ABSTRACT

MEYERS, EMILY ANN. Evaluation of Fungicide Sensitivity of United States *Blumeria graminis* f. sp. *tritici* Population. (Under the direction of Dr. Christina Cowger).

Wheat powdery mildew, caused by *Blumeria graminis* f. sp. *tritici*, is managed primarily with wheat (*Triticum aestivum*) cultivar resistance and foliar fungicides in the United States (U.S.). Both management strategies are prone to deterioration and defeat simply due to the biology and disease cycle of this pathogen. In the U.S., host resistance gene effectiveness has been measured once a decade for the past 30 years. Despite high levels of fungicide insensitivity in other global cereal powdery mildew populations, fungicide sensitivity in the U.S. *B. graminis* f. sp. *tritici* population has never been evaluated. Formulated fungicide products from three mode-of-action categories are labelled for use on wheat in the U.S.: demethylation inhibitors (DMIs), quinone outside inhibitors (QoIs), and succinate dehydrogenase inhibitors (SDHIs). The study presented here sought to measure the sensitivity of the U.S. *B. graminis* f. sp. *tritici* population to fungicides from each of the three categories and determine the genetic causation of any observed decreases in sensitivity.

Nearly 400 *B. graminis* f. sp. *tritici* isolates were collected from 27 fields in 15 states in the central and eastern U.S. in the 2013 and 2014 wheat growing seasons. Six fungicides were evaluated: 3 DMIs, (tebuconazole, prothioconazole, and metconazole), 2 QoIs (pyraclostrobin and picoxystrobin), and 1 SDHI (fluxapyroxad). Isolates were screened for fungicide sensitivity using a detached-leaf assay in which susceptible seedlings were sprayed with a concentrations of a formulated fungicide active ingredient, then cut into leaf segments which were floated atop water agar, and inoculated with a single *B. graminis* f. sp. *tritici* isolate. Leaf segments were later rated for fungal growth, then ratings were converted to EC_{50} values or estimates of the effective fungicide concentration at which an isolate's growth was inhibited by 50%. Regional mean

EC_{50}s were most significantly different among the DMIs, with isolates originating from the eastern U.S. (especially the Mid-Atlantic and Great Lakes regions) exhibiting decreased sensitivity to DMIs compared to those from the central U.S. (Plains region, Arkansas, and Missouri). U.S. *B. graminis* f. sp. *tritici* isolates exhibited a diverse range in QoI sensitivity; however, differences in regional sub-populations were not as evident. Interestingly, on average isolates from the central U.S. were more SDHI insensitive than those from the Great Lakes and Southeast regions.

Genetic sequencing of DMI and QoI fungicide target genes was completed to determine if any alterations correlated with observed differences in isolate sensitivity. In QoI target gene *cytb*, only synonymous mutations were found at previously identified mutation sites, further underlining the lack of major QoI insensitivity in the U.S. *B. graminis* f. sp. *tritici* population. In DMI target gene *CYP51*, 3 genotypes were found in the U.S. collection based on differences at the 136 locus: wildtype, Y136; mutant, F136; and heteroallelic (Het), Y136 & F136. In the DMI insensitive United Kingdom population, 2 CYP51 genotypes were identified involving changes at both the 136 and 509 loci: mutant, F136; Het-Het; Y136 & F136 + S509 & T509. Estimation of *CYP51* gene copy number revealed 2-4 copies in isolates from both countries; however, *CYP51* expression was twice as high in United Kingdom isolates as in U.S. isolates. Further research is necessary to fully understand the similarities and differences between these two populations.

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Evaluation of Fungicide Sensitivity of United States *Blumeria graminis* f. sp. *tritici* Population

by
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DEDICATION

To those that have supported me, especially when I needed it the most.

BIOGRAPHY

Emily was first exposed to agriculture while growing up on a family-run dairy farm in rural Wisconsin. After spending her childhood and teenage years feeding calves and picking stones, Emily attended Ripon College, a small liberal arts school in central Wisconsin, where she received a degree in Biology in 2013. Under the advisement of Dr. Mark Kainz, Emily attended two plant science summer research undergraduate internships where she was exposed to the field of plant pathology. She then joined the NC State plant pathology program in Fall 2013 and, after lab rotations, began her Ph.D. research on fungicide sensitivity of the U.S. wheat powdery mildew population under the direction of Dr. Christina Cowger.

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CHAPTER 1

Literature Review

1.1 Global importance of wheat

Cultivated wheat (*Triticum aestivum*) is planted on nearly 220 million hectares around the world, more arable land than any other crop (FAOStat). Wheat provides 20% of the daily caloric and protein consumption worldwide and is a staple food for 35% of the world population (FAOStat). The world population is projected to reach 9.6 billion by the year 2050. In 2017, more than 770 million metric tons of wheat was produced globally. But to support the growing population, wheat yield will need to increase by 70% by 2050 (National Association of Wheat Growers 2019). The United States is the fourth largest producer of wheat (after China, India, and Russia), yielding around 50 to 60 million metric tons on around 15 to 20 million hectares a year (FAOStat).

The demand for wheat will continue to rise not only because of the growing population but also because of an increase in consumption per capita. Wheat consumption in many Asian countries like Indonesia, Bangladesh, Thailand, and Vietnam has increased over the past 50 years (Curtis and Halford 2014). Currently global wheat production is increasing by about 1% annually; however, to meet demand by the year 2050, at least a 2.4% annual increase is needed (Ray et al. 2013). Though wheat yield potential is thought by some to have a 'yield ceiling', breeding and management efforts continue to be made to increase yield while combating yield-limiting threats like insect pests and diseases without using more land acreage (Jaggard et al. 2010).

1.2 Impact of *Blumeria graminis* on cereal crop production

Blumeria graminis (DC.) Speer (formally *Erysiphe graminis*) is an obligate, biotrophic ascomycete fungus that causes cereal powdery mildew and relies on plant tissue of grasses to complete its life cycle. This species is further broken down into 8 *formae speciales* (ff. spp.) based on host specificity (which grass genera it is able to infect) (Marchal 1902, Oku et al. 1985). Those ff. spp. pathogenic on cultivated cereal crops include: f. sp. *tritici* (wheat powdery mildew), f. sp. *hordei* (barley powdery mildew), f. sp. *secalis* (rye powdery mildew), and f. sp. *avenae* (oat powdery mildew). The other ff. spp. are able to cause disease on wild grasses: f. sp. *agropyri* (*Agropyron* spp. and *Elymus* spp.), f. sp. *bromi* (*Bromus* spp.), f. sp. *poae* (*Poa* spp.), and f. sp. *dactylidis* (*Dactylis* spp.). The first seven of these ff. spp. were designated by Marchal (1902) while the eighth was added by Oku et al. (1985). Unofficial designations are also periodically used for *B. graminis* infections of *Lolium* spp. (f. sp. '*lolii'*) (Carver et al. 1990) and *Triticale* spp. (f. sp. '*triticale*') (Troch et al. 2010, Walker et al. 2011).

Cereal-infecting *B. graminis* ff. spp. have a nearly annual sexual stage and a polycyclic asexual spore stage. The sexual cycle is thought to be triggered in order to survive between cereal growing seasons on leaf debris. Sexual ascospores develop and mature inside of dark, spherical chasmothecia (Cunfer 2002). Ascospores released from the protective chasmothecia are capable of infecting live host tissue and initializing the asexual cycle. Asexual oval-shaped conidia (8-35 µm), in conidial chains, from white to pink to tan pustules (that turn grey overtime) perpendicular to the plant surface (Cunfer 2002). These fluffy, powdery pustules are able to occupy all aboveground plant parts, often beginning in the lower leaves and progressing up the plant during a severe infection. Conidia are easily wind dispersed triggering new pustule production and completing the asexual cycle in 7 to 10 days (Beard 2019). Thus, if not controlled

at or near the time of disease onset, cereal powdery mildew can quickly spread across an entire field.

For infection, growth, and spore dispersal, *B. graminis* prefers cool temperatures (15 to 22 °C) and a humid (>70%) but not wet environment (Beard 2019). In eastern U.S.-grown winter wheat, these conditions often occur early in the growing season, allowing *B. graminis* f. sp. *tritici* infections to have a maximal detrimental impact of up to 45% on yield (Salgado and Paul 2016). Due to its obligate nature, *B. graminis* utilizes host-sequestered nutrients to support its own growth. When the cereal host's nutrients are limited early in the growing season (Feeke's growth stages 2 through 6), fewer tillers are produced leading to fewer heads and therefore lower yield. If the disease continues to progress, occupying more the green leaf and stem tissue, the rate of photosynthesis in the host is diminished reducing the number of kernels per head and the kernel size (Griffiths et al. 1975).

1.3 Cereal powdery mildew management and management challenges

Cereal powdery mildew, though able to occur in most regions worldwide where wheat is grown, can be controlled through the use of cultural, host resistance, and chemical management practices (Cunfer 2002). Cultural approaches seek to make the host-pathogen environment unfavorable for *B. graminis* growth yet maximize host yield (Katan 2010). Management through host resistance requires forethought by the grower, while chemical control options are often used after the disease has been sighted. Proper selection of host cultivar and chemical fungicide is important; their impact of economic return must be considered (Salgado and Paul 2016, Thompson et al. 2014, Cunfer 2002, Beard 2019).

B. graminis has a high evolutionary potential or ability to quickly adapt to overcome host resistance and chemical management strategies. The biological nature and disease cycle of this fungus lend themselves to rapid adaptation through many opportunities for genetic change (McDonald and Linde 2002). As explained earlier, *B. graminis* has both sexual and asexual spore stages. This allows for genetic recombination during the sexual cycle and, because the number of conidia produced per pustule is extremely high, the chance for random mutation is high during the asexual cycle. In addition, the aerial dispersion of conidia supports the rapid spread of *B. graminis* to interact with new plants, fields, and environments (McDonald and Linde 2002).

Further details on the three management strategies of *B. graminis* are discussed below.

1.3.1 Cultural management

B. graminis favors a cool and humid environment for infection. Therefore, primary infections often occur on the lower leaves near the base of the plant where the leaf density, and thus humidity, are the highest (Beard 2019). To help minimize humidity levels, seeding rates and nitrogen applications are evaluated and adjusted. A moderate seeding rate of 25-30 seeds per square foot results in fewer plants per acre, allowing more space between plants for better air flow thus cutting down on moisture accumulation and humidity level (Lee et al. 2009, Mansfield and Hawkins 1992). Applying nitrogen during wheat tillering (Feeke's growth stages 2 through 5) aids in production of additional tillers but also creates a high foliage density where *B. graminis* could thrive (Salgado and Paul 2016). A delicate balance between boosting tiller formation and suppressing humidity can be established by capping nitrogen applications at 70 pounds per acre (Salgado and Paul 2016).

Another strategy for minimizing cereal powdery mildew incidence is to limit the amount of inoculum in the field prior to planting (Beard 2019). Rotating to non-host crops between cereal growing seasons can create an inhospitable environment that conidia and, if maintained for long enough, even chasmothecia cannot survive. If a cereal crop must be planted in the same field for back-to-back seasons, then proper tillage of the soil is important to lessen the amount of inoculum. Tilling under the cereal stubble and residue produced at harvest limits the amount of active *B. graminis* inoculum that could infect the following newly planted cereal crop (Salgado and Paul 2016).

Additionally, if cereal powdery mildew infections occur, further spread of the disease could be prevented by unfavorable weather conditions. *B. graminis* conidia are unlikely to infect on or disperse from a wet leaf surface (Aust and Hoyningen-Huene 1986) or at temperatures above 25°C (QiOng et al. 2010, Beard 2019). Disease severity and incidence may quickly diminish following a rain or warming, preventing the need to deploy additional disease control tactics (Beard 2019). Growers may opt to rely on weather forecasting instead of using a control strategy if cereal powdery mildew epidemics are historically less likely or less severe in their region and if more typical control strategies do not create an economically beneficial situation (Cornell CALS).

1.3.2 Host resistance management

Wheat (*Pm*) and barley (*Ml*) powdery mildew resistance genes are bred into cultivars with agronomically beneficial backgrounds. These genes were either discovered in the genomes of the cultivated cereal or in related and ancient wild grass species. More than 90 wheat *Pm*-genes located at over 50 loci have been deployed in wheat varieties, while approximately 85 *Ml*-genes have been used in barley cultivars (Jorgenson and Wolfe 1994, McIntosh et al. 2013, Li et

al. 2019). In addition to *Pm*- and *Ml*-genes that correlate to *B. graminis* effector proteins, quantitative trait loci are also important in suppressing cereal powdery mildew (Miedaner and Flath 2007).

Host resistance is a commonly used and economically advantageous method of cereal powdery mildew management (Salgado and Paul 2016). However, maintaining durability of resistance genes can be difficult given the great evolutionary adaptation abilities of *B. graminis* populations (McDonald and Linde 2002). Historically, when the same major gene is heavily deployed, that gene becomes regionally defeated (Cowger et al. 2018). *B. graminis* sampling and monitoring studies revealed this to be the case for *Pm4a* and *Pm17* in U.S. wheat (Niewoehner and Leath 1998, Parks et al. 2008, Cowger et al. 2009) and several *Ml*-genes in the 1980s in European barley (Brown et al. 1990, Brown et al. 1991, Brown et al. 1993).

1.3.3 Chemical management

In times when resistance genes rapidly breakdown or susceptible cereal varieties are planted, chemical control options are essential for controlling cereal powdery mildew outbreaks. Growers often apply foliar fungicides after scouting a field and finding signs of powdery mildew infection on the lower leaves (Salgado and Paul 2016). Therefore, chemical management of cereal powdery mildew is usually expected to act as a protectant to prevent disease on upper leaves while also acting as a curative eradicant on the already diseased lower leaves (Beard 2019).

The Fungicide Resistance Action Committee (FRAC) categorizes fungicides based on their function and also ranks each category for their risk of the development of resistance (FRAC 2019). Fungicides from three chemical mode of action groups are labelled for treatment of wheat

powdery mildew in the U.S. (NCERA-184): demethylation inhibitors, quinone outside inhibitors, and succinate dehydrogenase inhibitors. The resistance risk and efficacy history of the three modes of action are discussed below.

1.3.3.1 Demethylation inhibitor fungicides

Demethylation inhibitor (DMI) fungicides (also called triazoles) target C14-demethylase, a cytochrome P450 involved in ergosterol biosynthesis. Because ergosterol is a key component of fungal cell membranes, DMIs are able to slow fungal growth. DMIs bind the heme of the enzyme inhibiting its ability to demethylate lanosterol, a ergosterol precursor (Ziogas and Malandrakis 2015).

DMIs comprise a very important group of fungicides. More than 40 different DMI molecules have been developed and released for use on agricultural plant pathogens since the 1970s (Ziogas and Malandrakis 2015). DMIs are also used for fungal management in humans and animals. Labelled as FRAC Code 3, DMIs are considered to be at moderate risk for resistance (FRAC 2019). Aggressive application of DMIs led to resistance beginning in the 1980s.

European *B. graminis* populations were overexposed to DMIs in the late 1970s and early 1980s in an attempt to control widespread cereal powdery mildew epidemics (Hoffmann 1986, Wolfe and Limpert 1987). DMI resistance followed the intensive applications in the Netherlands (de Waard et al. 1986), the United Kingdom (Fletcher et al. 1987), the Czech Republic (Svec et al. 1995), and France (Godet and Limpert 1998). A substantial decrease in DMI efficacy has also been recently found in Australian barley powdery mildew populations (Tucker et al. 2015, Tucker et al. 2019).

1.3.3.2 Quinone outside inhibitor fungicides

Quinone outside inhibitor (QoI) fungicides (also called strobilurins) disrupt the mitochondrial respiration electron transport chain by targeting the cytochrome bc1 complex (complex III) of fungi. This interference creates an energy deficiency, thereby halting any high energy processes such as spore germination or haustorial development (Sierotzki 2015). QoIs bind the outer quinol oxidation site of the bc1 complex, interacting with key amino acid residues to interrupt ATP production (Link et al. 2003).

QoIs are likely the second most important fungicide mode of action after the DMIs (Sierotzki 2015). The discovery of the original natural strobilurin occurred in 1977 from *Strobilurus tenacellus* (Anke 1995). The first formulated QoI was released in 1996 (Brasseur et al. 1996) and now 20 different QoI molecules exist (FRAC 2019). Labelled as FRAC Code 11, QoI fungicides are at the highest risk for developing resistance as well as cross-resistance (FRAC 2019). Historically in cereal powdery mildew populations resistance to QoIs has occurred soon after the release of the QoI product.

After only 2-3 years of QoI applications for control of European wheat powdery mildew, reduced QoI efficacy was evident (Chin et al. 2001). This makes it one of the pathogens with the most rapid evolution of QoI insensitivity (Fletcher et al. 2004). The same decrease in QoI sensitivity has also been detected in Australian *B. graminis* f. sp. *tritici* populations (Lopez and Kay 2017).

1.3.3.3 Succinate dehydrogenase inhibitor fungicides

Succinate dehydrogenase inhibitor (SDHI) fungicides (also called carboxamides), like QoIs, impact the mitochondrial respiration electron transport chain. SDHIs, though, target

complex II, succinate dehydrogenase (Mathre 1971, Ulrich and Mathre 1972). Complex II is comprised of four subunits: A, B, C, and D. SDHIs were found to occupy the ubiquinone binding pocket formed between the B, C, and D subunits (Huang et al. 2006).

Carboxin was the first SDHI released in 1969 (von Schmeling and Kulka 1966) followed by additional first generation SDHIs. With an adjustment of a phenyl group, the second generation of more broad spectrum SDHI fungicides began in 2003 with boscalid (Stammler et al. 2007). At least 10 additional second generations SDHIs have since been developed and more continue to be released (Stammler et al. 2015). SDHIs are labelled as FRAC Code 7 and are thought to have medium to high risk for development of resistance (FRAC 2019). At this time, insensitivity to SDHIs has not been detected in *B. graminis* (FRAC 2018).

1.4 Objectives of this study

Annual wheat powdery mildew epidemics occur in the Mid-Atlantic U.S. With the durability of resistant cultivars in question year-to-year, effective fungicides options are essential for controlling wheat powdery mildew. Despite decades of foliar fungicide use on wheat in the U.S., fungicide sensitivity levels have never been measured for the U.S. *B. graminis* f. sp. *tritici* population. Therefore, the objectives of this dissertation are to:

1. Evaluate the U.S. *B. graminis* f. sp. *tritici* population for sensitivity to DMI fungicides tebuconazole and prothioconazole (Chapter 2).
2. Determine the QoI sensitivity level and its genetic cause in the U.S. *B. graminis* f. sp. *tritici* using QoIs pyraclostrobin and picoxystrobin, as well as establish the baseline sensitivity to SDHI fluxapyroxad (Chapter 3).

3. Identify the underlying genetic causes of diversity in DMI sensitivity and assess these differences between United States and United Kingdom *B. graminis* f. sp. *tritici* isolates (Chapter 4).

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CHAPTER 2

Sensitivity of the U.S. *Blumeria graminis* f. sp. *tritici* population to demethylation inhibitor fungicides

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Abstract

Wheat powdery mildew, caused by *Blumeria graminis* f. sp. *tritici*, is managed in the United States with cultivar resistance and foliar fungicides. Despite high levels of fungicide sensitivity in other cereal mildew populations, fungicide sensitivity of U.S. *B. graminis* f. sp. *tritici* has never been evaluated. Almost 400 *B. graminis* f. sp. *tritici* isolates were collected from 15 U.S. states over two years and phenotyped for sensitivity to two widely used demethylation inhibitor (DMI) fungicides, tebuconazole and prothioconazole. A large range of sensitivity to both DMIs was observed, with more insensitive isolates originating from the eastern U.S. (Great Lakes, Mid-Atlantic, and Southeast regions) and more sensitive isolates from central states (Plains region, Arkansas, and Missouri). Cross-resistance was indicated by a positive although weak association between tebuconazole and prothioconazole sensitivities at all levels of analysis (EC_{50} values, $P < 0.0001$). A possible fitness cost was also associated with prothioconazole insensitivity ($P = 0.0307$) when analyzed at the state population level. This is the first assessment of fungicide sensitivity in the U.S. *B. graminis* f. sp. *tritici* population, and it produced evidence of regional selection for reduced DMI efficacy. The observation of reduced sensitivity to DMI fungicides in the eastern U.S. underlines the importance of rotating between chemistry classes to maintain the effectiveness of DMIs in U.S. wheat production. While cross-resistance was demonstrated, variability in the relationship of EC_{50} values for tebuconazole and prothioconazole also suggests that multiple mechanisms influence *B. graminis* f. sp. *tritici* isolate responses to these two DMI fungicides.

2.1 Introduction

Cereal powdery mildew is caused by host-specific formae speciales of *Blumeria graminis* (DC.) Speer, an obligately biotrophic ascomycete fungal plant pathogen with global incidence. The two most prominent formae speciales are *B. graminis* f. sp. *tritici*, which specializes on wheat (*Triticum aestivum*), and *B. graminis* f. sp. *hordei*, the barley (*Hordeum vulgare*) form. Epidemics are favored by cool, humid conditions, and are characterized by polycyclic development in which spore loads can build up to extremely high levels (Both and Spanu 2004). The pathogen population is characterized by great genetic diversity and regular sexual recombination (Wicker et al. 2013), giving it a high evolutionary potential (McDonald and Linde 2002, Cowger et al 2018). Powdery mildew can reduce grain yield by up to 30% if left unmanaged, as early-season infection leads to decreased production of additional stems and spikes (tillering) of cultivated cereal crops (Bowen et al. 1991).

Management of cereal powdery mildew has relied on cultivar resistance, both quantitative and qualitative, and fungicide applications. Worldwide, approximately 85 major barley powdery mildew (*Ml*) and 80 major wheat powdery mildew (*Pm*) resistance gene variants have been identified in barley, wheat, or other closely related wild grasses (Jorgensen and Wolfe 1994, McIntosh et al. 2013, Wiersma et al. 2017). When *Ml-* or *Pm*-genes with strong effects are deployed, *B. graminis* can rapidly defeat them due to its high adaptive capacity (Parks et al. 2008, Cowger et al 2018). Frequent monitoring and surveys of European cereal powdery mildew populations revealed a breakdown of several *Ml*-genes in the 1980s (Brown et al. 1990, Brown et al. 1991, Brown et al. 1993).

Demethylation inhibitor (DMI) fungicides are commonly used to reduce cereal powdery mildew severity. This group of chemicals hinders *B. graminis* growth by interfering with

production of ergosterol, a key component of fungal cell membranes (Siegel 1981). Just as with resistance genes, fungicide efficacy can erode rapidly due to the high adaptive capacity of *Blumeria*. The Fungicide Resistance Action Committee (FRAC) ranks DMI fungicides (FRAC Group 3) at moderate risk for the development of fungicide resistance (Brent and Holloman 2007). In combination with the pathogen's evolutionary potential, this creates a high likelihood that insensitivity will develop in *B. graminis* populations in geographic areas where DMI fungicides are repeatedly applied.

Not long after DMI fungicides were introduced, European cereal mildew populations became DMI-resistant. Several first-generation DMIs (such as triadimefon, triadimenol, and propiconazole) were approved for agricultural use in Europe in the late 1970s and early 1980s and were applied intensively to cereal crops to control widespread powdery mildew epidemics (Hoffmann 1986, Wolfe and Limpert 1987). Over the next decade, decreased DMI sensitivity was documented in *B. graminis* f. sp. *tritici* populations in the Netherlands (de Waard et al. 1986), the United Kingdom (Fletcher et al. 1987), the Czech Republic (Svec et al.1995), and France (Godet and Limpert 1998). In cases where multiple first-generation DMIs were evaluated, cross-resistance was noted Czech isolates and British isolates (Svec et al. 1995, Wyand and Brown 2005). The same reduction in DMI sensitivity and cross-resistance was observed in European *B. graminis* f. sp. *hordei* populations while DMI resistance was also found in progeny of resistant x sensitive isolate crosses (Blatter et al. 1998, Wyand and Brown 2005). In addition, Australia has seen a substantial decrease in the efficacy of some DMIs on barley powdery mildew (Tucker et al. 2015).

Highly intensive use of DMIs on European wheat and barley has led to the demise of these fungicides as a useful tool for powdery mildew control, and created a need for the

development and release of other fungicide chemistries. However, *B. graminis* f. sp. *tritici* quickly became well known for overcoming fungicides, evolving resistance to six different chemical classes in only two to five years after their initial release (FRAC 2013). In fact, FRAC has now ranked *B. graminis* in the highest risk category for development of fungicide resistance (FRAC 2013).

In the United States (U.S.), wheat powdery mildew occurs nearly annually in the cool, humid Mid-Atlantic states (Maryland, Virginia, North Carolina, and South Carolina). The disease can also be found fairly frequently across the winter wheat area east of the Mississippi River and sporadically on hard winter wheat in the Central Great Plains (Cowger et al. 2012). However, recent U.S. *B. graminis* f. sp. *tritici* epidemics have occurred as far northwest as Montana (M. Burrows, 2015 and 2016, *personal communication*) and as far southeast as Georgia and Alabama (J. Johnson, 2014, and K. Bowen, 2013 and 2014, *personal communication*). Geographic expansion of this disease suggests a need for more effective wheat powdery mildew management in the U.S. through deployment of *Pm*-genes in cultivars with quantitatively mildew-resistant backgrounds and regular monitoring of both *Pm*-gene efficacy and fungicide resistance development.

Understanding both the virulence profile of a regional *B. graminis* population and its fungicide sensitivity level is important to achieve durable and reliable cereal powdery mildew management. In the U.S., wheat *Pm*-gene efficacy has lately been assessed once every ten years, and rapid breakdown of a *Pm*-gene has been observed several times, e.g., *Pm4a* in 2002 and *Pm17* in 2009 (Niewoehner and Leath 1998, Parks et al. 2008, Cowger et al. 2009, Cowger et al. 2018). However, the fungicide sensitivity of the U.S. *B. graminis* f. sp. *tritici* population has

never been evaluated. Despite decades of fungicide use on wheat in the U.S., neither data on U.S. *B. graminis* f. sp. *tritici* baseline fungicide sensitivity nor periodic surveys have been published.

An increase in fungicide use on U.S. wheat has occurred over the last decade (Fernandez-Cornejo et al. 2014). U.S. wheat growers found an economic benefit in applying fungicides following a surge in the U.S. wheat market price in 2007 (Thompson et al. 2014, Lopez et al. 2015). Most eastern U.S. wheat growers continue to make at least one fungicide application per growing season, even though prices have returned to less favorable levels (C. Cowger, *personal observation*). Even with annual DMI applications on wheat in most regions of the country, DMI fungicide sensitivity of *B. graminis* f. sp. *tritici* has never been evaluated in the U.S.

With the goal of helping avert the high degree of DMI resistance present in European cereal mildew populations, we set out to determine current levels of U.S. *B. graminis* f. sp. *tritici* sensitivity to several fungicide chemistries. Here, we report on the sensitivity of U.S. *B. graminis* f. sp. *tritici* to two commonly applied DMI fungicides: 1) tebuconazole, a "first-generation" DMI registered in the U.S. in 1994 and approved for use on wheat in 1997 (FAO 1998), and 2) prothioconazole, a "second-generation" DMI registered in 2007 (FAO 2009). This study is the first evaluation of U.S. *B. graminis* f. sp. *tritici* sensitivity to any fungicide. To our knowledge, this is also the first evaluation of *B. graminis* f. sp. *tritici* sensitivity to prothioconazole internationally, and the second evaluation of tebuconazole, with the only other study being in 1993 in Central Europe (Godet and Limpert 1998).

We hypothesized that both DMI fungicides would be less effective at controlling *B. graminis* f. sp. *tritici* isolates from the Mid-Atlantic U.S. states, where wheat powdery mildew epidemics and fungicide applications occur each year. Additionally, we expected isolates from the Great Plains states, where wheat powdery mildew occurs only sporadically, to be

successfully controlled by both DMI fungicides. Across the U.S., we also anticipated *B. graminis* f. sp. *tritici* populations to be relatively less sensitive to the older DMI, tebuconazole, than to the newer DMI, prothioconazole.

2.2 Materials and Methods

2.2.1 *B. graminis* f. sp. *tritici* isolate collection. *B. graminis* f. sp. *tritici*-infected wheat plants were collected in the U.S. in 2013 and 2014 as described in Cowger et al. (2018). Approximately 390 of the over 1,000 genetically pure isolates in that collection were used in this study. These isolates were collected from 27 fields in 15 states and grouped into 5 geographic regions (Figure 2.1, Table 2.1) based on previous population subdivision analysis (Cowger et al. 2016). Although they are geographic neighbors, the Plains and AR-MO *B. graminis* f. sp. *tritici* populations proved to possess different virulence profiles, likely because the two regions cultivate hard and soft wheat, respectively (Cowger et al. 2018). Isolates were maintained on *B. graminis* f. sp. *tritici*-susceptible detached leaves of universally susceptible cultivars ‘Chancellor’ or ‘Jagalene’ and incubated at 17°C with 12 hours of light.

Historic European standard isolates JIW11 and Fel09 were kindly provided by B. Keller and maintained in live culture along with the U.S. isolates. DMI-sensitive isolate JIW11 was collected in the United Kingdom in 1985. DMI-resistant isolate Fel09 was collected in Germany in 1998. These isolates were also subjected to the same fungicide sensitivity assays as U.S. isolates and compared to U.S. isolates as controls. Both European isolates had been included in a prior study evaluating DMI (triadimenol and propiconazole) sensitivity (Wyand and Brown 2005).

2.2.2 Detached-leaf fungicide sensitivity assay. Formulated tebuconazole and prothioconazole were obtained from Bayer CropScience (Research Triangle Park, NC) in the form of Folicur and Proline, respectively. Stock solutions of 500 mg L^{-1} of active ingredient were prepared in water and used to make 11 concentration dilutions per fungicide. These tebuconazole (0.219, 0.438, 0.875, 1.75, 3.5, 7, 10, 14, 17, 20, and 24 mg L^{-1}) and prothioconazole (5, 10, 20, 40, 80, 110, 160, 200, 250, 300, and 350 mg L^{-1}) concentrations were determined in a preliminary pilot study using seven U.S. *B. graminis* f. sp. *tritici* isolates collected in 2013. In each case, the range was designed to capture the spectrum of *B. graminis* f. sp. *tritici* sensitivity by including high concentrations that prevented growth of all tested isolates, while still maintaining precision in predicting isolate sensitivity.

