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WHEAT DISEASE MANAGEMENT WITH FUNGICIDES: QOI-RESISTANT *PARASTAGONOSPORA NODORUM* AND *ZYMOSEPTORIA TRITICI*, APPLICATION TIMING FOR LEAF DISEASE MANAGEMENT, AND SPRAYER CONFIGURATIONS FOR FUSARIUM HEAD BLIGHT MANAGEMENT

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
College of Agriculture, Food and Environment
at the University of Kentucky

By

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Lexington, Kentucky

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Lexington, Kentucky

2021

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ABSTRACT OF THESIS

WHEAT DISEASE MANAGEMENT WITH FUNGICIDES: QOI-RESISTANT *PARASTAGONOSPORA NODORUM* AND *ZYMOSEPTORIA TRITICI*, APPLICATION TIMING FOR LEAF DISEASE MANAGEMENT, AND SPRAYER CONFIGURATIONS FOR FUSARIUM HEAD BLIGHT MANAGEMENT

Disease management in winter wheat can be improved by a properly implemented foliar fungicide program. The fungicide, application time, and application system should be selected based on the host, pathogens present, risk of fungicide resistance, and time of disease onset. These factors vary among environments and require region-specific research to determine appropriate practices. Trials were conducted to evaluate different aspects of fungicide applications for managing Fusarium head blight (FHB), caused by *Fusarium graminearum*, and the Septoria tritici leaf blotch complex, caused by *Zymoseptoria tritici* and *Parastagonospora nodorum*. A survey of *P. nodorum* isolates from Kentucky and Illinois and *Z. tritici* isolates from Kentucky showed that both pathogen populations included strains that were resistant to quinone outside inhibitor (QoI) fungicides. Trials were conducted to determine the optimal fungicide application time to manage the Septoria leaf blotch complex, and whether disease management programs benefited from adding a foliar fungicide. The effect that sprayer speed, nozzle type, and configuration had on coverage of wheat heads and FHB management was evaluated. Applying alternative fungicide chemistries to the QoI class at Feekes 9 along with an application at Feekes 10.51 made with an appropriate nozzle and application speed will provide a more effective fungicide program for winter wheat in Kentucky.

KEYWORDS: QoI fungicide resistance, *Parastagonospora nodorum*, *Zymoseptoria tritici*, *Fusarium graminearum*, fungicide application timing, fungicide coverage

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WHEAT DISEASE MANAGEMENT WITH FUNGICIDES: QOI-RESISTANT *PARASTAGONOSPORA NODORUM* AND *ZYMOSEPTORIA TRITICI*, APPLICATION TIMING FOR LEAF DISEASE MANAGEMENT, AND SPRAYER CONFIGURATIONS FOR FUSARIUM HEAD BLIGHT MANAGEMENT

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TABLE OF CONTENTS

ACKNOWLEDGMENTS iii

LIST OF TABLES vi

LIST OF FIGURES viii

CHAPTER 1. IDENTIFICATION OF QUINONE OUTSIDE INHIBITOR FUNGICIDE-RESISTANT ISOLATES OF PARASTAGONOSPORA NODORUM FROM ILLINOIS AND KENTUCKY 1

Abstract 1

Introduction 2

Materials and methods 6

Results 16

Discussion 19

CHAPTER 2. IDENTIFICATION OF QUINONE OUTSIDE INHIBITOR FUNGICIDE-RESISTANT ISOLATES OF *ZYMOSEPTORIA TRITICI* FROM KENTUCKY 31

Abstract 31

Introduction 32

Material and methods 34

Results 39

Discussion 40

CHAPTER 3. EFFECT OF FUNGICIDE APPLICATION TIMING ON LEAF BLOTCH DISEASE MANAGEMENT ON WINTER WHEAT CULTIVARS DIFFERING IN SUSCEPTIBILITY 48

Abstract 48

Introduction 49

Material and methods 52

Results 56

Discussion 58

CHAPTER 4. EVALUATION OF NOZZLE CONFIGURATION AND SPRAYER SPEED ON CONTROL OF FUSARIUM HEAD BLIGHT AND DEOXYNIVALENOL CONTAMINATION OF HARVESTED GRAIN AND COVERAGE OF WHEAT HEADS 68

Abstract 68

Introduction 68

Materials and Methods 70

Results 74
Discussion 76
CHAPTER 5. SUMMARY 88
REFERENCES 91
VITA 101

LIST OF TABLES

Table 1.1 Number of *Parastagonospora nodorum* isolates collected by state and county and percentage of isolates with the G143A mutation, which causes resistance to quinone outside inhibitor (QoI) fungicides.. 24
Table 1.2 Mean germination rate of conidia from QoI sensitive *Parastagonospora nodorum* isolates at different concentrations of salicylhydroxamic acid (SHAM). .. 25
Table 1.3 Effective concentration of azoxystrobin that inhibited 50 % of conidial germination relative to a non-amended control (EC50) for quinone outside inhibitor (QoI) fungicide-sensitive isolates of *Parastagonospora nodorum* at different concentrations of salicylhydroxamic acid (SHAM). 26
Table 1.4 Effective concentration of azoxystrobin that inhibits 50% of conidial germination relative to a non-amended control (EC50) for five *Parastagonospora nodorum* isolates resistant to quinone outside inhibitor (QoI) fungicides and five isolates of *P. nodorum* sensitive to QoI fungicide in the presence of 60 ppm SHAM. 27
Table 1.5 Relative control of leaf blotch caused by isolates of *Parastagonospora nodorum* with and without the G143A mutation when treated with the quinone outside inhibitor fungicide, pyraclostrobin, in a greenhouse trial. 28
Table 2.1 The number of *Zymoseptoria tritici* isolates examined by county and the percentage of isolates that have the G143A mutation, which causes resistance to quinone outside inhibitor (QoI) fungicides.. 44
Table 2.2 Effective concentration of azoxystrobin that inhibits 50% of conidial germination relative to a non-amended control (EC50) for quinone outside inhibitor (QoI) fungicide-sensitive isolates of *Z. tritici* with and without salicylhydroxamic acid (SHAM). 45
Table 2.3 Effective concentration that inhibits 50% of conidial germination relative to a non-amended control (EC50) for four Isolates of Zymoseptoria tritici resistant to quinone outside inhibitor (QoI) and four *Zymoseptoria tritici* sensitive to QoI fungicides in the presence of 60 ppm SHAM... 46
Table 3.1 Agronomic practices including planting date, harvest date, nitrogen rates, herbicide applications, insecticide applications, and soil type. 63
Table 3.2 Fungicide application timings and application dates...................................... 64
Table 3.3 Significance of cultivar, fungicide, and their interaction on area under disease progress curve (AUDPC), test weight (TW), yield, Fusarium head blight index, and deoxynivalenol (DON) content... 65
Table 3.4 The effect of moderately susceptible and moderately resistant winter wheat cultivars on area under disease progress curve (AUDPC), test weight (TW), and yield. 66
Table 3.5 The effect of fungicide application timing on Septoria leaf blotch complex (SLBC) area under disease progress curve (AUDPC), test weight (TW), yield, Fusarium head blight (FHB) index and deoxynivalenol (DON) concentration. 67
Table 4.1 Nozzle configurations and sprayer speeds evaluated. Pressure was adjusted to achieve similar spray volumes across treatments... 79

Table 4.2 Sprayer speed, nozzle configuration, and speed × nozzle configuration effects ($P > F$) on front coverage, back coverage, and total coverage of a simulated wheat head sprayed with water. 80
Table 4.3 Sprayer speed, nozzle configuration, and speed × nozzle configuration effects ($P > F$) on relative Fusarium head blight (FHB) control, relative deoxynivalenol (DON) control, relative test weight increase, and relative yield increase in wheat sprayed with Prosaro fungicide (prothioconazole + tebuconazole; Bayer Crop Science, St. Louis, MO) relative to a water control.......... 81
Table 4.4 Effect of sprayer speed on spray coverage on the back of simulated wheat heads. 82
Table 4.5 Effect of nozzle configuration front, back and total spray coverage of a simulated wheat head.......... 83
Table 4.6 Effect of nozzle configuration and sprayer speed on relative control of Fusarium head blight (FHB) with Prosaro fungicide (prothioconazole + tebuconazole; Bayer Crop Science, St. Louis, MO). 84
Table 4.7 Pearson correlation coefficients and probability values between relative control of Fusarium head blight (FHB) or deoxynivalenol (DON) and front, back, and total coverage of simulated wheat heads. 85

LIST OF FIGURES

Figure 1.1 Alignment of a section of the *Parastagonospora nodorum* cytochrome b amino acid sequence corresponding to codons 129, 137 and 143, wherein mutations may occur that confer resistance to quinone outside inhibitor fungicides. QoI fungicide-resistant *P. nodorum* isolates have a shift from glycine (G) to alanine (A) at codon 143 (G143A mutation. The reference sequence represents the six isolates from Arkansas that were collected in 1995, prior to the use of QoI fungicides in wheat. Representative additional sequences from resistant and sensitive isolates are included for comparison 29
Figure 1.2 Inhibition of conidial germination at different concentrations of azoxystrobin for *Parastagonospora nodorum* isolates resistant and sensitive to quinone outside inhibitor (QoI) fungicides. 30
Figure 2.1 Alignment of a section of the *Zymoseptoria tritici* cytochrome b amino acid sequence corresponding to codons 129, 137 and 143, where mutations may occur that confer resistance to quinone outside inhibitor fungicides. QoI fungicide-resistant isolates have a shift from glycine (G) to Alanine (A) at codon 143 (G143A mutation) of the cytochrome b of QoI sensitive and resistant isolates of *Zymoseptoria tritici* and the DNA sequence at codon 143 where a mutation occurred. 47
Figure 4.1 Orientation of spray nozzles in relation to the wheat head. The AITTJ60 and TTJ60 spray nozzles were tested at both the typical nozzle orientation and the 30° backward rotation orientation. 86
Figure 4.2 Water sensitive paper was wrapped around plastic tubes that were of similar diameter as wheat heads and were placed at the approximate height as surrounding wheat heads. These served as a simulated wheat heads used to measure coverage from different spray nozzle configurations and sprayer speeds in field trials conducted at Princeton, KY in 2017-2019. 87

CHAPTER 1. IDENTIFICATION OF QUINONE OUTSIDE INHIBITOR FUNGICIDE-RESISTANT ISOLATES OF *PARASTAGONOSPORA NODORUM* FORM ILLINOIS AND KENTUCKY

Abstract

Stagonospora leaf and glume blotch, caused by *Parastagonospora nodorum,* is a major disease of winter wheat (*Triticum aestivum*) in Kentucky and Illinois capable of significantly reducing grain yield and quality. Pathogens such as *P. nodorum* that overwinter in crop residue are often concerns in regions like Kentucky and Illinois that practice no-till farming. In addition, the lack of wheat cultivars with complete resistance to *P. nodorum* has led to the reliance on foliar fungicides for disease management. Quinone outside inhibitor (QoI) fungicides (Fungicide Resistance Action Committee, FRAC, Group 11) are one of the major classes used to manage foliar diseases in wheat. Use of the QoI class of fungicides tends to select isolates of fungal pathogens with resistance due to mutations in the fungal cytochrome b gene. Isolates of *P. nodorum* were collected from Illinois in 2014 and Kentucky in 2018, 2019 and 2020. Amplification and sequencing of a segment of the cytochrome b gene from these isolates revealed a mutation at codon 143 that confers a change from glycine to alanine in the amino acid sequence (known as the G143A mutation). This G143A mutation is the most common mutation in plant pathogenic fungi resistant to QoI fungicides. *In vitro* plate assays and greenhouse trials were used to confirm and characterize the QoI resistance caused by the G143A mutation. The frequency of the tested isolates with the G143A mutation was 46% (57 out of 123 isolates) and 5% (3 out of 60 isolates) for Kentucky and Illinois, respectively. This research is the first to identify the G143A mutation in *P. nodorum* isolates with resistance to QoI fungicides in Illinois and Kentucky. Wheat farmers in

these states and the surrounding region should utilize different practices to manage diseases caused by *P. nodorum*, including rotating wheat with non-host crops, and applying fungicide products that contain active ingredients from different FRAC groups or tank-mixing fungicides from different FRAC groups.

Introduction

Stagonospora nodorum leaf and glume blotch are common diseases of winter wheat (*Triticum aestivum*) in Kentucky and Illinois. The causal agent of both diseases is *Parastagonospora nodorum* (synonyms include *Phaseosphaeria nodorum*, *Stagonospora nodorum* and *Septoria nodorum*). Stagonospora leaf blotch often occurs together with leaf blotch caused by *Zymoseptoria tritici*. Collectively these are referred to as the Septoria leaf blotch complex (Eyal et al. 1987). Infection of wheat by *P. nodorum* can occur on most of the plant, notably producing elongated necrotic lesions with a chlorotic halo on foliage, and dark discolored glumes on grain heads (Eyal et al. 1987; Mehra et al. 2019)

When inoculum is abundant in warm humid environments, high incidence and severity of leaf and glume blotch are likely on susceptible wheat cultivars, which can result in yield losses as much as 20-50% (Bhathal et al. 2003; Eyal 1981; Eyal et al. 1987). Yield loss from *P. nodorum* is primarily due to the damage it causes to flag leaves, which reduces photosynthetic capacity and grain fill (Bhathal et al. 2003; Sylvester et al. 2018). Grain quality and test weight are reduced by glume infections that cause shriveled and discolored grain (Bhathal et al. 2003; Eyal et al. 1987; Mehra et al. 2019). The frequency of *P. nodorum* and yield loss varies by region, year, and environmental

conditions. Some of the difference in infection frequency can be explained by initial inoculum levels, which makes residue management an important strategy for reducing inoculum (Engle et al. 2006; Hardwick et al. 2001; Holmes and Colhoun 1974; Mehra et al. 2016; Mehra et al. 2015; Shah et al. 2000).

No-till farming poses a challenge for disease management and often results in an abundance of inoculum (Krupinsky and Tanaka 2001). The multiple hosts of *P. nodorum*, including barley and some wild grasses, and multiple inoculum sources add additional complexity to disease management (Eyal 1981; Eyal et al. 1987; Krupinsky and Tanaka 2001; Mehra et al. 2019; Solomon et al. 2006; Sommerhalder et al. 2010; Williams and Jones 1973). Although *P. nodorum* can be seed-borne, ascospores and conidia from crop residue are the main sources of inoculum (Caten and Newton 2000; Eyal 1981; Eyal et al. 1987; Krupinsky and Tanaka 2001; Mehra et al. 2019; Shah et al. 1995; Sommerhalder et al. 2010). Air-borne ascospores reduce the effectiveness of tillage for inoculum management and facilitate the long distance spread of *P. nodorum,* with small amounts capable of starting an outbreak (Arseniuk et al. 1998; Caten and Newton 2000; Mehra et al. 2015; Sommerhalder et al. 2010; Stover et al. 1996). Asexual conidia are produced quickly after infection, leading to the rapid spread of this pathogen over short distances (Eyal 1981; Eyal et al. 1987; Mehra et al. 2019; Shah et al. 2001). In addition to making *P. nodorum* a prolific pathogen, the polycyclic life cycle that includes a sexual and asexual stage provides a high level of adaptability, further increasing the difficulty of managing Stagonospora leaf and glume blotch (Eyal 1981; Eyal et al. 1987; McDonald and Linde 2002; Mehra et al. 2019; Shah et al. 2001).

In addition to managing inoculum levels, planting resistant cultivars, and applying foliar fungicides are disease management tactics that are often used. *Parastagonospora nodorum* produces several necrotrophic effectors that interact with the host to kill tissue, enhancing colonization which promotes disease. These effectors complicate breeding for resistant cultivars due their being multiple different effectors and the variability of their occurrence (Solomon et al. 2006). Currently, only cultivars with partial resistance are available. Depending on the cultivar, resistance can vary with environment and may not provide protection against both Stagonospora leaf and glume blotch (Fried and Meister 1987; Kim and Bockus 2003; Shah et al. 2000). The limitations of other management strategies increase the importance of foliar fungicide use. Fungicide applications are an effective way to protect photosynthetic capacity and yield potential in the presence of *P. nodorum* (Gooding et al. 2000; Sylvester et al. 2018).

