CHARACTERIZATION OF FUSARIUM IN WHEAT AND CORN GRAIN, AND MANAGEMENT OF WHEAT DISEASES IN MICHIGAN

By

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ABSTRACT

CHARACTERIZATION OF FUSARIUM IN WHEAT AND CORN GRAIN, AND MANAGEMENT OF WHEAT DISEASES IN MICHIGAN

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Fusarium spp. are widely distributed throughout the word, infecting numerous crops including wheat and corn, and are known for their production of mycotoxins hazardous to humans and animals. Historically, *Fusarium graminearum* sensu stricto was thought to be the primary *Fusarium* species infecting wheat and corn in the midwestern United States. Over 560 isolates of *Fusarium* were collected from 121 fields in Michigan and identified to a species level, confirming numerous species besides *F. graminearum* are infecting wheat and corn in Michigan. While *F. graminearum* comprised 82% of recovered isolates, members of the *Fusarium tricinctum* complex were also identified in nine fields of wheat. In corn, members of the *Fusarium fujikuroi* species complex comprised 50% of isolates recovered. *F. awaxy*, a species not yet reported in corn in the U.S., was identified in six fields, comprising 4.6% of the collection. Isolates of *F. graminearum* collected were also genotyped to determine the type of trichothecene mycotoxins they produce. The large majority, 413 isolates (92%) were the 15-acetyldeoxynivalenol (15-ADON) type and twenty-six (6%) were 3-acetyl-deoxynivalenol (3-ADON). In addition, seven isolates (1.5%) were classified as the NX-2 chemotype. Interestingly, most of the NX-2 and 3-ADON isolates were found in the same region, from five fields in the far norther eastern part of the state with less intensive agricultural land use.

Fungicides are an important tool in wheat and corn to manage *Fusarium* diseases and reduce toxin accumulation. Here, we utilized our collection of *Fusarium* isolates to characterize *in vitro* fungicide sensitivity to three Demethylation Inhibitor (DMI) chemistries (metconazole,

tebuconazole, and prothioconazole). All EC_{50} values were below 4 µg/mL for *F. graminearum*, and sensitivity between the three chemistries was highly correlated. A field trial was established to investigate sensitivity *in vivo* with eight isolates of differing *in vitro* sensitivities. No differences in fungicide efficacy were observed. While there may not be practical resistance in Michigan currently, monitoring should continue as there is variation in *in vitro* sensitivities present within and among species of *Fusarium*.

Work here also aimed to inform management of Fusarium head blight in wheat with field trials investigating the response of yield and disease to various inputs. A trial was established in East Lansing, MI (2014-2018) on soft white winter wheat cultivar 'Ambassador' to investigate the risks, benefits, and interactions of two nitrogen levels, three fungicide regimes, and a plant growth regulator trinexapac-ethyl. In some years, the high nitrogen treatments had significantly higher fungal disease and lower yield compared to base nitrogen treatments. This trial also demonstrated that lodging due to high nitrogen rates can increasing Fusarium head blight incidence and foliar disease.

In this trial and others, large variation in optimal fungicide regimes was observed. To evaluate which fungicide application timings or combination of timings were most effective over a large number of years, a meta-analysis of previously conducted fungicide trial data from Michigan was performed. Data from 46 trials from 2007-2020 was utilized to investigate six fungicide regimes, all in a mean positive yield response. There were statistically significant differences between some regimes. Probabilities of positive yield response and prediction intervals were calculated for all regimes to aide growers in making future fungicide application decisions.

I dedicate this dissertation to my late grandmother Frances Johnson. A woman who knew the power of education and instilled in her children and grandchildren the importance of higher education. From kindergarten to PhD, she supported me in every way possible, and was a shining example of strength and grace.

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Part 1: Wheat Management in Michigan

Role of nitrogen

Nitrogen is an essential nutrient for plants (Havlin et al. 2013). Higher nitrogen rates can lead to increased grain, forage, and straw yields in wheat (May et al. 2014; Cox et al. 1987; Kanampiu et al. 1997). However, there are diminishing returns as nitrogen rates increase (Knott et al. 2016; Brinkman et al. 2014) and the effect on yield and physiology can be cultivar specific (Knott et al. 2016). Yield response to nitrogen can also vary by site and by year (Kelley 1993; Howard et al. 1994; Quinn and Steinke 2019), indicating environmental factors can affect N loss or uptake and potential yield.

However, some studies have shown a significant decrease in yield from high rates of nitrogen (Kelley 1993; Brinkman et al. 2014; Roth et al. 1984). The cause is not clear, but explanations include harvestability problems from lodging or increased competition for resources after improved early vegetative growth. One significant risk that could also explain yield loss is exacerbation of disease by increased nitrogen rates, especially fungal infection (Veresoglou et al. 2013), which has been exhibited in a variety of pathosystems. Both host and pathogen can be impacted by nitrogen in way that may impact disease outcomes including leaf and canopy size, root length and activity, concentration of N in leaf tissue, and ability of plants to produce secondary metabolites and defense compound (Walters and Bingham 2007).

Nitrogen has been demonstrated to increase FHB disease in wheat and barley (Lemmens et al. 2004; Heier et al. 2005; Subedi et al. 2007; Krnjaja et al. 2015), but this result can be inconsistent across sites and years (Subedi et al. 2007; Krnjaja et al. 2015; Heier et al. 2005), and some studies find no effect on disease at different nitrogen levels (Yoshida et al. 2008; Hofer et al. 2016). The impact of nitrogen source is also variable, with one study finding increased

disease with ammonium nitrate applications rather than urea (Teich and Hamilton 1985), whereas others found no impact from source (Silva et al. 2019; Lemmens et al. 2004).

Some studies report higher FHB or foliar disease with lower nitrogen rates (Teich and Nelson 1984; Hofer et al. 2016; Yang et al. 2010; Tompkins et al. 1993); generally these studies had little to no nitrogen (below recommended rates) so this may be a result of nutrient stress. Another result of lower nitrogen rates can be differences in canopy coverage; with less biomass and shorter plants, fungal spores may be more effectively dispersed and likelier to reach the wheat head (Hofer et al. 2016).

There are also multiple examples in the literature of foliar disease worsening with increased nitrogen rates (Howard et al. 1994; Brinkman et al. 2014; Cox et al. 1987; Mascagni et al. 1997). One study across multiple varieties found that *Septoria tritici* (Simón et al. 2003) infection progressed faster with increased nitrogen rates. While another study found no effect on *Septoria* from nitrogen, but an increase of *Blumeria graminis* f. sp. *Tritici* (Tamburic-Ilincic, Brinkman, et al. 2015). Stripe rust (*Puccinia striiformis* f. sp. *tritici*) infection across multiple genotypes was demonstrated to be affected by increased N rates or application (Danial and Parlevliet 1995). There was a significant genotype x N interaction, which means disease susceptibility and cultivar N response reaction to nitrogen likely impact this disease response.

The relationship between nitrogen and foliar disease may be attributed to multiple factors. Nitrogen may modulate plant architecture. Architecture may affect air speed and dispersal through canopy, as well as temperature and humidity (Tompkins et al. 1993). In barley it has been demonstrated that canopies in high N treatments had higher relative humidity (Hofer et al. 2016). In wheat, one study demonstrated early season nitrogen treatments affected canopy

size by increasing shoot numbers and leaf area, leading to an increase in stripe rust disease (Neumann et al. 2004).

Nitrogen is also an essential nutrient for fungi, and nitrogen leaf status has been shown to correlate with disease in wheat, particularly in rust pathogens (Neumann et al. 2004; Robert et al. 2002; Olesen et al. 2003). Additional N applied can result in higher leaf N concentration even in the flag leaf later in the season (Kelley 1993). In experiments with *Puccinia striiformis* f. sp. *tritici*, leaves with low-nitrogen content had decreased spore production per lesion while lesion size was unaffected. Increasing nitrogen content led to increased spore production, however this effect plateaued suggesting once nitrogen was sufficient for the fungus it would not increase spore production. Nitrogen content of spores was also constant regardless of leaf N status (Robert et al. 2002, 2004). Furthermore, studies with lade season N applications not affecting canopy, still resulted in more severe disease, demonstrating the effects of nitrogen are more than just biomass or canopy related (Neumann et al. 2004).

Besides possible exacerbation of disease, greater nitrogen rates come with the additional risk of lodging. Lodging can occur when the culm breaks or bends, or plants lean from the crown (Pinthus 1974). Increased nitrogen rates have shown to increase height and reduce strength of the stem (Zhang et al. 2017; Crook and Ennos 1995) which can lead to lodging. Higher nitrogen rates also lead to less carbohydrate deposition, resulting in a more succulent stem, which is an additional factor contributing to lodging (Havlin et al. 2013). When stems break, translocation of carbon and minerals will be affected. Even if stems remain intact, photosynthetic capacity and carbon assimilation may be impacted as lodging increases shading(Pinthus 1974)(Pinthus 1974)(Pinthus 1974) , thus lodging has been shown to reduce straw yield, grain yield, test weight, and impact grain quality (Pinthus 1974). Lodging may also impact disease dynamics;

one study demonstrated elevated mycotoxin concentration in lodged wheat and barley (Nakajima et al. 2008).

Environmental sustainability is an additional concern with increasing nitrogen rates and applications. Increased nitrogen use may reduce NUE (Nitrogen Use Efficiency) and N losses to the atmosphere (Kanampiu et al. 1997). Nitrogen losses from run-off are also a concern where it can cause nitrate contamination of drinking water or eutrophication of inland surface waters (Havlin et al. 2013).

Wheat response to plant growth regulator products

Plant growth regulators (PGRs) are used in a variety of crops to modulate plant growth for more desirable physiology. In wheat, plant growth regulators are used to reduce plant height and prevent lodging. When the height is reduced, the force from the weight of the head is minimized (Crook and Ennos 1995). Growers often utilize a PGR to moderate the risk of lodging from higher nitrogen rates.

The growth regulator product trinexapac-ethyl (TE) is registered in the United States for wheat and has been demonstrated in some studies to decrease plant height, reduce leaf area, increase stem diameter, increase number of grains per spike, and increase straw strength (Espindula et al. 2009; Knott et al. 2016; Wiersma et al. 2011; Matysiak 2006). TE is an acylcyclohexanedione; compounds in this class work to inhibit gibberellin production by structurally mimicking 2-Oxoglutaric Acid - an important co-substrate involved in late stages of GA biosynthesis (Rademacher 2000). The main characterized functions of gibberellins in plants are “promotion of longitudinal growth, seed germination, induction of bolting in long-day plants, and promotion of fruit setting and development” according to (Rademacher 2000).

The response to PGR products in wheat is likely environment specific as many studies report inconsistent impacts on height and yield over different site-years (Quinn and Steinke 2019; Knott et al. 2016; Matysiak 2006). Some report a positive yield response; others a reduction in yield or no change at all. It is likely that PGR products only show an economic benefit in years when lodging occurs (Swoish and Steinke 2017). Factors that may impact the response include N rate and susceptibility of the cultivar to lodging (Swoish and Steinke 2017; Brinkman et al. 2014). For example, cultivars with increased coronal roots have been shown to be more lodging resistant (Crook and Ennos 1995).

Impact of plant growth regulators on disease

While not intended to affect disease, it is possible these PGR products may indirectly affect disease infection as they modulate plant physiology. In some other grass systems, including anthracnose (*Colletotrichum cereale*) and dollar spot (*Sclerotinia homoeocarpa*) on bluegrass, TE application significantly reduced disease severity (Inguagiato et al. 2009; Golembiewski and Danneberger 1998). There is also evidence TE can reduce apple scab infection (*Venturia inaequalis*) (Spinelli et al. 2010). One experiment with prohexadione-calcium in apple showed pathogenesis genes significantly upregulated with application upon pathogen challenge (Bini et al. 2008). One study in wheat demonstrated increased various *Fusarium* spp. infection of cereal grains with plant growth regulator Ethephon ((2-chloroethyl) phosphonic acid) (Martin et al. 1991).

It is not surprising PGRs may impact disease as gibberellins also interfere with levels of the plant hormones ethylene and ABA, which could impact defense responses. Additionally, gibberellins can interfere with sterol, brassinosteroid, and flavonoid metabolism (Rademacher 2000). PGRs may also impacting gibberellin synthesis in fungi. While this has not been

demonstrated for trinexapac-ethyl, which is commonly used on wheat, it has been shown in the onium compounds, such as chlormequat chloride, which are effective on cereal crops. Chlormequat chloride has been demonstrated to effect CPP-synthase in gibberellin producing fungus *Fusarium fujikuroi* (Rademacher 2000, 1992). In contrast, two other tested acylcyclohexanediones compounds did not affect gibberellin formation in the two fungal organisms tested.

Fungicide use for disease management

Fungicides are a key management tool in wheat, specifically for fusarium head blight control as there are few cultivars with full resistance to this disease. Fusarium head blight is a major risk for growers, as Fusarium produces mycotoxins, mainly Deoxynivalenol. The presence of these toxins can result in dockages at the elevator, or even unmarketable grain if levels are high enough. Besides toxin contamination, severe epidemics of FHB have demonstrated to reduce yields but up to 40-50% (Windels 2000).

While fungicides are an important and widely used tool, they still only suppress disease by about 50%, with meta-analysis of multi-state winter wheat trials showing Prosaro (Prothioconazole + tebuconazole) reduced disease index by 44.5% and DON by 37.5% (Paul et al. 2008). While an important benefit of these fungicides is DON toxin reduction from FHB and improvement of grain quality, there are also yield benefits and protection from foliar diseases. Meta-analyses show a 6.8% yield increase even for one of the least effective products (tebuconazole) applied at flowering. The more efficacious products (Prosaro and Caramba) averaged an 11% increase. If disease pressure is high (>30% index), there are even greater benefits, ranging from 9.84% to 21.6% yield increases (Paul et al. 2010). Another estimate put yield benefit from flowering fungicide at 330 kg/ha increase on average, with Prosaro showing

an average increase of 431.6 kg/ha (P.A. Paul et al. 2018). However, timing is key to providing control; ideally 4-6 days after the start of anthesis. Meta-analysis shows that 5 to 7 days later than anthesis can result in 10-20% less control depending on the chemistry, with estimates of 11% reduction in efficacy for caramba but 17% for Prosaro. Another analysis estimated a reduction of 100 kg/ha in the benefit from Prosaro if it was applied too early or too late (P.A. Paul et al. 2018).

Fungicides are also important to control leaf disease. Leaf diseases are omnipresent in Michigan but depending on the variety and the environment in a particular year, they may not be severe. When foliar diseases are severe, they can be a yield limiting factor. In wheat, fungicides can result in increased grain dry matter and grain nitrogen. This increased grain dry matter comes from increased green leaf retention during grain fill, which has a demonstrated relationship with yield (Cook et al. 1999). Work has been done to show fungicides impact the duration of grain fill, rather than accumulation (Gooding et al. 2005; Dimmock and Gooding 2002).

Timing is an important factor in determining the yield response to these fungicides and the protection they provide. Common timings for fungicide application include Feekes 6, Feekes 9 to protect the fully emerged flag leaf, and Feekes 10.5.1 during flowering to prevent Fusarium head blight infection. Flowering is the most important timing to protect against FHB and DON accumulation, however protection of the flag leaf from foliar disease is also a major benefit. Feekes 9 applications have shown to be the most efficacious at reducing flag leaf disease, as they protect the flag leaf immediately after emergence (Stephen N. Wegulo et al. 2011; Sylvester et al. 2018; Cook et al. 1999). One study suggested an estimated yield loss of 27.2 kg ha-1 per day for each day a fungicide application was delayed after flag leaf emergence if disease epidemics were already occurring (Cook et al. 1999). Feekes 10.5.1 applications can increase kernel

weights (Brinkman et al. 2014), and depending on when epidemics develop, may provide similar flag leaf protection as Feekes 9 applications (Sylvester et al. 2018). When epidemics occur later, at or after anthesis, Feekes 10.5.1 application may even provide superior flag leaf protection through grain fill.

Feekes 6 applications, while providing some benefit, are too early to provide flag leaf protection by the end of the season (Brinkman et al. 2014; Willyerd et al. 2015) and often aren't profitable. Early applications, such as Feekes 6, are commonly added in double pass programs, but don't always significantly impact yield (Sylvester et al. 2018). Only in years with more severe disease at early stages is a yield response seen from Feekes 6 application. At this stage, disease can impact seeds/spike yield and tillers per row, with demonstrated losses from powdery mildew and leaf rust. In severe mildew epidemics, number of tillers were reduced up to 30% (Green et al. 2014; Bowen 1991). Yet some environments allow for compensatory growth so early disease may not have as large of an effect on yield (Bowen 1991).

Strobilurin fungicides in particular are thought to provide greater yield response for the exact same amount of disease for two hypothesized reasons. They are known to maintain green area longer, and perhaps by preventing spore germination the plant doesn't have to elicit a defense response (Bartlett et al. 2002). However a study in wheat found any additional yield gain from Strobilurin could be explained by duration of green leaf area (Dimmock and Gooding 2002).

In seasons with low severity, there is not always a demonstrated yield benefit to result in a profitable application (Cox et al. 1987; Stephen N Wegulo et al. 2011; Quinn and Steinke 2019; Weisz et al. 2011). This can also be highly cultivar dependent (Loyce et al. 2008; Byamukama et al. 2019). Importance of fungicide for protecting yield may also differ by disease,

as they have different potential to impact yield. One study demonstrated *Septoria* was nine times more detrimental to yield than powdery mildew infection, even at similar severity levels (Olesen et al. 2003). However this can also be a cultivar specific effect underlying the importance of variety screening and selection (Green et al. 2014).

Interaction of nitrogen and fungicide applications

There is also suggestion in the literature of a synergistic interaction between fungicides and nitrogen, meaning a greater yield benefit is achieved from fungicides when higher nitrogen rates are used. This has been reported in several cases, however results are not always consistent across site years (Kelley 1993; Salgado et al. 2017; Quinn and Steinke 2019; Ishikawa et al. 2012; Brinkman et al. 2014). There are also studies reporting no interaction at all (May et al. 2014; Mascagni et al. 1997).

Part 2: Epidemiology and biology of Fusarium spp infecting wheat and corn

Members of the genus *Fusarium* are filamentous Ascomycetes and are some of the most important pathogens in the world. This genus is very large and complex with over 300 reported species, including many plant pathogens and even some human pathogens (Summerell et al. 2010; O'Donnell et al. 2018a; Starkey et al. 2007). Notably, these species infect agriculturally important crops of wheat (*Triticum aestivum* L.) and corn (*Zea mays* L.) grain, causing mycotoxin contamination. It is estimated that approximately forty species can cause Fusarium head blight of wheat or ear rot of corn around the globe (Bottalico and Perrone 2002; Xu et al. 2008; Logrieco et al. 2002; Aoki et al. 2014). The most common culprit being species the *Fusarium graminearum* species complex (FGSC), which includes 16 distinct species including *F. graminearum* sensu stricto. The FGSC resides inside the *Sambucinum* complex, which includes notable head blight pathogens *F. poae*, *F. culmorum*, and *F. cerealis* as well (Aoki et al. 2014). There are also numerous species outside of the *Sambucinum* complex that can also cause disease or toxin contamination, including the *Fusarium tricinctum* complex and the *Fusarium fujikuroi* complex (Logrieco et al. 2002)

Biology of *Fusarium* spp. causing head blight of wheat and ear mold of corn

There are diverse lifestyles across the *Fusarium* plant pathogens, but all species are thought to survive well dormant in soil or as saprotrophs on residue. Species produce asexual spores, micro or macro conidia often in masses on sporodochia. These spores are responsible for dispersal across short distances (Trail 2009; Fernando et al. 2021). Many produce chlamydospores under certain conditions, which are hardier resting spores responsible for longer term survival in soil. Species capable of sexual reproduction also produce ascospores in perithecia, flask shaped fruiting bodies which actively discharge the ascospores, which can

disperse over large distances (Prussin et al. 2014). F. *graminearum* is homothallic and is known to readily undergo perithecia formation and subsequent sexual recombination, making it a prolific ascospore producer. Other *Fusarium* species are heterothallic or do not have a known sexual state (Leslie and Summerell 2006). In *F. graminearum*, perithecium are readily produced on the plant or crop residue providing inoculum in season, and acting as survival structures providing inoculum in following seasons.

F. graminearum, and most other *Fusarium* species, are known to infect in a hemi-biotrophic manner. Spores attach to plant tissue and can infect through a variety of sites, depending on environmental conditions, time of infection, and the host tissue being infected. In wheat, extruded anthers provide an easy entry route for the pathogen and surfaces within the floral cavity have thin-walled, susceptible cells (Bushnell 2001; Lewandowski et al. 2006). In barley, paleal margins and prickle-type trichome cells have been shown to be important sites of pathogen invasion (Imboden et al. 2018). Similarly in wheat, specialized epidermal cells (papillae) also seem to be sites of infection (Rittenour and Harris 2010) as well as stomates in smaller proportions (Pritsch et al. 2000). Appressoria have also been demonstrated to directly penetrate caryopses and the inner surfaces of paleas, lemmas, and glumes (Boenisch and Schäfer 2011; Kang and Buchenauer 2000). Once inside, mycotoxins and enzymes disrupt plant cell components, and H_2O_2 release results in oxidative stress. Eventually this leads to plant cell death and increased nutrient availability to the fungus for more rapid growth, marking the necrotrophic stage of the fungus (Walter et al. 2010). The arsenal of enzymes used by *Fusarium* includes lipases, cutinases, poly-galacturonases, and xylanases. These play a role in initial infection, as well as virulence and movement of the pathogen through the spikelet and head (Ferrari et al. 2012; Paccanaro et al. 2017; Walter et al. 2010; Wanjiru et al. 2002). Mycotoxins, toxic

secondary metabolites produced the fungus, are also important for virulence. Deoxynivalenol (DON) is one of the most widely produced and well characterized. Important mycotoxins reported in the literature besides DON include: nivalenol, moniliformin, beauvericin, T2, HT2, diacetoxyscirpenol, fusarenone-X, neosolaniol, fumonisin B1, fusaproliferin, monoacetoxyscirpenol, and zearalenone (Logrieco et al. 2002). There is considerable variation between species in the arsenal of toxins produced, as well as variation between isolates of a given species, in which toxins are produced and in what amounts (O'Donnell et al. 2018a).

The amount of enzymes or trichothecene mycotoxins a strain produces correlated with virulence in multiple studies (Khaledi et al. 2017; Goswami and Kistler 2005a; Fabre et al. 2019). Virulence does not seem to be influenced by the type of mycotoxin produced in some crops (Goswami and Kistler 2005), but one study did find nivalenol-producing strains were more aggressive on certain hosts compared to DON-producing strains (Carter et al. 2002). Recent proteomic studies found that strains of *F. graminearum* largely express the same proteins during wheat infection, but the amount of those proteins vary significantly and correlate with aggressiveness (Fabre et al. 2019).

These same *Fusarium* species infecting wheat and corn can also infects other crops such as potatoes, dry bean, soybean, rice, hops, and oats across diverse tissues types such as roots, stalks, florets, and even pods (Jacobs et al. 2019; Goswami and Kistler 2004; Gachango et al. 2012; Pioli et al. 2004). These species are often found infecting roots, crowns, and inflorescence of weeds, with one study isolating *F. graminearum* from over 52 species of weeds (Sneideris et al. 2019). While these are diverse crops and tissues, there is currently no evidence to suggest genetic differentiation between the populations of *F. graminearum* isolated from these different hosts (Kuhnem, Spolti, et al. 2015; Burlakoti et al. 2008; Sneideris et al. 2019; Kuhnem, Del

Ponte, et al. 2015), and while aggressiveness of a strain may very between hosts, strains are usually pathogenic across a variety of hosts (Goswami and Kistler 2005).One study found virulence factors important for wheat infection, such as DON, lipases, and MAP kinases, are also important for soybean infection (Sella et al. 2014). There is also however evidence for preferential expression of particular genes depending on the host (Harris et al. 2016).

The *F. graminearum* genome is thought to be very polymorphic and have rapidly adapted throughout its evolutionary history. Genome analysis has revealed a "two speed" genome in *F. graminearum*, where regions related to pathogenicity and host adaption were more likely to be in the "faster" sections (Wang et al. 2017). Additional genomic studies found 80% of protein coded genes were polymorphic, and genes involving host adaptation had greater diversification rates (Laurent et al. 2016). *F. graminearum* also utilizes repeat induced point mutation, a mechanism that introduces transition mutations in sections of repeated DNA sequences, specifically in ascogenous hyphae during sexual reproduction. This mechanism increases genotypic diversity and allows rapid adaptation in the face of selection pressures (Hane et al. 2015; Cuomo et al. 2007).

Diversity of Species causing head blight and ear mold

There are over forty species of *Fusarium* that can infect cereals causing head blight or ear mold, all which produce secondary metabolite toxins with cross-kingdom affects on plants, humans, and animals. However, in North America, *Fusarium graminearum sensu stricto* is thought to be the primary pathogen infecting cereals and often grain elevators only test for deoxynivalenol (DON), the most common toxin produced by *F. graminearum*. DON is documented to cause reproductive and developmental toxicity, as well as acute symptoms such as vomiting and gastroenteritis (Pestka 2010) in humans and cattle. However, surveys in the

United States are beginning to reveal a greater diversity of species infecting cereals besides *F. graminearum* with the possibility of contamination with toxins besides DON.

In North Carolina, *F. graminearum* dominated most fields, but surprisingly, members of the *Fusarium tricinctum* species complex (FTSC) comprised the majority of isolates in four fields. FTSC members were found in proportions greater than 20% in 10 additional fields, which were largely in the coastal region (Cowger, Ward, et al. 2020). In a Kentucky survey of wheat, a majority of isolates were *F. graminearum*. However, in a single field four isolates from the FTSC were found, including *F. acuminatum* and *F. cf. reticulatum* (Bec et al. 2015). In North Dakota wheat surveyed in 2010, a collection of 120 isolates were all *F. graminearum* except for a single *F. culmorum* isolate (Puri and Zhong 2010). Similarly, in Wisconsin, *F. graminearum* dominated wheat, but five isolates of *F. culmorum* were identified. These were all found in a single field in the same year in Door county, a peninsula on lake Michigan (Mueller et al. 2021). A survey in Nebraska found solely *F. graminearum* in wheat (Panthi et al. 2014). Although occurrences of these other species in United States seem relatively low, many surveys have a relatively low number of isolates, and isolation protocols have been biased to collection of *F. graminearum* over other species that may not sporulate as readily or produce the characteristic macroconidia. Larger scale surveys of wheat globally have reported a greater diversity of *Fusarium* spp. A review of European literature reported at least 17 species associated with cereals in addition to the FGSC (Bottalico and Perrone 2002). Ontario, Canada has reported *F. avenaceum*, *F. culmorum*, and *F. pseudograminearum*, as well as toxins (HT-2 and HT) that are not produced by *F. graminearum* (Turkington et al. 2011; Tamburic-Ilincic, Wragg, et al. 2015) in wheat.

In corn, species distribution data from North America is even more sparse with very few studies investigating diversity of *Fusarium* spp. A 1990 survey of the southeastern and central United States found most samples of corn harboring *F. moniliforme, F. proliferatum, F. subglutinans* (Leslie et al. 1990). A survey of Colorado corn in 1985 found *F. moniliforme, F. subglutinans,* and *F. graminearum,* with *F. graminearum* being the most virulent. However, since these surveys additional species have been elucidated in the *Fusarium Fujikuroi* complex and many isolates have been re-assigned to different species. Many isolates in these early surveys were classified as *F. moniliforme,* which is no longer a recognized species. A survey in Ontario, Canada, a near neighbor to Michigan, found *F. subglutinans* infecting corn at the highest proportions, along with *F. graminearum*, *F. poae, F. sporotrichioides,* and *F. proliferatum* (Schaafsma et al. 2008). Across the globe, similar members of the *Fusarium fujikuroi* complex have been found infecting corn (Pfordt et al. 2020; Degraeve et al. 2016; Thrane et al. 2004) along with numerous members of the *Fusarium sambucinum* and *Fusarium tricinctum* complexes.

It is also important to note that these species often co-occur in the same field and even the same wheat head (Xu et al. 2008; Birr, Hasler, J. Verreet, et al. 2020) or corn stalk (Kommedahl 1979). There is not yet any evidence for synergism reported between species (Xu et al. 2008; Siou et al. 2015), but some observations of common co-occurrences. A Swedish survey revealed specific pairs of species are more likely to occur together such as, *F. culmorum* with *F. sporotrichioides* and *F. poae* with *F. tricinctum* (Karlsson et al. 2017). A survey of the Baltic sea region found that 21% of samples had 2-3 species per head detected. *F. culmorum* and *F. graminearum* were detected mainly alone, whereas *F. poae* and *F. avenaceum* were often detected in combination with another species (Beyer et al. 2006). It is not yet known if these co-

occurrences are result of specific biological interactions or rather common environmental variables favoring particular species, however numerous genes have been found to be upregulated when these species are co-inoculated (Walkowiak et al. 2015). Species co-occurrence can be relevant to disease and toxin outcomes. In corn, presence of other species can stimulate fumonisin or DON production in certain scenarios, or result in overall less disease (Giorni et al. 2019; Velluti et al. 2001). The effects of co-occurrence or competition may also be isolate specific, with one study finding certain isolates are less affected by competition than others (Siou et al. 2015).

Determinants of species composition

Many factors likely contribute to variation of species composition within a field. Most of these pathogens are thought to be globally distributed, as there is known long range dispersal of *Fusarium* species. Spores are known to move long distances through multiple means: in water through soil, air dispersed over continental distances, and through human movement and transport of infected tissues (Summerell et al. 2010). While most species can be found worldwide, their abundance in a region or field could be attributed to factors such as climate, soil type, residue, host availability, crop rotation, crop genotype, and culture practices (Xu et al. 2008).

The local climate or weather events can be important for each stage of the life cycle of *Fusarium*: spore germination and infection of host, sporulation and reproduction, and ability to survive in residue and overwinter. Thus, each year there are multiple ways in which weather could shift community composition. In a survey of wheat in Luxembourg, there was evidence for a shift in species composition from a single season of drought, favoring *F. culmorum* over *F. graminearum* (Beyer et al. 2014). However, the following year, *F. graminearum* dominated

again. Literature has also associated environmental conditions with specific species, such as drier, warmer conditions favoring *F. poae,* and cooler, wet conditions favoring *F. avenaceum,* (Xu et al. 2008) for example. A survey of *Fusarium* spp. infecting barley across three years found drastic changes in species composition that authors attributed to climate as well as variety (Beccari et al. 2017). Similarly, in analysis of *Fusarium* diversity on German corn, mean temperature and precipitation during flowering correlated most strongly with *Fusarium* composition, and September weather correlated strong with stalk infection and severity (Pfordt et al. 2020).

Rotation history or residue present in a field is thought to also be a major factor influencing composition of species. In the 1990s in Europe, a shift from *F. culmorum* to *F. graminearum* was observed, which was speculated to be caused by increase in maize acreage with *F. graminearum* producing abundant ascospores on maize residue compared to *F. culmorum* (Waalwijk et al. 2003). Similarly, in Asia and Louisiana, species such as *F. asiaticum* seem to be found on wheat in rice growing areas (van der Lee et al. 2015; Gale et al. 2011) more commonly than in non-rice growing regions. A mycotoxin survey in China also found certain crop rotations favored production of particular toxins in *F. graminearum*, Zearalenone was mainly detected in fields with a maize rotation, whereas as DON levels were higher in rice rotation fields (Qiu et al. 2016). Similarly, a study of *Fusarium* species infecting soybean roots found that intercropping with maize increased the diversity of species. Without maize intercropping, the more aggressive *F. oxysporum* was isolated more frequently from soybean roots (Chang et al. 2020). Altogether, these factors demonstrate that species composition can rapidly shift at a field level, and that abundance of particular species is likely heavily influenced by the year in which fields are sampled.

Genotypic diversity within *F. graminearum* related to chemotype

As previously mentioned, *F. graminearum* sensu stricto is a very diverse species. One important facet of diversity of *F. graminearum* is the mycotoxin profiles. While different isolates have potential to produce specific toxins, there is also different forms of a toxin that an isolate can produce, referred to as the "chemotype" of the isolate. In *F. graminearum* and closely related species, the genotypes at the TRI loci determine the form of type B trichothecene produced: 3-acetyl-deoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), or nivalenol (NIV) (McCormick et al. 2011). Recently, an additional novel type A trichothecene was found, the NX-2 chemotype. NX-2 isolates do not create the type B trichothecenes, rather the type A, which is attributed to variation in the *Tri1* gene (Varga et al. 2015).

Besides differences in chemical structure, these chemotypes may correspond with biological differences. Studies suggest 3-ADON isolates produce higher levels of DON compared to 15-ADON isolates (Gilbert et al. 2010; Puri and Zhong 2010; Ward et al. 2008; Liu et al. 2017); however, there are differing reports on overall aggressiveness of these isolates (Kuhnem, Spolti, et al. 2015; Malihipour et al. 2012). When the growth rate of twenty isolates of each chemotype from wheat fields in China was tested, 15-ADON had higher growth rates at 20°C and 25°C, but at 30°C 3-ADON isolates grew significantly faster. The 15-ADON isolates from China also produced perithecia early and ejected significantly more ascospores than 3-ADON or NIV isolates (Liu et al. 2017).

Chemotypes are also of interest as they can be a proxy for genetic populations. Previously, *F. graminearum* was thought to be one large panmictic population. This was in part due to the large variation seen within a single field, and relatively less variation attributed to differences between fields (Talas et al. 2011; Burlakoti et al. 2008). However, as more advanced

methods were used, a population division correlating with chemotype was discerned (Ward et al. 2008; Panthi et al. 2014). Recent analysis of whole genome sequences supports the division of three main populations of *F. graminearum* in the United States: NA1 corresponding to the 15-ADON chemotype, NA2 corresponding to the 3-ADON chemotype, and NA3 corresponding to the new NX-2 chemotype. NX-2 has the least diversity indicating a recent expansion (Kelly and Ward 2018a). NX-2 is only found in the US and Canada thus far and is thought to be endemic (Kelly et al. 2016). 3-ADON populations are also less diverse compared to 15-ADON populations and are thought to result in an introduction from Europe, likely Italy (Puri and Zhong 2010; Ward et al. 2008). Although there are three populations identified, there is admixture between them detected and the number of isolates with genetic backgrounds not correlating with their chemotype is increasing with time (Kelly et al. 2015; Kelly and Ward 2018a).

Geographic distribution of chemotypes

A survey of winter wheat in the northeastern US (NY, PA, MD, VA, KY, NC) collected 998 isolates of *F. graminearum*, and 92% of those were 15-ADON chemotype (Schmale et al. 2011). In Nebraska, all 73 isolates collected were 15-ADON (Panthi et al. 2014). Europe is also dominated by 15-ADON, with only 13% of isolates characterized as 3-ADON (Pasquali et al. 2016). However, there has been increase in 3-ADON populations observed in some regions (J. M. Liang et al. 2015; Puri and Zhong 2010). Isolates from North Dakota from 1980 were compared to those from 2000 in spring wheat and found a 15-fold increase in 3-ADON relative to 15-ADON (Puri and Zhong 2010). In Canada there is also a trend of increasing 3-ADON as you move west (Guo et al. 2008; Burlakoti et al. 2008; Ward et al. 2008). In Ontario, the closest province to Michigan, 96% of *F. graminearum* isolates from corn and 98% from wheat were found to be 15-ADON (Burlakoti et al. 2016).