Ten-day-old universally susceptible ('Chancellor' or 'Jagalene') wheat seedlings were sprayed with a fungicide concentration to runoff using an atomizer. A sprayed water control was also included. Plants were allowed to dry 20 minutes in the fume hood before being moved to a growth chamber overnight (8 to12 hours).

Seedlings to which fungicide had been applied were then cut into leaf segments approximately 3 cm in length. Four leaf segments sprayed with a given fungicide concentration were floated on benzimidazole-amended (50 mg L^{-1}) 0.5% water agar in a 60x15 mm plastic Petri plate. Twelve plates, one for each fungicide concentration plus the water control, were evenly inoculated with *B. graminis* f. sp. *tritici* conidia of a single isolate using a settling tower. Plates were incubated at 17°C with 12 hours of light.

Ratings were conducted at ten days post-inoculation. Leaf segments were rated for fungal growth based on pustule coverage and density. Each leaf segment was rated, producing four ratings per concentration, on a scale of 0 to 3 where 0 = no fungal growth, green leaf segment, or

1-2 sparsely sporulating pustules; 1 = less than 10 individual pustules covering between 5 and 20% of the leaf segment; 2 = multiple fluffy individual pustules covering between 20 and 60%; and 3 = many fluffy, dense, coalescing pustules covering more than 60% of the leaf segment (Appendix A: Supplementary Figure 2.1, Supplementary Figure 2.2). This detached leaf assay was completed once per *B. graminis* f. sp. *tritici* isolate*fungicide concentration combination. In a few cases, particular isolates were in poor condition or unavailable at the time of testing, such that the actual totals of isolates assayed for tebuconazole and prothioconazole sensitivity were 382 and 345, respectively. Hormetic effects (whereby low doses may have a beneficial effect while high doses have an inhibitory one) were not observed (Appendix A: Supplementary Figure 2.2).

2.2.3 Measuring sporulation as a component of fitness. In some pathosystems, isolates with reduced sensitivity (or full resistance) to a fungicide exhibit a fitness cost (Mikaberidze and McDonald 2015). Here, we evaluated *B. graminis* f. sp. *tritici* isolate sporulation as a quantitative component of fitness. Spores per pustule were quantified by collecting five samples per isolate of about 20 (18 to 22) individual, non-coalescing pustules each.

Detached susceptible ('Chancellor' or 'Jagalene') wheat leaf segments on benzimidazole-amended agar were inoculated lightly, about half of regular inoculation density, to ensure individual pustules could be visually counted and collected. Pustules were collected into tubes of 100% ethanol at 8 days post-inoculation. Ethanol was then evaporated from the samples using a SpeedVac. Dry, pelleted conidia were re-suspended in 100 µL of pure, white, light mineral oil by vortexing. Conidia were counted in the 25 innermost squares of a hemocytometer. The number of spores per sample and the number of spores per pustule were then calculated for each isolate as follows:

Spores per sample = (number of counted spores) x 100,000

Spores per pustule = (spores per sample) / (number of pustules collected)

The 100,000 in the first formula accounts for both the hemocytometer and the 100 µL suspension volume. The mean number of spores per pustule for all five collected samples and the standard error of that mean were calculated. In a few cases isolates died before being evaluated for fitness, such that sporulation data was collected for a total of 364 isolates out of the 382 total isolates.

2.2.4 Effective concentration calculations. Leaf segment ratings were used to calculate relative EC_{50} values, where EC_{50} is defined as a parameter estimate of the effective fungicide concentration at which an isolate's growth is inhibited by 50% (Noel et al. 2018). For this calculation, leaf ratings normalized by 3, where a rating of 3 represented full, uninhibited growth, acted as the dependent variable and the natural logarithm of the fungicide concentration for each rating was the independent variable. EC_{50} values along with standard errors were calculated in SAS 9.4 using a nonlinear mixed model (PROC NLMIXED), where normalized leaf ratings, as a function of fungicide concentration, followed a logistic curve and the random variation around the predicted curve followed a Gaussian distribution (Kiernan et al. 2012). The logistic curve equation was given by $y_i = \dfrac{1}{1+\left(\dfrac{1}{b_o}-1\right)\exp\left(-b_1 conc\right)}$, and the EC_{50} was calculated as $estimate\ EC50 = \dfrac{\log\left(\dfrac{1}{\dfrac{1}{b_o}-1}\right)}{-b_1}$. This model required that b_0 be greater than zero and b_1 be negative. Most EC_{50} value estimates were calculated using the SAS NLMIXED model with default settings. However, approximately 40 prothioconazole EC_{50} values required fine- tuning of the settings by increasing the maximum number of iterations, changing the integration

method, or changing the optimization technique. When using the default settings, these EC_{50} values were estimated with low precision and were not significantly different from zero. These estimated values were found to fall in gaps between two measured fungicide concentrations (e.g. between 40 and 80 mg L^{-1} prothioconazole), suggesting that additional concentrations may help increase the precision of estimates in these cases.

Reproducibility of the assay was verified by repeating it once each for 50 isolates for tebuconazole and 47 isolates for prothioconazole (Appendix A: Supplementary Figure 2.3). The calculated EC_{50} values of the analytical replicates were ranked and converted into normal quantiles (on a 0-to-1 scale) using the SAS 9.4 (Cary, NC) RANK procedure; because of skew, the Blom rank transformation option was applied to normalize the EC_{50} values and stabilize variances. The CORR procedure was then used to evaluate the rank concordance of the two replicates. For both DMIs, significant correlation coefficients confirmed that the assay was able to rank isolates reproducibly across the EC_{50} range (Appendix A: Supplementary Figure 2.3; tebuconazole: $P < 0.0001$, Spearman $S = 0.66$; prothioconazole: $P = 0.0001$, $S = 0.54$).

2.2.5 Geographical comparisons. EC_{50} values were compared at the three hierarchical geographic sampling levels (region, state, and field) for each fungicide using a generalized linear mixed model (PROC GLIMMIX), a log normal distribution, and the Kenward-Roger method for calculating denominator degrees of freedom in the testing of Type III hypotheses of no effect. Tebuconazole and prothioconazole EC_{50} values were the dependent variables, while region, state nested within region, and field nested within state and region were the independent variables. Collections from multiple fields were not available in all 15 states; however, both state- and field-level variables were included to account for variation among fields when evaluating at the field level. States represented by a single field did not contribute to the estimation of the effect of

field nested within state and region. Least square mean estimates of EC_{50} values were compared at each of the three sampling levels per DMI fungicide. Sporulation values were compared using the same PROC GLIMMIX model with spores per pustule as the dependent variable and region, state, and field as independent variables.

The possible presence of cross-resistance to tebuconazole and prothioconazole (isolates having diminished sensitivity to both DMIs), as well as correlation of EC_{50} of each fungicide with sporulation, were evaluated using a general linear model (PROC GLM) multivariate analysis of variance. In that analysis, region, state nested within region, and field nested within state and region were the experimental factors. EC_{50} values and spores per pustule, the dependent variables, were log-transformed to improve homogeneity of variances, and the experimental factors were used to determine the residuals for each dependent variable and calculate the partial correlation between the sets of residuals. Partial correlation coefficients from the error terms were used for correlation analysis after accounting for the experimental effects.

2.3 Results

2.3.1 Range in EC_{50} value compared to European controls. Observed tebuconazole EC_{50} values for the U.S. isolates ranged from 0.12 (± 0.02) mg L^{-1} to 18.7 (± 3.0) mg L^{-1}, a 155-fold difference in tebuconazole sensitivity, with a median value of 1.2 mg L^{-1} (Appendix A: Supplementary Figure 2.4A). The two European control isolates ranked as expected relative to each other, but with only a 2-fold difference in tebuconazole sensitivity: the DMI-resistant isolate Fel09 had a high EC_{50} value relative to the observed U.S. sensitivity range at 13.9 (± 1.0) mg L^{-1}, while DMI-sensitive isolate JIW11 had an EC_{50} of 7.1 (± 1.3) mg L^{-1}. Both European isolates were above the U.S. median, as well as the U.S. third quartile value of 2.4 mg L^{-1}.

Among the U.S. isolates, observed prothioconazole EC_{50} values ranged from 0.19 (± 0.09) mg L^{-1} to 295.7 (± 86.2) mg L^{-1}, a 1,556-fold difference with a median value of 32.6 mg L^{-1} (Appendix A: Supplementary Figure 2.4B). Again, there was a much smaller (58-fold) difference in prothioconazole sensitivity between the two European control isolates, although these isolates ranked as expected: DMI-resistant isolate Fel09 had a prothioconazole EC_{50} value of 463.0 (± 37.6) mg L^{-1}, while DMI-sensitive isolate JIW11 had an EC_{50} of 8.0 (± 4.8) mg L^{-1}. The Fel09 value exceeded the observed prothioconazole U.S. range, while the value for JIW11 was below the first quartile, 16.3 mg L^{-1}.

2.3.2 Geographic differences in DMI sensitivity. Significant differences in mean regional EC_{50} values were detected for both DMIs (Figure 2.2). A higher EC_{50} value indicates a reduction in fungicide sensitivity. Eastern regional populations (Mid-Atlantic, Southeast, and Great Lakes) had significantly higher mean tebuconazole EC_{50} values than central regional populations (AR & MO and Plains) ($P < 0.0001$) (Figure 2.2A). Among the eastern populations, the Mid-Atlantic and Southeast regions were also significantly higher in EC_{50} than the Great Lakes region. Isolates from Georgia, New York, and Mississippi exhibited a large and significant reduction in tebuconazole sensitivity (Table 2.2). Isolates from North Carolina and Virginia also had reduced sensitivity as compared to isolates originating from the most sensitive states Michigan, Nebraska, Oklahoma, and Missouri ($P < 0.0001$) (Table 2.2). At the field level, the most tebuconazole-insensitive populations were sampled from fields 11 and 12 in Georgia, field 19 in New York, and field 27 in North Carolina (Table 2.3). Isolates collected from field 6 in Missouri, field 1 in Nebraska, and fields 4 and 5 in Oklahoma were most sensitive to tebuconazole ($P < 0.0001$).

Within the geographic regions, there were some significant differences in tebuconazole sensitivity between states within a region (Table 2.2) and between fields within a state (Table 2.3). The differences were overall less in the central U.S. states: Arkansas isolates exhibited significantly more insensitivity than Missouri isolates, and there were no significant differences among the Plains states of Kansas, Nebraska and Oklahoma (Table 2.2) or among the three Oklahoma fields (Table 2.3). By contrast, in the Southeast region, Georgia isolates were more tebuconazole-insensitive than Florida and Alabama isolates, while Mississippi isolates were more insensitive Florida isolates. Within Georgia, fields 11 and 12 were significantly more insensitive than field 13. In the Great Lakes region, New York isolates were significantly more insensitive than Ohio, Pennsylvania, and Michigan isolates and there were several field-to-field differences within New York and Ohio. Mid-Atlantic states were not significantly different from each other; however, within North Carolina, field 27 was significantly more tebuconazole-insensitive than all other North Carolina fields.

The same east-west difference observed for tebuconazole sensitivity was detected in regional mean prothioconazole EC_{50} values ($P = 0.0001$) (Figure 2.2B). Isolates from the Great Lakes, Mid-Atlantic, and Southeast regions had significantly higher EC_{50} values than those from the AR-MO region, while isolates from the Plains were significantly more sensitive than Great Lakes and Mid-Atlantic isolates (Figure 2.2B). Reduced prothioconazole sensitivity was prominent in Georgia, New York, and North Carolina as well as Ohio, Arkansas, and Kansas ($P < 0.0001$) (Table 2.2). Much like tebuconazole, state populations most sensitive to prothioconazole were in Oklahoma, Nebraska, and Missouri. As with tebuconazole, Georgia fields 11 and 12 and New York field 19 exhibited reduction significant reduction in sensitivity to prothioconazole (Table 2.3) as compared to the most sensitive fields. In this case, they were

joined by New York fields 18 and 20, Ohio field 16, and North Carolina field 26. The fields most sensitive to prothioconazole were again Missouri field 6, Nebraska field 1, and Oklahoma fields 4 and 5, as well as New York field 17 ($P = 0.0013$).

There were also differences in prothioconazole sensitivity within regions (Table 2.2) and states (Table 2.3). In the Plains region, Kansas was significantly more insensitive than Nebraska and Oklahoma, while no Oklahoma fields were significantly different from one another. As with tebuconazole, Arkansas isolates exhibited significantly more prothioconazole insensitivity than Missouri isolates and, in the Southeast, Georgia isolates were significantly more insensitive than Florida and Alabama isolates. In Georgia, fields 11 and 12 were more insensitive than field 13 and in North Carolina field 26 was significantly more insensitive than field 25. In the Great Lakes, New York and Ohio isolates were significantly more prothioconazole-insensitive than Michigan isolates, while Mid-Atlantic states were not significantly different from each other.

2.3.3 Sporulation variability and relationship to DMI sensitivity. Sporulation was successfully quantified for 364 of the U.S. isolates screened for DMI sensitivity. Among isolates, sporulation ranged from 1,000 spores per pustule, the lower limit of the spore quantification method, to 636,658 spores per pustule. Geographic differences in sporulation were significant ($P = 0.0014$) at the state level (Table 2.2). On average, isolates from New York, Mississippi, and Nebraska produced significantly more spores per pustule than isolates from Georgia, Virginia, Pennsylvania, North Carolina, and Ohio. Sporulation was not significantly different at the regional ($P = 0.12$) or field ($P = 0.38$) levels (data not shown).

At the geographic level of state, where sporulation differed significantly, the correlation of sporulation to prothioconazole sensitivity was significant but weak (Figure 2.3). State mean prothioconazole EC_{50} values were significantly negatively correlated with state mean spores per

pustule, but with a low *r* value of -0.12 ($P = 0.03$). The same relationship was found to be significant at the field level (n = 27, $r = -0.15$, $P = 0.01$) but was not significant at the regional (n = 5, $r = -0.08$, $P = 0.15$) or isolate (n = 345, $r = -0.02$, $P = 0.89$) levels (data not shown). No significant relationships between tebuconazole EC_{50} values and sporulation were identified.

2.3.4 Cross-resistance to DMIs. The results showed evidence of cross-resistance to tebuconazole and prothioconazole (Figure 2.4). The EC_{50} values for the two DMIs were significantly associated at all four geographic levels: isolate (n = 345, $r = 0.36$, $P = 0.0344$), field (n = 27, $r = 0.46$, $P < 0.0001$), state (n = 12, $r = 0.47$, $P < 0.0001$), and region (n = 5, $r = 0.53$, $P < 0.0001$).

2.4 Discussion

Along with resistant wheat cultivars and another chemistry class (quinone outside inhibitors), DMI fungicides are presently one of just three tools for controlling epidemics of *B. graminis* f. sp. *tritici*. In the U.S., epidemics of wheat powdery mildew have expanded to new areas of the country in recent years, where commercial varieties tend to be susceptible because resistance to the disease has historically not been a breeding priority. Taken together, these facts highlight the need to quantify the current efficacy of, monitor, and protect available wheat powdery mildew control measures. However, although DMI fungicides have been registered on wheat for over 20 years in the U.S., this is the first study to monitor the sensitivity of the U.S. *B. graminis* f. sp. *tritici* population to any members of this chemistry class.

Our results reveal regional differences in sensitivity; however, without an earlier baseline of U.S. *B. graminis* f. sp. *tritici* DMI sensitivity for comparison, we can only infer that local populations with reduced sensitivity may be undergoing a shift toward reduced sensitivity due to

fungicide use. That is the case for populations of the fungus in Europe. Multiple applications of DMI fungicides per year have resulted in widespread DMI resistance in Europe. In comparison to the sensitivity of *B. graminis* f. sp. *tritici* populations in Europe, no portion of the U.S. population is strongly insensitive to either fungicide tested here, and the focus now should be on preserving the DMI efficacy that remains.

We observed a large range in *B. graminis* f. sp. *tritici* sensitivity to both tebuconazole and prothioconazole across the central and eastern U.S. The east-west divide in sensitivity to both DMIs is further evidence that *B. graminis* f. sp. *tritici* in the U.S. is not one large random-mating population, but instead consists of multiple sub-populations with isolate migration only occurring from west to east (Cowger et al. 2016). The same regional pattern was observed in virulence to wheat *Pm*-genes in a study that included the isolates used here (Cowger et al. 2018).

The DMI sensitivity that we identified in the western part of our sampling area was as hypothesized. However, we expected DMI insensitivity to be maximal in the Mid-Atlantic region due to the greater frequency of epidemics there, and instead, reduced sensitivity spanned the entire eastern U.S. wheat-growing area. This can be partially explained by the previously observed genetic similarity of the Mid-Atlantic and Great Lakes *B. graminis* f. sp. *tritici* populations (Cowger et al. 2016). Together with previous findings on migration patterns of U.S. *B. graminis* f. sp. *tritici* (Cowger et al. 2016), our results suggest that the *B. graminis* f. sp. *tritici* populations of the Plains and AR-MO regions will likely remain sensitive to DMI fungicides due to migration, as sensitive isolates may migrate from there to the Southeast, Great Lakes, and Mid-Atlantic regions, but less sensitive isolates should not migrate from those areas toward the west.

Although regional populations did have characteristic and significantly different DMI sensitivity levels, there were also significant differences between states in a region and between fields in a state. This was least apparent in the Plains where genetic diversity for DMI sensitivity is relatively low and fungicide applications to wheat are less frequent than in the eastern U.S. By contrast, there were more sensitivity differences within other regions. For example, significant differences in sensitivity to both DMIs were observed between fields in Georgia and North Carolina, where collection sites were within 175 and 50 miles, respectively, of each other. These local differences support the isolation-by-distance model of many small, separate yet intermixing *B. graminis* f. sp. *tritici* populations in the U.S. (Cowger et al. 2016). Altogether, our results suggest there is greater diversity in DMI sensitivity in the eastern U.S. states, providing the opportunity for more rapid gain in insensitivity through selection.

Our findings suggest that central U.S. *B. graminis* f. sp. *tritici* populations have had relatively less exposure to tebuconazole and prothioconazole. More frequent exposure is likely occurring in eastern populations, particularly those in New York, North Carolina, and Georgia fields, and producing a selective effect in the form of reduced sensitivity. The regional differences observed here are consistent with fungicide application data collected by the United States Geological Survey (USGS). Over the years of tebuconazole use, heavy application across all crops was reported in southern Alabama, Georgia, Florida, North Carolina, southern Virginia, western New York, and Ohio (Thelin and Stone 2013, Baker and Stone 2015). This fits with the reduction in tebuconazole sensitivity observed in our study. However, tebuconazole use in Kansas and Oklahoma increased greatly in 2010-2012 (Baker and Stone 2015) and in preliminary data in 2013 and 2014 the years leading up to collection of the present sample (http://water.usgs.gov/nawqa/pnsp/usage/maps/show_map.php?year=2013&map=TEBUCONA

ZOLE&hilo=L). This increase was not reflected in the performance of our *B. graminis* f. sp. *tritici* isolates from those Great Plains states. Due to low wheat powdery mildew incidence in these states during collection years 2013 and 2014, we had relatively few fields and isolates to represent the Plains region. Perhaps a more extensive sample in the future might reflect the effects of this increase in tebuconazole use. In the USGS fungicide use survey, prothioconazole use across crops was highest in Georgia, Florida, North Carolina, Virginia, and Ohio (Thelin and Stone 2013, Baker and Stone 2015). This is consistent with the decrease in prothioconazole sensitivity our study found in Georgia, North Carolina, and Ohio.

A degree of cross-resistance was suggested by the correlation of tebuconazole and prothioconazole EC_{50} values at all geographic levels. Though significant, the association was weak; indeed, several outlier isolates exhibited highly reduced sensitivity to one of the fungicides while remaining fully sensitive to the other. These results suggest that *B. graminis* f. sp. *tritici* adapts to overcome DMIs via two or more different mechanisms. In this case, there may be a shared pathway that increases both tebuconazole and prothioconazole insensitivity, but also one or more additional mechanisms that specifically enhance resistance to one of the active ingredients.

Tebuconazole and prothioconazole molecules likely do not interact with *B. graminis* f. sp. *tritici* using the same mechanism, as prothioconazole itself is a triazolinthione, while tebuconazole is a triazole. The antifungal effects of prothioconazole are due to its primary metabolite, prothioconazole-desthio (Parker et al. 2013). Prothioconazole-desthio is a true triazole and binds the same target protein in the ergosterol synthesis pathway as other DMIs (Parker et al. 2013, Price et al. 2015). Perhaps this chemical difference in the two DMIs requires *B. graminis* f. sp. *tritici* to utilize multiple pathways to become insensitive to both fungicides.

Despite this chemical difference and the ten-year difference in registration in the U.S., the geographic and individual patterns of insensitivity to tebuconazole and prothioconazole are similar. This finding was surprising, as we expected more insensitivity to the older product, tebuconazole, than the newer prothioconazole. Given that cross-resistance is present, the years of tebuconazole use (and perhaps the use of other older DMI fungicides) may have primed *B. graminis* f. sp. *tritici* isolates for reduced prothioconazole sensitivity before the latter was ever applied. However, without a true baseline for *B. graminis* f. sp. *tritici* prothioconazole sensitivity at the start of its commercial use on U.S. wheat, there is no way to know whether this priming hypothesis is correct.

In gaining the ability to overcome a fungicide, pathogen isolates may pay a fitness cost. Fitness is complex, being determined by many factors; therefore, we expected to observe a large variation in sporulation among isolates, and we did. Among states and isolates, sporulation was significantly associated with sensitivity to prothioconazole, but with very low correlation values. This weak relationship was presumably due to the influence of variables other than DMI sensitivity on sporulation capacity. Thus, the effect of reduced prothioconazole sensitivity on *B. graminis* f. sp. *tritici* fitness, if any, appears to be very small in proportion to overall isolate-to-isolate variation. Isolates from Georgia, Virginia, North Carolina, and Ohio exhibited reduced DMI sensitivity and relatively low spore production, but isolates from other states that also had reduced prothioconazole sensitivity, such as New York and Mississippi, had very high spore production (Table 2.2). This indicates that prothioconazole insensitivity in *B. graminis* f. sp. *tritici* is not necessarily accompanied by a measurable reduction in fitness, or the effect is so mild that it is overshadowed by other fitness determinants. Of course, other fitness components not assayed in this study could be affected by reduced DMI sensitivity.

Tebuconazole EC_{50} values did not significantly correlate with spore production at any geographic level of analysis, indicating that tebuconazole insensitivity does not engender a fitness cost associated with sporulation in U.S. *B. graminis* f. sp. *tritici* populations. Since tebuconazole has been applied to U.S. wheat for ten more years than prothioconazole, perhaps tebuconazole insensitivity was originally accompanied by a fitness reduction that later compensatory mutations allowed *B. graminis* f. sp. *tritici* populations to overcome while maintaining tebuconazole insensitivity.

The European *B. graminis* f. sp. *tritici* isolates Fel09 and JIW11 were used as DMI-insensitive and DMI-sensitive isolates, respectively, for comparison to U.S. isolates. While no prior information was available on their sensitivity to tebuconazole and prothioconazole, both isolates had been included in a study evaluating triadimenol and propiconazole sensitivity along with sequencing to identify the genetic basis of DMI insensitivity (Wyand and Brown 2005). Unsurprisingly, Fel09 ranked among the most insensitive U.S. isolates for both DMIs, actually exceeding the highest prothioconazole EC_{50} value observed in the U.S. collection. JIW11 exhibited a relatively low prothioconazole EC_{50} value when compared to the range of U.S. values but, surprisingly, performed similarly to the most tebuconazole-insensitive U.S isolates. These findings suggest that on the whole, the present U.S. *B. graminis* f. sp. *tritici* population has remained more sensitive to DMIs than European populations were in the mid-1980s, especially to tebuconazole. This makes sense, given the lower per-hectare application of fungicides to cereals in the U.S. compared to the UK and Europe.

In summary, we found evidence of reduced DMI sensitivity in eastern U.S. *B. graminis* f. sp. *tritici*, probably the result of selection, as well as evidence of a degree of DMI cross-resistance. While there was some indication of lower sporulation in populations with reduced

prothioconazole sensitivity, the effect was very small, and therefore unlikely to substantially affect reproduction of prothioconazole-insensitive isolates. The *B. graminis* f. sp. *tritici* population of the eastern U.S. soft wheat region appears to be at particular risk of evolving even greater DMI insensitivity. These results support a strategy of chemistry rotation for control of common fungal diseases in eastern U.S. wheat and crops rotated with it. To preserve current DMI efficacy, U.S. wheat growers should continue to rotate DMIs with other chemistries, and use mixed mode of action fungicide products, to lessen the selection pressure imposed on the *B. graminis* f. sp. *tritici* population (van den Bosch et al. 2014).

Moreover, with a high potential for evolution in *B. graminis* f. sp. *tritici*, and evidence of fungicide resistance in European *B. graminis* populations starting more than 20 years ago, the U.S. *B. graminis* f. sp. *tritici* population should be regularly evaluated for fungicide efficacy. The results of this study can be used as a basis against which to compare DMI sensitivity of future U.S. collections of *B. graminis* f. sp. *tritici* isolates. This first-ever screening of DMI fungicide sensitivity in U.S. *B. graminis* f. sp. *tritici* isolates has demonstrated the need for fungicide recommendations to be made on a regional rather than national level, and for greater attention to chemistry rotation and mixing. The earlier that insensitivity is detected in a fungal pathogen population and adjustments to application regimes are made, the more the development of resistance can be slowed (Zulak et al. 2018).

Understanding the molecular basis of DMI insensitivity is important for helping prevent the development of full DMI resistance. Mutations correlating with DMI insensitivity have been identified in both European and Australian *B. graminis* isolate populations. DMIs slow fungal growth by occupying the active site of the 14α-demethylase enzyme (CYP51) and thus not allowing enough ergosterol to be created to support fungal growth (Gisi et al. 2000). For *B.*

graminis f. sp. *tritici,* the *CYP51* gene of DMI-insensitive isolate Fel09 was sequenced by Wyand and Brown (2005) and found to have a Y136F amino acid mutation that was not found in *CYP51* of the DMI-sensitive isolate, JIW11. Y136F is the most common mutation associated with DMI resistance in many fungi and is the only *CYP51* mutation that has been documented in *B. graminis* f. sp. *tritici* thus far. DMI-insensitive *B. graminis* f. sp. *hordei* isolates were found to have the Y136F mutation as well as a K147Q mutation (Wyand and Brown 2005). More recently, an S509T mutation in *CYP51* was found, always in combination with Y136F, in DMI-insensitive Australian *B. graminis* f. sp. *hordei* isolates (Tucker et al. 2015). *B. graminis* f. sp. *hordei* isolates with the S509T mutation exhibited the greatest insensitivity to tebuconazole in particular (Tucker et al. 2015), whereas an S524T mutation (homologous to S509T in *B. graminis*) in the *CYP51* gene of another wheat pathogen, *Zymoseptoria tritici*, was more closely associated specifically with prothioconazole insensitivity (Cools and Fraaije 2013).

Although the Y136F and S509T mutations are common causes of DMI insensitivity in phytopathogens, many other genetic abnormalities have been linked to DMI resistance. Various influential single-site mutations have been identified in the *CYP51* gene of *Z. tritici* including L50S, D134G, V136A, and Y461S (Cools et al. 2011, Becher and Wirsel 2012, Cools and Fraaije 2013). Other causes besides SNPs in *CYP51* include an insertion in the promoter region of *CYP51* (Hamamoto et al. 2000, Schnabel and Jones 2001, Ma et al. 2006, Garcia-Effron et al. 2008, Villani et al. 2016), which is sometimes linked to overexpression of *CYP51* (Hamamoto et al. 2000, Ma et al. 2006, Villani et al. 2016, Rallos and Baudoin 2016), as well as overexpression of efflux drug transporters like ATP-binding cassette (ABC) transporters and major facilitator superfamily (MFS) pumps (Cannon et al. 2009, Leroux and Walker 2011).

With these possible mechanisms of reduced DMI sensitivity in mind, the U.S. *B. graminis* f. sp. *tritici* collection used in this phenotypic fungicide sensitivity study is currently under genetic analysis. Efforts focus on identifying mutations in *CYP51* or expression differences that may be correlated with reduced sensitivity to tebuconazole and prothioconazole.

