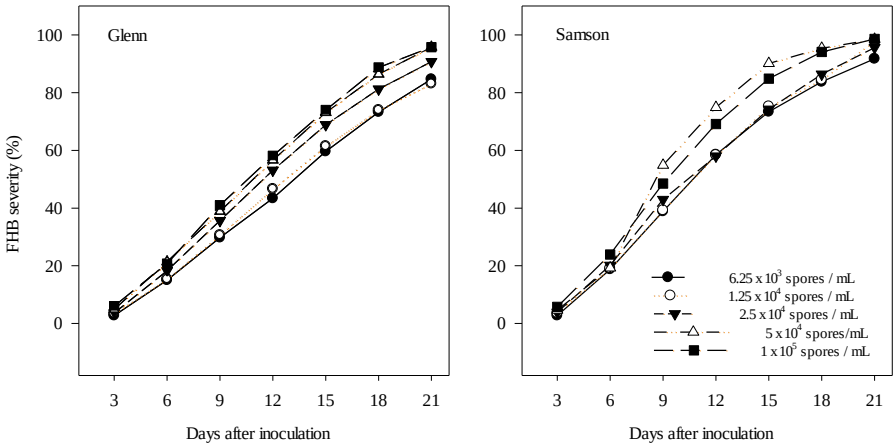

Fig. 1. Progression of Fusarium head blight severity in a moderately resistant (Glenn) and a susceptible (Samson) spring wheat cultivar. Plants were inoculated with spore suspensions of *Fusarium graminearum* —●— 6.25 x10^3, ···○··· 1.25x10^4, –▼– 2.5x10^4, –△·– 5x10^4, and ·–■– 1x10^5 spores / mL.

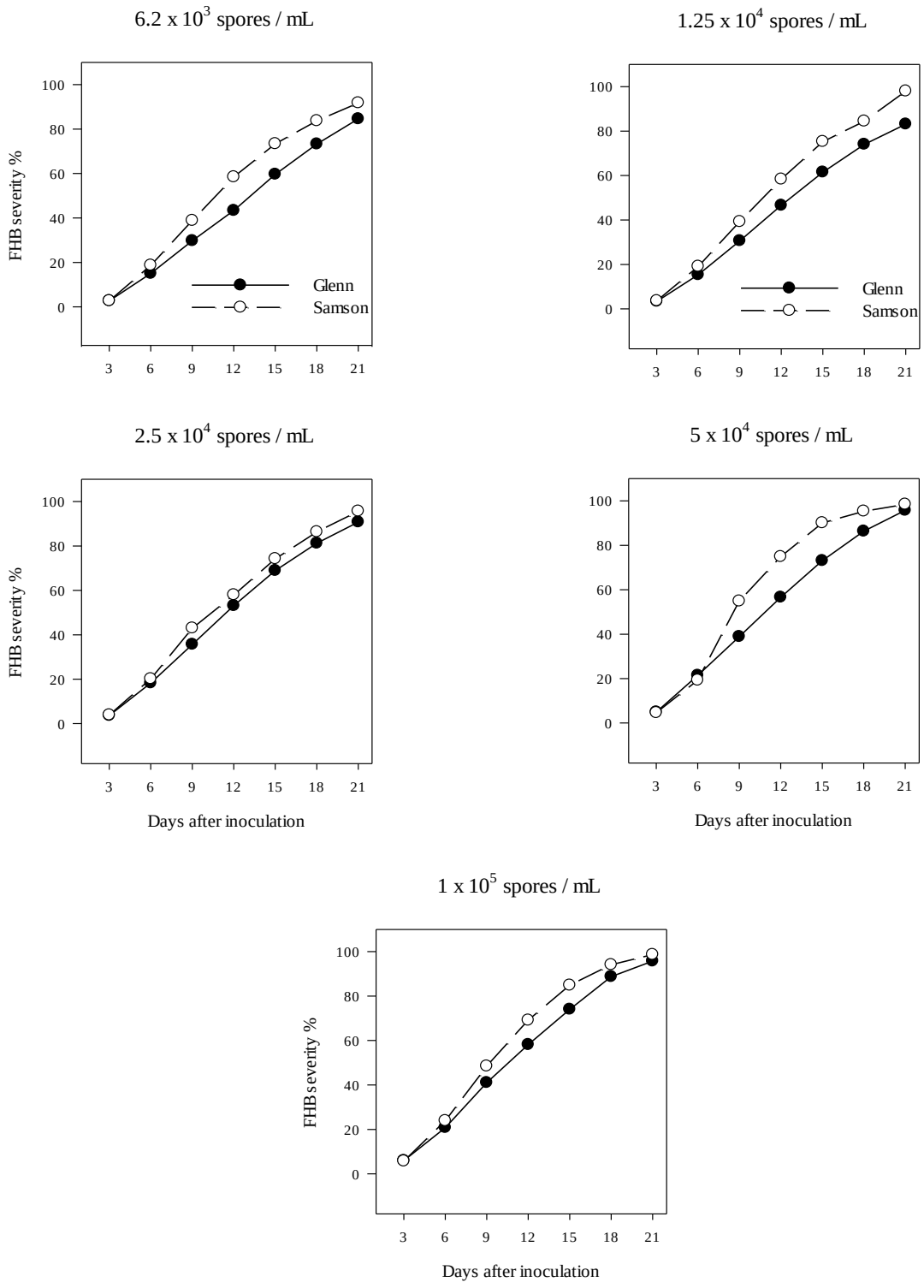

Fig. 2. Progression of Fusarium head blight (FHB) severity in a moderately resistant (Glenn) and a susceptible cultivar (Samson) at different concentrations of *Fusarium graminearum* inoculum. Evaluations of FHB severity were made from 3 to 21 days post inoculation at 3 day intervals.

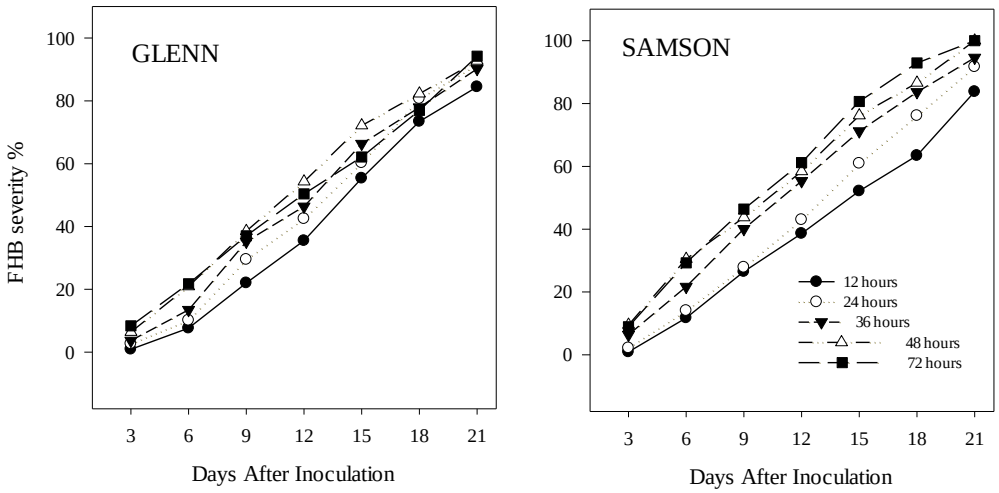

Fig. 3. Progression of Fusarium head blight (FHB) severity in a moderately resistant (Glenn) and a susceptible (Samson) spring wheat cultivar exposed to different spike bagging periods (hours). Plants were inoculated with a spore suspension of *Fusarium graminearum* (10^5 spores / mL) and spike bagging period was 12 hours, 24 hours, 36 hours, 48 hours, and 72 hours after inoculation.

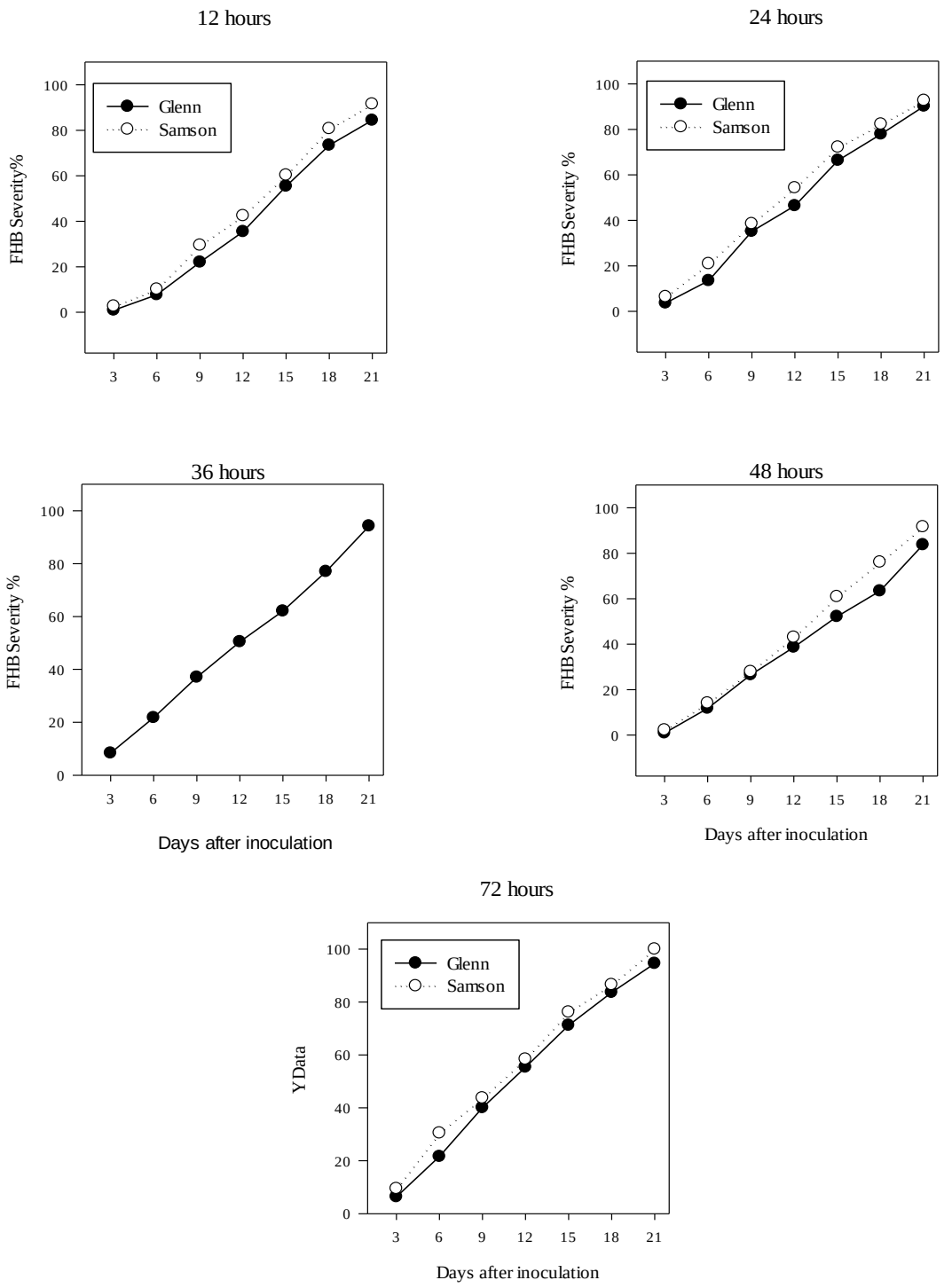

Fig. 4. Progression of Fusarium head blight (FHB) severity in a moderately resistant (Glenn) and a susceptible cultivar (Samson) exposed to different spike bagging periods following inoculation of wheat spikes. FHB severity was evaluated seven times at 3 day intervals following inoculation.