Some studies also seem to find concentrated areas where 3-ADON dominates. In the survey of the northeastern U.S., 7% of isolates were 3-ADON, with a majority found in New York, and 45% of them from a single field. Authors suggested that 3A populations in NY could have come from Canada which saw a recent incline in 3-ADON populations, perhaps by "punctuated episodes of atmospheric transport"(Schmale et al. 2011). A more in-depth study of New York found that while 15-ADON dominated in the western half of the state, population from Willsboro in the far eastern part of the state were 53% 3-ADON (Kuhnem, Spolti, et al. 2015). Similarly, a study in Manitoba found that in total, 15-ADON dominated, but in some locations 3-ADON was dominate, including one field where 97% of isolates were 3-ADON (Guo et al. 2008).

Part 3: Fungicides for *Fusarium* management

Importance of fungicides for *Fusarium* disease management

Fusarium graminearum is a major pathogen worldwide including the United States, causing economic losses from Fusarium Head Blight (FHB) in wheat and ear mold infections in maize (Nganje et al. 2004; Windels 2000). Besides yield losses, mycotoxin contamination of wheat and corn with deoxynivalenol (DON) can be hazardous for both humans and animals (Pestka 2010), resulting in crop losses if grain does not meet quality standards.

As complete genetic resistance to *F. graminearum* has not been fully elucidated nor integrated into all commercial varieties of wheat and maize, fungicides remain an important component of disease management (Wegulo et al. 2015; McMullen et al. 2012). In Michigan, fungicides are frequently used on soft white wheat (SWW) to manage FHB, with past survey results indicating at least 40% of large scale growers of SWW use a fungicide every year (Black and Nagelkirk 2014). In 2012, an analysis of wheat production in the European Union found that

without use of triazole fungicides member countries would not be able to satisfy internal demand for wheat by 2020. If they were to produce wheat without fungicides they would need to increase cultivated land by 13.9% (Di Tullion et al. 2012).

Historically, demethylation inhibitor (DMI) fungicides (FRAC Code 3) have been the only fungicide class available for management of head scab to wheat growers in the United States. They have been widely used since 1998 when Folicur (tebuconazole) was first introduced for FHB and DON reduction in some states (McMullen et al. 2012). Four DMI fungicides had full federal registration for FHB suppression on wheat by 2008: tebuconazole, prothioconazole, metconazole, and propiconazole. Similarly, in corn, Proline 480 SC (prothioconazole) and Caramba (metconazole), are the only choices for suppression of *Fusarium* ear molds and reduction of DON. That was until in 2019, when a new succinate dehydrogenase inhibitor (SDHI), pydiflumetofen (Syngenta, Switzerland), was registered for head scab management in wheat and ear rot in corn with the trademark name of Adepidyn™. It is marketed as Miravis™ Ace in wheat and Miravis™ Neo in corn as a pre-mix with additional chemistries.

Although registered for foliar disease control in wheat and corn, there are no quinone (QOI) products registered for FHB or ear mold control, as they are known to increase mycotoxin content. In meta-analysis of field studies in the United States, an application of QOI anytime between Feekes 9 and flowering increased DON compared to untreated checks, whereas DMI applications significantly decrease DON (P. A. Paul et al. 2018). Another study found significant increase as early as Feekes 7 depending on the variety (Ellner 2005). Even in studies where QOI products decreased FHB, applications did not control DON at levels comparable to triazole products (Marques et al. 2017; Blandino et al. 2009). Likewise, *Fusarium* has demonstrated shown inherent *in vitro* insensitivity to trifloxystrobin and azoxystrobin compared to triazole

products (Dubos et al. 2011; Broders et al. 2007). Multiple hypotheses have been proposed in the literature for this effect. These fungicides may be effective at controlling other fungi, who normally co-colonize the spike and reduce the impact of *Fusarium* (Simpson et al. 2001). Another hypothesis is that these fungicides provide an enhanced "greening effect", maintaining green leaf area and grain fill period, providing more time for toxin to accumulate (Bartlett et al. 2002). A third explanation is some type of stress response from the fungi, perhaps leading to an increase in efflux of toxins in response to the QOIs.

The widely used DMI class of fungicides is used not just in protection of wheat and other crop plants, but also in animal husbandry, human medicine, and industrial settings as a preservative for items such as wood, paint, and coolant systems (Parker et al. 2014). In human medicine, inherent resistance has limited many options and resistance is developing in human pathogens as well. This, coupled with the widespread use of DMIs, makes the resistance to these chemistries of worry and interest to many. Better understanding of resistance mechanisms will allow more informed development of future fungicides (Ribas e Ribas et al. 2016; Parker et al. 2014).

Mode of action and fungicide target of DMI fungicides

Ergosterol is an essential sterol in fungal cell membranes in most fungi. DMI fungicides inhibit the C14-demethylation step of ergosterol biosynthesis, by binding to the P450 monooxygenase encoded by CYP51 genes that mediate this step. In filamentous fungi, these DMI products have been demonstrated to cause "extensive vacuolization, accumulation of lipid bodies, thickening of hyphal cell walls, and progressing necrosis or degeneration of hyphal cytoplasm(Kang et al. 2001)."

In most filamentous ascomycetes, there are two or more paralogous CYP51 genes. *Fusarium graminearum* has three such paralogues CYP51A, CYP51B, and CYP51C (Cools et al. 2013). At least two of these, CYP51A and CYP51B are thought to be functionally redundant in ergosterol production. This redundancy might explain the observation that species with multiple CYP51 paralogues are intrinsically less sensitive to triazole fungicides.

There are differing reports of the relative functions and sensitivities of the three paralogues. One study of deletion mutants suggested CYP51C has no function in triazole sensitivity (Fan et al. 2013). Conversely, a similar study found CYP51A and CYP51C impact sensitivity, while B had no influence on sensitivity phenotype. Interestingly, this study tested multiple active ingredients and found the CYP51A deletion was more sensitive to triadimefon and propiconazole, whereas CYP51C deletion was increased in sensitivity to six other compounds, but not triamdimefon or propiconazole (Liu et al. 2011). This suggests there may be some selectivity of certain compounds targeting particular paralogues.

One clear conclusion from the literature however is the importance of CYP51A, which is the most differentially expressed paralogue when fungi are challenged with DMI fungicides (Fan et al. 2013; Liu et al. 2011; Yin et al. 2009a; Becher et al. 2011). When CYP51B or CYP51C was deleted CYP51A was significantly upregulated, whereas expression of CYP51B nor CYP51C varied with other deletions (Liu et al. 2011). Additionally, no mutations have been identified in CYP51B or CYP51C to confer resistance to azoles. Taken together, evidence suggests that CYP51A seems to be the most important however additional CYP51 proteins may be advantageous for azole resistance (Cools et al. 2013). Research in *A. fumigatus* found similar results, with both CYP51A and CYP51B able to functionally produce sterols but all mutations found in the CYP51A paralogue (Parker et al. 2014).

Mechanisms of resistance to DMI fungicides

There are four broad categories of mechanisms for resistance to DMI fungicides: (1) survival without methylated sterols, (2) mutations in CYP51 that prevent or reduce binding in the active site, (3) over expression of CYP51 genes, or (4) enhanced fungicide efflux. Survival with methylated sterols as an alternative in ergosterol biosynthesis has only been reported in budding yeast, and not suspected to be a mechanism in filamentous lant pathogens (Ziogas and Malandrakis 2015).

Mutations in CYP51 genes have been reported in plant pathogens, such as *Mycosphaerella graminicola* where more than 30 amino acid alterations have been discovered (Cools et al. 2012). These mutations prevent the azole from accessing the active site due to conformational changes, or allow access but prevent binding to the heme due to residue changes (Song et al. 2018). In *Fusarium graminearum* researchers have also found numerous mutations in CYP51 genes, however many times they cannot be attributed to a phenotypic change in sensitivity. Two such studies found several unique haplotypes among isolates with a range of sensitivities, but mutations seemed to be randomly distributed and did not correlate with sensitivity. Furthermore, isolates with some of the highest EC_{50} values had no mutations found, likewise some of the least sensitive isolates had mutations (Pasquali et al. 2020; Talas and Mcdonald 2015). This lack of correlation has been observed in other fungal pathogens besides *Fusarium* (Hulvey et al. 2012; Stammler et al. 2009). This does not mean CYP51 genotype does not play a role in resistance, as site directed mutagenesis has demonstrated a CYP51B mutation reducing affinity for binding (Qian et al. 2018). However the role of amino acid alterations in resistance is probably minor compared to other fungicide modes of action, as it has been hypothesized that the evolution of CYP51 proteins is very constrained (Cools et al. 2013).

Over expression of CYP51 is a more common culprit of resistance in numerous pathogens. By creating additional CYP51, more CYP51 is available to synthesize ergosterol even as some is bound to fungicide molecules. There are a variety of regulons for CYP51 that may mediate this overexpression (Song et al. 2018), as ergosterol production can be regulated both transcriptionally and post transcriptionally. It is found that this mechanism of resistance in many cases provides cross resistance to other DMI products. In *Puccinia triticina*, researches sampled 110 isolates and found that the *in vitro* EC_{50} correlated with constitutive up-regulation of CYP51 (Stammler et al. 2009). Similarly, this was demonstrated in *Venturia inaequalis* where practical resistance to DMIs was seen in apple orchards. The most resistant strains were overexpressing CYP51A, and in some strains this could be correlated to a 553 bp insertion located upstream of CYP51A (Schnabel and Jones 2001). In *F. graminearum*, experimental studies inducing resistance with cultivation on fungicide mended media, have also found mutations in CYP51A and overexpression of all three CYP51 paralogues (Duan et al. 2018).

Perhaps the most worrisome resistance mechanism is enhanced efflux of fungicides. In some cases, this type of resistance results in reduced sensitivity to multiple classes of fungicides rendering disease control very challenging. Studies of *Sclerotinia homoeocarpa* resistant isolates revealed increased expression of a known efflux transporter of DMI fungicides, which is upregulated constitutively, and further expressed upon fungicide treatment. This transporter also seemed to confer increased sensitivity to dicarboximides and SDHI fungicides (Sang et al. 2015; Hulvey et al. 2012). In *F. graminearum* a predicted pleiotropic drug resistance ABC transporter (FgABC1) was deleted and resulted in an increased sensitivity to some plant defense fungicidal compounds, but not all, suggesting some selectivity. No triazoles were tested, as researchers were interested in this transporters role as a virulence factor (Gardiner et al. 2013). However, in

Fusarium culmorum, another predicted pleiotropic drug resistance ABC transporter was found to be involved in fungicide resistance. Expression of the ABC transporter increased 30-fold in strains which had been adapted in the lab to be resistant to tebuconazole. Subsequently, researchers found this over expression in field isolates as well and did not find changes in sequences surrounding ABC1. Researchers did not observe fitness penalties in lab or greenhouse assays (Hellin et al. 2018), and the over expressing strain was less sensitive in greenhouse assays to tebuconazole, although the difference was not statistically significant.

An additional study deleting four different ABC transporters individually, found that two of those mutants (belonging to group I of ABC-G and to group V of ABC-C subfamilies) were involved in fungicide resistance. Their deletion significantly increased the sensitivity of the isolates to four different triazole fungicides as well as fenarimol, but not to the six other chemical groups they tested (Ammar et al. 2013). It has been hypothesized in the literature that this multi-drug resistance is unlikely to develop in field strains of *Fusarium*, as it has been known to greatly decrease saprotrophic fitness in some filamentous fungi (Ziogas and Malandrakis 2015).

Presumably, there is likely a combination of these factors in differing proportions within strains, and not one mechanism responsible for the variation of sensitivities seen in *Fusarium*. One study analyzing transcriptional response of *F. graminearum* to tebuconazole found multiple responses: an eleven fold increase in expression of an ABC transporter, upregulation of nine genes involved in biosynthesis of sterols, and a 50 fold increase in both CYP51A and CYP51B (Liu et al. 2010). Furthermore, there may be more than just these four mechanisms. A genome wide association study of 220 isolates of *F. graminearum* with differing sensitivities to propiconazole identified 51 quantitative trait nucleotides that were associated with sensitivity. The three loci which were statistically significant associated with sensitivity were all

uncharacterized, with predicted functions of regulating lipid metabolism, a transcription factor, and hypothetical protein with numerous predicted functions (Talas et al. 2016). There have also been studies identifying less sensitive isolates, but not found any variation in CYP51 genotype, or expression levels (Yin et al. 2009b) of CYP51, or explanation for resistance.

DMI sensitivities in natural populations

Luckily, in *Fusarium graminearum* there are not yet widespread reports of resistance in natural populations. Many survey studies did not report any large variation in sensitivities, and EC_{50} values reported were all below 1 µg/mL (Ivic et al. 2011; Avozani et al. 2014; Tateishi et al. 2010; Machado et al. 2017; Spolti et al. 2012). While two studies found significantly increasing EC_{50} values over time (Anderson et al. 2020; Klix et al. 2007), another did not find any relationship between year of collection and sensitivity (Tateishi et al. 2010). Of the studies that reported variation in sensitivities or suspected resistance, only one validated a reduction in efficacy *in planta* in a greenhouse setting. This was an isolate from a survey of New York, with an EC_{50} for tebuconazole of 8.09 ug/mL (Pierri Spolti et al. 2014). To date no suspected failure of field applications has been reported.

SDHI fungicides

The succinate dehydrogenase inhibitor (SDHI) group of fungicides works by inhibiting respiration. Previously, no other SDHI chemistries were effective for *F. graminearum* or closely related species, apart from fluopyram. The SDHI fluopyram is effective on a limited number of *Fusarium* root rot diseases, mainly caused by members of the *Fusarium solani* species complex. Fluopyram is in the pyridinyl-ethylbenzamides chemical group. Then, in 2019, it is notable when pydiflumetofen (Syngenta, Switzerland) was registered for FHB and ear mold suppression and a new mode of action was available.

Pydiflumetofen is a N-methoxy-(phenyl-ethyl)-pyrazole-carboxamide, with the chemical formula $C_{16}H_{16}Cl_3F_2N_3O_2$. Currently, there is no documented resistance to pydiflumetofen, but the fungicide resistance action committee classifies this fungicide as medium to high risk for resistance development (FRAC code list 2019, www.frac.info/publications), as resistance to SDHI fungicides has occurred in many pathosystems (Veloukas et al. 2011; Avenot et al. 2008; Popko, Jr. et al. 2018; Rehfus et al. 2016; Miyamoto et al. 2010; Avenot et al. 2012) resulting in loss of product efficacy. This class targets the SDH enzyme which has four subunits, three of which are involved in fungicide binding. Many mutations conferring resistance have been found in these three subunits (B, C and D). Different mutations within subunits, and even different amino acid changes at a single codon have been found within and between organisms (Stammler et al. 2015). In *Zymoseptoria tritici* alone, nine different mutations have been found (Sierotzki and Scalliet 2013). Resistance to SDHI has also been attributed to overexpression of efflux pumps in fungal plant pathogens as well (Fraaije et al. 2019).

A study investigating resistance risk of *F. graminearum* to pydiflumetofen generated resistant mutants in the laboratory and found four different mutations in the SDHC subunit, which caused reduced sensitivity (Sun et al. 2020). There has not yet been any population level sensitivity data published for *F. graminearum.* However, baseline *in vitro* sensitivity to pydiflumetofen in a few other pathosystems has been published (Ayer et al. 2019; Miller et al. 2020), including a closely related FHB pathogen *F. asiaticum* (Hou et al. 2017). In all cases, no resistance has been found in natural baseline populations.

Chapter 2 : **Characterization of species composition, chemotype, and in vivo and in vitro fungicide sensitivity of Fusarium isolates from wheat and corn in Michigan, USA**

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Abstract

Fusarium species are a major concern due to mycotoxin contamination of wheat and corn grains in North America. To characterize the population of *Fusarium* in Michigan, over 569 isolates were collected and species composition, chemotype (15-ADON, 3-ADON, NIV, NX) and fungicide sensitivity were determined. Members of *the Fusarium sambucinum complex* were the major species associated with wheat, but members of the *Fusarium tricinctum* complex (7%) were found as well. In corn, only 37% of isolates were identified as *F. graminearum* and multiple species from *Fusarium fujikuroi* complex were found in high proportions. *In vitro* sensitivity to triazole chemistries registered in the United States (metconazole, tebuconazole, and prothioconazole) were assessed with mycelial growth assays. Isolates were most sensitive to metconazole, and less sensitive to prothioconazole and tebuconazole. Species-specific differences in sensitivity were uncovered, with FTSC members significantly less sensitive than *F. graminearum*, and FFSC significantly more sensitive. A small portion of isolates within *F. graminearum* had EC_{50} values 10-fold greater than sensitive isolates. In order to determine if this reduced sensitivity *in vitro* would lead to practical resistance, a field trial was established in 2019. A subset of *F. graminearum* isolates were chosen for investigation, four identified as sensitive *in vitro*, and four with reduced sensitivity *in vitro* (approximately 10-fold greater EC_{50} values). Plots were inoculated with spore suspensions of each isolate 48 hours prior to fungicide applications in a factorial manner in a randomized complete block design. No differences in the relative fungicide efficacy were found, signaling no practical resistance currently exists despite differences *in vitro* and widespread use in wheat throughout Michigan for the last 10 years. While currently there may not be practical resistance, monitoring should continue as there is variation in *in vitro* sensitivities present within and among species of *Fusarium*.

Introduction

The genus *Fusarium* is very large and complex with over 300 reported species, including many economically important plant pathogens widely distributed across the world (van der Lee et al. 2015b; Summerell et al. 2010). These species are filamentous ascomycetes and many are prolific secondary metabolite producers of mycotoxins. The most notorious species reside in the *Fusarium sambucinum* species complex (FSAMSC) causing Fusarium head blight of wheat (*Triticum aestivum* L.) or ear rot of corn (*Zea mays* L.). These pathogens are devastating not only due to their impact on yield, but also the mycotoxin contamination of grain which may be used in human food, animal feed, or industrial products. Mycotoxin contamination can result in price dockages or leave grain completely un-marketable (Nganje et al. 2004; Windels 2000). In wheat, yield losses of 40-70% have been reported with severe FHB disease pressure (Singh et al. 2016). Members of the FSAMSC can also infect diverse tissue types such as roots and stalks on additional crops including dry bean, soybean, potatoes, and many weed species (Sneideris et al. 2019; Gachango et al. 2012; Bilgi et al. 2011; Jacobs et al. 2019; Broders et al. 2007; Al-Hatmi et al. 2016; Xue et al. 2007).

Globally, its estimated that as many as 40 species can infect cereals (Xu and Nicholson 2009; Bottalico 1998; Logrieco et al. 2002; Aoki et al. 2012). Historically, *Fusarium graminearum* sensu stricto was thought to be the primary pathogen in the United States. Grain elevators often only test for deoxynivalenol (DON), the most common mycotoxin produced by *F. graminearum*. DON is documented to cause reproductive and developmental toxicity, as well as acute symptoms such as vomiting and gastroenteritis (Pestka 2010) in humans and cattle. However, surveys in the United States are starting to find a greater diversity of species causing FHB besides *F. graminearum*. Literature suggests many different factors can affect the

composition of species in a field or region, including climate, soil type, host abundance and genotype, crop rotation, and cultural practices (Xu et al. 2008; Beccari et al. 2017; Beyer et al. 2014; Gale et al. 2011; Qiu et al. 2016; Karlsson et al. 2017; Chang et al. 2020). Many times multiple species are found within a single field or even a single plant (Xu et al. 2008; Birr, Hasler, J. A. Verreet, et al. 2020; Cowger, Ward, et al. 2020; Karlsson et al. 2017). Besides *F. graminearum* and other members of the FSAMSC, members of the *Fusarium tricinctum* species complex (FTSC) have been reported in some surveys of wheat in the United States, in a single field in Kentucky (Bec et al. 2015) and more recently in coastal regions of North Carolina in proportions greater than 20% in 14 different fields (Cowger et al. 2020). Surveys of wheat in Ontario, Canada have reported *F. avenaceum*, *F. culmorum*, and *F. pseudograminearum* (Turkington et al. 2011; Tamburic-Ilincic, Wragg, et al. 2015).

While *F. graminearum* populations of corn have been studied, no recent surveys examining the diversity of species infecting corn have been completed in the United States. The most recently published surveys date back more than 30-40 years, where *F. graminearum* along with members of the *Fusarium fujikuroi* species complex (FFSC) including *F. moniliforme*, *F. proliferatum*, and *F. subglutinans* were found infecting corn in the Southeastern U.S. and the state of Colorado (Leslie et al. 1990; Gilbertson et al. 1985). At that time, the FFSC was divided into nine species, but now contains over 50 phylogenetically distinct species (Aoki et al. 2014). More recently surveys of corn in Ontario, Canada, found *F. subglutinans* in the highest proportions, along with *F. graminearum*, *F. poae*, *F. sporotrichioides*, and *F. proliferatum* (Schaafsma et al. 2008). Across the globe, members of the FFSC, FSAMSC, and FTSC have all been reported infecting corn and causing mycotoxin contamination (Pfordt et al. 2020; Degraeve et al. 2016; Thrane et al. 2004).

These *Fusarium* species all have different profiles of mycotoxins they produce. Nivalenol, moniliformin, beauvericin, T2, HT2, diacetoxyscirpenol, fusarenone-X, neosolaniol, fumonisin B1, fusaproliferin, monoacetoxyscirpenol, and zearalenone are some of the potent mycotoxins besides deoxynivalenol produced by these fungi (Logrieco et al. 2002; O'Donnell et al. 2018a). Different species have differing abilities to produce these toxins, and there is considerable variation in toxin production within species (O'Donnell et al. 2018b). The variation in trichothecene toxin production of *F. graminearum* is particularly well studied, and isolates can be categorized by their *Tri* genotype, which influences the form of trichothecene they produce, referred to as the "chemotype". Chemotypes in *F. graminearum* include 3-acetyl-deoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), as well as nivalenol (NIV) (McCormick et al. 2011). Recently, a novel type A trichothecene was characterized, and called NX-2 (Varga et al. 2015; Kelly et al. 2016).

Differences in chemotype have been found to correlate with certain phenotypes and population structure. Studies suggest 3-ADON isolates produce higher levels of DON compared to 15-ADON isolates (Gilbert et al. 2010; Puri and Zhong 2010), however there are conflicting reports on relative aggressiveness of the two (Kuhnem, Spolti, et al. 2015; Malihipour et al. 2012; Spolti et al. 2012). In some areas of North America there was a rapid increase in the proportion of 3-ADON isolates relative to 15-ADON isolates, suggesting selection in favor of the 3-ADON chemotype(J. Liang et al. 2015; Puri and Zhong 2010). Phylogenetic studies of *F. graminearum* genomes have identified three populations (NA1,NA2, and NA3) with 15-ADON isolates generally belonging to the NA1 population, 3-ADON belonging to the NA2, and NX-2 belonging to the NA3 population. However these studies also detected admixture between these three populations, and the number of isolates with chemotypes not corresponding to the

previously assigned populations is increasing with time (Kelly et al. 2015b; Kelly and Ward 2018b).

No matter the species or chemotype, managing *Fusarium* diseases in wheat and corn remains a challenge and requires an integrated approach. While there is some cultivar resistance available, varieties often only provide partial resistance, and have a low grower adoption rate (Cowger, Smith, et al. 2020). Cultural practices such as tillage and rotation can help reduce inoculum production in residue, but not completely eradicate it (Teich and Hamilton 1985; Wegulo et al. 2015). Fungicides can reduce toxin accumulation and provide yield protection, and are an important tool in wheat and corn (McMullen et al. 2012; Wegulo et al. 2015; Paul et al. 2010; Limay-Rios and Schaafsma 2018). In the United States there are multiple Demethylation Inhibitor (DMIs) fungicides and one Succinate Dehydrogenase Inhibitor (SDHI) fungicide registered for FHB management in wheat and corn. Pydiflumetofen (Syngenta, Switzerland) is the only SDHI registered, and was recently introduced as Miravis™ products in 2018. DMI fungicides have been widely used since 1998 when tebuconazole (Folicur) was first registered in some states (McMullen et al. 2012). Four DMI active ingredients had full federal registration for FHB suppression on wheat by 2008: tebuconazole, prothioconazole, metconazole, and propiconazole. Similarly, in corn, prothioconazole and metconazole were the only registered active ingredients available before the introduction of pydiflumetofen in 2019.

In Michigan, fungicides are frequently used to manage FHB and foliar pathogens, with past survey results indicating at least 40% of large scale soft white wheat growers use a fungicide every year (Black and Nagelkirk 2014). Across the United States, 42% of growers had applied a fungicide to target FHB at least once in the last five years, and 13% had applied it every year (Cowger, Smith, et al. 2020). DMI fungicides inhibit the C14-demethylation step of ergosterol

biosynthesis, by binding to the P450 monoxygenase encoded by CYP51 genes. In most filamentous ascomycetes, there are two or more paralogous CYP51 genes. *Fusarium* has three such paralogues CYP51A, CYP51B, and CYP51C (Cools et al. 2013). While there are differing reports of the relative functions and sensitivities of these three paralogues, there is consensus in the literature that CYP51A is the most important and likely the main target (Fan et al. 2013; Liu et al. 2011; Yin et al. 2009a; Becher et al. 2011). CYP51A is where mutations are often found in *Fusarium* and other fungi, but when surveys of natural populations of *F. graminearum* have found mutations, none have correlated consistently with phenotypic changes in sensitivity to DMI fungicides (Pasquali et al. 2020; Talas and Mcdonald 2015). Other common mechanisms of DMI resistance include increased CYP51 expression, or enhanced efflux of fungicides (Ziogas and Malandrakis 2015) and both of these mechanisms have been demonstrated to decrease DMI sensitivity in lab strains (Hellin et al. 2018; Ammar et al. 2013; Duan et al. 2018; Fan et al. 2014).

DMI fungicides are considered a medium risk chemistry and *Fusarium* spp. are considered low-risk pathogens for resistance development by the Fungicide Resistance Action Committee (FRAC). However, DMI resistance continues to be a concern for multiple reasons. One, DMI fungicides are heavily relied upon for multiple plant diseases, and for human medicine and industrial applications. Second, the long history of agricultural use increases the risk for resistance selection. Finally, when DMI resistance does develop, pathogens often exhibit cross resistance between products. Researchers around the globe have investigated *in vitro* sensitivity in members of the *F. graminearum* species complex, and there are not yet widespread reports of resistance in natural populations or large variation in EC_{50} values for currently recommended products (Ivic et al. 2011; Avozani et al. 2014; Tateishi et al. 2010; Machado et al. 2017; Spolti

et al. 2012). Of the studies that have suspected shifts in sensitivity (Anderson et al. 2020; Klix et al. 2007), only one validated a reduction in efficacy *in planta,* where greenhouse assays demonstrated a reduction in fungicide efficacy on wheat with a *F. graminearum* isolate from New York state, with an EC_{50} for tebuconazole of 8.09 µg/mL (Pierri Spolti et al. 2014). Other *Fusarium* species are less studied than *F. graminearum,* but there are *in vitro* studies in the literature with low sample sizes and it appears there may be species specific differences in sensitivity to DMI fungicides (Ivic et al. 2011; Pierre Hellin et al. 2016; Gachango et al. 2012; Shin et al. 2014; Villafana and Rampersad 2020; Müllenborn et al. 2008).

The aim of this study was to survey *Fusarium* spp. infecting wheat and corn in Michigan to determine the composition of species, toxin chemotypes, and fungicide sensitivities to ultimately inform disease management strategies. A total of 560 *Fusarium* spp. isolates across Michigan were collected over multiple years, identified to a species level, and genotyped for their trichothecene chemotype. Fungicide sensitivity was assessed for the three primary DMI active ingredients, tebuconazole, metconazole, and prothioconazole. Although some *Fusarium* surveys have been conducted in the United States, none have been done in the state of Michigan, and few at this sample depth. Previous survey methods may have been biased toward recovery of *F. graminearum* as well. Identification of species from this survey will not only enhance our understanding of the ecology of *Fusarium* spp. in Michigan field crops, but will help to identify risks of potential mycotoxin contamination and fungicide resistance, and ultimately inform disease management strategies.

Methods

Sample collection

The collection of 569 isolates originated from Michigan wheat, corn, dry bean, and soybean surveys spanning 2011-2017, with a majority collected from wheat and corn in 2016 and 2017. In those years, wheat fields in major agricultural areas were visited and any symptomatic heads collected. Samples were also solicited from agribusiness professionals, grain elevators, university researchers, and extension personnel. Sampling focused on wheat and corn (n=513), crops with large acreage in Michigan and impacted by mycotoxin contamination, but *F. graminearum* isolates from root rot surveys of soybean (n=40) and dry bean (n=16) were also included to expand sampling. Samples originated from 72 unique wheat fields, 26 unique corn fields, 14 soybean fields, and 9 dry bean fields (Figure 2.1). Majority of fields sampled had 1-4 isolates per field collected. Any meta-data available was recorded, including field coordinates. For grain samples without known field locations, coordinates of the nearest town were recorded. K-means cluster analysis was used to divide fields into regions for analysis. The R function 'kmeans' was used to divide coordinate points into 6 clusters, and the number of clusters was chosen based on BIC criterion.

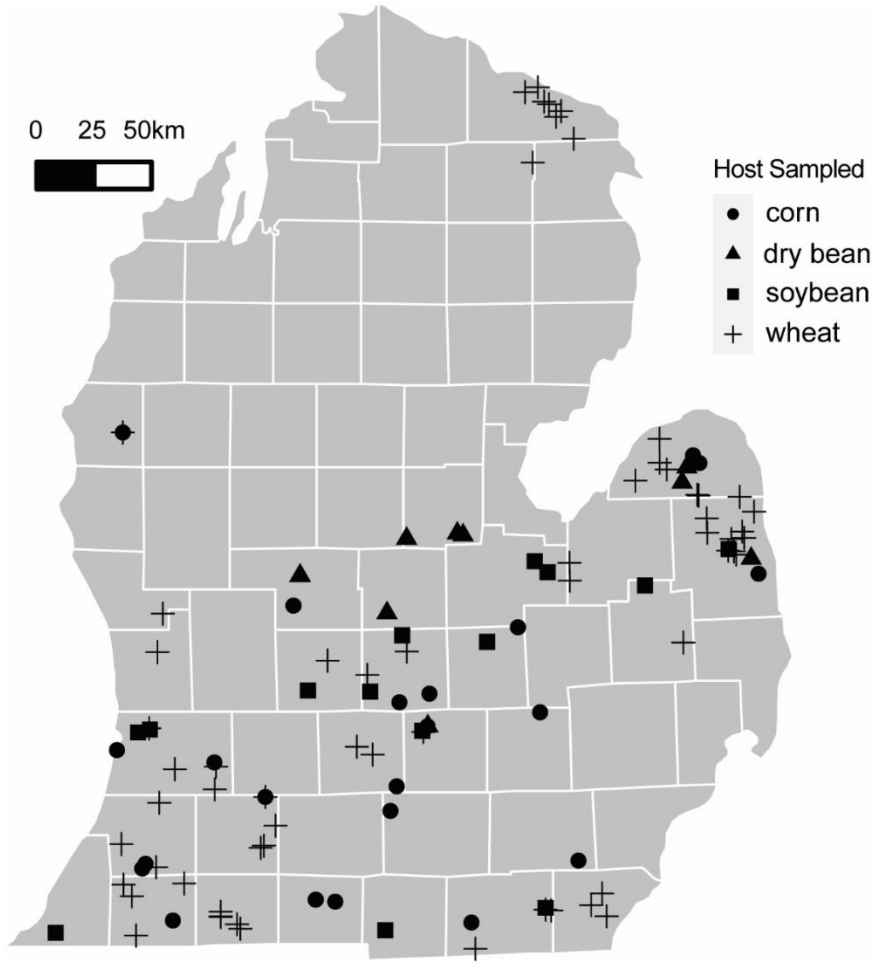


Figure 2.1. Map of sampled locations across the state of Michigan, with shape depicting the host sampled. A total of 569 isolates across all four hosts (Wheat, Corn, dry bean, and soybean), from 121 sites were utilized in this study.

Fusarium isolation

Samples were dried at room temperature and stored in paper bags until isolations could be completed. Both grain and head samples were incubated in moist chambers and checked for *Fusarium* for up to six days. In many cases sporodochia would form in the moist chamber. If not, hyphae would be transferred to Carnation Leaf Agar (CLA) to induce sporulation. CLA was prepared by rinsing, drying, UV-C irradiating carnation leaves that were subsequently placed onto WMS (2% water agar with 300mg/L of metalaxyl and 15mg/L of streptomycin). Wheat heads were surface sterilized prior to incubation, first in 70% ethanol for 1 min, then 1% NaOCl

for 1 minute, rinsed twice in sterile water, and finally dried in a laminar flow hood for 30 mins. All other material besides wheat heads was directly incubated in moist chambers or CLA without surface sterilization.

Sporodochia were removed with an insect pin under a stereo-microscope and deposited on the surface of WMS. Spores (macroconidia or microconidia) were then spread across the plate with sterile loop. Approximately 16-24 hours later, the WMS agar was inspected with the aid of a stereo-microscope, and an isolated germinating spore was transferred to a Potato Dextrose Agar Petri-plate (PDA; Acumedia, Neogen, Lansing, MI) amended with neomycin (125mg/L) using a dissecting needle. Morphology was observed and an isolate was put into long term storage if determined to be a *Fusarium* spp. based on gross morphology. Isolates were stored on sterile #1 Whatman filter paper in sterile coin envelopes at 4°C and as colonized agar plugs in 35% glycerol in cryovials at -80°C.

Additional isolates of *F. graminearum* collected in previous studies from soybean and dry bean roots were included in chemotype distribution and fungicide sensitivity analysis, in order to increase the number of isolates and sampled locations. A total of 16 isolates collected from a previous dry bean survey were utilized (Jacobs et al. 2019). An additional 40 *F. graminearum* isolates from soybean collected from two previous studies were also included. In 2015, soybean roots were sampled in a field study described in (Rossman et al. 2018), and isolated as described in (Rossman 2016). Soybean roots were also surveyed in 2011-2012 by Rojas et al (Rojas et al. 2017) with Komada's and WMS media used for isolations. Species identity of these isolates was reconfirmed as described below before use in the present study.

Identification of species and chemotype

DNA was extracted from mycelial tissue using commercially available kits (Qiagen DNeasy plant mini kit or ZymoQuick-DNA Fungal/Bacterial Miniprep kit) following manufacturer's instructions. Mycelia was disrupted with 2.3mm and 0.5 mm glass beads prior to lysis with a FastPrep machine. A multi-locus genotyping assay (MLGT) utilizing a Luminex 100 flow cytometer, was used to determine species and chemotype. The methods and probe sequences are described in Ward 2008 (Ward et al. 2008) and updated in Garmendia 2018 (Garmendia et al. 2018). The MLGT utilizes the trichothecene loci of Tr12, Tri1, and Tri3 to identify chemotype. Four markers were used for species identification, including an additional trichothecene locus (Tri101), translation elongation factor (EF-1a), reductase locus (RED), and mating type locus MAT1-1-1. For each species and chemotype, there were probes targeting sequences in two separate genes (Ward et al. 2008).

If the MLGT was not able to identify an isolate to the species level, the Translation Elongation factor 1-α was amplified using primers EF1 (ATGGGTAAGGA(A/G)GACAAGAC) and EF2 (GGA(G/A)GTACCAGT(G/C)ATCATGT) from (O'Donnell et al. 1998). The polymerase chain reaction (PCR) was performed with DreamTaq polymerase (ThermoScientific) in 25µl reactions at the following thermocycler parameters: 94°C for 3m, 35 cycles of 94°C for 30 s, 53°C for 30 s, 72°C for 1 min, followed by 72°C for 7 min. Then, enzymatic cleanup of amplified product was performed with 3 U of exonuclease I and 0.5 U of rSAP Shrimp Alkaline Phosphatase per sample (New England Biolabs). Samples were incubated for 45 min at 37°C, and enzymes were inactivated by incubation at 85°C for 5 min. Fragments were Sanger sequenced in forward and reverse directions using EF1 and EF2 primers. Sequences were trimmed for quality and assembled using codon-code aligner v.4.2. Trimmed sequences were

approximately 600 bp. The alignment tool at CBS-KNAW Fungal Biodiversity Centre's Fusarium MLST database was used to assign species if there was a 99% or greater similarity with known reference sequence (https://fusarium.mycobank.org/).