Quinone outside inhibitor (QoI) fungicides (Fungicide Resistance Action Committee, FRAC, Group 11) are one of the three main classes used to manage Stagonospora leaf and glume blotch in wheat (Sylvester and Kleczewski 2018). Strobilurin fungicides were the first type of QoI fungicide introduced in mid-1990s and were commonly used due to the effectiveness of their novel mode of action (Bartlett et al. 2002). Shortly after their introduction, strobilurin fungicides failed to control powdery mildew (caused by *Blumeria graminis*) on wheat in Northern Germany (Bartlett et al. 2002; Sierotzki et al. 2000). Initially the mechanism of resistance to QoI fungicides in *B. graminis* was unknown but suspected to be due to one or multiple mutations in the cytochrome b gene, as had been discovered in some nonpathogenic Basidiomycetes (Kraiczy et al. 1996; Sierotzki et al. 2000). It was determined that the reduced sensitivity

of *B. graminis* to QoI fungicides was conferred by a mutation resulting in a change of glycine to alanine at codon 143 (known as the G143A mutation) (Sierotzki et al. 2000). Two other mutations, F129L and G137R, confer partial resistance to QoI fungicides in other plant pathogens (Kim et al. 2003; Sierotzki et al. 2007). The G143A mutation confers the most complete resistance to QoI fungicides among the three mutations (Kim et al. 2003; Sierotzki et al. 2007; Sierotzki et al. 2000).

Quinone outside inhibitor fungicides work by binding to the cytochrome b and blocking electron flow in the mitochondria. Some fungi use an alternative oxidase to continue respiration in the presence of inhibitors (McDonald and Linde 2002; Wood and Hollomon 2003). Alternative respiration is thought to be inactive when the fungi are grown *in planta*; when studying QoI resistance *in vitro*, this pathway must be controlled with an inhibitor (Wood and Hollomon 2003). Salicylhydroxamic acid (SHAM) is commonly used to inhibit the alternative oxidase pathway when studying QoI resistance (Wood and Hollomon 2003).

Resistance to QoI fungicides in *P. nodorum* isolates with the G143A mutation that were collected from Sweden in 2003-2005 (Blixt et al. 2009) and from Virginia in the United States in 2017 (Kaur et al. 2021) has been reported previously. In both studies, some isolates without a mutated cytochrome b gene also displayed resistance to the QoI fungicide, azoxystrobin. SHAM was only used in the Virginia study, suggesting that alternative respiration is not the reason for the unexplained resistance, and that there may be a mechanism other than a mutation in the cytochrome b gene (Blixt et al. 2009; Kaur et al. 2021). Similar patterns of resistance have been reported in several other pathogens

where QoI resistance was not related to a mutation in the cytochrome b gene (Fernández-Ortuño et al. 2008; Pereira et al. 2017b).

The typical pattern of fungicide resistance indicated that isolates of *P. nodorum* lacking sensitivity to QoI fungicides would be found in other parts of the country (Hobbelen et al. 2014). The goal of this study was to determine the frequency of QoI fungicide-resistant *P. nodorum* isolates in winter wheat fields grown in Kentucky and Illinois. Additional objectives were to characterize the QoI-resistant isolates *P. nodorum* and determine the impact this has on the control of Stagonospora leaf blotch in winter wheat.

Materials and methods

Collection of *P. nodorum* isolates

Winter wheat leaves and heads with symptoms of Stagonospora nodorum blotch were collected from Kentucky during May and July 2018-2020. In 2019, some wheat leaves were pressed, dried, and stored before attempting to isolate *P. nodorum*. In other years, isolations were made from fresh plant material.

Wheat leaves were collected from fields and then placed in a humidity chamber for 24 to 48 h to induce the production of conidia formed in cirrhi, exuding from pycnidia. Using a sterile needle, cirrhi were lifted from the surface of a wheat leaf and were mixed with sterile water or equal parts rifamycin (MP Biomedicals., Irvine, CA) and sterile water. Ten to twenty microliters of conidial suspension were spread on water agar (20 g/liter agar, Alfa Aesar, Haverhill, MA) or potato dextrose agar (PDA) (BD bioscience, San Jose, CA) amended with 25 mg/liter rifamycin in Petri dishes (100 x 15-

mm; VWR International, Radnor, PA) and incubated under fluorescent light. After approximately 18 to 24-h, a single germinated conidium was transferred to V8 agar (Dhingra and Sinclair 1985), (modified to be more acidic by reducing the CaCO3 to 2 g/liter), amended with 25 mg/liter rifamycin. After 4-5 days, a single colony of *P. nodorum* was transferred to a new plate of V8 media and cultured under fluorescent light at room temperature (approximately 23°C) for 7 to 10 days. Once colonies were sporulating, they were preserved on silica at 20°C (Tuite 1969). Additionally, some isolates were preserved on agar plugs in 15% glycerol and stored in a -80 freezer.

In 2019, some wheat leaves were dried in a plant press for two weeks, then stored in paper envelopes for approximately 3-8 months. Lesions on the dried leaves were cut into approximately ½ cm squares including a mix of necrotic and non-diseased tissue. Squares of tissue were disinfested with a one min soak in a 10% NaOCl solution then rinsed twice in sterile water for two min each. Pieces of wheat leaves were dried between sterile paper towels for approximately 15 min. After the squares of tissue were dried, they were placed on selective media adapted from Manandhar and Cunfer (1991) by amending Fries minimal media (30 g/liter sucrose, 5 g/liter NH_4 tartrate, 1 g/liter NH_4N0_3, 1 g/liter KH_2PO_4, 0.48 g/liter $MgSO_4.7H_2O$, 1 g/liter NaCl and 884 µl of a 1 M $CaCl.2H_2O$ solution) with 5 mg/ml chloroneb (Sigma-Aldrich, St. Louis, MO), 5 mg/ml cupric hydroxide (Beantown Chemical, Hudson, NH), 5 mg/ml dicloran (Sigma-Aldrich, St. Louis, MO), 3.13 mg/ml chloramphenicol (Gold Biotechnology, Inc., St. Louis, MO), 3.13 mg/ml erythromycin (Gold Biotechnology, Inc., St. Louis, MO), 12.5 mg/ml tetracycline hydrochloride (Gold Biotechnology, Inc., St. Louis, MO), and 10 mg/ml neomycin sulfate (Gold Biotechnology, Inc., St. Louis, MO). Colonies of *P. nodorum*

were fluorescent under ultraviolent light when grown on Fries minimal media. The fungicides and antibiotics were added to select against other microbes. Wheat leaves on Fries minimal media amended with the selective agents were kept under ultraviolent light (black light) at 25°C for 7 to 10 days. Petri dishes (100 x 15 mm) containing the wheat leaves on Fries minimal media were checked daily for fluorescence. Fragments of agar containing hyphae from fluorescent colonies were transferred to V8 media amended with 50 mg/liter ampicillin (Gold Biotechnology, Inc., St. Louis, MO). From these cultures, single-spored isolates were prepared and stored as previously described on silica at -80°C.

One hundred and twenty-three isolates of *P. nodorum* were collected from six Kentucky counties during 2018-2020. In 2018 isolates of *P. nodorum* were only collected from Caldwell County. The number of isolates in 2020 was reduced compared to previous years due to the lower observed levels of Stagonospora nodorum leaf blotch. Sixty isolates collected from seven Illinois counties that were part of a collection made by the Bradley lab in 2014 at the University of Illinois were included in this study. Six isolates from Arkansas that were collected in 1995 by Dr. Eugen Milus (University of Arkansas, Fayetteville, AR) and provided by Dr. Christina Cowger (United States Department of Agriculture – Agricultural Research Service, Raleigh, NC) were also included. These isolates collected in 1995 were used as "baseline" isolates, since QoI fungicides would not have been registered for use on wheat at that time. These isolates also were part of a previous study published by Crook et al. (2012).

DNA extraction and amplification of a cytochrome b gene sequence

DNA was extracted from mycelia harvested from 7 to 10-day old cultures of *P. nodorum* grown on V8 media. FastDNA spin kits (MP Biomedicals, Irvine, CA) were used to extract DNA from mycelia, following the manufacturer's instructions. Five microliters of RNase A (Thermo Fisher Scientific, Waltham, MA) were added per 100 µl of genomic DNA and incubated at 37°C for 30 min then for 10 min at 55°C. Polymerase chain reaction (PCR) primers developed by Blixt et al. (2009) were used to amplify a 515 base pair section of the cytochrome b gene that spanned a region including codons 129, 137, and 143. Twenty-five µl reactions were carried out with one µl of template, 0.2 µM of forward and reverse primers, 12.5 µl of GoTaq Green PCR master mix (Promega Corporation., Madison, WI) and 10.5 µl of nuclease free water (Promega Corporation., Madison, WI). The thermocycler (T100; Bio-Rad Laboratories, Hercules, CA) was set according to the protocol reported by Blixt et al. (2009), with the only modification being that 35 cycles were run instead of 30 cycles. For every PCR experiment, a previously confirmed sample was used as the positive control and a reaction lacking template DNA served as the negative control. Products were visualized on a 1% agarose gel (Avantor Performance Materials, Center Valley, PA) with a UVP MultiDoc-it Digital Imaging System (Analytikjena, Jena, Germany) to confirm amplification.

Sequencing and alignment

Polymerase chain reaction products were purified by using Zymo Research DNA Clean and Concentrator Kits (Irvine, CA) and sent to Eurofins Scientific (Louisville, KY) for sequencing. Geneious R6 bioinformatics software (Geneious Biologics, Auckland, New Zealand) was used to process, translate, and align sequencing results. To confirm

the identity of the PCR products and to detect the presence of the F129L, G137R, or G143A mutations that confer QoI resistance, a sequence of a wild type (QoI-sensitive) isolate from Gen Bank (ID: 5522516; National Center for Biotechnology Information, Bethesda, MD) that lacked these three mutations was included in the alignment. Isolates were categorized as likely to be QoI fungicide-resistant, or sensitive to QoI fungicides, based on the presence or absence of one or more of these mutations previously reported to confer resistance to this class (F129L, G137R and G143A).

Determining concentration of SHAM for conidial germination assay

Eight isolates of *P. nodorum* (18Sn106, 19Sn010, 19Sn018, 19Sn024, 19Sn788, 19Sn815, 19Sn827, and 19Sn829) determined as likely to be sensitive to QoI fungicides by sequencing their cytochrome b gene, were randomly chosen by using the RAND function in Microsoft Excel 2016 (Microsoft Corp., Redmond, WA). These isolates were used to test different concentrations of SHAM (Alfa Aesar, Haverhill, MA) to determine if it affected conidial germination of *P. nodorum*. SHAM was dissolved in methanol and added to PDA after the media had cooled to approximately 50°C after autoclaving, for a final concentration of 40, 60, 80, 100 and 120 parts per million (ppm). A control lacking SHAM, to which an equivalent amount of ethanol had been added, was included. Cultures were incubated for 12, 24 and 36 h in the dark at 24°C. At each time point, two samples of 50 conidia each were examined with a compound microscope at 100× magnification. PDA plates were examined for conidia starting in the middle and moving toward one edge of the plate in a serpentine pattern. The number of conidia that had germinated out of the first 50 observed was recorded, and this was repeated for the other half of the plate. A conidium was considered to be germinated if the total length of its

germ tube was longer than the conidium. The trial was conducted once. The data from the period that showed the greatest differentiation between the different concentrations of SHAM was selected for analysis.

For analysis of conidial germination at different concentrations of SHAM the trial was considered a random effect. The effects of SHAM, isolate, and interaction between the two factors were considered fixed and evaluated using the PROC GLM function in SAS software (version 9.4; SAS INC., Cary, NC).

Testing for alternative respiration

The eight isolates used previously plus 19Sn827 were used to test for alternative respiration. All of these isolates were determined as likely to be QoI-sensitive by sequencing of their cytochrome b genes. Conidial germination assays were conducted on PDA amended with technical grade azoxystrobin (Syngenta Crop Protection, Greensboro, NC) and SHAM in Petri dishes (60 x 15-mm, VWR International, Radnor, PA). After the media was autoclaved, the azoxystrobin dissolved in acetone was added for a final concentration of 0, 0.001, 0.01, 0.1, 1 and 10 ppm. Three sets of azoxystrobin-amended PDA were made, each with 0, 60, or 100 ppm SHAM. The trial was conducted twice on different days. Plates were incubated at 24°C for 24 h in the dark, and conidia were counted as previously described. The effective concentration that inhibited 50% of conidial germination relative to a non-amended control (EC50) was calculated for each isolate of *P. nodorum* using linear spline interpolation in SAS software (version 9.4; SAS INC., Cary, NC) (Zhang et al. 2021).

The effect that SHAM had on EC50 values was analyzed by using the GLIMMIX procedure of SAS software (version 9.4; SAS INC., Cary, NC). Trials were considered random effects, while isolate, SHAM, and their interaction were treated as fixed. Least Square means (lsmeans) of the EC50 values were calculated and compared at ($\alpha = 0.05$) by using the Lines statement. Comparison of lsmeans only occurred if the F test was significant ($P \leq 0.05$).

Establishing EC50 values for resistant and sensitive isolates

In vitro plate assays were used to confirm that *P. nodorum* isolates with the G143A mutation were resistant to QoI fungicides. Five isolates of *P. nodorum* from Kentucky (20Sn254, 19Sn847, 19Sn832, 19Sn824, and 18Sn096) with the G143A mutation and five without (20Sn248, 19Sn826, 18Sn070, 19Sn848, and 19Sn841) were randomly selected using the RAND function in Microsoft Excel 2016. Conidial germination assays were used to compare EC50 values between isolates that had the G143A mutation and those that did not. The trial was conducted twice, with three replications for each isolate. Replications consisted of six PDA plates, each amended with one of the six previously described concentrations of azoxystrobin and 60 ppm SHAM. From each replication. EC50 values were calculated by using the method previously mentioned.

Analysis of the affect that isolate had on EC50 values for azoxystrobin analysis was conducted by using the GLIMMIX function of SAS software (version 9.4; SAS INC., Cary, NC). Trials were listed as random effects, and isolate was considered a fixed effect. Least Square means (lsmeans) of the EC50 values were calculated and compared

at ($\alpha = 0.05$) by using the Lines statement. Comparison of lsmeans only occurred if the F test was significant ($P \leq 0.05$).

Determining the discriminatory dose of azoxystrobin

Discriminatory dose assays are useful to quickly screen isolates for fungicide resistance without conducting a full set of *in vitro* plate assays (Russell 2004). To determine the discriminatory dose of azoxystrobin, 15 QoI fungicide-resistant and 21 QoI fungicide-sensitive isolates of *P. nodorum* were screened using conidial germination assays as previously described. The only difference was that replication of isolates within the two trials was not included. The percent inhibition of conidial germination at the different concentrations of azoxystrobin was graphed in Microsoft Excel 2016 (Microsoft Corp., Redmond, WA) to visualize the dose that would differentiate between QoI fungicide-resistant and -sensitive isolates.

Greenhouse trials

Experimental design and plant material

Greenhouse trials were conducted to investigate the ability of QoI fungicides to control leaf blotch caused by isolates of *P. nodorum* with the G143A mutation. Trials consisted of a QoI fungicide treatment and a nontreated control. The treatment and control groups were evaluated by inoculating plants with two QoI fungicide-resistant or two -sensitive isolates of *P. nodorum*. The trial used a completely randomized design and was conducted twice. The treatment consisted of a QoI fungicide and an untreated control. Each treatment was tested with four different inocula. The treatment x inoculum was replicated three times in the first trial and six times in the second. ‘Pembroke 2016’ (Van Sanford et al. 2018) soft red winter wheat was planted in 13-cm round plastic pots,

Pro-Mix BX Mycorrhizae general purpose potting soil (Quakertown, PA) was used without the addition of fertilizer. Plants were watered every two to three days and thinned to four per pot. Greenhouse conditions were between 20°C and 27.6°C, with 16 h of light. Plants in the first trial were four weeks old when the experiment began. The plants were trimmed to fit inside humidity chambers made from 3-liter plastic bottles. In the second trial, 2-week-old plants were used to avoid trimming.

Inoculum preparation

Twenty isolates of *P. nodorum*, 10 with the G143A mutation and 10 without, were randomly selected by using the RAND function in Microsoft Excel 2016. From among the 20 isolates, two isolates with the G143A mutation (18Sn074 and 19Sn824) and two isolates without (19Sn015 and 19SN018) were chosen based on which had the most visible sporulation. Isolates were cultured for 10 days on V8 media amended with 25 mg/liter rifamycin under constant light before they were used to make inoculum according to the method reported by Kim et al. (2004).