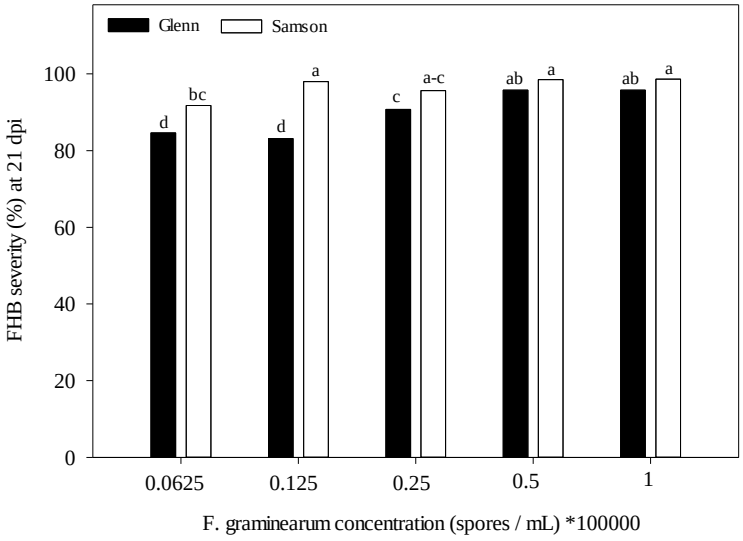

Fig. 5. Effect of *Fusarium graminearum* inoculum concentration on Fusarium head blight (FHB) severity in a moderately resistant (Glenn) and a susceptible (Samson) spring wheat cultivar 21 days post inoculation in the greenhouse.

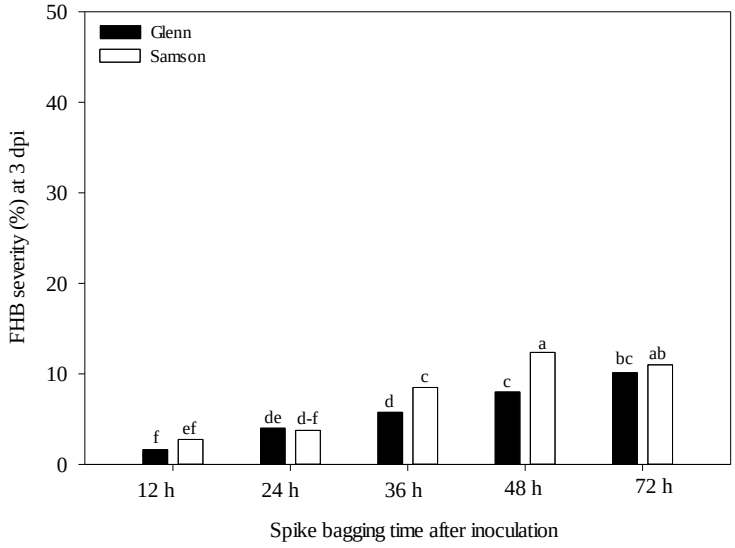

Fig. 6. Effect of *Fusarium graminearum* spike bagging period on Fusarium head blight (FHB) severity in a moderately resistant (Glenn) and a susceptible (Samson) spring wheat cultivar 3 days post inoculation in the greenhouse.

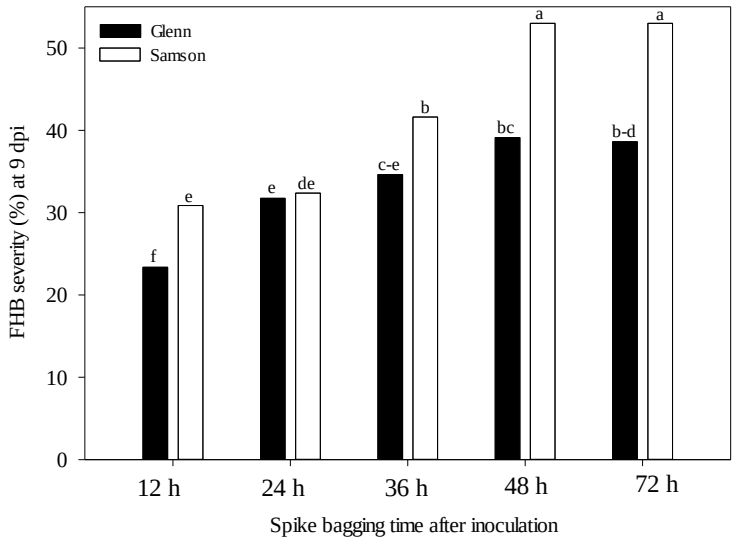

Fig. 7. Effect of *Fusarium graminearum* spike bagging period on Fusarium head blight (FHB) severity in a moderately resistant (Glenn) and a susceptible (Samson) spring wheat cultivar 9 days post inoculation in the greenhouse.

CHAPTER VI

TIMING OF FUNGICIDE APPLICATIONS FOR MANAGEMENT OF FOLIAR FUNGAL DISEASES OF WINTER WHEAT

1. Abstract

Foliar fungal diseases of wheat are associated with significant economic losses every year. This study was carried out to determine the effect of the fungicides Prosaro™ (prothioconazole + tebuconazole) and Headline® (pyraclostrobin) applied at different times on foliar disease severity and yield in winter wheat. In 2015 and 2016, field trials were conducted using two winter wheat cultivars Overley and Overland under dryland and irrigated field conditions. There was a highly significant effect ($P < 0.0001$) of fungicide treatments on the area under disease progress curve (AUDPC) in both years and environments. Overall, AUDPC was significantly higher in plots treated with Prosaro at or after anthesis compared to plots treated prior to anthesis. In addition, there was a significant reduction in yield in the applications timed at 6 and 12 days after anthesis, without regard to the fungicide chemical class. Critical point models, as well as the trends in disease progress curves, were variable among years and environments. Critical point models showed that foliar disease severity at anthesis and a few days after anthesis more accurately predicted yield loss in an epidemic year (2015) compared to a dryer year (2016). Foliar disease severity assessment later in the 2016 growing season (milk stage of wheat grain development) provided a more reliable relationship with yield in both environments. AUDPC and yield were highly and negatively correlated ($R = -0.98$; $n = 8$; $P < 0.0001$ in 2015, and $r = -0.84$; $n = 9$; $P = 0.0089$ in 2016). Applications of Prosaro™ at the flag and boot stages of wheat development were more effective in protecting yield

from foliar fungal diseases in both years and environments than applications made at later growth stages.

2. Introduction

Wheat (*Triticum aestivum*) is planted on more acreage than any other crop in the world (Baenziger et al. 2009; Bishwajit et al. 2017). In 2003, the total acreage of wheat was 215 million hectares on five continents (Chrispeels and Sadava 2003). Wheat is the primary source of protein and calories for 35% of the world's population (Food and agriculture organization of the United Nations 2015), and plays a significant role in food security and food sovereignty in many countries.

Foliar fungal diseases of wheat are associated with significant losses of the photosynthetic area. Frequency and severity of foliar fungal disease epidemics have increased over the years. In wheat, rust epidemics (stripe, leaf, and stem) have increased in severity after higher winter temperatures and lower spring temperatures in the Pacific Northwest of the United States (Caubel et al. 2017; Luck et al. 2011). Emerging infectious diseases pose a grave risk to plant health. Monoculture and the effects of climate change have caused increases in incidence and severity for many foliar fungal diseases; therefore, the use of fungicides has expanded widely. In wheat fields of the United States, in the central Great Plains, major foliar fungal diseases are leaf rust (*Puccinia triticina*), stripe rust (*Puccinia striiformis*), tan spot (*Pyrenophora tritici-repentis*), powdery mildew (*Blumeria graminis*), spot blotch (*Cochliobolus sativus;* anamorph: *Bipolaris sorokiniana*), and Septoria tritici blotch (*Septoria tritici*) (Wegulo et al. 2011). Wheat rusts include stripe or yellow rust, leaf or brown rust, and stem or black rust (*Puccinia graminis* f.sp. *tritici*). Stripe rust is associated with losses totaling over 5 MMT per year, and 88% of the cultivars planted worldwide are susceptible to the disease

(Schierenbeck et al. 2016). Early epidemics of leaf rust affecting the flag leaf can cause 60 - 70% yield losses (Huerta-Espino et al. 2011). The group of stem rust races known as "Ug99" constitutes a major threat to wheat production worldwide. Most of the bread wheat varieties planted are susceptible to stem rust Ug99 (Singh et al. 2015).

Tan spot is a foliar disease that can cause losses of up to 50% of the crop (Shabber and Bockus 1988). The disease has been increasing in severity in wheat fields due to no-tillage practices, causing serious damage especially when conditions are warm and humid (Wegulo et al. 2012).

Powdery mildew of wheat is one of the most common foliar diseases, and it is frequently reported in Western and Southern Europe and South America (Caubel et al. 2017; Morgounov et al. 2012). *Blumeria graminis* reduces yield and grain quality, and infects wheat leaves primarily in the spring when air temperatures begin to warm. (Morgounov et al. 2012).

Foliar disease severity can be used to estimate yield loss by models. Models that help to simplify the relationships between disease severity and yield loss are important tools for decision-making in the field to optimize disease management strategies. Three models frequently used in plant disease epidemiology are the critical points, the multiple points, and the area under disease progress curve (AUDPC) (Campbell and Madden 1990; James 1974; Teng et al. 1979). Critical point models use disease severity at a particular point in time to predict future yield loss using simple linear regression analysis (Zadoks and Schein 1979). Critical point models provide the best fit to estimate yield loss due to rust epidemics (Teng et al. 1979). In Brazil, a critical point model was used to relate yield and severity of foliar blast (*Pyricularia oryzae*), brown spot (*Bipolaris oryzae*), and scald (*Gerlachia oryzae*) of rice (Bordin et al. 2016). In winter wheat, in four studied locations,

the highest relationship between tan spot/spot blotch severity and yield was at the flowering stage (Wegulo et al. 2009). In Iran, prediction of crop loss showed that disease assessments from Zadoks growth stage 39 (GS39) (Zadoks et al. 1974) to GS60 were good indicators of yield loss in the bread wheat-stripe rust pathosystem (Eslahi and Mojerlou 2016). A model to estimate the effect of the wheat diseases *Septoria tritici* blotch (STB) and leaf rust on plant growth and yield predicted the effect of these diseases on crop growth with less than 10% differences between modeled versus experimental data (Robert et al. 2004).

Studies on crop physiology have shown the importance of the flag leaf of wheat as it contributes a large proportion of photosynthates for grain filling (Borrill et al. 2015; Chrispeels and Sadava 2003; Stoy 1963). Fungicides are applied to wheat leaves to maximize yield. The fungicides Headline® (pyraclostrobin) and Prosaro^{TM} (prothioconazole + tebuconazole) are registered for controlling foliar fungal diseases on wheat (Wegulo 2010). Fungicide chemical class and application timing have shown inconsistent results in the control of foliar diseases of wheat (Cromey et al. 2004; Wegulo et al. 2009, Wegulo et al. 2011). A fundamental question in this research is, how do pre-anthesis (flag and boot), anthesis, or post-anthesis fungicide applications (6 or 12 days post-anthesis) impact yield and foliar disease severity?

The objectives of this study were to determine the effect of two fungicides (Headline® and Prosaro^{TM}) applied at pre-anthesis, anthesis, and post-anthesis on foliar fungal disease severity and yield. Critical point model analysis was also employed to find the best relationship between foliar disease severity and yield loss.