EC_{50} determination for *F. graminearum* isolates

Three DMI fungicides were tested as commercial formulations: Folicur 3.6F (38.7% tebuconazole, Bayer Crop Science), Caramba 0.75 SL (8.6% metconazole, BASF), and Proline 480 SC (41% prothioconazole, Bayer Crop Science). Table 2.1 lists additional information about these chemistries. EC_{50} values (effective concentration to reduce growth by 50%) were determined by mycelial growth assays on fungicide amended medium in 100mm x 15mm Petri plates for 47 isolates in the collection. This set of 47 isolates was chosen to represent diversity across crop host, time period collected, and geography, representing 34 unique fields. Media was prepared and assays performed in the same manner as previously described (Breunig and Chilvers 2021). Briefly, ½ strength Potato Dextrose Agar was used and six doses (0.003, 0.03, 0.3, 1, 3, or 30 µg/mL final concentration in assay plates) were tested along with a non-treated control. These concentrations were chosen based on previous literature (Pierri Spolti et al. 2014), and preliminary studies with additional concentrations. Plates were measured after 5 days in an incubator at 24°C in darkness. There were three replicates per isolate at each concentration, with one replicate in each of three separate experimental runs. All three products were tested simultaneously. Analysis was conducted in R (R Core Team 2018), and function 'drm' in package 'drc' was used to fit the dose response model. Relative growth values were used as the response values, determined by dividing the average radial growth of the same isolate on the non-treated control plates for that experimental run. The 3-parameter log-logistic model (LL.3) was chosen to calculate EC50 values as it best fit the greatest proportion of isolates. All code for

data processing and analysis is publicly available on https://github.com/mikbreunig/Fusarium_triazole.

Table 2.1 Demethylase inhibitor fungicide products tested in this study

Active Ingredient (A.I.)	**Chemical group name**	**Commercial formulation Tested (% A.I)**
Tebuconazole	Triazoles	Folicur 3.6F, 38.7%
Metconazole	Triazoles	Caramba 0.75 SL, 8.6%
Prothioconazole	Triazolinthiones	Proline 480 SC, 41%

Selection of screening dose

Data from EC_{50} estimations was analyzed in order to choose a single informative dose to screen the remaining collection in a less resource intensive manner. The correlation between relative growth values from each dose with the EC_{50} estimation for that isolate was determined across all three chemistries. While multiple doses had significant correlations, the strongest correlation was found to be at 1µg/mL (Pearsons correlation, rho=0.704, $P < 0.0001$). Additionally, 1µg/mL had a less continuous response with most isolates falling below 50% relative growth, indicating it may provide better differentiation than lower doses (Supplementary Figure 2.1).

Screening isolate collection for *In vitro* sensitivity at 1µg/mL

A total of 445 isolates were assayed, in 13 sets of approximately 35-40 isolates. For assay inoculum, isolates were grown on PDA plates for seven days at room temperature. Media was prepared and assays performed in the same manner as previously described for EC_{50} determination. An isolate was screened twice, using plates from two separately prepared medium bottles for each product tested, but in a single run. All three chemistries were tested simultaneously. To investigate variability between sets, multiple isolates were included in more

than one set. The residual variance and variance due to isolate were each 10-fold greater than the variance from set, therefore all sets were combined in further analysis and visualizations, and set was included as a random factor in all models.

Evaluation of EC_{50} of diverse *Fusarium* spp.

EC_{50} values for tebuconazole and metconazole were determined for additional *Fusarium* species found in significant proportions in the survey of wheat and corn. This included *F. poae* (n=8), *F. sporotrichioides* (n=8), *F. subglutinans* (n=9), and members of the *Fusarium tricinctum* Complex (n=11). Seven isolates of *F. graminearum* previously characterized and representing a range of EC_{50} values were included for comparison purposes. All isolates were grown on PDA plates for inoculum production for six days, then transferred to assay plates and allowed to grow for four days incubated in the dark at 24°C. Doses, media preparation, and data collection were same as described above.

In vivo characterization of *F. graminearum* fungicide sensitivity in wheat field plots

In order to evaluate if there was a reduction in product efficacy when *F. graminearum* isolates have greater EC_{50} values, a field experiment was established at the Michigan State University Plant Pathology Farm in East Lansing, Michigan in 2019 and 2020 on susceptible cultivar 'Ambassador' to compare fungicide efficacy in wheat plots. Plots were inoculated with one of eight isolates, four which were among the most "sensitive" isolates, and four of the least sensitive that might be characterized as "resistant" based on *in vitro* data. These isolates and their EC_{50} are listed in Table 2.2. The eight isolates, and a mock-inoculation (sprayed with water and Tween 20), were tested against two fungicide treatments (metconazole and tebuconazole) in a factorial manner, in a randomized complete block design with four replications. The mock-

inoculated plots were included in the trial to determine baseline disease pressure. Spores were applied at Feekes 10.5.1 and fungicides applied approximately 36 hours later.

Ascospores and macroconidia were used at approximately equal proportions for inoculum. Ascospores were produced on carrot agar (300g boiled and blended carrots per liter), and macroconidia were produced on mung bean agar (40g mung beans boiled and strained per liter). Inoculated plates were kept at room temperature under constant light, and after significant sporulation was achieved, plates were washed with sterile water, scraped, and filtered through three layers of sterile miracloth (MilliporeSigma). Spores were stored at 4°C for up to one week prior to use in the field. Prior to application, spores were quantified with a hemocytometer. Then, in the field, spores were diluted with water in a 3-gallon spray tank to approximate concentration of 90,000 spores/mL of ascospores and 90,000 spores/mL macroconidia in 2019. In 2020, approximately 1.1×10^5 ascospores/mL and 7×10^4 macroconidia/mL were used. Tween 20 (tank concentration of 0.25%) was also added to the solution as a surfactant.

Fungicide treatments consisted of untreated plots receiving no fungicide, tebuconazole (Folicur 3.6F) applied at 0.292 L ha^{-1}, or metconazole (Caramba) at 0.986 L ha^{-1}. Fungicides were applied with a hand-held spray boom consisting of four nozzles spaced 0.5 meters apart, pressurized with CO_2 at 275 kPa and calibrated to apply 140.3 L ha^{-1}. Teejet nozzle D6TJ60-110015VS (Teejet Technologies, Wheat, IL) was used to apply fungicides. Adjuvant Induce (Non-ionic surfactant; Helena Chemical Company, Collierville, TN) at 0.125% v/v was applied with both fungicides. Spores of *F. graminearum* isolates were applied with the same boom as fungicides, but with DGTJ60-11002VS nozzles, and calibrated to apply 305ml of inoculum per plot. The boom was rinsed with 3L of water between each isolate application.

Fusarium head blight was rated 21 days after anthesis when wheat started to turn color by evaluating the incidence of infection in 100 wheat heads, and rating the severity of those infected heads by visually estimating the percentage of the head discolored. In 2020, when disease pressure was quite low, ratings were done on the entire plots rather than 100 heads. The entire plot area was harvested (2.1 m wide by 6.1 m long) utilizing a small plot combine to determine yield. Grain subsamples were collected and 100g were sent to the U.S. Wheat and Barley Scab Initiative mycotoxin testing laboratory (Dr. Yanhong Dong, University of Minnesota, St. Paul, MN) for deoxynivalenol concentration. Subsamples of 200 kernels were also collected to count the number of Fusarium damaged kernels (FDK), and measure kernel weight.

Table 2.2 *F. graminearum* isolates recovered from wheat used to investigate fungicide efficacy *in vivo* some of the most sensitive and least sensitive isolates *in vitro,* and their EC_{50} estimates.

Isolate name	EC_{50} Estimate Tebuconazole (µg/mL)	EC_{50} Estimate Metconazole (µg/mL)
21A	2.24	0.18
Kmiso	2.92	0.29
3-C	3.94	0.55
107M	0.89	0.06
24A	0.07	0.01
93D	0.06	0.01
Ph-1	0.09	0.02
76J	0.09	0.02

Statistical analysis

All data analysis was conducted in R (R Core Team 2018). All linear mixed modeling was done in 'lme4' package in R (Bates et al. 2015) and significance of factors tested with ANOVA, and mean comparisons made with 'emmeans'. When analyzing EC_{50} values, isolate was a random effect and chemistry as a fixed effect. In modeling of relative growth data, meta-data factors of (ID, chemotype, region, year, host) were tested as fixed effects, and isolate and

set included as random effects. All code for data processing and analysis is publicly available on https://github.com/mikbreunig/Pydiflumetofen.

Results

Identification of species infecting wheat

A total of 384 isolates were recovered from 75 wheat fields and identified to a species or species complex level using translation elongation factor sequences (*Table 2.3*). *F. graminearum* dominated wheat samples, representing 82.5% of samples. Additional members of the Fusarium Sambucinum Species Complex (FSAMSC) were found at low levels (1-2 isolates per field) including *F. cerealis* (n=1), *F. culmorum* (n=1), and *F. sporotrichioides* (n=11), and *F. poae* (n=16). *Fusarium tricinctum* species complex members were also present in 11 unique fields of wheat, representing 6.8% of isolates infecting wheat. *F. avenaceum* was the most prevalent with 14 isolates found, followed by *F. acuminatum* (n=9), then *F. tricinctum* (n=2). A majority of the FTSC species were found in a single field, at the Saginaw Valley Research and extension center, from which grain was sampled at harvest. In that field, all nine *F. acuminatum* isolates were found along with additional FTSC members and several *F. graminearum* isolates.

Identification of species infecting corn

Corn grain and ears were sampled from conventional corn fields as well as two popcorn fields. A total of 129 isolates recovered from 30 sites. *F. graminearum* was still an abundant species found in corn, however in a lower percentage than in wheat samples, representing only 37% of isolates. The second most abundant species were members of the *Fujikuroi* Species Complex (FFSC), with *F. subglutinans* representing 33.3% of isolates and found in 13 out of 26 sampled sites. FFSC members *F. fujikuroi*, *F. proliferatum*, *F. awaxy*, and *F. verticillioides* together represented another 16% of isolates from corn. *F. verticillioides* was the least abundant

FFSC species, with only two isolates identified from two field sites. Members of the *Fusarium incarnatum-equiseti* species complex were also found in wheat and corn at low levels (2% and 7%, respectively). Two isolates in the *Fusarium oxysporum* species complex were found in corn in two field sites. The abundance and distribution of species across hosts and fields are visualized in Figure 2.2.

Figure 2.2. Tree plot depicting abundance of *Fusarium* species recovered across hosts and fields in Michigan from 2014-2018. Thick black squares depict individual fields sampled, with the colors within depicting the number of isolates of a particular species recovered from that field. Box size is scaled to number of isolates recovered, smallest box in lower left-hand corner represents one isolate.

Table 2.3 identification and abundance of *Fusarium* spp. recovered from wheat and corn samples from 2015-2018 in the state of Michigan.

	Isolate Origin							
Species Complex	Wheat				Corn[a]			
Species identification	No. of isolates		% of total	No. of fields	No. of isolates		% of total	No. of fields
***Fusarium sambucinum* complex (FSAMSC)**	**346**				**53**			
F. graminearum	317		82.5 %	63	49		37 %	19
F. cerealis	1		0.3 %	1	-		-	-
F. sporotrichioides	11		2.8 %	6	4		3.1 %	3
F. culmorum	1		0.3 %	1	-		-	-
F. poae	16		4.2 %	14	-		-	-
***Fusarium incarnatum-equiseti* complex (FIESC)**	**8**		2.1 %	6	**9**		6.9 %	5
***Fusarium tricinctum* species complex (FTSC)**	**27**							
F. tricinctum	2		0.5 %	2	-		-	-
F. avenaceum	15		3.9 %	9	-		-	-
F. acuminatum	9		2.3 %	2	-		-	-
Not identified	1		0.3 %	1	-		-	-
***Fusarium fujikuroi* Complex (FFSC)**	**3**				**65**			
F. fujikuroi	-		-	-	8		6.2 %	4
F. subglutinans	3		0.8 %	3	43		33.3 %	14
F. proliferatum	-		-	-	6		4.6 %	5
F. verticillioides	-		-	-	2		1.5 %	2
F. awaxy	-		-	-	6		4.6 %	4
***Fusarium oxysporum* species complex (FOSC)**	**-**		**-**	**-**	**2**		1.5 %	**2**
Total	**384**			**72**	**129**			**26**

[a] corn includes isolates from commercial popcorn and field corn

Chemotype identification

A majority (92%) of *F. graminearum* isolates in Michigan were genetically identified as 15-acetyldeoxynivalenol (15-ADON). Only 26 isolates (6%) were 3-acetyldeoxynivalenol (3-ADON), and seven (1.6%) identified as the NX-2 chemotype. Of the 26 3-ADON isolates, 24 were isolated from the far northeastern region, along with all NX-2 isolates (Figure 2.3). The 3-ADON isolates were found across five of the six sampled fields in this region in 2017. Two 3-ADON isolates were found in a single field in the eastern "thumb" region. All the 3-ADON and NX-2 isolates were found in samples from wheat. A single *F. culmorum* isolate was identified in the collection and was identified as 3-ADON chemotype.

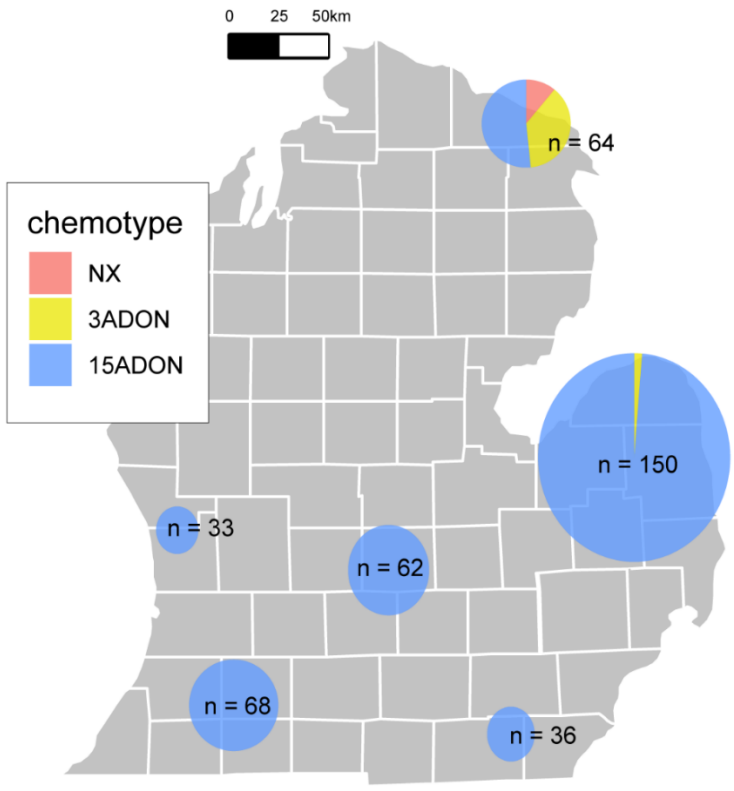


Figure 2.3 Distribution of genotypically determined chemotypes of *F. graminearum* isolates collected across six regions in Michigan. Regions were determined by Kmeans clustering of the geographical coordinates from each sampled field.

EC50 determination for 46 *F. graminearum* isolates

The EC_{50} values of 46 isolates of *F. graminearum* were determined for three DMI chemistries using mycelial growth assays in petri plates amended with commercial formulations of fungicides. The EC_{50} values for tebuconazole ranged from 0.064 to 3.945 µg/mL (variation factor = 61.6), with mean 0.646 µg/mL. The EC_{50} of isolates to prothioconazole ranged from 0.167 to 1.142 (variation factor = 6.8) with mean 0.421 µg/mL. Isolates were most sensitive to metconazole, with EC_{50} values ranging from 0.009 to 0.548 µg/mL (variation factor = 60.8) and mean 0.075 µg/mL (Figure 2.4). EC_{50} values were significantly different between all three active ingredients ($P < 0.0001$), being most sensitive to metconazole, followed by prothioconazole, and least sensitive to tebuconazole. While isolates had differing levels of sensitivity to the three chemistries, the EC_{50} values between different chemistries significantly correlated between all combinations of the three (Figure 2.5), with sensitivity of tebuconazole and metconazole having the strongest correlation (pearsons, $r = 0.94$, $P < 0.0001$) and metconazole and prothioconazole the weakest (pearsons, $r=0.81$, $P < 0.0001$).

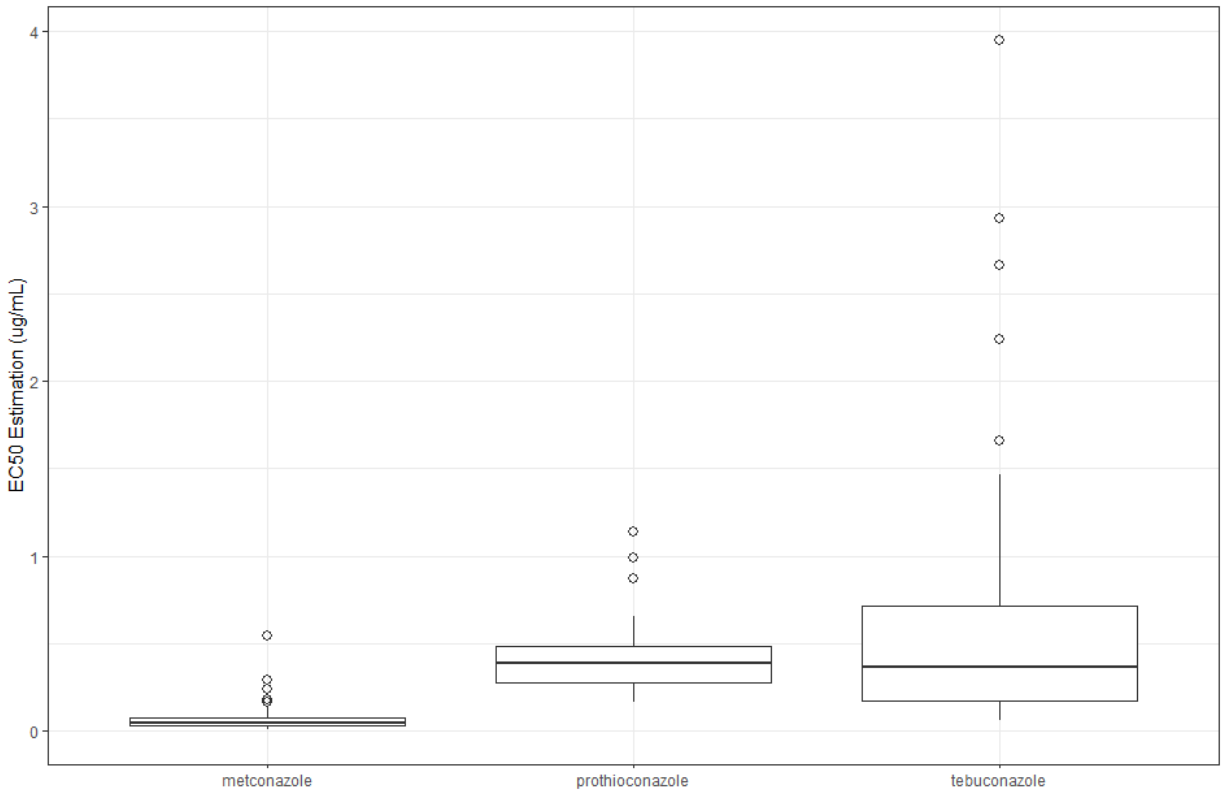


Figure 2.4. Boxplot summarizing distribution of EC_{50} values (effective concentration reducing growth by 50%) of 46 isolates of *F. graminearum* across three DMI fungicides metconazole (Caramba), prothioconazole (Proline), and tebuconazole (Folicur) determined by mycelial growth assay in ½ strength PDA amended with commercially formulated fungicides.

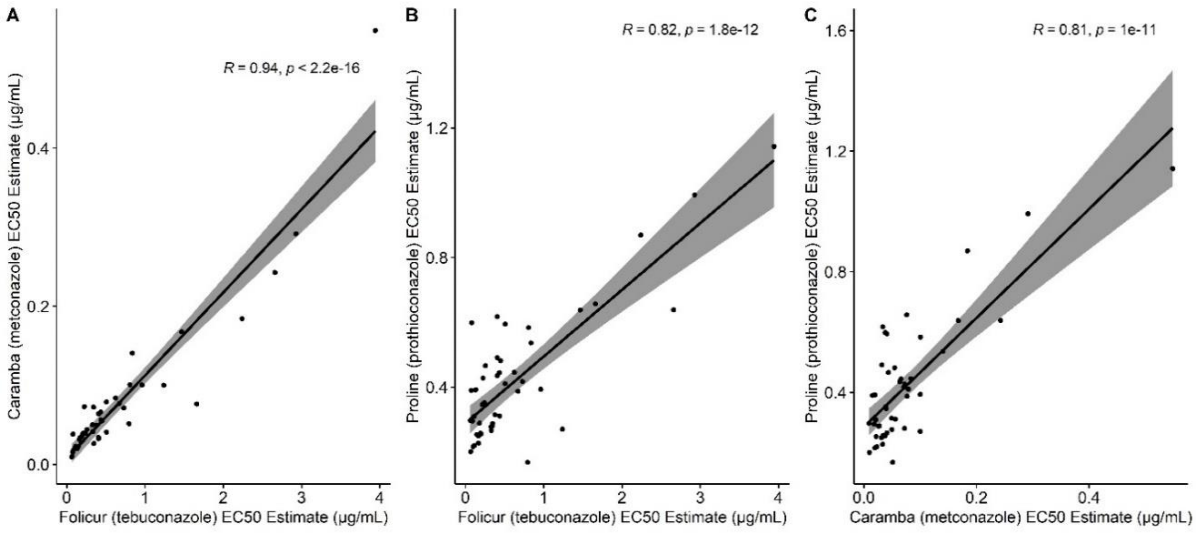


Figure 2.5. Scatterplots depicting relationship between EC_{50} values between three DMI chemistries from 46 *F. graminearum* isolates determined by poison-plate mycelial growth assays. A) Relationship between metconazole and tebuconazole, B) relationship between tebuconazole and prothioconazole, and C) relationship between metconazole and prothioconazole. All were strongly correlated and statistically significant relationships as determined by Pearson's correlation coefficient, demonstrating significant cross-sensitivity between chemistries. Line depicts linear regression of values, with 95% confidence interval shaded in grey.

Screening isolate collection for *in vitro* DMI sensitivity at 1µg/mL

Approximately 445 isolates were screened at 1µg/mL across the three DMI chemistries metconazole, tebuconazole, and prothioconazole (Supplementary Figure 2.3). Consistent with the initial EC_{50} results from 46 isolates, metconazole was significantly more effective. Isolates had 11% greater relative growth on 1ug/mL of prothioconazole ($P < 0.0001$) and 18% greater growth on tebuconazole ($P < 0.0001$) relative to metconazole. Relative growth of isolates on prothioconazole and tebuconazole were not significantly different from one another. Species was also a significant factor affecting relative growth ($P < 0.0001$) as observed in Figure 2.6. *F. awaxy*, *F. verticillioides*, and F. *subglutinans* were all significantly more sensitive than *F. graminearum* (Figure 2.6Figure 2.4). Members of FTSC and FIESC were both less sensitive,

having significantly greater relative growth values compared to *F. graminearum*, as determined by Tukey's test (α=0.05).

Of the 303 *F. graminearum* isolates tested 35 isolates had at least one replication with relative growth over 50%, and 10 isolates had relative growth values greater than 50% in two or more replication, which could mean their EC_{50} values would likely be near or above 1µg/mL. These 10 isolates were then further tested to determine the actual EC50 value, along with references isolates from the original set. None of the EC_{50} values exceeded the range of EC_{50} values found in the original set of 46 *F. graminearum* isolates tested (Figure 2.4).

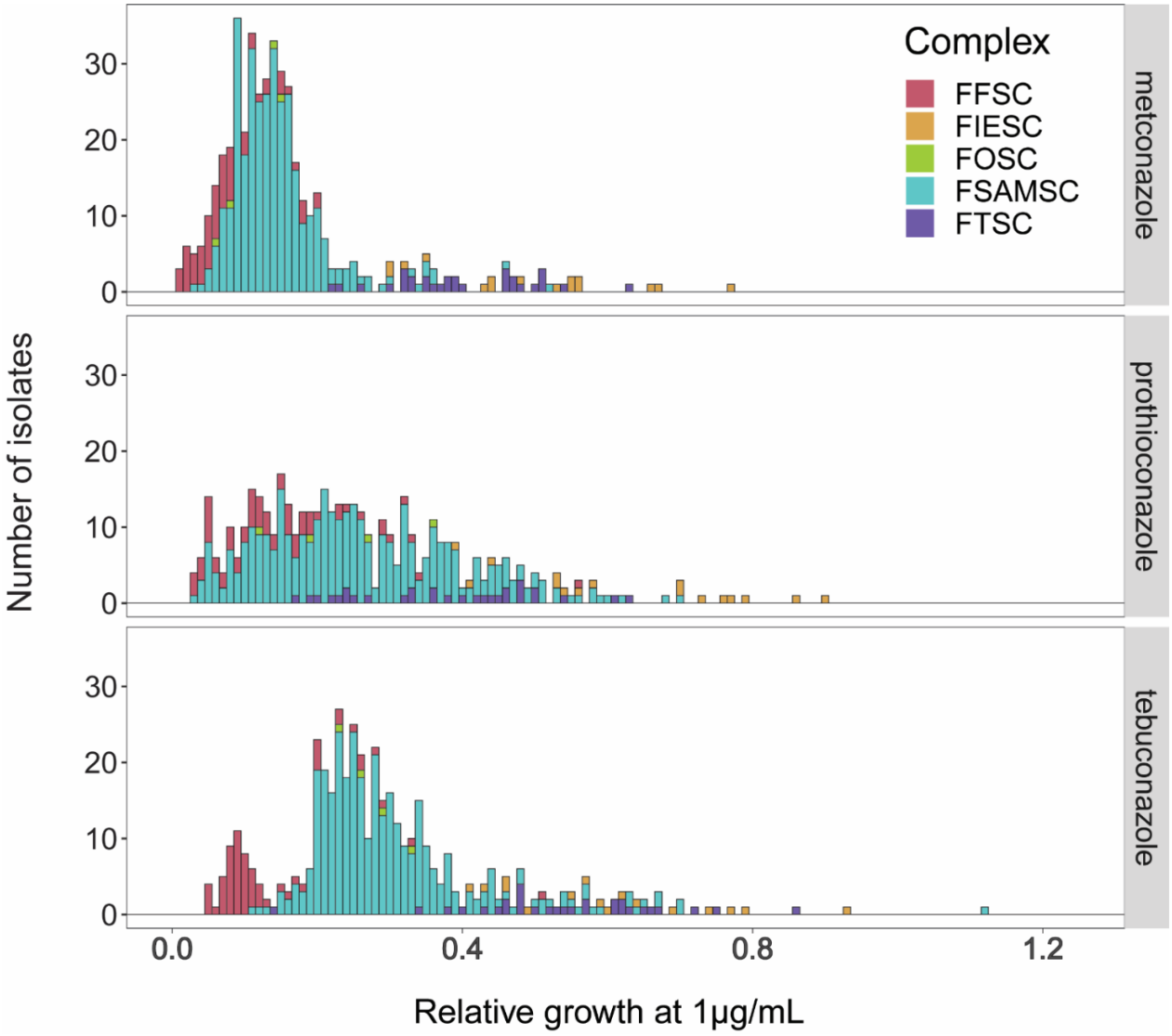


Figure 2.6. Distribution of mean relative growth values from two replicates of 445 *Fusarium* isolates tested in mycelial growth assays in ½ strength PDA plates amended with 1µg/mL commercial formulation of metconazole (Caramba), prothioconazole (Proline), and tebuconazole (Folicur). Different colors represent different species complexes FFSC (*Fusarium fujikuroi*), FIESC (*Fusarium incarnatum-equiseti*), FOSC (*Fusarium oxysporum*), FSAMSC (*Fusarium sambucinum*), and FTSC (*Fusarium tricinctum*).

Influence of chemotype, year, host, and region on *In vitro* DMI sensitivity of *F. graminearum*

Within the set of *F. graminearum* isolates tested at 1µg/mL, there appeared to be differences in relative growth between isolates corresponding with different chemotypes. Chemotype was a significant factor ($P < 0.0001$) in ANOVA testing, with 3-ADON isolates significantly less sensitive to DMI fungicides (Figure 2.7). Isolates of 3-ADON chemotype had an average of 30% relative growth across all three chemistries, whereas 15-ADON isolates averaged 22%, representing an 8% increase in relative growth. There was a significant chemistry by chemotype interaction ($P<0.0001$), as evident by the lack of differences across metconazole, but statistically significant differences between chemotypes within prothioconazole and tebuconazole (Figure 2.7).

Year of collection was a marginally significant factor ($P=0.03$), but there did not seem to be a trend of decreasing sensitivity between 2011 and 2017 (Figure 2.8). Region ($P= 0.56$) nor host ($P=0.33$) significantly influenced sensitive of the *F. graminearum* isolates screened at 1 µg/mL.

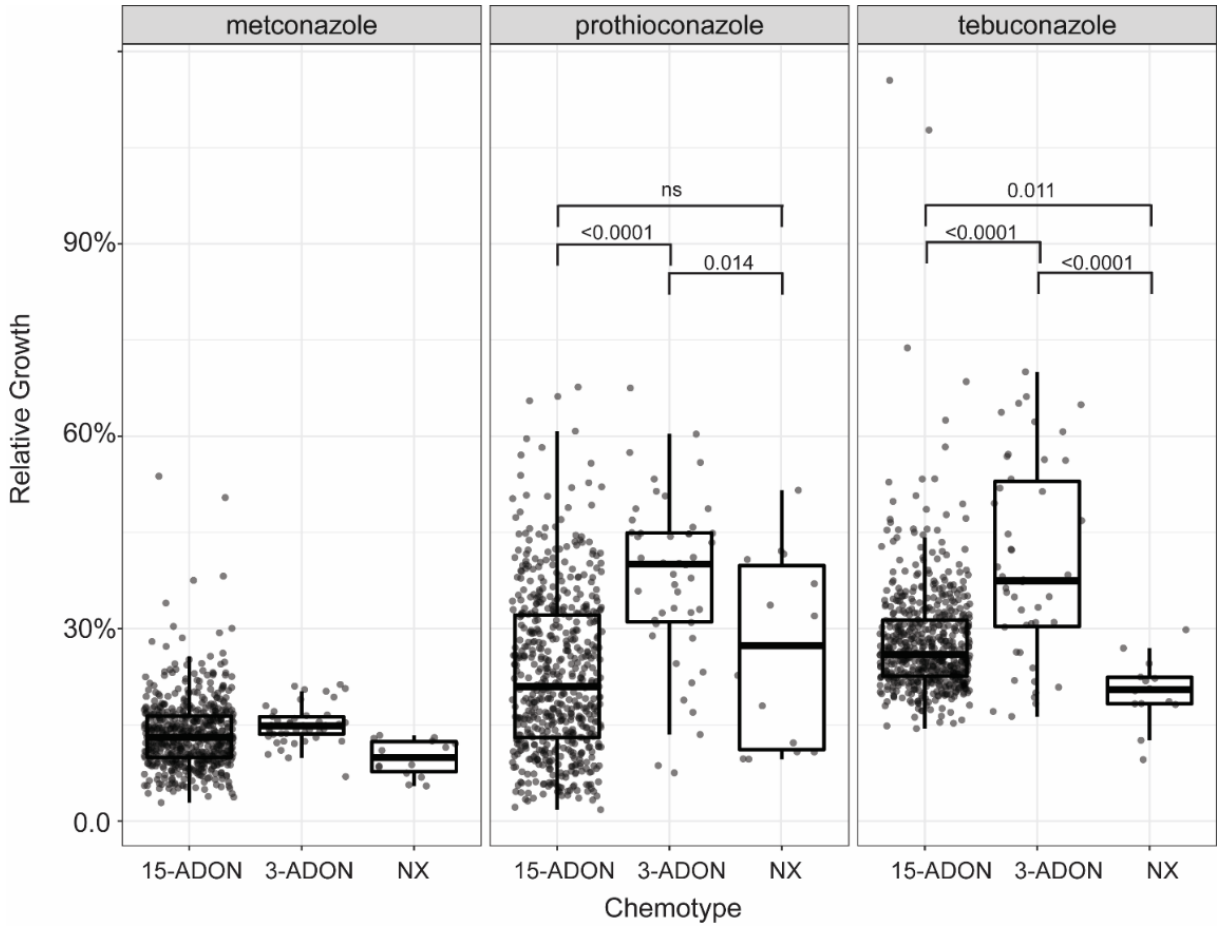


Figure 2.7. boxplots with points representing each isolate overlayed to display the distribution of relative growth values of 303 *F. graminearum* isolates from in vitro mycelial growth assays at 1 µg/mL of three DMI fungicides, genotypically determined to be three different chemotypes, 15A-DON, 3A-DON, or NX-2. Chemotype was a significant factor for prothioconazole and tebuconazole, with p-values displayed above comparisons of each chemotype, however there were no significant differences between chemotypes within metconazole.