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Table 2.1. Isolates of *Blumeria graminis* f. sp. *tritici* collected during 2013 and 2014 in five United States wheat-growing regions and assayed for tebuconazole sensitivity, prothioconazole sensitivity, and sporulation

Region	Year	Field[a]	State	Town	Cultivar	N[b]
Plains	2014	1	Nebraska (NE)	Lincoln	Unknown	13
Plains	2014	2	Kansas (KS)	Manhattan	2137	19
Plains	2013	3	Oklahoma (OK)	Hinton	Jackpot	7
Plains	2013	4	Oklahoma (OK)	Stillwater	OK Bullet	10
Plains	2014	5	Oklahoma (OK)	Stillwater	Pete	14
AR-MO	2014	6	Missouri (MO)	Bronaugh	MFA2525	15
AR-MO	2014	7	Arkansas (AR)	Fayetteville	Ricochet	13
Southeast	2013	8	Mississippi (MS)	Greenwood	Coker 9553/Armor Ricochet	20
Southeast	2014	9	Alabama (AL)	Athens	Unknown	18
Southeast	2014	10	Florida (FL)	Gretna	AGS2060	18
Southeast	2013	11	Georgia (GA)	Pine Mountain	Magnolia	26
Southeast	2014	12	Georgia (GA)	Thomasville	AGS2060	14
Southeast	2014	13	Georgia (GA)	Sandersville	AGS2000	12
Great Lakes	2014	14	Michigan (MI)	Rogers City	MSU-D6234	12
Great Lakes	2013	15	Ohio (OH)	Jeromesville	Croplan 8309	20
Great Lakes	2014	16	Ohio (OH)	Wooster	Bravo	14
Great Lakes	2013	17	New York (NY)	Brockport	Otsego	9
Great Lakes	2013	18	New York (NY)	Aurora	Pioneer 25R34	5
Great Lakes	2014	19	New York (NY)	Brockport	Otsego	17
Great Lakes	2014	20	New York (NY)	Groveland	Otsego	13
Great Lakes	2013	21	Pennsylvania (PA)	Pennsylvania Furnace	Growmark 820	18
Great Lakes	2014	22	Pennsylvania (PA)	Pennsylvania Furnace	Chemgro DH75	14
Mid-Atlantic	2013	23	Virginia (VA)	Saluda	Southern States 8404	12
Mid-Atlantic	2013	24	North Carolina (NC)	Four Oaks	Featherstone	12
Mid-Atlantic	2013	25	North Carolina (NC)	Calypso	Coker Oakes	15
Mid-Atlantic	2014	26	North Carolina (NC)	Clayton	USG 3120	15
Mid-Atlantic	2014	27	North Carolina (NC)	Mt. Olive	Coker Oakes	11

[a]Approximate field locations are shown in Figure 2.1.

[b]N = maximum number of isolates assayed per field. For 16 field*assay combinations, there were missing data for 1 to 4 isolates, such that a total of 378 isolates were analyzed for tebuconazole sensitivity, 369 for prothioconazole sensitivity, and 364 for sporulation.

Table 2.2. Mean tebuconazole and prothioconazole sensitivities and sporulation of *Blumeria graminis* f. sp. *tritici* isolates sampled in 2013-2014 from 15 U.S. states[w]

		EC_{50}[x]		
State	**N**[y]	Tebuconazole	Prothioconazole	**Sporulation**[z]
Georgia (GA)	51	2.52 a	44.74 ab	25,132 a
New York (NY)	42	2.01 ab	48.18 a	64,854 cd
Mississippi (MS)	20	1.82 abc	28.67 bcd	72,446 cd
North Carolina (NC)	53	1.66 bc	39.80 ab	37,718 ab
Virginia (VA)	12	1.36 bcd	26.51 abcde	30,209 ab
Alabama (AL)	18	1.20 cde	22.05 cde	61,445 bcd
Ohio (OH)	35	0.97 def	46.25 ab	38,330 ab
Pennsylvania (PA)	32	0.96 def	30.04 abcd	37,142 ab
Arkansas (AR)	13	0.89 def	42.81 abc	49,183 abcd
Florida (FL)	18	0.86 def	23.26 cde	56,562 bcd
Kansas (KS)	19	0.75 defg	36.35 abc	42,719 abc
Michigan (MI)	12	0.65 efg	20.01 cde	51,606 bcd
Nebraska (NE)	13	0.57 fg	14.84 e	98,037 d
Oklahoma (OK)	31	0.49 g	20.04 de	53,162 bcd
Missouri (MO)	15	0.45 g	5.12 f	57,240 bcd

[w] Within a column, means followed by the same letter are not significantly different at $P < 0.05$ using a pairwise t-test.

[x] EC_{50} = effective concentration of fungicide at which isolate growth is decreased by 50%. Mean mg L^{-1} of fungicide active ingredient.

[y] N = maximum number of isolates assayed per state.

[z] Mean spores per pustule.

Table 2.3. Mean tebuconazole and prothioconazole phenotypic sensitivity values of *Blumeria graminis* f. sp. *tritici* isolates per field sampled in 2013-2014[x]

			EC_{50}[y]	
State	**Field**	**N[z]**	Tebuconazole	Prothioconazole
Georgia (GA)	11	26	4.24 a	50.22 abc
North Carolina (NC)	27	11	3.50 ab	49.01 abc efg
New York (NY)	19	17	3.39 ab	76.14 ab
Georgia (GA)	12	13	3.13 abc	70.53 abc
New York (NY)	20	13	2.14 bcd	77.82 ab
Mississippi (MS)	8	19	1.82 cd	28.67 defghij
North Carolina (NC)	24	12	1.65 cde	39.18 abcdefgh
New York (NY)	18	5	1.52 bcdef	56.33 abcd
Ohio (OH)	16	15	1.51 de g	50.56 abc ef
New York (NY)	17	9	1.48 defg	16.15 ijk
North Carolina (NC)	26	15	1.47 de g	52.44 abc e
Virginia (VA)	23	12	1.36 de gh	26.51 defghijk
Pennsylvania (PA)	22	14	1.22 defgh	40.31 abcdefghi
Georgia (GA)	13	12	1.21 defgh	25.28 d fghijk
Alabama (AL)	9	18	1.20 defgh	22.05 hijk
Arkansas (AR)	7	13	0.89 efghi	42.81 abcdefgh
North Carolina (NC)	25	15	0.88 efghi	24.91 d ghijk
Florida (FL)	10	18	0.86 fghi	23.26 d ghijk
Kansas (KS)	2	19	0.75 f hij	36.35 cdefgh
Pennsylvania (PA)	21	18	0.75 f hij	22.38 d hijk
Michigan (MI)	14	12	0.65 f ij	20.01 hijk
Oklahoma (OK)	3	7	0.65 f hijk	29.80 bcdefghi k
Ohio (OH)	15	20	0.62 ij	42.30 abc efg
Nebraska (NE)	1	13	0.57 ijk	14.84 k
Oklahoma (OK)	4	10	0.54 ijk	16.99 ijk
Missouri (MO)	6	15	0.45 jk	5.12 l
Oklahoma (OK)	5	14	0.33 k	15.89 jk

[x] Within a column, means followed by the same letter are not significantly different at $P < 0.05$ using a pairwise t-test.

[y]EC_{50} = effective concentration of fungicide at which isolate growth is decreased by 50%. Mean mg L^{-1} of fungicide active ingredient.

[z]N = maximum number of isolates assayed per field.

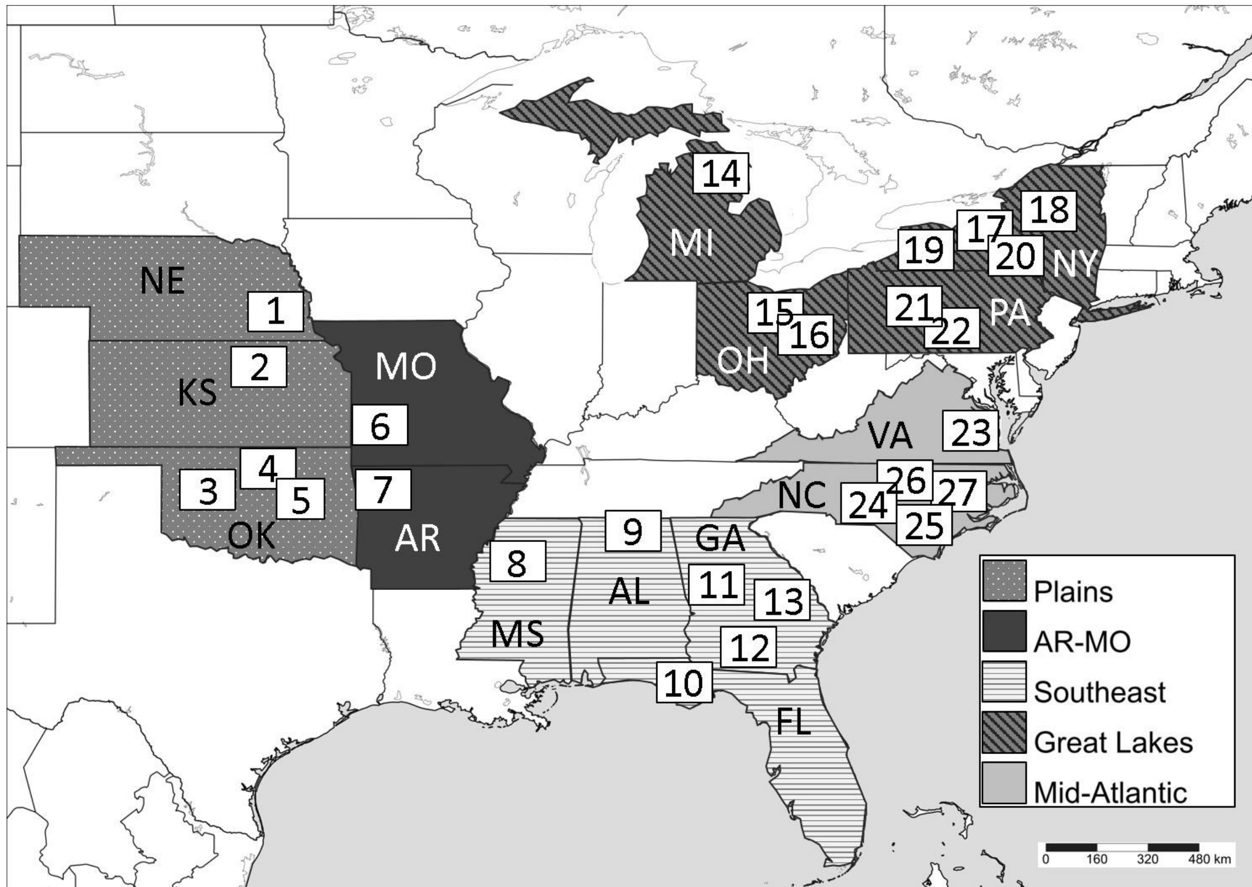

Figure 2.1. Map showing approximate locations where samples of wheat with powdery mildew were collected in 2013 and 2014 (numbers indicate fields; see Table 2.1 for details). States were grouped in five geographic regions based on wheat market class and previous population analyses (Cowger et al. 2016).

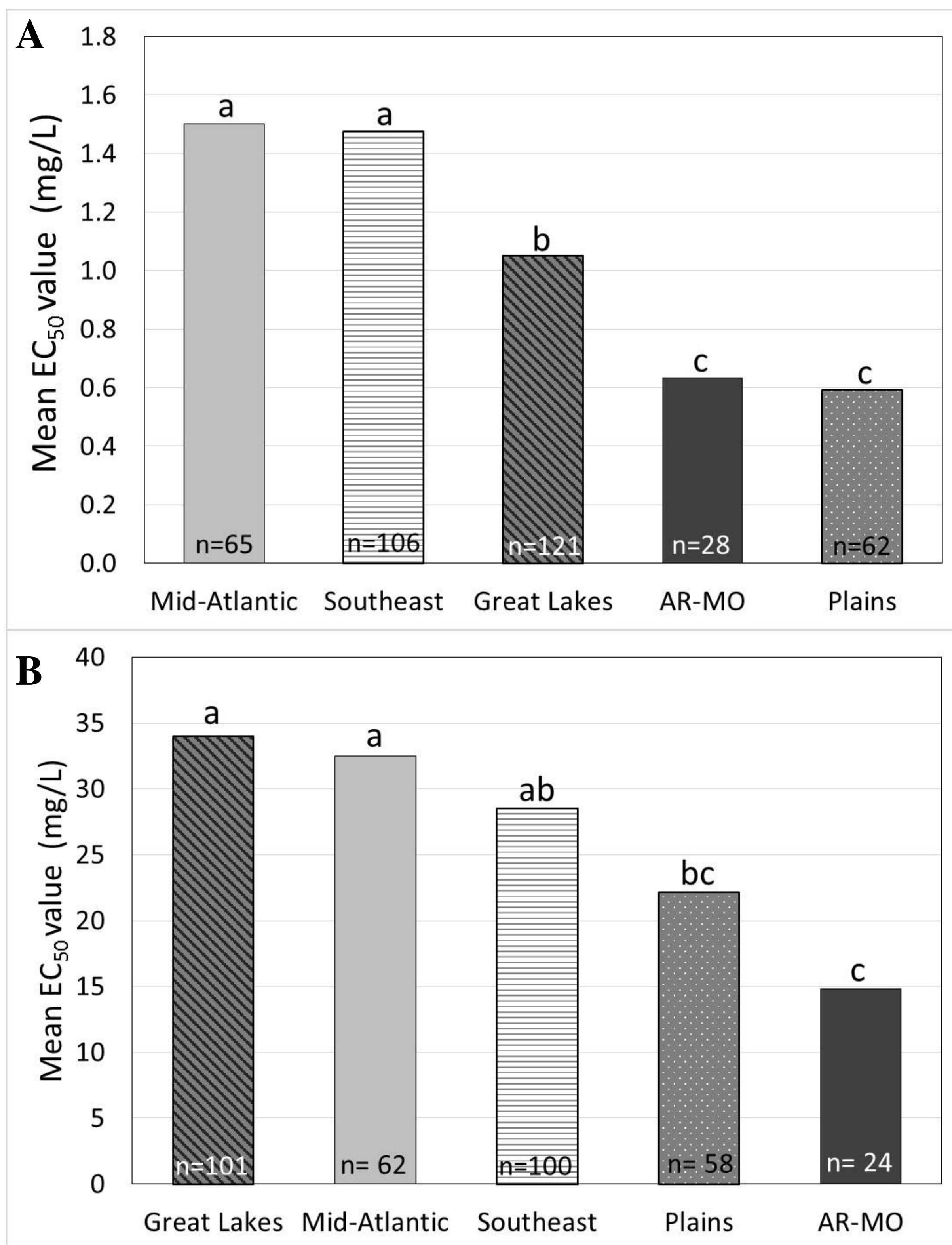

Figure 2.2. Regional mean EC_{50} values of tebuconazole (A) and prothioconazole (B). Sample sizes per region are shown in each bar. Bars topped by the same letter are not significantly different ($P < 0.05$) using a pairwise t-test.

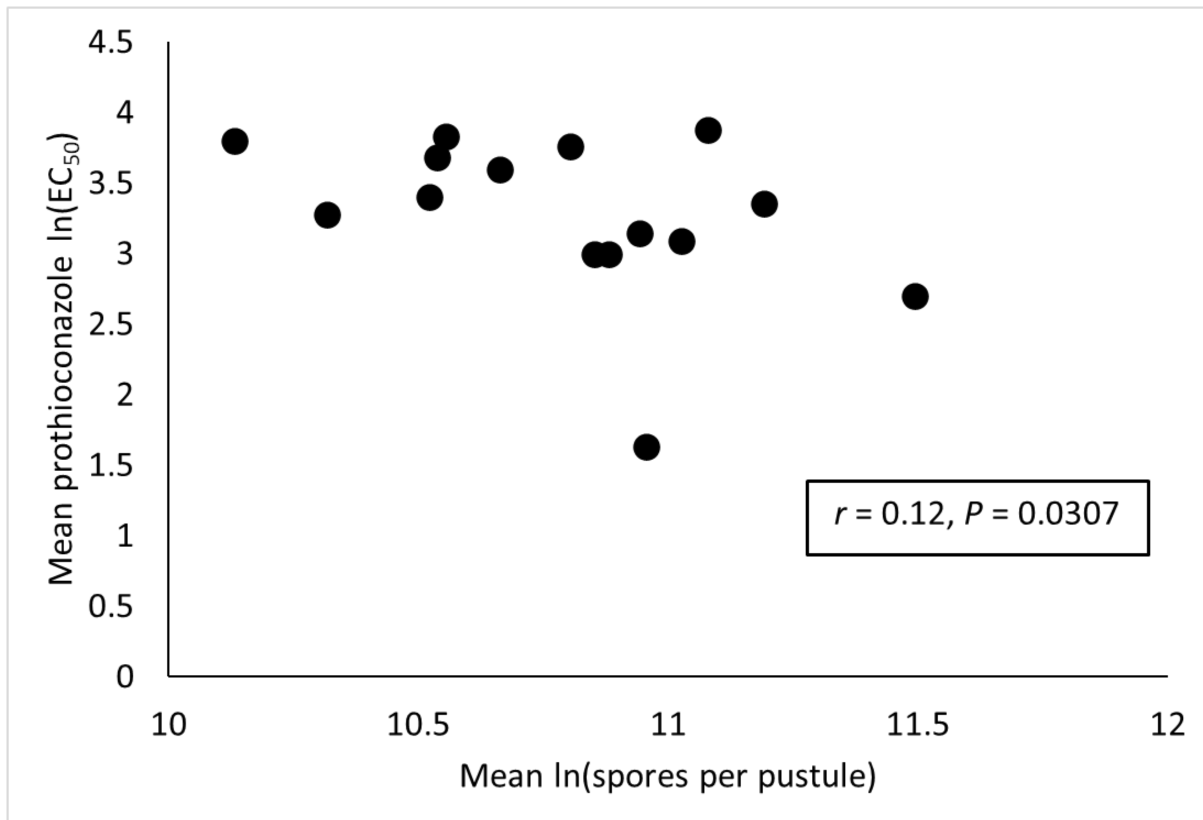

Figure 2.3. Correlation of mean log-transformed prothioconazole EC_{50} values with *Blumeria graminis* f. sp. *tritici* sporulation, a component of fitness; each data point is a state mean.

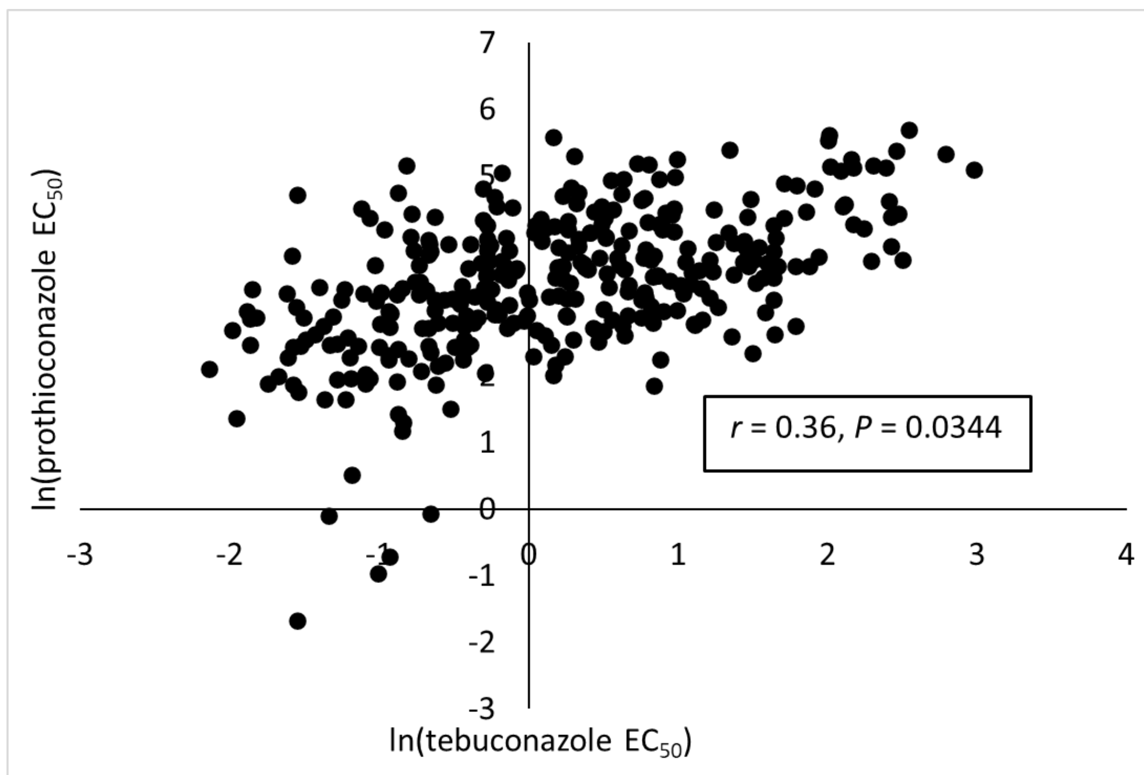

Figure 2.4 Correlation of log-transformed sensitivity (EC_{50}) to tebuconazole and prothioconazole in U.S. *Blumeria graminis* f. sp. *tritici* isolates.

CHAPTER 3

United States wheat powdery mildew population sensitive to QoI and SDHI fungicides

ABSTRACT

Wheat powdery mildew, caused by *Blumeria graminis* f. sp. *tritici*, is managed primarily with cultivar resistance and foliar fungicides in the United States. Formulated fungicide products from three mode-of-action categories are labelled for use on wheat in the United States: demethylation inhibitors (DMIs), quinone outside inhibitors (QoIs), and succinate dehydrogenase inhibitors (SDHIs). European populations of *B. graminis* f. sp. *tritici* are widely insensitive to DMI and QoI fungicides, and an early stage of reduced DMI sensitivity has been discovered in the U.S. population as well. Despite years of QoI use on U.S. wheat and the recent release of second-generation SDHI fungicides, the sensitivity of the U.S. population has not been evaluated to either mode of action. Approximately 375 *B. graminis* f. sp. *tritici* isolates collected from 15 states in the central and eastern U.S. were screened for sensitivity to QoIs pyraclostrobin and picoxystrobin along with the SDHI fluxapyroxad. A large range of sensitivity was observed to both QoIs; however, resistance factor values were, at maximum, 11.2 for pyraclostrobin and 5.3 for picoxystrobin, indicating only a relatively low level of insensitivity. Additionally, only synonymous mutations were found at the F129L, G137R, and G143A loci in cytochrome *b*. Thus, QoI insensitivity is not yet pronounced in the U.S. *B. graminis* f. sp. *tritici* population. A baseline for fluxapyroxad sensitivity was established which will allow comparison of future SDHI sensitivity levels.

3.1 Introduction

Wheat powdery mildew, caused by the fungal pathogen *Blumeria graminis* (DC.) Speer f. sp. *tritici* (Em. Marchal), can infect all aboveground parts of wheat (*Triticum aestivum*) worldwide and can decrease yield by 30% when the disease is severe (Johnson et al. 1979). This pathogen thrives in cool, humid weather and grain yield is most negatively impacted when *B. graminis* f. sp. *tritici* infections occur early in the growing season. *B. graminis* f. sp. *tritici* is among the plant pathogens with the highest potential for genetic adaptation due to its biological properties. It undergoes approximately annual sexual recombination, and engages in polycyclic asexual reproduction during the wheat growing season, with fluffy pustules bearing conidia that mature in only seven to ten days. The pathogen has a very large effective population size, given the thousands of asexual spores per pustule, and these spores are easily dispersed by wind (Meyers et al. 2019, Limpert et al. 1999). Altogether, these traits allow *B. graminis* f. sp. *tritici* to rapidly adapt to disease control strategies.

Available wheat powdery mildew control methods include resistant wheat varieties and foliar fungicide applications. More than 90 major powdery mildew (*Pm*) resistance alleles at more than 50 loci, plus additional quantitative trait loci, have been identified and introgressed into modern wheat varieties (McIntosh et al. 2013, Li et al. 2019). However, many *Pm* genes have been defeated across large geographic regions due to adaptation by *B. graminis* f. sp. *tritici*, especially when the genes are deployed in backgrounds lacking quantitative resistance (Parks et al. 2008, Cowger et al. 2018, Brown et al. 1990, Brown et al. 1991, Brown et al. 1993). Also, widespread use of susceptible cultivars can lead to severe powdery mildew epidemics in areas where the disease was not previously a problem. In either case, the result may be a reliance on fungicides for management.

Formulated fungicides from three chemical categories are currently labelled for application to control *B. graminis* f. sp. *tritici* in the United States (U.S.): demethylation inhibitors (DMIs), quinone outside inhibitors (QoIs), and succinate dehydrogenase inhibitors (SDHIs) (NCERA-184 2019). Unfortunately, repetitive applications of fungicides from the same category impose a selective pressure on *B. graminis* f. sp. *tritici*, resulting in loss of fungicide efficacy in some cases (Brent and Hollomon 2007). The Fungicide Resistance Action Committee (FRAC) ranks fungicide modes of action by their likelihood of selecting for resistance development. Since all three fungicide categories available for wheat powdery mildew control in the U.S. have single-site modes of action, they are all rated as having at least moderate risk of resistance (FRAC 2019). Thus, as risk is elevated due to both fungicide chemistries and pathogen biology, fungicide sensitivity monitoring becomes especially important.

Reduced efficacy of DMIs against *B. graminis* f. sp. *tritici* was identified 25-30 years ago in Europe after less than a decade of aggressive DMI applications (de Waard et al. 1986, Fletcher et al. 1987, Svec et al. 1995, Godet and Limpert 1998). Reduced DMI sensitivity was also recently observed in *B. graminis* f. sp. *tritici* in southeastern Australia (Lopez and Kay 2017). In the U.S., reduced DMI sensitivity has been detected in eastern U.S. *B. graminis* f. sp. *tritici* populations compared to their central U.S. counterparts (Meyers et al. 2019). This discovery has made it more urgent to investigate the status of the other two modes of action available for control of powdery mildew in wheat. For that reason, we turned our attention to understanding the current efficacy status of QoIs and establishing a sensitivity baseline for a new SDHI active ingredient in U.S. wheat.

Reduced QoI efficacy in *B. graminis* f. sp. *tritici* was identified in Europe approximately 20 years ago, after only 2 to 3 years of QoI use (Chin et al. 2001), and more recently in Australia

(Lopez and Kay 2017). *B. graminis* f. sp. *tritici* was one of the pathogens to most rapidly evolve to QoI insensitivity in Europe (Fisher et al. 2004). FRAC lists QoI fungicides in the highest risk category for evolution of resistance, since cross resistance often occurs across all QoI chemistries (FRAC 2019, Vincelli 2002). QoI fungicides target the quinol outer (Qo) oxidation site of the cytochrome bc_1 enzyme complex, and QoI-resistant plant pathogens often carry a G143A point mutation in the cytochrome *b* (*cytb*) gene (Fernandez-Ortuno et al. 2008). This mutation, a substitution of alanine for glycine at amino acid position 143, usually results in a high level of QoI insensitivity. It was detected in European QoI-resistant *B. graminis* f. sp. *tritici* isolates (Sierotzki et al. 2000), and has been found in several other powdery mildews (Fernandez-Ortuno et al. 2008). Two other *cytb* mutations (F129L and G137R) that confer a quantitative reduction in QoI sensitivity have been identified: F129L in *Pyricularia grisea*, *Pythium aphanidermatum*, *Pyrenophora teres*, and *Pyrenophora tritici-repentis;* and G137R only in *Pyrenophora tritici-repentis* (Gisi et al. 2002, Sierotzki et al. 2007).

QoI-resistant isolates with the G143A mutation often have resistance factors (RFs, ratios of isolate sensitivity compared to a standard sensitive isolate) of 100 or more (Sierotzki 2015). Isolates with F129L or G137R mutations often have much lower RFs; instead, these mutations have been found to have a greater influence on isolate fitness (Esser et al. 2004, Sierotkzi et al. 2007). In past studies, *B. graminis* f. sp. *tritici* isolates insensitive to QoIs and possessing the G143A mutation did not manifest reduced fitness (Heaney et al. 2000, Chin et al. 2001). In other pathogens, like *Venturia inaequalis,* G143A mutant isolates exhibited a decrease in cytochrome bc_1 enzyme activity resulting in slowed growth (Fisher et al. 2004).

Some plant pathogens have remained sensitive to QoIs despite aggressive applications of the products (Sierotzki 2015, Grasso et al. 2006). In these species, a type I intron immediately

following the 143 locus prevents the G143A mutation from evolving, as the results of splicing render the mutation-intron combination lethal. In addition to many rust pathogens, the type I intron has been detected in various ascomycetes (Sierotzki 2015), including *Alternaria solani* (Grasso et al. 2006), *Monilinia fructicola* (Luo et al. 2010), and *Fusicladium effusum* (Standish et al. 2016), but not in *B. graminis* (Grasso et al. 2006).

In some pathosystems, on the other hand, highly QoI-resistant isolates were found to lack the commonly found *cytb* mutations. This was the case for several isolates of *V. inaequalis* (Steinfeld et al. 2002), *Podosphaera fusca* (Fernandez-Ortuno et al. 2008), and *Botrytis cinerea* (Ishii et al. 2009). The G143A mutation was even detected in a few QoI-sensitive *B. cinerea* isolates, indicating that this mutation is not the only determining factor of QoI sensitivity level in that pathogen (Ishii et al. 2009). Because the influence of *cytb* alterations differs greatly between pathogens, evaluations of both the QoI sensitivity and fitness levels of *B. graminis* f. sp. *tritici* populations along with their *cytb* genotypes are important to consider given the likelihood of development of QoI insensitivity in this pathosystem.

Despite nearly 20 years of use of QoI fungicides on wheat in the U.S., QoI sensitivity in the U.S. *B. graminis* f. sp. *tritici* population has not been evaluated phenotypically or genotypically. Since a potential breakdown of QoI efficacy could occur quickly, understanding the current QoI sensitivity of the U.S. *B. graminis* f. sp. *tritici* population is both important in its own right and may help wheat growers and their advisors tailor a resistance management plan to optimize remaining efficacy across fungicide classes.

Second-generation SDHI chemistries are becoming an additional option for wheat powdery mildew control. SDHI fungicides are rated at moderate to high risk for resistance by FRAC (FRAC 2019). Fungicides in this chemical category target the succinate dehydrogenase

(sdh) enzyme in the electron transport chain (FRAC 2019). In other fungi, mutations for SDHI insensitivity have been found on the B, C, and D subunits of *sdh* (FRAC SDHI Working Group 2015). However, neither reduced SDHI efficacy nor *sdh* mutations have been discovered in *B. graminis* f. sp. *tritici* worldwide (FRAC SDHI Working Group 2019, Graf et al. 2017).

The second-generation SDHI fluxapyroxad was registered for use on wheat in the U.S. in 2012 (FAO 2013a). In order to establish a proper fungicide baseline to use in future studies, a survey of the sensitivity of the fungal population at the time of release of a new fungicide active ingredient is necessary (Russel 2004). The U.S. *B. graminis* f. sp. *tritici* isolates evaluated for DMI sensitivity (Meyers et al. 2019) were collected in 2013 and 2014, making them a useful resource for determining the baseline fluxapyroxad sensitivity level in this population.

