TAR SPOT OF CORN: POPULATION DYNAMICS, ECONOMIC IMPACT AND MANAGEMENT IN MIDWESTERN UNITED STATES

by

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This dissertation is dedicated to my loving family. A special gratitude to my belated mom, Hulda Heywood Browne, who is my source of daily motivation, and has kept me pursuing my academic ventures with excellence. Although she has departed this world earlier than expected, her memories, and core values continue to guide my life. "Make me proud, make yourself proud, and make your country proud."

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!! Cheers to the First Doctor in the Family!!
WE DID IT!

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OVERVIEW.

Tar spot is a new foliar disease of corn in the United States. Tar spot was first detected in 2015 and is now among the most important corn diseases in the Midwest. Tar spot is caused by the obligate biotrophic fungus, *Phyllachora maydis* Maubl, from the genus, *Phyllachora* which consist of over 1,200 species of host-specific fungi. Due to the recent emergence, studies relating to *P. maydis* population dynamics in the U.S. are limited. How much genetic diversity, variation, and level of gene flow are occurring within and among these populations? Knowledge of the population dynamics is imperative for understanding the pathogen's biology, ecology, epidemiology, and management. Currently, no corn hybrid is fully resistant to tar spot. Foliar fungicides are currently the most effective option for disease management, but best practices for fungicide management remain unknown. Better information is needed on fungicide efficacy and fungicide application timing to reduce tar spot severity, protect yield, and increase profitability for Indiana corn growers.

This research dissertation presents four chapters to answers those questions and bridge the gaps between the knowns and unknows of this novel corn-*Phyllachora maydis* pathosystem. **Chapter 1** presents a literature review on tar spot of corn, its economic impact, the causal pathogen, its host, lifecycle, distribution, and known management strategies as a resource for understanding the pathosystem in the U.S. **Chapter 2** examines the genetic population structure, diversity, geneflow and mode of reproduction in Midwest U.S. by employing microsatellite (SSR) markers. **Chapter 3** presents results from multi-year, multi-location, small-plot field trials on the net return of foliar fungicides and fungicide timing on tar spot management in Indiana. Lastly, **Chapter 4** concludes by evaluating of an integrated management strategy for tar spot by examining the integration of tillage, corn hybrids and fungicide application in reducing tar spot severity while protecting yields.

Results provided in this research dissertation will be used to guide future studies and provide stakeholders such as researchers, corn growers, extension personnel in academia and industry with valuable information needed to guide effective disease management decisions.

CHAPTER 1. LITERATURE REVIEW - TAR SPOT: AN UNDERSTUDIED DISEASE THREATHENING CORN PRODUCTION IN THE AMERICAS.

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1.1 Tar spot: a new foliar disease of corn with significant economic impact in the U.S.

Tar spot caused by *Phyllachora maydis* Maubl, an obligate fungus, is a major foliar disease of corn. Tar spot can reduce grain yield, and quality of silage, stover, and husks (Maublanc 1904; Hock et al. 1989; Bajet et al. 1994). In Latin America, economic damage of up to 50 percent has been documented when epidemics are severe early in the crop's reproductive phases. *P. maydis* is endemic to Latin America, where it was first identified in Mexico in 1904 (Maublanc 1904; Abbott 1931; Malaguti and Subero 1972; Liu 1973; Bajet et al. 1994). Beginning in 2015, *P. maydis* appeared and has spread in the Midwestern United States (Bissionnette 2015; Ruhl et al. 2016; McCoy et al. 2018; Dana Lana et al. 2019; Malvick 2020).

In Latin America, tar spot is purportedly associated with two additional fungi (Hock et al. 1995): *Monographella maydis* Müller and Samuels, a necrophyte, and *Coniothyrium phyllachorae* Maubl, a fungal hyperparasite (Hock et al. 1995). In the U.S., however, only *P. maydis* has been documented in association with tar spot (McCoy et al. 2019). The disease can cause corn grain yield losses ranging from 11 to 46 percent in Latin America (Hock et al. 1989; Pereyda-Hernández et al. 2009). Corn grain yield losses of up to 25 to 30 percent were recently reported in the Midwestern U.S. (Telenko et al. 2019; Mueller et al. 2019). Due to a lack of information about this pathosystem and the dire threat tar spot poses to U.S. corn production, there is a pressing need for research on the biology, ecology, epidemiology, and management of the organism(s) that cause tar spot. This Feature Article reviews the available literature on tar spot of corn and the other

species associated with this disease to help guide current and future research on this economically important pathosystem.