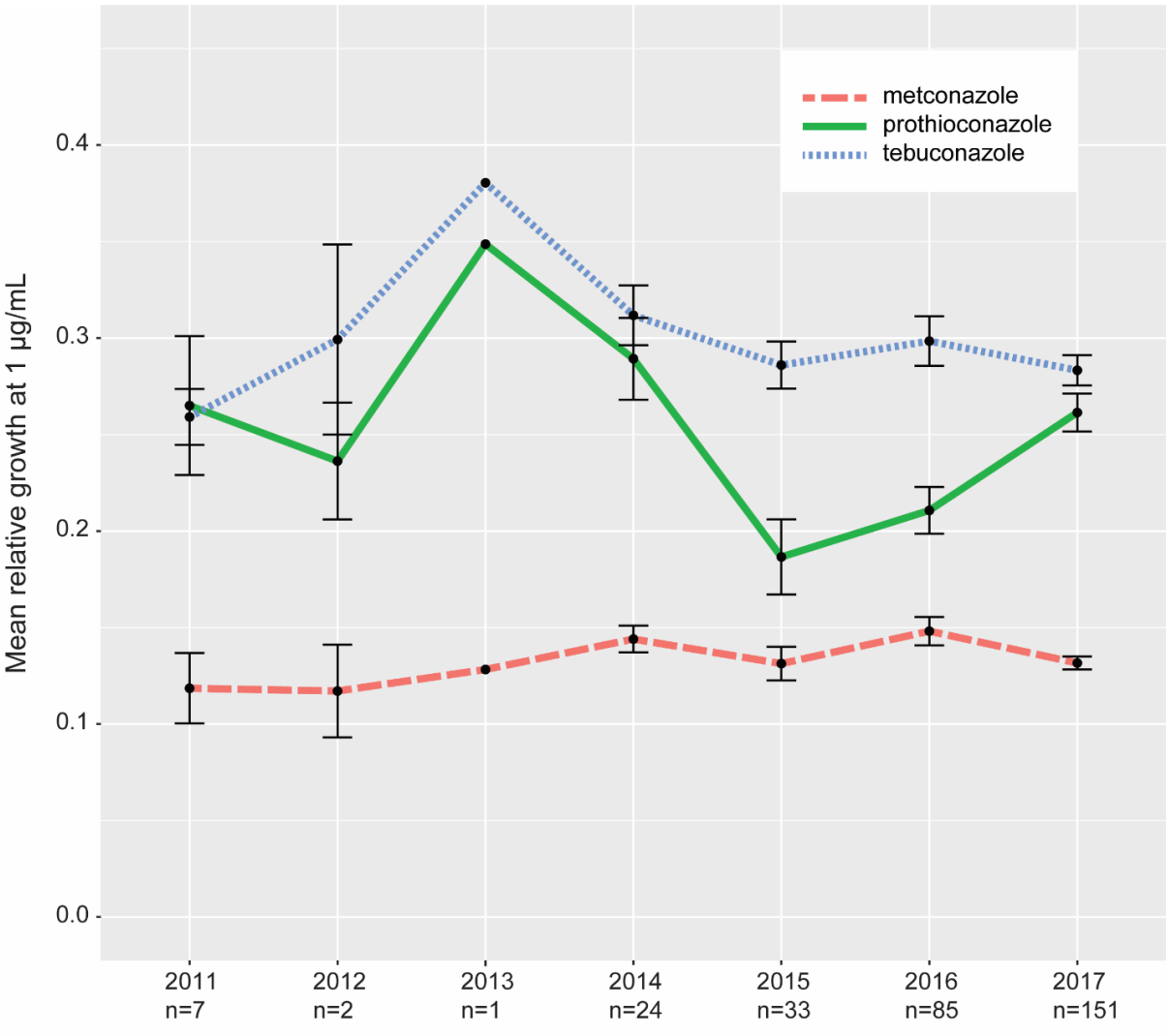


Figure 2.8. Mean relative growth values of *F. graminearum* isolates collected from 2011 to 2017 as determined by mycelial growth assay on media amended with 1 µg/mL metconazole (red dashed line), prothioconazole (green solid line), or tebuconazole (blue dotted line)

Evaluation of EC_{50} of diverse *Fusarium* spp.

To confirm the variation in sensitivity between species observed in the 1µg/mL screen, EC_{50} values were determined for a subset of isolates of differing species (Table 2.4). *F. acuminatum* isolates had significantly greater EC_{50} values compared to all species but *F. tricinctum*. All member of the FTSC (*F. acuminatum*, *F. avenaceum*, and *F. tricinctum*) had the largest ranges of EC_{50} values, as well as the greatest standard deviations compared to other species (Table 2.4).

Even though 1µg/mL as a screening dose was original chosen for *F. graminearum*, results here indicate it is also an informative dose for diverse *Fusarium* species, with the EC_{50} values of the other six species tested significantly correlating with their relative growth at 1µg/mL as well (**Error! Reference source not found.**). However, EC_{50} values of *F. subglutinans* and *F. sporotrichioides* correlated less strongly (Rho=0.55 and 0.76 respectively). This can be attributed to their increased sensitivity resulting in low variation of relative growth values when tested at 1µg/mL.

Table 2.4 Mean EC_{50} values of isolates from seven *Fusarium* spp. from mycelial growth assays using ½ strength PDA amended with commercial formulations of DMI fungicides metconazole and tebuconazole.

Species	N[a]	Metconazole (Caramba) EC_{50} (µg/mL)				Tebuconazole (Folicur) EC_{50} (µg/mL)			
		Mean	Standard deviation	Min	Max	Mean	Standard deviation	Min	Max
F. acuminatum	5	0.78	0.41	0.29	1.29	3.01	2.14	0.83	6.40
F. avenaceum	6	0.29	0.43	0.02	1.16	0.85	1.25	0.05	3.39
F. graminearum	7	0.10	0.18	0.02	0.51	0.30	0.20	0.09	0.71
F. poae	8	0.07	0.06	0.03	0.21	0.30	0.25	0.07	0.90
F. sporotrichioides	8	0.05	0.01	0.03	0.07	0.13	0.03	0.08	0.16
F. subglutinans	7	0.01	0.00	0.00	0.01	0.02	0.00	0.01	0.02
F. tricinctum	2	0.49	0.65	0.03	0.95	1.14	1.44	0.13	2.16

[a]Number of isolates tested for each species to determine the presented mean. Each isolate was tested in mycelial growth assays run three times to determine individual EC_{50}

[b]isolates chosen based on data from previous assays to represent the range of *F. graminearum* EC_{50} values and included in experiment as reference points

In vivo characterization of *F. graminearum* fungicide sensitivity in wheat field plots

To confirm differences in disease pressure between mock-inoculated and inoculated treatments, untreated plots were analyzed. For all disease parameters, isolate was a significant factor and the mock inoculated treatment was nominally lower than all the inoculated plots (Figure 2.9). To explore differences in pathogenicity and aggressiveness between isolates, data was further separated to include only non-fungicide treated inoculated plots. Disease was modeled with sensitivity as a fixed factor and isolate as a random factor. Sensitivity was not a significant factor ($P > 0.05$) for any disease parameters, yield, or test weight. However there was a trend of less sensitive isolates being less aggressive, particularly with disease incidence, where mean incidence was 11.7% lower in less sensitive isolates.

Next, a reduced model was tested with isolate as the only fixed factor to explore virulence differences between individual isolates. In most cases isolate was a significant factor. Notably had a highly significant effect on deoxynivalenol concentration in grain ($P = 0.008$). As depicted in Figure 2.9, there was a large variation in DON concentration in non-treated plots, some isolates producing levels of approximately 10ppm, with others as high as 30ppm. However, for most parameters there was not a high degree of statistical separation between isolates, and only the lowest and the highest isolates were significantly different.

In order to test differences in fungicide efficacy, the reduction in disease parameters as well as yield and test weight for each fungicide plot was calculated as: (1-(plot observation/average in untreated))*100. The reduction due to fungicide of each disease parameter was modeled with sensitivity and chemistry as a fixed effect, and block as a random effect. In all cases chemistry was a significant factor and sensitivity was not. For all parameters, metconazole resulted in a significantly greater reduction of FHB compared to Folicur (Figure

2.10). There was no significant interaction between sensitivity and chemistry. Another model was explored, testing isolate and chemistry as fixed effects and rep as a random effect. Isolate did not have a significant effect on any response variable except for reduction of disease incidence. In that case, isolate was a significant factor (P = 0.029) however the only significant differences were between an aggressive isolate, 21A, relative to two less aggressive isolates (kmiso and 3-C) as seen in Figure 2.10.

In 2020, the same experiment was performed, however conditions were not favorable for FHB development, and inoculated treatments did not have significantly greater disease index or deoxynivalenol levels compared to mock-inoculated plots. Therefore, relative fungicide efficacy could not be evaluated.

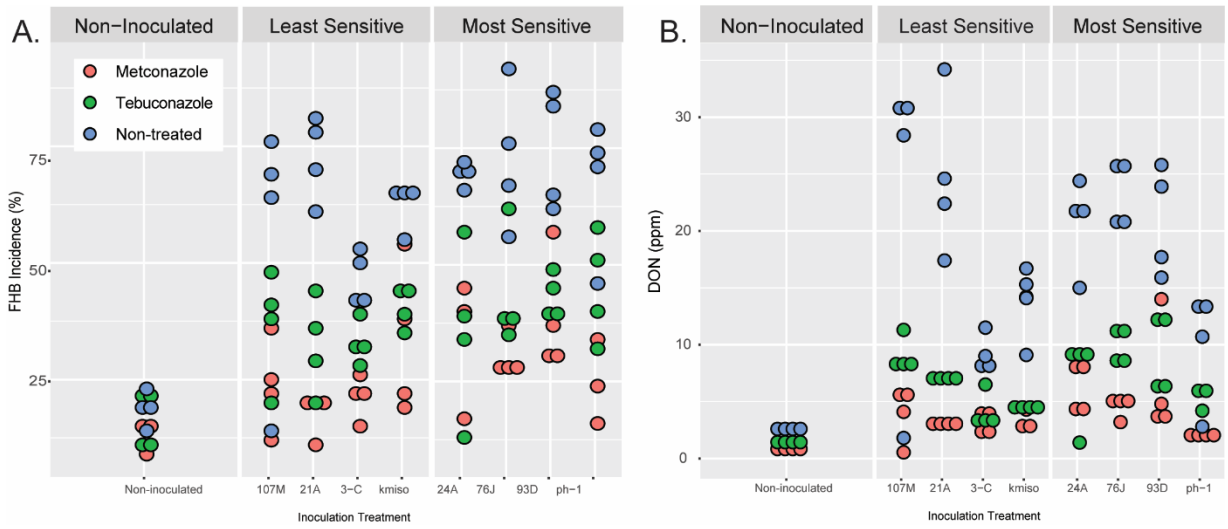


Figure 2.9. Dot-plots depicting measures of FHB from plots inoculated with isolates characterized *in vitro* as either the most sensitive isolates or the least sensitive with (A) head blight incidence measured by the number of diseased heads out of 100, and (B) level of deoxynivalenol (DON) contamination of wheat grain. from Each dot represents value from one plot (replicate) with red depicting metconazole (Caramba) treated plots, green representing plots treated with tebuconazole (Folicur), and blue the non-treated plots.

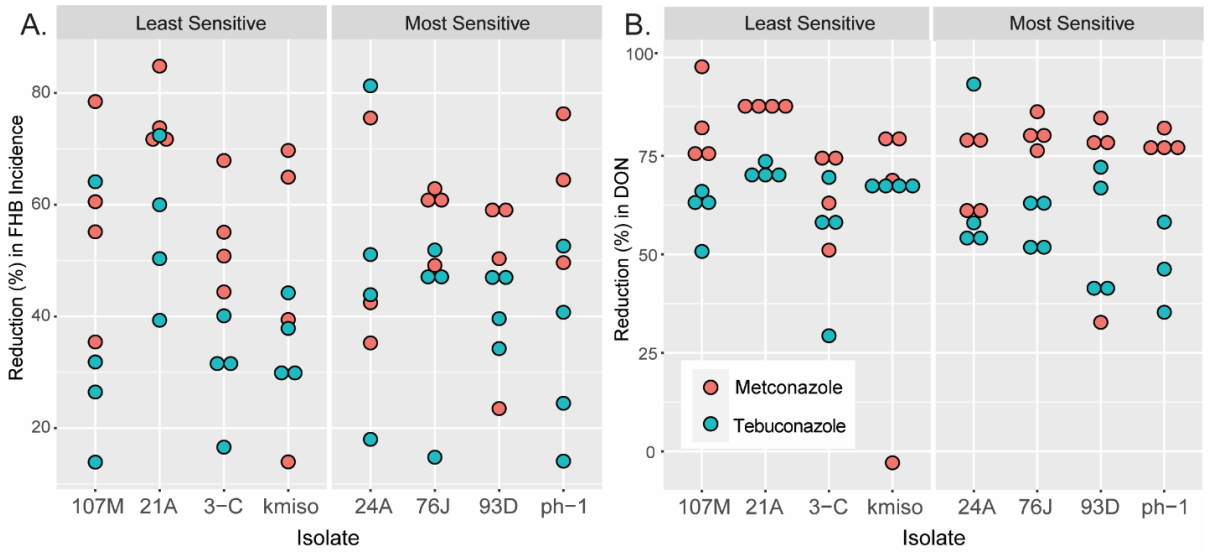


Figure 2.10. Dot-plots depicting fungicide efficacy against eight isolates of *F. graminearum* in a small plot field trial, expressed as the percent that the fungicide application reduced (A) head blight incidence and (B) level of deoxynivalenol (DON) contamination of grain in ppm compared to the untreated control in plots inoculated with a single isolate. These isolates were previously characterized *in vitro* as either the most sensitive isolates or the least sensitive. Each dot represents value from one plot (replicate) with red depicting metconazole (Caramba) treated plots and light blue representing plots treated with tebuconazole (Folicur).

Discussion

This collection of over 590 isolates from Michigan allowed us to investigate the species composition, toxin chemotypes, and fungicide sensitivities of *Fusarium* spp. infecting wheat and corn to ultimately inform disease management strategies. Over 98 fields were sampled across Michigan between 2011 and 2019, allowing us to confirm the presence of lesser-studied species in the Midwest and estimate the variation of DMI fungicide sensitivity phenotypes present. This study also marks one of the first surveys of *Fusarium* spp. in corn in recent history in the United States, the few studies available (Leslie et al. 1990; Gilbertson et al. 1985) all occurred prior to 2000. In both wheat and corn, numerous species besides *F. graminearum* were found. Particularly in corn, where *F. graminearum* sensu stricto only represented 37% of the collection. Fungicide sensitivity was assessed for 491 total isolates for three DMI active ingredients, tebuconazole, metconazole, and prothioconazole. Although variation in sensitivities was found, with EC_{50} values ranging from 0.009 to 3.945 µg/mL, isolates least sensitive *in vitro* still appeared to be insensitive *in vivo.*

F. graminearum composed the majority of isolates in wheat, representing 83% of the collection, but only 37% of isolates in corn. Members of the FFSC composed the majority of isolates recovered from corn, mainly *F. subglutinans (33%),* however several isolates of other members including *F. fujikuroi* senso stricto, *F. proliferatum, F. verticillioides,* and 6 isolates of *F. awaxy* were recovered. This is the first report of *F. awaxy* in the United States on corn, a species recently described by Petters-Vandresen et al as closely related to *F. subglutinans* and morphologically indistinguishable (Petters-vandresen 2019). Isolates identified as *F. awaxy* were found in five sites out of the 72 sampled across the state, three which were commercial fields and two which were university research sites. These sites were from disparate regions,

suggesting these were not likely a single recent introduction, and with deeper sampling and molecular identification *F. awaxy* isolates may be more widely found.

Members of the FFSC found here are known to produce moniliformin, beauvericin, bikaverin, fusaproliferin, and fumonisins, with the exception of *F. subglutinans* which has not been documented to produce the carcinogenic fumonisin toxins (Logrieco et al. 2002; O'Donnell et al. 2018b). The high proportion of *F. subglutinans* found here is consistent with a 2008 survey of corn in the near neighbor, Ontario, where authors found that 39% of total isolates were *F. subglutinans* (Schaafsma et al. 2008). Three isolates of *F. subglutinans* were also recovered from wheat, which can be sporadically recovered from small grains as well according to European surveys (Bottalico and Perrone 2002).

Notably, 26 isolates identified as *Fusarium tricinctum* complex were recovered from wheat, but none from corn. The majority of these were identified as *F. avenaceum*, representing 3.7% of the collection, followed by *F. acuminatum* at 2.4%. In our pathogenicity testing (Appendix C), as well as the literature, these species did not appear to spread through the rachis, nor bleach the outer part of the kernels (Stack and McMullen 1985; Bec et al. 2015). Notably, in a single field the majority of FTSC isolates were found (11 isolates). Grain samples were received for this field, not heads. This could suggest a potential bias against FTSC members if only bleached heads are used to identify and collect survey samples. Even if these species are not highly aggressive in spreading throughout a wheat head, damage can still occur if conditions are favorable for toxin accumulation, or infection occurs at a high incidence. Species in the FTSC are known to produce moniliformins and enniatins (Abramson et al. 2001; O'Donnell et al. 2018b) and previous studies have observed high moniliformin concentrations in fields with known natural infection of FTSC (P. Hellin et al. 2016; Cowger, Ward, et al. 2020). In an

inoculated study *F. avenaceum* result in yield loss and moniliformin accumulation (Vogelgsang et al. 2008). A survey of North Carolina wheat fields found similar results, with *F. graminearum* the main causal agent, but surprisingly, FTSC members comprised greater than 20% of isolates in 10 fields (Cowger, Ward, et al. 2020). Deeper sampling may reveal a greater proportion of FTSC species causing head blight in wheat in the U.S., and further investigation is needed to elucidate what factors might be contributing to high proportions of FTSC species rather than *F. graminearum* in specific fields.

In both hosts a small number of FIESC isolates were recovered (wheat = 2%, corn=6%). These are generally regarded as non-pathogens and commonly isolated saprotrophs from environmental samples (Leslie and Summerell 2006), however this complex is known to produce atleast 10 different mycotoxins (O'Donnell et al. 2018a). Our greenhouse inoculations of wheat with two isolates found they led to damage of inoculated kernel but did spread to adjacent kernels. Future work could include investigating the pathogenicity and virulence of these species to determine their relative importance and contribution to mycotoxin accumulation in grain.

To further investigate the diversity within the dominant species *F. graminearum*, the chemotype of all *F. graminearum* isolates in this study from wheat, corn, soybean, and dry bean were predicted by genotype at the TRI loci. Despite varying crop diversity and climactic conditions, the 15-ADON chemotype dominated all regions comprising 92% of the total collection. No NIV *F. graminearum* isolates were found in this survey, but one *F. cerealis* isolate recovered from wheat was identified as the NIV genotype. This is consistent with previous surveys that only found NIV producing *F. graminearum* in the southern United States (Gale et al. 2011; Schmale et al. 2011).

The only area with a significant portion of 3-ADON isolates was in the far northeastern corner of the state, a coastal area with less agriculture intensive land use and more natural areas. Over 37% of isolates from this region were 3-ADON chemotype and 10% were NX-2. This observation supports the hypothesis of researchers in New York State, who found a similar increase in 3ADON and NX-2 genotypes in non-agricultural environments, and increased admixture between TRI genotype-defined populations. These non-agricultural environments may be able to harbor different genotypes and act as sources of inoculum to nearby agricultural fields either due to the lower proportion of agricultural hosts, or increase in diversity of hosts (Fulcher et al. 2019). The 'thumb' region of the state, which is an intensive agricultural area, was the only other region with 3ADON, with only two isolates found.

One shortcoming of this study is the non-systematic approach to sampling. In only a few sites was a location sampled over multiple years. The northeastern area seemed to harbor more diverse TRI genotypes, but this area was only successfully sampled in one year. A smaller number of grain sample were taken in 2016 from this region, but no *F. graminearum* was recovered. Thus, we are unable to conclude the stability of this trend in TRI genotypes over time. Likewise, there was a significant impact of year on the composition of species, but this was not a reliable test as only a few locations were sampled over multiple years.

Another main objective of the present survey was to determine the variation of fungicide sensitivity present within *F. graminearum* and across diverse species. The three most widely used DMI active ingredients in the United States for wheat and corn were tested: metconazole, tebuconazole, and prothioconazole. First, EC_{50} values for 46 isolates of *F. graminearum* were estimated, then relative growth assays screening isolates at 1 ug/mL were performed on the remaining 445 isolates. Isolates of *F. graminearum* with relative growth values greater than 50%

at 1 ug/mL, as well as additional species, were further investigated with an additional experiment to determine EC_{50} values to metconazole and tebuconazole. This represents the largest study of fungicide sensitivity to *Fusarium* spp. in the United States.

We found variation in EC_{50} values of *F. graminearum*, and significantly different levels of *in vitro* sensitivity to the three chemistries tested. Isolates were most sensitive to metconazole (mean 0.075 µg/mL), then prothioconazole (mean 0.421 µg/mL) and least sensitive to tebuconazole (mean 0.646 µg/mL), which is concordant with other surveys of *F. graminearum* DMI sensitivity (Klix et al. 2007; Pierri Spolti et al. 2014; Anderson et al. 2020). Evidence from previous literature, as well as our *in vivo* work here, showed this relationship is reflected in relative efficacy in the field. A published meta-analysis of fungicide efficacy data reported metconazole 17.3% more effective at reducing FHB index and 28.8% more effective at reducing DON relative to tebuconazole (Paul et al. 2008). Here, we also report species in the FFSC, FTSC, and FSAMC were also more sensitive to metconazole relative to tebuconazole (Table 2.4). Since this relationship has been reported for a number of years, it may suggest inherently greater activity of metconazole compared to tebuconazole, rather than declining sensitivity to tebuconazole. To test this hypothesis, one would need a collection of historical isolates from Michigan. Our study did include isolates dating back to 2011, however tebuconazole was introduced in the U.S prior to that in approximately 2006.

In this set of isolates, we did not observe differences of *in vitro* sensitivity based on host, region, or year of collection. There were however statistically significant differences between genetically determined chemotypes. Isolates identified as 3A-DON were significantly less sensitive to fungicides in mycelial growth assays at 1 µg/mL (Figure *2.7*). However, considering the low sample size of 3-ADON isolates (n=26), this should be interpreted with caution and

further investigated. The difference here was modest, only an 8% mean difference in relative growth. Additional investigation across wider geographies would be needed to confirm if a difference in sensitivity is related to chemotype and population structure.

The *in vitro* DMI sensitivity of *F. graminearum* was further investigated to determine if this phenotype translated to differences in field efficacy. A small plot field study was established in 2019 and 2020 comparing strains that were the most sensitive and least sensitive *in vitro* and evaluating the efficacy of metconazole (Caramba) and tebuconazole (Folicur). Adequate disease did not develop in 2020, but in 2019 no differences in fungicide efficacy was found between plots inoculated with isolates from these two groups (Figure 2.10), indicating isolates with EC_{50} values in the range found here are not yet practically resistant. This study also revealed the large variation in DON production (10-30ppm) between isolates in a field setting and underlines the importance of isolate selection for inoculated field trials.

Development of DMI resistance in *Fusarium* should continue to be of concern however, as our study demonstrated there is variation in populations of *F. graminearum* in Michigan. This variation suggests there are likely genetic differences in the population, which further DMI use could continue to select for. Evidence from a 2014 study suggests that practical resistance may not be far off, as authors reported an isolate from New York with an EC_{50} to tebuconazole of 8.09 µg/mL. This isolate displayed reduced fungicide sensitivity *in vivo* when treated with fungicides in greenhouse assays. There is considerable inter-lab variation in fungicide sensitivity assays, so while this EC_{50} values is not directly comparable to data presented here, their data certainly suggest that *in vitro* sensitivity is a good indicator of *in vivo* sensitivity in *F. graminearum*, and resistance development may not be far in the future.

This dataset also demonstrated a strong and highly significant correlation between EC_{50} values of all three DMI chemistries, suggesting if sensitivity were to continue to decrease, cross-resistance to multiple DMI active ingredients would likely occur. This has been previously reported in *F. graminearum* (Piérri Spolti et al. 2014; Becher et al. 2010). DMI resistance is also a challenge in other wheat pathogens in the United States, notably *Zymoseptoria tritici* (Augusti et al. 2019; Sykes et al. 2018). These issues underscore the importance of continued monitoring and resistance management to preserve the use of DMI chemistries for *Fusarium* management in wheat and corn. Prior to 2019, DMI products were the only products registered for head blight and ear mold control. Now a new SDHI active ingredient, pydiflumetofen, is available. A study with a subset of this collection demonstrated *F. graminearum* populations in Michigan are sensitive to this chemistry (Breunig and Chilvers 2021), indicating it could be a rotational partner and reduce selection pressure for DMI resistance.

This study also revealed species-specific differences in DMI sensitivity. These differences were apparent from relative growth values at 1 µg/mL, and were further investigated with EC_{50} values of select species. Members of the FTSC were less sensitive than *F. graminearum* to DMI chemistries. *F. acuminatum* had the highest EC_{50} values to (0.83 – 6.40 µg/mL to tebuconazole) across all *Fusarium* species tested. *F. subglutinans* was the most sensitive, with EC_{50} values 10-fold lower than *F. graminearum, F. poae,* or *F. sporotrichioides*. With the exception of *F. subglutinans,* 1 µg/mL was also an adequate screening dose or diverse species as EC_{50} values of these correlated strongly to the relative growth data at 1 µg/mL. *F. subglutinans* was so sensitive however, that there was little to no variation in relative growth values at 1 µg/mL, leading to a relatively lower correlation with EC_{50} values (Supplementary Figure 2.2).

The practical significance of the EC_{50} values from diverse species outside of *F. graminearum* remains undetermined. The target of DMI fungicides, the CYP51 gene, have three known paralogues in *Fusarium* spp. Previous studies have also shown there are species specific differences in CYP51 sequences (Yin et al. 2009a; Fernández-Ortuño et al. 2010), which could lead to inherent differences in DMI sensitivity. Further work is needed to elucidate the mechanisms behind species specific differences *in vitro* and determine relative fungicide performance across these additional species of importance.

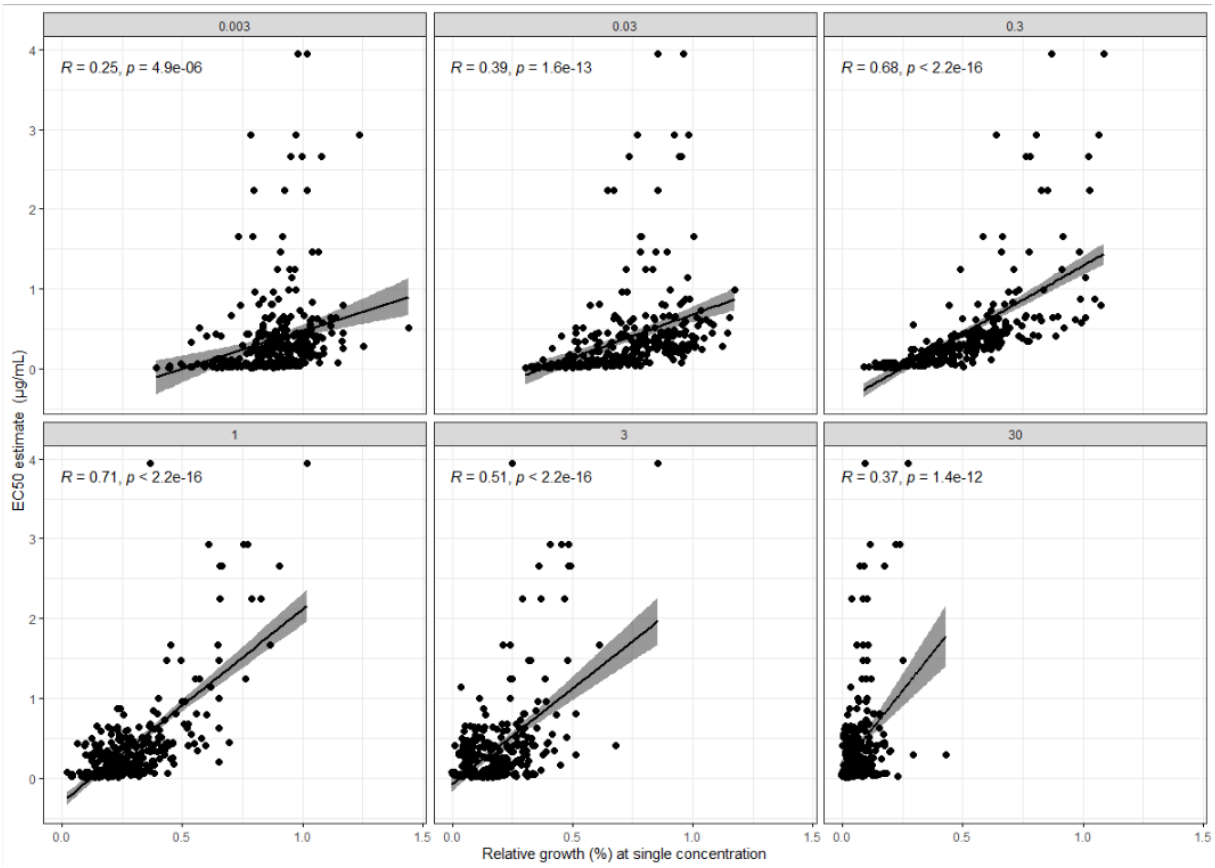
Combination evidence from *in vitro* and *in vivo* studies presented here suggest there is currently no resistance to DMI fungicides in Michigan populations of *F. graminearum*. There is however variation within *F. graminearum*, and between other *Fusarium* species. Specifically, the *Tricinctum* complex species appears to be less sensitive to fungicides, and they should be included in future fungicide sensitivity monitoring efforts. The relative importance of FTSC remains to be determined, while only comprising 7% of the isolates here, more in depth surveys and methods that do not bias for *F. graminearum* may reveal higher proportions across the United States, as they did in North Carolina (Cowger, Ward, et al. 2020).

Prior to our characterization of 569 *Fusarium* isolates, little was known about populations of *Fusarium* infecting wheat, and particularly corn in the Upper Midwest of the United States. Here we confirm that numerous species are infecting these crops with the potential to cause mycotoxin contamination, and *F. graminearum* must not be thought of as the only culprit. The results presented here also underscore the impact of accurate species identification on management and epidemiology of Fusarium head blight and ear mold. Agriculturalists such as breeders and agronomist should consider utilizing additional species besides *F. graminearum* when screening germplasm or inoculating management trials. Species diversity is also an

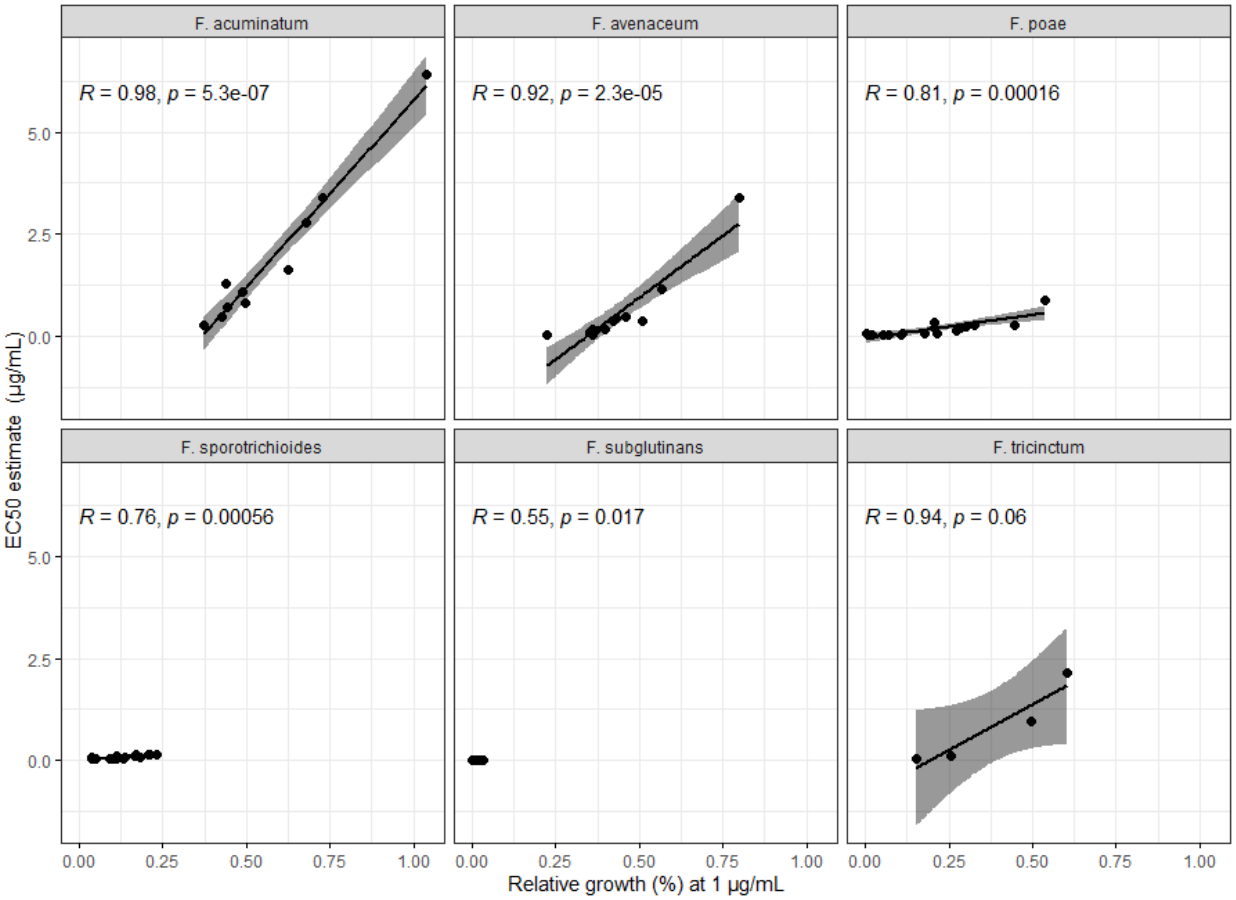
important determinant of potential toxin contamination, here we identified several species capable of producing mycotoxins beyond deoxynivalenol, including moniliformin or fumonisins. Finally, this data demonstrated that while no practical resistance to DMI chemistries was found in Michigan, despite widespread use for 10-20 years, monitoring should continue as there is variation present within and among species of *Fusarium*.

APPENDICES

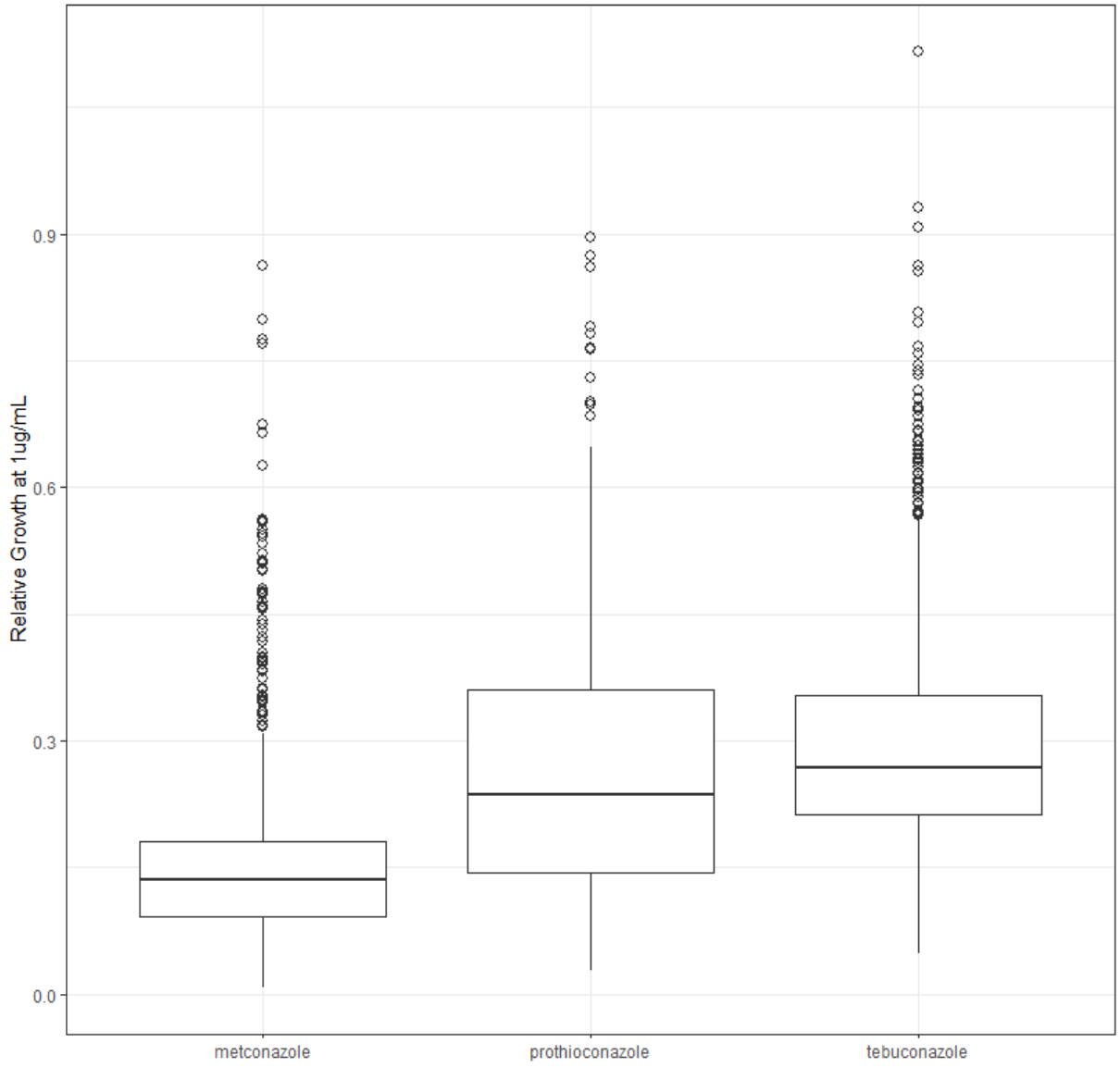
APPENDIX A: Supplemental figures and tables



Supplementary Figure 2.1. Relationship between relative growth at single concentrations with EC_{50} values estimated using all six concentrations for 46 isolates of *F. graminearum* tested across three DMI products (tebuconazole, metconazole, and prothioconazole). Pearsons correlation statistics displayed, and linear regression line in black with 95% confidence interval shaded in grey. All concentrations were significantly correlated with the EC_{50} value.