Here, we report how this collection of approximately 375 *B. graminis* f. sp. *tritici* isolates from 15 U.S. states was utilized to estimate current sensitivity levels to two QoI fungicides, pyraclostrobin and picoxystrobin, and establish a baseline of sensitivity to fluxapyroxad. In the U.S., wheat powdery mildew epidemics have greater incidence and severity in the eastern states, where yields and inputs are generally higher than in the central Plains states. Thus, we expected eastern U.S. isolates to have reduced sensitivity to QoI fungicides as compared to central U.S. isolates, in line with the findings on DMI fungicides in Meyers et al. (2019). We sequenced a portion of the *cytb* gene to search for previously reported mutations in relation to observed reductions in QoI sensitivity. We expected to observe only natural variability in SDHI sensitivity, as fluxapyroxad had not yet been applied to wheat in the U.S. to any significant extent.

3.2 Materials and Methods

3.2.1 *B. graminis* f. sp. *tritici* isolate collection. More than 375 *B. graminis* f. sp. *tritici* isolates were collected in 15 U.S. states (Table 3.1) from 27 fields (Appendix B: Supplementary Table 3.1) in 2013 and 2014 as described in Meyers et al. (2019). A map of regions, states, and numbered field collection sites that will be discussed in this paper can also be found in Meyers et al. 2019. Pustules from infected whole plant field samples were transferred to fresh detached leaves of a universally susceptible wheat cultivar (‘Chancellor’ or ‘Jagalene’) that were floated atop benzimidazole-amended water agar (50 mg L^{-1}) and single-spored for isolate purity. Isolates were then maintained by transferring them to fresh detached leaves every 9-11 days until their use in this study. European *B. graminis* f. sp. *tritici* isolates Fel09 and JIW11 were kindly provided by B. Keller, maintained in the same manner, and utilized as QoI-resistant and -sensitive controls (Robinson et al. 2002), respectively.

3.2.2 Fungicide sensitivity assay. Formulated fungicides with single active ingredients were obtained from their manufacturers for use in this study: pyraclostrobin in the form of Headline (BASF), picoxystrobin in the form of Aproach (Corteva Agriscience / DowDupont), and fluxapyroxad in the form of Sercadis (BASF). Pyraclostrobin, a methoxy-carbamate chemical, was first registered for use on U.S. wheat in 2002 (FAO 2004) and picoxystrobin, a methoxy-acrylate, was registered in 2012 (FAO 2013b). Both Headline and Aproach are labelled for use on wheat in the U.S., but Sercadis is not. At the time of this study, a dual active ingredient formulation that included fluxapyroxad (Priaxor, BASF) was labelled for use on wheat; however, in order to confine the study to single active ingredients, Sercadis was used instead. Experimental concentrations of each fungicide were determined with a subset of 7 U.S.

B. graminis f. sp. *tritici* isolates in order to establish a concentration range capable of capturing the growth abilities of the isolate collection.

All isolates were screened using a wheat detached-leaf sensitivity assay as described in Meyers et al. (2019). For each fungicide, a water control plus 11 fungicide concentrations were used as follows: pyraclostrobin 0.5, 1.5, 3, 7, 10, 14, 20, 45, 75, 110, and 150 mg L^{-1}; picoxystrobin 0.5, 1, 2, 4, 6, 8, 10, 12, 14, 16, and 18 mg L^{-1}; and fluxapyroxad 1, 10, 30, 60, 100, 150, 175, 200, 225, 250, and 275 mg L^{-1}. Briefly, susceptible wheat seedlings (cultivar 'Jagalene') were sprayed to runoff with a particular fungicide concentration. The next day, leaves were segmented, placed atop water agar amended with benzimidazole (50 mg L^{-1}), and inoculated with a single *B. graminis* f. sp. *tritici* isolate. This assay yielded four leaf ratings (0, 1, 2, or 3) per concentration per fungicide for each isolate evaluated. Estimates of effective concentration values (EC_{50} values) were calculated from leaf ratings using a nonlinear mixed model (SAS PROC NLMIXED, Cary, NC) and mean EC_{50} values were compared based on geographic levels of region, state, and field using a generalized mixed model (PROC GLIMMIX) as previously described (Meyers et al. 2019).

This assay was successfully completed for 370 isolates for pyraclostrobin, 374 for picoxystrobin, and 332 for fluxapyroxad. To confirm the repeatability of the experiment, the assay was repeated for 27 isolates for pyraclostrobin, 32 for picoxystrobin, and 29 for fluxapyroxad. SAS PROC RANK was used to confirm that ranks of EC_{50} values were correlated between the two replicate trials, respectively, of pyraclostrobin ($P = 0.049$, r = 0.38), picoxystrobin ($P = 0.004$, r = 0.49) and fluxapyroxad ($P = 0.049$, r = 0.37).

3.2.3 Evaluation of isolate fitness and cross-resistance. To assess a possible relationship between reduced fungicide sensitivity and fitness, sporulation (as a component of

fitness) was evaluated for 364 isolates included in this study as described in Meyers et al. (2019). Mean spores per pustule were calculated from five replicates of 20 pustules per replicate and analyzed for correlation along with fungicide EC_{50} values using a multivariate analysis of variance (PROC GLM) as explained in Meyers et al. (2019).

The EC_{50} values of the U.S. *B. graminis* f. sp. *tritici* isolates were analyzed for possible cross-resistance, or correlated response to the two QoI fungicides, pyraclostrobin and picoxystrobin. Cross-resistance was evaluated using a PROC GLM variance correlation analysis as previously described (Meyers et al. 2019).

3.2.4 QoI genetic analysis of *cytb* gene.

3.2.4.1 DNA extraction. *B. graminis* f. sp. *tritici* conidia were collected into 2 mL screwcap tubes by dipping pustule-covered leaf segments in 100% ethanol. Conidial samples were then centrifuged for 20 minutes and ethanol was decanted from the tubes. The conidial samples were then frozen at -80°C and lyophilized to remove all remaining ethanol. Dried spores were stored at -80°C until their DNA extraction. Three replicate tubes of *B. graminis* f. sp. *tritici* conidia were collected per isolate, with each tube containing approximately equal volumes of dried conidia. Samples were ground with five nickel-plated beads per tube and agitated with a Vortex Genie attachment. Each sample tube received two 30-second grinding intervals, with samples being returned to liquid nitrogen between intervals. Then DNA was extracted from both U.S. and European standard *B. graminis* f. sp. *tritici* isolates using an E.Z.N.A.® Plant DNA extraction kit (Omega Bio-Tek, Norcross, GA) and an adapted version of the kit protocol.

3.2.4.2 PCR. Possible presence of the QoI resistance-causing mutation G143A in the *cytb* gene was analyzed for a subset of 14 U.S. isolates using allele-specific PCR amplification. Isolates were selected to represent the range of QoI sensitivity, having the highest and lowest

pyraclostrobin and picoxystrobin EC_{50} values. European isolates Fel09 and JIW11 were used as controls, since JIW11 has the G143 genotype and Fel09 has G143A (Robinson et al. 2002, Wyand and Brown 2005). Primers and protocol for this procedure were adapted from Fraaije et al. (2002). Primers 143GF and PMR4 were used to detect the presence of the wild-type sensitive allele, while primers 143CF and PMR5 were used for detection of the resistant allele. If amplified, the sensitive allele reaction should yield a fragment of 439 base pairs in length and the resistance allele reaction a 409-bp fragment (Fraaije et al. 2002). The standard PCR protocol described by Fraaije et al. (2002) was used for 10 µL reactions with 55°C and 62°C for annealing temperatures for the sensitive and resistant reactions, respectively.

3.2.4.3 Sequencing. To detect the possible occurrence of qualitative *cytb* mutations F129L and G137R, a 675-bp portion of the *cytb* gene was amplified and sequenced for an additional 16 *B. graminis* f. sp. *tritici* isolates selected based on extremeness of QoI EC_{50} values. European control isolates Fel09 and JIW11 were also included in this experiment as standards. Primers CBF1 and CBR3 from Fraaije et al. (2002) were utilized for both PCR amplification and sequencing. Post-amplification fragments were cleaned up using ExoSAP (ThermoFisher) and Sanger sequencing was performed by the NC State University Genomic Sciences Laboratory (Raleigh, NC).

3.3 Results

3.3.1 Observed pyraclostrobin and picoxystrobin EC_{50} values. A range in sensitivity was observed for both QoI fungicides tested (Figure 3.1). Pyraclostrobin EC_{50} values ranged from 0.04 to 31.32 mg L^{-1}, a 783-fold difference, with median and mean values of 1.80 and 3.51 mg L^{-1}, respectively (Fig. 1A). Picoxystrobin EC_{50} values ranged from 0.09 to 10.68 mg L^{-1}, a

119-fold difference, with median and mean values of 2.20 and 2.63 mg L^{-1} respectively (Fig. 1B). For both QoIs, mean values were greater than median values indicating that both sets of observations were right skewed.

The European QoI-sensitive control isolate, JIW11, was found to have QoI sensitivity near that of the mean of the U.S. sample population, with a pyraclostrobin EC_{50} value of 2.79 mg L^{-1} and a picoxystrobin EC_{50} value of 2.05 mg L^{-1}. As was to be expected, efstimation of QoI EC_{50} values for the European QoI-resistant isolate Fel09 was unsuccessful for both pyraclostrobin (Appendix B: Supplementary Figure 3.1A) and picoxystrobin (Appendix B: Supplementary Figure 3.1B), as the isolate grew at levels comparable to the water-sprayed check on all experimental concentrations. QoI-sensitive control JIW11 EC_{50} values were used to calculate RF, defined as the ratio of a U.S. EC_{50} value over the JIW11 EC_{50} for a given QoI. JIW11 was chosen as the sensitive reference isolate because it was collected before the application of QoI products in Europe. Pyraclostrobin RFs ranged from 0.01 to 11.2. Picoxystrobin RFs ranged from 0.04 to 5.3.

The U.S. isolates collected from 15 states were grouped into 5 geographic regions (Plains, AR-MO, Great Lakes, Mid-Atlantic and Southeast) as described in Meyers et al. (2019). Mean regional EC_{50} values were not significantly different for pyraclostrobin (P = 0.85, Figure 3.2A); however, regional differences were evident for picoxystrobin (P = 0.03, Figure 3.2B). Isolates from the Mid-Atlantic and Great Lakes regions had larger picoxystrobin EC_{50} values as compared to isolates from the Southeast region (Figure 3.2B).

At the state level, significant differences in sensitivity were observed for both QoIs (Table 3.1). For pyraclostrobin (P = 0.008), isolates from Arkansas, Kansas, and Georgia had significantly reduced sensitivity while isolates from Alabama, Florida, Michigan, and Missouri

were the most sensitive. In contrast, for picoxystrobin (P = 0.0003), isolates from Ohio, New York, Pennsylvania, and Mississippi had significantly reduced sensitivity and Missouri, Nebraska, Michigan, and Alabama isolates were the most sensitive. Significant differences between states within a region were also identified: Arkansas had reduced pyraclostrobin sensitivity compared to Missouri, and in the Southeast, Georgia isolates had reduced sensitivity compared to Alabama and Florida. For picoxystrobin, isolates from Great Lakes states Ohio, New York, and Pennsylvania had significantly reduced sensitivity compared to Michigan isolates, and Southeast states Mississippi and Georgia had significantly reduced sensitivity compared to Alabama isolates.

When comparing fields, significant differences in pyraclostrobin sensitivity were found, with isolates collected from New York field 19, Arkansas field 7, and Georgia field 12 having reduced sensitivity and isolates from Florida field 10, New York field 17, and Missouri field 6 having the greatest sensitivity (Appendix B: Supplementary Table 3.1). Similarly, there were field-level differences for picoxystrobin, with reduced sensitivity found in collections from Ohio field 16, New York field 18, and Pennsylvania field 21, and maximal sensitivity found in North Carolina field 26, Michigan field 14, and Alabama field 9. Significant differences between fields within a state were found in New York for pyraclostrobin and in North Carolina for picoxystrobin.

3.3.2 QoI fungicide cross-resistance and fitness cost to reduced sensitivity. For many plant pathosystems, cross-resistance to QoI fungicides is present, and sometimes a decrease in fitness is observed in isolates with QoI resistance or reduced sensitivity to QoIs. A correlation analysis of the U.S. *B. graminis* f. sp. *tritici* EC_{50} values for pyraclostrobin and picoxystrobin revealed a significant ($P < 0.0001$) but very weak ($R^2 = 0.0939$) association (Figure 3.3). The

previously reported assay of fitness for 364 of these isolates indicated that differences in sporulation were significant only at the geographic level of state, and not different among regions or fields (Meyers et al. 2019). In the present study, both pyraclostrobin ($P < 0.0001$) and picoxystrobin ($P = 0.0008$) EC_{50} values were significantly negatively correlated with sporulation at the state level; i.e. sporulation tended to be higher in states with greater QoI sensitivity (Figure 3.4). However, both associations were weak ($R^2 = 0.26$ and 0.20, respectively). Though the relationship between fluxapyroxad EC_{50} value and state mean sporulation was also significant ($P = 0.0111$) and the association was in this case positive, it was very weak ($R^2 = 0.1238$, data not shown).

3.3.3 *cytb* gene mutation analysis. Sequencing of a portion of the *cytb* gene covering three SNP sites of interest (F129L, G137R, and G143A) was completed for 16 selected isolates with origins in Kansas, Missouri, Arkansas, Georgia, Michigan, New York, Pennsylvania, and North Carolina (Table 3.2). For F129L, the wildtype codon is TTC and mutant codons found in other pathosystems have been TTA, TTG, or CTC. For all isolates sequenced for this study, the wildtype TTC codon was found (data not shown). For G137R, the wildtype codon is GGG and mutants in other studies were either CGG or AGG. Neither of these non-synonymous codons were found in the U.S. isolates; however, the synonymous GGT codon was found in 4 of the isolates evaluated. For the G143A mutation that may confer full QoI resistance, the wildtype codon is GGT and the mutant codon is GCT. As expected, the mutant GCT codon was found in QoI-resistant European control isolate Fel09; it was not found in any U.S. isolates, including the most QoI-insensitive ones. Synonymous codons GGG and GGA were found in several isolates originating from New York, Pennsylvania, and North Carolina.

Allele-specific PCR amplification of the 143 locus was conducted on a different set of isolates, also chosen to represent the extremes of the range of QoI sensitivity in this study, and the results were consistent with the sequencing results. For the wild-type G143 allele reaction, amplification occurred for all U.S. isolates examined and JIW11, the QoI-sensitive UK control isolate. The mutant G143A allele was only amplified in Fel09, the QoI-resistant German control isolate; it was not amplified in any of the U.S. isolates.

3.3.4 Fluxapyroxad geographic baseline sensitivity. Fluxapyroxad EC_{50} values of the U.S. isolates ranged from 0.20 to 124.46 mg L^{-1}, a 613-fold difference in sensitivity. The mean EC_{50} value across all evaluated U.S. isolates was 13.93 mg L^{-1}. European isolates JIW11 and Fel09 were found to have EC_{50} values of 0.75 and 19.03 mg L^{-1}, respectively.

Isolates from the AR-MO and Plains regions had reduced fluxapyroxad sensitivity compared to isolates from the Great Lakes and Southeast regions ($P = 0.0022$, Figure 3.2C). At the state level, Arkansas, Kansas, and Nebraska isolates were more insensitive to fluxapyroxad, while isolates from Missouri, Florida, Georgia, Alabama, Oklahoma, Michigan, and Mississippi were more sensitive ($P = 0.0012$, Table 3.1).

When comparing fields, there was significantly less fluxapyroxad sensitivity in Arkansas field 7, North Carolina field 26, New York field 19, and Kansas field 2 than in North Carolina field 25, Georgia field 11, Mississippi field 8, and New York field 17 ($P < 0.0001$; Appendix B: Supplementary Table 3.1). Significant differences between fields in the same state were frequent for fluxapyroxad sensitivity, with differences among North Carolina, New York, Ohio, and Pennsylvania fields (Appendix B: Supplementary Table 3.1).

3.4 Discussion

Foliar fungicide applications are an integral tool for wheat powdery mildew control where susceptible wheat cultivars are planted or *Pm* resistance genes have become ineffective. The present study was the first QoI sensitivity screening of such a large number of U.S. *B. graminis* f. sp. *tritici* isolates. It provided evidence that the pathogen populations sampled in 2013-2014 were sensitive to the QoI fungicides pyraclostrobin and picoxystrobin. There was also no evidence of *cytb* gene mutations previously shown to confer either large or small reductions in QoI sensitivity. However, considerable differences among individual isolates for sensitivity to both fungicides and modest regional differences in picoxystrobin sensitivity differences were observed.

These findings contradicted our hypothesis that, like DMI sensitivity, QoI sensitivity would be broadly diminished in the eastern U.S., where wheat powdery mildew outbreaks occur most often and most wheat crops receive at least one fungicide application. In comparison to the QoI fungicide sensitivity breakdown that occurred in only a few years in the European *B. graminis* f. sp. *tritici* population, the U.S. population has remained sensitive to QoIs despite almost 20 years of applications. Therefore, QoI fungicides appear to remain a generally effective and important wheat powdery mildew control option in the U.S., especially in the eastern states where sensitivity to DMI fungicides has begun to decrease.

Nevertheless, this large-scale survey revealed a large range in observed QoI sensitivity. The detection of a 119-fold range in pyraclostrobin sensitivity and a 783-fold range in picoxystrobin sensitivity showed that substantial variation in QoI response exists within the U.S. *B. graminis* f. sp. *tritici* population and is not accounted for by the known insensitivity-conferring mutations. With maximum RFs of 11.2 and 5.3 for pyraclostrobin and picoxystrobin,

respectively, the population is still at a low level of insensitivity by international standards, but sufficient sustained pressure in the form of regular QoI applications could allow the population to make rapid gains in insensitivity from selection.

Surprisingly, there was little congruence in sensitivity to the two QoIs, with only weak evidence of cross-resistance. This suggests the U.S. *B. graminis* f. sp. *tritici* population may have somewhat different mechanisms of adaptation to pyraclostrobin and picoxystrobin. Although some isolates with high insensitivity to one QoI were relatively sensitive to the other, isolates from Alabama, Missouri, and Michigan remained the most sensitive to both pyraclostrobin and picoxystrobin. Isolates from North Carolina field 25 were among the most sensitive to pyraclostrobin, but among the least sensitive to picoxystrobin. This differential response is uncommon for QoI fungicides in most pathosystems, but not unheard of in *B. graminis* f. sp. *tritici*; for example, no cross-resistant relationship was found between azoxystrobin, kresoxim-methyl, and trifloxystrobin in a German population (Heaney et al. 2000).

Isolate JIW11 was collected in the United Kingdom in 1985, prior to major QoI use there, and was used in this study as a QoI-sensitive control. JIW11 was found to have EC_{50} values slightly below the average of the U.S. samples for both pyraclostrobin and picoxystrobin. This implies that the mean QoI sensitivity of the U.S. *B. graminis* f. sp. *tritici* population may be similar to that of the ‘old,’ pre-QoI European population. However, 65% of U.S. isolate pyraclostrobin EC_{50} values and 47% of pyraclostrobin values yielded RFs less than one, indicating they are more sensitive than JIW11. Pyraclostrobin and picoxystrobin EC_{50} values for European QoI-resistant isolate Fel09, which was collected in Germany in 1998, could not be calculated using the screening assay and estimation procedure presented here due to the complete insensitivity of the isolate to all fungicide concentrations utilized (Appendix B: Supplementary

Figure 3.1). The only conclusion possible was that Fel09 had a pyraclostrobin EC_{50} of >150 mg L^{-1} and a picoxystrobin EC_{50} >18 mg L^{-1}. As the maximum observed EC_{50} values of U.S. isolates were 31 mg L^{-1} for pyraclostrobin and 11 mg L^{-1} for picoxystrobin, the U.S. collection was clearly far more sensitive than isolate Fel09.

The comparatively low level of QoI resistance found in the U.S. *B. graminis* f. sp. *tritici* isolates was consistent with the *cytb* sequence results. DNA sequence mutations were found at two of three mutation sites of interest; however, none of the identified mutations conferred amino acid changes (Table 3.2). Additionally, the presence of these synonymous mutations seemed to have no relationship to magnitude of EC_{50} for either QoI fungicide. With variation in QoI sensitivity not explained by changes in the sequence of the three evaluated mutation sites, further *cytb* sequence analysis will need to be conducted in search of other changes in relation to QoI sensitivity in *B. graminis* f. sp. *tritici*.

There is some evidence that when a QoI is occupying the binding pocket of cytb, overexpression of the alternative oxidase pathway can be utilized to bypass the cytb complex entirely (Ishii 2010, Sierotzki 2015). This mechanism could account for *B. graminis* f. sp. *tritici* isolates having reduced QoI sensitivity without *cytb* alterations. In another powdery mildew fungus, *Podosphaera fusca,* experiments with and without salicylhydroxamic acid (SHAM), an inhibitor of the alternative oxidase pathway, produced the same results, indicating no alternative oxidase activity (Fernandez-Ortuno et al. 2008). To evaluate if the same is true for *B. graminis* f. sp. *tritici,* SHAM could be added to the QoI application on wheat plants prior to subjecting them to fungal inoculation.

In further experiments, consideration should be given to the methodology for confirming the *cytb* genotypes found in this study. Heteroplasmy, the presence of differing mitochondrial

genomes in the same cell, is known to exist in *B. graminis* f. sp. *tritici* (Fraaije et al. 2002). When the proportion of G143A (or any *cytb* mutation) to the wildtype G143 is low, the mutation may not be detectable (Robinson et al. 2002, Ishii 2010). Both of the mutation detection methods used in this study involved PCR, which can be insufficient for low frequency mutation detection when heteroplasmy is present (Ishii et al. 2009). Instead, fluorescent probe-based qPCR and ddPCR methods with higher precision could be used to evaluate whether G143A, F129L, and G137R are present in a low percentage in several U.S. *B. graminis* f. sp. *tritici* isolates with reduced QoI sensitivity.

Additionally, the presence of *cytb* mutations may have been missed due to instability of heteroplasmy. It is thought that mitochondrial heteroplasmy is only maintained when necessary and reversion to homoplasmy is able to occur sometimes in only a few generations (Sierotzki 2015). QoI resistance was lost by *Podosphaera xanthii* and *Plasmopora viticola* after lack of QoI exposure (Ishii et al. 2007, Sierotzki et al. 2005, Genet et al. 2006). If this is also the case for *B. graminis* f. sp. *tritici*, then isolates might have been maintained for too many generations on QoI-free leaf tissue and reverted to wildtype mitochondrial homoplasmy prior to spore DNA extraction. To test this theory and prevent any changes in mitochondrial genetics, spore collection for DNA extraction and QoI sensitivity screening should be completed as soon as possible after field sampling to minimize the number of serial transfers and generations.

Newly developed SDHI fungicides have recently become a popular option for crop disease control. Fluxapyroxad was first registered for use in the U.S. in 2012, and several other new SDHI active ingredients have followed. We hypothesized that, since fluxapyroxad was a newly released product at the time of fungal collection, *B. graminis* f. sp. *tritici* isolates from all sampled U.S. regions would be equally sensitive to the fungicide. However, we found isolates

from Arkansas, Kansas, Nebraska, and Virginia to have reduced fluxapyroxad sensitivity. With a rapid increase in use of second-generation SDHI containing fungicide formulations expected, it is important to have this geographically linked sensitivity baseline with which to compare in future years.

The development of new products containing SDHI active ingredients provides options for wheat disease management, potentially lessening the selection pressure exerted by DMIs and QoIs. For wheat powdery mildew control in Europe, new SDHI formulations may be the only effective fungicides, as the European *B. graminis* f. sp. *tritici* population is resistant to both DMIs and QoIs. In the U.S., three second-generation SDHIs are now registered for use on wheat: fluxapyroxad, benzovindiflupyr, and pydiflumetofen (NCERA-184 2019). All three are formulated along with a QoI and/or a DMI active ingredient (NCERA-184 2019). With fluxapyroxad, *B. graminis* f. sp. *tritici* inhibition was not as strong as that of QoIs and DMIs (E. Meyers, personal observation); therefore, mixed mode-of-action formulations may be necessary for full disease control. In the present study, most leaf segments bearing the higher concentrations of fluxapyroxad produced a flecking phenotype that resulted in a rating of 1 instead of a clean, green leaf with a rating of 0, while QoI and DMI products fully controlled the isolates with ratings of 0. Because of this, fluxapyroxad EC_{50} values could only be estimated for 332 isolates instead of the approximately 380 isolates evaluated.

This study found the baseline sensitivity of fluxapyroxad in the U.S. to be uneven across the geographic areas that were sampled. Unexpectedly, isolates from the central U.S. were on average less sensitive to fluxapyroxad than isolates from the southeastern U.S. Isolates from Arkansas, Kansas, and Nebraska were least sensitive to the SDHI, and also were collected from university research farms. SDHIs were applied to wheat on both the Arkansas (personal

communication, Gene Milus and David Moon) and Kansas (personal communication, Erick DeWolf) research farms between the years of 2010 and 2014. On the other hand, there was no record of SDHI application in the Nebraska research field prior to isolate collection (personal communication, Stephen Wegulo). Both in Arkansas and in Kansas, the research farm fungicide trials records included just one or two SDHI entries among many other products; therefore, it seems unlikely that the relatively small amount of penthiopyrad or fluxapyroxad applied in either location could have had a large selective effect. However, to confirm whether the *B. graminis* f. sp. *tritici* populations in Arkansas, Kansas, and Nebraska have broadly reduced sensitivity to SDHIs, samples from commercial wheat fields in those areas should be evaluated.

Since the 2013-2014 U.S. *B. graminis* f. sp. *tritici* isolate collection was created, SDHI use has nearly doubled in the central U.S. (USGS 2015) due to recommendations for controlling frogeye leaf spot (caused by *Cercospora sojina*) on soybean and sheath blight (caused by *Rhizoctonia solani*) on rice. With the possibility of chasmothecia on wheat stubble remaining in fields being exposed to fungicides, application of SDHIs of other crops must also be considered. Therefore, as additional SDHI products are applied to wheat and the crops rotated with wheat, it will be important to take into account the existing SDHI sensitivity level prior to major SDHI use. The fluxapyroxad baseline determined in this study will help decipher changes in the *B. graminis f. sp. tritici* population in future U.S. fungicide sensitivity evaluations.

Overall, this study is the first evaluation of pyraclostrobin, picoxystrobin, and fluxapyroxad sensitivities in the U.S. *B. graminis* f. sp. *tritici* population. These data will provide a useful comparison at the national as well as regional and state levels for QoI and SDHI studies in years to come. Though we did not detect a G143A or other known QoI sensitivity-influencing mutations in the *cytb* gene, we did find evidence of isolates and regional populations with

reduced QoI sensitivity. Future scouting and isolate sampling could be planned accordingly. Most importantly, these results suggest that QoI fungicides generally remain effective for control of *B. graminis* f. sp. *tritici* in the U.S. Wheat growers in the U.S. should continue to use QoI and SDHI active ingredients in mixed-chemistry products to further prolong the efficacy of these control options and delay emergence of fungicide resistance that has evolved in other *B. graminis* f. sp. *tritici* populations around the world.

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Table 3.1. Mean pyraclostrobin, picoxystrobin, and fluxapyroxad sensitivities of *Blumeria graminis* f. sp. *tritici* isolates samples in 2013-2014 from 15 U.S. states[a]

		EC$_{50}$ values[b]		
State	**N**	**Pyraclostrobin**	**Picoxystrobin**	**Fluxapyroxad**
Arkansas (AR)	13	3.62 a	2.26 abcd	27.49 a
Kansas (KS)	18	3.05 a	2.11 abcd	15.15 ab
Georgia (GA)	52	2.39 a	1.98 bcd	5.31 d
Pennsylvania (PA)	32	2.21 ab	2.49 ab	7.64 cd
New York (NY)	40	2.16 ab	2.50 ab	6.96 cd
Ohio (OH)	30	2.15 ab	3.07 a	7.57 cd
North Carolina (NC)	55	1.74 ab	2.09 bcd	7.08 cd
Oklahoma (OK)	30	1.72 abc	1.76 bcde	4.57 d
Virginia (VA)	12	1.72 abc	2.38 abcd	8.36 bcd
Mississippi (MS)	20	1.56 abc	2.46 abc	4.29 d
Nebraska (NE)	12	1.34 abc	1.42 def	15.05 abc
Alabama (AL)	18	1.27 bc	1.02 f	5.13 d
Florida (FL)	17	1.15 bc	1.53 cdef	5.49 d
Michigan (MI)	12	1.11 bc	1.20 ef	4.02 d
Missouri (MO)	20	0.87 c	1.43 def	5.83 d

[a] Within a column, means followed by the same letter are not significantly different at $P < 0.05$ using a pairwise t-test.
[b] EC$_{50}$ = effective concentration of fungicide at which isolate growth is decreased by 50%. Mean mg/L of fungicide active ingredient.