3. Materials and methods

Field experiments were conducted under rainfed (one experiment) and irrigated (one experiment) conditions during the 2015 and 2016 wheat growing seasons at the Eastern Nebraska Research and Extension Center (ENREC), formerly the Agricultural Research and Development Center (ARDC) near Mead, Nebraska (41.2286° N, 96.4892° W). Winter wheat cultivars Overland and Overley were planted in the fall of 2014 and 2015. The size of the experimental plots was 1.22 m by 6.10 m in 2014 and 1.22 m by 4.57 m in 2015. Seeding rate was 60 kg/ha in both years. The Nebraska Certified Seed Book characterizes the reaction of Overland as moderately resistant (MR) to moderately susceptible (MS) to leaf rust, and MR to stem and stripe rust (Nebraska Crop Improvement Association). Overley is characterized as highly susceptible to stripe rust, MS reaction to leaf rust and powdery mildew, MR to tan spot, and highly susceptible to Fusarium head blight (DeLange Seed Inc. Girard, Kansas) and highly resistant (R) to leaf rust (Fritz et al. 2004).

The irrigation system consisted of a fixed-superficial rectangular framework. Sprinklers (full circle Impact Sprinklers of 1.9 cm model 30H, Rain Bird, Azusa, CA) were spaced 6.7 m x 4.6 m apart. Irrigation cycles were programmed using an ICC-commercial irrigation controller (Hunter Industries, San Marcos, CA). Plots were irrigated with a run cycle of 5 minutes ON and 15 minutes OFF from 10 A.M. to 8 P.M. seven days a week.

Weather data were collected using Watchdog® portable weather stations (Spectrum Technologies, Thayer Court, IL, USA). Stations were placed on dryland and irrigated plots close to the borders. Weather data (temperature, relative humidity, leaf wetness, and rainfall) were recorded daily at one-hour intervals. In the irrigated plots, rainfall values

represent the sum of precipitation and water collected by the rain bucket when the irrigation system was turned on from 30 May, 2015 to 22 June, 2015 and from 23 May, 2016 to 14 June, 2016. Leaf wetness (a representation of the quantity of water freely available on the surface of leaves (Rowlandson et al.2015)) was measured using gold-plated leaf wetness sensors (Spectrum technologies) on a scale from 1 (dry) to 15 (wet).

Eight fungicide treatments were applied to generate different levels of foliar disease intensity. Fungicide treatments consisted of two chemicals (a triazole and a strobilurin) applied at different stages of wheat development. Tebuconazole + prothioconazole (triazole-DMI), commercially available as Prosaro™ (Bayer Ag life science, Kaiser-Wilhelm-Allee, Leverkusen, Germany), was sprayed at GS 39 or flag leaf stage, GS 45 or boot stage, anthesis (GS 60) when 30-40% of the anthers of the wheat head were extruded, 6 days, and 12 days post-anthesis (DPA) at a rate of 0.47 L/ha. Pyraclostrobin (strobilurin-QoI), commercially available as Headline® (BASF Ag Products, Research Triangle Park, NC, USA), was sprayed at GS 60, 6 DPA, and 12 DPA at a rate of 0.66 L/ha. Untreated check plots were also included.

Fungicides were applied using a CO_2-powered-backpack sprayer, equipped with four Tee-jet 800-1 VS nozzles (TeeJet Technologies, Dillsburg, PA), spaced at 30.5 cm apart, and set at 241 kPa, delivering a volume rate of 150 L/ha. A non-ionic spray adjuvant NIS 90-10 (Precision laboratories, Waukegan, IL, USA) was added at a rate 0.125% vol/vol to improve the performance of the fungicides.

At weekly intervals, foliar disease severity was visually estimated as the percentage of necrotic foliage in each plot. The first assessment was made at approximately GS 37 - 39 (when the ligule of the flag leaf was visible), and the final assessment was made at

approximately late milk to dough development (GS 77 – 80; when the kernels began to fill with the photosynthetic products of the flag leaf) (Kearney 2006).

Foliar disease severity was estimated as the percentage of necrotic tissue over the total leave tissue, and it was used to calculate the area under the disease progress curve (AUDPC) according to the trapezoidal integration method (Campbell and Madden 1990). Plots were harvested when grain moisture content dropped to 15% using a small plot combine (Wintersteiger, Dimmelstrasse, Austria) and yield was estimated (kg/ha).

Data analysis was carried out using SAS software version 9.4. Disease ratings and AUDPC were analyzed using generalized linear mixed model PROC GLIMMIX (SAS Inc, Cary, NC, USA). Repetitions and the interaction Repetition by Cultivar (Error A) were specified as random effects. Least square means (LS-means) were compared using Fisher-LSD (alpha=0.05).

Type I results of the analysis of variance were obtained using timing of application of Prosaro^{TM} fungicide as a quantitative factor. A sequential sum of squares was used to determine linear, quadratic, cubic, and quartic relationships among Prosaro^{TM} application timing and yield and AUDPC. The model proposed was:

$$y=\beta_0+ \beta_1x+ \beta_2x_2 + \beta_3x_3 + \beta_4x_4$$

where: y is the response (yield); x is the quantitative factor (Prosaro^{TM} application timing); β_0 is the intercept; β_1, β_2, β_3, β_4, are the linear, quadratic, cubic and quartic coefficients relating the timing to the response (yield/AUDPC), respectively.

Regression analysis was used to relate foliar disease severity and yield. Critical point models of the foliar disease severity were obtained using PROC REG. Treatment LS-

means were used in regression analysis. Coefficients of determination (R2) and the t-test and its associated P-value for the slope were used to determine the growth stage at which disease severity was most strongly related to yield.

Correlation coefficients among AUDPC and yield values were determined using PROC CORR. LS-means by cultivar were selected and compared with pairs by growing season at each variable (AUDPC and yield) and between AUDPC and yield for each growing season. Data were separated by cultivar (Overley and Overland) and environmental conditions (dryland and irrigated).

4. Results

4.1 Environmental conditions. Climatological data were taken from dryland and irrigated environments at the experimental plots. Rainfall was higher in 2015 than in 2016 (Table 2). In irrigated plots, rainfall values represent the sum of precipitation and water from the sprinkler system in May and June (periods of active growth and grain filling). Total rainfall values in May and June were 3 and 2.3 times higher in irrigated versus dryland plots in 2015 and 2016, respectively (Table 2). Additionally, in June, leaf wetness values were 1.3 times and 1.9 times higher in irrigated versus dryland plots in 2015 and 2016, respectively (Table 2).

4.2 Foliar diseases on winter wheat. Overall, AUDPC was higher in 2015 than in 2016 (Tables 3 and 4). Additionally, in both years Overley matured more quickly than Overland. There was greater variation in foliar disease severity in the first year than in the second year. In 2015, Overley showed higher AUDPC than Overland. Also, Overland had higher yield than Overley (Tables 3 and 4).

4.3 Dryland conditions. Overall, under dryland conditions, there was a highly significant effect ($P < 0.0001$) of fungicide treatments on foliar disease severity, AUDPC and yield, except for foliar disease severity 1 and 2 in 2016 which were not significant. AUDPC was statistically higher in plots treated with Prosaro at anthesis compared to plots treated with Prosaro at GS 45 (Table 3). In Overley, Prosaro- and Headline- treated plots sprayed at 12 DPA were not significantly different from each other or from the untreated check plots (Table 3).

The highest yield was in the Prosaro-treated plot sprayed at anthesis (for Overley, and 2015).The lowest AUDPC was in the Prosaro-treated plots sprayed at GS 45. In the fungicide-treated plots, the highest reductions in yield were in the Prosaro-treated plots sprayed at anthesis (1484 kg/ha) compared to the Prosaro-treated plots sprayed at 12 DPA (1114 kg/ha; 25% yield reduction), and Headline-treated plots sprayed at 12 DPA (933 kg/ha; 37% yield reduction) (Table 3). In Overley, untreated check plots showed the lowest yield (677 kg/ha).

In 2015 for Overland, Prosaro-treated plots at GS 39 and GS 45 showed significantly lower foliar disease severity (Table 3, Figure 1b) compared to all other treatments. Yield showed similar trends as AUDPC in Overland; however, the Prosaro-treated plots at GS 39 (3314 kg/ha) and GS 45 (3441 kg/ha) and Headline treated plots at anthesis (3088 kg/ha) showed significantly higher yields compared to all other treatments (Table 3).

In 2016 for Overley, AUDPC in the untreated check plots was significantly higher compared to fungicide-treated plots (Table 3). AUDPC was highest in the untreated check plots; inversely, Prosaro-treated plots sprayed at GS 45 had the lowest AUDPC value (Table 3).

In 2016 for Overland, spraying Prosaro and Headline at 6 and 12 DPA did not provide adequate control for foliar diseases. Although Prosaro- and Headline- treated plots sprayed at anthesis showed better control, these treatments were not as good as the earlier applications of Prosaro (GS 39 and GS 45). Prosaro-treated plots sprayed at GS 39 and GS 45 yielded 4665 kg/ha and 4702 kg/ha, respectively. In Overland, untreated check plots yielded higher as compared to sprayed plots with Headline at 6 DPA and Prosaro at 12 DPA (Table 3). Both treatments reached 100% foliar disease severity by the end of the experiment (Figure 2).

4.4 Irrigated conditions. Under irrigated conditions, effects of fungicide treatments on AUDPC and yield were highly significant in both years. Also, there was a significant effect of cultivar on AUDPC in 2016 ($P = 0.0241$) (Table 4). In 2015 for Overley, Prosaro-treated plots sprayed at GS 45 showed the lowest level of foliar disease severity, which was approximately four times less than the untreated check plots in the same experiment (AUDPC = 1837 in the untreated check plots versus 397 in the Prosaro – treated plots at GS 45) (Table 4). In Overland, the lowest levels of AUDPC were detected in the Prosaro-treated plots at GS 39 and GS 45 (Table 4).

In 2016, in Overland, AUDPC in the untreated check plots had ranked equally as Prosaro-treated plots sprayed at 6 and 12 DPA, as well as Headline-treated plots sprayed at 6 and 12 DPA (Fisher-LSD; $\alpha = 0.05$) (Table 4). In Overland, yield in the untreated check plots overlapped in rank with all fungicide treatments, except for Prosaro-treated plots at anthesis. Prosaro-treated plots at anthesis reached the highest yield with 3498 kg/ha (Table 4).

4.5 Disease progress curves. In general, disease progression showed different patterns in the growing season of 2015 than in 2016. Under dryland conditions, disease

progress in 2015 (Figure 1) showed greater variation in foliar disease severity from the first to the fifth assessment than disease progress in 2016 (Figure 2). Likewise, under irrigated conditions disease progression increased more quickly in 2015 (Figure 3) compared to disease progression in 2016 (Figure 4). Among all the treatments evaluated in this study, Prosaro-treated plots at GS 39 consistently showed the lowest foliar disease severity in all combinations of year by environment for both cultivars (Figures 3 and 4). However, there was an exception for the growing season 2015 in the cultivar Overley where Prosaro was not sprayed in plots at GS 39 due to rain (Figures 1a and 2a).

In 2015 under dryland as well as under irrigated conditions for Overley, disease progress curves showed inconsistent results of foliar disease severity with several intersecting points between lines of progression (Figures 1a and 2a). A significant or highly significant effect of the interaction cultivar by fungicide was present on many evaluations of foliar disease severity (Tables 3 and 4).

In Overland, progress curves showed that Prosaro applications at GS 39 and GS 45 effectively controlled foliar diseases (Figures 1b and 2b). Foliar disease severity was reduced by 56% and 48% in foliar disease progression in Prosaro treated plots at GS 45 compared to untreated check plots under dryland and irrigated conditions, respectively.