Supplementary Figure 2.2. Pearsons correlation between relative growth at 1µg/mL and the EC_{50} estimations based of six doses, across seven diverse *Fusarium* spp. from mycelial growth assays performed on ½ strength PDA amended with commercial formulation of tebuconazole (Folicur). Line depicts linear regression with 95% confidence interval shaded in grey.



Supplementary Figure 2.3. Boxplots depicting the distribution of relative mycelial growth values of *Fusarium* isolates (n=445) on ½ strength PDA amended with DMI fungicides metconazole (Caramba), prothioconazole (Proline), and tebuconazole (Folicur).

Table A2.1 Pearsons correlation between EC_{50} values of an isolate and the relative growth at 1µg/mL for the same isolate, for mycelial growth assays across tebuconazole and metconazole.

Species	Rho	P	n isolates evaluated
F. acuminatum	0.98	< 0.00001	5
F. avenaceum	0.92	< 0.00001	6
F. poae	0.81	0.00016	8
F. sporotrichioides	0.76	0.00056	8
F. subglutinans	0.55	0.017	7
F. tricinctum	0.94	0.06	2

Table A2.2 EC_{50} (effective concentration to reduce growth by 50%) for 46 *F. graminearum* isolates determined from poison plate mycelial growth assays for three DMI chemistries: metconazole, tebuconazole, and prothioconazole, with associated meta-data for each isolate.

Isolate	Chemo-type	Year	Sample type	Metconazole		Tebuconazole		Prothioconazole		County isolated
				EC_{50} Estimate	Standard Error	EC_{50} Estimate	Standard Error	EC_{50} Estimate	Standard Error	
10-C	15	2014	wheat head	0.1	0.022	1.241	0.424	0.27	0.048	Sanilac
102A	15	2017	wheat head	0.026	0.015	0.348	0.213	0.288	0.059	Presque isle
102C	3	2017	wheat head	0.073	0.012	0.226	0.059	0.427	0.036	Presque isle
106B	15	2017	wheat head	0.077	0.019	0.673	0.251	0.387	0.037	Presque isle
15-A	15	2014	wheat head	0.243	0.07	2.66	0.557	0.638	0.071	Sanilac
21A	15	2016	wheat head	0.184	0.042	2.242	0.368	0.869	0.432	Sanilac
24A	15	2016	wheat head	0.01	0.003	0.069	0.033	0.201	0.016	Huron
27A	15	2016	wheat head	0.168	0.065	1.468	0.405	0.638	0.098	Sanilac
2D	15	2016	wheat head	0.1	0.046	0.963	0.419	0.393	0.08	Kalamazoo
3-A	15	2014	wheat head	0.032	0.013	0.407	0.346	0.491	0.152	Sanilac
44B	15	2016	wheat grain	0.056	0.016	0.438	0.127	0.311	0.052	Cass
53F	15	2016	wheat grain	0.023	0.004	0.116	0.045	0.219	0.029	Van Buren
57G	15	2016	wheat grain	0.028	0.008	0.182	0.064	0.288	0.022	Van Buren
66C	15	2016	wheat grain	0.034	0.007	0.17	0.054	0.227	0.029	Allegan

Table A2.2 (cont'd)

67F	**15**	**2016**	**wheat grain**	**0.02**	**0.004**	**0.102**	**0.023**	**0.216**	**0.021**	**Muskegon**
69G	15	2016	wheat grain	0.041	0.025	0.509	0.381	0.594	0.111	Muskegon
73L	15	2015	wheat grain	0.072	0.032	0.344	0.098	0.28	0.06	Tuscola
73S	15	2015	wheat grain	0.016	0.009	0.076	0.046	0.39	0.027	Tuscola
76D	15	2017	wheat head	0.038	0.009	0.202	0.095	0.256	0.025	Lenawee
78A	15	2017	wheat head	0.039	0.014	0.242	0.11	0.351	0.078	Lenawee
78B	15	2017	wheat head	0.076	0.019	1.662	0.596	0.657	0.06	Lenawee
7H	15	2016	wheat head	0.084	0.029	0.625	0.231	0.445	0.064	St joseph
80A	15	2017	wheat head	0.101	0.037	0.808	0.293	0.583	0.116	Ingham
82B	15	2017	wheat head	0.066	0.029	0.435	0.222	0.444	0.069	Tuscola
82D	15	2017	wheat head	0.071	0.024	0.731	0.18	0.417	0.044	Tuscola
89B	15	2017	wheat head	0.141	0.062	0.839	0.217	0.536	0.067	St joseph
8D	15	2016	wheat head	0.035	0.006	0.192	0.043	0.257	0.042	St joseph
93A	15	2017	wheat head	0.055	0.019	0.451	0.258	0.481	0.035	Sanilac
93D	15	2017	wheat head	0.009	0.004	0.064	0.029	0.297	0.048	Sanilac
99A	15	2017	wheat head	0.05	0.015	0.327	0.105	0.276	0.05	Sanilac
C13E12A	15	2016	corn ear	0.02	0.008	0.133	0.053	0.392	0.051	Eaton

Table A2.2 (cont'd)

C1E3B	**15**	**2016**	**corn ear**	**0.031**	**0.007**	**0.165**	**0.045**	**0.249**	**0.026**	**branch**
C5-3D	15	2014	corn grain	0.021	0.007	0.11	0.066	0.309	0.033	Kalamazoo
C6B	15	2016	corn ear	0.05	0.013	0.38	0.138	0.314	0.018	Allegan
C9E4C	15	2016	corn ear	0.079	0.015	0.508	0.239	0.41	0.053	Jackson
K-MISO2_2-17	15	2013	soy root	0.292	0.173	2.929	0.963	0.993	0.181	Livingston
K-MISO2_4-18	15	2014	soy root	0.038	0.011	0.082	0.018	0.598	0.067	Clinton
P4-R13-2	15	2017	corn root	0.044	0.014	0.258	0.109	0.466	0.041	Van Buren
P5-R7-3	15	2017	corn root	0.04	0.015	0.215	0.049	0.346	0.048	Van Buren
ph-1	15	1995	corn stalk	0.019	0.004	0.093	0.029	0.295	0.045	Ingham
F_15_27	15	2015	dry bean root	0.064	0.017	0.407	0.089	0.435	0.054	Gratiot
F_15_10	15	2015	dry bean root	0.034	0.013	0.408	0.255	0.617	0.129	Huron
10-B	15	2014	wheat head	0.022	0.006	0.149	0.061	0.253	0.113	Sanilac
3-C	15	2014	wheat head	0.549	0.29	3.945	2.47	1.142	0.048	Sanilac
89C	15	2017	wheat head	0.041	0.019	0.332	0.325	0.265	0.184	St joseph
99B	15	2017	wheat head	0.051	0.033	0.796	0.745	0.168	0.067	Sanilac

APPENDIX B: Pathogenicity confirmation of subset of recovered isolates

Various species isolated from wheat were inoculated on the *Fusarium* susceptible spring wheat variety (cv. Wheaton) in order to confirm pathogenicity. Three pots with 3-4 plants per pot were used for each isolate in a single experiment. Two runs (unless otherwise specified in Table A2) were completed in each experiment.

Conidia of each species was prepared by growing isolates on mung bean agar or Carboxymethyl cellulose medium to induce sporulation (Cappellini and Peterson 1965). Spores were harvested from CMC broth by centrifugation and rinsed with water, or scraped and rinsed off of mung bean plates. Spore suspensions were filtered through three layers of sterile miracloth, then quantified with a hemocytometer. Spore solutions were diluted to a standard concentration of 1×10^5 spores/mL, and stored in 35% glycerol in -80°C until use.

At the time of inoculation, spore suspensions were removed from the freezer and stored on ice. At the beginning of anthesis plants were inoculated by pipetting 10ul of the spore suspension between the lemma and palea on a kernel in the midsection of a wheat head. After inoculation, plants were moved to a misting chamber, where they were misted for 10 seconds every five minutes, for 72 hours. After 72 hours they were returned to the greenhouse bench.

Approximately 14 days after inoculation wheat heads were inspected for necrosis and kernels damage. The number of spikelets affected per head were counted and recorded. If only a single kernel was damaged and infection had not spread to entire spikelet, a score of 0.5 was recorded.

Greenhouse data was analyzed in R with a linear mixed model using package ‘lmer’, with treatment as a fixed factor, and isolate and rep nested in run as random factors. Species identification significantly affected number of diseased spikelets at 14 days post inoculation

(p=0.005). Comparison of estimated marginal means with package 'emmeans' demonstrated *F. graminearum* was significantly more virulent than all other species tested. All other species were not significantly more virulent than one another. While they were all capable of kernel damage, they were much less virulent than *F. graminearum*, often never progressing past the point of inoculation. In some cases, the kernel was not affected (so received a score of zero) but the lemma was discolored or necrotic.

Table A2.3 Mean disease rating at 14 days post inoculation for isolates inoculated on susceptible cultivar 'wheaton' in the greenhouse to confirm pathogenicity.

Species	Isolate ID[a]	Mean	Standard Deviation	Number of plants tested	Standard Error	95% CI.min	95% CI.max
F. acuminatum	110B	0.1	0.2	8	0.1	-0.1	0.3
F. acuminatum	110B	0.5	0.1	12	0.0	0.4	0.6
F. acuminatum	83A	0.5	0.5	11	0.2	0.2	0.9
F. acuminatum	83Q	0.5	0.3	12	0.1	0.3	0.6
F. avenaceum	105L	0.5	0.2	10	0.1	0.3	0.6
F. avenaceum	121E	0.5	0.3	11	0.1	0.4	0.7
F. avenaceum	121E	0.5	0.0	9	0.0	-	-
F. avenaceum	50A	0.5	0.0	12	0.0	-	-
F. avenaceum	61B	0.3	0.5	12	0.1	0.0	0.6
F. avenaceum	61B	0.5	0.2	10	0.1	0.3	0.6
F. avenaceum	71C	0.5	0.1	12	0.0	0.4	0.6
F. avenaceum	83N	0.5	0.2	10	0.1	0.3	0.6
F. avenaceum	83O	0.5	0.0	9	0.0	-	-
F. avenaceum	83O	0.4	0.2	10	0.1	0.2	0.5
F. graminearum	10-A	7.9	4.3	12	1.2	5.2	10.6
F. graminearum	107H	8.3	5.3	12	1.5	4.9	11.6
F. graminearum	107M	10.3	1.8	12	0.5	9.1	11.4
F. graminearum	107U	11.4	4.4	11	1.3	8.4	14.3
F. graminearum	117A	11.0	1.0	11	0.3	10.3	11.7
F. graminearum	15-A	11.1	3.2	11	1.0	8.9	13.3
F. graminearum	23D	10.9	1.8	10	0.6	9.6	12.2
F. graminearum	23D	9.4	3.9	10	1.2	6.6	12.2
F. graminearum	24A	9.7	3.5	12	1.0	7.5	11.9
F. graminearum	3-A	11.0	2.0	12	0.6	9.7	12.3
F. graminearum	3-C	4.7	2.3	11	0.7	3.2	6.3
F. graminearum	3-C	9.7	2.7	12	0.8	7.9	11.4
F. graminearum	66B	12.4	3.9	12	1.1	9.9	14.8

Table A2.3 (Cont'd)

F. graminearum	66B	5.7	3.4	12	1.0	3.5	7.9
F. graminearum	76J	10.7	3.1	11	0.9	8.6	12.8
F. graminearum	76O	7.8	5.2	12	1.5	4.4	11.1
F. graminearum	76O	9.6	5.1	11	1.5	6.2	13.1
F. graminearum	78B	11.5	1.9	10	0.6	10.1	12.9
F. graminearum	78B	8.2	3.5	11	1.1	5.8	10.6
F. graminearum	93D	10.4	3.4	12	1.0	8.2	12.5
F. graminearum	93D	7.8	4.3	11	1.3	4.9	10.7
F. graminearum	99C	11.6	4.0	12	1.1	9.1	14.1
F. graminearum	Ph1	6.3	4.6	12	1.3	3.3	9.2
F. graminearum	Ph1	11.2	2.8	11	0.9	9.3	13.1
F. graminearum	Ph1	8.9	5.2	12	1.5	5.6	12.2
F. graminearum	Ph1	9.1	4.9	12	1.4	6.0	12.2
F. graminearum	Ph1	4.3	3.9	10	1.2	1.5	7.1
F. graminearum	Ph1	8.1	3.6	11	1.1	5.6	10.5
F. poae	110A	0.5	0.0	11	0.0	-	-
F. poae	26A	0.1	0.3	4	0.1	-0.3	0.5
F. poae	27D2	0.1	0.3	12	0.1	-0.1	0.3
F. poae	36A	0.0	0.1	12	0.0	-0.1	0.1
F. poae	90F	0.4	0.2	7	0.1	0.3	0.6
F. poae	90F	0.4	0.2	10	0.1	0.2	0.5
F. sporotrichioides	7F	0.4	0.2	11	0.1	0.3	0.5
F. sporotrichioides	91C	0.5	0.2	12	0.1	0.4	0.6
F. sporotrichioides	91C	0.6	0.2	10	0.1	0.4	0.7
F. subglutinans	83V	0.4	0.3	4	0.1	0.0	0.8
F. subglutinans	C13E2	0.2	0.3	10	0.1	0.0	0.4
F. tricinctum	57A	0.5	0.0	11	0.0	-	-
FIESC	63A	0.4	0.2	6	0.1	0.2	0.6
FIESC	80E	0.1	0.2	11	0.1	0.0	0.3

[a] if multiple experimental runs were performed for that isolate, they were listed in separate lines

APPENDIX C: Comparison between prothioconazole and prothioconazole-desthio

This appendix represents a brief submitted to Plant Health Progress

Abstract

The active ingredient prothioconazole is a demethylase inhibitor (DMI) widely used in numerous commercial fungicide formulations for crop protection. However, prothioconazole converts *in planta* to prothioconazole-desthio, which was found to be the primary active form inhibiting CYP51 activity in fungi. Here, experiments were performed on *F. graminearum* to compare the use of technical grade prothioconazole-desthio, technical grade prothioconazole, and formulated product Proline in poison plate mycelial growth assays to investigate the relevancy of using prothioconazole for sensitivity testing. Prothioconazole-desthio was significantly more efficacious at mycelial growth inhibition than prothioconazole, but mycelial growth and EC_{50} values between them correlated significantly. While care should be taken in interpreting EC_{50} values of prothioconazole, the compound is still suitable for monitoring sensitivity rather than purchasing the more expensive prothioconazole-desthio.

Introduction

The active ingredient prothioconazole is a demethylase inhibitor (DMI) widely used in numerous commercial fungicide formulations for crop protection and is the primary component of the fungicide products Proline and Prosaro. DMI fungicides inhibit the C14-demethylation step of ergosterol biosynthesis, by binding to the cytochrome P450 monoxygenase encoded by CYP51 genes. Prothioconazole is a particularly important compound for the management of Fusarium head blight of wheat and ear mold of corn, as well as reduction of mycotoxin accumulation in these crops (Paul et al. 2010; Limay-Rios and Schaafsma 2018). Prothioconazole is known to rapidly convert *in planta* and in the environment to

prothioconazole-desthio and additional metabolites (Lehoczki-Krsjak et al. 2013; Dong et al. 2019). The product prothioconazole-desthio was found to be the primary active form inhibiting CYP51 activity in fungi, not prothioconazole (Parker et al. 2013). The speed of this breakdown may depend on environmental factors such as weather and cultivar effects. The half-life of prothioconazole has been reported to be between two to ten days in field settings (Lehoczki-Krsjak et al. 2013; Dong et al. 2019).

Pathogen resistance to DMI fungicides is of wide concern due to their frequent use, their importance in human medicine, as well as the known risk of cross-resistance between DMI products. Thus, many researchers engage in monitoring of *in vitro* sensitivity of fungal species including the plant pathogen *F. graminearum*, the main causal agent of Fusarium head scab in wheat and Fusarium ear mold in corn. Use of technical grade prothioconazole-desthio would allow testing of the exact amount of the active form, but it is costly to procure in significant amounts for large scale monitoring. To investigate the relevancy of using prothioconazole for *in vitro* sensitivity testing, two experiments were performed to compare the use of technical grade prothioconazole-desthio to the same concentration of technical grade prothioconazole.

Mycelial growth assays

Mycelial growth assays first compared growth of 46 isolates on media amended with 1 ug/mL of Proline 480 SC (41% prothioconazole, Bayer Crop Science), technical grade prothioconazole (Sigma-Aldrich, St. Louis, MO, USA), and technical grade prothioconazole-desthio (Sigma-Aldrich, St. Louis, MO, USA). These isolates were collected from 38 fields across Michigan from diverse hosts and time periods. EC_{50} values (effective concentration to reduce growth by 50%) were determined for seven of the isolates to directly compare technical grade prothioconazole and prothioconazole-desthio. Media was prepared and assays performed

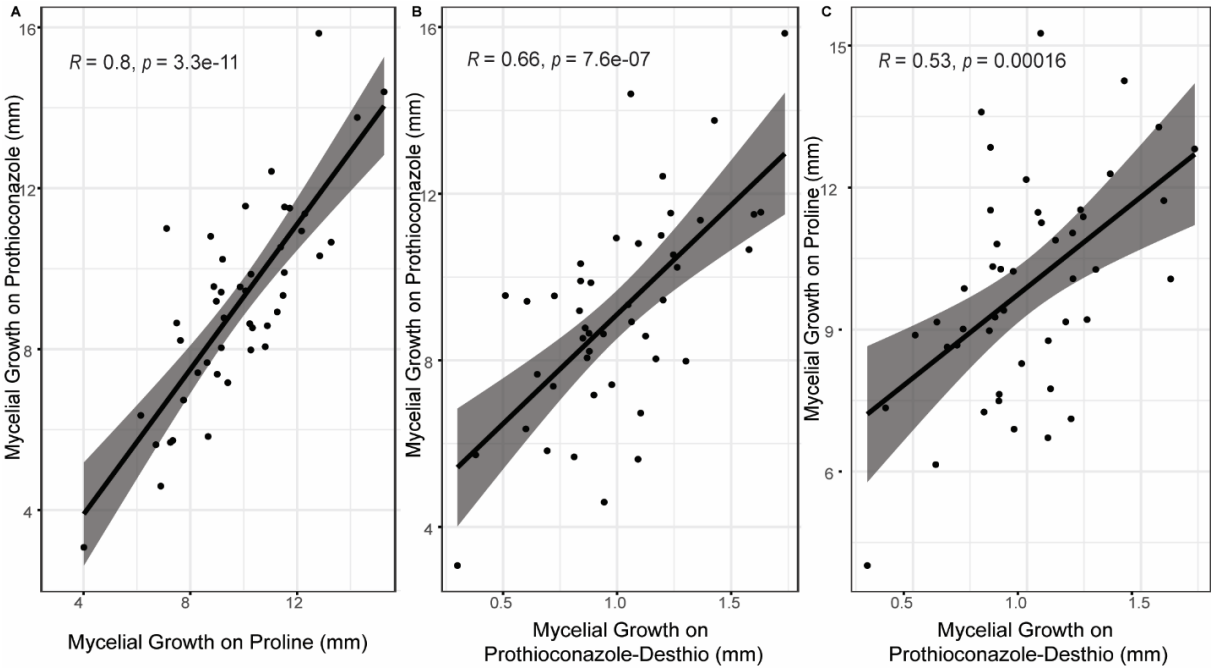
in the same manner as described in Breunig and Chilvers (2021). Briefly, ½ strength Potato Dextrose Agar was used (PDA; Acumedia, Neogen, Lansing, MI) and six doses (0.003, 0.03, 0.3, 1, 3, or 30 µg/mL final concentration in assay plates) were tested. Technical grade active ingredients were dissolved in methanol and a methanol control was included. Plates were inoculated with *F. graminearum* colonized 5mm agar plugs and assay plates were incubated in the dark at 24°C for 5 days, then measured in two perpendicular directions with digital calipers. Products were tested simultaneously, and the experiment was run three separate times. Analysis was conducted in R, and function 'drm' in package 'drc' was used to fit the dose response model and determine EC_{50} estimations.

Correlation of mycelial growth

When *F. graminearum* mycelial growth was compared at 1µg/mL prothioconazole-desthio was significantly more efficacious than technical grade prothioconazole ($P < 0.0001$) and formulated Proline 480 SC ($P < 0.0001$). Isolates grew on average 0.2mm per day when exposed to prothioconazole-desthio, but 1.8 and 1.9 mm per day when exposed to prothioconazole or Proline 420 EC, respectively. Pearson correlation revealed significant correlation between the two technical products (rho=0.661, $P < 0.0001$). The highest correlation was seen between technical grade prothioconazole and formulated Proline (rho=0.80266, $P < 0.0001$). Proline and prothioconazole-desthio also significantly correlated but the relationship was not as strong (rho=0.5278, $P = 0.0001$) (Figure 1).

A set of seven *F. graminearum* isolates were tested with additional fungicide doses to determine EC_{50} values to directly compare technical grade prothioconazole and prothioconazole-desthio (Table 1). These seven isolates were chosen to represent a range of EC_{50} values from previous experiments. EC_{50} values for prothioconazole were significantly greater, with a mean

difference of 0.226 µg/mL (paired t-test, P = 0.013). EC_{50} values did correlate but the relationship was only marginally significant (spearman's rank correlation, rho=0.7142, P = 0.088). Taken together with the data from additional isolates tested at 1 µg/mL, it can be assumed trends in sensitivity could certainly be detected using technical grade prothioconazole and formulated product (Proline), but results may not perfectly align with prothioconazole-desthio.



Supplementary Figure 2.4 Scatter plots depicting the relationship between mycelial growth of 46 *F. graminearum* isolates tested on Petri-plates amended with 1ug/ul of active ingredient A) Proline and Prothioconazole B) prothioconazole and prothioconazole-desthio and C) Proline and prothioconazole-desthio. All chemistries were tested simultaneously in three runs, with the average of those replications presented. Pearsons correlation statistics displayed, and linear regression line in black with 95% confidence interval shaded in grey.

Table A2.4 EC_{50} estimations of seven *F. graminearum* isolates determined by poison plate mycelial growth assays

Isolate	Prothioconazole EC_{50} (µg/mL)		Prothioconazole-desthio EC_{50} (µg/mL)	
	Estimate	SE	Estimate	SE
21A	0.15	0.09	0.03	0.02
24A	0.21	0.11	0.04	0.01
27A	0.40	0.19	0.06	0.02
57G	0.12	0.08	0.05	0.02
73S	0.16	0.10	0.05	0.01
89B	0.27	0.15	0.06	0.02
Kmiso2217	0.69	0.24	0.12	0.06

Relevancy of prothioconazole use *in vitro* sensitivity assays

Ultimately, care should be taken when interpreting and comparing EC_{50} values from prothioconazole to other DMI chemistries, as in this study EC_{50} values of prothioconazole are 5-fold greater than the active form prothioconazole-desthio. In terms of relative sensitivity, here we show that sensitivity determined from prothioconazole and prothioconazole-desthio does correlate. Prothioconazole is most likely readily converted in petri-plates into the active prothioconazole-desthio, as significant reduction in growth was seen in these *in vitro* assays. However, this conversion is likely highly environmentally driven and could introduce additional variability into poison plate assays. For this reason, prothioconazole may not be suitable for investigate of small differences in sensitivity or work investigating resistance mechanisms. However, the results here demonstrate technical grade prothioconazole or formulated Proline would still be suitable for identifying large differences or shifts in sensitivity.

Chapter 3 : Baseline Sensitivity of *Fusarium graminearum* from Wheat, Corn, Dry Bean and Soybean to Pydiflumetofen in Michigan, USA

Source:

Breunig, M., and Chilvers, M. I. 2021. Baseline sensitivity of *Fusarium graminearum* from wheat, corn, dry bean and soybean to pydiflumetofen in Michigan, USA. Crop Prot. 140 Available at: https://doi.org/10.1016/j.cropro.2020.105419.

Chapter 4 **Effects of Fungicide, Nitrogen, and Growth Regulator on Fusarium Head Blight, Foliar Disease, and Yield in Michigan Wheat**

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Abstract

Michigan growers are increasingly interested in intensive management of winter wheat (*Triticum aestivum* L.) to increase yield, quality, and profitability- utilizing increased nitrogen rates, fungicide applications, and growth regulators. However, these inputs come with agronomic risks and increased costs. A trial was established in East Lansing, MI (2015-2018) on soft white winter wheat cultivar 'Ambassador' to investigate the risks, benefits, and interactions of these inputs. Two nitrogen levels, "base" (100.88 kg ha^{-1} in early spring) and "high" (additional 56.04 kg ha^{-1} at Feekes 6), were combined in a factorial manner with a fungicide at Feekes 6, fungicide at anthesis (Feekes 10.5.1), or combination of both. High nitrogen treatments were also replicated with the plant growth regulator Palisade EC at Feekes 6. Presence and magnitude of response varied across years, as did disease pressure. There was no additional grain yield increase from the higher nitrogen rate. In some years, the high nitrogen treatments had significantly higher fungal disease and lower yield (by as much as 511.1 kg ha^{-1}) compared to base treatments. High nitrogen rates also increased lodging significantly, in turn increasing Fusarium head blight incidence and foliar disease. A fungicide at anthesis significantly increased yield in two years, by 802.98 kg ha^{-1} and 359.79 kg ha^{-1} in 2015 and 2016, respectively, and consistently reduced foliar disease and Fusarium head blight. This study also found no significant fungicide x nitrogen interaction resulting in yield gain as previously reported. While these inputs may have value, here we demonstrate the risks as well.

Introduction

In 2020, Michigan wheat yields averaged 5044 kg ha^{-1} compared to the national average of 3423 kg ha^{-1}(Crop Production 2020 Summary 2021). Considering this high yield potential, more intensive management regimes are becoming increasingly common in Michigan.

Additionally, receiving price premiums or prices dockages due to the level of grain quality can also incentivize growers to use increased inputs. Some of these practices include prophylactic pesticide applications, nitrogen rates greater than university recommendations, and use of plant growth regulators. While these inputs have benefits, they also come with risks, as well as increased cost of production.

Increased nitrogen rates can lead to increases in grain, forage, and straw yields in wheat (May et al. 2014; Cox et al. 1987; Kanampiu et al. 1997), but have diminishing returns as rates increase (Knott et al. 2016; Brinkman et al. 2014), and the effect on yield and physiology can be specific to the cultivar, site, and year (Kelley 1993; Howard et al. 1994; Quinn and Steinke 2019; Knott et al. 2016). Some studies have even reported a significant decrease in yield from increased rates of nitrogen (Kelley 1993; Brinkman et al. 2014; Roth et al. 1984). Nitrogen impacts both plants and plant pathogens in multiple ways that can impact disease outcomes: leaf and canopy size, root length and activity, concentration of nitrogen in leaf tissue, and ability of plants to produce secondary metabolites and defense compounds (Walters and Bingham 2007). Nitrogen has been demonstrated to increase disease in a variety of pathosystems (Veresoglou et al. 2013), including Fusarium Head Blight (FHB) in wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) (Lemmens et al. 2004; Heier et al. 2005; Subedi et al. 2007; Krnjaja et al. 2015). However, this effect can be inconsistent across sites and years, and some studies observing no effect on disease from nitrogen applications at all (Yoshida et al. 2008; Hofer et al. 2016). Multiple studies also demonstrate increasing foliar disease with increased nitrogen rates in wheat (Howard et al. 1994; Brinkman et al. 2014; Cox et al. 1987; Mascagni et al. 1997; Simón et al. 2003; Tamburic-Ilincic, Brinkman, et al. 2015). This effect could be attributed to changes in plant architecture and biomass, which can affect air speed, temperature, and humidity

within the canopy (Tompkins et al. 1993; Hofer et al. 2016; Neumann et al. 2004). Additionally, nitrogen levels in plant tissue can also correlate with disease in wheat, particularly in rust pathogens (*Puccinia* spp.) as nitrogen is also required for fungal growth and spore production (Neumann et al. 2004; Robert et al. 2002; Olesen et al. 2003).

Excessive nitrogen rates can increase plant height and reduce stem strength, increasing the risk for lodging (Zhang et al. 2017; Crook and Ennos 1995). Lodging has been shown to reduce straw yield, grain yield, test weight, and decrease grain quality. Plant growth regulators reduce plant height and prevent lodging and reduce risk from high nitrogen rates in wheat (Crook and Ennos 1995). The plant growth regulator trinexapac-ethyl is registered as Palisade EC (Syngenta Crop Protection, Switzerland) in the United States for wheat and can decrease plant height, reduce leaf area, increase stem diameter, increase number of grains per spike, and increase straw strength (Espindula et al. 2009; Knott et al. 2016; Wiersma et al. 2011; Matysiak 2006). Trinexapac-ethyl is an acylcyclohexanedione which inhibit gibberellin production by structurally mimicking 2-Oxoglutaric Acid, an important co-substrate involved in the late stages of gibberellic acid biosynthesis (Rademacher 2000). Plant growth regulators seem to have an inconsistent impact on height and yield over different site-years (Quinn and Steinke 2019; Knott et al. 2016; Matysiak 2006), and may only show an economic benefit in years when lodging occurs (Swoish and Steinke 2017). While not intended to affect disease, it is possible growth regulators may indirectly reduce disease as seen in other pathosystems utilizing different PGR compounds perhaps due to modulation of plant physiology or effect on fungal gibberellins (Inguagiato et al. 2009; Golembiewski and Danneberger 1998; Spinelli et al. 2010; Bini et al. 2008; Martin et al. 1991).

Fungicides are a key management tool in wheat, specifically for FHB control as there are few cultivars with full resistance to this disease. FHB is a major risk for growers; severe epidemics have demonstrated yield reductions up to 50% (Windels 2000) and the pathogen produces mycotoxins, mainly deoxynivalenol (DON). Presence of these toxins can result in sale price reductions, or even unmarketable grain. Meta-analysis of multi-state winter wheat trials shows products applied at anthesis can reduce FHB by 40 to 50% while providing protection from foliar diseases and average yield increases of 330 kg ha^{-1}(Paul et al. 2008). Foliar diseases are omnipresent in Michigan, but there is considerable variation in severity and yield losses each year. Fungicide applications are demonstrated to protect leaf area, increasing grain dry matter coming from retention of green leaf area during grain fill (Gooding et al. 2005; Dimmock and Gooding 2002). However, in seasons with low disease severity, there is not always a demonstrated yield response from fungicide applications (Cox et al. 1987; Quinn and Steinke 2019; Weisz et al. 2011; Stephen N. Wegulo et al. 2011). Cultivars can also vary in their disease resistance and fungicide response (Loyce et al. 2008; Byamukama et al. 2019).

Timing is a very important factor in determining the yield response to fungicide applications. Besides application at anthesis, fungicides are sometimes applied early in the season, between Feekes 5 and Feekes 8. However, these applications are well before full flag leaf emergence and would not have lasting protection into grain fill (Brinkman et al. 2014; Willyerd et al. 2015). Early fungicide applications are often made in combination with an anthesis application, but may not impact yield (Sylvester et al. 2018).

Synergism between fungicide applications and nitrogen may occur in which a greater yield increase is achieved from fungicides when higher nitrogen rates are applied. However, this interaction between fungicides and nitrogen is not consistent across site years (Kelley 1993;

Salgado et al. 2017; Quinn and Steinke 2019; Ishikawa et al. 2012; Brinkman et al. 2014), and some studies report no synergistic interaction (May et al. 2014; Mascagni et al. 1997). This synergism is often cited as a reason to increase nitrogen rates and fungicide usage, but this may only result in a reduction of profitability and sustainability, if this synergism does not occur.

Although nitrogen, fungicides, and a plant growth regulators have been investigated across wheat growing areas in the United States, here we aim to specifically evaluate the effects of these inputs alone and in combination on disease and yield in Michigan. Ultimately, this study will inform better disease management and agronomic strategies.

Materials and Methods

Trial design and field details

Field trials were conducted at the Michigan State University Plant Pathology Farm in East Lansing, MI (42°69'14.12" N lat., -84°48'57.89" W long). Soft white winter wheat (cultivar 'Ambassador', D.F. seeds, Inc.) was planted with 19.1 cm row spacing at a rate of 4.4 million seeds per ha^{-1}. Plots were 2.1 m wide by 6.1 m long, with five to eight replications in a randomized complete block design (number in each year listed in Table A4.1). Ambassador is cultivar susceptible to Fusarium head blight as well as many foliar diseases, with moderate height and high yield potential. The previous crop for all trials was soybeans (*Glycine max* L.). Fields were non-irrigated and conventionally tilled prior to planting and contained subsurface drainage. The soil was a Capac loam.

Two nitrogen fertility levels "base", 100.88 kg ha^{-1} in early spring (Feekes 2 to 3), and "high", with an additional 56.04 kg ha^{-1} at Feekes 6, were combined in a factorial manner with an early fungicide at Feekes 6, fungicide at anthesis (Feekes 10.5.1), or combination of both applications. High nitrogen treatments were also replicated with a Feekes 6 application of the

plant growth regulator Palisade EC. This resulted in 12 treatment combinations, listed in Table 4.1. Exact dates of planting, treatment applications, and harvest are reported in Table A4.1.

Table 4.1.Trial factors and their combinations, resulting in the 12 different treatments to examine the role of nitrogen fertility, fungicide and plant growth regulator application on plant disease, agronomic and yield parameters in winter wheat.

Treatment	Nitrogen regime	Fungicide regime[b]		Growth regulator (Palisade EC)[c] at Feekes 6
		Stratego YLD Feekes 6	Prosaro 421 SC Feekes 10.5.1	
1	**Base:** 100.88 kg ha^{-1} of nitrogen at in early spring	-	-	-
2		+	-	-
3		-	+	-
4		+	+	-
5	**High:** 100.88 kg ha^{-1} of nitrogen at in early spring and additional 56.04 kg ha^{-1} at Feekes 6 [a]	-	-	-
6		+	-	-
7		-	+	-
8		+	+	-
9		-	-	+
10		+	-	+
11		-	+	+
12		+	+	+

[a] With the exception of 2016, where only 25.76 kg ha^{-1} of additional nitrogen was applied due to error.
[b]Stratego YLD (10.8% prothioconazole + 32.3% trifloxystrobin) was applied at 0.292 L ha^{-1} at Feekes 6. Prosaro 421 SC (19% tebuconazole + 19% prothioconazole) was applied at 0.475 L ha^{-1} at Feekes 10.5.1.
[c]Palisade EC (12% trinexepac-ethyl) was applied at 0.803 L ha^{-1} at Feekes 6.