Table 3.2. Cytochrome *b* codon sequences of *Blumeria graminis* f. sp. *tritici* isolates selected for varying levels of sensitivity (EC_{50} values) to QoI fungicides pyraclostrobin and picoxystrobin

			EC_{50} values (mg/L)		**cytb codon sequence at amino acid position[a]**	
Isolate[b]	**Field[c]**	**State**	**Pyraclostrobin**	**Picoxystrobin**	**137**	**143**
JIW11	--	--	2.8	2.0	GGG	GGT
Fel09	--	--	>150	>18	GGG	**GCT**
KSM-C-3-5	**2**	**KS**	6.4	0.27	GGG	GGT
KSM-D-1-5			6.7	0.33	**GGT**	GGT
MOB(14)-D-2	**6**	**MO**	0.21	0.35	GGG	GGT
ARF-C-1-1	**7**	**AR**	0.43	8.6	GGG	GGT
GAT-E-1-1	**12**	**GA**	10.3	8.3	**GGT**	GGT
GAS-B-1-2	**13**		0.50	10.7	GGG	GGT
GAS-D-1-3			15.7	5.8	GGG	GGT
MIR(14)-C-1-2	**14**	**MI**	2.2	0.28	GGG	GGT
MIR(14)-C-2-1			0.24	2.3	GGG	GGT
NYB(14)-E-3-1	**19**	**NY**	14.3	0.91	GGG	**GGG**
NYG-E-3-5	**20**		31.3	2.6	**GGT**	**GGA**
PAF-A-2-5	**21**	**PA**	1.4	8.0	GGG	GGT
PAF(14)-D-1-5	**22**		20.8	4.4	GGG	**GGA**
NCF-B-3-3	**24**	**NC**	0.22	0.24	GGG	GGT
NCC-B-1-3	**25**		0.19	5.5	GGG	**GGG**
NCC-D-1-1			0.90	8.2	**GGT**	**GGA**

[a] QoI-sensitive control isolate JIW11 had wild-type codon sequences at both positions, and QoI-resistant control isolate Fel09 had the QoI insensitivity-conferring mutation G143A (codon sequence GCT). All other mutations shown were synonymous. At amino acid position 129, all isolates had codon sequence TTC.
[b] QoI-sensitive control isolate JIW11 was collected in the United Kingdom in 1985. QoI-resistant control isolate Fel09 was collected in Germany in 1998.
[c] Field numbers correspond to the map in Meyers et al. (2019).

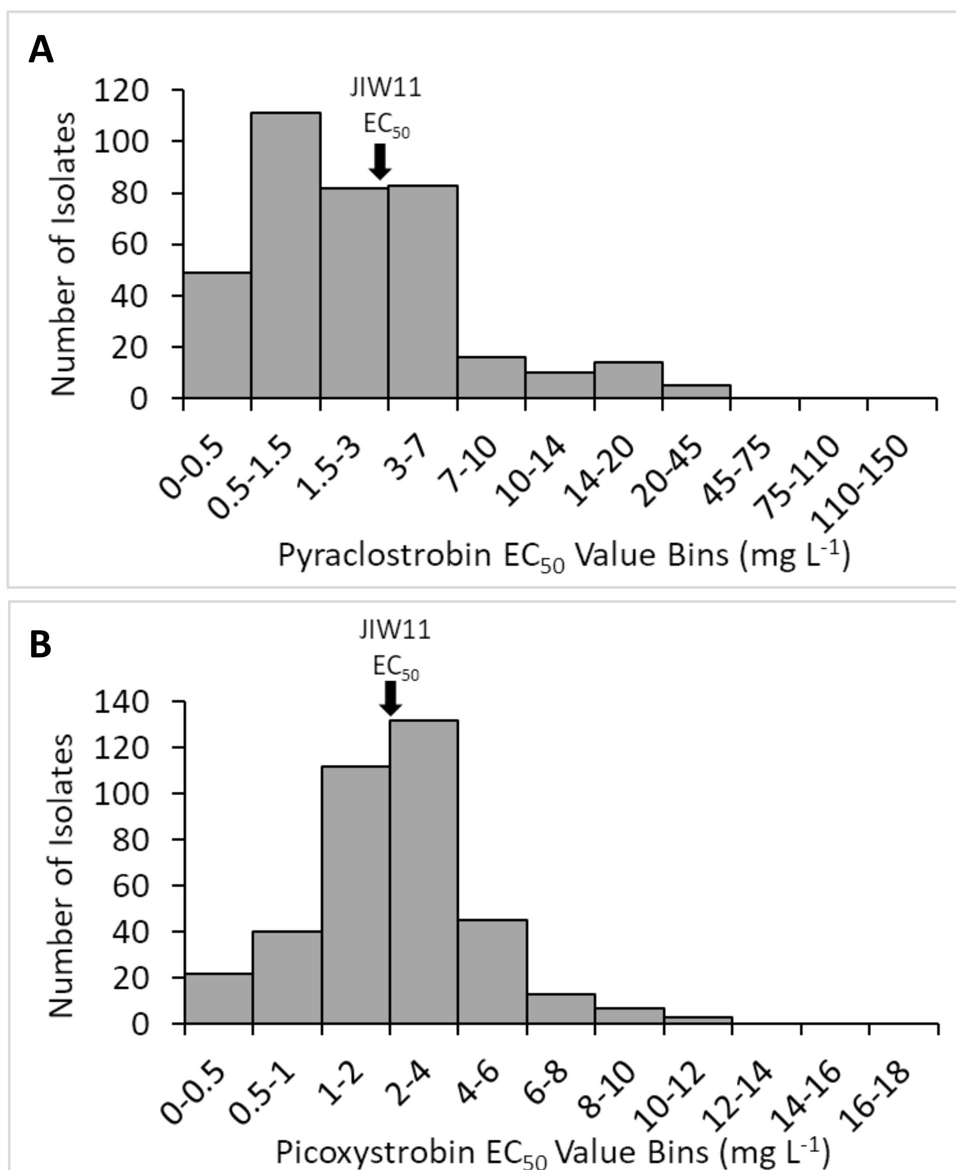

Figure 3.1. Sensitivity (EC_{50}) of *B. graminis* f. sp. *tritici* isolates from the central and eastern U.S. to the QoIs **A)** pyraclostrobin (370 isolates), with EC_{50} range 0.04 - 31.32 mg L^{-1}, a 783-fold difference, and **B)** picoxystrobin (374 isolates), with EC_{50} range 0.09 - 10.68 mg L^{-1}, a 119-fold difference. Isolates are assigned to EC_{50} value bins based on their calculated EC_{50} values; bins are delineated by experimental concentrations used to measure QoI sensitivity. EC_{50} values of European QoI-sensitive control isolate JIW11 have been indicated by arrows.

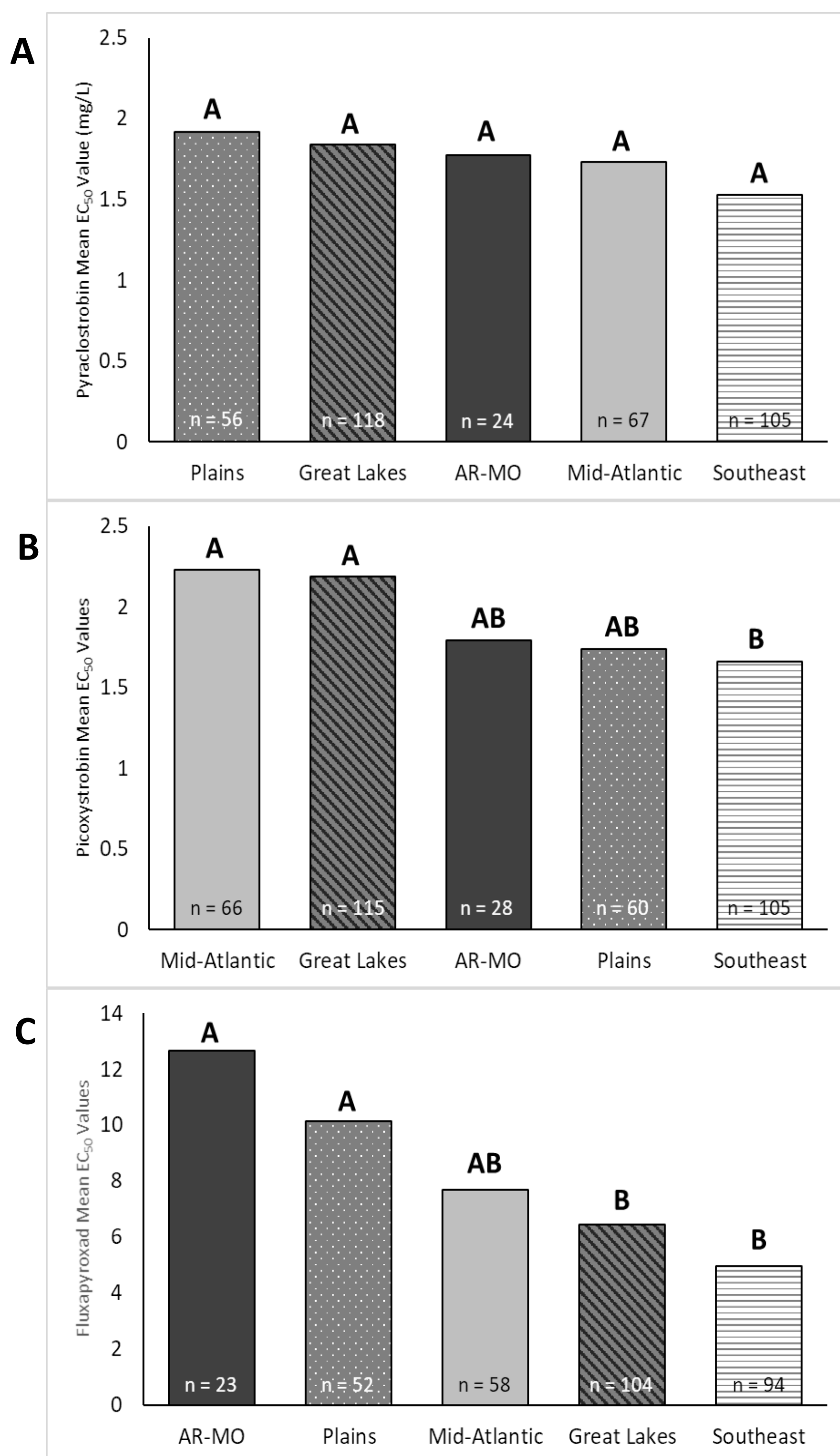

Figure 3.2. Regional mean EC_{50} values for **A**) pyraclostrobin, **B**) picoxystrobin, and **C**) fluxapyroxad in a national sample of *Blumeria graminis* f. sp. *tritici* from 2013-2014. Sample sizes per region are shown in each bar. Bars topped by the same letter are not significantly different ($P < 0.05$) using a pairwise t-test.

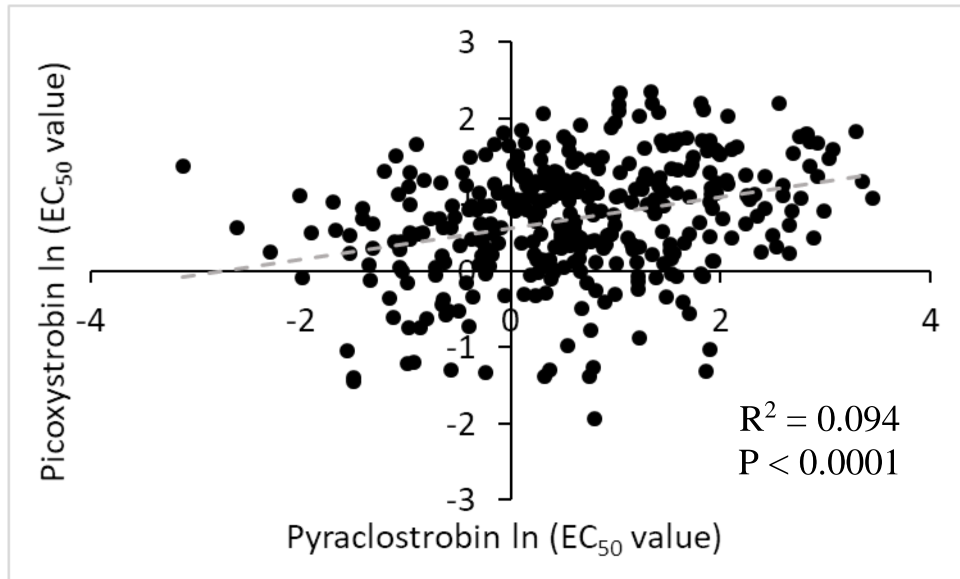

Figure 3.3. Correlation of log-transformed sensitivity (EC_{50}) to pyraclostrobin and picoxystrobin in U.S. *Blumeria graminis* f. sp. *tritici* isolates.

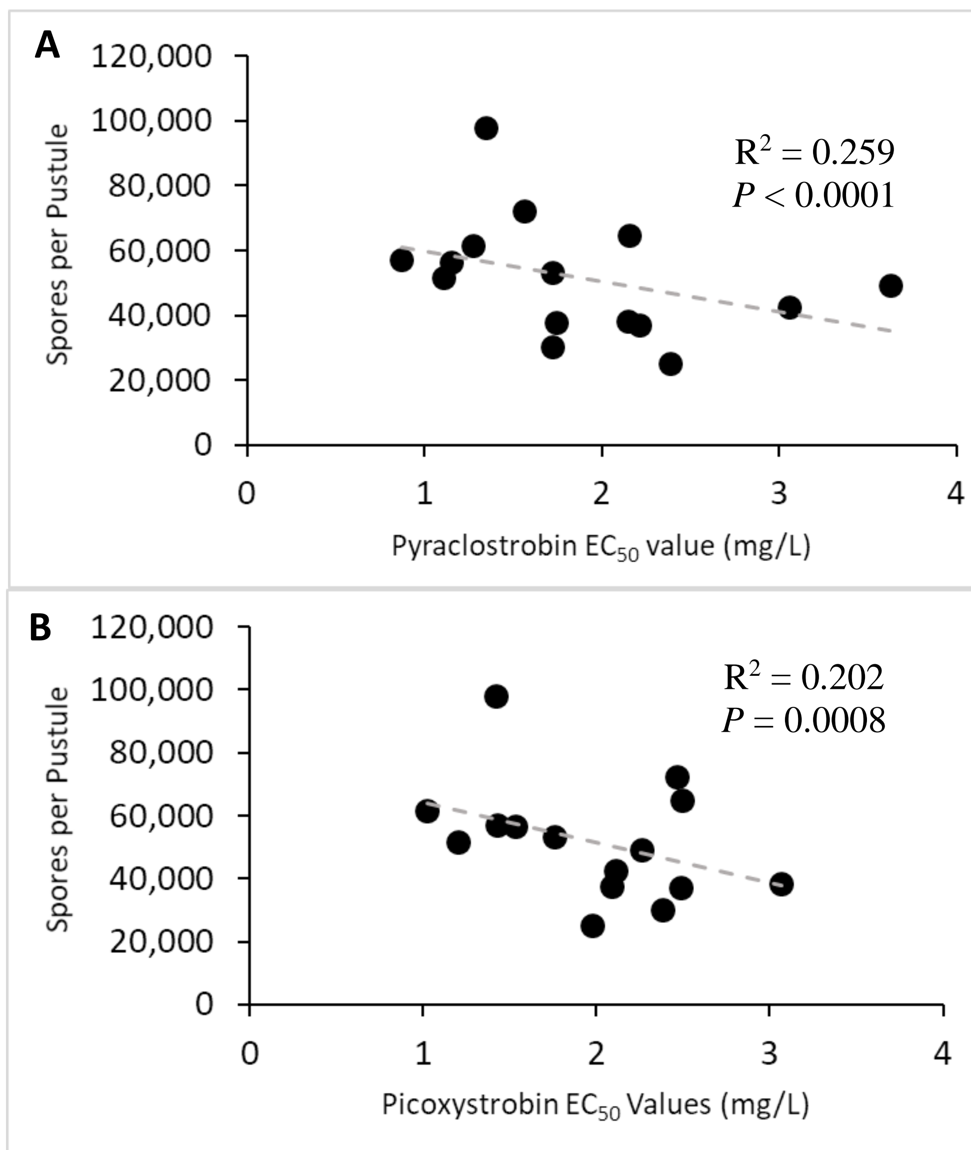

Figure 3.4. Correlation of EC_{50} values for **A**) pyraclostrobin and **B**) picoxystrobin with *Blumeria graminis* f. sp. *tritici* sporulation, a component of fitness. Each data point is the mean of isolates collected in a state

CHAPTER 4

Relationship of DMI fungicide sensitivity to *CYP51* genetic and expression differences in U.S. and U.K. *Blumeria graminis* f. sp. *tritici* isolates

ABSTRACT

Sensitivity to demethylation inhibitor (DMI) fungicides can be quite complex, as it is potentially influenced by several mechanisms in the same organism. A Y136F point mutation in the target C14α-demethylase protein (CYP51) was identified in DMI-insensitive isolates of the plant pathogen *Blumeria graminis* f. sp. tritici in both European and Australian populations. Recently, a reduction in DMI sensitivity was discovered in *B. graminis* f. sp. *tritici* sub-populations in the eastern wheat growing region of the United States. A comparison with other more insensitive populations gives insight into the basis of this change in sensitivity and could potentially help prevent further development of DMI resistance in the U.S. *B. graminis* f. sp. *tritici* population. *CYP51* gene sequencing, expression analysis, and copy number variation estimation were conducted for a selection of U.S. isolates and compared to more DMI-insensitive isolates from the United Kingdom. Heteroallelism, the presence of two genotypes in the same isolate, was discovered at the CYP51 136 locus in both sets of isolates. Additionally, heteroallelism involving the CYP51 S509T mutation was detected in several U.K. isolates and was linked specifically to a reduction in tebuconazole sensitivity. The evolutionary role of the heteroallelic state has yet to be determined. *CYP51* expression was twice as high in U.K. isolates as in U.S. isolates; however, the number of *CYP51* gene copies (2-4) estimated using digital droplet PCR (ddPCR) were similar in both isolate samples. This suggests that alterations in *CYP51* transcriptional regulators and promoter elements may be involved in the large reduction in DMI insensitivity observed in U.K. isolates. Further investigation of the location within the genome, function, and regulation of the multiple *CYP51* gene copies, as well as analysis of the effect of specific alleles in heteroallelic isolates, will help extend our understanding of the evolution of DMI resistance.

4.1 Introduction

Over the past 50 years, dozens of unique demethylation inhibitor (DMI) fungicide molecules have been developed for use in protecting both humans and plants from fungal infections (Sheehan et al. 1999, Ziogas and Malandrakis 2015). DMI fungicides limit fungal growth by interfering with the production of ergosterol, a key component in fungal cell membranes. Specifically, DMIs target C14α-demethylase (*CYP51*) and occupy this enzyme's active site to block the demethylation of lanosterol in the sterol production pathway (Gisi et al. 2000). Without a large enough supply of ergosterol to produce new cells, DMIs are able to halt the growth of pathogenic fungi. However, decades of use of DMI-containing fungicide products have exerted a selective effect, reducing the efficacy of disease control and inciting DMI resistance in many fungi.

Several different types of genetic and biochemical changes pertaining to *CYP51* have been linked to DMI insensitivity in fungal populations, including *CYP51* mutations, copy number variation, and overexpression (Ziogas and Malandrakis 2015). Discovering which of these alterations is the cause of a pathogen's DMI insensitivity is key to understanding what approach to take to control the disease. Knowing if a mechanism works to reduce sensitivity to a specific DMI active ingredient, perhaps based on binding site alterations, can help determine which currently available formulations or how to design new DMI molecules to obtain durable DMI fungicide efficacy.

Many different point mutations in the target *CYP51* gene have been identified in relation to variability in DMI sensitivity, the most common of which is the Y136F amino acid alteration (Becher and Wirsel 2012). Many of these mutations impact the structure and shape of the enzyme's binding pocket such that the enzyme is still functional but DMI molecules are unable

to bind correctly (Becher and Wirsel 2012, Tucker et al. 2019). Often, specific mutations influence the binding ability of a particular DMI; the accumulation of multiple *CYP51* mutations in a single fungal isolate can create cross resistance among DMIs (Cools et al. 2013).

In a few genera of plant pathogens, an increased number of *CYP51* copies have been discovered. Of those, *Fusarium*, *Aspergillus*, and *Rhynchosporium* species are the best studied, although it is not entirely clear whether the gene copies are functionally redundant (Cools et al. 2013, Hawkins et al. 2014). In *A. fumigatus* and *A. nidulans*, two *CYP51* copies have been identified, while three copies were found in *A. flavus* and *F. graminearum* (Song et al. 2018). Studies have also revealed an additional pseudo-*CYP51* gene in *R. commune* (Brunner et al. 2016, Hawkins et al. 2014) and evaluations of the grape powdery mildew fungus *Erysiphe necator* suggested three, four, or even nine *CYP51* copies in DMI insensitive isolates (Jones et al. 2014, Rallos and Baudoin 2016).

In the case of *E. necator*, the presence of multiple *CYP51* copies correlated with increased *CYP51* expression (Rallos and Baudoin 2016). In other fungi, like *Penicillium digitatum, Blumeriella jaapii, A. fumigatus,* and *Venturia inaequalis*, an insertion in the promoter region of *CYP51* was found to cause the overexpression (Hamamoto et al. 2000, Schnabel and Jones 2001, Ma et al. 2006, Garcia-Effron et al. 2008, Villani et al. 2016). With an excess of functional CYP51 protein, DMI molecules may be too scarce to effectively inhibit ergosterol production. Overexpressed *CYP51* genes are frequently of the wildtype genotype without point mutations, indicating that the overexpression itself is the mechanism of DMI insensitivity, rather than mutated gene transcripts (Ziogas and Malandrakis 2015).

Experiments evaluating all three of these potential mechanisms are necessary to understand the genetic cause and evolutionary development of DMI insensitivity in plant

pathogens. In situations where DMI insensitivity has been identified, knowing which mechanism is behind the loss in sensitivity may help to keep the insensitivity from becoming widespread.

Blumeria graminis (DC.) Speer f. sp. *tritici* (Em. Marchal), the causal agent of wheat powdery mildew, can easily overcome management strategies due to its high evolutionary potential (FRAC, McDonald and Linde 2002). This obligate ascomycete proliferates asexually via polycyclic production of easily windblown conidia leading to a large effective population size. Due to random mutation in asexual conidia, along with frequent sexual recombination via asci in chasmothecia, *B. graminis* f. sp. *tritici* is able to quickly overcome both powdery mildew (*Pm*) resistance genes and foliar fungicide chemistries. As DMI fungicides are one of the few wheat powdery mildew management options, they were applied aggressively to European cereal crops in the mid-to-late 1980s. This resulted in widespread DMI insensitivity in the European *B. graminis* f. sp. *tritici* population. DMI-insensitive isolates have also been found in the southeast Australian *B. graminis* f. sp. *tritici* population. The Y136F mutation in CYP51 was found in the populations of both continents (F. Lopez-Ruiz, personal communication, Wyand and Brown 2005). Additionally, the S509T mutation was found in combination with Y136F in the *CYP51* genes of extremely DMI-insensitive Australian isolates of the sister species *B. graminis* f. sp. *hordei* (cause of barley powdery mildew) (Tucker et al. 2015).

With the recent identification of reduced DMI sensitivity in the *B. graminis* f. sp. *tritici* population of the eastern United States (U.S.) (Meyers et al. 2019), it is important to better understand the genetic and biochemical mechanisms behind DMI insensitivity in this pathosystem. This knowledge could help to redesign DMI molecules to delay the loss of efficacy of this chemistry. Here we utilize molecular techniques to examine *CYP51* of U.S. *B. graminis* f. sp. *tritici* isolates for point mutations, gene copy number differences, and expression levels in

relation to sensitivity to tebuconazole, prothioconazole, and metconazole fungicides. A well-characterized German isolate along with a collection of United Kingdom (U.K.) isolates were used for reference. By comparing the genetic and cellular bases of DMI insensitivity in these two populations, we hoped to gain a better understanding of the evolution of DMI insensitivity, as well as insights into the prevention of widespread DMI resistance in the U.S. *B. graminis* f. sp. *tritici* population.

4.2 Materials and Methods

4.2.1 *B. graminis* f. sp. *tritici* isolates. U.S. *B. graminis* f. sp. *tritici* isolates were collected from 27 commercial wheat fields in 15 states in 2013 and 2014 as described in Cowger et al. (2018) and Meyers et al. (2019) (Figure 4.1). As a recent sample of the U.K. *B. graminis* f. sp. *tritici* population, 13 isolates were collected from the natural environment near Norwich, and an additional 5 isolates were collected in greenhouses at the John Innes Centre, Norwich, for a total of 18 isolates (all collected in 2014 and 2015, and kindly provided by J.K.M. Brown). Additional European isolates were included to act as references. DMI-sensitive isolate JIW11 was collected in 1985, DMI-resistant isolate Fel09 was collected in 1998, and the genome sequence isolate 96224 was collected in 1996 (Wyand and Brown 2005, Wicker et al. 2013).

All isolates were maintained on detached leaf plates and transferred to fresh susceptible wheat leaf material (‘Chancellor’ or ‘Jagalene’) every 8-11 days as previously described (Meyers et al. 2019). Results of tebuconazole and prothioconazole sensitivity screening were published for more than 360 isolates (Meyers et al. 2019).

4.2.2 DMI fungicide sensitivity screening and EC_{50} value calculation. A detached-leaf assay (Meyers et al. 2019) was used to test tebuconazole and prothioconazole sensitivity of the

18 U.K. isolates. Briefly, susceptible wheat seedlings (cultivar 'Jagalene') were sprayed to runoff with a particular fungicide concentration. The next day, leaves were segmented, placed atop water agar amended with benzimidazole (50 mg L^{-1}), and inoculated with a single *B. graminis* f. sp. *tritici* isolate. This assay yielded four leaf segment ratings (0, 1, 2, or 3) per concentration per fungicide for each isolate evaluated. However, after initial tests, DMI fungicide concentrations were increased to capture the higher insensitivity levels of the U.K. isolates. Tebuconazole concentrations of 0, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, and 220 mg L^{-1} and prothioconazole concentrations of 0, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 mg L^{-1} were used. Isolates JIW11 and Fel09 were evaluated using the original concentrations presented in Meyers et al. (2019).

Sensitivity to a third, less widely-used DMI, metconazole, was also evaluated for both U.S. and U.K. isolate collections. Metconazole was first registered for use in the U.S. in 2007 (US-EPA 2007), and the formulated, single-active ingredient product Caramba (BASF) was used for the sensitivity assay. At the time of the metconazole assessment, some isolates were no longer alive. Therefore, metconazole sensitivity was evaluated for 59 U.S. isolates and 15 U.K. isolates using the detached-leaf methodology previously described (Meyers et al. 2019). For both U.S. and U.K. isolates, metconazole concentrations were as follows: 0, 0.25, 0.5, 1, 2, 3, 4, 5, 6, 8, 10, and 12 mg L^{-1}.

EC_{50} values, or estimates of effective concentrations causing a 50% reduction in fungal growth, were calculated for each isolate-by-fungicide combination from the 0, 1, 2, and 3 leaf rating values of the sensitivity assay using PROC NLMIXED (SAS, Cary, NC) as described in Meyers et al. (2019). Mean EC_{50} values were used to compare isolates by geography, sporulation, *CYP51* genotype, *CYP51* copy number, and *CYP51* expression using PROC

GLIMMIX (SAS) as previously described. PROC REG (SAS) was also used to evaluate the correlations between levels of sensitivity to the different DMIs for each isolate collection.

4.2.3 U.S. isolate subset for expression and copy number analysis. A subset of 30 U.S. isolates was selected for *CYP51* expression and copy number analysis (Table 4.1). From the isolates remaining alive and vigorous, selection was based on geographic location and *CYP51* genotype*DMI phenotype combination. The 30 isolates represented all 15 states where isolates were originally collected. Genotype*phenotype combinations were divided into 9 categories. Three genotypes were observed among U.S. isolates: Y136, F136, and a heteroallelic (Het) type with both alleles present (Table 4.2; further discussed below). DMI sensitivity phenotypes were divided into high and low groups, with the high EC_{50} values being above the 80th percentile of the observed sensitivity range. For prothioconazole, the cutoff EC_{50} value between high and low categories was 80 mg L^{-1} and for tebuconazole 4 mg L^{-1} (Table 4.1). Each genotype*phenotype category observed in a state was included in the isolate subset, and all living isolates with the Het genotype were also included. Thus, there was one isolate from a state where all isolates had the same genotype and phenotype levels, and there were multiple isolates from states where multiple genotypes and phenotype levels were observed.

4.2.4 DNA extraction, RNA extraction, and cDNA synthesis. Conidial DNA was extracted from all 378 U.S. isolates evaluated in Chapter 2 using an E.Z.N.A.® Plant DNA extraction kit (Omega Bio-Tek, Norcross, GA) as described in Chapter 3. Additional DNA extractions, using the same kit and protocol, were completed for the subset of 30 U.S. isolates. For these additional extractions, three biological replicates per isolate were completed and DNA was quantified using a Qubit 2.0 fluorometer (ThermoFisher).

B. graminis f. sp. *tritici* RNA was extracted from the subset of 30 U.S. isolates, the 18 U.K. isolates, and DMI-sensitive control isolate JIW11. Fungal material from these isolates were collected in the form of epidermal peels using a 5% cellulose acetate solution as described by Arnold (2018). Peels were collected into 15 mL tubes, with three biological replicate tubes per isolate, and kept at -80°C until extraction. For extraction, tubes were kept on liquid nitrogen, ten nickel-plated beads were added per tube, and peels were ground using a Vortex Genie attachment in 3 rounds of 30-second intervals. Tubes were returned to liquid nitrogen between grinds. An RNeasy Plant Mini kit (Qiagen) was used following the protocol, employing the RLC buffer in place of the RLT buffer. RNA was extracted to a total volume of 70 uL per biological replicate.

To remove residual DNA, samples were subjected to a TURBO™ DNase (Invitrogen) treatment. The 'intense' version of the protocol was applied, using double the amount of DNase in order to achieve the purest RNA sample possible. Afterwards, RNA quality and concentration were evaluated using a DeNovix DS-11 spectrophotometer and a Qubit 2.0 fluorometer. As a secondary precaution to filter out DNA, an additional genomic DNA WipeOut Buffer (Qiagen) was used before reverse transcribing RNA to cDNA with a QuantiTect Reverse Transcription Kit (Qiagen). cDNA was evaluated for quality and concentration, using a DeNovix DS-11 spectrophotometer and a Qubit 2.0 fluorometer.

4.2.5 *CYP51* gene sequencing of U.S. isolates. For 363 of the U.S. isolates, along with European isolates 96224, JIW11, and Fel09, the entire *CYP51* gene and its upstream promoter region were sequenced using amplicon sequencing. Two pools of primers (Appendix C: Supplementary Table 4.1) were used to sequence the gene in 173 to 275 bp overlapping fragments. Sequencing and amplicon processing were completed in the lab of L. Cadle-Davidson

at Cornell University. Amplicons were aligned to the reference genome of Swiss isolate 96224 (Wicker et al. 2013) in order to identify *CYP51* mutations in the U.S. isolates and group them into genotypes. Genotypes of the 18 U.K. isolates were determined and provided by C. Arnold.