Fungicide treatment and inoculation

Pyraclostrobin fungicide (Headline SC; BASF Corp., Research triangle Park, NC) was selected for the QoI fungicide treatment and was applied at a rate of 0.16 kg a.i./ha with a carrier volume of 140 liters/ha. Applications were made with a Generation iii Research Sprayer (DeVries Manufacturing Inc., Hollandale, MN) using a TeeJet 8002EvS (Wheaton, IL) to all plants except the nontreated control. After the fungicide was applied, plants were allowed to dry approximately 24 h before they were inoculated with a suspension containing 4.5×10^5 conidia/ml prepared from the previously

mentioned isolates of *P. nodorum*. The inoculum was applied by using a hand trigger spray bottle to coat the surfaces of the wheat leaves till runoff. Humidity chambers constructed from 3-liter clear plastic bottles were placed on the plants after inoculation and removed after a week in the first trial, and after four days in the second trial. Plants were checked daily and misted to sustain humidity, plants were misted when condensation was not covering a majority of the humidity chamber, or daily after the humidity chamber was removed.

Disease ratings and statistical analysis

Plants were evaluated for Stagonospora leaf blotch severity 10 days after inoculation. One leaf from each of the four plants in a pot was rated for severity by visually estimating the area covered by lesions. The leaf that was visually representative of incidence and severity across the whole plant. The mean severity was determined for each replicated treatment and the relative control was calculated using the average severity of the untreated control according to the following equation.

$$\text{Relativ control} = \left(1 - \left(\frac{\text{mean severity of treatment}}{\text{mean severity of nontreated control}}\right)\right) \times 100$$

Analysis of the % relative control provided by the QoI fungicide application in the greenhouse trials for individual isolates was conducted by using the GLIMMIX function of SAS software (version 9.4; SAS INC., Cary, NC). Trial was considered a random effect, and isolate was fixed. Least Square means (lsmeans) of the % relative control was calculated and compared at (α = 0.05) by using the Lines statement. Comparison of lsmeans only occurred if the *F* test was significant ($P \leq 0.05$).

Results

Amplification, sequencing, and alignment of a segment of the cytochrome b gene

Polymerase chain reaction was used to amplify a segment of the cytochrome b gene that spanned codons 129, 137 and 143 for all 183 isolates collected from Kentucky and Illinois, along with the baseline isolates from Arkansas. Consensus sequences were compiled from forward and reverse sequences for all isolates except for Sn1452, Sn1418, 20Sn253, 19Sn010, Sn11426, Sn1449, 19Sn813, 19Sn831, 19Sn844 and 18Sn835. For these isolates, either the forward or reverse sequence was used. Sequences used in this study were at the Q50 quality level and ranged from 315 to 511 base pairs in length with the region of interest being a minimum of 78 nucleotides from the end.

Aligning PCR products and corresponding amino acid sequences revealed that some isolates had guanine replaced by cytosine, producing a predicted shift from glycine to alanine at position 143 in the cytochrome b gene (Fig. 1.1). This was the only alteration observed (Fig. 1.1). The sequences of the isolates shown in figure 1.1 were uploaded to GenBank. Three of these isolates had the G143A mutation (19Sn847, 18Sn104, and 18Sn090). The 3 other isolates (19Sn841, 18Sn107,19Sn788) and the reference sequence representative of the six pre-QoI isolates form Arkansas (20Sn444-20Sn449) did not have the G143A mutation (accession No. OK504518, OK504514, OK504513, OK504517, OK504515, OK504516, OK504519, OK504520, OK504521, OK504522, OK504523, and OK504524 respectively).

In total, 57 isolates out of the 123 from Kentucky, and three out of the 60 from Illinois, had the G143A mutation. The baseline isolates from Arkansas had no mutations in the amplified segment of their cytochrome b gene. *Parastagonospora nodorum* isolates

with the G143A mutation were detected in all Kentucky counties sampled, but not in every year they were sampled. For Illinois *P. nodorum* isolates with the G143A mutation were detected in only one of the seven Illinois counties surveyed. The percentage of isolates with the G143A mutation from each county and year sampled ranged from 0 to 100%, and 0 to 30%, for Kentucky and Illinois respectively (Table 1.1). Fulton County in 2020 and Todd County had the highest rate at 100% (Table 1.1). When data from counties with isolates from multiple years were combined and counties with only one isolate were excluded, the percentage of Kentucky isolates with the G143A mutation by county ranged from 20% to 75%. The overall percentage of the *P. nodorum* isolates examined with the G143A mutation was 46.3%, and 5%, for Kentucky and Illinois, respectively.

Effect of SHAM on conidial germination and alternative respiration

The effect of SHAM on conidial germination was tested with eight QoI fungicide-sensitive isolates using concentrations ranging from 40 to 120 ppm, in increments of 20. SHAM did not have a statistically significant effect on conidial germination ($P = 0.6758$). Conidial germination of *P. nodorum* isolates on SHAM-amended media ranged from 76.6 to 83.64%, while the control had 83.8% germination (Table 1.2). Since no inhibition of conidial germination was observed, 60 and 100 ppm SHAM were selected for use in trials to evaluate if alternative respiration in *P. nodorum* was potentially present. Concentrations of SHAM below 60 ppm were not selected for further evaluation to help ensure that alternative respiration was inhibited. One hundred parts per million of SHAM was selected to provide an enough of an increase in SHAM that a difference in EC50 could be seen if inhibition of alternative respiration increased with concentration.

EC50 values for azoxystrobin were calculated for nine isolates without the G143A mutation at 0, 60 and 100 ppm SHAM. The effect of SHAM and isolate were both significant (*P* = <0.0001), the interaction between the two was significant (*P* = 0.0036). For all but two isolates (18Sn080 and 19Sn010) no significant difference in EC50 values were observed between no SHAM or SHAM at 60 or 100 ppm (Table 1.3). For the isolates 18Sn080 and 19Sn010, EC50 values were significantly greater when SHAM was not included compared to when SHAM was included at either 60 or 100 ppm. Based on these results, 60 ppm SHAM was used for conidial germination assays for subsequent trials.

Determining EC50 values for isolates with and without the G143A mutation

To verify that the G143A mutation conferred QoI resistance to *P. nodorum,* conidial germination assays were conducted for five isolates with the mutation (20Sn254, 19Sn847, 19Sn832, 19Sn824, and 18Sn096) and five without (20Sn248, 19Sn826, 18Sn070, 19Sn848, and 19Sn841). EC50 values were significantly affected by isolate (*P* = <0.0001). The average EC50 value for *P. nodorum* isolates with the G143A mutation was ≥10 ppm azoxystrobin (Table 1.4). Isolates with the G143A mutation had significantly greater EC50 values than isolates without the G143A mutation, which had EC50 values that ranged from 0.053 to 0.172 ppm (Table 1.4).

Establishing a discriminatory dose assay to identify QoI fungicide-resistant isolates

Inhibition of conidial germination was graphed for the concentrations of azoxystrobin previously described. This was done to visually show the optimal dose that provided differential inhibition of conidial germination between QoI fungicide-resistant

and -sensitive isolates of *P. nodorum*. Azoxystrobin at 10 ppm inhibited greater than 98% of conidial germination for sensitive isolates but, at the same concentration less than 20% inhibition of conidial germination was observed for resistant isolates (Fig. 1.2). Azoxystrobin at one ppm inhibited conidial germination of sensitive isolates by more than 99%, while inhibition of germination of resistant isolates was less than 16% (Fig. 1.2). Based on these results, one ppm of azoxystrobin was selected as the discriminatory dose to distinguish between QoI fungicide-resistant and -sensitive isolates of *P. nodorum*.

Greenhouse trial

A greenhouse trial was conducted to assess the efficacy of the QoI fungicide, pyraclostrobin, for controlling Stagonospora leaf blotch, caused by *P. nodorum* isolates with and without the G143A mutation. The main effect of isolates was significant for relative control of leaf blotch ($P = \leq 0.0001$). Pyraclostrobin fungicide provided significantly less control of leaf blotch caused by *P. nodorum* isolates with the G143A mutation compared with leaf blotch caused by isolates without the G13A mutation (Table 1.5).

Discussion

The objective of this study was to survey Kentucky and Illinois wheat fields for QoI fungicide-resistant isolates of *P. nodorum*. Additionally, QoI-resistant isolates were characterized to determine the mechanism of resistance and how management of Stagonospora nodorum leaf blotch is affected. This is the first report of *P. nodorum* isolates from Kentucky and Illinois with the G143A mutation. The isolates collected from

Illinois in 2014, are the oldest known North American isolates of *P. nodorum* with resistance to QoI fungicides.

Sequencing of the cytochrome b gene of *P. nodorum* isolates revealed that the G143A mutation was present in isolates from Kentucky and Illinois. The G143A mutation was the only mutation in the segment of the cytochrome b gene that was amplified. This is consistent with the findings of Blixt et al. (2009) and Kaur et al. (2021), in which isolates of *P. nodorum* with the G143A mutation were resistant to QoI fungicides, and the F129L and G137R mutations were absent.

The occurrence of *P. nodorum* isolates with the G143A mutation in Kentucky and Illinois varied within each county surveyed (0-100% and 0-30% for Kentucky and Illinois, respectively). This is consistent with the previous two reports that documented variation in the frequency of the G143A mutation by location, 50% to 98% in Sweden and 5% to 32% in Virginia (Blixt et al. 2009; Kaur and Mehl 2021). The G143A mutation occurred more frequently in isolates from Kentucky than those from Illinois. The disparity in the frequency of the G143A mutation between *P. nodorum* isolates from Kentucky and Illinois is likely due to the four-year period between isolate collection in the two states. During this period, it is likely that more QoI-resistant *P. nodorum* isolates were selected due to additional years of exposure to QoI fungicides, which is a pattern that is well documented in other fungal plant pathogens (Beyer et al. 2011; Genet et al. 2006; Wise et al. 2009). Additionally, selection pressure can vary between sample locations due to differences in environment and management practices (Leadbeater et al. 2019). While specific management practices and environmental conditions are not known for sample locations, these factors may influence the frequency of the G143A mutation.

Alternative respiration occurs in some plant pathogens and affects EC50 values unless an inhibitor like SHAM is used (Amand et al. 2003; Bradley and Pedersen 2011; Miguez et al. 2004). The inclusion of SHAM reduced the EC50 values for two isolates of *P. nodorum*. These results indicate that alternative respiration is present in *P. nodorum* isolates, and that SHAM should be used in conidial germination assays.

Conidial germination assays confirmed that the G143A mutation conferred resistance to QoI fungicides. The EC50 values for all the *P. nodorum* isolates with the G143A mutation were significantly greater than the EC50 values of isolates without the G143A mutation. Plant pathogens with the G143A mutation usually have a resistance factor ≥ 100 (Brent and Hollomon 2007b). In our research, specific resistance factors could not be calculated since exact EC50 values were not obtained for isolates with the G143A mutation, but they were in the expected range. The previous reports of QoI-resistant isolates of *P. nodorum* (Blixt et al. 2009; Kaur et al. 2021) reported higher EC50 values for azoxystrobin than those found in this study. This is likely due to the use of mycelial growth assays instead of conidial germination assays to calculate EC50 values. In general, azoxystrobin and other QoI fungicides have a greater inhibitory effect on conidial germination than mycelial growth (Bartlett et al. 2002). Additionally, the EC50 values reported by Blixt et al. (2009) were calculated without the use of an alternative respiration inhibitor, such as SHAM, which can affect EC50 values.

Results of research conducted in the greenhouse demonstrated that QoI fungicides were less effective at controlling Stagonospora leaf blotch caused by *P. nodorum* isolates with the G143A mutation. Based on our results, field efficacy of QoI fungicides would be poor in managing Stagonospora leaf blotch if large numbers of QoI-resistant isolates are

present within a field. Frequencies of the G143A mutation as low as one precent can lead to a significant level of resistance within a few applications of a QoI fungicide (Brent and Hollomon 2007a). To avoid yield loss, wheat farmers may need to utilize other management practices, such as the use of moderately resistant cultivars, crop rotation and other forms of inoculum management (Bergstrom 2010). If a foliar fungicide application is needed for Stagonospora leaf blotch management, farmers should apply fungicide products that contain active ingredients from multiple chemistry classes or tank-mix fungicides from different chemistry classes (Leadbeater et al. 2019). Succinate dehydrogenase inhibitors (SDHI) and demethylation inhibitors (DMI) fungicide (FRAC Group 7 and 11 respectively) are labeled for use on wheat and are effective at controlling Stagonospora leaf blotch (Crop Protection Network 2021b). Use of QoI fungicides in combination with either of these modes of action could lead to increased selective pressure and a higher risk of resistance. The FRAC considers SDHI and DMI fungicides to have a medium to high and medium risk of selecting fungal isolates with resistance, respectively (Anonymous 2021). Application of single ingredient SDHI or DMI products could lead to selection of *P. nodorum* isolates with resistance to these chemistry classes (Mueller et al. 2017). Isolates of *P. nodorum* from Europe and China have already been documented as being resistant to DMI fungicides (Pereira et al. 2017a). Currently, isolates of *P. nodorum* from North America have not been found that are resistant to DMI or SDHI fungicides. However, since QoI fungicides are ineffective for managing Stagonospora nodorum leaf blotch caused by isolates with the G143A mutation, practices should be put in place that protect the fungicides that remain effective. These practices

should include efforts to monitor *P. nodorum* populations for resistance to other fungicide classes, such as SDHIs and DMIs.

Table 1.1 Number of *Parastagonospora nodorum* isolates collected by state and county and percentage of isolates with the G143A mutation, which causes resistance to quinone outside inhibitor (QoI) fungicides.

State	County	Year	No. of isolates	Isolate with G143A mutation (%)
Kentucky	Caldwell	2018	47	68.1
Kentucky	Caldwell	2019	17	47.1
Kentucky	Fayette	2019	14	64.3
Kentucky	Fulton	2019	4	0.0
Kentucky	Logan	2019	4	75.0
Kentucky	Fayette	2020	8	12.5
Kentucky	Fulton	2020	1	100.0
Kentucky	Todd	2020	1	100.0
Kentucky	Woodford	2020	27	7.4
Illinois	Clay	2014	4	0.0
Illinois	Effingham	2014	10	0.0
Illinois	Gallatin	2014	8	0.0
Illinois	Pike	2014	1	0.0
Illinois	Pope	2014	10	30.0
Illinois	Wayne	2014	7	0.0
Illinois	White	2014	20	0.0
Arkansas [z]	Unknown	1995	6	0.0

[z] Arkansas isolates are considered "baseline" isolates since they were collected prior to QoI fungicides being registered for use on wheat in the U.S.

Table 1.2 Mean germination rate of conidia from QoI sensitive *Parastagonospora nodorum* isolates at different concentrations of salicylhydroxamic acid (SHAM).

SHAM concentration (ppm)	Germination [z] (%)
0	83.7 A
40	83.6 A
60	79.1 A
80	76.6 A
100	78.8 A
120	77.6 A

[z] Mean germination values followed by the same letter are not significantly different from each other (alpha = 0.05).

Table 1.3 Effective concentration of azoxystrobin that inhibited 50 % of conidial germination relative to a non-amended control (EC50) for quinone outside inhibitor (QoI) fungicide-sensitive isolates of *Parastagonospora nodorum* at different concentrations of salicylhydroxamic acid (SHAM).

Isolate	SHAM concentration (ppm)	EC50 [z] (ppm)
18Sn080	0	0.088 A
18Sn080	60	0.042 B
18Sn080	100	0.034 BC
18Sn106	0	0.034 BC
18Sn106	60	0.029 BC
18Sn106	100	0.030 BC
19Sn010	0	0.080 A
19Sn010	60	0.038 B
19Sn010	100	0.028 BC
19Sn015	0	0.030 BC
19Sn015	60	0.014 C
19Sn015	100	0.027 C
19Sn018	0	0.044 B
19Sn018	60	0.029 BC
19Sn018	100	0.028 BC
19Sn024	0	0.044 B
19Sn024	60	0.035 B
19Sn024	100	0.029 BC
19Sn788	0	0.039 B
19Sn788	60	0.030 BC
19Sn788	100	0.036 B
19Sn815	0	0.039 B
19Sn815	60	0.032 BC
19Sn815	100	0.029 BC
19Sn829	0	0.029 BC
19Sn829	60	0.027 BC
19Sn829	100	0.039 B

[z] EC50 values followed by the same letter are not significantly different from each other (alpha = 0.05 level).

Table 1.4 Effective concentration of azoxystrobin that inhibits 50% of conidial germination relative to a non-amended control (EC50) for five *Parastagonospora nodorum* isolates resistant to quinone outside inhibitor (QoI) fungicides and five isolates of *P. nodorum* sensitive to QoI fungicide in the presence of 60 ppm SHAM.

Isolate	G143A mutation	Mean EC50 [z] (ppm)
18Sn096	Present	>10 A
19Sn824	Present	>10 A
19Sn832	Present	>10 A
19Sn847	Present	>10 A
20Sn254	Present	>10 A
20Sn248	Absent	0.172 B
18Sn070	Absent	0.097 C
19Sn826	Absent	0.110 C
19Sn841	Absent	0.053 D
19Sn848	Absent	0.065 D

[z] EC50 values followed by the same letter are not significantly different from each other (alpha = 0.05 level).