1.2 Signs, symptoms, causal agent(s), and host range.

Tar spot is characterized by the formation of black stromata, the fruiting bodies of *P. maydis*, on the foliage. The stromata resemble spots of tar (Figure 1.1). Like other species in the genus, *P. maydis* is an obligate biotroph, requiring a living host to grow and reproduce (Cannon 1991). In fields with infested corn residue, initial signs and symptoms of tar spot may appear in the lower canopy of the corn plant (Bajet et al. 1994). In the U.S., however, "top down" patterns of symptom development, in which upper portions of the plants exhibit symptoms first, occur frequently in sites where new outbreaks occur, suggesting long distance transmission of inoculum. For instance, plants of any age, leaves, leaf sheaths, and husks are susceptible to infection (Bajet et al. 1994; Hock et al. 1995).

Infection by *P. maydis* results in the development of glossy structures (masses of black fungal tissue) known as stromata (Figure 1.2) (Hock et al. 1995; Carson 1999; CIMMYT 2003). Stromata are embedded in host tissue and scattered across or clustered on both leaf surfaces, occasionally coalescing into stripes (Liu 1973). Stromata are sometimes enclosed by brown, elliptical, necrotic halos referred to as "fisheye lesions" (Figure 1.3). In severe cases, necrotic halos coalesce, causing extensive necrosis and leaf blight leading to premature senescence and death of plants (Ceballos and Deutsch 1992; Hock et al. 1995; Carson 1999). The host range for *P. maydis* appears to be restricted to *Zea mays* (Cline 2005), although other *Phyllachora* species cause tar spot on a wide range of grass species and other hosts (Parbery 1967, 1971).

Figure 1.1. Fungal fruiting bodies of *Phyllachora maydis* on the foliage resemble spots of tar.

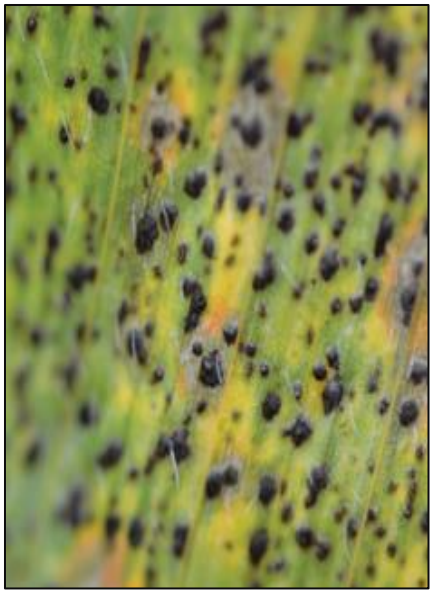

Figure 1.2. Slightly- raised, semi-circular, dark brown to black glossy structures known as stromata are shielded by clypeus.

Figure 1.3. Stromata can be enclosed by brown, elliptic, necrotic halos known as "fisheye lesions" (indicated by arrows).

Older literature indicated that fisheye lesions were always associated with the presence of the fungus Monographella maydis (Muller and Samuels 1984; Ceballos and Deutsch 1992; Hock et al. 1992; Bajet et al. 1994; Hock et al. 1995). However, these results were based on limited surveys, identification was based solely on morphological characteristics, and no voucher specimens were deposited. In Latin America, infection by *P. maydis* or *M. maydis* alone was initially considered to be of minor importance (Müller and Samuels 1984; Hock et al. 1991). Dual infection with *M. maydis* and *P. maydis* was implicated in significant leaf necrosis and yield loss (CIMMYT 2003). In field conditions where both fungi were present, researchers speculated that *M. maydis* entered plants following infection by *P. maydis* and subsequently produced a toxin that caused the fisheye lesions. However, in Mexico, Ecuador, Honduras, and the U.S., fisheye symptoms were sometimes present but *M. maydis* was absent (Ceballos and Deutsch 1992; Ruhl et al. 2016; McCoy et al. 2019). McCoy et al. (2019) carried out a Next-Generation sequencing analysis to determine if *M. maydis* was present in fisheye lesions on samples collected in Michigan, and to identify the different fungi found in tar spot lesions with and without fisheye symptoms. Two *Microdochium spp.* operational taxonomic units (OTU) were identified; however, neither was

abundant nor associated consistently with fisheye symptoms. No evidence of *M. maydis* was found among U.S.-associated fisheye samples (McCoy et al. 2019).