Treatment applications

In early spring (Feekes 2-3) 100.9 kg ha^{-1} of nitrogen was broadcast applied as urea (46-0-0). An additional 56.04 kg ha^{-1} of nitrogen for treatments 5 to 12 was applied as urea with hand spreaders at Feekes 6 (jointing). However, in 2016, only an additional 25.8 kg ha^{-1} was applied due to calculation error discovered later in the year. Early fungicide applications of Stratego YLD (10.8% prothioconazole + 32.3% trifloxystrobin; Bayer Crop Science, St. Louis, MO) were applied at Feekes 6 at a rate of 292 mL ha^{-1}. Plant growth regulator product Palisade EC (12%

Trinexepac-ethyl; Syngenta Crop Protection, Switzerland) was also applied at Feekes 6 at 803 mL ha^{-1}. Fungicide applications of Prosaro 421 EC (19% tebuconazole + 19% prothioconazole; Bayer Crop Science, St. Louis, MO) were applied at anthesis (Feekes 10.5.1) at a rate of 475 mL ha^{-1}. Fungicides were applied with a hand-held spray boom consisting of four nozzles spaced 50.8 cm. apart, pressurized with CO2 at 40 psi, and calibrated to apply 140.3 L ha^{-1}. Flat fan nozzles (XR TeeJet 8001VS) were used for Feekes 6 foliar applications and forward and rearward dual spray nozzles (Teejet D6TJ-60 110015VS, Teejet Technologies, Wheaton, IL) were used for the Feekes 10.5.1 treatments. The adjuvant Induce (Non-ionic surfactant; Helena Chemical Company, Collierville, TN) at 0.125% v/v was applied with both fungicide applications and Palisade EC.

Data collection

Foliar diseases were visually assessed as plot-wide estimates of the percentage of flag leaf area impacted by lesions, which were collected 3 weeks post anthesis. Fusarium head blight was rated approximately 21 days after anthesis, as plants began to lose green coloration, by evaluating 100 wheat heads (50 heads from two randomly selected portions of the plot) and counting the incidence of effected spikes. FHB disease severity was a visual estimate of the proportion of the head discolored. Lodging ratings were plot-wide visual estimates of the percentage of plot affected. If a plot had significant lodging, 100 heads from each of the non-lodged and lodged portions were rated and recorded separately. In analysis of lodged portions in 2018, only plots with lodging were included in mean calculations and hypothesis testing of treatment effect.

Height was assessed by measuring three plants from three separate portions of each plot after anthesis. Grain yield was determined by harvesting the center rows of each plot, using a

small plot combine, and adjusting plot weights to 13% moisture. Grain subsamples were collected and sent to the U.S. Wheat and Barley Scab Initiative mycotoxin testing laboratory (Dr. Yanhong Dong, University of Minnesota, St. Paul, MN) for deoxynivalenol analysis. From grain subsamples 100 or 200 kernels were counted to determine the percentage of *Fusarium* damaged kernels (FDK) and record kernel weight. In years when 200 kernels were evaluated, weights and FDKs were divide by two in order to normalize all data to 100 kernels for comparison.

Data analysis

Each year was analyzed and presented separately, as there was a significant year x treatment interaction for most response variables. Analysis was performed in SAS (version 9.4, SAS institute, Cary, NC) using PROC Mixed. Since the PGR application was only tested against one level of nitrogen, a traditional factorial analysis could not be conducted. Rather, mixed model with treatment as a single fixed effect with 12 levels, and block as a random factor was fit, and contrast testing was used to test estimate and test for differences in marginal mean. Only high N without PGR treatments were used to compare "high" versus "base" nitrogen. Significance testing of interactions was completed by simultaneously testing a set of three contrasts. All P-values presented in text are results of contrast tests, unless otherwise specified. In all analyses, block was treated as a random factor, with treatment as a fixed effect. All code for contrast testing s is publicly available on a GitHub repository at https://github.com/mikbreunig/NxFxPGR.git.

Results

Yield response to nitrogen, fungicide, and PGR

While various factors had a significant effect in different years, none of the interactions between factors were significant. In 2015 and 2017, nitrogen level did have a significant effect on yield, but base nitrogen treatments had significantly greater yields. High nitrogen treatments reduced yield by 507.74 kg ha^{-1} ($P < 0.001$) in 2015 and 513.98 kg ha^{-1} in 2017 ($P = 0.032$). PGR application resulted in a numeric increase in yield each year, ranging from 15.6 kg ha^{-1} to 280.5 kg ha^{-1}, with an average of 191.67 kg ha^{-1}, however, this was not a statistically significant regardless of year (Table 4.2).

Fungicide applications significantly increased yields in 2015 and 2016 but had no significant effect on yield in 2017 or 2018. Mean yield of all treatment combinations for each year can be found in Table A4.2. In 2015, Feekes 10.5.1 applications contributed most to yield, when applied alone significantly increasing yield by 802.98 kg ha^{-1} ($P < 0.001$). A Feekes 6 application alone, nor added to the Feekes 10.5.1, did not significantly impact yield in three out of four years (Figure 4.1). However, in 2016, the early application did significantly increase yield by 359.79 kg ha^{-1} ($P=0.015$). It provided less value when added to the Feekes 10.5.1 application, with only a 243.44 kg ha^{-1} difference, which was not a statistically significant increase ($P=0.097$). The Feekes 10.5.1 treatment alone provided a 359.79 kg ha^{-1} increase ($P=0.016$), and the application of both resulted in 604.24 kg ha^{-1} increase ($P < 0.001$), compared to the non-fungicide treated plots (Fig 1).

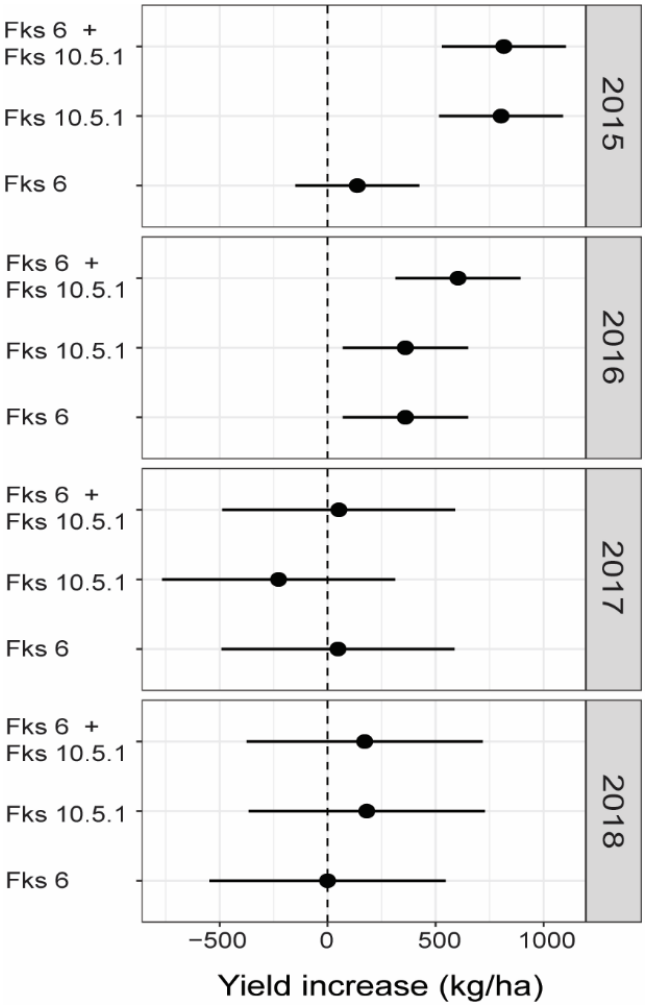


Figure 4.1 Contrast estimates of winter wheat yield difference (kg ha^{-1}) between plots receiving no fungicide and the specified fungicide treatment in East Lansing, MI. Each regime, as well as non-treated plots, had at least five replications each year. Dots represent the mean, with lines representing standard error of the estimate.

Table 4.2 Estimated winter wheat grain yield of base nitrogen, high nitrogen, and high nitrogen with PGR treatments from four years of trials in East Lansing, MI.

Nitrogen Level[z]	Mean Yield (kg ha^{-1})							
	2015		2016		2017		2018	
base	6,858	A[y]	4,461	ns	4,844	A	6,159	ns
high	6,350	B	4,521	ns	4,240	B	5,944	ns
high with PGR	6,559	B	4,537	ns	4,594	AB	6,224	ns

[y] Means sharing a letter are not significantly different (α=0.05), as determined by contrast testing between nitrogen levels within each year

[z]Nitrogen treatment base: 100.88 kb/ha, high: 56.04 kg ha^{-1}, or high nitrogen with addition of PGR treatment at Feekes 6.

Increase in kernel weight from nitrogen and fungicide

Kernel weight response to inputs varied based on year, with mean weights of each treatment combination reported in Table A4.2. In 2015, an estimated 0.21 g increase resulted from PGR application (P = 0.04), however no significant response from PGR was observed in the other three years. Nitrogen resulted in significant differences in 2015 and 2018. In two years, base nitrogen treatments had greater kernel weights than high nitrogen treatments, by 0.31 g in 2015 (P=0.004) and 0.259 g in 2018 (P=0.068). The fungicide at anthesis did result in an estimated 0.22 g increase (P= 0.011) in 2015, and in 2016, a 0.16 g increase (P<0.001). In 2016, the Feekes 6 application alone also resulted in a significant increase of 0.11 g (P = 0.035), compared to the non-fungicide treated plots. Added to the Feekes 10.5.1, the Feekes 6 application resulted in a 0.11 g (P = 0.026) increase. However, a benefit from the Feekes 6 application was not observed in any other year. The double application in 2016 resulted in 0.27 g gain (P < 0.001). In 2017 and 2018, no significant differences were detected from any factors.

Foliar fungal pathogens response

Foliar diseases were present each year but varied in the predominate diseases and their severities. In 2015, there was significant Stagonospora nodorum blotch (caused by *Parastagonospora nodorum*) and Septoria tritici blotch (caused by *Zymoseptoria tritici*). In 2016, there was a stripe rust epidemic (caused by *Puccinia striiformis* f. sp. *tritici*), which started unusually early for the region and was the predominate foliar disease. In 2017, there was powdery mildew early in the season (caused by *Blumeria graminis* f. sp. *tritici*), and stripe rust appeared at low levels around anthesis. In 2018, there was severe leaf disease, with Septoria tritici blotch predominating, and low levels of stripe rust and powdery mildew.

Nitrogen and fungicide had a significant effect on final foliar disease severity in 2015, and also significantly interacted with eachother ($P < 0.001$). While nitrogen increased disease significantly ($P = 0.009$), the effect was largest in the treatments without fungicide applications (Table 4.3). Disease pressure was moderated by fungicide applications, in both high and base nitrogen treatments. The results were similar in 2016 where, in the absence of fungicides, disease was greater in high nitrogen plots compared to base nitrogen plots. However, neither the effects of nitrogen nor the nitrogen x fungicide interaction was statistically significant factors that year. In 2018, nitrogen also significantly increased foliar disease ($P = 0.001$), but the nitrogen x fungicide interaction was not significant ($P = 0.536$). Nitrogen resulted in a range of 3.7 to 12.5% increase in foliar disease across the fungicide programs (Table 4.3).

In all study years, fungicides significantly decreased foliar disease, with applications at anthesis the most effective. Aside from 2016, the double application at both Feekes 6 and Feekes 10.5.1 did not provide additional benefit compared to a single application at Feekes 10.5.1, apart from 2016. In 2016, the Feekes 6 application added to the Feekes 10.5.1 application resulted in

an additional 13% reduction to end of season disease severity (P=0.005). While the early Feekes 6 alone reduced final foliar disease in all four years, the early timing provided much less benefit compared to the Feekes 10.5.1 alone (Table 4.3).

PGR application significantly reduced foliar disease in 2015 by 6.5% (P=0.002). However, looking at individual treatment means, this difference was only of practical significance in the plots without fungicide applications, where disease was reduced by 17%. In plots receiving fungicides, the effect was much smaller or not present, which would explain the significant fungicide by PGR interaction in 2015 (P=0.029). These affects were not seen in the other three study years, where there was no significant difference from PGR application, nor significant interactions.

Table 4.3 Visually estimated percentage of flag leaf with foliar lesions, at final rating (approximately 3 weeks post anthesis) from trials conducted from 2015 to 2018 in East Lansing, MI on 'Ambassador' soft white wheat, a susceptible variety.

Year	Fungicide Regime[x]	Nitrogen Treatment[y]		
		Base N	High N	High N + PGR
2015	Non-treated	51.0 c[z]	73.0 a	56.0 bc
	Feekes 6	60.0 b	52.0 c	51.0 c
	Feekes 10.5.1	6.6 d	8.8 d	4.6 d
	Feekes 6 + Feekes 10.5.1	3.8 d	9.0 d	5.4 d
2016	Non-treated	72.2 ab	78.8 a	80.0 a
	Feekes 6	70.3 ab	65.3 abc	60.8 bc
	Feekes 10.5.1	52.5 dc	37.8 ed	51.7 cd
	Feekes 6 + Feekes 10.5.1	42.2 ed	30.0 e	30.2 e
2017	Non-treated	31.0 ab	35.6 a	32.8 a
	Feekes 6	28.4 abc	17.8 cde	19.8 bcd
	Feekes 10.5.1	8.3 de	7.9 e	7.1 e
	Feekes 6 + Feekes 10.5.1	6.0 e	6.4 e	6.9 e
2018	Non-treated	32.5 ab	39.2 a	40.0 a
	Feekes 6	19.2 c	31.7 ab	28.3 b
	Feekes 10.5.1	5.8 d	12.2 cd	11.8 cd
	Feekes 6 + Feekes 10.5.1	3.8 d	7.5 d	6.8 d

[x]Fungicide regime: Non-treated plots receiving no fungicide applications, Stratego YLD (292 ml/ha) at Feekes 6, Prosaro 421 SC (475 ml/ha) at Feekes 10.5.1, or combination of both applications.

[y]Nitrogen treatment base: 100.88 kb/ha, high: 56.04 kg ha^{-1}, or high nitrogen with addition of PGR treatment at Feekes 6.

[z]lsmeans estimates, treatments sharing the same letters signifies they are not significantly different, comparison made within a year across all treatments (α=0.05)

Impact of nitrogen, fungicides, and PGR on Fusarium head blight

Fusarium head blight was only prevalent at significant levels in 2015 and 2018. In 2016 and 2017, there was negligible visual evidence of FHB in the field. Grain samples from harvest were still tested for DON in those years, but levels were low (0.1 ppm or less) and FDK at 1% or less. In 2016 and 2017 treatments did not have a significant effect on DON concentration ($P = 0.576$ in 2016, and $P = 0.125$ in 2017) nor on FDK ($P = 0.329$ in 2016, $P= 0.082$ in 2017). In both 2015 and 2018, when there was significant disease pressure, there were no significant interactions between any factors for DON, FDK, visual disease severity, or incidence. PGR only had a significant effect on FHB in 2015, resulting in a 5% reduction of FDK, compared to the high nitrogen treatments that did not receive PGR ($P = 0.003$). However, no other FHB parameters besides FDK were significantly impacted by the PGR treatment, nor was any affect observed in 2018 (Table 4.3).

In 2018, nitrogen level had no significant effects on FHB. In 2015, however, DON was significantly greater in the high nitrogen treatments, by 1.5 ppm ($P < 0.001$). There was also a 5.9% increase ($P = 0.014$) in disease incidence and 5.1% increase in FDK observed ($P = 0.006$). There were no differences in severity observed between nitrogen treatments (Table 4.4). In 2018, there did not seem to be an affect from nitrogen, with no significant difference seen except for a small (1.3%) increase in FDK in the base nitrogen treatment (Table 4.4). While lodging did impact FHB incidence scores in 2018, nitrogen did not seem to have a significant effect on FHB in non-lodged portions of plots (Table 4.4). Fungicide did have a significant effect on FHB, as expected. The anthesis fungicide application, alone or in combination, reduced DON by at least 1 ppm in 2015, and by over 2 ppm in 2018. An early fungicide alone, did not affect FHB, except for variation in DON levels. In 2015, the Feekes 6 application increased DON by 1.96 ppm ($P <$

0.001), compared to non-fungicide treated plots, but in 2018, the same treatment only slightly lowered DON concentrations (Table 4.4).

Table 4.4 Estimates of Fusarium Head Blight parameters (FDK, Disease Severity, Disease incidence, DON) across the two factors of Nitrogen and Fungicide regime, across two trial years when FHB levels were significant (2015 and 2018).

Year	Factor	Level	Fusarium Damaged Kernels (%)	FHB Severity (%) (non-lodged / lodged)[c]	FHB Incidence (%) (non-lodged / lodged)[c]	DON[c] (ppm)
2015	Nitrogen[a]	High	15.4	29.8	10.2	6.1
		High + PGR	9.7***	28.5	9.3	5.7
		Base	10.3***	35.3	4.3**	4.6***
	Fungicide[b]	Non-treated	13.9	42.6	9.2	5.6
		Feekes 6	13.5	38.0	11.7	7.5***
		Feekes 10.5.1	10.2*	20.3***	5.8	4.3***
		Feekes 6 + Feeke 10.5.1	9.6*	23.9***	5.0	4.5***
2018					**(non-lodged / lodged)[c]**	
	Nitrogen[a]	High	3.3	39.8 / 39.5	19.1 / 55.3	2.7
		High + PGR	3.2	40.0 / 39.0	18.6 / 49.8	2.7
		Base	4.6*	37.2 / NA[c]	20.2 / NA	2.8
	Fungicide[b]	Non-treated	5.1	46.5 / 53.5	23.0 / 61.0	4.1
		Feekes 6	4.2	52.1 / 59.1	23.2 / 62.8	3.7**
		Feekes 10.5.1	2.8***	28.1*** / 23.2***	15.3***/ 42.5***	1.5***
		Feekes 6 + Feeke 10.5.1	2.7***	29.7*** / 21.2***	15.5***/ 44.1*	1.7***

*Significance level of contrast test comparing each level within factors, comparing N levels to the high nitrogen treatment, and fungicide regimes to the non-fungicide treated plots (*P<0.1, **P<0.01, ***P<0.001).

[a] levels include Stratego YLD (292 ml/ha) at Feekes 6, Prosaro 421 SC (475 ml/ha) at Feekes 10.5.1, or combination of both.

[b] Nitrogen treatment considered the base or "base" treatment of 100.88 kb/ha, or "high" of 100.88 kb/ha with an additional 56.04 kg ha^{-1} at Feekes 6. "High + PGR" received the high nitrogen treatment and application of Palisade EC (0.803L ha^{-1}) at Feekes 6.

[c]In 2018 only, data was collected separately on non-lodged and lodged portions of the same plot. No lodging occurred in base nitrogen plots.

[d]deoxynivalenol concentration.

Impact of nitrogen and PGR on lodging

There were no significant interactions between factors, nor any significant effect from fungicide applications on the amount of lodging, in any year. High nitrogen treatments had significantly greater lodging at the end of the season, with a mean increase of 38% in 2015 ($P < 0.001$) and 13% in 2018 ($P = 0.010$). In 2017, there was less lodging overall (mean 13%) and no significant differences between treatments. In 2016, there was no measurable lodging. End of season lodging values for all treatment combinations in each year are listed in Table A4.2. In 2018, significant lodging occurred at anthesis on 31 May associated with high winds (up to 30.5 kph). In total, 29 plots had measurable lodging from that event, all of which had received the high nitrogen rate; there was no lodging found in the base nitrogen plots. PGR did not seem to prevent this lodging, as 13 of the lodged plots received PGR and 16 had not. However, PGR application significantly reduced the amount of lodging by 9.3% the time of lodging ($P = 0.040$). At the end of the season after additional lodging occurred, the difference in lodging scores in plots with and without PGR diminished to 6.6% ($P = 0.202$). With minimal lodging in 2017, there was no significant benefit to the use of PGR with only a 3% difference ($P = 0.405$). In 2015, PGR did have a more significant effect, reducing the amount of lodging by 18% ($P = 0.044$). However, lodging was still significantly higher in the PGR treated high nitrogen plots than the base nitrogen plots with mean lodging scores at harvest of 36 % and 15 % ($P = 0.027$), respectively.

Relationships between lodging and disease

In 2018, there was an opportunity to measure the effect of lodging on disease development, as lodging occurred earlier in the season at anthesis. Disease ratings were taken in both lodged and non-lodged portions of the same plots. Incidence of FHB was significantly

increased in the lodged portion of plots compared to the non-lodged, with a mean difference 33.5% (paired t-test, $P < 0.001$). There was not a significant influence by treatment on the magnitude of difference in FHB incidence between lodged and non-lodged portions of the plot ($P = 0.133$). However, the magnitude of difference in FHB severity did significantly vary with treatment ($P = 0.001$), as illustrated in Figure 4.2. In plots that received Prosaro 421 EC at anthesis, non-lodged portions had increased disease severity compared to the lodged portions. Whereas plots without Prosaro 421 EC had increased severity in the lodged portions as expected.

Foliar disease was also significantly impacted by lodging. Lodged portions of plots had on average 27.3% higher disease ratings of the flag leaves at the end of the season ($P <0.001$). Foliar disease correlated better with the early season lodging rating ($rho=0.371$, $P = 0.002$), rather than the final rating of lodging at harvest ($rho= 0.101$, $P = 0.400$). This effect could not be tested in 2015, as ratings were only assessed on a whole plot basis.

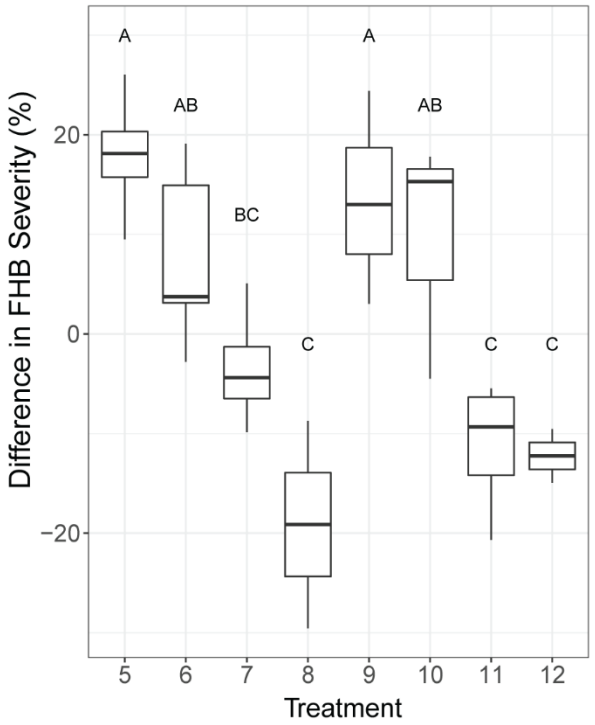


Figure 4.2 Difference in Fusarium head blight (FHB) severity between lodged portion and non-lodged portions of the same plot, in 2018, when severe lodging took place early in the season at anthesis. No lodging occurred in base nitrogen treatments 1-4, so only high nitrogen treatments 5-12 are illustrated which received an additional 56.04 kg ha^{-1}. Treatments sharing letters denotes they are not significantly different ($\alpha = 0.05$). Treatment 5 = no additional input; Treatment 6 = fungicide at Feekes 6; Treatment 7 = fungicide at Feekes 10.5.1; Treatment 8 = fungicide at Feekes 6 and 10.5.1; Treatment 9 = growth regulator; Treatment 10 = fungicide at Feekes 6 and growth regulator; Treatment 11 = fungicide at Feekes 10.5.1 and growth regulator; Treatment 12 = fungicide at Feekes 6 and 10.5.1 and growth regulator.

Plant height response to nitrogen, fungicides, and PGR

Plant height was only measured in 2017 and 2018, and treatments only had a significant effect in 2017. There was a marginally significant nitrogen and fungicide interaction (P = 0.057), and no interaction between PGR and fungicide was found (P = 0.381). Contrasts test between high nitrogen treatments with and without PGR did not reveal a significant effect on height in 2017 (P = 0.164) nor in 2018 (P = 0.188), but some treatments with PGR did trend lower than their non-treated comparison. There were significant differences between fungicide and nitrogen levels, but no clear trends (Table A4.3).

Grain test weight increase from PGR and nitrogen

In all years PGR numerically increased test weight, however only in 2015 and 2017 was it a statistically significant difference when there was a 12.16 kg/m^3 and 5.74 kg/m^3 increase respectively (P = 0.011 and P = 0.035). In two years, high nitrogen treatments had nominal increases in test weight but were not statistically significant differences. The exception was 2015, where high nitrogen treatment had significantly lower test weights (P < 0.001) compared to the basenitrogen treatments by 19.14 kg/m^3.

No increase in test weight resulted from the early Feekes 6 fungicide application in any year, but the application at anthesis alone did benefit test weight in two years. In 2015 there was a 14.03 kg/m^3 increase (P = 0.011) and in 2018 an 8.94 kg/m^3 (P = 0.001) increase from the application of Prosaro at anthesis. Interestingly though, in 2017 there was a significant decrease in test weight from the application at 10.5.1 of 7.29 kg/m^3 (P = 0.019). No differences were found between fungicide treatments in test weight in any other years. There were not any significant interactions between inputs in any of the four years impacting test weight. Test weights of all treatment combinations in each year are presented in Table A4.2.

Discussion

This study aimed to investigate the impacts of nitrogen, fungicide, and a growth regulator application on fungal disease, grain quality, and yield in winter wheat in Michigan, USA. These inputs were investigated alone and in combination, to evaluate their individual effects as well as any interactions or synergisms that may occur. The study measured responses over four years in East Lansing, Michigan on a high yielding but disease susceptible soft white winter wheat cultivar ‘Ambassador’. While previous studies in Michigan and across the United States have tested these inputs, few have studied the combination of all three. While there were some

benefits, this study found numerous risks from these inputs. Nitrogen exacerbated fungal diseases and lodging. Lodging itself also increased disease, an additional risk from lodging besides the harvestability challenge. Fungicides did provide a yield benefit in some years, but not consistently, posing a risk to profitability if used on a prophylactic basis. While plant growth regulator applications did not seem to present any yield or disease risks, the responses were variable and never completely managed lodging from high nitrogen rates.

While several studies in the literature suggest a synergistic interaction between fungicides and nitrogen on yield (Kelley 1993; Salgado et al. 2017; Quinn and Steinke 2019; Ishikawa et al. 2012; Brinkman et al. 2014), this study did not demonstrate any interaction. Two previous studies also reported no interaction, even when a positive response was seen from both fungicide and increased nitrogen rates individually (Mascagni et al. 1997; May et al. 2014). Further investigation is needed to determine what conditions are necessary to achieve this synergism.

In the current study, the benefit of nitrogen alone was not demonstrated either. Nitrogen had no significant benefit to yield in any year. In fact, in 2015 and 2017, the high nitrogen treatment had an estimated 470.76 kg ha^{-1} lower yield than the base nitrogen treatments. This could be due to lodging, which was severe in 2015, albeit mild in 2017. Nitrogen increased fungal disease, which could also contribute to this yield reduction, but no yield reduction from nitrogen was observed in 2016, when foliar disease was the most severe. However, in 2016, due to calculation error, the additional nitrogen applied to "high" treatments was much lower (25.76 kg ha^{-1} instead of 56.04 kg ha^{-1}), which could also explain the lack of response in that year. In 2015 and 2018, the high nitrogen treatments also had reduced kernel weights. The additional nitrogen application could have increased other yield components, such as kernel number, thereby compromising the weight per kernel. Additionally, there may be other factors limiting yield, in that case additional

nitrogen would not result in an increase in yield. These possibilities likely all contribute in different proportions to the lack of yield response or losses from higher nitrogen treatments depending on the year. Likewise, there was not a low or no nitrogen check plot included, so it cannot be determined if the site was nitrogen responsive.

Nitrogen losses could also explain the lack of response, as nitrogen can be lost to the environment due to leaching, volatilization, or denitrification. These losses can also underscore the additional risk of high nitrogen applications: environmental concerns (Kanampiu et al. 1997), especially where nitrogen losses may impact water quality. Greenhouse gas emissions also increase with increasing nitrogen rates from synthetic sources, related to manufacturing, transport, and direct NO_2 emissions to the atmosphere (Kindred et al. 2008). A limitation to this study is neither plant tissue nor residual nitrogen in the soil was tested, so nitrogen losses are unknown.

This study also provided evidence that lodging can significantly affect disease dynamics. Specifically, in 2018, where lodging occurred close to anthesis, FHB incidence and foliar disease were significantly greater by the end of the season in lodged portions of plots compared to non-lodged portions of the same plot. The final foliar disease ratings correlated more strongly with the early season lodging ratings, than the end of season ratings. This would suggest that an earlier lodging event would have much greater impact on disease then lodging occurring towards the end of the season.

Ratings of FHB were taken from heads of both non-lodged and lodged portions of the same plot, in order to directly test the effects of lodging on FHB. Incidence of FHB was consistently greater in the lodged portions (mean 33% increase). However, differences in FHB severity varied greatly. Upon closer analysis, the magnitude of the difference between non-lodged and lodged

portions corresponded with the treatment each plot had received. In non-fungicide treated plots, FHB severity was greater in lodged portions, as it was with FHB incidence. However, in fungicide treated plots, the trend was reversed, with non-lodged portions having higher FHB levels, compared to the lodged portion of the same plot.

This would suggest that fungicides performed better on lodged portions of the plots than the non-lodged portions. Coverage, movement of the fungicide, or duration of activity could have been altered. As lodging happened the day preceding fungicide application, the wheat spikes in lodged portions would have been in a more horizontal position, where perhaps fungicide coverage would be improved over vertical, standing spikes. This is an interesting phenomenon that deserves further investigation.

Because DON testing was done from grain sub-samples from the total plot, unfortunately the effect of lodging on toxin accumulation could not be directly tested, but in 2015 there was a significant increase in DON in high nitrogen treatments. This could be from lodging or from effects of nitrogen unrelated to lodging. However, lodging alone has been previously reported to cause an increase in DON and nivalenol toxin in small grains from *F. graminearum*, with toxin concentration increasing with duration of lodging (Nakajima et al. 2008). In our study, the relationship between nitrogen and DON was not consistent, as no affect was seen in DON concentrations in 2018. Even though lodging greatly increased FHB incidence in 2018, when looking at non-lodged portions of plots, nitrogen did not affect observable levels of FHB (Table 4.4). Likewise, FDK were slightly decreased by nitrogen, and no significant difference was detected in DON concentration. Perhaps lodged portions were less likely to be harvested completely, therefore reducing their proportion in final grain samples and negating any increases the lodged portions would have caused. More work needs to be done to tease apart nitrogen's

role in FHB, to determine what other factors may be causing the inconsistencies. While there is evidence that nitrogen can influence FHB, other studies have not reported any correlation, and many have found a variable relationship depending on the year (Yoshida et al. 2008; Hofer et al. 2016; Krnjaja et al. 2015; Lemmens et al. 2004; Heier et al. 2005).

Nitrogen significantly increased foliar disease in three out of the four years and was a statistically significant increase in two of those years, 2015 and 2018. This affect was most pronounced in non-fungicide treated plots, with increases from the high nitrogen rate between 3.7 to 12.5%. However, there was a significant nitrogen by fungicide interaction in 2015, and this increase from nitrogen was much smaller or non-existent in fungicide treated plots, suggesting fungicides can effectively manage the disease risk from increased nitrogen rates. Increases in 2015 and 2018 also coincided with high lodging rates, so again, the effects of lodging caused by nitrogen versus the nitrogen itself on disease dynamics cannot be separated.

Along with lodging, there are numerous possible ways in which nitrogen may be affecting disease dynamics, many of which have been postulated in the literature but not experimentally investigated, including differential biomass accumulation, amount and timing of tillering, and synchronicity of anthesis. Canopy architecture would be affected, which has been demonstrated in small grains to impact wind speed, temperature, relative humidity, and leaf wetness (Tompkins et al. 1993; Hofer et al. 2016). These factors could affect pathogen growth and dispersal, providing a more or less favorable environment for spore production or release.

Fungicides showed clear disease protection across both nitrogen rates, significantly reducing foliar disease in all years. However, statistically significant yield benefits were only seen in 2015 and 2016. A single application at anthesis was sufficient for disease protection and yield protection in 2015. In 2017 and 2018, the single application at anthesis also provided the same

level of disease reduction as the double application at the end of the season. However, 2016 was the exception, where the early application led to significantly lower disease and greater yield protection compared to the plots receiving no fungicide. The double application in 2016 also performed significantly better than the single application at anthesis. This could be explained by the severe epidemic of stripe rust, which started early in the season, approximately 1 May at Feekes 6. In this case, the early Feekes 6 application would have reduced initial infection and subsequent inoculum in those plots, resulting in reduced disease at the end of season. This severe disease early in the season would also explain the kernel weight increase that was seen in 2016 from the early application, which was not observed in any other years.

Ultimately, the Feekes 10.5.1 application proved most effective over the four years, both at controlling foliar diseases, as well as reducing FHB and DON accumulation, and two applications were unnecessary in most years. It is widely supported in the literature that the anthesis Feekes 10.5.1 timing is the most effective for FHB management (Paul et al. 2010; P.A. Paul et al. 2018). This study provides additional evidence of the yield benefit from foliar disease protection at the Feekes 10.5.1 timing (Sylvester et al. 2018). Additionally, this study suggests that in the absence of a severe epidemic, there is no significant benefit from two fungicide applications, and likely a profit loss.

As shown by the epidemic in 2016, however, there is a chance for this early treatment to be beneficial. Ultimately, a grower will have to decide what is right for their production system, based on yield potential, weather forecast, disease risk, and application cost. Costs may be diminished if a grower is already planning to apply an insecticide or herbicide at this timing. Cultivar is also an important factor in this decision. Cultivars can not only differ in their disease resistance, but also their tolerance to yield losses (Byamukama et al. 2019; Green et al. 2014);

this study used a cultivar very susceptible to FHB and moderately susceptible to foliar diseases. If a cultivar more resistant to fungal disease was used, then the yield gain from either of these fungicide applications may be diminished, and previous literature demonstrates the likelihood of a positive net return is reduced (Weisz et al. 2011; Stephen N. Wegulo et al. 2011; Marburger et al. 2015; Cox et al. 1987).

Although beneficial in 2016, 2015 demonstrated a risk of using a fungicide in the strobilurin class. The Feekes 6 application of Stratego YLD, which contained the strobilurin active ingredient trifloxystrobin, increased DON compared to treatments with no fungicide in 2015, by 1.96 ppm. Previous studies have demonstrated this effect from a strobilurin fungicide (P. A. Paul et al. 2018; Ellner 2005; Marques et al. 2017; Blandino et al. 2009). However, few have documented this effect from a fungicide applied so early in the season at Feekes 6. Fungicides in general, but especially strobilurins, are known to prolong greening (Bartlett et al. 2002). Perhaps in 2015, the decreased leaf disease and prolonged greenness provided longer grain fill and increased moisture content, creating conditions for DON to accumulate longer, compared to the non-fungicide treated plots.

In this study, the PGR Palisade EC (active ingredient trinexepac-ethyl) showed variable effects. In some years, it did reduce lodging, but not completely. In 2015, Palisade EC influenced FDK (but not other FHB parameters) and foliar disease, reducing disease in both cases. However, these reductions were modest, and not observedin any other year. This could signal that only in certain environments is there a biological effect on pathogen or plant defense from this growth regulator. A disease reduction from trinexepac-ethyl has been reported in other pathosystems, including anthracnose (*Colletotrichum cereale*) and dollar spot (*Sclerotinia*

*homoeocarp*a) on turf, and in apple orchards infected with *Venturia inaequalis* (Inguagiato et al. 2009; Golembiewski and Danneberger 1998; Spinelli et al. 2010).