Table 1.5 Relative control of leaf blotch caused by isolates of *Parastagonospora nodorum* with and without the G143A mutation when treated with the quinone outside inhibitor fungicide, pyraclostrobin, in a greenhouse trial.

Isolate	G143A mutation	Relative control of leaf blotch [z] (%)
19Sn015	Absent	62 A
19Sn018	Absent	52 A
18Sn074	Present	2 B
19Sn824	Present	0 B

[z] Values followed by the same letter are not significantly different from each other (alpha = 0.5 level)

	Isolate	Accession No.	129 ↓ 137 ↓ 143 ↓
	Reference	OK504519-OK50424	MATAFLGYVLPYGQMSLW**G**ATVITNLM
QoI-sensitive	19Sn841	OK504517	. . . . **G** . . . .
	18Sn107	OK504515	. . . . **G** . . . .
	19Sn788	OK5045416	. . . . **G** . . . .
			GGT
QoI-resistant	19Sn847	OK504518	. . . . **A** . . . .
	18Sn104	OK504514	. . . . **A** . . . .
	18Sn090	OK504513	. . . . **A** . . . .
			GCT

Figure 1.1 Alignment of a section of the *Parastagonospora nodorum* cytochrome b amino acid sequence corresponding to codons 129, 137 and 143, wherein mutations may occur that confer resistance to quinone outside inhibitor fungicides. QoI fungicide-resistant *P. nodorum* isolates have a shift from glycine (G) to alanine (A) at codon 143 (G143A mutation. The reference sequence represents the six isolates from Arkansas that were collected in 1995, prior to the use of QoI fungicides in wheat. Representative additional sequences from resistant and sensitive isolates are included for comparison.

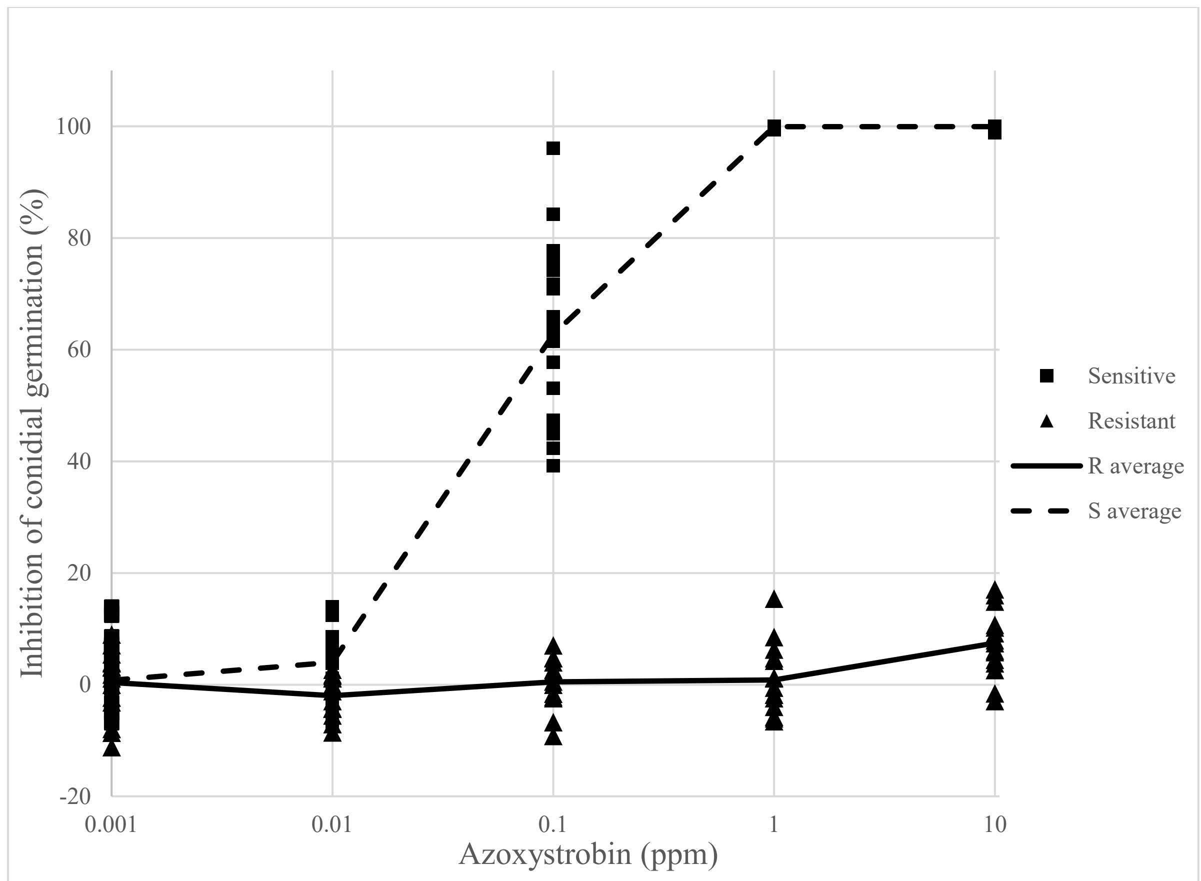

Figure 1.2 Inhibition of conidial germination at different concentrations of azoxystrobin for *Parastagonospora nodorum* isolates resistant and sensitive to quinone outside inhibitor (QoI) fungicides.

CHAPTER 2. IDENTIFICATION OF QUINONE OUTSIDE INHIBITOR FUNGICIDE-RESISTANT ISOLATES OF *ZYMOSEPTORIA TRITICI* FROM KENTUCKY

Abstract

Winter wheat (*Triticum aestivum*) production in Kentucky often utilizes no-till farming, a practice that can be favorable for fungal plant pathogens that overwinter in crop residue. One such pathogen, *Zymoseptoria tritici,* is the causal agent of Septoria tritici blotch and a prominent pathogen of wheat in Kentucky. The limitations of inoculum management and lack of fully resistant wheat cultivars increase the importance of foliar fungicide applications as a tool for management of Septoria tritici leaf blotch. Quinone outside inhibitor (QoI) fungicides (Fungicide Resistance Action Committee, FRAC, Group 11) are one of the main classes of foliar fungicides used in wheat. Active ingredients in the QoI fungicide class act on a single target in the fungal cytochrome b, which increases the risk of fungal pathogens developing resistance. A single mutation in the fungal cytochrome b gene can confer resistance to this class of fungicides. Isolates of *Z. tritici* resistant to QoI fungicides have been documented in Europe, Africa, and North America. In these cases, QoI fungicide-resistant *Z. tritici* isolates had a mutation in the cytochrome b gene at codon 143 that shifted glycine to alanine (known as the G143A mutation) in the amino acid sequence. In this current study, 79 *Z. tritici* isolates collected from eleven Kentucky counties during 2020 were screened for QoI resistance. Polymerase chain reaction was used to amplify a section of the cytochrome b gene spanning a section in which mutations that can cause QoI resistance are known to occur. Sequencing the amplified section of the cytochrome b gene revealed that 19 out of the 79 isolates tested (24.1%) had the G143A mutation. *In vitro* assays confirmed that isolates with the G143A mutation were resistant to QoI-fungicides. This study is the first to

identify isolates of *Z. tritici* with the G143A mutation in Kentucky. Wheat producers in Kentucky and the surrounding areas should implement management practices that include rotating to non-host crops, planting wheat cultivars with moderate resistance to Septoria tritici blotch, and applying fungicides that contain active ingredients from other FRAC groups.

Introduction

Septoria tritici blotch is a common foliar disease of winter wheat (*Triticum aestivum*) in Kentucky. The causal agent of Septoria tritici blotch, *Zymoseptoria tritici* (syn. *Mycosphaerella graminicola* and *Septoria tritici*) has been a documented pathogen of wheat since the 19th century but only became a major threat to wheat production in the last 60 years (Eyal 1987). The implementation of no-till and conservation tillage, along with the use of cultivars susceptible to *Z. tritici*, facilitated Septoria tritici blotch becoming one of the most consequential pathogens in the world (Dean et al. 2012; Eyal 1987).

Leaf symptoms caused by *Zymoseptoria tritici* often occur along with symptoms caused by *Parastagonospora nodorum*. These symptoms are similar and are collectively referred to as the Septoria leaf blotch complex (Eyal 1987). Together, these diseases can cause up to 50% yield reduction (Eyal 1981; Eyal 1987). Part of what makes *Z. tritici* hard to manage is that its life cycle that includes both a sexual and an asexual stage. Wind-borne ascospores facilitate the spread of *Z. tritici* over long distances, and water-borne conidia allow rapid increase of this pathogen within wheat fields (Eyal 1987; Ponomarenko et al. 2011).

The level of resistance of individual wheat cultivars is a major determining factor in the severity and incidence of disease (Gladders et al. 2001). Host resistance is a useful tool for managing Septoria tritici leaf blotch (Eyal 1981; Ponomarenko et al. 2011). However, qualitative host resistance to *Z. tritici* can be quickly overcome (Cowger et al. 2000). The highly adaptable nature of this pathogen conferred by its polycyclic life cycle that includes a sexual and an asexual stage facilitates the breakdown of host resistance and reduced sensitivity to fungicides (Dean et al. 2012; McDonald and Linde 2002). The lack of durable highly resistant cultivars, and limitations in inoculum management, has resulted in frequent fungicide use in high-risk environments (Dean et al. 2012; Torriani et al. 2015).

The introduction of quinone outside inhibitor (QoI) fungicides (Fungicide Resistance Action Committee, FRAC, Group 11) provided a novel mode of action that controlled a wide range of fungal pathogens (Bartlett et al. 2002). *Blumeria graminis*, causal agent of powdery mildew on wheat, was the first plant pathogen reported with resistance to QoI fungicides (Bartlett et al. 2002; Sierotzki et al. 2000). Isolates of *B. graminis* from Germany had guanine replaced by cytosine at codon 143 of the cytochrome b gene (known as the G143A mutation), shifting glycine to alanine in the amino acid sequence, which conferred resistance to QoI fungicides (Sierotzki et al. 2000). Two other mutations in the fungal cytochrome b gene have been associated with QoI resistance in other fungi, which are the F129L and G137R mutations where shifts from phenylalanine to leucine and from glycine to arginine occur, respectively (Kim et al. 2003; Sierotzki et al. 2007).

The first QoI-resistant isolates of *Z. tritici* were found in the United Kingdom in 2001 and 2002. These isolates had the G143A mutation (Fraaije 2003, 2004). Since then, isolates of *Z. tritici* resistant to QoI fungicides have been found across Europe, spreading from several different areas where isolates with the G143A mutation arose independently (Amand et al. 2003; Beyer et al. 2011; Drabešová et al. 2013; Siah et al. 2010; Torriani et al. 2009). Shortly after the discovery of QoI-resistant *Z. tritici* isolates in Europe, they were also found in Africa and North America (Allioui et al. 2016; Estep et al. 2013; Siah et al. 2014; Taher et al. 2014). North American isolates of *Z. tritici* with the G143A mutation were first detected in Oregon in 2012 (Estep et al. 2013). Population genetics in Oregon and Europe and field trials in Europe have shown that the G143A mutation has spread via windborne ascospores, and independent selection (Allioui et al. 2016; Estep et al. 2015; Fraaije et al. 2005; Siah et al. 2014; Torriani et al. 2009).

Since the discovery of the *Z. tritici* isolates from Oregon with the G143A mutation, no additional findings of QoI resistant *Z. tritici* have been reported in North America. The goal of this study was to survey Kentucky wheat fields for QoI-resistant isolates of *Z. tritici* and if found, determine the mutation responsible for resistance in Kentucky isolates.

Material and methods

Collection of *Z. tritici* Isolates

Winter wheat leaves displaying symptoms of Septoria tritici leaf blotch were collected from Kentucky wheat fields during May and June 2020. Wheat leaves were examined under a dissecting scope for pycnidia exuding cirrhi. If conidiation was not

occurring, leaves were placed in a humidity chamber for 24 to 48 h to induce the production of conidia. Cirrhi from several pycnidia were lifted from the surface of a wheat leaf with a sterile needle and suspended in 20 µl of sterile water. The conidial suspension was then spread onto potato dextrose agar (PDA) (BD bioscience, San Jose, CA) amended with 25 mg/liter rifamycin (MP Biomedicals., Irvine, CA) in 100 x 15-mm plastic Petri dishes (VWR International, Radnor, PA). Cultures were incubated under fluorescent light in a 12 h light/dark cycle. After approximately 24 h at room temperature (approximately 23°C) a single germinated conidium was transferred to V8 agar. V8 agar was made according to Dhingra and Sinclair (1985) but modified to be more acidic by reducing the CaCO3 to 2 g/liter and amended with 25 mg/liter rifamycin. After four to seven days, conidia from a culture of *Z. tritici* that originated from a single conidium were spread on V8 agar amended with 25 mg/liter rifamycin and grown for 10 days at room temperature under 12 h light/dark cycle of fluorescent light. Conidia from 10-day old cultures were stored on silica at -4°C (Tuite 1969).

Isolates of *Z. tritici* were collected from 34 wheat fields across nine counties in western Kentucky and from two counties in the north central part of the state. From the 11 counties surveyed, 221 isolates were collected in total. The RAND function in Microsoft Excel 2016 (Microsoft Corp., Redmond, WA) was used to select up to three isolates from each field, for a total of 79 isolates of *Z. tritici* that were used in this study.

DNA isolation and amplification of a cytochrome b gene sequence

Mycelia was harvested from 10-day old cultures of *Z. tritici* grown on PDA. DNA was extracted from the mycelia using FastDNA spin kits (MP Biomedicals, Irvine, CA) according to the manufacturer's instructions. RNase A (Thermo Fisher Scientific,

Waltham, MA) (5 µl/100 µl genomic DNA) was added and incubated at 37°C for 30 min then for 10 min at 55°C. Polymerase chain reaction (PCR) primers developed by Torriani et al. (2009) were used to amplify a 652-base section of the cytochrome b gene that spanned codons 129, 137 and 143 where mutations known to confer QoI resistance can occur. Reactions (25 µl) were conducted with 1 µl of template DNA, 0.05 µM of forward and reverse primers, 12.5 µl of GoTaq Green PCR master mix (Promega Corporation, Madison, WI), and 11.5 µl sterile water. The thermocycler (T100; Bio-Rad Laboratories, Hercules, CA) was set at 96°C for two min then 35 cycles of 96°C for 30 s, 58°C for 30 s, 72°C for 1min and a final five min extension at 72°C. For every PCR run, a negative control lacking template DNA was included. After the first successful reaction, a positive control consisting of DNA that had previously been amplified successfully was included. Polymerase chain reaction products were visualized on 1% agarose (Avantor performance materials, Center Valley, PA) gel using the UVP MultiDoc-it Digital Imaging System (Analytikjena, Jena, Germany). Polymerase chain reaction products were purified by using Zymo Research Clean and Concentrate kits (Irvine, CA) according to the manufacturer's instructions.

Sequencing and alignment

Purified PCR products were sent to Eurofins Scientific (Louisville, KY) for sequencing. Geneious R6 bioinformatics software (Geneious Biologics, Auckland, New Zealand) was used to align and translate PCR products. To confirm the identity of the PCR products and determine if the F129L, G137R, or G143A mutations were present, a *Z. tritici* cytochrome b sequence from a wildtype (QoI-sensitive) isolate from GenBank was included (ID: MH699754.1; National Center for Biotechnology Information,

Bethesda, MD). The presence or absence of mutations known to confer QoI-resistance was used to categorize isolates as either likely sensitive or resistant to QoI fungicides.

Finding the appropriate concentration of salicylhydroxamic acid (SHAM) for *in vitro* assays

Previous studies that utilized SHAM to inhibit alternative respiration in *Z. tritici* when conducting *in vitro* assays used concentrations ranging from 1 to 100 ppm. (Amand et al. 2003; Drabešová et al. 2013; Estep et al. 2015). In this study, 60 ppm of SHAM (Alfa Aesar, Haverhill, MA) was used since it was within the range described, and has successfully been used to limit alternative respiration in *Cercospora sojina* (Zhang et al. 2012).