Under irrigated conditions in Overley, plots treated with Prosaro at GS 45 showed a slow progression compared to the untreated check (Figure 3a). In Overland, Prosaro treatment at GS 39 and GS 45 was effective in controlling foliar disease severity (Figure 3b).

In the growing season 2016 under dryland and irrigated conditions, both cultivars showed low levels of disease severity until day 150 (Figures 3 and 4). Under irrigated

conditions for Overley, untreated check plots showed higher levels of foliar disease severity compared to the other treatments at the end of the evaluation period. This trend was evident beginning at day 147 (Figure 4a). In Overland, the last evaluation (day of the year 164) showed higher levels of disease for all the treatments starting at the fourth evaluation. Overland plots were similar in their disease level with prominent differentiation starting at day 155 (Figure 4b).

4.6 Correlations between AUDPC and yield. Correlation analyses using LS-means of fungicide treatments by year and by cultivar between AUDPC and yield, revealed high correlations between yield and foliar disease severity. Correlation coefficients for AUDPC were R = -0.98 (n= 8; P <0.0001) in 2015, and R = -0.84 (n= 9; P = 0.0089) in 2016. In Overley under irrigated conditions, foliar diseases were highly and positively correlated in both years (R = 0.89; n=8; P = 0.0028) as well as yield for both years (R = 0.67; n = 8; P = 0.0697). In Overland under irrigated conditions, Pearson's correlation coefficient for AUDPC was R = 0.92 (n=8; P = 0.0005) among years (2015 versus 2016). In 2015, AUDPC and yield were poorly correlated (R = – 0.35; n=8; P =0.3552). However, in 2016 foliar diseases and yield were highly and negative correlated R = -0.91 (n=9; P = 0.0009).

Under dryland conditions, Pearson's correlation coefficients were high and positive for AUDPC, R = 0.95 (n = 8; P <.0001) among years (2015 versus 2016), and for yield, R = 0.87 (n = 8; P = 0.0019), among years. For the relationships AUDPC versus yield, correlations coefficients were R= -0.93 (n=8; P 0.0003) and R= -0.97 (n=9; P <0.0001) in the growing seasons 2015 and 2016, respectively.

4.7 Critical point models. Analysis of critical point models was conducted using linear regression with foliar disease severity as the independent variable, and yield as the

dependent variable. Considering the coefficients of determination (R^2) and the P-values for regression, the best relationships of yield versus foliar disease severity were reached at the second and third evaluation in the 2015 growing season under dryland and irrigated conditions, respectively. Plants were close to the flowering growth stage (GS 60) and a few days after flowering (GS 60-65) at the timing of the second and third evaluation. Meanwhile, the best relationships of yield and foliar disease severity were reached at the fourth evaluation in the 2016 growing season under both dryland and irrigated conditions (Table 5). AUDPC and yield were significantly linearly related in irrigated plots in 2015 ($R^2 = 0.6480$, $P < 0.0001$) and in 2016 ($R^2 = 0.6544$, $P < 0.0001$). In contrast, the lowest relationship between AUDPC and yield was in the dryland environment in 2016 ($R^2 = 0.1117$, $P = 0.1752$) (Table 5).

4.8 Prosaro application timing on yield and AUDPC. Polynomial regression models were fit using the method of least squares to describe the relationships yield-Prosaro application timing and AUDPC-Prosaro application timing (Table 6). First (linear) and second (quadratic) order polynomials were detected as significant ($P<0.05$) for both relationships (timing-AUDPC) and (timing-yield). In two of the combinations of the environment by growing season, the linear relationship offered the best fit for Prosaro-application timing versus yield for irrigated 2016 and dryland 2015 (Figure 5 and Table 6). On the other hand, for the dryland 2016, and irrigated 2015, the quadratic relation offered the best fit to the data. Therefore, yield reached an inflection point a few days before anthesis and decreased at 6 and 12 DPA (Figure 5 and Table 6). For AUDPC, ANOVA (type-I) showed that the linear effect offered the best fit between Prosaro application timing and AUDPC (Figure 6 and Table 6).

5. Discussion

Foliar fungal diseases in the 2015 and 2016 growing seasons caused damage to the canopy of the crop and reduced yield. Overall, foliar disease severity was higher in 2015 than in 2016. Models based on regression analysis such as critical point models (Zadoks and Schein 1979), and trapezoidal integration (Madden et al. 2007) were used to determine the relationships yield and foliar disease severity by years and environments. Fungicides were effective in controlling foliar diseases and provided gradients of foliar disease severity for disease modeling. As it was demonstrated in this study, early-timed applications (GS 39 – GS 45) were effective in controlling foliar diseases and protecting yield better than the anthesis and post-anthesis applications in the winter wheat pathosystem.

Critical point models and AUDPC were used to interpret yield loss on field plots of wheat with artificially-created differential levels of disease using fungicides. In 2015, the critical point for foliar disease severity assessment was in the flowering stage up to few days after (GS 60 – 65). This approach also showed that measurements of foliar disease severity early in the growth development (GS 37 - 39) were highly variable and poorly related to yield (Table 5). These results are in agreement with Wegulo et al. (2009) who demonstrated that tan spot and spot blotch severity in winter wheat were strongly related to the yield when severity was assessed at the flowering stage (GS 60).

Applying Prosaro early in the growing season significantly reduced foliar disease severity. The protective-curative effect of the triazole-fungicide application timed at GS 39 and GS 45 positively impacted on yield in the two growing seasons and both environments without regard to the cultivar. Prosaro-treated plots at GS 45 showed a 78% reduction in AUDPC compared to the untreated check plots. Headline and Prosaro

applied at anthesis did not differ in the AUDPC in either growing season or environment. Fungicides applied at anthesis showed equal efficacy in controlling foliar fungal diseases and protecting yield. However, slightly higher yields were detected in the Headline-treated plots than in the Prosaro-treated plots at anthesis. This fact was evident when comparing AUDPC values for both fungicide chemistries versus the untreated checks in each combination of year by environment. In a similar study, the application of a triazole only, and a triazole combined with a strobilurin fungicide at anthesis significantly delayed flag leaf greenness; however, this increase in green leaf area during maturation was not correlated with higher yields (Blandino and Reyneri 2009). In contrast, this study showed that applications of either fungicide chemical class at anthesis increased yields compared to the untreated checks. Inversely, post-anthesis applications (6 and 12 DPA) of Prosaro and Headline did not improve control of foliar fungal diseases. Post-anthesis applications of both chemical classes were as poor as the untreated check plots at controlling foliar fungal diseases.

AUDPC was significantly and negatively correlated with yield in both growing seasons. These results are indicative that foliar disease severity affected yield in both years. In addition, correlations suggested a higher relationship between foliar disease severity and yield loss in the 2016 growing season as correlations were more robust ($r \leq -0.90$) and reliable ($P < 0.0001$). Linear regression analysis for AUDPC-yield showed stronger relationships in the irrigated environment than in the dryland environment in both growing seasons. Therefore, results of this study indicate that environmental conditions can affect not only the estimation of foliar disease severity and their relationships with yield loss, but also, the effectiveness of controlling foliar diseases in winter wheat.

The significant differences of fungicide treatments on AUDPC and yield compared to the untreated check plots implied that a fungicide application was needed during both growing seasons. Climatic conditions in 2015 and 2016 were conductive to a high incidence and severity of foliar fungal diseases in winter wheat fields in southeastern Nebraska. In 2016, Creech and Werle reported that grain yield was decreased in 2015 and quality was low in 2016. The yield-protective effect of triazole-fungicide applied early in the growing season was demonstrated in both years in both environments. Similar results were found by Wegulo et al. (2009), and Wegulo et al. (2012) when evaluating the economic impact of foliar disease control in winter wheat and the yield response to foliar fungicide application in winter wheat, respectively. It was found that applications of fungicides at GS 39 or later had the highest yield benefit and net return. Earlier applications (GS 31) can be counterproductive (Wegulo et al. 2012), which emphasizes the importance of well-timed applications of fungicides in this pathosystem. In the cultivar Overland, Prosaro applications at GS 39 and GS 45 effectively controlled foliar diseases. Reductions of 56% and 48% in foliar disease development were detected in Prosaro treated plots at GS 45 compared to untreated check plots under dryland and irrigated conditions, respectively.

Yield loss can be estimated by subtracting the yield of the untreated check plots from the attainable yield of the fungicide-treated plots. In 2016, under dryland conditions, the highest yield was registered in the plots treated with Prosaro at GS 45 (late boot) in both cultivars. In these scenarios, yield losses of 29% and 34% were detected in Overley and Overland, respectively. Our results are similar to those reported in the wheat-yellow spot and *Septoria nodorum* blotch pathosystem in Western Australia, where under favorable

and continuous disease development both diseases were associated with losses of around 30% loss in grain yield (Bhathal et al. 2003).

Disease progress curves indicated epidemic development patterns in both years and environments. However, disease progress curves showed inconsistent results of foliar disease severity with several intersecting points between lines of progression. Foliar disease progression reached a peak after the day of the year 150 (2015) and 160 (2016) in dryland and irrigated environments. These results are similar to leaf rust epidemics of barley in New Zealand where the epidemic showed low severity early in the season and became more severe as the season progressed (Whelan et al. 1997). Results from this study showed high variability at the end of the growing season (middle of June in 2015 and end of June in 2016) expressed as high coefficients of variation in the late evaluations of foliar disease severity (data not shown). Similar results were reported by Wegulo et al. (2009) who found that the disease severity assessment made after GS 71 (watery ripe) at the day of the year 176 had a weak relationship to yield (Wegulo et al. 2009).

Models for crop loss assessment are tools used to understand the relationships occurring in a foliar disease-plant pathosystem. However, there are some constraints of using these models to interpret biological results. Disease forecasting and prediction of the amount of foliar disease severity in a pathosystem involve several interactions between the host, the pathogen, and the environment, including the human interventions that aim to control the pathogen. Fungicide application timing showed inconsistent results by cultivar and year. Although fungicides applied at GS 39 and GS 45 were effective in controlling foliar diseases and protecting yield, their efficacy depended on the cultivar.

Overland showed higher yield with fungicides applied at GS45 than GS39, while the inverse relationship was true for Overley.

In the absence of disease, Overley is a higher yielder than Overland. The lower yield in Overley in this study was due to the 2015 severe epidemics of Fusarium head blight and stripe rust; Overley is highly susceptible to both diseases.

Inconsistency of results from application timing was reported by Cromey et al. (2004) in the *Didymella exitialis*-wheat pathosystem, where they found that tebuconazole and azoxystrobin applied at different growth stages provided no consistent effect on green leaf area retention. Additionally, the effect of Prosaro application timing on yield was related to linear and quadratic polynomials depending on the year and environment. Bockus et al. (1997) had proposed a quadratic model to relate moderately severe tan spot epidemics and increased seed yields. According to this model, an inflection point between boot and full heading constitutes the optimum time where the effectiveness of fungicide application is at its maximum. However, neither linear nor quadratic models fit the data of application timing under high disease pressure (Bockus et al. 1997).

This study demonstrated that earlier fungicide applications at growth stages GS 39 and GS 45 more effectively controlled foliar fungal disease in winter wheat than later applications. Analysis of critical point models showed a stronger relationship between foliar fungal diseases and yield when disease severity was evaluated close to flowering under warm and wet weather conditions, whereas the best relationship to predict yield loss by foliar diseases was obtained using disease severity readings around the milk growth stage under drier conditions.