Another fungus, *Coniothyrium phyllachorae* Maubl was also speculated to be associated with stroma of *P. maydis* (Maublanc 1904; Müller and Samuels 1984). *C. phyllachorae* is a fungal hyperparasite that destroys perithecia produced by *P. maydis* (Maublanc 1904), suggesting that *C. phyllachorae* may be used as a biological control for tar spot rather than being responsible for tar spot symptoms. However, this potential management strategy has not been tested (Hock et al. 1995). The observation that tar spot lesions containing *C. phyllachorae* are usually smaller than lesions containing *M. maydis* (Hock et al. 1989, 1995) has not been explained.

1.3 Biology of spores.

P. maydis is an ascomycete, producing sexual spores (ascospores) and asexual spores (conidia) (Figures 1.4 and 1.5). Ascospores are formed in single-walled asci within a single perithecium covered by stromata. Eight ovals to ovoid ascospores, 10-14 µm by 5.5-8 µm, are produced per ascus (Maublanc 1904; Liu 1973; Hock et al. 1992). Ascospores are discharged through the perithecial ostiole in a mucilaginous mass (Figure 1.6). A single perithecium will discharge spores repeatedly over the course of several days, occasionally producing pale cirrhi (Parbery 1963). Ascospores require a temperature range of 20 to 25 ^{0}C for optimal germination with relative humidity >75% and prolonged periods of leaf wetness (Maublanc 1904; Hock et al. 1989; Bajet et al. 1994; Pereyda-Hernández et al. 2009; Groves et al. 2020). Dittrich et al. (1991) found that in laboratory studies ascospore germination can occur in as little as 2 h in distilled water at 24 ^{0}C. These researchers also indicated that ascospore germination and formation of appressoria by *P. maydis* occurred between 10 and 20 ^{0}C, with appressoria forming within 12 to 24 h, which is consistent with other members of the genus (Parbery 1963).

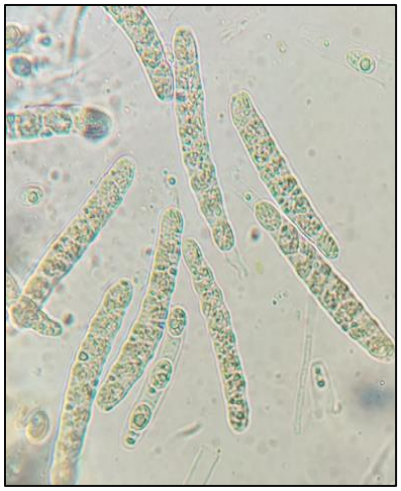

Figure 1.4. *Phyllachora maydis* sexual spores (ascospores).

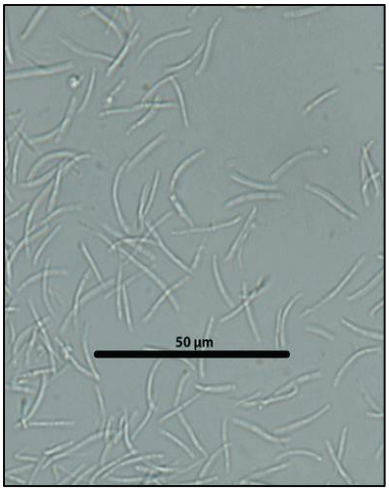

Figure 1.5. *Phyllachora maydis* asexual spores (conidia).

Figure 1.6. Sexual spores (ascospores) of *Phyllachora maydis* can be discharged through a perithecial ostiole in a mucilaginous mass.

Phyllachora maydis can overwinter on plant residue. In Mexico, on infected corn material that was left uncovered on the soil surface for 3 months, ascospores had a maximum germination rate of 3%. Recent studies by Kleczewski et al. (2019) and Groves et al. (2020) showed that ascospores overwintered in the Midwestern U.S. on corn residue despite harsh weather conditions (a low of-34 ^{0}C air temperature); these ascospores were able to germinate and infect seedlings under controlled conditions. Nevertheless, neither the mechanisms of overwintering nor the existence of alternative plant hosts of *P. maydis* is known (Mottaleb et al. 2019, Groves et al. 2020).