Four isolates of *Z. tritici* (20St2039, 20St069, 20St133, and 20St177) without the G143A mutation were selected to test the ability of 60 ppm SHAM to inhibit alternative respiration if it was present. Technical grade azoxystrobin (Syngenta Crop Protection, Greensboro, NC) was dissolved in acetone and added to PDA after it cooled to approximately 50°C. Final azoxystrobin concentrations of 0, 0.001, 0.01, 0.1, 1, and 10-ppm were made and poured into Petri dishes (60 x 15-mm, VWR International, Radnor, PA). Each trial consisted of two sets of azoxystrobin amended media, in which SHAM was dissolved in methanol for one set at a final concentration of 60 ppm. The other set had an equivalent amount of methanol added and served as the 0 ppm SHAM control. The trial was repeated twice, with two replications of each isolate included. Two samples of 50 conidia were observed per repetition at 100x magnification. Conidia were counted by starting in the center of the plate and moving toward one edge of the Petri dish in a serpentine pattern. The number of germinated conidia was recorded, and then the process

was repeated on the other half of the plate. A conidium was considered germinated when the length of its germ tube was longer than the conidium. Germination data were used to calculate percent inhibition at each concentration of azoxystrobin tested. From this, the effective concentration that inhibited 50% of conidial germination relative to a nonamended control (EC50) was calculated for each isolate of *Z. tritici* using SAS software (version 9.4; SAS Institute Inc., Cary, NC) to perform linear interpolation as described by Zhang et al (2021).

The effect that SHAM had on EC50 values was evaluated by using the PROC GLIMMIX function in SAS software. Trials were considered random, and isolates and the interaction between the two factors were considered fixed effects. Least squares means (lsmeans) of the EC50 values were compared by using the lines statement (α = 0.05). Lsmeans comparisons were only made if the *F*-test was significant ($P \leq 0.05$).

Determining EC50 values for isolates with and without the G143A mutation

Four isolates of *Z. tritici* with the G143A mutation (20St118, 20St247, 20St153, 20St090) and four without (20St039, 20St078, 20St207, 20St159) were selected for use in conidial germination assays to confirm that this mutation confers resistance to QoI fungicides *in vitro*. Azoxystrobin and SHAM were added to PDA as previously described and poured in 60 x 15-mm Petri dishes. The trial was conducted three times with three replications per isolate. The number of germinated conidia were recorded and EC50 values were calculated as previously described. The EC50 values above or below the range of azoxystrobin tested were listed as the maximal or minimal concentration evaluated.

EC50 values for QoI resistant and sensitive isolates were evaluated using the PROC GLIMMIX function in SAS software. Trials were considered random, and isolates were treated as fixed effects. Lsmeans of the EC50 values were compared using the lines statement ($\alpha = 0.05$). Lsmeans comparisons were only made if the F-test was significant ($P \leq 0.05$).

Results

Amplification, sequencing, and alignment of the cytochrome b gene

The 79 isolates in this study were screened for QoI-resistance by amplifying and sequencing a section of their cytochrome b gene that spanned codons 129, 137, and 143. Consensus sequences used for analysis were assembled from the forward and reverse strands for all isolates except for 20St099, 20St228, 20St205, 20St151, 20St128, and 20St218. For these isolates either the forward or reverse sequence was used. The sequences used for alignment were at the Q50 quality level and ranged from 644 to 520 base pairs in length, and the area of interest was a minimum of 182 bases from the end.

Alignment of the sequenced PCR products revealed that 19 out of 79 (24.1%) of the Kentucky *Z. tritici* isolates examined had the G143A mutation. No other mutations were present in the segment of the cytochrome b gene examined (Fig. 2.1). The sequences of the six isolates shown in Fig. 2.1 were uploaded to GenBank. Three of these isolates had the G143A mutation (20St201, 20St153 and 20St077), while the other three isolates did not (20St033, 20St178 and 20St123) (accession No. OK020134, OK020136, OK020137, OK020139, OK020135 and OK020138 respectively). The percentage of isolates collected from western Kentucky with the G143A mutation ranged from 11.1%

for Warren County to 50% for Christian County (Table 2.1). The G143A mutation was not detected in any of the isolates collected from the two counties surveyed in the north central part of the state.

In vitro plate assay

The effect that SHAM had on EC50 values was determined by calculating EC50 values with and without SHAM. EC50 values of azoxystrobin were significantly affected by isolate (*P* = <0.0001), SHAM (*P* = 0.0004), and the interaction between the two (*P* = <0.0001). The EC50 values for two (20St039, and 20St177) of the four isolates of *Z. tritici* were significantly reduced by the addition of 60 ppm SHAM (Table 2.2).

Isolates of *Z. tritici* with the G143A mutation were confirmed to be resistant to QoI fungicides by calculating EC50 values of four isolates with the G143A mutation (20St118, 20St247, 20St090 and 20St153) and four without (20St039, 20St078, 20St207, and 20St159). The main effect of isolate had a significant effect on EC50 values (*P*= <0.0001). Isolates with the G143A mutation had significantly greater average EC50 values than those without (Table 2.3). Isolates with the G143A mutation had EC50 values ranging from 2.581 to ≥6.595 ppm, while isolates without the G143A mutation had EC50 values ranging from ≤0.008 to 0.150 ppm (Table 2.3).

Discussion

The goal of this study was to collect isolates of *Z. tritici* from Kentucky and test them for QoI resistance. QoI-resistant isolates of *Z. tritici* were detected by sequencing a segment of the cytochrome b gene, and *in vitro* plate assays were used to further

characterize the QoI-resistant isolates. This study is the first to identify Kentucky isolates of *Z. tritici* with the G143A mutation.

The G143A mutation was present in multiple isolates of *Z. tritici* and was the only mutation in the segment of the cytochrome b gene sequenced. This is consistent with previous reports, which found that QoI-resistant isolates of *Z. tritici* had the G143A mutation but not the F129L or G137R mutations (Drabešová et al. 2013; Estep et al. 2013; Fraaije 2004; Kildea et al. 2010; Siah et al. 2010; Siah et al. 2014; Taher et al. 2014).

The frequency of *Z. tritici* isolates with the G143A mutation in Kentucky varied by county ranging from 0% to 50%. This is consistent with previous reports, where the frequency of *Z. tritici* isolates varied by location, from 0% to 100% in the Czech Republic (Drabešová et al. 2013), 12% to 88 % in the United Kingdom (Fraaije 2004), 0% to 70% in France (Siah et al. 2010), 0% to 66% in Morocco (Siah et al. 2014), and 9.7% to 83.4% in Oregon (Estep et al. 2013). The G143A mutation was only detected in isolates of *Z. tritici* from western Kentucky, which may be due to the smaller number of isolates collected from counties in the north central part of the state. Increasing the sample size may have resulted in finding the G143A mutation in these two counties. Alternatively, differences in environment and management practices may provide inadequate selection pressure to elevate the G143A mutation to a detectable level in this region (Brent and Hollomon 2007b; Leadbeater et al. 2019).

The inclusion of SHAM decreased the EC50 values for two of the *Z. tritici* isolates, indicating that alternative respiration may be active, and that SHAM should be used in conidial germination assays. This is consistent with previous studies that

demonstrated alternative respiration affecting *Z. tritici* sensitivity to QoI fungicides (Amand et al. 2003; Miguez et al. 2004; Ziogas et al. 1997).

Isolates of *Z. tritici* with G143A mutations were resistant to QoI fungicides, with EC50 values ranging from 2.581 to greater than 6.595 ppm of azoxystrobin. Previous studies reported a much larger range of EC50 values, from <10 to >100 ppm azoxystrobin (Allioui et al. 2016; Amand et al. 2003; Cheval et al. 2017; Siah et al. 2010; Siah et al. 2014). Similarly, some studies reported EC50 values for sensitive isolates significantly greater than what was observed in this study (Amand et al. 2003; Siah et al. 2010). The higher EC50 values reported in previous studies may be due to the use of mycelia inhibition assays instead of conidial germination assays to determine EC50 values. Azoxystrobin and other QoI fungicides generally inhibit conidial germination to a greater extent than mycelial growth (Bartlett et al. 2002).

Resistance factors of ≥100 are typically associated with fungal plant pathogens that have the G143A mutation (Brent and Hollomon 2007a). The resistant factors for isolates of *Z. tritici* with the G143A were in the resistant range but <100. Wheat producers in Kentucky and the surrounding area should use other management practices including rotating to non-host crops, and planting cultivars with moderate resistance to Septoria leaf blotch (Shaner 2010.). If a foliar fungicide is needed to control Septoria tritici blotch, products with multiple modes of action or tank mixes of fungicides from different chemistry classes should be used (Mueller et al. 2017). Alternative fungicide classes to QoI fungicides that are labeled for wheat include succinate dehydrogenase inhibitors (SDHI) and demethylation inhibitors (DMI) (FRAC Group 7 and 11, respectively) (Crop Protection Network 2021b). Isolates of *Z. tritici* resistant to DMI and

SDHI fungicides have been reported in Europe, and DMI resistant isolates have been reported in the United States (Dooley et al. 2016; Estep et al. 2015; Leroux et al. 2007). While SDHI and DMI resistance have not been reported for *Z. tritici* isolates in Kentucky, management practices should be put in place to reduce the risk of resistance developing. This should include efforts to monitor *Z. tritici* for resistance to SDHI and DMI fungicides.

Table 2.1 The number of *Zymoseptoria tritici* isolates examined by county and the percentage of isolates that have the G143A mutation, which causes resistance to quinone outside inhibitor (QoI) fungicides**.**

County	Region of Kentucky	Year	No. of isolates	Isolates with the G143A mutation (%)
Caldwell	Western	2020	9	44.4
Christian	Western	2020	8	50.0
Fayette	North central	2020	2	0.0
Fulton	Western	2020	9	22.2
Graves	Western	2020	9	22.2
Logan	Western	2020	8	25.0
Simpson	Western	2020	7	28.6
Todd	Western	2020	8	12.5
Trigg	Western	2020	8	12.5
Warren	Western	2020	9	11.1
Woodford	North central	2020	2	0.0

Table 2.2 Effective concentration of azoxystrobin that inhibits 50% of conidial germination relative to a non-amended control (EC50) for quinone outside inhibitor (QoI) fungicide-sensitive isolates of *Z. tritici* with and without salicylhydroxamic acid (SHAM).

Isolate	**SHAM concentration (ppm)**	**EC50 [z] (ppm)**
20St039	60	0.049 C
20St039	0	0.192 A
20St177	60	0.032 C
20St177	0	0.133 B
20St133	60	0.017 C
20St133	0	0.020 C
20St069	60	0.007 C
20St069	0	0.023 C

[z] Values followed by the same letter are not significantly different from each other (alpha = 0.05 level).

Table 2.3 Effective concentration that inhibits 50% of conidial germination relative to a non-amended control (EC50) for four Isolates of *Zymoseptoria tritici* resistant to quinone outside inhibitor (QoI) and four *Zymoseptoria tritici* sensitive to QoI fungicides in the presence of 60 ppm SHAM.

Isolate	G143A mutation	Mean EC50 [z] (ppm)
20St118	Present	> 6.595 A
20St247	Present	> 4.572 B
20St090	Present	3.238 C
20St153	Present	2.581 C
20St039	Absent	0.150 D
20St078	Absent	0.033 D
20St207	Absent	0.013 D
20St159	Absent	< 0.008 D

[z] Values followed by the same letter are not significantly different from each other (alpha = 0.05 level).

	Isolate	Accession No.	129 ↓ 137 ↓ 143 ↓
QoI-sensitive	20St033	OK020139	MATAFLGYVLPYGQMSLW**G**ATVITNL
	20St178	OK020135	. . . . **G** . . . . . .
	20St123	OK020138	. . . . **G** . . . . . .
			GGT
QoI-Resistant	20St153	OK020136	MATAFLGYVLPYGQMSLW**A**ATVITNL
	20St201	OK020134	. . . . **A** . . . . . .
	20St077	OK020137	. . . . **A** . . . . . .
			GCT

Figure 2.1 Alignment of a section of the *Zymoseptoria tritici* cytochrome b amino acid sequence corresponding to codons 129, 137 and 143, where mutations may occur that confer resistance to quinone outside inhibitor fungicides. QoI fungicide-resistant isolates have a shift from glycine (G) to Alanine (A) at codon 143 (G143A mutation) of the cytochrome b of QoI sensitive and resistant isolates of *Zymoseptoria tritici* and the DNA sequence at codon 143 where a mutation occurred.

CHAPTER 3. EFFECT OF FUNGICIDE APPLICATION TIMING ON LEAF BLOTCH DISEASE MANAGEMENT ON WINTER WHEAT CULTIVARS DIFFERING IN SUSCEPTIBILITY

Abstract

Efficient winter wheat (*Triticum aestivum*) production requires intensive disease management to prevent a reduction in yield and grain quality. Disease management programs should start with inoculum management, including rotating with a non-host crop and tillage if it is region appropriate, then planting a resistant wheat cultivar. In addition to these practices, fungicides are frequently applied to manage foliar and head diseases of wheat caused by fungal pathogens. Results of several research trials have shown that a fungicide application at early anthesis (Feekes 10.51) is the optimal application timing for best management of Fusarium head blight (FHB) and the associated mycotoxin, deoxynivalenol (DON), that can contaminate grain. The optimal application time for management of foliar diseases has not been as clear. The efficacy of early (before the flag leaf is visible at Feekes 8) vs late (Feekes 8 and later) foliar fungicide applications has differed among studies. For a fungicide to provide optimal protection, it should be applied at or just prior to disease onset. The time of disease onset varies with the pathogen and environment, making it difficult to determine the optimal time to apply a foliar fungicide. Since onset of foliar diseases can vary considerably among environments, the most reliable recommendations on foliar fungicide applications will account for regional differences. Two of the main foliar pathogens of wheat in Kentucky are *Zymoseptoria tritici* and *Parastagonospora nodorum*. Both produce similar leaf blotches and are collectively referred to as the Septoria leaf blotch complex (SLBC). In this study, field trials were conducted to evaluate the management of the SLBC using

different fungicide application timings and two winter wheat cultivars that differed in susceptibility to this complex. Fungicides were applied at Feekes 6 (jointing stage), Feekes 9 (flag leaf ligule and collar visible), or 10.51, and in different combinations. The largest difference in severity of the SLBC was between the resistant and susceptible cultivars, while fungicide timing did not significantly affect foliar disease severity. When only a Feekes 6 fungicide application was made, it resulted in lower test weights and yields compared to other fungicide application timings. The greatest yields and test weights were achieved when a Feekes 6 or 9 application was followed by a Feekes 10.51 application.

Introduction

The profitability of winter wheat (*Triticum aestivum*) grown in Kentucky relies on protecting yield and grain quality by implementing an integrated disease management strategy. Proper disease management plans combine the use of resistant cultivars, appropriate planting dates, inoculum management, and fungicide applications (Lee et al. 2009). Fungicides are a beneficial management tool when used properly. However, they bring along a significant cost that must be offset by increased yield to be economically beneficial (Cook et al. 1999). Fungicide applications made after the initiation of infection are not optimal and may provide little or no benefit (Cook et al. 1991; Cook et al. 1999). For the greatest protection, fungicide applications should be made prior to the onset of infection (Cook et al. 1991; Cook et al. 1999).

The best fungicide application time for disease management in winter wheat varies by disease and has recently become a topic of interest due to the necessity of

maximizing control of Fusarium head blight (FHB) (Cook et al. 1991). Refining application timing has proven to be a successful strategy for increasing fungicide efficacy for managing FHB and deoxynivalenol (DON) (Yoshida et al. 2012; D'Angelo et al. 2014; Freije and Wise 2015; Paul et al. 2019; Bolanos-Carriel et al. 2020). Results of field trials have demonstrated that applying a suitable fungicide at early anthesis (Feekes 10.51) and up to six days after provided improved protection from FHB than applications made earlier or later (Yoshida et al. 2012; D'Angelo et al. 2014; Freije and Wise 2015; Paul et al. 2019; Bolanos-Carriel et al. 2020).

Determining the optimum application time for a foliar fungicide is challenging, since initiation of infection occurs at different growth stages depending on the pathogen and environmental conditions (Cook et al. 1991; Ransom and McMullen 2008; Wegulo et al. 2009; Sylvester et al. 2018). Disease lesions on the flag leaf reduces photosynthetic potential and has been demonstrated to be highly corelated to yield loss (Lupton 1972; Gooding et al. 2000; Bhathal et al. 2003; Sanchez-Bragado et al. 2016). Traditionally, fungicides used to manage foliar diseases were applied from Feekes 9 to Feekes 10.5 (Milus 1994). Fungicides applied at these growth stages were intended to protect the flag leaf and thus yield (Cook et al. 1999; Gooding et al. 2000; Dimmock and Gooding 2002). Before the flag leaf has fully emerged, there is little photosynthetic capacity directed towards grain fill (Lupton 1972; Sanchez-Bragado et al. 2016). Thus, flag leaf emergence may be the earliest time to see direct benefit in yield from a foliar fungicide application. The difference in efficacy of fungicide applications made at flag leaf emergence and anthesis has been demonstrated to be minimal (Guy et al. 1989; Cook et al. 1991;

Cromey et al. 2004; Wegulo et al. 2011a; Willyerd et al. 2015; Sylvester and Kleczewski 2018; Sylvester et al. 2018).