4.2.6 *CYP51* copy number variation assessment of U.S. isolates. Variation in *CYP51* copy number was evaluated among the 30 selected U.S. isolates, the 18 U.K. isolates, and the DMI-sensitive control isolate JIW11 using digital droplet PCR (ddPCR) performed with a BioRad QX200™ Droplet Generator and a BioRad QX200™ Droplet Reader. The associated protocols were followed as described in Arnold (2018). For each isolate, three biological replicates of DNA with two technical replicates were completed for the *CYP51* gene. The β-tubulin gene was used as a reference under the assumption of one β-tubulin gene per genome (Eastman et al. 2011). Forward and reverse primers for each gene were designed by C. Arnold (Table 4.3). The annealing temperature used for both primer sets was 60°C. Briefly, 22-uL reactions were prepared using 11 uL of 2x QX200™ ddPCR™ EvaGreen® Supermix (BioRad), 0.44 uL of 10-uM forward primer, 0.44 uL of 10-uM reverse primer, 0.25 uL of 20 units/uL enzyme NcoI HF® (New England BioLabs), 2 uL of 1.5-ng/uL template DNA, and 7.87 uL water. The restriction enzyme was included for more precise copy number determination in case of tandem *CYP51* genes, and 3 ng total isolate DNA was used to be within the optimal detection range of the assay based on the genomic weight calculations by C. Arnold (2018).

BioRad QuantaSoft software was used to view the droplet counts and manipulate thresholds. A medium threshold was manually applied to each reaction to delineate a boundary between the positive and negative droplet clouds. Any reading with less than 10,000 total droplets was repeated. A ratio of positive droplets to total number of droplets was computed for each reaction (technical replicate). A mean ratio was then calculated for each biological replicate

for each gene. To estimate the number of *CYP51* copies relative to β-tubulin copies, the mean *CYP51* ratio was divided by the mean β-tubulin ratio for each biological replicate. Those values were then averaged per isolate and multiplied by 2 to estimate the number of relative *CYP51* copies per isolate. PROC GLIMMIX (SAS) was used to determine if any significant differences exist between gene copy number and U.S. isolate selection category.

4.2.7 *CYP51* gene expression analysis. Quantification of *CYP51* gene expression was completed using quantitative reverse transcription PCR (qRT-PCR). Triplicate biological replicates of cDNA with two technical reps per biological rep were analyzed for each of the 30 U.S. selected isolates, the 18 U.K. isolates, and the DMI-sensitive control isolate JIW11. SYBR Green Supermix (BioRad)was used to estimate expression of the *CYP51*, β-tubulin, and actin genes. β-tubulin and actin expression were used to standardize *CYP51* expression per biological replicate because the two reference genes were previously found to be the most stable (Hacquard et al. 2013). Primer sequences and primer efficiencies were determined by C. Arnold (Table 4.4, Arnold 2018). Reactions were assayed using a CFX96 Real-Time System C1000 Thermal Cycler (BioRad) in 10 uL volumes: 5 uL SsoAdvanced universal SYBR Green Supermix (BioRad), 0.3 uL of 10-uM forward primer, 0.3 uL of 10-uM reverse primer, 2 uL of 2-ng/uL cDNA, and 2.4 uL water.

Quantification cycle (Cq) values were calculated by the BioRad CFX software. Technical replicates that differed by > 1 Cq per biological replicate were discarded; in those cases, *CYP51* expression was calculated from two biological replicates instead of three. Relative *CYP51* expression was calculated using the Pfaffl method (Pfaffl 2001), where mean JIW11 Cq was used to calibrate the Cq value of each biological replicate of each isolate. Then the mean of actin

and β-tubulin expression was used as a reference to normalize each isolate (Bustin et al. 2009, Vandesompele et al. 2002). *CYP51* expression was computed as follows:

(1) $Bio\ Rep\ Mean\ Cq = \frac{(Cq\ tech\ rep\ 1 + Cq\ tech\ rep\ 2)}{2}$.

Performed for each biological replicate per isolate per gene (CYP51, actin, and β-tubulin).

(2) $Mean\ JIW11\ Cq = \frac{(JIW11\ Bio\ Rep\ 1\ Mean\ Cq + JIW11\ Bio\ Rep\ 2\ Mean\ Cq + JIW11\ Bio\ Rep\ 3\ Mean\ Cq)}{3}$.

Performed for each gene (CYP51, actin, and β-tubulin).

(3) $\Delta Bio\ Rep\ Mean\ Cq = Bio\ Rep\ Mean\ Cq - Mean\ JIW11\ Cq$.

Performed for each biological replicate per isolate per gene (CYP51, actin, and β-tubulin).

(4) To take into account primer efficiency (E), was computed $E^{\Delta Bio\ Rep\ Mean\ Cq}$ per biological replicate per isolate per gene (CYP51, actin, and β-tubulin). Primer efficiencies calculated by C. Arnold using serial dilution curves can be found in Table 4.4.

(5) $Bio\ Rep\ CYP51\ Expression = \frac{CYP51\ E^{\Delta Bio\ Rep\ Mean\ Cq}}{\left[\frac{(actin\ E^{\Delta Bio\ Rep\ Mean\ Cq} + \beta{-}tubulin E^{\Delta Bio\ Rep\ Mean\ Cq})}{2}\right]}$.

Performed for each biological replicate per isolate.

(6) $Isolate\ CYP51\ Expression =$

$$\frac{(Bio\ Rep\ 1\ CYP51\ Expression + Bio\ Rep\ 2\ CYP51\ Expression + Bio\ Rep\ 3\ CYP51\ Expression)}{3}.$$

Performed for each isolate.

The level of correlation between copy number and gene expression for U.S. isolates was evaluating using PROC REG (SAS), while PROC GLIMMIX (SAS) was used for determining relationships between *CYP51* gene expression and DMI EC_{50} values for both the U.S. and U.K. isolate collections. Differences in gene copy number based on isolate category were also evaluated using PROC GLIMMIX (SAS).

4.3 Results

4.3.1 DMI sensitivity of U.S. and U.K *B. graminis* f. sp. *tritici*. Mean EC_{50} values differed dramatically between the U.S. and U.K. samples for all three DMIs evaluated (Figure 4.2; $P \leq 0.0005$). Prothioconazole sensitivity ranged from 0.19 to 295.7 mg L^{-1} for the U.S. isolates, while U.K. prothioconazole EC_{50} values ranged from 309.8 to 921.4 mg L^{-1} (Figure 4.2A). For tebuconazole, U.S. isolate EC_{50} values ranged from 0.12 to 18.7 mg L^{-1} and U.K. isolates ranged from 14.0 to 121.3 mg L^{-1} (Figure 4.2B).

Metconazole sensitivity was evaluated for fewer isolates from both countries (59 U.S. and 15 U.K. isolates), and the ranges for the two countries overlapped considerably more than for the other two DMIs, but the assay still revealed a significant difference between means of the two samples ($P = 0.0005$), with U.S. EC_{50}s ranging from 0.41 to 6.5 mg L^{-1} and U.K. EC_{50}s from 1.2 to 9.9 mg L^{-1} (Figure 4.2C).

Differences by U.S. geographic region in the prothioconazole and tebuconazole sensitivities of the U.S. *B. graminis* f. sp. *tritici* collection were previously reported (Meyers et

al. 2019). Geographic differences in mean U.S. metconazole EC_{50} values were not previously reported, and were significant at both the regional and state levels. Isolates from the Mid-Atlantic and Great Lakes regions had significantly greater EC_{50} values and thus reduced metconazole sensitivity as compared to isolates from the Plains region ($P = 0.01$, Supplementary Figure 4.1A). At the state level, isolates originating from Virginia, New York, North Carolina, Arkansas, and Florida have significantly reduced metconazole sensitivity as compared to isolates from Oklahoma and Alabama ($P = 0.01$, Supplementary Figure 4.1B). Metconazole sensitivity differences were not significant at the field level ($P = 0.08$, data not shown).

As described in Meyers et al. (2019), a significant but weak association between prothioconazole and tebuconazole sensitivity was observed at all U.S. geographic levels: isolate ($r = 0.36$, $P = 0.03$), field ($r = 0.46$, $P < 0.0001$), state ($r = 0.47$, $P < 0.0001$), and region ($r = 0.53$, $P < 0.0001$). No significant correlations between tebuconazole and metconazole or between prothioconazole and metconazole were found at any U.S. geographic level ($P \geq 0.18$). For the U.K. isolates, tebuconazole sensitivity significantly but weakly correlated with both prothioconazole ($P = 0.04$, $r = 0.42$) and metconazole ($P = 0.05$, $r = 0.44$), but prothioconazole and metconazole EC_{50} values did not correlate significantly.

4.3.2 U.S. *CYP51* genotypes: geographic and sporulation differences. Sequencing of the *CYP51* gene of 365 U.S. *B. graminis* f. sp. *tritici* isolates revealed three distinct genotypes for amino acid 136 (Table 4.2). Wildtype Y136 (codon TAT) and mutant F136 (codon TTT) were found in 62.5% and 28.5% of the collection, respectively. The third, heteroallelic (Het) genotype possessing both TAT and TTT codons was found in the remaining 9% of the sample. The Het genotype was first observed as a double peak on Sanger sequencing chromatographs of a few isolates. These results were confirmed not to be due to isolate cross-contamination by growing

five single-spored subsamples from each of three isolates and sequencing each subsample. All subsamples showed the same double peak on the chromatograph, indicating both Y136 and F136 were present in these isolates. The Het genotype was further confirmed during amplicon sequencing.

The F136 genotype was particularly common in the Atlantic seaboard states (Figure 4.1). By contrast, the Plains and AR-MO regional samples were dominated by wildtype Y136 *CYP51* isolates. The same was true for Southeast states Mississippi, Alabama, and Florida. Similarly, the majority of Michigan and Ohio isolates had the Y136 genotype. Fields in Georgia (fields 11, 12, and 13) differed in their *CYP51* genotypic proportions, with those in western Georgia predominantly of the F136 type, while field 13 isolates in eastern Georgia mostly had the Y136 genotype.

The greatest *CYP51* genotype diversity was found in North Carolina, New York, and Pennsylvania, where all three genotypes were usually found in each field. However, the Het *CYP51* genotype was widely distributed outside those states as well. It was found in 13 of the 27 fields in four of the five U.S. geographic regions. Het isolates were found in Kansas, Mississippi, North Carolina, Virginia, Ohio, Pennsylvania, and New York.

DMI EC_{50} values were averaged based on the three U.S. *CYP51* genotypes, and a consistent pattern was evident across the three DMI fungicides (Figure 4.3A). Isolates possessing the F136 genotype had a significantly greater reduction in prothioconazole sensitivity than Y136 genotype isolates, while mean prothioconazole EC_{50} of Het isolates was not significantly different than that of Y136 or F136 isolates ($P = 0.002$, Figure 4.3A). For both tebuconazole ($P < 0.0001$) and metconazole ($P < 0.0001$), isolates with the Y136 genotype were significantly more sensitive than either F136 and Het isolates (Figure 4.3A).

Sporulation, a component of fitness, was measured for 325 U.S. *B. graminis* f. sp. *tritici* isolates (. Het genotype isolates were found to have significantly greater sporulation than F136 isolates (Figure 4.4). Sporulation of Y136 genotype isolates did not differ significantly from either of the other two genotypes.

4.3.3 U.K. *CYP51* genotypes. Using both sequencing of overlapping PCR amplicons and sequencing of gene clones, two *CYP51* genotypes were discovered in the U.K. *B. graminis* f. sp. *tritici* population by C. Arnold (2018). The two genotypes differed for CYP51 amino acids 136 and 509 (Table 4.2). The wildtype Y136 genotype was not found in the U.K. collection; however, the mutant F136 genotype identified in U.S. isolates was also found in U.K. isolates. The second *CYP51* genotype in the U.K. population was heteroallelic at both the 136 and 509 loci (Het-Het); i.e., both the wildtype (Y136 and S509) and mutant (F136 and T509) loci were found in these Het-Het isolates. Clones revealed that the Y136 and S509 wildtype loci were linked in one allele in these isolates, while the mutant F136 and T509 loci were together in a second allele (Arnold 2018).

Mean DMI EC_{50} values were computed for each U.K. genotype. Sensitivity to prothioconazole (P = 0.52) and metconazole (P = 0.34) did not differ significantly based on *CYP51* genotype (Figure 4.3B). However, Het-Het isolates had a significantly reduced sensitivity to tebuconazole compared to F136 isolates (P = 0.006).

4.3.4 *CYP51* copy number variation in U.S. isolates. *CYP51* gene copy number estimates ranged from 1.5 to 4 copies per isolate (Figure 4.5A). For purposes of discussion, estimates were rounded to the nearest whole copy number. From the perspective of the 9 genotype-phenotype categories used to select the 30 isolates, there was no significant relationship between category and copy number (P = 0.35, data not shown). Collapsing the nine

categories, there was no significant difference between mean copy number based on *CYP51* genotype (P = 0.30, Figure 4.5B). However, looking at the relationship of copy number to DMI sensitivity by U.S region, a significant correlation was found to tebuconazole EC_{50} values (Figure 4.6A, P = 0.03, r = 0.67) and a near-significant correlation to prothioconazole EC_{50} values (Figure 4.6B, P = 0.06, r =0.60). The same relationship was not found for metconazole (P = 0.56, data not shown).

4.3.5 U.S. and U.K. *CYP51* expression. For the U.S. *B. graminis* f. sp. *tritici* isolates, *CYP51* expression was 0.4- to 2.7-fold higher than the mean of actin and β-tubulin expression, while the U.K. isolates ranged from 1.3- to 14.7-fold higher (means different at $P < 0.0001$, Figure 4.7A). In the U.S. sample, there was no significant relationship between gene expression and the 9 genotype-phenotype categories used to select the isolates (P = 0.48, data not shown), and the *CYP51* expression of U.S. isolates was only marginally different by *CYP51* genotype (P = 0.05, Figure 4.7B). In contrast, U.K. F136 isolates had significantly greater relative *CYP51* gene expression than Het-Het isolates (P = 0.0001, Figure 4.7B).

For the U.S. isolates, *CYP51* expression was significantly correlated with tebuconazole sensitivity (P = 0.007, r = 0.78), but not with sensitivity to the other two DMIs (Table 4.5). For the U.K. isolates, expression was marginally correlated with prothioconazole sensitivity (P = 0.06, r = 0.44), and not with sensitivity to tebuconazole or metconazole.

A significant positive correlation was observed between *CYP51* expression and copy number among U.S. isolates (P = 0.002, r = 0.72, Figure 4.8). All Het isolates had relatively high *CYP51* copy numbers (3-4) and relatively high expression (1.4-3). On the other hand, F136 and Y136 isolates were scattered across the observed ranges (Figure 4.8).

4.4 Discussion

Comparisons of plant pathogen populations with different histories of exposure to a stress can help characterize the adaptive status of each population by reference to the other. Here we compared three genetic and molecular mechanisms of DMI resistance in samples of *B. graminis* f. sp. *tritici* from two populations that differed in historic exposure to DMI fungicides. Because of that different history of selection, the U.K. isolates were much more DMI-insensitive than those from the U.S. The contrast between the samples continued with differences in the genotypes of *CYP51* detected in the two countries and the respective expression levels of that gene. However, *CYP51* heteroallelism and copy number variation were found in both samples. These findings may help illuminate the order of steps in the development of fungicide resistance.

DMI EC_{50} values revealed highly significant differences between the sensitivity of U.S. and U.K. isolates. For both prothioconazole and tebuconazole, which required an expanded range of experimental fungicide concentrations to estimate U.K. EC_{50}s, the maximum observed U.S. EC_{50} value was near that of the minimum observed U.K. value. For metconazole, the U.S.-U.K. difference was less; however, metconazole is rarely used on wheat in either country (http://water.usgs.gov/nawqa/pnsp/usage/maps/show_map.php?year=2016&map=METCONAZOLE&hilo=L&disp=Metconazole; Garthwaite et al. 2016). Even so, the difference in metconazole sensitivity between the two populations was also significant, with the U.K. population again more insensitive. Decreased metconazole sensitivity in the U.K. could be due to tebuconazole-metconazole cross resistance. These data reflect the effects of selection by heavy DMI use on the U.K. population. Despite what may be approximately annual DMI applications on wheat in the U.S., the results indicate that the U.S. *B. graminis* f. sp. *tritici* population is far from fully DMI-resistant; however, it displays diversity in DMI sensitivity.

CYP51 sequencing revealed the presence of four distinct *CYP51* genotypes among the U.S. and U.K. isolates. However, not all genotypes were detected in both collections. The wildtype Y136 genotype was only found in the U.S., while the mutant F136 genotype was found in isolates from both samples. A small proportion (9%) of U.S. isolates were heteroallelic (Het), having both Y136 and F136 alleles. The additional feature possessed by heteroallelic (Het-Het) U.K. isolates was the occurrence of wildtype and mutant nucleotides at both the 136 and 509 loci. Because *B. graminis* f. sp. *tritici* conidia from which the DNA was derived were haploid, heteroallelism indicated a minimum of two *CYP51* gene copies in these particular isolates. Due to the small size of the U.K. sample, it is not possible to infer the proportions of F136 and Het-Het genotypes in the U.K. population as a whole. Further, it is possible that a broader sample of the U.K. *B. graminis* f. sp. *tritici* population would have included the Y136 genotype.

In the U.S., Y136 isolates were more sensitive to all three DMIs evaluated, while isolates with either the F136 or Het genotypes were less sensitive to DMIs. It appears that the F136 mutation caused the same reduction in DMI sensitivity in the Het isolates as in those with a pure F136 genotype. In the U.K. sample, F136 and Het-Het isolates did not differ in prothioconazole or metconazole sensitivity, but Het-Het isolates did have significantly elevated tebuconazole EC_{50} values as compared to F136 isolates. The fact that *CYP51* heteroallelism conferred extra tebuconazole insensitivity beyond that provided by the F136 mutation in the U.K. but not the U.S. isolates indicates that the S509T mutation is likely specifically conducive to insensitivity to that DMI. Indeed, the same association of S509T with decreased tebuconazole sensitivity was detected in an Australian barley powdery mildew (*B. graminis* f. sp. *hordei)* sample (Tucker et al. 2019). Analyzing the CYP51 binding pocket, those authors discovered that the Y137F + S524T genotype (homologous to Y136F + S509T in *B. graminis* f. sp. *tritici*) caused the largest

increase in the binding cavity volume and the largest decrease in the diameter of the channel to the cavity. This suggests that tebuconazole, a relatively small DMI molecule, may not be able to properly span the cavity and bind, given the volume and orientation of the mutated protein (Tucker et al. 2019).

A higher frequency of F136 isolates, a mutant state associated with reduced sensitivity to all 3 DMIs, was found in the U.S. Atlantic seaboard than farther west. This fits with our previous finding that the U.S. *B. graminis* f. sp. *tritici* population has reduced DMI sensitivity in the eastern U.S. as compared to the AR-MO and Plains regions (Meyers et al. 2019). The F136 mutation appears to be an important factor explaining these regional differences. Given that the migratory pattern is from west to east in the U.S. *B. graminis* f. sp. *tritici* population (Cowger et al. 2016), it seems likely that migration will not increase the frequency of the F136 mutation in the AR-MO and Plains regions.

Het genotype isolates were scattered throughout the mid-Atlantic and Northeast states, as well as in Mississippi and Kansas fields, where they were unlikely to have arrived by migration from the east. It was striking to find this genotype in so many widely separated locations. This indicates either that the U.S. founding *B. graminis* f. sp. *tritici* population, hypothesized to have arrived with European colonists (Parks et al. 2009), included Het isolates when it was first established and has since expanded due to human activity, or that there have been convergent gene duplication and mutation events in different regional subpopulations. While Het isolates are widely distributed in the U.S., they are currently a small minority of the population. Thus, Het isolates may lack a selective advantage that the more commonly found F136 isolates possess under the typical U.S. fungicide regime. One possibility is that the Het genotype represents a transitional stage between the Y136 and F136 genotypes. The increased sporulation of Het

isolates compared to isolates with the F136 genotype may be responsible for maintaining the former in the population, despite their retention of the presumably disadvantageous Y136 allele. Therefore, the Het genotype may be advantageous, permitting growth and undiminished sporulation despite contact with DMI fungicides. However, given that sporulation is only one component of isolate fitness, other aspects of fitness (latent period, pustule size, effectiveness of dispersion, etc.) should be evaluated to further understand what genotypic state is most beneficial. In addition, it remains unclear what function, if any, the wildtype Y136 allele has in the U.S. Het isolates (or the wildtype Y136 + S509 allele has in the U.K. Het-Het isolates).

If the Het genotype, heteroallelic at the 136 locus, migrated to the U.S. from the U.K. when the U.S. *B. graminis* f. sp. *tritici* population was initially established, it may have since been replaced in the U.K. by the Het-Het genotype with heteroallelism at both the 136 and 509 loci. A larger U.K. sample would be needed to corroborate the absence of the Het (as opposed to Het-Het) genotype there. In any case, the present study indicates that at this time, Het-Het isolates have not migrated to the U.S., and hopefully U.S. fungicide management programs can prevent their emergence.

Given that the U.K. Het-Het genotype isolates only had an advantage over their F136 compatriot strains in confronting tebuconazole, and not prothioconazole or metconazole, it appears that intensive tebuconazole applications in particular may have driven the emergence of the Het-Het U.K. genotype. From the present data, it appears that, while T509 has conferred additional insensitivity to tebuconazole on U.K. Het-Het strains, higher *CYP51* expression is instead the driving force for F136 and Het-Het isolates to overcome other DMI applications (more on this below). Indeed, our data suggest that CYP51 expression of U.K. isolates is modestly correlated with insensitivity to prothioconazole but not with insensitivity to

tebuconazole, where T509 is explanatory. It is intriguing that the T509 mutation only appears in linkage to the F136 mutation and in U.K. heteroallelic isolates, suggesting a relatively recent emergence of this mutation.

This is not the first discovery of *CYP51* heteroallelism influencing DMI sensitivity in a powdery mildew. The same phenomenon was recently found in *Erysiphe necator* (causal agent of grape powdery mildew) isolates which were also heteroallelic (Y and F) at the 136 locus (Rallos and Baudoin 2016). The heteroallelic *E. necator* isolates had both greater *CYP51* gene expression and greater DMI insensitivity than pure mutant and pure wildtype isolates. Copy number variation analysis of *E. necator* isolates revealed 1 to 14 *CYP51* gene copies. While 96% of isolates with a single copy were Y136, 94% of isolates with multiple copies had F136 copies, suggesting gene duplication events and increased copy number are key to DMI resistance in *E. necator* (Jones et al. 2014).

U.S. *B. graminis* f. sp. *tritici* isolates were found to possess from 2 to 4 *CYP51* copies in their genomes. The range in copy number observed in the U.S. collection did not differ much from that of the U.K. *B. graminis* f. sp. *tritici* isolates, among which 2 to 5 copies were found (Arnold 2018). It should be noted that the isolates evaluated in Arnold 2018 and the current set overlapped, but were not identical: just three were in common between the two sets. Unlike in *E. necator*, no significant difference in copy number was observed between genotype groups. Differences in *CYP51* copy number did, however, correlate to tebuconazole sensitivity which could indicate that increased gene copy number specifically influences tebuconazole sensitivity in the U.S. Unexpectedly, our findings indicated at least 3 gene copies in 47% of the Y136 and 67% of the F136 isolates evaluated, when multiple copies were only anticipated to occur in Het

isolates. Further allele-specific analyses will be needed to determine if the additional *CYP51* gene copies are redundant or individually functional in each of the three genotypes.

CYP51 expression levels differed drastically between the two sets of isolates, with the mean expression of U.K. isolates being twice (and the maximum over four times) that of U.S. isolates. Unlike *E. necator* isolates, expression did not differ significantly by genotype in the U.S. *B. graminis* f. sp. *tritici* isolates. Expression in U.K. F136 genotype isolates, on the other hand, was three times that of the U.K. Het-Het isolates, which had expression levels near those of the U.S. Het isolates. This result was noteworthy given that *CYP51* copy number variation is similar in the U.S. and U.K. isolates and there was no significant difference in copy number between U.K. F136 and Het-Het isolates (Arnold 2018). The evidence supports the hypothesis that *CYP51* overexpression is responsible for the high EC_{50} values in U.K. F136 isolates, rather than the F136 mutation itself, because U.S. F136 isolates (with up to 10 times lower EC_{50} values) had much lower *CYP51* expression, close to that of the reference genes. Further investigation of *CYP51* promoters and transcription factors in U.K. F136 isolates is needed to understand the mechanism of this overexpression.

In both U.S. and U.K. samples, *CYP51* expression was significantly or nearly significantly correlated with sensitivity to prothioconazole, and uncorrelated with response to metconazole. The relationship of expression to sensitivity was strongly divergent for tebuconazole: as was discussed above, T509 rather than expression was the main explanatory factor in the U.K. sample, while in the U.S. sample, without T509, expression was significantly related to sensitivity. The lack of association between *CYP51* expression and metconazole sensitivity in both samples may be due to the relatively minor extent of adaptation to this DMI, and suggests that cross-resistance involving it is limited.

In the U.S. sample, a significant correlation of both *CYP51* copy number and gene expression with tebuconazole sensitivity was observed along with a significant, positive relationship between gene copy number and expression. This relationship would be expected when all gene copies present in a genome were being expressed. Further insight could be gained by examining allele-specific copy number and allele-specific gene expression in these isolates.

Other future experiments that could add to our knowledge of the genetics of DMI insensitivity in *B. graminis* f. sp. *tritici* include expression analysis of other genes. RNA-sequencing of isolates in this study could verify the qPCR *CYP51* expression levels presented here and shed light on possible changes in expression of other genes besides *CYP51*. In addition to mechanisms involving the *CYP51* target gene, overexpression of drug transporters has also been associated with DMI insensitivity (de Waard et al. 2006). These membrane-bound efflux pumps are ATP-binding cassette (ABC) and major facilitator superfamily (MFS) transporters that expel fungicide molecules from fungal cells. When in sufficient abundance, the pumps can decrease the accumulation of DMI molecules enough to limit their ability to occupy the CYP51 active site (de Waard et al. 2006). This phenomenon is most often found in human-infecting fungi (Lupetti et al. 2002, Cannon et al. 2009); however, the correlation between efflux-pump overexpression and DMI resistance has also been established in prominent phytopathogens like *Mycosphaerella graminicola* and *Botrytis cinerea* (Leroux and Walker 2011, 2013).

Gene copy number estimation could also be verified by performing whole genome sequencing (WGS) on selected isolates. WGS could reveal whether multiple *CYP51* gene copies are located in tandem or are dispersed in the genome. Tandem gene copies could indicate a recent duplication event where both gene copies remain functional under the same regulatory elements, while dispersed copies may indicate loss or change in gene function or expression. The

more we understand about the locations of the gene copies, the better we can hypothesize how and why the copies arise in the evolution of DMI insensitivity.

Overall, this study suggests that the U.S. and U.K. *B. graminis* f. sp. *tritici* populations are at different evolutionary stages of development of DMI resistance. The discovery of multiple *CYP51* gene copies and heteroallelism in the U.S. *B. graminis* f. sp. *tritici* population provides the first characterization of the basis for reduced DMI sensitivity in this country. In the U.S., tebuconazole-specific reduced sensitivity may be associated with both *CYP51* copy number and expression. By comparative analysis of a small sample of the less sensitive U.K. population, we developed the hypotheses that U.K. insensitivity stems from *CYP51* overexpression in F136 genotype isolates for general DMI-insensitivity and an S509T mutation in Het-Het isolates for tebuconazole-specific insensitivity. Further study of the differences between these populations in different stages of evolution toward DMI resistance could help define a more precise approach to slowing the process, not only in the U.S. *B. graminis* f. sp. *tritici* population but in other pathosystems as well.

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Table 4.1. Nine selection categories of the 30 U.S. *Blumeria graminis* f. sp. *tritici* isolates used to analyze *CYP51* gene expression and copy number variation.

Selection category	*CYP51* genotype[a]	Prothioconazole EC_{50} level[b]	Tebuconazole EC_{50} level[c]	Isolates
1	Y136	Low	Low	ALA2-E-1-1 ARF-A-1-5 FLG-B-1-3 GAS-B-1-2 KSM-B-1-5 MIR(14)-C-2-1 MOB(14)-A-2 MSG-C-3-4 NCF-D-1-1 NEL-6 NYB(14)-C-3-1 OHJ-C-3-3 OKS-A-2-2 PAF(14)-B-1-4
2	Y136	High	High	OHW-D-3-5
3	F136	High	High	NCM-B-3-4
4	F136	Low	High	GAP-B-2-2 GAT-D-3-3
5	F136	High	Low	NYG-B-1-5
6	F136	Low	Low	GAT-B-2-4 MIR(14)-D-3-3 NCM-A-1-1 NYA-E-3-3 NYG-D-1-1
7	Het	Low	Low	KSM-B-3-1 VAS-B-1-3 VAS-B-3-2 VAS-D-2-1
8	Het	Low	High	KSM-D-1-5
9	Het	High	High	NCM-E-1-2

[a] U.S. *CYP51* genotypes are explained in Table 4.2.
[b] High EC_{50} values were > 80 mg L^{-1}; low values were < 80 mg L^{-1}.
[c] High EC_{50} values were > 4 mg L^{-1}; low values were < 4 mg L^{-1}.