The pycnidial stage of *P. maydis* (*Linochora maydis*) may also be present in the form of filiform spermatia. Spermatia are 10-15 µm by 0.5 µm and are produced in pycnidial fruiting bodies, which are often found with perithecia in stromata. According to Parbery (1967) and Muller and Samuels (1984), these spores may fulfill the role of conidia in the *Phyllachora* life cycle. The genus *Microdochium* spp. includes important plant pathogens, particularly on grasses and small grain cereals (Von Arx 1987). *Microdochium* spp. are recognized as Fusarium-like fungi due to similar spore morphology. However, the conidigenous cells in *Microdochium* spp. are not phialidic as in true *Fusarium* species and the conidia have a truncate base rather than 'foot-cells' (Von Arx 1987). *Monographella maydis* (*Syn. Microdochium maydis* E. Müll. and Samuels) was first described in 1984 from leaf tissue in Mexico (Muller and Samuels 1984; Von Arx 1987; Hock et al. 1992; Bajet et al. 1994). Both the teleomorph and anamorph of *M. maydis* were recovered from

fisheye lesions, and inoculation of corn plants naturally infected with *P. maydis* and *M. maydis* conidial suspensions caused the characteristic fisheye lesions and significantly increased disease severity (Hock et al. 1992). However, a lack of methodological details in the Hock et al. (1992) study limits the credibility of these observations.

Monographella maydis forms single-walled asci within perithecia immersed in host tissue, eventually erupting through the epidermis. Eight fusiform ascospores, 18 to- 22 µm by 3.5 to 5 µm and containing 1 to 3 transverse septa, are produced per ascus. Conidia produced in sporodochia are hyaline, elongate, mostly curved, 20-46 µm by 3-4 µm with 3-9 transverse septa. The sexual stage of the pathogen is rarely found in the field. Conidial germination was greatest at 25°C in darkness (Dittrich et al. 1991). In inoculation trials during this research, infection of corn with Monographella maydis by itself was achieved in only one of eight attempts under 38/18^{0}C day/night temperatures and 80-100% relative humidity. *Monographella maydis* persists on infected crop residue, with conidia remaining viable for 109 days on detached leaves at room temperature (Hock et al. 1992).

1.4 Disease cycle.

The disease cycle of tar spot is not fully understood. However, ascospores and conidia of *P. maydis* can overwinter in stromata on decaying corn leaves or residue in fields (Kleczewski et al. 2019; Groves et al. 2020). Hence, infested residue with propagules are likely the source of primary inoculum. According to Hock et al. (1992), ascospores are released from stromata and disperse either by wind or rain splash to foliage during periods of moderate temperature (16 to 23^{0}C), leaf wetness duration of greater than 7 hours per night, and relative humidity >75% (Hock et al. 1995). Long-distance spore dispersal is another possible source of primary inoculum. However, ascospore dispersal has been documented to only as far as 31 m from the source of the inoculum (Liu 1973). Ascospores infect nearby corn plants and this cycle will repeat multiple times per growing season under conducive conditions (Hock et al. 1989; Bajet et al. 1994). In the U.S., in fields with no previous history of the disease, tar spot symptoms appeared first in the upper crop canopy (Robertson and Malvick 2020- personal communication). These observations raise questions about the possibility of long-distance dispersal. Neither the incubation period (time from inoculation to symptom development) nor latent period (time from inoculation to onset of reproductive structures) (Parlevliet 1979) has been clearly established for *P. maydis*. Preliminary data from two of our labs

indicated that the latent period can be variable, between 14 and 20 days at 16 to 23°C (Cruz and Kleczewski 2020 unpublished data). The duration of latent periods can be strongly influenced by growing degree days (GDD) and host resistance level (Precigout 2020). Symptoms of tar spot are observed 14 days after infection and new ascospores are produced in stromata soon thereafter (Hock et al. 1995). A schematic representation of the presumed disease cycle of tar spot in the U.S. is shown in Figure 1.7.