Disease on the top three leaves has been negatively correlated with yield and protecting these leaves is a well-documented strategy for improving grain production (Thomas et al. 1989; Cook et al. 1999; Gooding et al. 2000; Bhathal et al. 2003; Cromey et al. 2004). However, as Sylvester et al. (2018) noted, there is a trend towards earlier fungicide applications with few research studies evaluating this practice. Early fungicide applications occur at pseudo stem erection (Feekes 4/5), tillering (Feekes 2) and even in the fall after winter wheat is planted (Ransom and McMullen 2008; Weisz et al. 2011; Turkington et al. 2016; Sylvester and Kleczewski 2018; Sylvester et al. 2018). Early fungicide applications are aimed at reducing disease pressure on the flag leaf by targeting residue-borne pathogens and disrupting polycycle disease cycles (Cook et al. 1999; Friskop et al. 2018; Sylvester and Kleczewski 2018). Another reason for early fungicide applications is reducing application cost by combining them with an herbicide application already taking place (Willyerd et al. 2015).

The efficacy and benefit of early (before Feekes 8) and late (Feekes 8 and later) foliar fungicide applications vary between studies. Often, studies report that results are affected by environment, pathogen, disease pressure, and host resistance (Cook et al. 1999; Ransom and McMullen 2008; Wegulo et al. 2009; Wegulo et al. 2011a; Weisz et al. 2011; Lopez et al. 2015). Fungicide applications made at or after flag leaf emergence were more likely to provide better yield and disease control than those made before this growth stage (Cook et al. 1991; Ransom and McMullen 2008; Wegulo et al. 2011a; Sylvester and Kleczewski 2018). When an early fungicide application was beneficial,

Septoria leaf blotch was present, caused by *Zymoseptoria tritici* a residue-borne polycyclic pathogen (Marroni et al. 2006). However, Cook et al. (1991) and Thomas et al. (1989) did not find an early fungicide application beneficial in managing *Z. tritici*. Split applications combining an early and late fungicide treatment were effective but not always advantageous (Milus 1994; Willyerd et al. 2015; Sylvester and Kleczewski 2018; Cook et al. 1991; Wegulo et al. 2009; Sylvester et al. 2018)

The optimal time to apply a foliar fungicide varies with location due to the presence and prominence of different pathogens and variability of disease onset. In Kentucky, the Septoria leaf blotch complex (SLBC), caused by *Z. tritici* and *Parastagonospora nodorum*, is a prominent foliar disease complex of wheat. The goal of this study was to evaluate different foliar fungicide application timings and provide region-specific recommendations. Trials were conducted in Kentucky to evaluate different fungicide application timings and combinations for their effects on SLBC management, test weight, and yield of winter wheat.

Material and methods

Field trials were conducted at two locations (Caldwell and Logan Counties, KY) each year in 2020 and 2021. The trials in Caldwell County were in different fields each year at the University of Kentucky Research and Education Center in Princeton, KY. The trials in Logan County were located in a farmer's fields that were different each year.

In all locations, winter wheat was planted following corn harvest (*Zea mays*) at a seeding rate of 3.7 million seeds per hectare. In Caldwell County, plots were seven rows wide on 17.8 cm spacing and 6.1 m long. Plots were later trimmed to between 3.6 and 4.7

m. Plots in Logan County were six rows wide on 17.8 cm spacing and 4.6 m in length, and then trimmed to 3.7 m in length. Agronomic practices, including nitrogen applications, herbicide applications, soil type, planting dates, and harvest dates are summarized in Table 3.1. Different fungicide application timings were evaluated on cultivars 'Pembroke 2016' (Van Sanford et al. 2018), and 'AgriMaxx 496' (AgriMaxx Wheat Company, Mascoutah, IL). Pembroke 2016 is moderately susceptible to Septoria leaf blotch, while AgriMaxx 496 is moderately resistant to several common fungal foliar diseases including Septoria leaf blotch and Stagonospora nodorum leaf blotch. Plots were arranged as a 2 × 7 factorial (cultivar × fungicide) in a randomized complete block design with four replications (blocks) within each location and year (with location and year combined to represent a unique environment).

To evaluate the efficacy and profitability of fungicides, applications were made at three individual timings (Feekes 6, 9, and 10.51) and combinations of timings (Feekes 6 + 10.51 or Feekes 9 + 10.51). Fungicide treatments and application dates are shown in Table 3.2. A non-treated control was also included. For the Feekes 6 and 9 applications, Tilt fungicide (Syngenta Crop Protection, Greensboro, NC) was applied at 292 ml/ha (0.12 kg a.i./ha propiconazole). For the Feekes 10.51 application, Miravis Ace (Syngenta Crop Protection) was applied at 1,001 ml/ha (0.15 kg a.i./ha pydiflumetofen + 0.13 kg a.i./ha propiconazole). All fungicides were applied with a carrier volume of 187.1 liters/ha, with a nonionic surfactant (Induce; Helena Chemical Company, Collierville, TN) added at 0.125% v/v. Fungicide applications were made by using a CO_2-powered backpack sprayer with four TwinJet-60 8002 nozzles (TeeJet Technologies, Wheaton, IL) spaced 50.8 cm apart.

Foliar disease severity evaluations were conducted using the double-digit rating scale described by Eyal et al. (1987). The only modification made was rating disease severity in increments of 5% instead of 10%. To accommodate this the third digit of the disease rating was shown in the tenths place. The numbers in the tenths and ones place represented % disease severity and the value in the hundreds place represented the vertical progression of the disease on the plant on a 1 to 10 scale. Disease ratings were collected from five places in each plot. Ratings were taken from the middle rows of each plot and were spaced approximately an equal distance from each other. Ratings focused only on foliar diseases caused by fungal pathogens, ignoring viral and bacterial diseases. Disease ratings began two weeks after each plot received its first fungicide application in 2020, and the week of the first application in 2021. Nontreated control plots were rated on the same schedule as those receiving the first fungicide application except for the first rating in 2021 when only the nontreated control plots were rated. Plots were rated every two weeks after the first evaluation until Feekes 10.51, where they were then evaluated weekly until around the soft dough growth stage (approximately Feekes 11.2). An area under disease progress curve (AUDPC) was calculated for each plot according to the following formula, where y_i is disease severity and t_i is the day of observation (Shaner 1977).

$$AUDPC = \sum_{i=0}^{n}\left(\frac{y_i + y_{i+1}}{2}\right)(t_{i+1} - t_i) \qquad \text{(Equation 1)}$$

FHB ratings were collected three to five weeks after Feekes10.51 at approximately the soft dough growth stage (Feekes 11.2). Fifty wheat heads were evaluated per plot in total by evaluating ten wheat heads at five different locations within each plot. FB disease ratings were taken from the middle rows of each plat and were

spaced approximately equal distance from each other. FHB severity and incidence was rated according to Stack and McMullen (2011), where % symptomatic area of each wheat head was estimated and FHB index was calculated, based on the following formula.

$$\text{FHB index} = \frac{(\text{severity})(\text{incidence})}{100} \quad \text{(Equation 2)}$$

Plots in Caldwell County were harvested with a Kincaid 8-XP small plot combine (Kincaid Equipment Manufacturing Company, Haven, KS) with a grain gauge (HarvestMaster H2; Juniper Systems, Logan, UT) used to measure grain weight, grain moisture concentration, and test weight. Logan County trials were harvested with a Wintersteiger Classic combine (Salt Lake City, UT). In 2020, whole plot grain samples were collected, and sample weight, test weight and grain moisture were measured with a grain analysis computer (GAC 2500-UGMA; Dickey-John Corp., Auburn, IL). In 2021 a combine-mounted Harvest Master Grain Gauge was used to measure harvested grain weight, test weight, and moisture concentration. Grain weights were adjusted to 13.5% standard moisture, then yields were calculated by dividing the adjusted harvested grain weight by plot area. Grain samples were sent to Hartwick College Center for Craft Food and Beverages (Oneota, New York) where DON levels were measured by using enzyme linked immunosorbent assays (ELISA).

Data were subjected to analysis with the GLIMMIX procedure in SAS software (version 9.4; SAS Institute Inc., Cary, NC). The model included the fixed effects of fungicide and cultivar and their interaction, and the random effects of environment and replications nested within environment. Least squares means (lsmeans) were compared

using the lines statement in SAS software, where $\alpha = 0.05$. Lsmean comparisons were made only if the F-test was significant ($P \leq 0.05$).

Results

Effect of wheat cultivar and fungicide timing on AUDPC

Foliar disease ratings collected throughout the season were used to calculate AUDPC. Cultivar had a significant effect on AUDPC ($P = <0.0001$) (Table 3.3). Neither fungicide application timing nor the interaction between fungicide timing and cultivar were significant ($P = 0.0769$ and $P = 0.9603$, respectively) (Table 3.3). The moderately susceptible wheat cultivar Pembroke 2016 had a significantly greater AUDPC than the moderately resistant cultivar AgriMaxx 496 (Table 3.4).

Effect of wheat cultivar and fungicide timing on test weight

Fungicide and cultivar both had a significant effect on test weight ($P = 0.0001$ and $P = <0.0001$, respectively) (Table 3.3). There was no significant interaction between cultivar and fungicide timing ($P = 0.2741$) for test weight (Table 3.3). The test weight of Pembroke 2016 grain was greater than that of AgriMaxx 496 grain (Table 3.4). The lowest test weight was from the nontreated control, which was not significantly different from the test weight of grain treated with a foliar fungicide at Feekes 6 or Feekes 6 + 9 (Table 3.5). The greatest test weight was from plots treated with a foliar fungicide at Feekes 6 followed by an application at Feekes 10.51. This was not significantly different from plots treated with a foliar fungicide at Feekes 9 alone, or at Feekes 9 followed by an application at Feekes 10.51 (Table 3.5).

Effect of wheat cultivar and fungicide timing on yield

Yield of wheat was affected by cultivar (*P* = < 0.0001) and fungicide application timing (*P* = 0.0373) but not by the interaction between the two (*P* = 0.8084) (Table 3.3). AgriMaxx 496 wheat had a significantly greater yield than Pembroke 2016 (Table 3.4). The lowest yield occurred in the plots treated with a foliar fungicide at Feekes 6. This was not significantly different from the yield of nontreated control plots (Table 3.5). Yield of the nontreated control plots was only significantly different from plots that received a fungicide application at Feekes 6 or Feekes 9, each followed by an application at Feekes 10.51 (Table 3.5). The greatest yield occurred for plots treated with a foliar fungicide at Feekes 9 followed by an application at Feekes 10.51. This was not significantly different from plots treated with a foliar fungicide at Feekes 9, 6 + 9, or Feekes 6 followed by an application at Feekes 10.51 (Table 3.5).

Effect of wheat cultivar and fungicide application on FHB index and DON

Fusarium head blight index was not affected by wheat cultivar (*P* = 0.5930), but was affected by fungicide application timing (*P* = <0.0001) (Table 3.3). There was no significant interaction between cultivar and fungicide timing (*P* = 0.6202) for FHB index (Table 3.3). The FHB index was the greatest in nontreated control plots but was not significantly different from plots treated with a single fungicide application at either Feekes 6 or 9 (Table 3.5). Plots treated with a foliar fungicide at Feekes 9 followed by an application at Feekes 10.51 had the lowest FHB index (Table 3.5). This was not significantly different from the FHB index of plots treated with a foliar fungicide at Feekes 6 followed by a Feekes 10.51 application, or a Feekes 10.51 application by itself (Table 3.5).

Deoxynivalenol concentration was not affected by cultivar (*P* = 0.5345) but was affected by fungicide application timing (*P*= <0.0001) (Table 3.3). Additionally, there was not a significant interaction between fungicide timing and cultivar (*P* = 0.7796) (Table 3.3). Deoxynivalenol concentration was the greatest in harvested grain from the nontreated control plots at 1.38 ppm. The DON concentration of harvested grain from plots that received a fungicide application at Feekes 6, or 6 + 9 was not significantly different from the nontreated control (Table 3.5). Grain harvested from plots that received a Feekes 10.51 fungicide application had significantly lower DON concentration than plots that did not receive an application at Feekes 10.51 (Table 3.5).

Discussion

Fungicide applications on winter wheat at Feekes 6, 9, and 10.51 were evaluated individually and in combination in this trial. The different foliar fungicide application timings were evaluated in combination with a fungicide application for FHB. Fungicide treatments were evaluated on a wheat cultivar moderately resistant, and one susceptible to, the SLBC. The objective of this trial was to provide recommendations on foliar fungicide use specific to Kentucky and the surrounding area.

Progression and severity of the SLBC throughout the growing season was represented using AUDPC. The use of a wheat cultivar with moderate resistance to SLBC resulted in lower AUDPC compared with the moderately susceptible cultivar. Planting cultivars with resistance to the SLBC is a general disease management practice that is foundational to any disease management plan (Eyal et al. 1987; Bergstrom 2010; Shaner 2010.).

Fungicide application timing did not have a significant effect on AUDPC. In both years, there was significant foliar damage caused by factors other than fungal pathogens. In 2020 there was freeze damage in both locations, and in 2021 the Caldwell County location had a high level of bacterial leaf spot. An effort was made to prevent these factors from affecting foliar disease ratings, but it is probable that they led to an over estimation of foliar disease, which may have affected the significance of fungicide timings on AUDPC.

The test weight of grain was greater for Pembroke 2016 than AgriMaxx 496. Increased disease levels can affect test weight (Sylvester and Kleczewski 2018). However, test weight is determined by both genetic potential and environmental conditions (Yabwalo et al. 2018), thus cultivar difference may explain the difference in test weight.

Plots that received a Feekes 10.51 fungicide application had improved test weights over the nontreated control, which is consistent with several previous studies (Ransom and McMullen 2008; Fernandez et al. 2014; Sylvester and Kleczewski 2018; Paul et al. 2019). In this study, the impact of foliar fungicide applications on test weight was dependent on application timing. This is contrary to two studies that reported test weight increased with a fungicide application regardless of the application timing (Milus 1994; Sylvester and Kleczewski 2018). Treating plots with a fungicide at Feekes 6 or 9 along with an application at Feekes 10.51 resulted in greater test weights than a Feekes 10.51 application alone. This is different from previous studies that concluded test weight was not enhanced by combining a Feekes 10.51 fungicide application with an earlier one (Fernandez et al. 2014; Sylvester and Kleczewski 2018). However, Sylvester and

Kleczewski (2018) found that combining an early fungicide application with a Feekes 10.51 fungicide application did not significantly improve test weight over a single Feekes 9 treatment, which is consistent with our findings.

In 2020, freeze damage occurred at both locations and likely reduced overall yields, especially in Logan County where the yield was lower than expected. In 2021, yields in Logan County were low, likely due to a problem that occurred with the nitrogen application. Pembroke 2016 had significantly lower yield than AgriMaxx 496. This is consistent with the greater disease levels recorded from Pembroke 2016 but could also be due to a difference in the ability of the two cultivars to endure freezing or nitrogen stress.

The ability of a fungicide to improve yield was dependent on application timing. This is different from several studies that reported an increase in yield for plots treated with a fungicide, regardless of timing (Cromey et al. 2004; Wegulo et al. 2011a; Sylvester and Kleczewski 2018; Sylvester et al. 2018). Our findings agreed with several previous reports, that a lone foliar fungicide application at Feekes 6 provided no benefit in yield (Cook et al. 1991; Sylvester et al. 2018; Wegulo et al. 2011a; Willyerd et al. 2015). The other individual application timings provided better yield than Feekes 6, but were not better than the nontreated control. Only plots that received a fungicide application at Feekes 6 or 9 and an application at Feekes 10.51 had significantly greater yield than the control. This is different from previous studies that found a single application of a foliar fungicide, or an Feekes 10.51 application for FHB could improve yield (Cook et al. 1999; Cromey et al. 2004; Marroni et al. 2006; Wegulo et al. 2011a; Fernandez et al. 2014; Willyerd et al. 2015; Sylvester et al. 2018). Our findings indicate that a foliar fungicide and a fungicide application at Feekes 10.51 were needed to prevent

yield loss. This is partially supported by Sylvester and Kleczewski (2018), which reported combining a fungicide application at Feekes 5 or 8 with an application at Feekes 10.51 could improve yield but was not significantly different from a lone Feekes 8 treatment.