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Table 1. Dates for evaluation of foliar disease severity

Evaluation	Growing season	Date of evaluation	Date of year
Severity 1	2015	April 24	144
	2016	May 11	132
Severity 2	2015	May 31	151
	2016	May 20	141
Severity 3	2015	June 7	158
	2016	May 28	149
Severity 4	2015	June 11	162
	2016	June 4	156
Severity 5	2015	June 19	170
	2016	June 12	164

Table 2. Summary of weather conditions at the experimental plots

Environment		Temperature °F Minimum		Maximum		Average		Rainfall[a] (mm)		Dew Point °F		Leaf wetness[b] 1-15		Relative Humidity %	
		2015	*2016*	*2015*	*2016*	*2015*	*2016*	*2015*	*2016*	*2015*	*2016*	*2015*	*2016*	*2015*	*2016*
Rainfed	**May**	33.3	33.7	88.2	87.2	60.8	60.1	193.4	186.2	48.8	45.0	3.3	4.1	66.4	60.0
	June	52.1	50.3	96.4	101.1	72.5	78.6	104.1	68.1	61.9	62.2	4.3	2.7	71.3	60.0
Irrigated	**May**	35.2	33.5	92.5	87.8	60.7	60.5	200,7	252.2	47.8	45.6	3.0	3.4	64.4	60.6
	June	51.7	50.0	96.8	99.1	72.3	77.1	599.4	571.5	60.9	62.1	5.8	5.1	69.7	63.7

Values were taken from portable weather stations model WatchDog 1450 (Spectrum Technologies Inc., Thayer Court, Aurora, IL)

[a] Rainfall (mm) for May 2015- 2016 and June 2015, 2016 on irrigated plots represent the sum of precipitation and the quantity of water collected by the rain bucket when the sprinkler system was turned on.

[b] Leaf wetness is measured in a Range/Resolution on a scale from 0 (dry) to 15 (wet) (Spectrum technologies).

Table 3. Foliar Damage associated with leaf rust, stripe rust, and tan spot, in wheat cultivars Overland and Overley under dryland conditions evaluated in Mead, NE, 2015 and 2016.

	Treatment[2]	Foliar disease severity %										AUDPC		YIELD (kg/ha)	
		1		2		3		4		5					
		2015	2016	2015	2016	2015	2016	2015	2016	2015	2016	2015	2016	2015	2016
Overley	Check	29.7 ab[2]	5.1 a-d	58.9 a	11.0 d-g	65.3 ab	37.5 a-c	88.8 a	54.7 a	100 a	99.8 a	2086 a	1362 ab	677 i	2730 e-g
	Prosaro GS 39	N/A	4.4 b-d	N/A	5.8 g	N/A	18.2 f	N/A	20.8 g	N/A	65.0 b-d	N/A	700 f	N/A	3275 c-e
	Prosaro GS 45	8.8 f	4.1 cd	13.5 fg	8.3 e-g	18.7 h	17.7 f	19.5 k	23.4 fg	36.2 h	52.0 ef	563 h	683 f	1233 gh	3861 bc
	Prosaro GS 60	16.7 e	4.4 b-d	30.4 c-e	8.4 e-g	31.7 g	22.5 ef	36.3 ij	24.8 fg	30.8 h	64.3 b-f	909 g	794 ef	1484 e-g	3542 b-d
	Prosaro 6 DPA	26.9 bc	4.4 b-d	46.8 ab	10.8 d-g	65.9 ab	25.4 d-f	62.2 ef	29.6 e-g	56.2 ef	57.8 d-f	1599 c	853 ef	1284 f-h	3160 d-f
	Prosaro 12 DPA	28.3 b	4.4 b-d	58.1 a	12.1 c-f	73.8 a	29.6 b-e	85.6 ab	43.8 b-d	100 a	74.8 bc	2106 a	1097 cd	1114 g-i	2575 fg
	Headline GS 60	20.3 de	6.2 a-d	25.2 ef	12.9 c-f	36.0 fg	22.8 ef	30.6 j	25.9 fg	45.7 g	60.2 c-e	936 g	839 ef	1468 e-g	4062 ab
	Headline 6 DPA	22.8 cd	2.8 d	32.4 c-e	7.5 fg	57.2 bc	29.6 b-e	65.6 de	38.3 c-e	57.3 e	74.8 bc	1439 de	976 c-e	1428 e-h	2756 e-g
	Headline 12 DPA	27.0 bc	3.6 cd	57.9 a	11.0 e-g	69.5 a	35.0 bc	84.5 ab	40.8 b-e	100 a	76.9 b	2065 a	1112 c	933 hi	2208 g
Overland	Check	29.9 ab	6.3 a-d	41.3 b-d	19.5 a-c	56.9 b-d	46.8 a	78.2 bc	56.2 a	99.8 a	100 a	1817 b	1543 a	1942 c-e	3113 d-f
	Prosaro GS 39	9.8 f	7.2 a-d	11.2 g	15.3 a-d	19.2 h	21.1 ef	16.2 k	22.4 g	30.7 h	49.4 f	506 h	774 ef	3314 a	4665 a
	Prosaro GS 45	10.4 f	9.1 a	12.2 g	14.7 a-e	14.2 h	27.2 c-f	15.2 k	24.6 fg	20.8 i	58.8 d-f	430 h	889 d-f	3441 a	4702 a
	Prosaro GS 60	26.6 bc	8.1 a-c	23.3 e-g	16.5 a-d	42.9 fe	36.8 bc	55.8 gh	33.1 d-f	47.1 g	75.2 bc	1175 f	1130 c	2531 b	3958 bc
	Prosaro 6 DPA	27.6 bc	9.1 a	29.7 de	15.2 a-e	53.2 cd	36.8 bc	43.1 ih	51.9 ab	66.0 d	99.5 a	1292 ef	1392 a	2159 b-d	3590 b-d
	Prosaro 12 DPA	29.9 ab	8.7 ab	33.1 c-e	20.5 ab	52.1 c-e	38.8 ab	66.2 de	50.8 ab	91.0 b	99.3 a	1596 cd	1448 a	2340 bc	3077 d-f
	Headline GS 60	20.3 de	5.7 a-d	30.9 c-e	19.4 a-c	43.9 d-f	34.4 b-d	48.9 gh	41.2 b-e	50.0 fg	68.7 b-d	1182 f	1164 bs	3088 a	3745 b-d
	Headline 6 DPA	33.8 a	7.8 a-c	32.8 c-e	21.2 a	67.2 a	39.3 ab	72.2 cd	49.7 a-c	75.1 c	99.5 a	1678 bc	1445 a	1874 c-f	3077 d-f
	Headline 12 DPA	30.2 ab	8.2 a-c	43.1 bc	14.2 b-f	55.0 c	36.1 bc	74.7 cd	51.9 ab	93.9 ab	100 a	1768 b	1374 a	1694 d-g	3313 c-e
		P-value													
	Cultivars (C)	0.0006	0.0653	0.0144	0.0027	0.0199	0.0820	0.2299	0.0516	0.0905	<.0001	0.0427	0.0127	0.0046	0.0333
	Fung. Trt. (F)	<.0001	0.9592	<.0001	0.1499	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
	C x F	0.0232	0.3030	0.0040	0.5280	<.0001	0.1741	<.0001	0.0976	<.0001	<.0001	<.0001	0.0250	0.0008	0.0479

[1]LS-means with the same letter in each row are not significantly different (LSD α =0.05).

Foliar disease severity is the percent of necrotic tissue over the total leaf tissue

Dosage of commercial products: Prosaro (Prothioconazole+ Tebuconazole) and Headline (Pyraclostrobin) were at 6.5 and 9 fluid ounces per acre, respectively.

Fungicide treatments were applied at different stages of wheat development.

Table 4. Foliar Damage associated with leaf rust, stripe rust, and tan spot, in wheat cultivars Overland and Overley under irrigated conditions evaluated in Mead, NE, 2015 and 2016.

	Fungicide	Foliar disease severity (%)										AUDPC		YIELD (kg/ha)	
		1		2		3		4		5					
		2015	2016	2015	2016	2015	2016	2015	2016	2015	2016	2015	2016	2015	2016
Overley	Check	16.0 e-f[1]	4.1 ab	53.0 bc	20.5 a-e	59.8 ab	45.6 a-d	72.9 a	76.9 a	100 a	100 a	1837 a	1585 ab	1122 de	1865 e
	Prosaro GS 39	N/A	3.6 b	N/A	7.2 g	N/A	15.2 h	N/A	23.5 ef	N/A	54.8 de	N/A	620 f	N/A	3382 ab
	Prosaro GS 45	6.8 g	6.2 a	11.0 j	14.8 ef	10.3 f	22.8 gh	11.3 g	30.6 d-f	30.2 f	59.7 c-e	397 h	863 d-f	1802 b-e	3098 a-d
	Prosaro GS 60	16.9 d-f	4.4 ab	26.0 ih	19.8 a-e	31.5 e	22.8 gh	22.6 f	50.2 bc	31.7 f	72.8 bc	782 fg	946 de	1656c-e	3492 a
	Prosaro 6 DPA	24.6 bc	4.2 ab	44.5 cd	20.5 a-e	43.2 c-e	24.2 gh	40.7 e	67.9 a	61.3 de	73.4 b	1296 e	1023 d	1272 c-e	2605 a-e
	Prosaro 12 DPA	22.4 b-e	4.9 ab	64.2 a	19.6 b-e	68.7 a	38.2 b-f	70.1 ab	72.0 a	100 a	96.4 a	1991 a	1325 c	988 e	2928 a-d
	Headline GS 60	19.7 c-e	4.1 ab	29.8 d-g	14.3 ef	33.5 de	23.9 gh	23.3 f	44.2 cd	49.3 e	67.2 b-d	920 f	919 de	1525 c-e	3044 a-d
	Headline 6 DPA	20.6 b-e	4.1 ab	35.8 ef	18.8 c-f	57.1 a-c	33.7 e-g	63.6 bc	72.0 a	75.5 b-d	96.8 a	1525 b-d	1230 c	1324 c-e	2798 a-e
	Headline 12 DPA	24.8 bc	4.8 ab	54.6 b	17.4 d-f	68.3 a	37.0 c-f	63.2 bc	71.0 a	100 a	100 a	1873 a	1309 c	1147 de	2376 b-e
Overland	Check	24.8 bc	4.2 ab	34.7 e-g	23.8 a-d	60.2 ab	42.4 a-e	62.5 bc	62.6 ab	97.8 a	100 a	1646 b	1712 a	1819 b-e	2332 c-e
	Prosaro GS 39	23.0 b-d	4.7 ab	9.3 j	14.5 ef	12.0 f	18.3 h	22.7 f	18.4 f	55.2 e	52.4 e	652 g	752 ef	1895 b-d	3498 a
	Prosaro GS 45	11.8 fg	4.2 ab	11.2 j	12.4 fg	10.8 f	29.8 fg	14.4 fg	27.0 ef	29.9 f	48.4 e	442 h	870 d-f	2545 ab	3234 a-c
	Prosaro GS 60	22.3 b-e	5.0 ab	22.9 i	25.2 a-c	55.0 a-c	41.4 a-e	70.1 ab	25.2 ef	72.1 cd	89.9 a	1445 c-e	1448 bc	2914 a	2817 a-e
	Prosaro 6 DPA	31.9 a	4.4 ab	32.4 e-h	22.4 a-d	47.2 b-d	47.4 a-c	48.9 de	31.9 de	78.3 bc	92.9 a	1389 de	1643 a	2050 bc	2662 a-e
	Prosaro 12 DPA	21.5 b-e	5.2 ab	27.3 f-i	21.7 a-d	56.2 a-c	51.1 a	62.1 bc	41.9 cd	85.5 ab	100 a	1490 b-d	1728 a	1481 c-e	2166 bc
	Headline GS 60	21.7 b-e	4.6 ab	36.6 de	25.8 ab	55.6 a-c	33.9 d-g	56.0 cd	30.1	58.2 e	89.1 a	1395 de	1332 bc	2079 bc	2945 a-d
	Headline 6 DPA	19.9 c-e	4.1 ab	22.8 i	22.0 a-d	46.3 b-d	49.0 a-c	63.9 a-c	36.5 c-e	61.1 de	99.2 a	1285 e	1667 a	1864 b-e	2823 a-e
	Headline 12 DPA	26.9 ab	5.4 ab	26.2 fg	26.5 a	54.4 a-c	50.2 ab	67.8 ab	41.6 cd	94.7 a	100 a	1572 bc	1759 a	1491 c-e	2079 de
		P – value													
	Cultivars (C)	0.0031	0.7390	<.0001	0.0234	0.6195	0.0436	<.0001	0.0110	0.4558	0.0144	0.8595	0.0241	0.1040	0.5445
	Fung. trt. (F)	<.0001	0.6350	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0057
	F x C	0.3373	0.6741	<.0001	0.1283	0.0012	0.0615	<.0001	0.0017	<.0001	0.0105	<.0001	0.0002	0.5861	0.7547

[1]LS-means with the same letter in each row are not significantly different (LSD $\alpha = 0.05$).