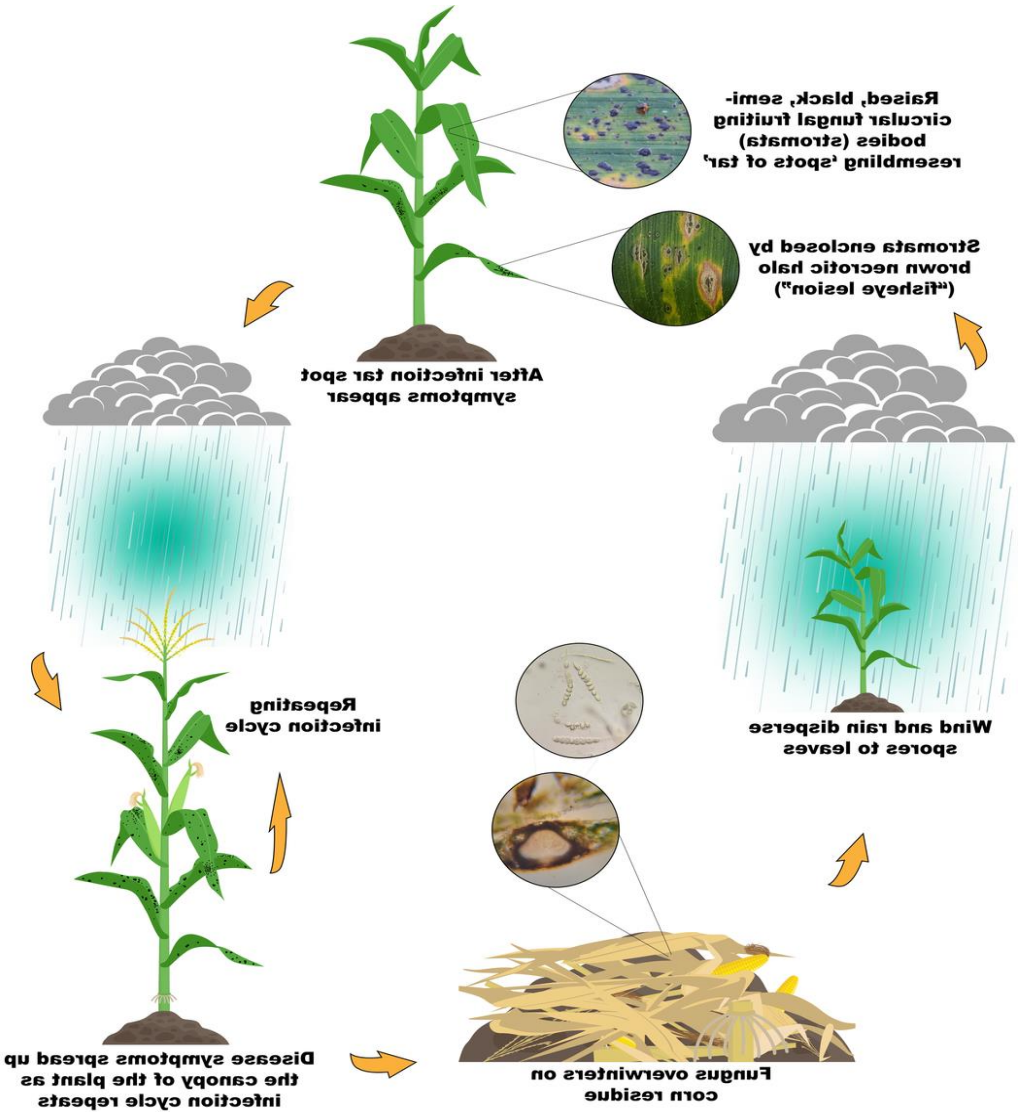

Figure 1.7. Schematic representation of the presumed tar spot disease cycle in the United States. *Phyllachora maydis* is capable of overwintering in corn residue and generating secondary infections. Symptoms of tar spot can be observed 14 days after infection and new ascospores are produced in stromata soon thereafter. Ascospore dispersal has been documented to only as far as 31 m from source. However, anecdotal evidence suggests that long-distance dispersal may also occur.