Low to moderate levels of FHB were present in the first year of the trial, but very little was observed in the second year. Cultivars were not significantly different for FHB index and DON. This is likely due to the cultivars having similar resistance ratings for FHB, and overall low levels of FHB. DON levels in this trial were low overall, remaining below two ppm for the nontreated control. However, a fungicide application at Feekes 10.51 with or without a foliar fungicide application significantly lowered the FHB index and DON levels compared to the nontreated control. This is consistent with the current recommendation for applying a fungicide for managing FHB and DON (Yoshida et al. 2012; D'Angelo et al. 2014; Freije and Wise 2015; Paul et al. 2019; Bolanos-Carriel et al. 2020).

The largest difference in AUDPC for the SLBC was between wheat cultivars and not fungicide treatments. This emphasizes the importance of selecting a wheat cultivar with resistance to prominent foliar pathogens and not relying on fungicide applications. The greatest improvement in test weight and yield occurred for plots treated with a fungicide at Feekes 6 or 9 and a fungicide application at Feekes 10.51 for FHB. When tested individually, the Feekes 9 foliar fungicide application provide better yield and test weight than an application at Feekes 6. Based on the results of this study, a foliar fungicide should be applied at Feekes 9 followed by an FHB application at Feekes 10.51.

However, this recommendation is solely based improvement in yield and test weight and does not consider the economic aspect of foliar fungicide applications.

Table 3.1 Agronomic practices including planting date, harvest date, nitrogen rates, herbicide applications, insecticide applications, and soil type.

	Caldwell 2019/20	**Caldwell 2020/21**	**Logan 2019/20**	**Logan 2020/21**
Planting date	Oct 15th	Oct 13th	Oct 15th	Oct. 15th
Harvest date	June 26th	June 6th	June 15th	June 19th
1st nitrogen application	44.8 kg/ha Feb 27th	44.8 kg/ha Mar. 9th	33.6 kg/ha Feb	33.6 kg/ha Feb
2nd nitrogen application	89.7 kg/ha Apr 1st	89.7 kg/ha Apr. 7th	67.3 kg/ha Mar	67.3 kg/ha Mar
Herbicide and insecticide application	Harmony SG 65.8 ml/ha + Warrior II 109.6 ml/ha + 0.25% NIS v/v on Mar. 23rd	Harmony SG 65.8 ml/ha + Warrior II 109.6 ml/ha + 0.25% NIS v/v on Mar. 3rd	Harmony Extra	Harmony Extra
Soil type	Crider silt loam	Crider silt loam	Pembroke silt loam	Crider silt loam / Pembroke silt loam

Table 3.2 Fungicide application timings and application dates.

			Application date			
Treatment	**Timing**	**Fungicide**	**Caldwell 2020**	**Logan 2020**	**Caldwell 2021**	**Logan 2021**
1	Nontreated	Nontreated	.	.	.	.
2	Feekes 6	Tilt	3-12	3-12	4-2	3-24
3	Feekes 9	Tilt	4-6	4-6	4-15	4-13
4	Feekes 10.51	Miravis Ace	4-27	4-21	5-4	5-2
5	Feekes 6 + 9	Tilt + Tilt	3-12 + 4-6	3-12 + 4-6	4-2 + 4-15	3-24 + 4-13
6	Feekes 6 + 10.51	Tilt + Miravis Ace	3-12 + 4-27	3-12 + 4-21	4-2 + 5-4	3-24 + 5-2
7	Feekes 9 +10.51	Tilt + Miravis Ace	4-6 + 4-27	4-6 + 4-21	4-15 + 5-4	4-13 + 5-2

Table 3.3 Significance of cultivar, fungicide, and their interaction on area under disease progress curve (AUDPC), test weight (TW), yield, Fusarium head blight index, and deoxynivalenol (DON) content.

Effect	AUDPC	Test weight	Yield	FHB index	DON
Cultivar	<0.0001	<0.0001	<0.0001	0.5930	0.5345
Fungicide	0.0769	0.0001	0.0373	<0.0001	<0.0001
Fungicide*cultivar	0.9603	0.2741	0.8084	0.6202	0.7796

Table 3.4 The effect of moderately susceptible and moderately resistant winter wheat cultivars on area under disease progress curve (AUDPC), test weight (TW), and yield.

Cultivar	AUDPC [x]	Test weight [x] (kg/hl)	Yield [x] (kg/ha)
AgriMaxx 496 [y]	2647 B	75.6 B	5339 A
Pembroke 2016 [z]	3128 A	76.5 A	4198 B

[x] Values followed by the same letter are not significantly different from each other (alpha = 0.05 level).

[y] Moderately resistant to the Septoria leaf blotch complex

[z] Moderately susceptible to the Septoria leaf blotch complex

Table 3.5 The effect of fungicide application timing on Septoria leaf blotch complex (SLBC) area under disease progress curve (AUDPC), test weight (TW), yield, Fusarium head blight (FHB) index and deoxynivalenol (DON) concentration.

Timing	Fungicide	AUDPC [z]	TW [z] (kg/hl)	Yield [z] (kg/ha)	FHB index [z] (0-100)	DON [z] (ppm)
Nontreated	Nontreated	2965 A	75.5 E	4546 BC	1.29 A	1.38 A
Feekes 6	Tilt	2901 A	75.6 DE	4496 C	1.15 A	1.23 AB
Feekes 9	Tilt	2875 A	76.2 ABC	4849 AB	1.19 A	1.20 B
Feekes 10.1	Miravis Ace	2928 A	76.2 BCD	4818 AB	0.70 BC	0.89 C
Feekes 6 + 9	Tilt + Tilt	2817 A	76.0 CDE	4835 AB	0.97 AB	1.22 AB
Feekes 6 + 10.51	Tilt + Miravis Ace	2879 A	76.8 A	4907 A	0.45 C	0.76 C
Feekes 9 + 10.51	Tilt + Miravis Ace	2847 A	76.7 AB	4929 A	0.43 C	0.84 C

[z] Values followed by the same letter are not significantly different from each other (alpha = 0.05 level).

CHAPTER 4. EVALUATION OF NOZZLE CONFIGURATION AND SPRAYER SPEED ON CONTROL OF FUSARIUM HEAD BLIGHT AND DEOXYNIVALENOL CONTAMINATION OF HARVESTED GRAIN AND COVERAGE OF WHEAT HEADS

Abstract

Fusarium head blight (FHB) of wheat, (caused primarily by *Fusarium graminearum* in the United States) is the most important disease of wheat in Kentucky and the surrounding region. In addition to reducing grain yields, grain quality also can be reduced by FHB because of lower test weights and contamination of harvested grain by mycotoxins, especially such as deoxynivalenol (DON), that are produced by *F. graminearum*. Although application of a foliar fungicide during anthesis is one of the primary practices used to manage FHB and DON, a major limiting-factor in achieving greater efficacy has been coverage of the wheat head with fungicides. This research evaluated different nozzle configurations and sprayer speed for their effect on coverage of simulated wheat heads and relative control of FHB and DON in field trials conducted in 2017-2019 in Princeton, KY. This research showed that only minor differences in simulated wheat head coverage were observed across nozzle configurations and sprayer speeds. Depending on which nozzle configuration is in use, applicators may need to adjust their sprayer speed to achieve the greatest control of FHB.

Introduction

Fusarium head blight (FHB) of wheat, (caused primarily by *Fusarium graminearum* in the United States) is the most important disease of soft red winter wheat grown in Kentucky and the surrounding region that includes Illinois, Indiana, Missouri, Ohio, and Tennessee. From 2018 to 2020, estimated annual losses caused by FHB in this

region ranged from 27,780 to 72,743 metric tons, resulting in annual economic losses ranging from $5.2 to $13.7 million (Crop Protection Network 2021a). Losses caused by FHB include reductions in grain yield and grain quality. Infected grain may be shriveled, resulting in lower test weights. In addition, *F. graminearum* can produce mycotoxins, such as deoxynivalenol (DON), that can contaminate grain (Dill-Macky 2010). The U.S. Food and Drug Administration (2010) has a 1 ppm advisory level for DON in finished wheat products that may potentially be consumed by humans. Farmers with wheat grain contaminated with high DON levels may receive a discounted price for their wheat by grain handlers or processors, or in some cases, may not be able to sell their grain at all.

FHB and DON can be managed by rotating to non-host crops, planting moderately resistant wheat cultivars, and applying foliar fungicides at anthesis (Bergstrom 2021). Combining moderately resistant cultivars with foliar fungicides has been a practice that has provided the most consistent control of FHB and DON (Wegulo et al. 2011b; Willyerd et al. 2012; Paul et al. 2019). Until the recent registration of Miravis Ace (Syngenta Crop Protection, Greensboro, NC), which contains a demethylation inhibitor (DMI) and a succinate dehydrogenase inhibitor (SDHI) active ingredient, fungicide products that provide the best management have contained only DMI active ingredients (Bolanos-Carriel et al. 2020; Crop Protection Network 2021b; Mesterhazy 2003; Paul et al. 2008; Paul et al. 2010). Although DMI fungicides are known to be xylem-systemic (Mueller et al. 2021), effective translocation of DMI fungicides from wheat leaves to heads was not detected in greenhouse and field trials (Lehoczki-Krsjak et al. 2013; Lehoczki-Krsjak et al. 2015). In addition, redistribution of DMI fungicides within the wheat heads did not occur at a level that would provide

adequate protection against *F. graminearum* infection (Lehoczki-Krsjak et al. 2013). Without translocation or adequate redistribution of these DMI fungicides within wheat heads, coverage is critically important for providing a high level of efficacy against FHB and DON.

Several research studies have focused on improved fungicide coverage of wheat heads by evaluating different nozzles and their orientations. Applying fungicides with nozzles designed to deliver the spray at an angle or rotating nozzles so that sprays are angled, compared to applying fungicides with nozzles that are oriented straight down, may improve fungicide deposition and coverage on a vertical target like a wheat head. Derksen et al. (2012) reported that directing spray 30° forward increased spray coverage of droplets on wheat heads. Halley et al. (2008) reported that nozzles angled to face forward or forward and backward provided greater fungicide deposition on wheat heads than nozzles that were spraying straight down. Parkin et al (2006) found that spray deposition on wheat heads increased by angling spray nozzles backwards. Ozkan et al. (2012) reported that nozzles with double spray patterns that delivered spray at both a forward and backward angle provided better spray deposition on vertical targets than single-flow patterns.

Materials and Methods

Field trials were conducted during the 2017, 2018, and 2019 growing seasons at the University of Kentucky Research and Education Center in Princeton, KY. The soft red winter wheat (*Triticum aestivum*) cultivar ‘AgriMaxx 446’ (AgriMaxx Wheat Company, Mascoutah, IL) was no-till planted using a John Deere 1590 grain drill (Deere & Company, Moline, IL) into corn (*Zea mays*) stubble at an approximate seeding rate of

3.7 million live seeds/ha in October 2016, 2017, and 2018. The grain drill had 14 rows, spaced 19 cm apart. Wheat was grown using agronomic, fertility, weed, and insect pest management practices typical for Kentucky (Lee et al. 2009.). No foliar fungicides were applied, except for fungicide treatments that were part of the experiment.

The treatments evaluated consisted of five different spray nozzle configurations and three different sprayer speeds (Table 4.1). Three different nozzles were tested: air induction dual pattern flat spray tip nozzles (AI3070), air induction turbo twinjet flat spray tip nozzles (AITTJ60), and turbo twinjet flat spray tip nozzles (TTJ60) (TeeJet Technologies, Wheaton, IL). The AI3070 is an asymmetric dual flat fan air induction nozzle designed for foliar fungicide applications on small grain crops with a 30° forward and 70° reverse facing spray fan (Fig. 4.1). The AITTJ60 is a symmetric air induction dual flat fan nozzle, and the TTJ60 is a non-air induction version of the same nozzle. In addition to the three nozzle types, the AITTJ60 and TTJ60 were tested facing either straight down or rotated 30° backwards, for a total of five nozzle configurations. Each configuration was tested at sprayer speeds of 13, 19 and 24 km/h. These speeds were selected to approximate common application speeds used by growers. Nozzle size and pressure was adjusted across the treatments to maintain a similar spray volume and droplet diameter according to manufacturer recommendations. The target spray volume was 112 L/ha with a coarse droplet size (Table 4.1).

In 2017, only water was applied through a tractor-mounted sprayer to measure coverage. The sprayer boom had three independently controlled sections with five nozzles on each of the left-hand and right-hand side section and four nozzles on the middle section. Nozzles were spaced 50.8 cm apart. Only the left-hand section of the

boom was used to apply water in 2017, and the other sections were turned off. The applications were made when wheat was at the Feekes 10.51 growth stage (anthesis) (Lee et al. 2009). To measure coverage, simulated wheat heads were constructed by wrapping water sensitive paper (76 × 26 mm; Spraying Systems Co., Wheaton, IL) around 15 ml cylindrical plastic tubes that were placed on metal rods at the same approximate height as surrounding wheat heads (Fig. 4.2). For each treatment, four simulated wheat heads with water sensitive paper were used in the same sprayer pass. Each treatment was applied using a different sprayer pass. Each pass was 30 m long, but simulated wheat heads were placed towards the middle of the pass to ensure that the sprayer had reached the appropriate speed. Water sensitive papers were allowed to dry for approximately 5 min before being collected in individual sealed plastic bags and then stored at 4°C in the laboratory refrigerator until analyzed a few days later.

The water sensitive papers were digitally scanned (Perfection V600 Photo scanner; Epson America, Inc., Los Alamitos, CA) and each image was uploaded to a computer as a JPG file. The image was then cropped in ImageJ software (National Institutes of Health, Bethesda, MD). MATLAB software (MathWorks, Natick, MA) was used to separate the images so that they represented the front and back of the simulated wheat heads. Coverage for the front and back images of the simulated wheat head were analyzed separately using ImageJ. Total coverage was calculated using the dimensions of the front and back spray cards along with the area stained by droplets. In 2019, the background of some spray cards changed from yellow to brown during storage. The contrast between the droplet stains and the background was not sufficient for coverage to be accurately calculated without additional processing. Adobe Photoshop (Adobe Inc.,

San Jose, CA) was used to index the colors of the spray cards, increasing contrast allowing ImageJ to recognize the droplets.

In 2018 and 2019, the sprayer was modified to have two spray tanks so that the left-hand section of the boom applied water, while the right-hand section of the boom applied Prosaro fungicide (prothioconazole + tebuconazole; Bayer Crop Science, St. Louis, MO) at 475 ml/ha (0.1 kg a.i./ha prothioconazole + 0.1 kg a.i./ha tebuconazole). The middle section of the boom was turned off, which provided a small buffer between the water-applied and Prosaro-applied areas. This modification allowed coverage to be measured in the water control (using the same methods previously described), but also allowed the effects of fungicide application on FHB, DON, test weight, and yield to be measured and compared to the water control. Coverage was measured from the water application using the methods described previously. In both the fungicide treated and water control plots, 40-50 wheat heads were evaluated (10 wheat heads in four or five different areas per plot, wheat heads were selected by grabbing a handful of wheat heads at each location and repeating until 10 wheat heads had been observed) for FHB severity and incidence, which was used to calculate FHB index as described by Stack and McMullen (2011).

From the middle of each plot, an 8-10 m long × 1.5 m wide section was harvested with a small plot research combine (Wintersteiger Delta; Wintersteiger, Salt Lake City, UT) equipped with a HarvestMaster Classic Grain Gauge (Juniper systems, Logan, UT) to measure total grain weight, seed moisture, and test weight. Harvested grain weights were adjusted to 13.5% moisture and converted to kg/ha. Grain samples were collected from each subplot at harvest and were sent to the University of Minnesota DON Testing

Laboratory (St. Paul, MN) where they were subjected to gas chromatography-mass spectrometry analysis to determine the DON concentrations present.

Relative control (%) of FHB and DON and relative increase (%) of test weight and yield were calculated for each fungicide-treated plot using the data from the water control as the base. These data from 2018 and 2019 and the coverage data from 2017-2019 were used for statistical analysis. For statistical analysis, each year was considered a replication, which was considered a random effect. The effects of speed, nozzle, and their interaction (speed × nozzle), were considered fixed and were evaluated for their effects using the GLIMMIX procedure of SAS software (version 9.4; SAS Institute Inc., Cary, NC). Least squares means (lsmeans) were computed and compared ($\alpha = 0.05$) by using the LINES statement to compare all pairwise differences among lsmeans. Comparison of lsmeans only occurred if the F-test was significant ($P \leq 0.05$). Pearson correlation analysis was conducted on the 2018-2019 data using SAS software (PROC CORR) to evaluate for relationships between relative control of FHB or DON and front, back, and total coverage.