Foliar disease severity is the percent of necrotic tissue over the total leaf tissue

Fungicide treatments were applied at different stages of wheat development.

Dosage of commercial products: Prosaro (Prothioconazole+ Tebuconazole) and Headline (Pyraclostrobin) were at fluid ounces per acre.

Table 5. Coefficient of determination (R^2), intercepts, and slopes from regressions of yield (kg/ha) versus disease severity assessment at different growth stages, and on area under disease progress curve (AUDPC) in the study of fungicide application timing on foliar disease severity in Nebraska during 2015 and 2016

	Intercept		**Slope**		**R^2**		**P > F**	
	Dryland	*Irrigated*	*Dryland*	*Irrigated*	*Dryland*	*Irrigated*	*Dryland*	*Irrigated*
2015								
May 24	2864	1913	-41.82	-9.97	0.1578	0.0128	0.1143	0.6650
May 31	3166	2418	-37.55	-22.36	0.4807	0.4648	0.0020	0.0026
June 7	3126	2193	-25.69	-10.80	0.3410	0.1667	0.0138	0.1037
June 11	2921	2001	-18.71	-6.02	0.3179	0.0665	0.0184	0.3176
June 19	2863	2362	-15.14	-9.47	0.2657	0.2171	0.0342	0.0594
AUDPC	3064	2341	-1.29	-0.49	0.3435	0.2231	0.0134	0.0556
2016								
May 11	2335	3350	181.80	-123.86	0.3043	0.0263	0.0176	0.5207
May 20	2947	3848	36.51	-55.06	0.0635	0.3396	0.3129	0.0111
May 28	4352	3915	-29.45	-32.44	0.1319	0.6157	0.1384	0.0001
June 4	4510	3683	-28.09	-19.72	0.2769	0.6308	0.0249	<.0001
June 12	4669	4413	-16.11	-19.62	0.1988	0.6052	0.0636	0.0001
AUDPC	4308	4102	-0.87	-1.04	0.1117	0.6585	0.1752	<.0001

Table 6. Linear and quadratic regressions between AUDPC and yield versus Prosaro application timing under dryland and irrigated conditions in 2014-2015 and 2015-2016

Linear regression analysis						
			Linear		Regression parameters	
	Variable	Intercept	Slope	R^2	F - value	P > F
Dryland 2015	Yield	2164	-56	0.88	22.77	0.0175
Irrigated 2015		1903	-21	0.27	1.09	0.3724
Dryland 2016		3596	-44	0.74	8.31	0.0633
Irrigated 2016		2955	-33	0.90	27.47	0.0135
Dryland 2015	AUDPC	1123	51	0.90	28.17	0.0131
Irrigated 2015		1090	44	0.81	13.46	0.0350
Dryland 2016		1030	20	0.94	50.99	0.0057
Irrigated 2016		1129	36	0.96	74.60	0.0033

Quadratic regression analysis							
			Slope			Regression parameters	
	Variable	Intercept	Linear	Quadratic	R^2	F - value	P > F
Dryland 2015	Yield	1944	-47	2.38	0.99	227.96	0.0044
Irrigated 2015		2255	-36	-3.78	0.91	9.55	0.0947
Dryland 2016		3864	-56	-2.88	0.96	25.19	0.0382
Irrigated 2016		3004	-35	-0.53	0.92	11.25	0.0816
Dryland 2015	AUDPC	997	58	1.68	0.97	38.55	0.0253
Irrigated 2015		927	51	1.76	0.91	10.15	0.0897
Dryland 2016		982	22	0.52	0.99	99.49	0.0100
Irrigated 2016		1121	36	0.09	0.96	25.18	0.0382

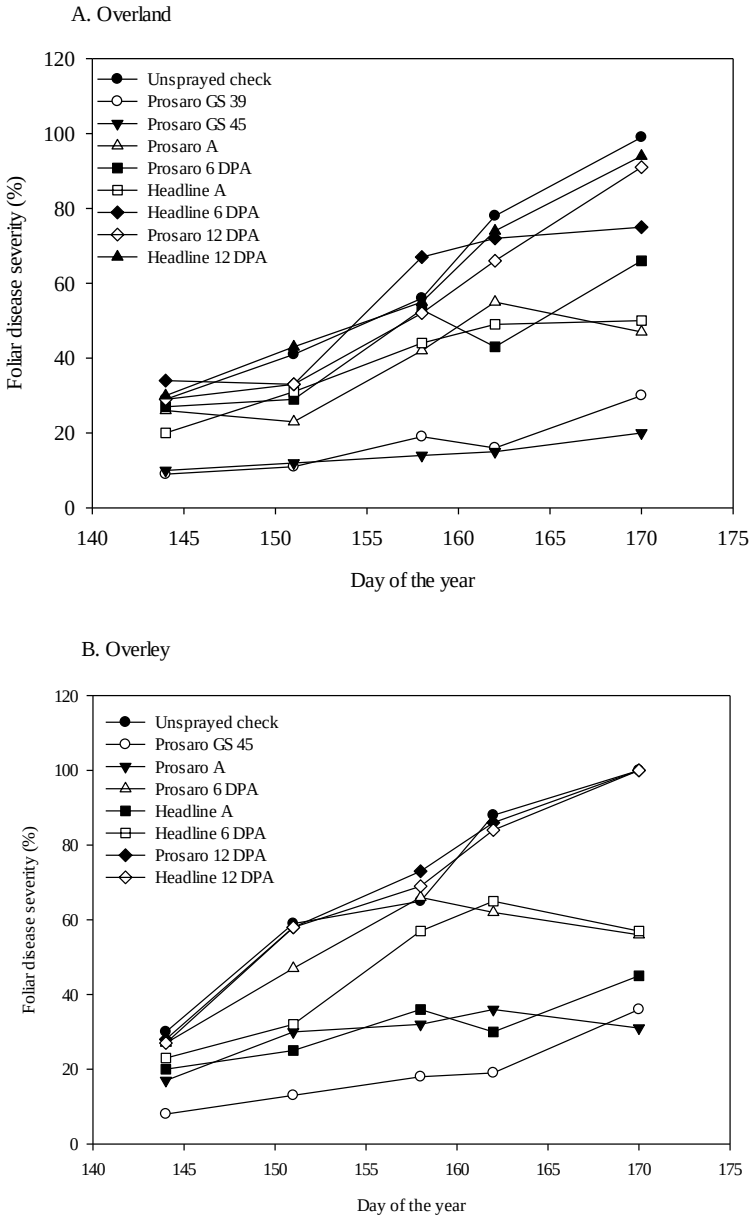


Fig. 1. Foliar disease severity in cultivars A) Overland and B) Overley under rainfed conditions during the 2014-2015 growing season. Plots were treated with Prosaro™ (triazole) and Headline® (strobilurin) at different application timing (GS 39, GS 45, GS 60-anthesis, 6 and 12 days post anthesis). Fungicides were applied at full label rates. Evaluations of foliar disease severity were conducted at day of the year: 144 (May 24), 151 (May 31), 158 (June 7), 162 (June 11) and 170 (June 19). Prosaro was not sprayed in plots of the cultivar Overley at GS 39 due to rain.

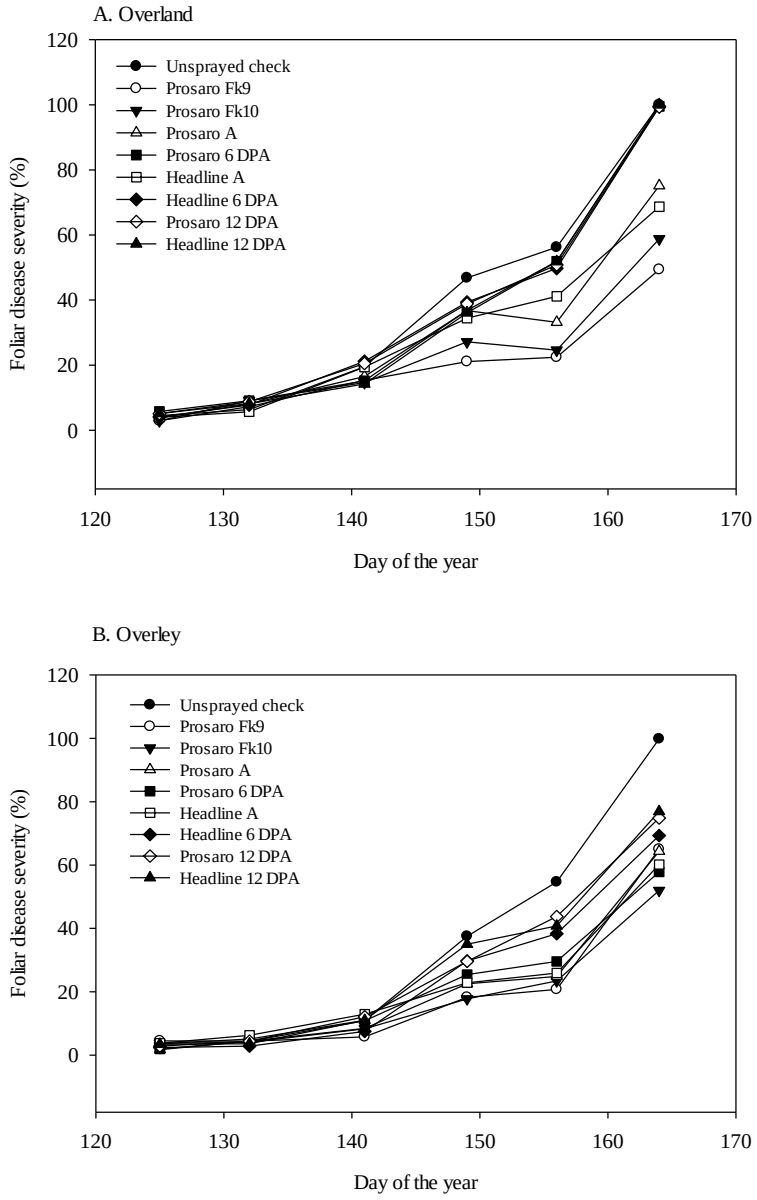


Fig. 2. Foliar disease severity in cultivars A) Overland and B) Overley under rainfed conditions of the 2015-2016 growing season. Plots were treated with Prosaro™ (triazole) and Headline® (strobilurin) at different application timing (GS 39, GS 45, GS 60-anthesis, 6 and 12 days post anthesis). Fungicides were applied at full label rates. Evaluations of foliar disease severity were conducted at the day of the year: 132 (May 11), 141 (May 20), 149 (May 28), 156 (June 4) and 164 (June 12).