Table 4.2. *CYP51* genotypes found in *Blumeria graminis* f. sp. *tritici* isolates collected from commercial fields in the United States and from both natural environments and greenhouses in the United Kingdom[a]

Genotype	Amino acid 136 codon[b]	Amino acid 509 codon[c]	% of U.S. Sample[d]	% of U.K. Sample[e]
Y136	TAT	TCC	62.5	----
F136	TTT	TCC	28.5	50.0
Het	TAT & TTT	TCC	9.0	----
Het-Het	TAT & TTT	TCC & ACC	----	50.0

[a] U.S. genotypes are differentiated from one another by the codon sequence at amino acid 136, while U.K. genotypes are differentiated by codon sequences at amino acids 136 and 509.
[b] Wildtype = TAT, Mutant = TTT
[c] Wildtype = TCC, Mutant = ACC
[d] Total of 363 isolates in U.S. sample.
[e] Total of 18 isolates in U.K. sample.

Table 4.3. Primers for detecting *CYP51* copy number variation in U.S. isolates *of Blumeria graminis* f. sp. *tritici* using digital droplet PCR (ddPCR)[a]

Gene[b]	Primer Name	Primer Sequence 5' -> 3'	Annealing Temp (°C)
CYP51	qCYP51_F2	TTTCATGCTTCACTGGGCAC	60
	qCYP51_R2	CAGTTTCTTTCTGCGTCCGA	
β-tubulin	qPCR_tub_F3	AGAACATGATGGCAGCCTC	60
	qPCR_tub_R3	GCATGCGTATAAAACGTGCAG	

[a] Primers were designed and tested by C. Arnold (2018).
[b] *CYP51* is the gene of interest; β-tubulin the reference gene.

Table 4.4. Primers for determining relative *CYP51* gene expression in *Blumeria graminis* f. sp. *tritici* via quantitative PCR (qPCR)[a]

Gene[b]	Primer Name	Primer Sequence 5'->3'	Annealing Temp (°C)	Primer Efficiency[c]
CYP51	cyp51_x12b_F	GAATCCCAAGCCAAGTAC	60	97%
	cyp51_x_splice_R	GCATTAACATCCCTCAGTT		
Actin	ACT_x12a_F	CGAGCTGTTTTCCCATC	54	93%
	ACT_2a_R	TATCTAAGAGTCAGAATACCA		
β-tubulin	TUBb_x12a_F	ACATGCTCTGCTATTTTCCG	58	93%
	TUBb_1a_R	TGGAATCCACTCAACAAAGT		

[a] cDNA-specific primers were designed by C. Arnold, R. Whetten, and E. Meyers.
[b] *CYP51* is the gene of interest; actin and β-tubulin were used as reference genes.
[c] Primer efficiencies were determined by C. Arnold using a cDNA dilution series (Arnold 2018).

Table 4.5. Correlations between relative *CYP51* gene expression and DMI sensitivity levels for *Blumeria graminis* f. sp. *tritici* isolates originating from the United States and the United Kingdom

Isolate Collection [a]	**Prothioconazole**		**Tebuconazole**		**Metconazole**	
	r	***P***	***r***	***P***	***r***	***P***
United States	0.56	0.09	0.78	0.007	-0.05	0.89
United Kingdom	0.44	0.06	0.09	0.74	-0.07	0.80

[a] U.S. collection: n = 30 selected isolates (Table 4.1); U.K. collection n = 18 isolates

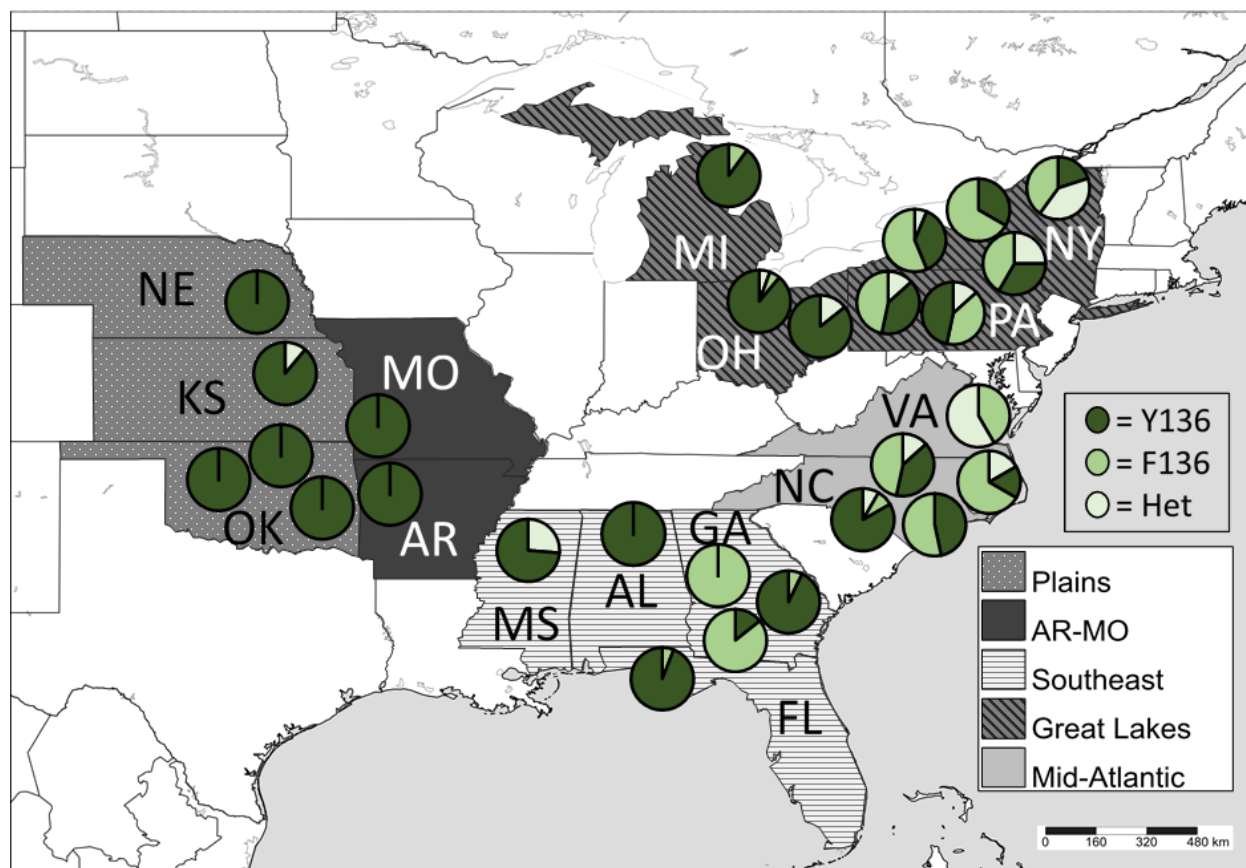


Figure 4.1. Geographic distribution of *CYP51* genotypes in the United States *Blumeria graminis* f. sp. *tritici* collection. The breakdown by *CYP51* genotype in samples from each of 27 fields is shown. Sample sizes are listed in Appendix C: Supplementary Table 4.2.

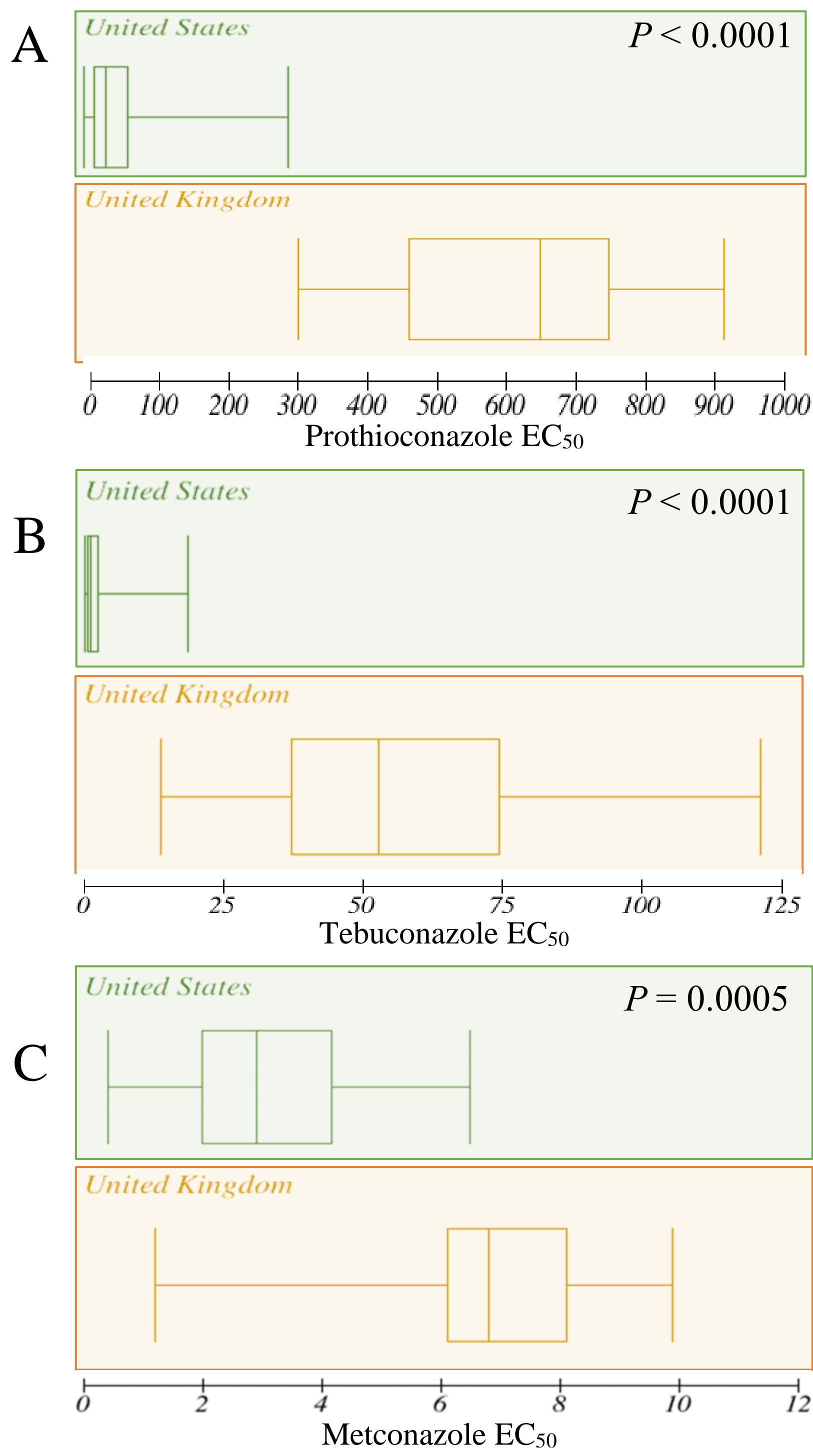


Figure 4.2. Range of EC_{50} values in the United States and United Kingdom collections of *Blumeria graminis* f. sp. *tritici* isolates. **A**) Prothioconazole sensitivity was assessed for 369 U.S. isolates and 18 U.K. isolates, **B**) tebuconazole sensitivity for 378 U.S. isolates and 18 U.K. isolates, and **C**) metconazole sensitivity for 59 U.S. isolates and 15 U.K. isolates. P-values < 0.05 indicate a significant difference between mean EC_{50}s based on comparative t-tests. Note that the scale differs for each fungicide EC_{50} plot.

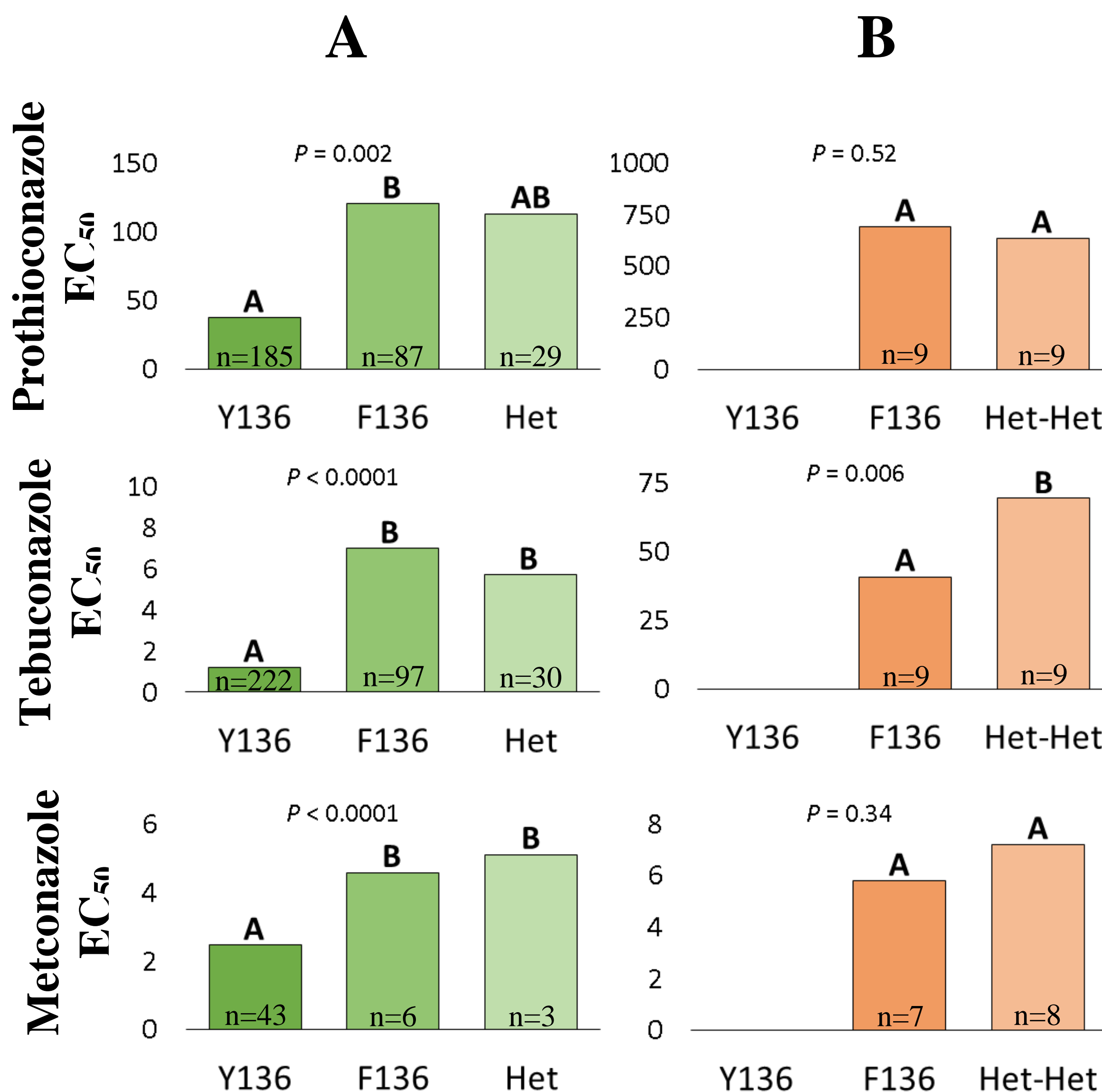


Figure 4.3. Mean DMI EC_{50} values of *Blumeria graminis* f. sp. *tritici* isolate collections categorized by *CYP51* genotype. **A**) U.S. isolates and **B**) U.K. isolates. *P*-values indicate significance of differences among means; within a graph, bars topped by the same letter are not different at $P < 0.05$.

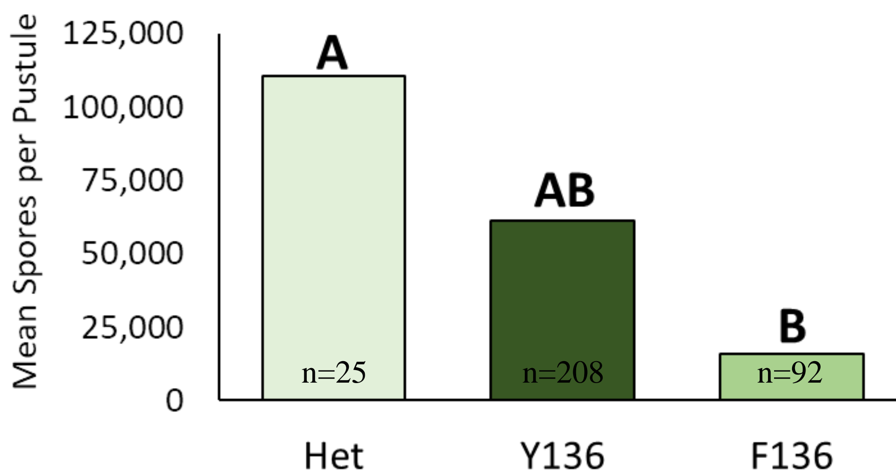


Figure 4.4. Mean sporulation, as a measurement of fitness, per *CYP51* genotype of United States *Blumeria graminis* f. sp. *tritici* isolate collection. Bars topped by the same letter are not significantly different ($P < 0.05$) using a pairwise t-test.

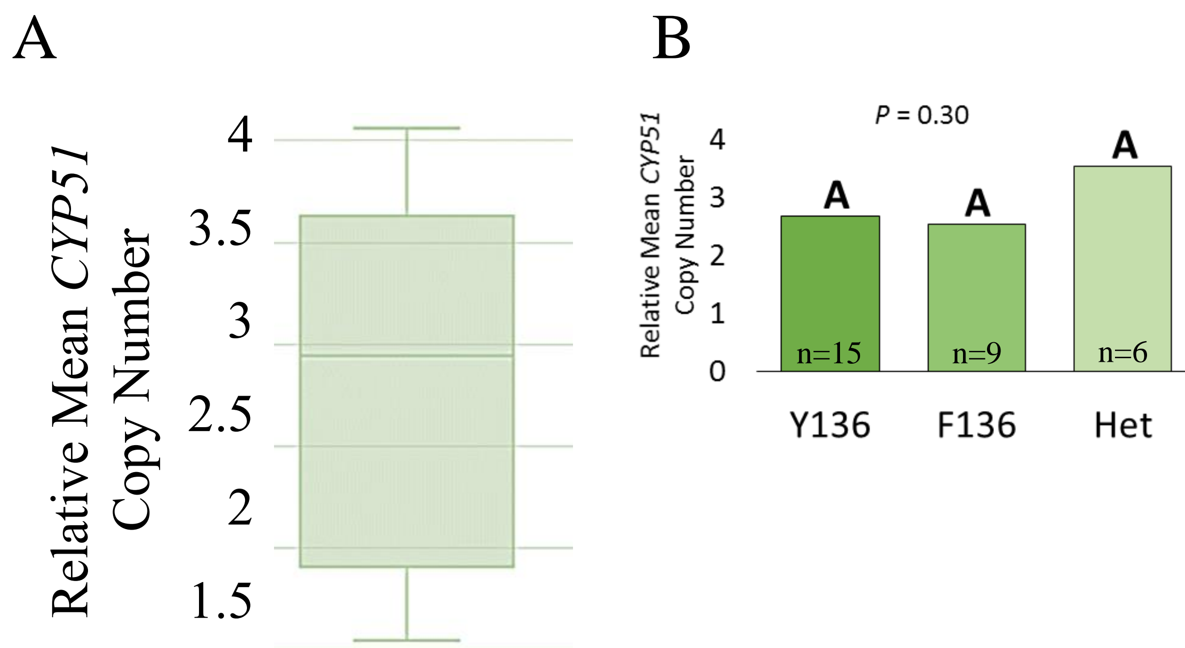
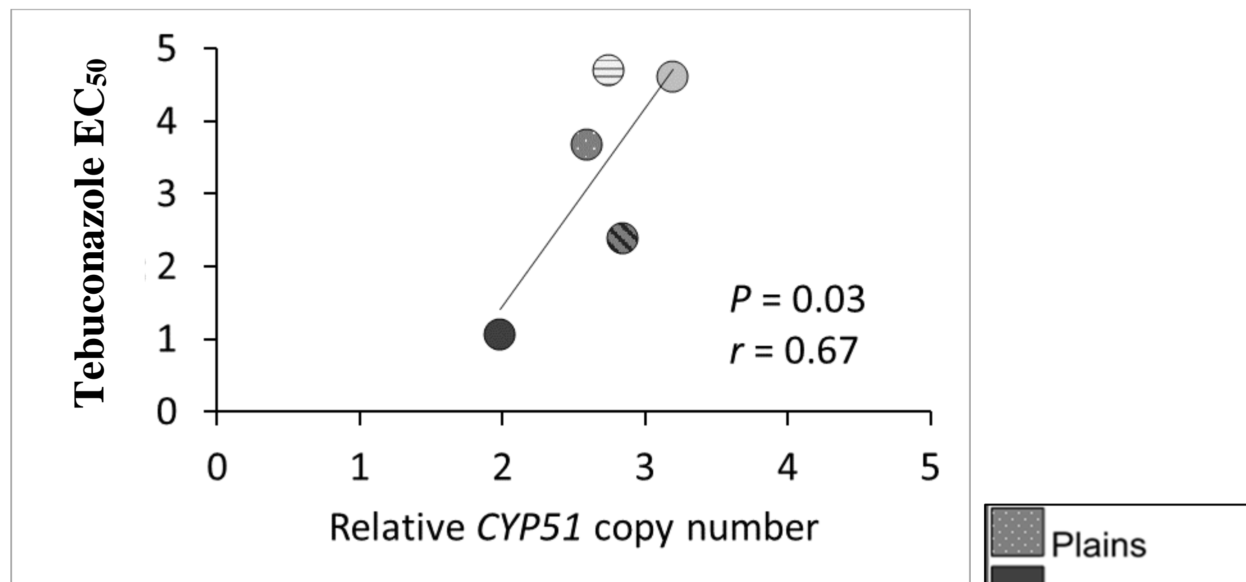


Figure 4.5. Variation in *CYP51* copy number estimates for 30 *Blumeria graminis* f. sp. *tritici* isolates collected in the United States. **A**) Observed range of *CYP51* copy number, and **B**) mean *CYP51* copy number estimate by genotype. *P*-value reflects comparison of means; bars topped by the same letter are not different at $P < 0.05$.

A



B

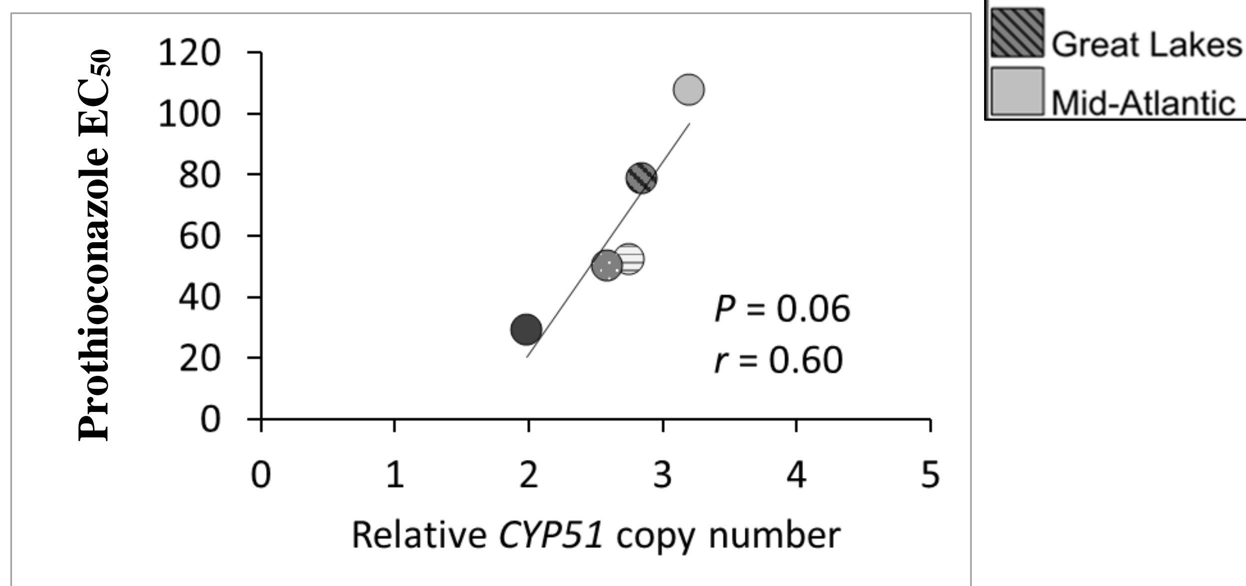
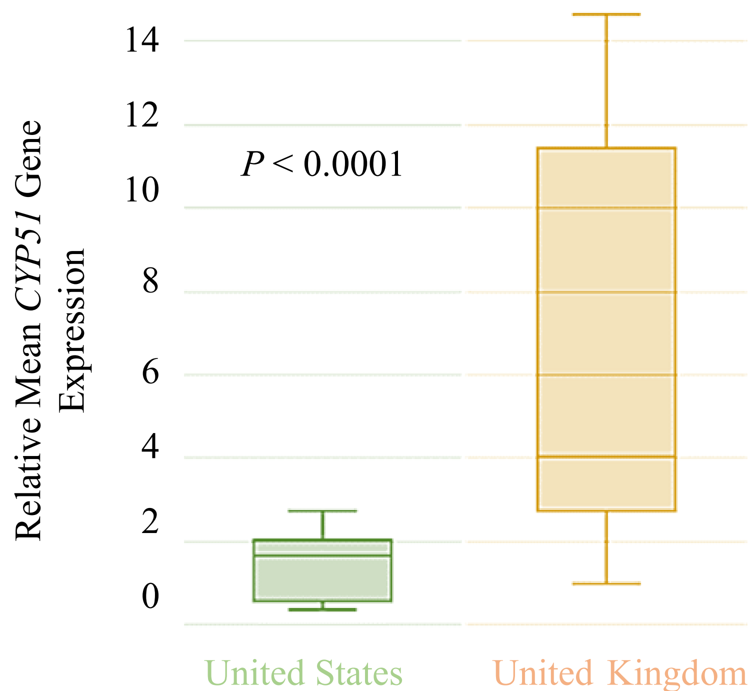


Figure 4.6. Correlation between relative mean *CYP51* copy number estimates and DMI sensitivity level of 30 *Blumeria graminis* f. sp. *tritici* isolates by U.S. region of origin. Significance and correlation coefficient values for **A**) tebuconazole and **B**) prothioconazole are indicated. Isolate sample sizes: Plains, N = 5; AR-MO, N = 2; Southeast, N = 7; Great Lakes, N = 9; and Mid-Atlantic, N = 7.

A



B

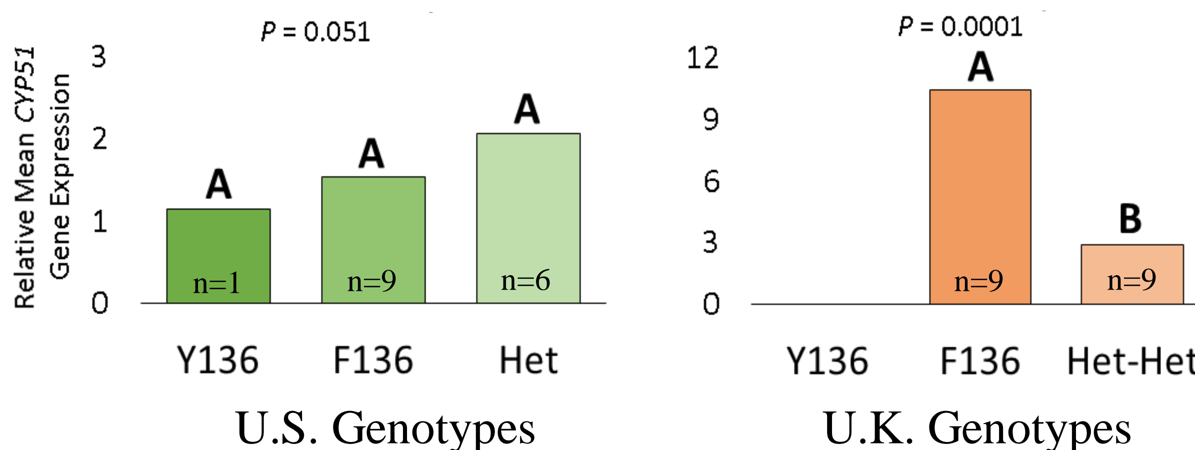


Figure 4.7. Relative *CYP51* expression estimates of 30 *Blumeria graminis* f. sp. *tritici* isolates collected from the United States and 18 from the United Kingdom. **A**) Observed range in *CYP51* expression and **B**) mean *CYP51* expression by genotype. *P*-values indicate significance of differences among means; within a graph, bars topped by the same letter are not different at $P < 0.05$.

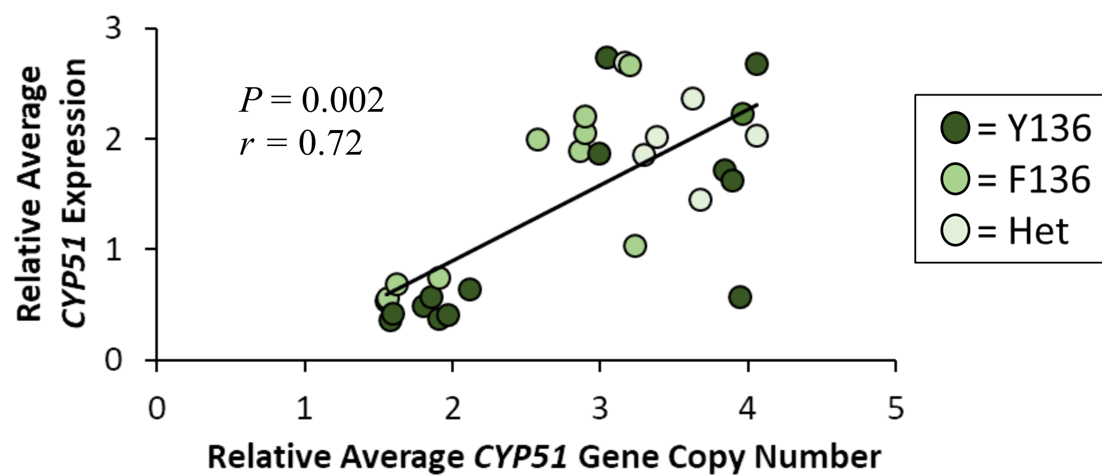


Figure 4.8. Relationship between *CYP51* copy number variation and *CYP51* expression in 30 United States *Blumeria graminis* f. sp. *tritici* isolates. Each data point is a single isolate.