Yield benefits from using plant growth regulators has been reported (Matysiak 2006; Espindula et al. 2009; Brinkman et al. 2014), and all four years in this study, there was a nominal increase in yield with the PGR treatment ranging from 15.6 kg ha^{-1} to 280.5 kg ha^{-1}, but none of these increases were statistically significant. In 2015, an increase in the weight of 100 kernels (0.211g) was also seen from PGR. Previous studies in Michigan had not reported significant yield response from PGR (Nagelkirk 2013; Quinn and Steinke 2019). If the increase in yield seen in our study is a true effect, it probably suggests that any yield benefit would be modest.

In this study, PGR application never reduced lodging rates to the levels found in base nitrogen plots. Here, foregoing the high nitrogen rates resulted in less lodging overall than high nitrogen rates with the PGR application. A reported risk posed by plant growth regulators is over-regulation, resulting in a yield decrease or a delay in maturity. To avoid this, adequately high nitrogen rates must be used (Espindula et al. 2009; Knott et al. 2016; Swoish and Steinke 2017). Even if the maximum yield benefit from this study (269 kg ha^{-1}) was achieved, that would have to compensate for the added cost of the additional nitrogen, chemical cost, and application costs. Ultimately, environment and cultivar may be important in determining the response to PGR applications, and characteristics such as susceptibility to lodging or plant height would be important factors to consider (Knott et al. 2016; Brinkman et al. 2014; Swoish and Steinke 2017).

While there are some clear conclusions from this study, there also remains limitations to this work. Yield, an important response variable in assessing utility of these inputs, is quite variable,

especially in small plot research. In this study, the variation present meant we would not have the power to statistically detect small changes in yield.

Ultimately, this study demonstrates that for winter wheat growers in Michigan, high intensity wheat management with additional inputs may be unnecessary, and result in profit loss as well as environmental risks. The challenge of lodging should not be underestimated, as it also presents a disease risk. A fungicide at Feekes 10.5.1 to manage FHB, DON, and fungal diseases was the only input that consistently showed a positive response, with significant differences in three out of the four study years. We did not detect a synergistic interaction between nitrogen and fungicides positively effecting yield, so usage of one should not encourage use of the other. Cultivar selection, location, and yield potential are additional factors to consider when making input decisions. The risks associated with each input need to be evaluated when making management decisions such as nitrogen rate or variety, not just the potential yield benefits.

APPENDIX

APPENDIX: Supplemental Figures

Table A4.1 Detailed timetable of applications, treatments, and data collection in studies from 2015-2018.

	2015	2016	2017	2018
Planting	10/10/14	10/7/15	9/27/16	9/25/17
Initial N	4/15/2015	4/16/16	Lost record	3/23/18
Additional N (for trts 5-12)	5/11/15	4/30/16	4/21/17	5/2/18
Palisade EC	5/13/15	5/3/16	4/26/17	5/4/18
Stratego YLD	5/13/15	5/3/16	4/26/17	5/4/18
Prosaro 421 SC	6/2/15	5/31/16	5/29/17	5/31/18
FHB rating	6/24/15	Not rated, only trace amounts of HS	Not rated, only trace amounts of HS	6/21/18
Harvest	7/25/15	7/19/16	7/14/17	7/12/18
Number of replications	5	6	8	6

Table A4.2 Mean estimates of grain yield, test weight, kernel weight, and end of season lodging ratings for each treatment combination in each year (treatment combinations listed in Table 4.1).

Year	Treatment combination	Yield (kg ha-1)		Test Weight (kg m^{-3})		Kernel Weight (g)		Lodging at Harvest[b] (%)	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
2015	1	6402.8	207.3	683.34	6.37	3.65	0.14	12.60	12.91
	2	6588.3	207.3	687.13	6.37	3.74	0.14	9.00	12.91
	3	7140.0	207.3	696.32	6.37	3.92	0.14	18.00	12.91
	4	7304.8	207.3	700.64	6.37	3.99	0.14	21.00	12.91
	5	5790.5	207.3	667.53	6.37	3.37	0.14	44.00	12.91
	6	6165.3	207.3	667.61	6.37	3.42	0.14	48.00	12.91
	7	6686.3	207.3	675.28	6.37	3.60	0.14	66.00	12.91
	8	6760.1	207.3	680.43	6.37	3.67	0.14	57.00	12.91
	9	6257.3	207.3	678.53	6.37	3.66	0.14	21.60	12.91
	10	6101	207.3	675.28	6.37	3.63	0.14	37.60	12.91
	11	7035.1	207.3	699.89	6.37	3.77	0.14	47.00	12.91
	12	6834.7	207.3	685.79	6.37	3.85	0.14	35.60	12.91
2016	1	4057.9	433.5	681.35	12.20	3.96	0.07	15.00	5.64
	2	4631.3	433.5	675.41	13.35	3.96	0.07	14.00	5.64
	3	4553.7	433.5	698.30	12.20	4.01	0.07	15.63	5.64
	4	4600.2	433.5	688.86	12.20	4.12	0.07	13.13	5.64
	5	4334.1	433.5	694.44	12.20	3.88	0.07	26.88	5.64
	6	4354.7	433.5	704.31	12.20	3.95	0.07	3.13	5.64
	7	4334.1	433.5	695.51	12.20	4.07	0.07	13.13	5.64
	8	5066.2	433.5	694.65	12.20	4.14	0.07	13.75	5.64
	9	4135.3	433.5	690.36	12.20	3.77	0.07	7.19	5.64
	10	4622.6	433.5	719.54	12.20	4.01	0.07	8.13	5.64
	11	4720.2	433.5	717.61	12.20	4.00	0.07	13.75	5.64
	12	4673.6	433.5	713.10	12.20	4.16	0.07	15.00	5.64
2017	1	4685.1	546	700.87	4.94	4.43	0.11	-	-
	2	5380.4	546	703.93	4.94	4.12	0.11	-	-
	3	4603	546	689.45	4.94	4.31	0.11	-	-
	4	4708.2	546	690.58	4.94	4.34	0.11	-	-
	5	4778.5	546	698.14	4.94	4.40	0.11	-	-
	6	4285.6	546	691.86	4.94	4.06	0.11	-	-
	7	3811.9	546	692.02	4.94	4.31	0.11	-	-
	8	4444.7	546	692.83	4.94	4.35	0.11	-	-
	9	4399.8	546	701.52	4.94	4.14	0.11	-	-
	10	4342.1	546	700.23	4.94	4.39	0.11	-	-
	11	4767.2	546	697.17	4.94	4.38	0.11	-	-
	12	4867.1	546	698.62	4.94	4.48	0.11	-	-

Table A4.2 (Cont'd)

2018	1	6216.6	337	778.75	4.43	4.14	0.20	2.00	8.07
	2	6308.38	337	783.47	4.43	4.17	0.20	9.50	8.07
	3	6263.44	337	787.33	4.43	4.33	0.20	22.00	8.07
	4	5850.32	337	785.18	4.43	4.21	0.20	20.50	8.07
	5	5826.91	337	783.68	4.43	3.93	0.20	27.17	8.07
	6	5504.18	337	780.68	4.43	4.13	0.20	27.83	8.07
	7	6048.72	337	789.04	4.43	3.79	0.20	29.83	8.07
	8	6396.84	337	793.34	4.43	3.96	0.20	23.67	8.07
	9	6020.22	337	781.32	4.43	4.13	0.20	23.33	8.07
	10	6250.65	337	790.98	4.43	4.11	0.20	10.17	8.07
	11	6296.10	337	794.19	4.43	4.03	0.20	24.67	8.07
	12	6331.71	337	794.41	4.43	4.09	0.20	23.83	8.07

[b] Estimated percentage of the plot lodged just prior to harvest

Table A4.3 Mean winter wheat plant height (cm) in 2017, East Lansing, MI. Three plants were randomly selected from three different sections of each plot and measured from soil line to the tip of the ear.

Fungicide Regime	Height (cm)[z]		
	BaseN	**High N**	**High N + PGR**
Non-treated	84.4 a	84.4 ab	79.4 abcd
Feekes 6	86.3 a	76.2 cd	73.7 d
Feekes 10.5.1	83.1 ac	74.5 d	77.2 cd
Feekes 6 and 10.5.1	80.9 abcd	82.9 abc	77.1 cd

[z] Treatment means with the same letter are not significantly different (α=0.05)

Chapter 5 **: Meta-analysis of yield response to applications of fungicides made at different crop growth stages in Michigan winter wheat**

Authors who contributed to this study were: Mikaela Breunig, Martin Nagelkirk, Adam M. Byrne, Jaime Wilbur, Kurt Steinke, and Martin I. Chilvers

Abstract

Various foliar fungal pathogens, as well as Fusarium head blight are a reoccurring threat to Michigan wheat production. Fungicides are a key management tool for these diseases, and many products are efficacious. However, the timing of application is thought to be a key determinant of yield response and disease prevention. A meta-analysis of previously conducted fungicide trial data from Michigan was performed to evaluate which fungicide application timings individually or in combination were most effective across years. Data from 46 trials (2007-2020) were utilized to examine six fungicide regimes. All regimes analyzed resulted in a mean positive yield response, and at least an 87% probability of a positive response in future applications. The combination treatment of Feekes 5-7 plus an application Feekes 10.5.1 had the highest mean yield response of 10.5 bu/ac. Early season applications (Feekes 5-7) resulted in the lowest response, with a mean yield increase of 4 bu/ac. Probabilities of positive yield response and prediction intervals were calculated for all regimes to aide growers in making future fungicide application decisions.

Introduction

Michigan has over 8,000 wheat farmers growing more than 500,000 acres of wheat annually, a high percentage of which is soft white and red varieties (Michigan Wheat Program 2017). There is a large demand for high quality soft wheat in Michigan driven by a strong milling industry in the state. One constant threat to wheat quality and yield are fungal diseases. Fungicides are a key management tool for these diseases, especially for Fusarium head blight management (FHB) as there are few cultivars with complete resistance. *Fusarium* spp. produce mycotoxins, mainly deoxynivalenol (DON), and contamination of grain with these toxins can result in reductions in sale price at the elevator, or even unmarketable grain if levels are severe.

Epidemics of FHB can also reduce yield by up to 40-50% (Windels 2000). Foliar diseases such as powdery mildew, Stagonospora leaf blotch, Septoria leaf spot, stripe rust, and leaf rust are also common in Michigan and present a large threat to yield (Figueroa et al. 2018; Michigan Wheat 101 2021). The incidence and severity of these disease will vary depending on when inoculum is present and environmental conditions throughout the season.

Numerous fungicide products are registered for wheat with efficacy across a variety of fungal diseases. Fungicides can provide yield benefits in wheat by protecting green leaf area during grain fill, resulting in increased grain dry matter (Gooding et al. 2005; Dimmock and Gooding 2002; Cook et al. 1999). Products in the strobilurin, demethylase inhibitor, and succinate dehydrogenase inhibitor classes are all registered and used on wheat in the United States. Since most products are highly efficacious across these diseases (De Wolf 2020), one of the key determinants of yield response to a fungicide is the timing of application relative to plant growth and pathogen development. Common application timings include early in the season at jointing (Feekes 5-7), at emergence of the flag leaf (Feekes 9), and during flowering to protect exposed anthers from *Fusarium* infection as well as provide flag leaf protection (Feekes 10.5.1).

In some studies, Feekes 9 applications have shown to be the most efficacious at reducing flag leaf disease as the fungicide protects the flag leaf immediately after emergence (Wegulo et al. 2011; Sylvester et al. 2018; Cook et al. 1999). Depending on onset of epidemics, Feekes 10.5.1 applications may provide similar flag leaf protection as Feekes 9 (Sylvester et al. 2018). Feekes 6 applications may be too early to provide any protection during grain fill (Brinkman et al. 2014; Willyerd et al. 2015). However, studies have demonstrated severe early season diseases such as powdery mildew can impact the yield components of seeds per spike yield or tillers per row (Green et al. 2014; Bowen 1991). Two applications per season is also a practice increasing

in frequency as some growers aim to maximize returns in high yielding areas. However, in years without severe disease pressure, two applications may not be profitable and does not always provide significantly greater disease protection than a single well timed application (Sylvester et al. 2018; Brinkman et al. 2014).

When fungicides are applied unnecessarily, not only is there potential profit loss, but also increased environmental risks. Environmental risks include additional greenhouse gas emissions resulting from added trips across the field, potential off target effects, and elevated selection pressure for fungicide resistance. Fungicide resistance is an increasing challenge for combating the wheat pathogen *Zymoseptoria tritici* in Europe and more recently in the United States (Hayes et al. 2016; Augusti et al. 2019; Sykes et al. 2018). Preliminary studies of two Michigan fields has found 25% of Michigan isolates resistant to azoxystrobin due to the G143A mutation and some isolates with resistance to SDHI chemistries (Augusti et al. 2019). Globally, fungicide resistance to additional foliar pathogens has been documented as well (Blixt et al. 2009; Castroagudín et al. 2015; Leroux et al. 2013; Cook et al. 2021)

The objective of this study was to perform a meta-analysis of fungicide trial data in Michigan to evaluate which fungicide application timings individually or in combination were most effective across many years. The results of this data set will characterize the range of yield responses observed from these different timings and combinations, ultimately enabling data driven fungicide application decisions by the grower.

Methods

Study and treatment selection

Fungicide efficacy trials have been carried out in the state of Michigan by academic researchers and extension personnel for many years. While some of this data has been published

in Plant Disease Management Reports or academic journals, much of it remains unpublished and only analyzed and presented for extension outreach purposes. Plot level data was available from three investigators, each

All available fungicide data was gathered from three investigators. Studies that included a non-treated control and at least one fungicide application were included. All trials utilized were randomized complete block design ranging from 3-8 replicates, with the majority having four replications. Only timings that had been investigated at more than one site, and on more than one variety and year were included. This resulted in six timings of interest, listed in Table 5.1. "T1" treatments consisted of spring applications made between Feekes growth stage 5 and 7. Treatments classified as "T2" were targeting the flag leaf emergence, at Feekes 8 or 9. Applications at Feekes 10.5.1 made from the start of flowering up to 4 days into flowering were considered "T3." Applications made 5 days after flowering or later were considered late T3 applications and referred to as "T3_L." Five days was chosen as a cut-off value, as the efficacy for FHB prevention begins to decline at 5 days post flowering based on a meta-analysis of multi-state FHB timing trials (P.A. Paul et al. 2018).

Many studies tested multiple products across a single timing. Only products that are presently registered for application on wheat in Michigan, and that were rated "fair" or better on the fungicide efficacy guide (De Wolf 2020) were selected. We assumed all products to have adequate efficacy, so product was not considered a factor in the analysis. All products applied at the same timing were combined as a single treatment in each study.

In some trials, additional factors such as nitrogen or variety were investigated. For those trials, data was divided by levels of the additional factor, and each level considered separate

studies, so that the non-treated comparison used to compute yield response was equal in all ways to the fungicide treatment.

Table 5.1 Description of fungicide application timings investigated and the number of studies and observations for each timing.

Timing	Abbreviation	Number of studies Evaluated	Number of years that timing was evaluated	Number of plots observations
Feekes 5-7 (early jointing)	T1	30	8	235
Feekes 8-9 (flag leaf emergence)	T2	17	14	161
Feekes 10.5.1 (first flower up to 4 days)	T3	87	12	688
Post Feekes 10.5.1 (5+ days after flowers)	T3_L	11	5	61
Feekes 5-7 + Feekes 10.5.1	T1 + T3	41	10	273
Feekes 8-9 + Feekes 10.5.1	T2 + T3	9	8	90
				Total: 1954

Trial Information

Trials were conducted in three regions by co-authors of this study: Michigan State University research farms in Ingham county, commercial fields in Sanilac county, and at the Saginaw Valley Research and Extension Center (SVREC) in Frankenmuth, Michigan. While trials had different plot sizes, all trials were arranged in blocks and had at least four replications. All trials were established in the fall, and 1.8 million seeds/ac were planted in 7.5-inch rows. A total of 16 varieties of soft white or soft red winter wheat were utilized. Fungicide treatments at SVREC and campus trials were made with handheld spray booms pressurized with CO_2, whereas trials in Sanilac county were sprayed with a tractor mounted boom sprayer. Additional details on trial methods are presented in Table 5.2.

Table 5.2. Trial details of the three investigators who contributed to the dataset.

Principal Investigator	***Locations & years conducted***	***Plot size(s) utilized***	***Available published reports with additional details***	***Harvest details***
Martin Nagelkirk MSUE wheat specialist	Deckerville, MI (various commercial fields) 2007-2008, 2010-2019	18 x 65 ft 15 x 60 ft 20 x 75 ft	(Nagelkirk et al. 2017; Nagelkirk 2015)	International 2144 combine equipped with a Juniper Harvest Master system
Dr. Kurt Steinke Soil Fertility Lab	Saginaw Valley Research and Extension Center, Frankenmuth, MI and MSU agronomy farm, East Lansing, MI 2016 – 2019	8 x 25 ft	(Quinn and Steinke 2019; Purucker and Steinke 2021, *in submission*)	Small-plot combine (Almaco, Nevada, IA)
Dr. Martin Chilvers Field Crop Pathology Lab	MSU plant pathology farm, East Lansing, MI 2015 - 2020	7 x 12 ft 7 x 14 ft	(Breunig et al. 2017; Byrne et al. 2016)	Small-plot combine with HarvestMaster system

Analysis of individual studies

The trial criteria and treatment selection described in the methods resulted in 93 studies from 46 projects, for a total of 1,954 total plot observations over 13 years. Raw plot level data for treatments of interest were extracted from individual field studies. An analysis of variance (ANOVA) was conducted on each study separately in PROC GLIMMIX in SAS (versions 9.4; SAS institute Inc., Gary, NC) to calculate the mean difference in yield from the non-treated control. Application timing was a fixed effect, and block was a random effect. Means were obtained with the LSMEANS statement. Means for each timing, the non-treated mean, their standard errors, as well as the residual variance estimate for the mixed-effect model were recorded.

For each study, the mean difference in yield for timing, *t*, in a trial with non-treated control, *nt*, was calculated by the equation

$$D_t = \bar{X}_t - \bar{X}_{nt}$$

The within-study variance for the difference, D, was calculated for each pair of timing *t* and non-treated control *c* :

$$Var_D = V_Y \left(\frac{1}{n_t} + \frac{1}{n_c}\right)$$

Where V_Y is the yield variance, or the residual estimate obtained from the covariance parameter in the PROC GLIMMIX output, n_t is the number of replications (plots) of the timing, and n_c is the number of replications of the non-treated control. Standard error of $\bar{D}_t$ was estimated as the square root of Var_D. $\bar{D}_t$ with Var_D became a single observation for each timing that was extracted and utilized in the network meta-analysis.

Network meta-analysis

Meta-analysis allows synthesis of many different trials by using weighted averages of effect size, with studies of lower residual variance having more weight in estimating the expected effect size (Madden and Paul 2011). The difference between the mean yield plots treated at a specific timing and the non-treated control was used as the effect size $\overline{D}_t$ in a network meta-analysis to investigate effect size differences between different fungicide application timings. A linear model with random study effect and unequal between-study total variance was fit according to code outlined in Madden et al 2016, using SAS PROC GLIMMIX. A heterogeneous variance-covariance structure for $\mathbf{\Sigma}$ was used, based on the heterogeneous compound symmetry (CSH) model. The within-study variances were incorporated by weighting each study by the inverse of the variance ($1/Var_D$) (Madden and Paul 2011; Paul et al. 2010; Madden et al. 2016). In the parameter statement, the initial estimate for the correlation term was 0.5 and the residual variance was held constant at 1 (Madden et al. 2016). For exploration of moderator variables, variables of interest were added as to the model statement as an interaction term with timing.

The standard type III test of fixed effects was performed to evaluate whether the application timing and moderator variables were significant. The LSMEANS statement was used to obtain estimates of $\overline{D}_t$, which represent the mean effect sizes of the timing across all studies, as well as corresponding standard errors and 95% confidence intervals. Mean comparisons were tested using Fisher's least significant difference ($\alpha = 0.05$).

Prediction and probability of response

Prediction intervals for 95% and 50% were calculated according to equation 14 in Madden et al 2011, utilizing the among study variance for each timing (covariance parameter

estimate), and the standard error of $\overline{D}_t$ from the lsmeans function. Probabilities of yield response were calculated according to equation 15b from Madden and Paul 2011 utilizing the among study variance (covariance parameter estimate) from each timing.

Results

Responses from individual studies

Boxplots in Figure 5.1 display the variation in yield response from individual studies and numeric means of each timing. Across all timings $\overline{D}_t$ ranged from 19 bu/ac loss to a 42 bu/ac gain. Of the 195 responses from 93 studies investigated, 173 (88%) had a yield response greater than zero and 153 (78%) had a response greater than 2 bu/ac (Figure 5.2).

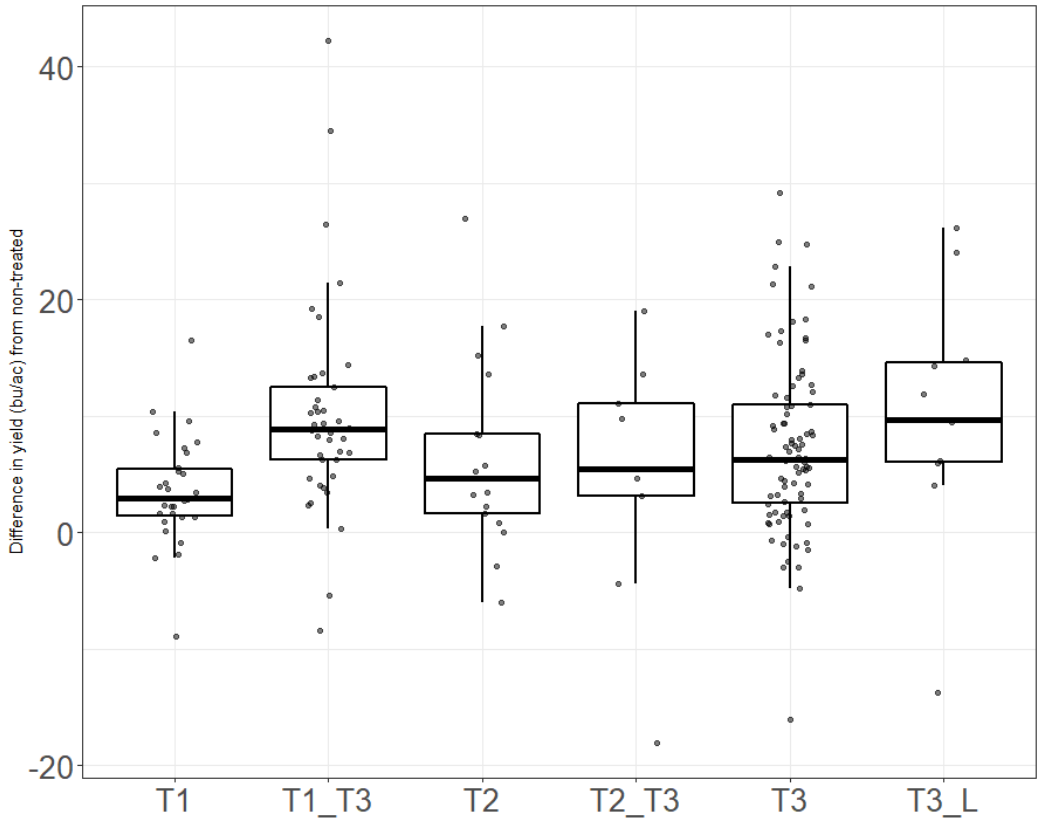


Figure 5.1 Boxplots displaying distributions and numeric means of yield responses from fungicide application made across six timings from 93 studies utilized in the meta-analysis.

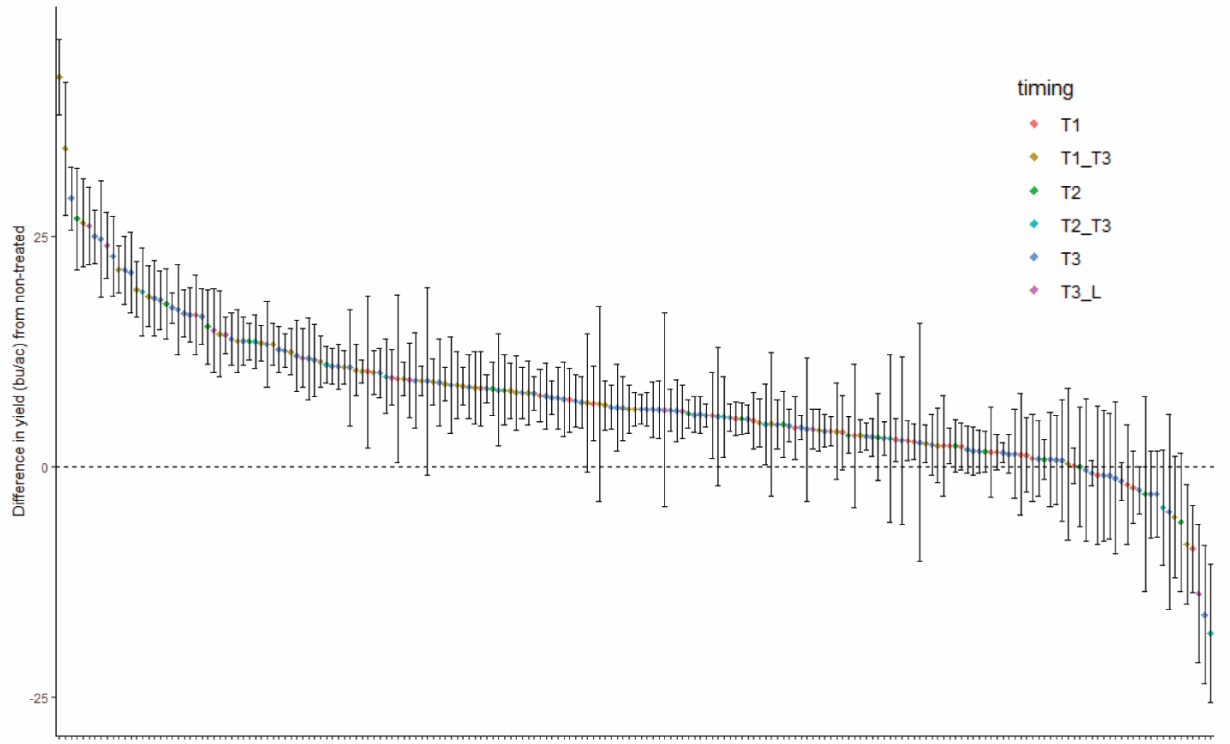


Figure 5.2. Forest plot representing distribution of all yield response observations utilized in the network meta-analysis. Diamonds represent the estimated effect size (mean of non-treated control subtracted from the mean of fungicide treated plots at specified timing) for 195 responses from 93 winter wheat fungicide efficacy studies conducted in the state of Michigan from 2007-2020. Error bars depict +/- the standard error of the difference between the non-treated and treated plots.

Network meta-analysis

Application timing significantly impacted the yield response from a fungicide application (type III test, P<0.0001). All application timings significantly increased yield (P <0.0001). The largest yield response was observed where two applications were made, with estimated response of 10.5 bu/acre for T1 + T3 applications, and a 9.5 bu/ac response to the T2 + T3 applications. T3, late T3, and T2 resulted in a 6.9, 7.4, and 6.6 bu/ac yield response, respectively. These three applications were not significantly different from each other nor the T2+ T3 application (Table 5.3). The T1 was significantly lower than all other treatments, with a 4.1 bu/ac yield response (Table 5.3). The addition of the T1 application to a T3 timing increased yield 3.1 bu/ac compared

to a T3 application alone. The addition of the T2 to the T3 also numerically increased yield by 2.06 bu/ac, but was not a statistically significant increase from the T3 application alone.

Table 5.3 Estimated effect size of the mean yield response from applications at specified timings

Timing	Effect Size ($\overline{D}_t$) [y]					Among-study variance $\hat{\sigma}^2$ (SE($\hat{\sigma}^2$))
	Estimate $\overline{D}_t$		SE($\overline{D}_t$)	95% CI_{Lower}	95% CI_{Upper}	
T1	4.0	A[z]	0.6	2.7	5.3	8.6 (6.0)
T2	6.9	B	1.0	8.5	12.5	37.2 (14.1)
T3	7.4	B	0.8	4.9	8.9	40.8 (8.3)
T3_L	6.7	B	1.3	6.1	12.9	31.7 (11.1)
T1_T3	10.5	C	1.0	5.1	8.9	60.8 (15.1)
T2_T3	9.5	CB	1.7	4.2	9.1	64.1 (38.3)

[y]Effect size ($\overline{D}$) is the mean yield response across all studies for each fungicide timing generated from the network meta-analysis, calculated as the difference between the fungicide treatment and the non-treated control for each study

[z]Means evaluated using Fisher's least significant difference; means followed by the same letter are not different at the $\alpha = 0.05$ significance level.

Prediction of yield response to each timing

The probability of a yield response greater than 0, 2.5, 5, or 10 bu/acre was estimated for each fungicide timing and presented in Table 5.4. Probability of a yield response greater than zero in a new individual study was 87% or higher for all timings. Applications at T1 have the lowest probability of achieving a substantial response, with only a 37% probability of achieving at least a 5 bu/ac yield gain. In contrast, applications made at T2, T3, or late T3 have a 77-78% chance of achieving a 5 bu/acre or greater response.

The 50% and 95% prediction intervals for a yield response at each timing were also estimated and presented in Figure 5.3. Although the combination treatments had the highest estimated effect size, they also have the widest prediction interval due to their relatively larger variance (Figure 5.3). While the T2 + T3 application had a relatively low sample size, which is reflected in the high standard error of the among study variance, the T1 + T3 had a large sample size, and high variance reflects the large variation in observed responses.

Table 5.4. Estimated probabilities of a response greater than 0, 2.5, 5 or 10 bu/acre to each fungicide timing or combination of timings, based on mean effect size $\bar{D}_t$ and the among study variance of each timing determined from network meta-analysis.

Timing	Probability of specified yield response (bu/acre)			
	<0	**2.5 +**	**5 +**	**10 +**
T1	.91	.70	.37	.02
T2	.87	.77	.62	.31
T3	.87	.78	.65	.57
T3_L	.88	.77	.61	.28
T1_T3	.91	.85	.76	.53
T2_T3	.88	.81	.71	.41

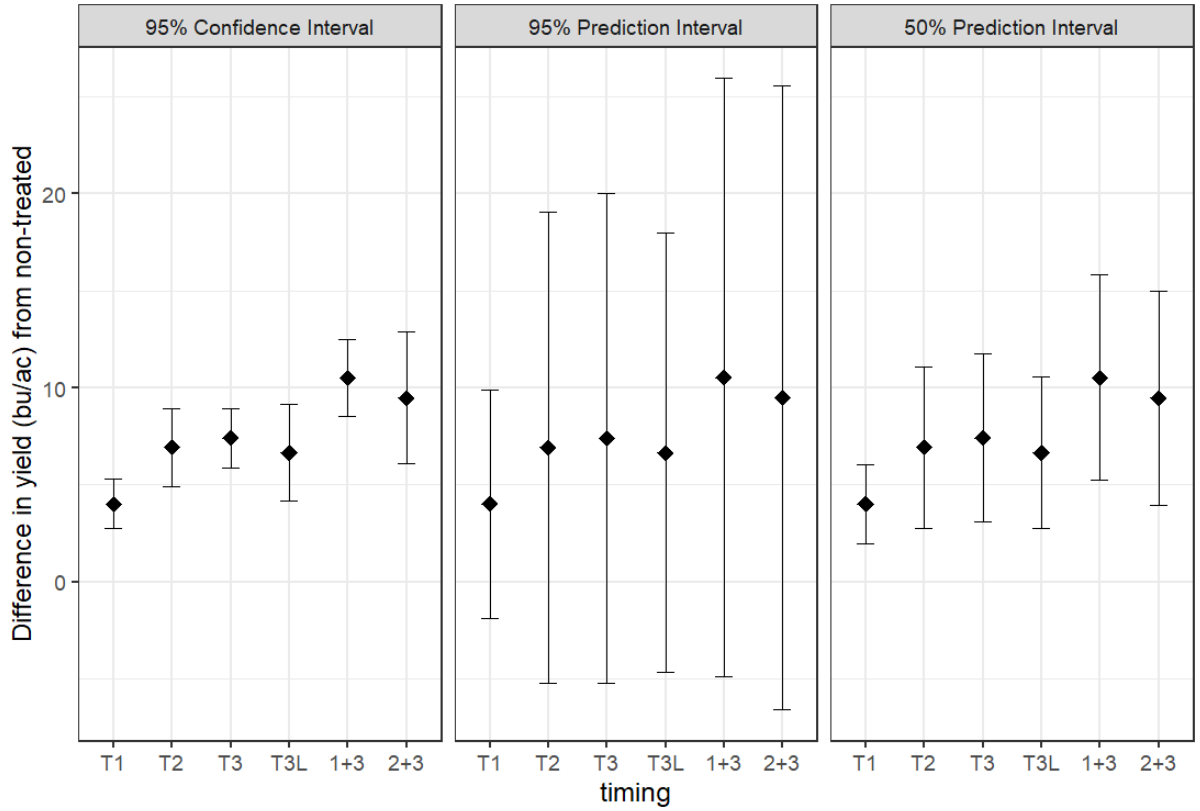


Figure 5.3 Display and comparison of 95% confidence interval for effect size $\bar{D}_t$ for each timing, as well as the 95% prediction interval and 50% prediction interval. If a new study was completed, there would be a 95% and 50% probability of a response within that interval, respectively.

Exploration of moderator variables

There was noticeable variation in yield responses within each timing therefore moderator variables were explored to investigate this heterogeneity. Interaction of timing and year was significant (P = 0.0013), however not all parameters were estimable since not every treatment was represented in every year. Visual representation of the variation across years can be observed in Supplementary Figure 5.1. Variety was not a significant moderator (P = 0.5102) and inclusion in the meta-analysis increased the among study variance estimates in the model. Site was not a significant variable (P = 0.0832). In exploratory visual analyses, it appeared that responses were more variable when yields were low. Thus, an additional categorical variable of base yield was created, classifying studies with non-treated yields below 91 bu/ac classified as "low" and those greater than 91 bu/ac as "high." This cut-off was chosen based on visual separation that appeared around 91 bu/acre (Supplementary Figure 5.2). When included in the model, base yield was only a marginally significant moderator (P = 0.0362) and led to increases in the among-study variance for two treatments, so the original model without any moderators was used for all analysis. Boxplots summarizing the distribution of response across "high" and "low" yielding studies can be observed in Supplementary Figure 5.3.

Discussion

The relative efficacy and yield response from different fungicide application timings has been evaluated over 13 years by multiple investigators in the state of Michigan. While anecdotally the variability of yield response to various fungicide timings between different years has been discussed, this is the first meta-analysis of Michigan fungicide efficacy data in winter wheat, utilizing 1,945 plot level observations. The network meta-analysis approach used here allows comparisons across different fungicide application timings, even if individual studies did

not include all timings. Meta-analysis also increases power for hypothesis testing and offers a more informative approach than simply counting the number of individual studies where p-values are significant.

Here we demonstrated that winter wheat in Michigan is responsive to fungicide applications, with a significant yield benefit from all fungicide application timings (Feekes 5 through late flowering), and an 87% probability of a positive response. Out of the six timings evaluated in the current analysis, the T1 timing provided the least benefit, with a mean yield increase of 4.01 bu/acre, significantly lower than all other applications. The T2, T3, and late T3 timings were not significantly different from each other with mean increases between 6.6-7.4 bu/ac. Results are similar to another multi-state meta-analysis, estimating a 6.8% - 11% yield increase from applications made at the T3 timing (Paul et al. 2010). While there was not a significantly greater yield response from the T3 application compared to the T2 or late T3 applications, a T3 application at flowering has the added benefit of controlling FHB and DON. These benefits were not quantified in this study, only yield, but the concentration of DON is an important for profitability. Previous literature demonstrates significantly less DON control is achieved from fungicide applications made 5 or more days after flowering (P. A. Paul et al. 2018).