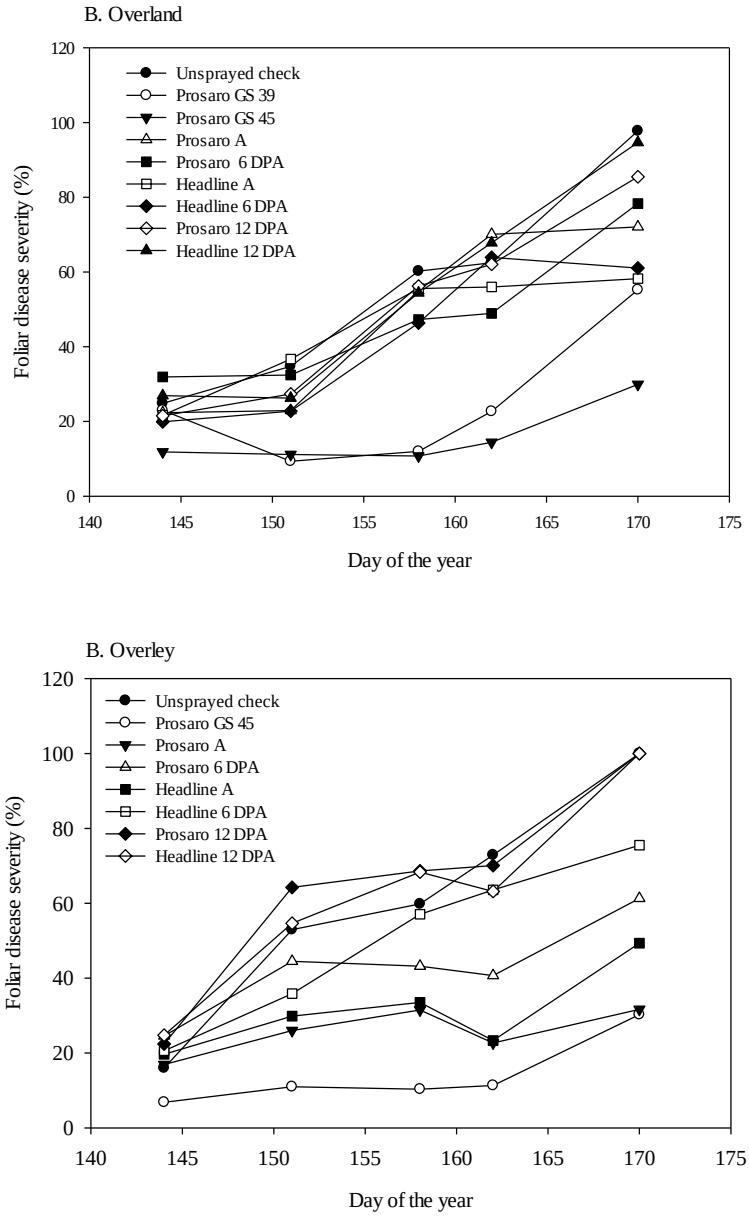


Fig. 3. Foliar disease severity in cultivars A) Overland and B) Overley under irrigated conditions of the 2014-2015 growing season. Plots were treated with Prosaro™ (triazole) and Headline® (strobilurin) at different application timing (GS 39, GS 45, GS 60-anthesis, 6 and 12 days post anthesis). Fungicides were applied at full label rates. Evaluations of foliar disease severity were conducted at the day of the year: 144 (May 24), 151 (May 31), 158 (June 7), 162 (June 11) and 170 (June 19). Prosaro was not sprayed in plots of the cultivar Overley at GS 39 due to rain.

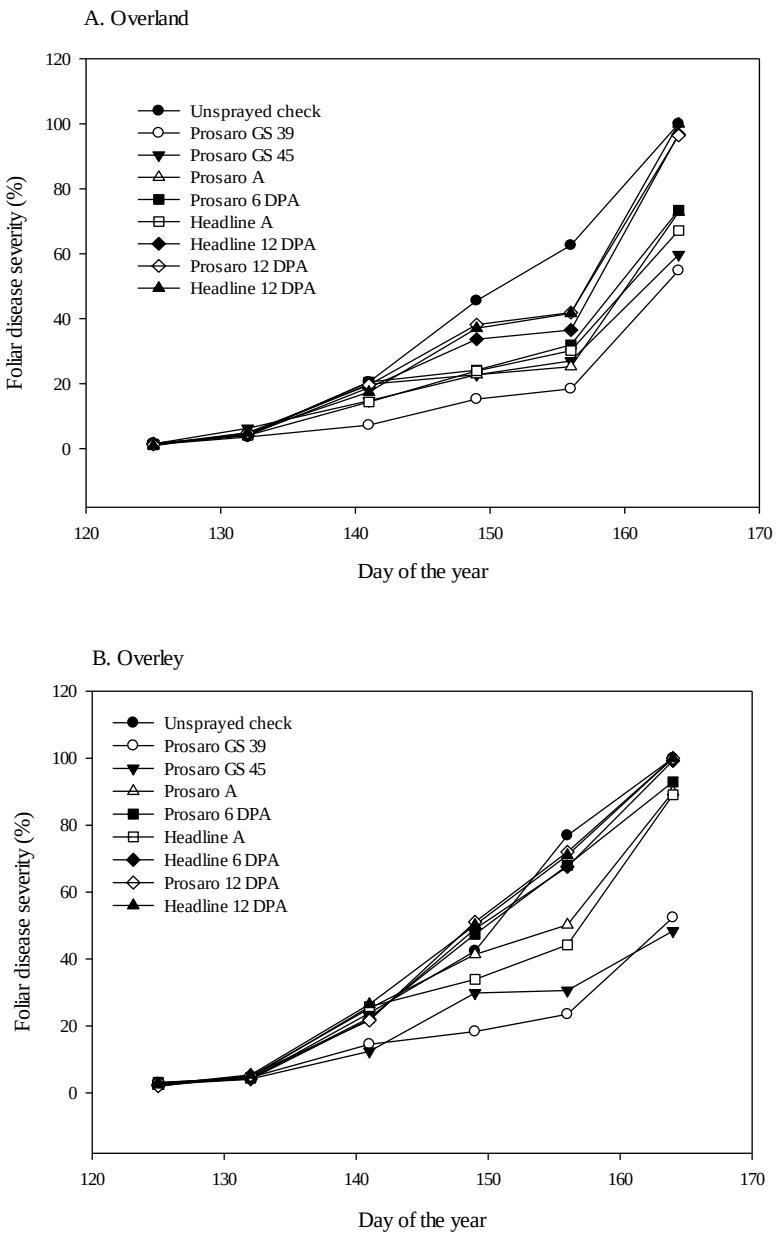


Fig. 4. Foliar disease severity in cultivars A) Overley and B) Overland under irrigated conditions of the 2015-2016 growing season. Plots were treated with ProsaroTM (triazole) and Headline® (strobilurin) at different application timing (GS 39, GS 45, GS 60-anthesis, 6 and 12 days post anthesis). Fungicides were applied at full label rates. Evaluations of foliar disease severity were conducted at the day of the year: 132 (May 11), 141 (May 20), 149 (May 28), 156 (June 4) and 164 (June 12).

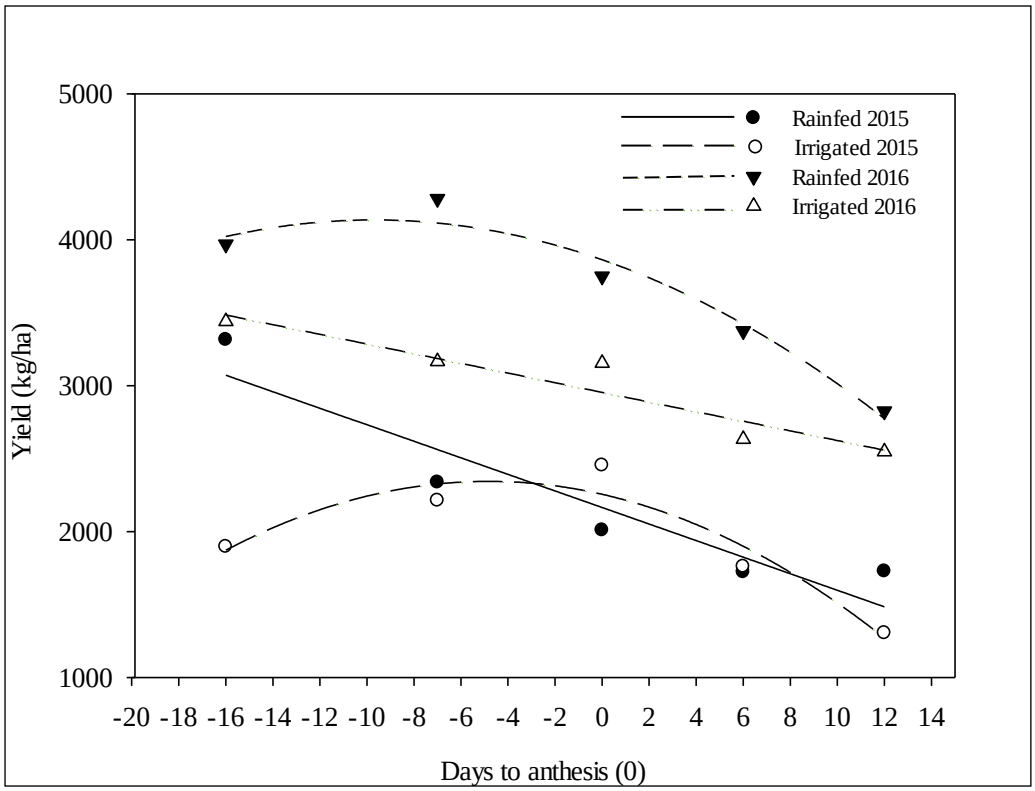


Fig. 5. Linear and quadratic effect of Prosaro application timing on yield. Prosaro was applied at GS 39 (-16 days to anthesis), GS45 (-7 days to anthesis), GS60 (anthesis = 0), 6 and 12 days post anthesis.

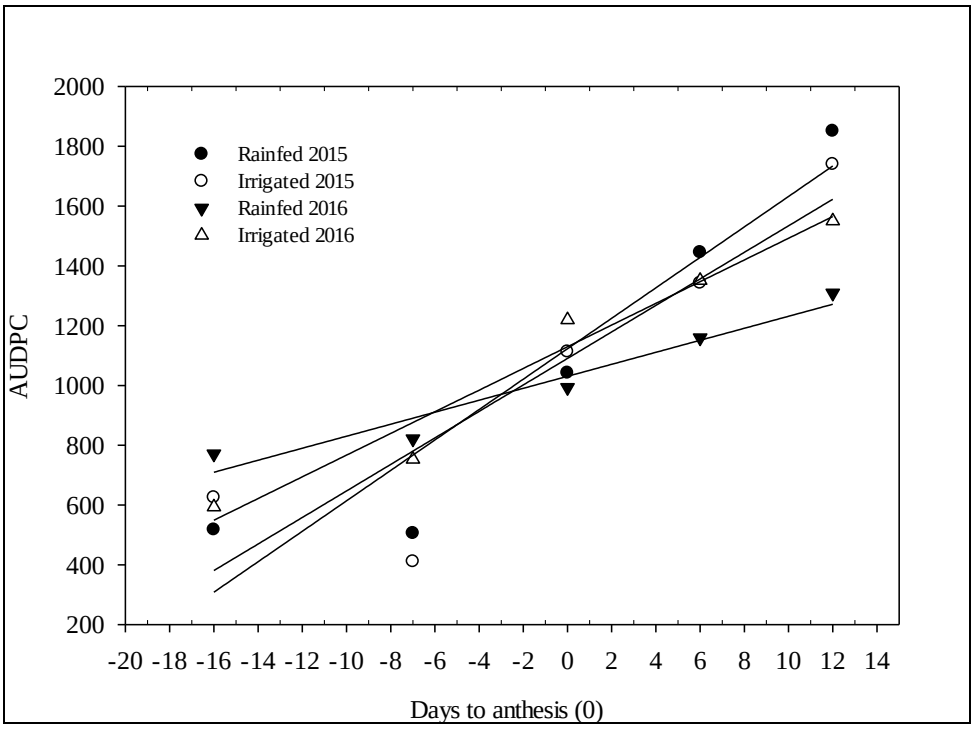


Fig. 6. Linear effect of Prosaro application timing on AUDPC. Prosaro was applied at GS39 (-16 days to anthesis), GS45 (-7 days to anthesis), GS60 (anthesis = 0), 6 and 12 days post anthesis.