Results

The only observed significant effect ($P \leq 0.05$) of speed was on back coverage of simulated wheat heads (Tables 4.2 and 4.3). The greatest coverage of the back of the simulated wheat head was achieved when the sprayer was traveling 12 km/h, which was significantly greater than the coverage achieved when traveling 24 km/h, but not different than traveling 19 km/h (Table 4.4).

Significant effects of nozzle were observed for coverage of the front, back, and total simulated wheat head, but not for relative control of FHB or DON or for relative

increase of test weight or yield (Tables 4.2 and 4.3). The greatest coverage of the front of the simulated wheat heads was achieved with the AI3070 nozzle, the AITTJ60 nozzle with no rotation, and the TTJ60 nozzle with no rotation, which all were greater than coverages achieved with the AITTJ60 or TTJ60 nozzles with backwards rotation (Table 4.5). The greatest coverage of the back of the simulated wheat heads was achieved with the TTJ60 nozzle with backwards rotation, which was not significantly different than the coverages achieved with AITTJ60 with backwards rotation or the TTJ60 with no rotation (Table 4.5). The greatest total coverage of the simulated wheat heads was achieved with the AI3070 nozzle and the TTJ60 nozzle with no rotation, which were not significantly different from each other, but significantly better than the coverage achieved with all other nozzle configurations (Table 4.5).

The significant interactive effect of speed × nozzle was observed for relative control of FHB, but not for any of the other measured variables (Tables 4.2 and 4.3). Within each nozzle configuration, relative FHB control was not affected by speed for the AI3070 nozzle or the AITTJ60 with or without backwards rotation; however, speed did affect the relative control of FHB for the TTJ60 nozzle in both the non-rotated and rotated configurations (Table 4.6). For the non-rotated configuration of TTJ60, the greatest relative control of FHB was achieved when traveling 13 km/h, which was greater than when traveling 24 km/h, but not different than traveling 19 km/h. The opposite was observed for the backwards rotated configuration of TTJ60, where the greatest relative control of FHB was achieved when traveling 24 km/h, which was greater than when traveling 13 km/h, but not different than traveling 19 km/h.

Pearson correlation analysis did not reveal any significant ($P \leq 0.05$) relationships between relative control of FHB or DON and front, back, and total coverage of simulated wheat heads (Table 4.7).

Discussion

Coverage of simulated wheat heads varied by nozzle type and configuration, which is consistent with the results of previous studies (Powell et al. 2004; Wolf and Caldwell 2004; Parkin et al. 2006; Halley et al. 2008; Vajs et al. 2008; Mesterházy et al. 2011; Derksen et al. 2012; Ozkan et al. 2012; Sreš et al. 2015; Vučajnk et al. 2018; Mur et al. 2020; Vučajnk et al. 2021). There was a statistical difference in coverage between the air induction and traditional symmetric dual flat fan nozzles in the down position but not the backwards orientation. The difference could be attributed to air induction nozzles producing larger droplets. However, this was controlled for by changing orifice size and pressure to regulate droplet size. While the results were mixed, our findings agreed with previous studies that air induction nozzles do not necessarily provide less coverage (Wolf and Caldwell 2004; Parkin et al. 2006; Ozkan et al. 2012). Rotating the nozzle 30° backwards was not beneficial for overall coverage, which is consistent with the findings of Mur et al. (2020) and contrary to those of Parkin et al. (2006). The backwards orientation of nozzles produced better coverage on the rear of simulated wheat heads than the down configuration. This indicates that coverage on the backside of the wheat head is improved by shifting some of the droplets from the front to the back. The asymmetric dual flat fan nozzle (AI3070) supplied equivalent total coverage as the best performing symmetric dual pattern nozzle (TTJ60 with no rotation). This agrees with previous studies that there is not an advantage to asymmetric style nozzles in terms of overall

coverage but differs in that the coverage was equivalent and not reduced (Vučajnk et al. 2018; Mur et al. 2020).

The effect of sprayer speed on coverage appears to be determined by the associated change in application rate (Wolf and Caldwell 2004; Sreš et al. 2015). The results presented here mostly agree with this, whereas the application rate was held close to constant across the different sprayer speeds. We found no statistically significant effect of speed on front or overall coverage of the simulated wheat heads. However, the coverage on the back of the simulated wheat head was influenced by travel speed even though the spray volume was relatively consistent. The added forward momentum of the droplets associated with the increased sprayer speed may have changed the trajectory of the spray pattern, which possibly explains the reduced coverage on the back of the simulated wheat head.

There was not a statistically significant relationship between coverage and DON reduction, FHB index, or yield. This is different from other studies that found yield, FHB reduction, and DON reduction could be improved by increasing fungicide coverage of wheat heads (Sreš et al. 2015; Vučajnk et al. 2021). The variation of coverage between nozzle types and configurations was 1.9 %, which did not change the deposition of fungicide enough to affect disease management. This is supported by the findings of Lehoczki-Krsjak et al. (2015) in which small differences in fungicide deposition did not change overall efficacy. Trials that demonstrated a statistically significant relationship between coverage and disease management typically had a variation of 10% or more between the best and worst treatments. (Sreš et al. 2015; Vučajnk et al. 2021).

The interaction between nozzle type and sprayer speed had a statistically significant effect on relative FHB control, even though there was not an associated change in coverage or distribution. Using an alpha of 0.1 instead of 0.05 results in a weak correlation between coverage on the back of the simulated wheat heads and reduction in FHB index. While not statistically significant the top three values for relative FHB control were achieved by nozzles that provided the best coverage on the rear of the simulated wheat heads. The greatest relative control of FHB occurred when the sprayer was traveling 13 km/h, which provided greater coverage on the back of the simulated wheat heads than faster speeds. From correlation analysis, no relationship between relative FHB control and coverage was observed. While this is somewhat perplexing, it is important to remember that coverage values are from simulated wheat heads and not from real wheat heads. The actual coverage of wheat heads can be underestimated when measured by spray cards (simulated wheat heads) due to wheat heads often having a slight angle away from vertical unlike a simulated wheat head. (Halley et al. 2008). This may lead to an incomplete understanding of a complex relationship between fungicide applications and FHB management.

Coverage was not demonstrated to be a major determinant of fungicide efficacy; this is believed to be from the low variation among treatments. Increasing coverage enough to have a biological effect may be difficult but is believed to be an effective strategy. In this study, nozzle type was less important for FHB management than using the right configuration and speed for the chosen nozzle to maximize the benefits of a fungicide application. Alternatively, if sprayer speed is a primary concern, using the proper nozzle might mitigate the loss of any potential efficacy.

Table 4.1 Nozzle configurations and sprayer speeds evaluated. Pressure was adjusted to achieve similar spray volumes across treatments.

Nozzle	Rotation	Speed (km/h)	Pressure (kPa)	Spray volume (L/ha)
AI3070	None	13	345	118
		19	414	113
		24	621	111
AITTJ60	None	13	483	115
		19	414	113
		24	621	111
AITTJ60	30° backward	13	483	115
		19	414	113
		24	621	111
TTJ60	None	13	345	118
		19	414	113
		24	414	113
TTJ60	30° backward	13	345	118
		19	414	113
		24	414	113

Table 4.2 Sprayer speed, nozzle configuration, and speed × nozzle configuration effects ($P > F$) on front coverage, back coverage, and total coverage of a simulated wheat head sprayed with water.

Effect	Front coverage	Back coverage	Total coverage
Speed	0.3010	0.0069	0.5514
Nozzle	0.0001	0.0050	0.0009
Speed × Nozzle	0.8603	0.9233	0.9827

Table 4.3 Sprayer speed, nozzle configuration, and speed × nozzle configuration effects ($P > F$) on relative Fusarium head blight (FHB) control, relative deoxynivalenol (DON) control, relative test weight increase, and relative yield increase in wheat sprayed with Prosaro fungicide (prothioconazole + tebuconazole; Bayer Crop Science, St. Louis, MO) relative to a water control.

Effect	Relative FHB control	Relative DON control	Relative test weight increase	Relative yield increase
Speed	0.7006	0.5768	0.4515	0.1375
Nozzle	0.2219	0.0942	0.7135	0.7790
Speed × Nozzle	0.0377	0.2451	0.3418	0.9070

Table 4.4 Effect of sprayer speed on spray coverage on the back of simulated wheat heads.

Speed (km/h)	Back coverage [z] (%)
13	8.1 A
19	7.3 AB
24	6.5 B

[z] Least squares means followed by the same letter are not significantly different ($\alpha = 0.05$).

Table 4.5 Effect of nozzle configuration front, back and total spray coverage of a simulated wheat head.

Nozzle	Rotation	Front coverage [z] (%)	Back coverage [z] (%)	Total coverage [z] (%)
AI3070	None	12.6 A	6.9 BC	9.7 A
AITTJ60	None	11.3 A	5.6 C	8.5 B
AITTJ60	30° backward	8.2 B	7.6 AB	7.9 B
TTJ60	None	11.9 A	7.7 AB	9.8 A
TTJ60	30° backward	7.8 B	8.5 A	8.1 B

[z] Least squares means followed by the same letter are not significantly different ($\alpha = 0.05$).

Table 4.6 Effect of nozzle configuration and sprayer speed on relative control of Fusarium head blight (FHB) with Prosaro fungicide (prothioconazole + tebuconazole; Bayer Crop Science, St. Louis, MO).

Nozzle	Rotation	Speed (km/h)	Relative FHB control [z] (%)
AI3070	None	13	53.5 CDE
		19	76.7 ABCD
		24	67.2 ABCDE
AITTJ60	None	13	51.5 DE
		19	75.3 ABCD
		24	55.9 BCDE
AITTJ60	30° backward	13	87.1 AB
		19	77.2 ABCD
		24	73.7 ABCDE
TTJ60	None	13	99.6 A
		19	68.3 ABCDE
		24	64.2 BCDE
TTJ60	30° backward	13	41.0 E
		19	66.7 ABCDE
		24	86.4 ABC

[z] Least squares means followed by the same letter are not significantly different ($\alpha = 0.05$).

Table 4.7 Pearson correlation coefficients and probability values between relative control of Fusarium head blight (FHB) or deoxynivalenol (DON) and front, back, and total coverage of simulated wheat heads.

Variables		**Pearson correlation coefficient**	**Probability**
Relative FHB control	Front coverage	-0.19	0.3145
	Back coverage	0.31	0.0961
	Total coverage	-0.02	0.9126
Relative DON control	Front coverage	0.19	0.3118
	Back coverage	0.23	0.2182
	Total coverage	0.29	0.1138

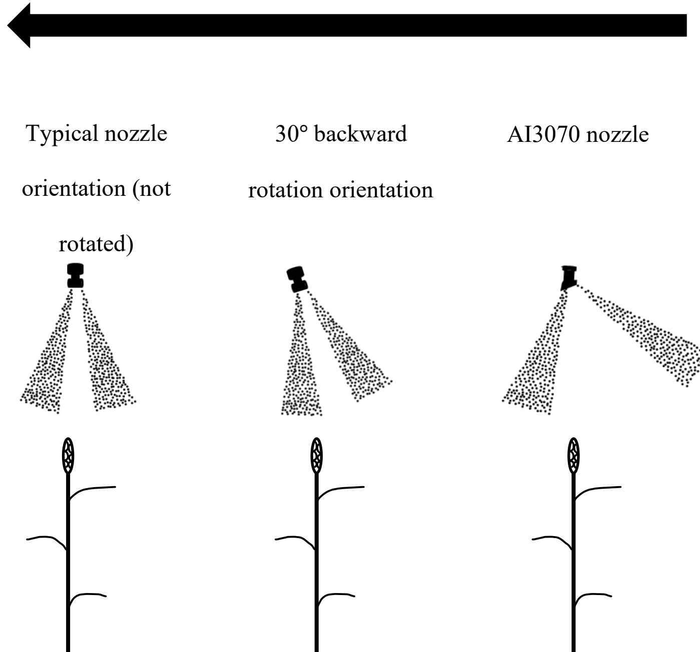

Figure 4.1 Orientation of spray nozzles in relation to the wheat head. The AITTJ60 and TTJ60 spray nozzles were tested at both the typical nozzle orientation and the 30° backward rotation orientation.

Figure 4.2 Water sensitive paper was wrapped around plastic tubes that were of similar diameter as wheat heads and were placed at the approximate height as surrounding wheat heads. These served as a simulated wheat heads used to measure coverage from different spray nozzle configurations and sprayer speeds in field trials conducted at Princeton, KY in 2017-2019.

CHAPTER 5. SUMMARY

Fungicides are commonly used to manage fungal diseases of winter wheat in Kentucky. Several choices follow the decision to apply a fungicide that affect disease management provided by the fungicide. Proper fungicide applications use an appropriate fungicide, application time, and sprayer configuration for the pathogens present. Some decisions can be appropriately made by consulting product labels or other readily available information. Other decisions should be based on region specific recommendations. In the absence of this guidance, growers can struggle to make appropriate decisions regarding fungicide applications. The objective of my thesis was to answer some of these questions for three common wheat pathogens in Kentucky. Fungicide management practices for Stagonospora nodorum leaf blotch, caused by *Parastagonospora nodorum*, Septoria tritici leaf blotch, caused by *Zymoseptoria tritici*, and Fusarium head blight, caused by *Fusarium graminearum* were examined. Isolates of *P. nodorum,* and *Z. tritici* were surveyed to determine if quinone outside inhibitor (QoI) fungicides remained effective against these pathogens. Field trials were conducted to evaluate the appropriate time to apply fungicides to manage the Septoria leaf blotch complex. Additionally, trials were conducted to examine sprayer application parameters for managing FHB with a fungicide.

Leaf blotches caused by *Z. tritici* and *P. nodorum*, collectively known as the Septoria leaf blotch complex are commonly managed with QoI fungicides (Fungicide Resistance Action Committee (FRAC) Group 11). Prior to this research it was unclear if QoI resistant isolates of *Z. tritici* and *P. nodorum* were present in Kentucky and how this would affect management of the Septoria leaf complex, as low levels of resistance can

lead to QoI fungicides being ineffective after just a few applications (Brent and Hollomon 2007a). Isolates of both pathogens had been documented as resistant to QoI fungicides in other parts of the United States (Estep et al. 2013; Kaur et al. 2021). However, neither pathogen population in Kentucky had been examined for resistance to QoI fungicides.

Identification of QoI resistant isolates of *Z. tritici* in Kentucky, and QoI resistant isolates of *P. nodorum* in Kentucky and Illinois, will help growers in this region avoid applying ineffective fungicides. The QoI class of fungicides is expected to become increasingly less effective at managing the Septoria leaf blotch complex. Due to this, growers should implement alternative management practices and take steps to protect the chemistries that remain effective. Additionally, the *P. nodorum* and *Z. tritici* populations in Kentucky need to be monitored for resistance to other fungicide classes, such as demethylation inhibitors (DMI) and succinate dehydrogenase inhibitors (SDHI).

Management of the Septoria leaf blotch complex and other fungal pathogens can be improved by applying suitable fungicides at the optimum time. Previously the most applicable recommendations for foliar fungicide application timing in Kentucky came from research conducted in other states (Willyerd et al. 2015). The work presented in my thesis support updated recommendations on foliar fungicide application timing specific to the environment and pathogens of Kentucky. Fungicides provided the best management of the Septoria leaf blotch complex when applied at flag leaf emergence (Feekes 9), followed by a fungicide application at early anthesis (Feekes 10.51).

Applying a fungicide at Feekes 10.51 has been shown to be the optimal application time for managing FHB and reducing deoxynivalenol (DON) contamination

in wheat (Bolanos-Carriel et al. 2020; D'Angelo et al. 2014; Freije and Wise 2015; Paul et al. 2019; Yoshida et al. 2012). Improving fungicide coverage of wheat heads has been identified as an additional way to improve the effectiveness of a fungicide application in managing FHB (Lehoczki-Krsjak et al. 2013). This study evaluated the effect that different nozzle types commonly used or available in Kentucky along with different sprayer speeds, and nozzle orientations had on FHB management. The coverage provided by the different nozzle types was similar, likely resulting in coverage not having a significant impact on FHB management or DON content. However, selecting the appropriate application speed for the nozzle being used was shown to be an effective way of improving FHB management.

The research presented in my thesis support improved recommendations for foliar fungicide applications. Management of the Septoria leaf blotch complex in Kentucky and surrounding regions can be improved by applying fungicides alternative to the QoI class at the Feekes 9 growth stage. Combining this with a fungicide application at early anthesis using an appropriate application speed will improve the management of the Septoria leaf blotch complex and FHB. Implementing these recommendations will help wheat farmers in Kentucky avoid reduction in grain quality and yield from fungal diseases.

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