CHAPTER 5

Conclusions

This dissertation reports the first evaluation of fungicide sensitivity in the United States population of *Blumeria graminis* f. sp. *tritici,* the causal agent of wheat powdery mildew. Despite decades of fungicide use on wheat in the U.S. and regional breakdown of *Pm* host resistance genes, efficacy of registered fungicides had not been completed prior to this study. Using a collection of almost 400 *B. graminis* f. sp. *tritici* isolates sampled from 15 states in the eastern U.S., sensitivity was measured for three fungicide modes of actions: demethylation inhibitors (DMIs), quinone outside inhibitors (QoIs), and succinate dehydrogenase inhibitors (SDHIs).

A range in sensitivity was found for all active ingredients tested in this study, indicating diversity in the U.S. population. Reduced sensitivity to DMI fungicides, in particular, was evident in Mid-Atlantic and Great Lakes sub-populations as compared to the central U.S. sub-population. Genetic investigation of the target gene, *CYP51*, further confirmed this geographic difference with the occurrence of the mutant F136 genotype in eastern sub-populations that was not found in the central U.S. sub-population. However, the mechanism by which DMI sensitivity is diminished seems to differ based on individual active ingredient as well as by continental population. In the U.S., the presence of the F136 allele (in either pure F136 or Het genotype isolates) was able to reduce sensitivity to all DMIs tested, while increased *CYP51* expression specifically caused reduced tebuconazole sensitivity. In the DMI-insensitive United Kingdom *B. graminis* f. sp. *tritici* population, increased *CYP51* expression influenced prothioconazole sensitivity and the presence of the T509 mutation in Het-Het genotype isolates caused a reduction in tebuconazole sensitivity. Further investigation into the mechanisms of DMI sensitivity (specifically causes of increased *CYP51* expression and copy number) in the world *B.*

graminis f. sp. *tritici* population is necessary. Protein docking studies with specific DMI active ingredients in the CYP51 binding pocket will also be beneficial in determining why multiple mechanisms may be necessary to overcome the DMIs.

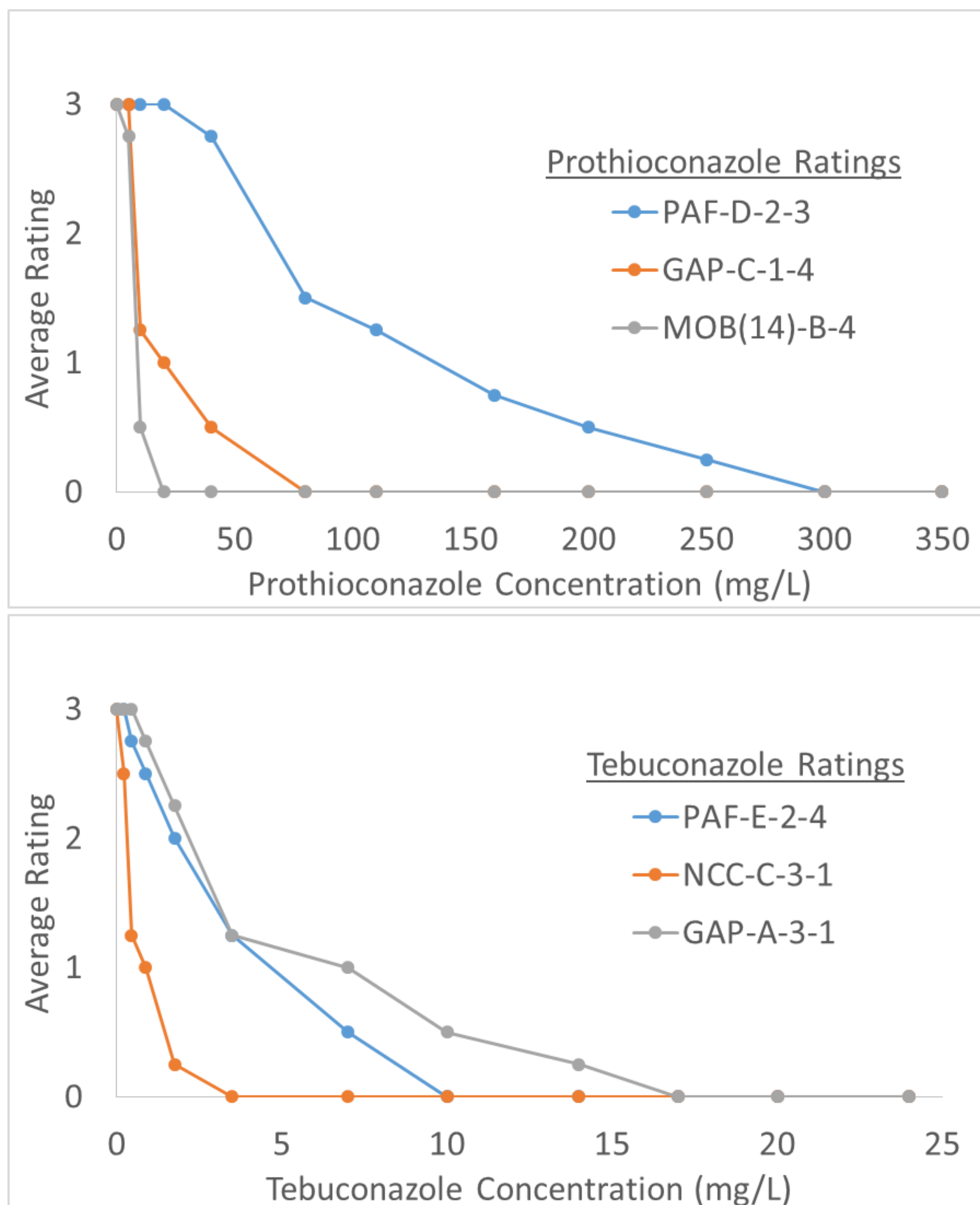
The diversity in sensitivity observed to QoI and SDHI fungicides in this study was less than that of DMIs. However, given the greater risk for resistance development in QoIs and SDHIs, sensitivity to them is expected to breakdown quickly, instead of gradually like what is likely occurring for the DMIs. Because of this, future isolate sampling and sensitivity analyses are recommended. The data from this dissertation should be used as a baseline to which future studies can be compared. Additional samples will be important in order to evaluate change in fungicide sensitivity and rate of adaptation over time.

The knowledge gained from this dissertation has confirmed foliar fungicides are an effective wheat powdery mildew control mechanism in the U.S. This is encouraging because it indicates that thus far growers have practiced responsible fungicide use. To maintain fungicide effectiveness and durability, mixed mode of action formulations should continue to be used to minimize the selection pressure on any one fungicide category. With the new information gained from this study, more tailored and regional-specific fungicide recommendations can begin to be made. However, continued sampling and monitoring of the U.S. *B. graminis* f. sp. *tritici* population will be key in preventing the DMI and QoI insensitivity found in the Europe in the U.S. population.

APPENDICES

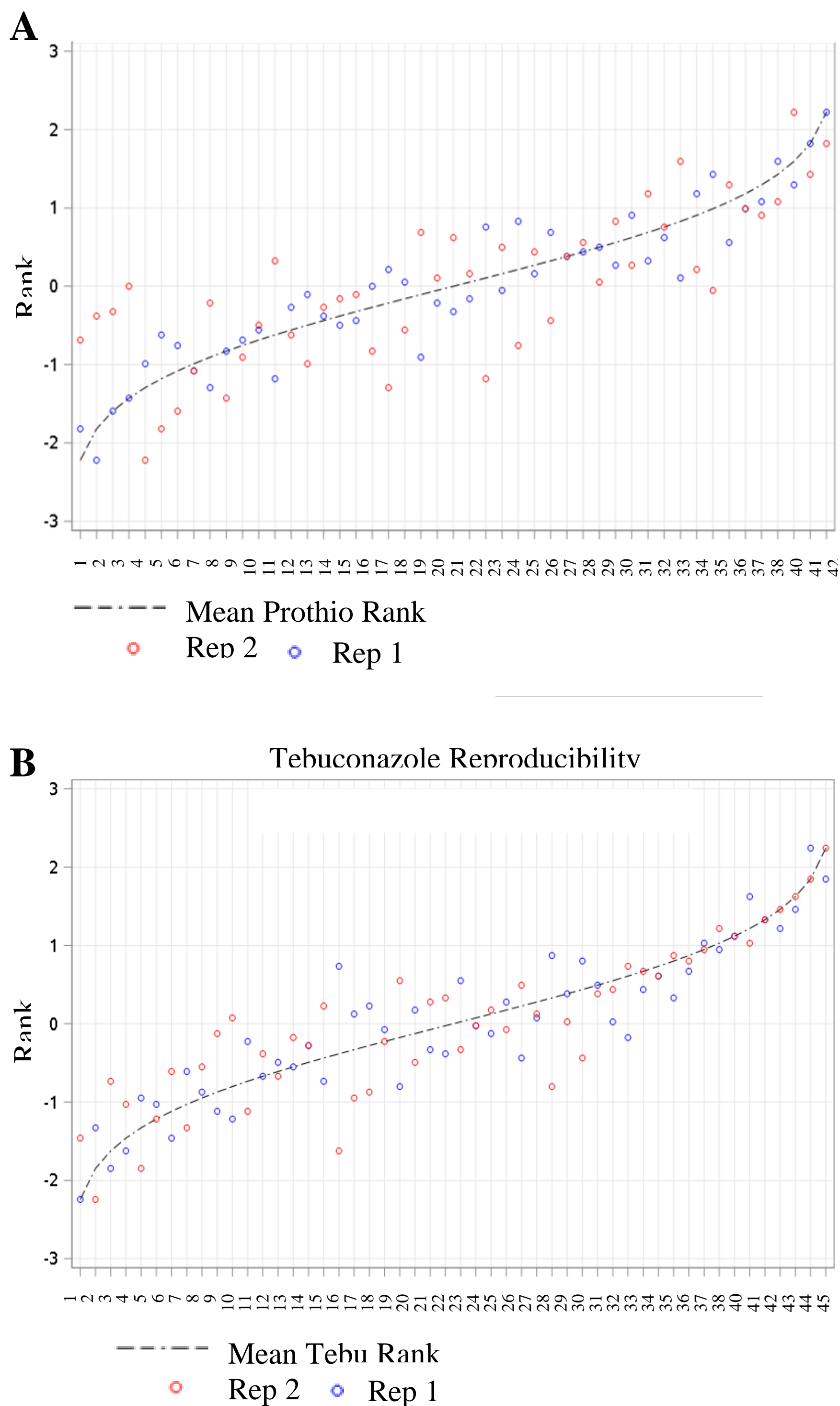
Appendix A

Supplementary Figure 2.1. Illustration of rating scale of 0, 1, 2, and 3 for detached-leaf fungicide sensitivity assay.



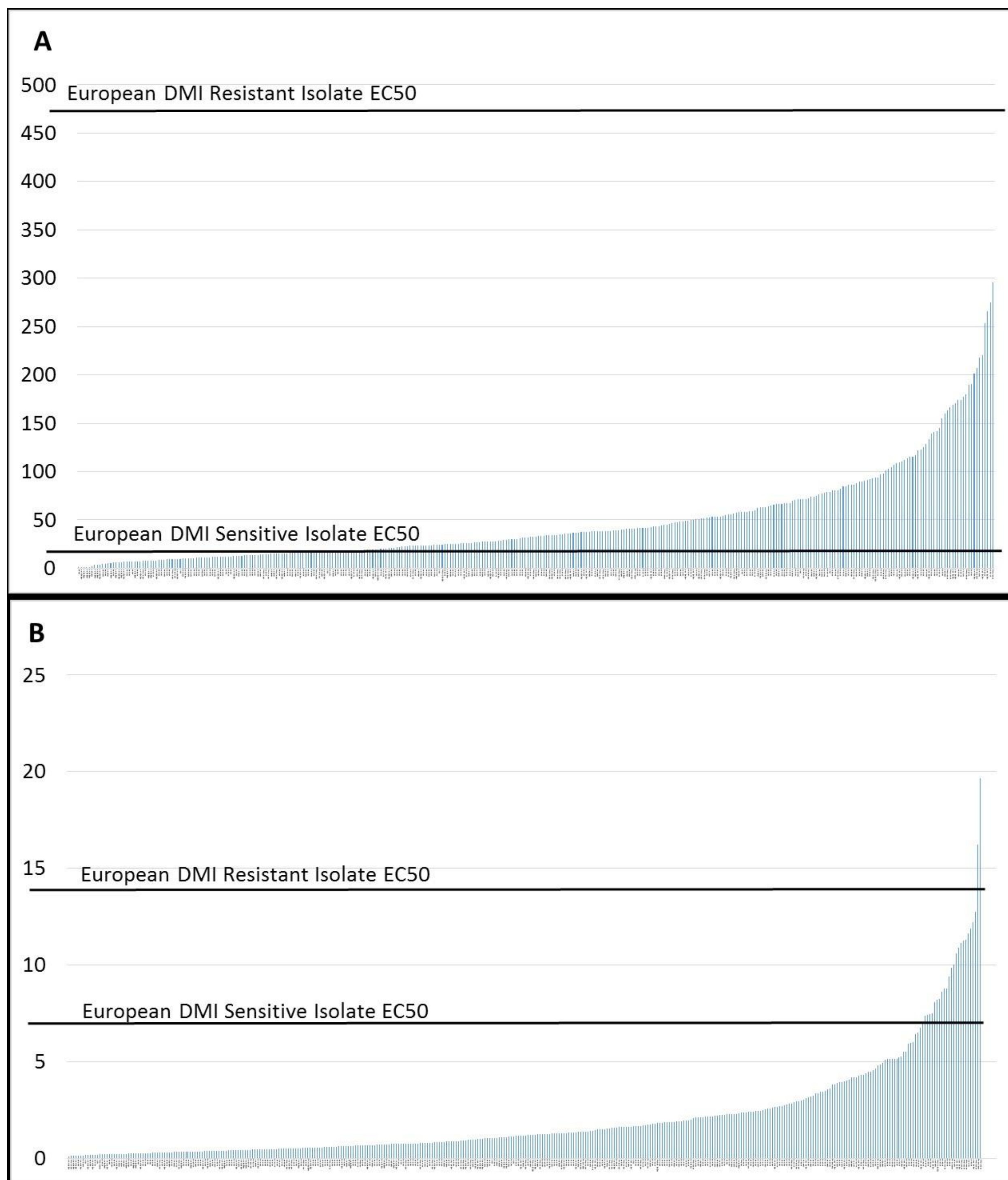
Supplementary Figure 2.2. Examples of sensitivity ratings averaged across 4 replicate leaf segments when each of three individual *Blumeria graminis* f. sp. *tritici* isolates was exposed to 13 concentrations of prothioconazole (top panel) or tebuconazole (bottom panel).[a]

[a]In 2013 or 2014, isolates PAF-D-2-3, GAP-C-1-4, and MOB(14)-B-4 were sampled from Pennsylvania Furnace, Pennsylvania, Pine Mountain, Georgia, and Bronaugh, Missouri, respectively. Isolates PAF-E-2-4, NCC-C-3-1, and GAP-A-3-1 were sampled from Pennsylvania Furnace, Pennsylvania, Calypso, North Carolina, and Pine Mountain, Georgia, respectively (Cowger et al. 2018).



Supplementary Figure 2.3. Plots of EC_{50} ranks in reproducibility analysis when a subset of *Blumeria graminis* f. sp. *tritici* isolates were twice each exposed to increasing concentrations of **A**) prothioconazole, 47 isolates and **B**) tebuconazole, 50 isolates.[a]

[a]Replicate 1 is shown in blue and replicate 2 in red



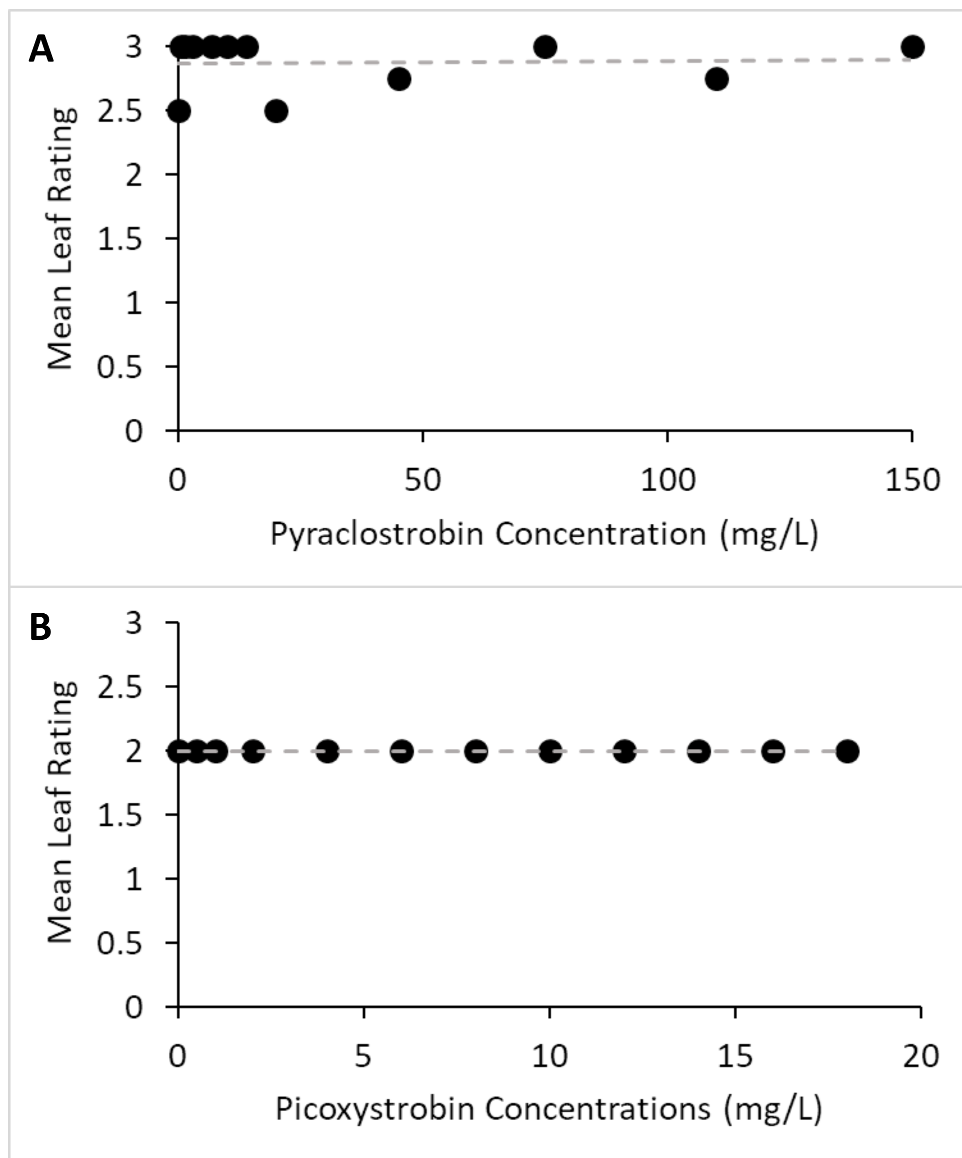
Supplementary Figure 2.4. EC_{50} values of all U.S. *Blumeria graminis* f. sp. *tritici* isolates evaluated for sensitivity to **A**) prothioconazole (345 isolates) and **B**) tebuconazole (382 isolates). For comparison, the EC_{50} values of European DMI-resistant isolate Fel09 and DMI-sensitive isolate JIW11 are also indicated.

Appendix B

Supplementary Table 3.1. Mean pyraclostrobin, picoxystrobin, and fluxapyroxad phenotypic sensitivity values of *Blumeria graminis* f. sp. *tritici* isolates per field sampled in 2013-2014 [a]

			EC_{50} Values (mg/L)[b]		
State	**Field**	**N**	**Pyraclostrobin**	**Picoxystrobin**	**Fluxapyroxad**
New York (NY)	**19**	15	5.06 a	1.96 bcdefgh	19.22 ab
Arkansas (AR)	**7**	13	3.62 ab	2.26 abcdef	27.50 ab
Georgia (GA)	**12**	15	3.51 a c	2.08 abcdefgh	6.66 cdefgh
Kansas (KS)	**2**	18	3.05 abc	2.11 abcdefg	15.15 ab
New York (NY)	**20**	11	2.98 abc	2.25 abcdefgh	10.12 bcdefg
North Carolina (NC)	**27**	11	2.73 abcd	3.01 abcd	11.84 abcdef
Ohio (OH)	**16**	14	2.46 abcde	3.51 ab	13.11 abcd
Pennsylvania (PA)	**22**	14	2.40 abcdef	2.10 abcdefgh	12.58 abcde
Georgia (GA)	**11**	25	2.06 bcdef	2.17 abcdef	4.43 hi
Pennsylvania (PA)	**21**	18	2.04 bcdef	2.94 abc	4.64 ghi
Georgia (GA)	**13**	13	1.88 bcdefg	1.72 cdefghi	5.29 efghi
Ohio (OH)	**15**	20	1.88 bcdef	2.68 abcd	4.37 ghi
Oklahoma (OK)	**4**	10	1.88 bcdefg	2.02 abcdefgh	3.65 ghi
North Carolina (NC)	**24**	12	1.84 bcdefg	1.65 defghi	4.33 ghi
Virginia (VA)	**23**	12	1.72 bcdefg	2.38 abcdef	8.36 bcdefgh
Oklahoma (OK)	**5**	13	1.71 bcdefg	1.41 fghi	6.51 cdefgh
Oklahoma (OK)	**3**	7	1.60 bcdefg	1.91 a cdefghi	4.02 fghi
Mississippi (MS)	**8**	20	1.56 b defg	2.46 abcde	4.02 hi
North Carolina (NC)	**26**	17	1.51 b defg	1.29 ghi	20.05 ab
New York (NY)	**18**	5	1.51 bcdefg	4.13 ab	3.53 fghi
Nebraska (NE)	**1**	12	1.34 b defg	1.42 fghi	15.04 abc
Alabama (AL)	**9**	18	1.27 defg	1.02 i	5.13 fghi
North Carolina (NC)	**25**	15	1.21 defg	2.96 abc	2.45 i
Florida (FL)	**10**	17	1.15 efg	1.53 efghi	5.49 defghi
Michigan (MI)	**14**	12	1.11 defg	1.20 hi	4.29 ghi
New York (NY)	**17**	9	0.95 fg	2.14 abcdefgh	3.42 hi
Missouri (MO)	**6**	15	0.87 g	1.43 fghi	5.83 defgh

[a] Within a column, means followed by the same letter are not significantly different at $P < 0.05$ using a pairwise t-test.
[b] EC_{50} = effective concentration of fungicide at which isolate growth is decreased by 50%.



Supplementary Figure 3.1. Mean leaf ratings of QoI-insensitive control isolate Fel09 for **A)** pyraclostrobin and **B)** picoxystrobin. With little to no change in ratings as QoI concentrations increase, the PROC NLMIXED (SAS, Cary, NC) program was unable to estimate EC_{50} values for this isolate.

Appendix C

Supplementary Table 4.1. Overlapping primers used to sequence the entirety of the *Blumeria graminis* f. sp. *tritici CYP51* gene[a]

Primer Name	Primer Sequence (5' to 3')	Forward Primer Position
51-01b_F	CATACTTCATGGCGAGCGG	-597
51-01b_R	ACACATGTAATCCTCCATAACAGC	-597
B51-02F	TTTTCCAACCTAGAGGCACTG	-486
B51-02R	CGAAACTGATTTCCCGTTTT	-486
A51-03F	CGCTCATAGCACGAATTTCA	-313
A51-03R	GTAATATGTTTCCAGCGGTCTGTG	-313
51-03b_R	GTTTCCAGCGGTCTGTGATT	-313
B51-04F	GGATTGAACTCCGCCGACTA	-117
B51-04R	AGTAGCTGCTTCAATACATTCAAGA	-117
A51-05F	CGTTGGCTAGTGGAATTATAAGTTTAT	68
51-05b_R	TCCCCAGAATATATCAATGGCA	68
B51-06F	AATCCCAAGCCAAGGTCAGT	233
B51-06R	ACATCAGTCCCGAAGACAGG	233
51-07b_F	ACTGAGGGATGTTAATGCTGAAG	387
51-07b_R	AAAGCTCTCCACTTCATTTTGG	387
B51-08F	GCCTTCCGCTCTTATGTACCT	575
B51-08R	GCATGAAATTGATTGGGGTAA	575
A51-09F	TTGGCAGTTTTGTATCATGACC	755
A51-09R	GGGAGTGCCATCTTTGTAGG	755
B51-10F	GTGGCAATTAATGCGCTCTT	938
B51-10R	ATTTAACAGGAGGTAATTCTGATCC	938
51-11b_F	TGAAGAACTCTACCAAGAACAGC	1094
51-11b_R	CTGGAACGGGCATTGGATTC	1094
51-12b_F	GTGAAAGAGGTCCTCCGTCT	1191
51-12b_R	CTTGCTGCCCCTGTGCTAA	1191
A51-13F	AGGCACGGATATGGAGGATG	1400
A51-13R	CCATTGGCCGAGAAAACATACT	1400
B51-14F	CAACGGTGCAATTAGTTACAATCA	1519
B51-14R	AGCTTGTAATCTAATCATCTCCAAACT	1519

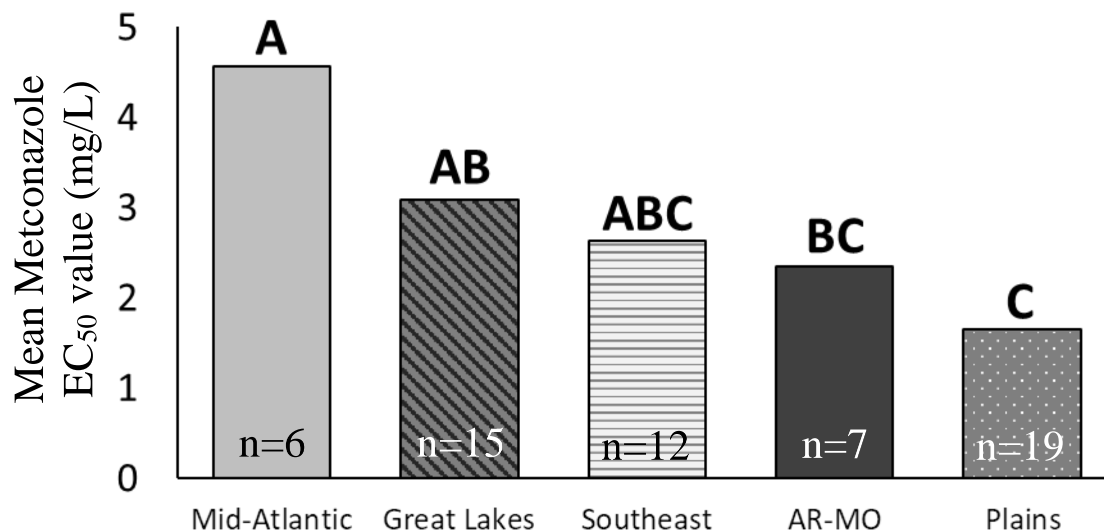
[a] Primers designed by R. Whetten.

Supplementary Table 4.2. Number of isolates genotyped per field

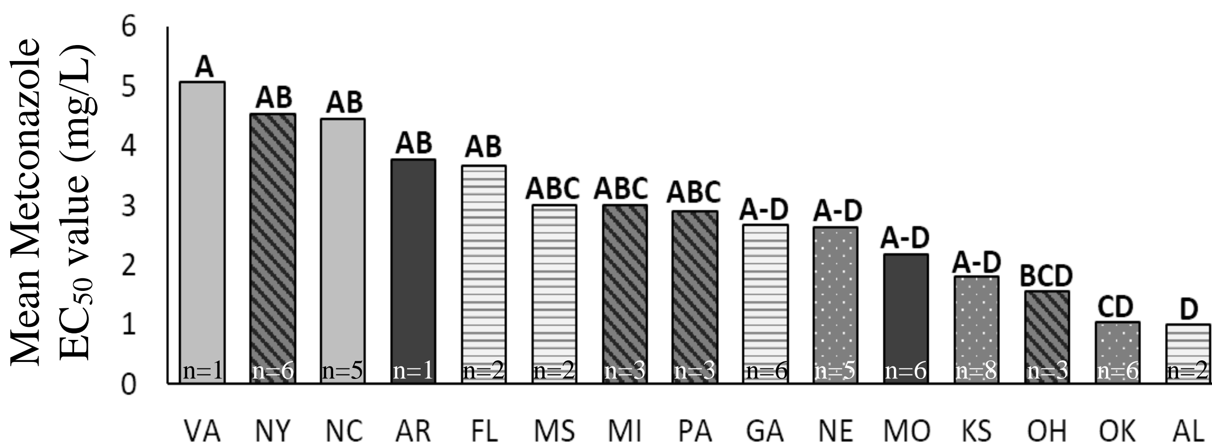
State	Field Number[a]	N[b]
NE	1	11
KS	2	18
OK	3	6
	4	9
	5	9
MO	6	15
AR	7	12
MS	8	19
AL	9	16
FL	10	17
GA	11	23
	12	14
	13	14
MI	14	10
OH	15	18
	16	14
NY	17	9
	18	5
	19	16
	20	12
PA	21	15
	22	15
VA	23	12
NC	24	12
	25	15
	26	15
	27	12

[a] Field numbers correspond to those in Meyers et al. (2019), where there is a table of locations (nearest towns). See Figure 4.1 for approximate field locations.
[b] N = number of isolates.

A



B



Supplementary Figure 4.1. Mean metconazole EC_{50} values per **A**) region and **B**) state from a national sample of *Blumeria graminis* f. sp. *tritici* from 2013-2014. Sample sizes are displayed in each bar. Bars topped by the same letter are not significantly different ($P < 0.05$) using a pairwise t-test.