APPENDICES

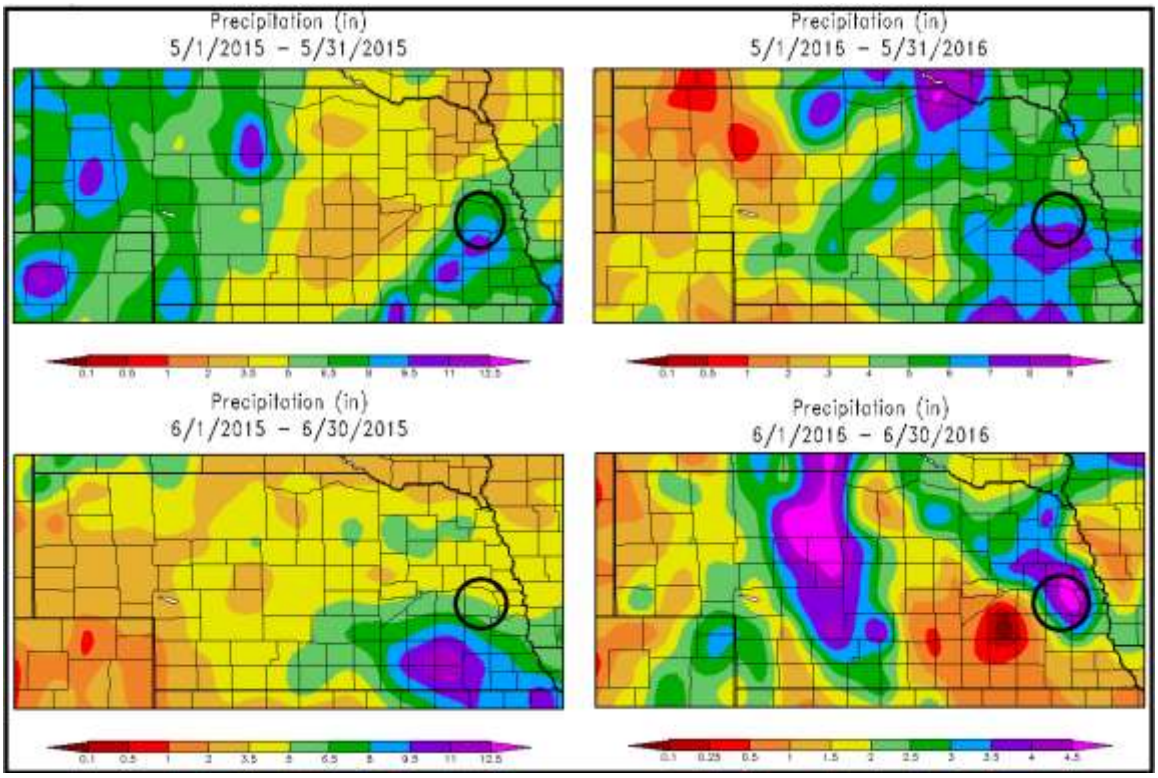
Appendix 1. Pearson correlation coefficients and probability under null hypothesis r = 0 in the evaluation of fungicide chemical class, fungicide application timing, and cultivar resistance on FHB, DON, weight of one thousand kernels (TKW), pounds of a bushel of grain (TWG), and Yield.

	2014-2015				2015-2016				Combined	
	Rainfed		Irrigated		Rainfed		Irrigated			
	R	P > |r|	R	P > |r|	R	P > |r|	R	P > |r|	R	P > |r|
IND/FDK	0.959	<.0001	0.821	0.0003	0.730	0.0030	0.850	0.0001	0.985	<.0001
IND/DON	0.976	<.0001	0.735	0.0028	0.859	<.0001	0.658	0.0199	0.862	0.0126
IND/TKW	-0.914	<.0001	-0.663	0.0098	0.606	0.0215	0.357	0.2101	-0.906	0.0050
IND/TWG	-0.895	<.0001	-0.863	<.0001	0.572	0.0324	0.155	0.5971	-0.922	0.0031
IND/Yield	-0.848	0.0001	-0.767	0.0014	-0.769	0.0013	-0.523	0.0550	-0.894	0.0066
FDK/DON	0.969	<.0001	0.804	0.0005	0.679	0.0075	0. 747	0.0022	0.824	0.0137
FDK/TKW	-0.942	<.0001	-0.692	0.0061	0.304	0.2910	0.281	0.3315	-0.872	0.0106
FDK/TWG	-0.989	<.0001	-0.835	0.0002	0.255	0.3785	0.047	0.8710	-0.899	0.0059
FDK/Yield	-0.918	<.0001	-0.847	0.0001	-0.411	0.1444	-0.549	0.0416	-0.893	0.0068
DON/TKW	-0.927	<.0001	-0.659	0.0104	0.679	0.0076	0.860	<.0001	-0.699	0.0803
DON/TWG	-0.968	<.0001	-0.681	0.0074	0.759	0.0016	0.592	0.0257	-0.690	0.086
DON/Yield	-0.879	<.0001	-0.557	0.0384	-0.508	0.0636	0.068	0.8175	-0.589	0.1641
TKW/TWG	0.895	<.0001	0.729	0.0030	0.880	<.0001	0.852	0.0001	0.942	0.0015
TWG/Yield	0.878	<.0001	0.892	<.0001	-0.267	0.3562	0.486	0.0780	0.973	0.0002
TKW/Yield	0.940	<.0001	0.510	0.0622	-0.267	0.3562	0.400	0.1559	0.929	0.0024

Appendix 2. Means of weight in pounds of a bushel of grain (TWG) evaluated under irrigated and rainfed conditions of southeastern Nebraska (Mead-ARDC), growing seasons 2015 and 2016.

Cv.	Fungicide treatments	Test weight (lb/bu)			
		Irrigated		Rainfed	
		2015	2016	2015	2016
Overley	Check	40.2 f-h	48.9 b-d	34.1 f	53.3 cd
	Prosaro A	46.7 b-d	51.5 ab	43.7 c	55.6 a
	Prosaro 6 DPA	44.8 d-g	48.9 b-d	43.2 c	54.8 ab
	Prosaro 12 DPA	38.2 h	49.5 a-d	38.2 ef	55.4 a
	Headline A	45.7 c-f	50.8 a-c	40.9 cd	55.8 a
	Headline 6 DPA	42.3 e-h	52.1 a	42.1 c	54.0 bc
	Headline 12 DPA	39.1 gh	48.8 b-d	35.5 de	55.2 ab
Overland	Check	44.5 e-g	48.4 cd	47.6 b	53.0 cd
	Prosaro A	53.5 a	48.5 cd	52.1 a	52.2 de
	Prosaro 6 DPA	50.7 a-d	44.3 f	52.6 a	51.4 e
	Prosaro 12 DPA	46.1 c-f	45.5 ef	50.6 ab	52.1 de
	Headline A	51.1 a-c	50.0 a-c	51.3 a	53.8 bc
	Headline 6 DPA	51.9 ab	45.0 ef	52.0 a	52.1 de
	Headline 12 DPA	45.1 d-g	45.6 ef	50.0 ab	51.9 de

Appendix 3. Maps of precipitation in the state of Nebraska during the months of May and June of 2015 and 2016. Black circles highlight the location of the Saunders County (one inch of precipitation represents 25.4 mm) (Source: High Plains Regional Climate Center (HPRCC))



Appendix 4. Effects of cultivar resistance and fungicide treatments on acetylated derivatives of deoxynivalenol 3-ADON and 15-ADON in winter wheat under two environmental conditions, in the growing seasons 2015 and 2016

Source	*3A-DON*	*15A-DON*
	P>F	
Rainfed, 2015		
Cultivar (C)	0.0036	0.0205
Fung. trt. (F)	0.5499	0.7353
C x F	0.5499	0.5683
Anthesis vs. Post-Anth.	0.4612	0.1136
Factorial vs. Additional	0.7497	0.5257
Irrigated, 2015		
Cultivar (C)	0.0681	0.0096
Fung. trt.(F)	0.9907	0.7535
C x F	0.8475	0.9117
Anthesis. vs. Post-Anth.	0.9676	0.5570
Factorial vs. Additional	0.8052	0.1271
Rainfed, 2016		
Cultivar (C)	NA	NA
Fung. trt.(F)	NA	NA
C x F	NA	NA
Anthesis vs. Post-Anth.	NA	NA
Factorial vs. Additional	NA	NA
Irrigated, 2016		
Cultivar (C)	NA	NA
Fung. trt.(F)	NA	NA
C x F	NA	NA
Anthesis vs. Post-Anth.	NA	NA
Factorial vs. Additional	NA	NA

Appendix 5. Means for acetylated relatives (3-ADON and 15-ADON) evaluated under rainfed and irrigated field conditions of southeastern Nebraska (Mead-ARDC), growing seasons 2015 and 2016.

Cv.	Fungicide Treatment	3-ADON (µg/g)		15-ADON (µg/g)		3-ADON (µg/g)		15-ADON (µg/g)	
		Rainfed				Irrigated			
		2015	2016	2015	2016	2015	2016	2015	2016
Overley	Check	1.0 ab	0.1	2.0 a	0.1	0.6 a-c	0.1	1.5 ab	0.1
	Prosaro A	0.9 ab	0.1	1.1 b-d	0.1	0.7 a-c	0.1	1.2 ab	0.1
	Prosaro 6 DPA	0.9 ab	0.1	1.6 ab	0.1	0.7 a-c	0.1	1.9 ab	0.1
	Prosaro 12 DPA	1.2 a	0.1	1.6 ab	0.1	0.8 a-c	0.1	2.6 ab	0.1
	Headline A	0.8 ab	0.1	1.3 a-c	0.1	0.7 a-c	0.1	1.3 ab	0.1
	Headline 6 DPA	0.9 ab	0.1	1.4 ab	0.1	0.9 ab	0.1	2.6 a	0.1
	Headline 12 DPA	0.6 bc	0.1	1.4 ab	0.1	0.8 a-c	0.1	2.3 ab	0.1
Overland	Check	0.1 c	0.1	0.5 cd	0.1	0.4 a-c	0.1	0.8 ab	0.1
	Prosaro A	0.1 c	0.1	0.6 cd	0.1	0.1 c	0.1	0.7 b	0.1
	Prosaro 6 DPA	0.1 c	0.1	0.4 d	0.1	0.1 c	0.1	0.9 ab	0.1
	Prosaro 12 DPA	0.1 c	0.1	0.4 d	0.1	0.4 a-c	0.1	0.7 ab	0.1
	Headline A	0.1 c	0.1	0.5 cd	0.1	0.3 a-c	0.1	0.8 ab	0.1
	Headline 6 DPA	0.1 c	0.1	0.5 cd	0.1	0.1 c	0.1	1.0 ab	0.1
	Headline 12 DPA	0.1 c	0.1	0.5 cd	0.1	0.2 bc	0.1	1.4 ab	0.1