The combination treatments of either T1 or a T2 in addition to a T3 application, had the greatest mean yield. The T1 + T3 application had greatest than all other application timings. However, the effect sizes were only 2-3 bu/ac greater than the T3 alone. The modest yield increase may not result in a positive net return considering the costs associated with an additional application. The combination treatments also had the largest among-study variances. This could be explained by these treatments having some of the largest responses in certain years, likely

under severe disease pressure. However, in some years the response from these combination treatments was equivalent to the single applications, leading to larger variance among studies compared to the single applications.

Numerous studies demonstrated that the protection of the flag leaf and penultimate leaf are crucial for yield protection from foliar pathogens (Bhathal et al. 2003; Sylvester et al. 2018; Cook et al. 1999). Fungicide applications made at the T1 timing do not provide lasting efficacy into grain fill which contributes to the lower yield response from this application later applications. However if there was severe disease early in the season (Feekes 4-7) yield components of tiller production and kernel per head may be important can be affected(Bowen 1991). However in many years in Michigan tillers produced in spring do not contribute to yield. Early applications may also have a role in reducing inoculum production and infection rate in seasons with early disease onset, which warrants further investigation. While T2, T3, and late T3 applications did not have significantly different yield responses in the network meta-analysis, individual studies used in the analysis did find significant differences between these timings. For example, in 2016 when severe stripe rust developed early in the season in MSU campus plots, the T2 application provided significantly greater yield response compared to the T3 application. The relative efficacy of these timings will likely vary with the individual year. This underscores the importance of practicing integrated pest management strategies when making fungicide application decisions, such as scouting each year to determine which pathogens are present and when. Integrating use and knowledge of varietal resistance is also a key IPM principle that can aide in profitable and sustainable fungicide application decisions.

Year, site, variety, and base yield potential were explored for their possible role in moderating the yield response to these fungicide treatments. Neither site nor variety were found

to be significant moderators of yield response in this data set. However previous literature has demonstrated yield response to fungicides can be highly dependent on variety, with cultivars varying in disease susceptibility, yield tolerance, and responsiveness to fungicide applications (Loyce et al. 2008; Green et al. 2014; Byamukama et al. 2019; Salgado et al. 2014; Bingham et al. 2009). However, this data set was not well suited to test the effects of variety. Although a total of 16 different varieties were tested, these were un-balanced across the other factors, with many only tested at a particular site or a particular year, preventing us from modeling and separating the effect of site year versus variety. Likewise, not all varieties were tested across all six timings.

Year was a significant moderator variable, but other factors including variety and site were confounded with year, and not all model parameters were estimable since not every treatment was represented in every year. From visualizing trends in the data, there appeared to be more variation in responses when base yield (mean yield of non-treated plots) for a study was lower than 91 bu/acre. The categorical variable of "high" or "low" base yield was marginally significant ($P= 0.036$) and there did appear to be a trend of greater response seen in "low" yielding treatments. Higher disease pressure resulting in a low base yield could explain the trend of an increased yield response observed in these trials (Supplementary Figure 5.3), but further exploration of this phenomenon is needed as several factors besides disease impact yield potential. These include soil moisture and weather variables, planting date, soil fertility, and more, which may or may not interact with fungicide response.

Disease pressure as a moderator variable could be useful here in explaining the variable response. Disease pressure is a common moderator variable explored in meta-analysis of plant pathology management trials (Paul et al. 2010; Ngugi et al. 2011; Kandel et al. 2018; Willbur et

al. 2019). Literature has demonstrated in seasons with low disease severity, there is not always a demonstrated yield benefit to wheat fungicide application (Cox et al. 1987; Stephen N Wegulo et al. 2011; Weisz et al. 2011). However, disease incidence and severity data were not available for all studies included in the analysis. Incorporating disease pressure into this model would be further complicated by the fact that multiple pathogens (both foliar and fusarium head blight) were causing yield losses in many site years. Different pathogens may have varying effects on yield, in that equivalent lesion severity may not result in equal yield reductions (Olesen et al. 2003; Green et al. 2014).

Although both year and yield potential may aide in more accurately characterizing yield responses, these factors are unknown prior to the season and ultimately may not assist growers in the decision making process. Additionally, the effects of year and yield potential are likely biologically attributed to weather variability and environmental factors that are difficult predict. This underlines the need for large sets of data to evaluate epidemiological variables and create accurate prediction tools. Prediction tools modeling disease risk or yield potential, could aide farmers in making in-season fungicide application decisions. Future meta-analytical and trial work should also focus on responses across varieties, a factor growers can control pre-season and consider in their application decisions.

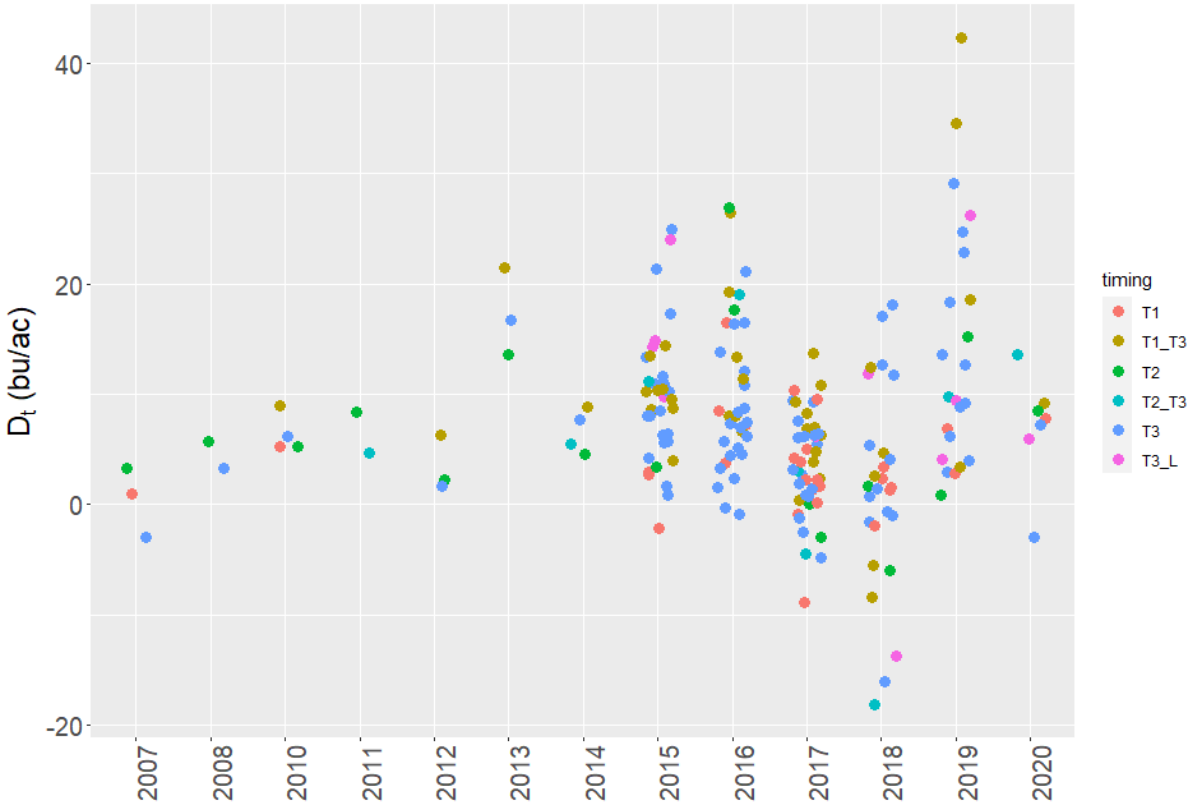
This data provides a first step in characterizing the range of yield responses across different fungicide regimes and estimating the probability of response across various fungicide timings. The wide prediction intervals (Figure 5.3) seen here indicate that the range of responses from most application timings is large, with 95% prediction intervals spanning 20-25 bushels, and 50% prediction intervals 10 bushels or wider. Likewise, almost all timings having significant

overlap in confidence and prediction intervals meaning there is likely not a single optimal timing for fungicide application each year.

Analysis here provides probabilities and ranges of realistic yield responses growers can expect in Michigan from various fungicide regimes. Integrating this information with individual costs and risk management strategies, can aide fungicide application decisions. This work along with future studies may aide in more strategic or conservative use of fungicide applications in Michigan wheat, which would not only improve grower profitability, but also reduce off-target effects from fungicide applications, and delay development of fungicide resistance.

APPENDICES

APPENDIX A: Supplemental Figures

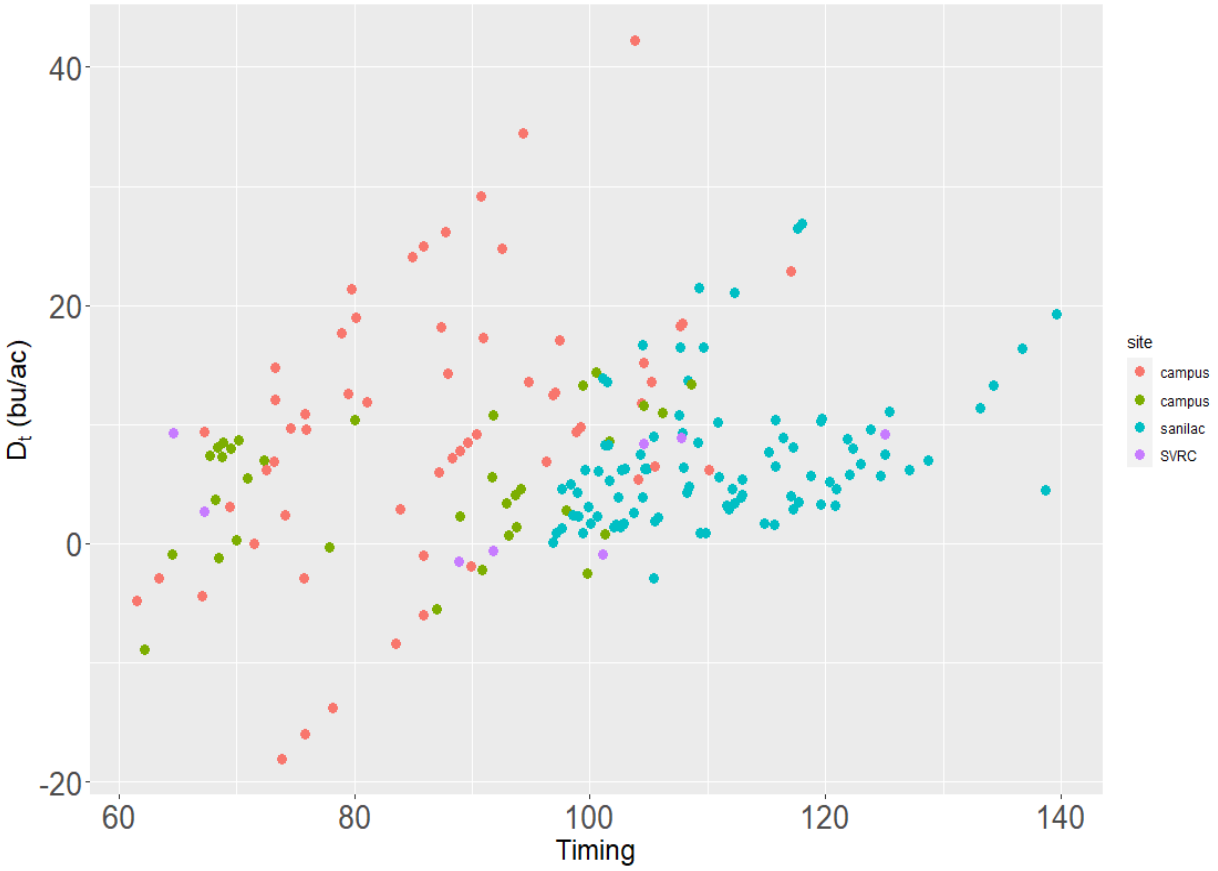


Supplementary Figure 5.1.Distribution of yield response across years, with each point representing the mean response from a single timing from a single study, with color representing the application timing.

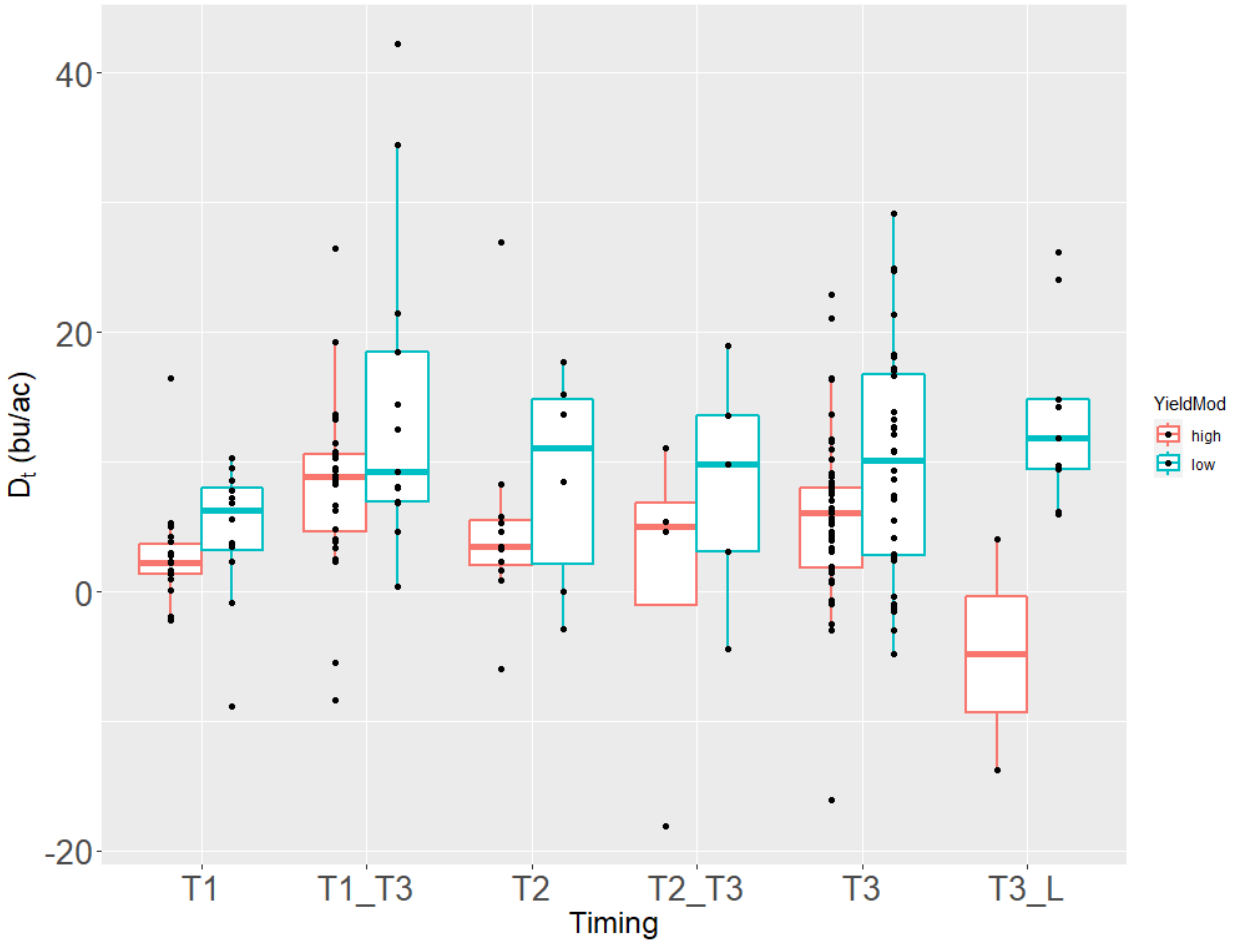
Table 5.5 Mean estimate of yield response from each timing and base yield combination, from random effect meta-analysis including base yield as a moderator variable.

Timing	Base yield[a]	Effect Size ($\overline{D}_t$) [b]				Among-study variance $\hat{\sigma}^2$ (SE($\hat{\sigma}^2$))
		Estimate $\overline{D}_t$	SE($\overline{D}_t$)	95% CI_{Lower}	95% CI_{Upper}	
T1	high	3.08	0.74	1.57	4.59	6.24 (5.17)
T1	low	6.02	1.46	3.13	8.90	
T2	high	5.34	1.23	2.79	7.88	29.70 (15.22)
T2	low	9.98	1.99	5.94	14.02	
T3	high	5.74	0.86	4.02	7.45	32.44 (7.28)
T3	low	10.95	1.25	8.46	13.43	
T3_L	high	6.90	2.72	1.52	12.28	36.60 (16.68)
T3_L	low	8.16	1.78	4.52	11.79	
T1_T3	high	8.76	1.19	6.34	11.17	56.13 (15.31)
T1_T3	low	13.34	1.92	9.52	17.17	
T2_T3	high	8.11	2.98	1.87	14.34	72.28 (46.3)
T2_T3	low	11.97	3.14	5.38	18.577	

[a]Base yield is categorical moderator variable, with trials categorized as "high" base yield if the mean yield of the untreated plots was greater than 91 bu/acre.
[b]Effect size ($\overline{D}$) is the mean yield response across all studies for each fungicide timing generated from the network meta-analysis, calculated as the difference between the fungicide treatment and the non-treated control for each study.



Supplementary Figure 5.2. Relationship between yield (bu/ac) of non-treated plots in a given trial, and the yield reponse seen from a fungcide application in that trial. Points are colored according to their location and investigator.



Supplementary Figure 5.3 Boxplots summarizing the distribution of yield responses across the six analyzed fungicide application timings, divided by base yield to explore yield potential as a moderator variable. Red boxplots represent “high” base yield, classified as studies where the mean yield of non-treated plots was greater than 91 bu/ac, and blue representing “low” base yielding studies. Boxplots are overlayed with black points of responses of individual studies.

APPENDIX B: Fungicide Timing Trials

Objective and methods

Trials investigating fungicide application timing were conducted at the Michigan State University Plant Pathology Farm in East Lansing, MI from 2017 to 2020. Treatments tested are listed in Table A5.1. In order to directly compare the impact of application timing, Prosaro 421 SC at 6.5 fl oz/ac (19% prothioconazole + 19% tebuconazole) was used for all treatments, with the exception of treatment 10 which was used as an industry standard comparison, with Stratego YLD (10.8% prothioconazole + 32.3% trifloxystrobin) at 4 fl oz/ac applied at T1 rather than Prosaro.

In all trial years the previous crop was soybean. Ambassador soft white winter wheat was planted at 7.5-in. row spacing and at a rate of 1.8 million seed/A. Plots were 7 ft wide and between 14 ft long. The entire trial area received broadcast urea fertilizer (90 lb N/A) at green up. In 2017 an additional 30 lbs N/A was applied on April 19. The experimental design was a randomized complete block with at least five replicates per treatment. Fungicides were applied with a hand-held spray boom pressurized with CO_2 at 40 psi. The boom consisted of four nozzles (Teejet D6TJ-60 110015VS) spaced 20-in. apart and was calibrated to apply 15 gal/A. Foliar disease ratings were plot-wide estimates of the percentage of flag leaf area impacted. In 2019 and 2020, ratings of severity (percentage of leaf affected on infected plants) and incidence (proportion of plants with infection) were taken separately. Plots were harvest, and yield adjusted to 13% moisture. Data were analyzed using SAS 9.4 PROC GLIMMIX (SAS Institute, Cary, NC), with ANOVA type three test for fixed effect used to determine if treatment was a significant factor. Each response variable was modeled with treatment as a fixed effect and block as a random effect. LSMEANS was used to generate mean values presented in tables.

Table A2.1 Description of treatments tested in fungicide timing trial from 2017-2020 and the dates applications were made in each year of the trial.

Treatment number	Treatment timing(s)[a]	Date of application			
		2017	2018	2019	2020
1	Non-treated	-	-	-	-
2	T1 (Feekes 5-6)	4/19/2017	5/4/2018[b]	5/10/2019	5/1/20
3	T1.5 (Feekes 7)	5/10/2017	5/15/18	NA[c]	NA
4	T2 (Feekes 9)	5/15/2017	5/21/2018	5/27/2019	5/23/20
5	T3 (Feekes 10.5.1)	5/29/2017	5/31/2018	6/11/2019	5/29/20
6	T3.5 (5-7 days post flowering)	6/6/2017	6/5/2018	6/18/2019	6/3/20
7	T1 + T3				
8	T2 + T3				
9	T1 + T2 + T3				
10	T1 (Stratego YLD 4 fl oz/ac) + T3 (Prosaro 6.5 fl oz/ac)				

[a]The product Prosaro (6.5 fl oz/ac) was used for all treatments except for treatment 10 which was used as a standard industry recommended comparison. Non-ionic surfactant Induce was applied at 0.125% v/v with all applications.
[b]In 2018, the combination treatments were not sprayed at T1 as labelled because of error, instead the T1 applications in combinations (7 and 9) were sprayed at T1.5
[c]The T3.5 treatment was not included in all years

Results summary

Mean estimates of yield, leaf disease ratings, and other data collected are displayed in subsequent supplementary tables along with P value of type three test for fixed effect from ANOVA testing for each response variable. In 2017, there was moderate leaf disease pressure. Stripe rust was found in the trial area on May 27 and persisted at low levels. There was no significant difference in yield between treatments in 2017, but treatments 5-10 did significantly reduce flag leaf disease compared to treatments 1, 2 and 4. In 2018 disease pressure was low, with final leaf area impacted all below 12%. At the end of the season, the percentage of plots lodged was estimated and interestingly, lodging was significantly greater in plots that had received a T3 fungicide application. An explanation for this was not determined. In 2019, all treatments significantly reduced severity of flag leaf disease at the end of the season, but incidence was not statistically reduced across all treatments. In 2020, all treatments significantly reduced foliar disease compared to the non-treated control, except for treatment 2. At the end of the season treatment 2 did not result in a reduction of disease compared to the non-treated control, but

Table A2.2 Mean estimates and ANOVA results of yield, lower leaf fungal disease ratings, and flag leaf fungal disease ratings for 2017 fungicide application timing trial in East Lansing, Michigan.

Treatment	Yield (bu/ac)	Lower leaf rating*		Flag leaf disease ratings									
		May 9	18May	May 25	May 27	Jun 1	Jun 8		Jun 15 stripe rust		Jun 15 other**	Jun 20 stripe rust		Jun 20 other**	
1	66.3	16.4	15.6	5	7	10	22.8	ab	20.4	ab	10.4	26.6	ab	14.8	a
2	74.1	17.8	15.6	6	8.6	13.6	25.2	ab	30.4	ab	8.2	39.6	ab	13	ab
3	77.6	18.2	9.8	0.8	3	3.2	3.6	c	0.6	c	6.8	2	c	9	abc
4	63.4	15.2	12.2	6	6.2	7.8	11.8	bc	10.4	bc	10	22	b	13.8	a
5	61.5	16.2	12.2	2.6	5.2	5.6	9.6	c	3.2	c	6.8	2.2	c	5.6	c
6	72.5	17.2	12.4	3.6	4.2	4.8	9.4	c	6.6	c	6.4	5.6	c	6.2	c
7	74.9	15.8	11.8	3	6.8	8.2	11.2	c	3.6	c	7	3.4	c	7.2	bc
8	64.0	16.6	11.8	3.8	3.4	6.4	9.8	c	3.8	c	8	1.8	c	5.8	c
9	67.5	16.8	13.2	2.8	4.4	5.4	6.8	c	1.2	c	6.6	1.2	c	6.6	c
10	73.1	14.6	13.2	3.2	5.6	5.8	6.2	c	3.2	c	9.8	2.2	c	4.4	c
*P****	0.807	0.447	0.059	0.09	0.153	0.099	0.006		0.001		0.686	0.0001		0.007	

* these were ratings of the L2 and L3, not flag leaf

**rating of all other fungal pathogens excluding stripe rust which was rated and presented separately

***type III test of fixed effects with mean separation determined by LSD at α=0.05 represented by letters

Table A2.3 Mean estimates of yield, lodging, test weight, and end of season flag leaf fungal disease ratings for 2018 fungicide application timing trial in East Lansing, Michigan.

Treatment	Yield	Lodging*		Test Weight		Flag leaf disease (Jun 21)	
1	91.8653	7	D	59.66	B	10	A
2	88.7164	10.2	DC	59.28	C	9.4	AB
3	91.1905	9.8	DC	59.64	B	11	A
4	85.8921	11.4	DC	59.64	B	3.4	ABDC
5	75.8346	31.2	BA	60.38	A	0.6	D
6	78.1097	25.4	BAC	60.28	A	9	BCD
7	75.4872	34	A	60.36	A	2	BDC
8	73.8083	36	A	60.5	A	1.2	CD
9	78.6569	30	BA	60.3	A	1.6	BCD
10	91.4588	17.8	BDC	60.38	A	1	D
*P***	0.0977	0.0149		<.0001		0.023	

*lodging at harvest evaluated by visual estimation of the percentage of the plot with any lodging

**type III test of fixed effects with mean separation determined by tukey's test at α=0.05 represented by letters

Table A5.4 Mean estimates of yield, test weight, grain moisture, FDK, kernel weight, and DON for 2019 fungicide application timing trial in East Lansing, Michigan.

Treatment	Yield (bu/acre)	Test weight	Moisture (%)	FDK*	200 kernel Weight (g)	DON** (ppm)
1	89.4 B	51.0 C	14.5 D	12.6 A	8.2 C	6.7 A
2	96.3 AB	50.8 C	14.6 CD	14.8 A	8.3 BC	7.5 A
4	104.6 A	51.5 BC	14.8 BCD	14.4 A	8.7 AB	7.9 A
5	107.7 A	52.6 A	15.4 ABC	6.6 B	8.9 A	2.3 B
6	98.8 AB	52.4 AB	15.4 ABC	5.4 B	8.8 AB	2.9 B
7	106.1 A	52.5 AB	15.4 ABC	6.6 B	9.3 A	2.5 B
8	99.2 AB	52.8 A	15.6 AB	5.4 B	9.2 A	2.6 B
9	107.4 A	52.6 A	15.7 A	4.4 B	9.2 A	2.0 B
10	109.7 A	52.2 AB	15.7 A	4.2 B	9.1 A	2.6 B
*P****	0.00120	<0.0001	<.0001	<.0001	<0.0001	<0.001

*number of fusarium damaged kernels out of 200 kernels rated

**Deoxynivalenol concentration in grain determined by USWBSI testing lab (Dr. Dong, University of Minnesota)

***type III test of fixed effects with mean separation determined by tukey's test at α=0.05 represented by letters

Table A5.5 Mean estimates of fungal disease ratings of lower leaves early in the season and flag leaves on Jun 14 and July 11 in 2019 fungicide application timing trial in East Lansing, Michigan.

Treatment	Lower leaf rating May 21		Flag leaf rating Jun 14		Flag leaf rating July 11	
	Incidence	Severity	Incidence	Severity	Incidence	Severity
1	50.5	7.6 AB*	30.5 AB	6.5 BC	100.0 A	35.5 A
2	24.0	5.4 AB	15.6 B	3.4 D	100.0 A	21.0 B
4	17.0	5.6 AB	41.0 AB	2.8 D	90.0 ABC	13.4 BC
5	42.0	7.2 AB	27.0 AB	15.0 A	92.0 ABC	12.0 BC
6	68.0	9.8 A	35.0 AB	9.4 B	97.0 AB	16.4 BC
7	16.2	4.6 AB	12.6 B	5.0 CD	64.0 C	6.4 C
8	19.8	3.4 B	55.0 A	5.0 CD	82.0 ABC	5.0 C
9	31.4	3.0 B	39.0 AB	5.6 CD	70.0 BC	5.4 C
10	32.0	3.2 B	14.4 B	3.0 D	78.0 ABC	6.8 C
P[*]	0.023	0.0009	0.0001	0.0001	0.0002	<.0001

[*]type III test of fixed effects with mean separation determined by tukey's test at α=0.05 represented by letters

Table A5.6 Mean estimates of yield, test weight, moisture, and fungal disease ratings for 2020 fungicide application timing trial in East Lansing, Michigan.

Trt	Yield (bu/ac)		Test Weight		% Moisture		Lower leaf disease May 8		lower leaf disease May 21		Flag leaf disease Jun 15				flag leaf disease Jun 24			
							incidence	severity	severity	incidence	incidence		severity		incidence		severity	
1	81.2	C	54.7	B	12.1	B	48	14	14	44	15.8	A	4.6	A	98	A	19	A
2	88.9	AB	54.6	B	12.2	AB	54	16	12.4	47	9.6	AB	4.2	AB	92	A	14.4	A
4	89.6	AB	55.4	A	12.3	AB	62	9	15	53	4	B	2.2	CD	23	BCD	3.8	BC
5	88.3	B	55.6	A	12.4	A	56	11	12.4	50	8.4	AB	4.2	AB	28	BC	4.4	BC
6	87.1	BC	55.6	A	12.4	A	70	16	11.4	35	14	A	4.6	A	35	B	6.2	B
7	88.8	AB	55.4	A	12.4	A	56	13	13	40	3.8	B	3.4	ABC	10.6	CDE	2	BC
8	94.7	A	55.7	A	12.3	AB	48	21	11	50	2.2	B	2.6	BCD	3.6	E	1.4	C
9	87.9	B	55.5	A	12.2	AB	52	12	13	40	2.6	B	1.4	D	3.8	E	2	BC
10	92.0	AB	55.3	A	12.3	AB	62	14	13	40	4.2	B	2.6	BCD	9	DE	3	BC
P^*	0.011		0.001		0.088		0.632	0.261	0.739	0.441	0.029		0.003		<.0001		<.0001	

*type III test of fixed effects with mean separation determined by tukey's test at α=0.05 represented by letter

Chapter 6 **:Concluding Statement**

Main conclusions

Fusarium spp. are a global threat to grain quality, animal health, human health, and producer profitability due to their impact on yield and mycotoxin contamination in wheat and corn. In order to elucidate which species are infecting Michigan wheat and corn, 560 isolates of *Fusarium* were collected from 121 fields in Michigan characterized genotypically and phenotypically in order to inform disease management strategies.

F. graminearum comprised 82% of recovered isolates in wheat, but only 37% of isolates recovered from corn. Members of the *Fusarium tricinctum* species complex were also identified in nine wheat fields, and warrant further investigation moving forward. In corn, *F. subglutinans* represented 33.3% of the collection. *F. awaxy*, a species not yet reported in the U.S., was identified in six corn fields, comprising 4.6% of the collection.

Trichothecene chemotypes were determined for all *F. graminearum* isolates, and the majority were the less toxigenic 15-ADON chemotype. However, twenty-six (6%) were identified as 3-ADON, and seven isolates (1.5%) as NX-2. Most of these NX-2 and 3-ADON isolates were found in the same region, from five fields in the far northeastern part of the state with less agricultural land use, suggesting non-agricultural areas may act as an inoculum source of diverse genotypes.

Fungicides are an important tool in wheat and corn to manage *Fusarium* diseases and reduce toxin accumulation, thus the *in vitro* and *in vivo* sensitivity to demethylation inhibitor (DMI) fungicides was characterized. While a wide variation of *in vitro* responses was observed, no differences in efficacy of metconazole or tebuconazole were observed in a field trial comparing the isolates most sensitive and least sensitive to DMI fungicides. Baseline sensitivity to a new succinate dehydrogenase inhibitor active ingredient, pydiflumetofen, was also

established and demonstrated Michigan populations of *F. graminearum* are very sensitive to this active ingredient.

An additional aim of this work was investigating the response of Fusarium head blight, foliar diseases, and yield to various management inputs. A trial was established in East Lansing, MI (2014-2018) on soft white winter wheat cultivar 'Ambassador' to investigate the risks, benefits, and interactions of two nitrogen levels, three fungicide regimes, and a plant growth regulator trinexapac-ethyl. Presence and magnitude of responses varied across years, as did disease pressure. In some years, the high nitrogen treatments had significantly higher fungal disease and lower yield compared to low nitrogen treatments. This trial also provided evidence that lodging due to high nitrogen rates in wheat can increase Fusarium head blight incidence and foliar disease, which has not been previously well documented in the literature, although discussed anecdotally.

Finally, a meta-analysis of previously conducted fungicide trials from 2007 to 2020 in Michigan was performed to investigate the variation in yield responses to single and double fungicide applications made at various timings. All timings had an 87% probability of a positive yield response in future applications. There were statistically significant differences between some application timings. Probabilities of positive yield response and prediction intervals were calculated for all applications to aide growers in making future fungicide application decisions.

Potential directions of future work

Our survey of wheat and corn recovered numerous besides *F. graminearum*, which could suggest mycotoxin contamination with toxins besides deoxynivalenol could be occurring. Members of the FTSC and FFSC found here can produce T2 and HT-2 toxin, moniliformins, beauvericins, fumonisins, and diacetoxyscirpenols, among others. Future work could more

systematically sample grain to determine if these mycotoxins are being produced in Michigan cereals and their prevalence. While we found species that are reported to produce these diverse toxins, we do not yet know if they are being produced at significant levels in Michigan fields. These studies also revealed differences in fungicide sensitivity between these diverse species, with FTSC species significantly less sensitive to DMI fungicides. Further investigation *in vivo* should work to characterize efficacy of fungicides across these diverse species. This work also demonstrated *F. graminearum* was very sensitive to the new active ingredient, pydiflumetofen, however other species have not been characterized. Investigation into the sensitivity of FFSC and FTSC to pydiflumetofen would be informative. These future studies together could lead to implementation of species specific fungicide application recommendations.

Finally, the characterization of *F. graminearum* chemotypes generates many questions about the role and distribution of the three trichothecene chemotypes, 15-ADON, 3-ADON, and NX-2. What, if any, role does this diversity play in the biology and epidemiology of these diseases? All NX-2 isolates and a majority of the 3-ADON isolates were found in a single region in the state, across 6 fields. One hypothesis in the literature suggests that the high prevalence of more diverse natural landscapes may be driving this diversity; sampling of non-hosts plants in across regions could help elucidate drivers of this diversity.

The meta-analysis of fungicide yield responses underscores the need for more predictive modeling tools to support fungicide application decisions making in-season. While there is a high probability of a positive yield response, that response may not be profitable in every year, and overuse of fungicides reduces sustainability of high-intensity wheat management. Results here underscore the variability of responses over site years, and future work can investigate

under which crop and disease conditions fungicide applications are needed for efficient wheat production.

Impacts

The sum of this work not only further informs the biology and ecology of Fusarium diseases, but will allow for more precise management of *Fusarium* in wheat and corn. Species diversity is not only important for accurate management recommendations, but also for monitoring of mycotoxin contamination in wheat and corn. Baseline sensitivity data for pydiflumetofen and characterization of DMI sensitivity provide a valuable reference point to monitor future shifts in fungicide sensitivity. This work also validated the use of a 1 ug/mL screening dose for effective evaluation of DMI sensitivity in *Fusarium* spp.

Results presented here also impact wheat management strategies. The field trial investigating fungicide regimes, nitrogen, and a plant growth regulator provide new information on management of wheat, with an emphasis on how nitrogen can affect FHB directly and indirectly through the process of lodging. Meta-analysis of fungicide efficacy trials from 2007-2020 allowed us to determine the range of yield responses and compare performance of applications made at different growth stages. Probabilities of positive yield response and prediction intervals were calculated for all regimes to aid growers in making future fungicide application decisions.

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