1.5 Geographical distribution.

Phyllachora maydis is endemic to parts of Mexico as well as Central and South America (Figure 1.8, Table 1.1), where it was apparently restricted for >100 years (Hock et al. 1995; Cline 2005). However, in 2015 it was detected for the first time in the U.S. and has spread significantly in the U.S. since then (Bissonnette 2015; Ruhl et al. 2016). In the U.S., *P. maydis* is now established in the states of Illinois, Indiana, Iowa, Michigan, Minnesota, Missouri, Ohio, Wisconsin, and Florida (Figure 1.8) (Bissonnette 2015; Ruhl et al. 2016; McCoy et al. 2018; Dalla Lana et al. 2019; Malvick et al. 2020). Multiple pathways have been proposed for the introduction of *P. maydis* into the U.S. (Ruhl et al. 2016; Mottaleb et al. 2019). Although *P. maydis* is not known to be seedborne, imported grains contaminated with leaf/husk residue can be a source of inoculum (Richardson 1990).

Figure 1.8. *Phyllachora maydis* was reported for the first time in Mexico in 1908 and currently is present in 14 additional countries. In the U.S. was reported in 2015 and is now established in nine states.

Table 1.1. Geographical distribution of tar spot based on available reports.

Continent/Country	Year documented	Source
Central, South America and Caribbean		
Peru	1931	Abbott (1931)
Dominican Republic, Guatemala	1944	Orton (1944); Bajet et al. (1994)
Bolivia	1949	Stevenson and Cárdenas (1949); Bajet et al. (1994)
Trinidad and Tobago	1951	Baker and Dale (1951)
U.S. Virgin Islands	1951	Stevenson (1975)
Honduras, Nicaragua, Panama	1967	McGuire and Crandall (1967)
Cuba	1968	Arnold (1986)
Colombia	1969	Castaño (1969); Bajet et al. (1994)
Venezuela	1972	Malaguti and Subero (1972); Bajet et al. (1994)
Puerto Rico	1973	Liu (1973); Bajet et al. (1994)
El Salvador, Haiti, Ecuador, Costa Rica,	1994	Bajet et al. (1994)
North America		
Mexico	1904	Maublanc (1904); Hock et al. (1989)
U.S. (Indiana and Illinois)	2015	Bissonnette (2015); Ruhl et al. (2016)
U.S. (Florida, Iowa, Michigan, Wisconsin)	2016	McCoy et al. (2018)
U.S. (Ohio)	2018	Dalla Lana et al. (2019)
U.S. (Minnesota, Missouri)	2019	Bissonnette (2019)- personal communication; Malvick (2020)

1.6 Molecular diagnostics.

The biotrophic nature of *P. maydis* makes it difficult to study in the laboratory, as it has never been cultured on synthetic medium (Muller and Samuels, 1984). The use of genetic technologies such as DNA diagnostics (amplification and sequencing) can compensate for this difficulty and provide a better understanding of the fungus. Prior to 2015, no *Phyllachora* spp. genomes had been sequenced, and hence no comparative sequence data were available in GenBank, NIH genetic sequence database, or the U.S. National Fungus Collection (BPI) (Ruhl et al. 2016). Phyllachora spp. were diagnosed mainly via symptom and morphological characters (Maublanc 1904; Parbery 1963; Muller and Samuels 1984; Hock et al. 1995; Ruhl et al. 2016). However, due to the recent documentation of *P. maydis* in the U.S., molecular diagnostic data are now available in GenBank and the NIH genetic sequence database. DNA was extracted from stromata that had been aseptically removed from corn leaves collected in each of the affected U.S. states, sequenced, and deposited in the U.S. National Fungus Collections (BPI) (McCoy et al. 2018). Currently, sequences for the internal transcribed spacer (ITS) regions of the ribosomal RNA gene are the only genetic sequences available for *P. maydis* in GenBank (Ruhl et al. 2016; McCoy et al. 2018). There are 67 specimen records for *Phyllachora maydis* and its synonyms in the U.S. National Fungus Collection (BPI), of which only five specimens were deposited based on molecular identification via ITS sequence confirmation. The current ITS sequences reported in the GenBank for identification of *P. maydis* are listed in Table 1.2.

Table 1.2. GenBank's available sequences for *P. maydis* identification.

Collection location	(NCBI Voucher) GenBank ID	Source
Indiana	(BPI 893231) No. KU184459	Ruhl et al. (2016)
Iowa	(BPI 910561) No. MG881848.1	McCoy et al. (2018)
Michigan	(BPI 910562) No. MG881847.1	McCoy et al. (2018)
Ohio	(18AP065) No. MK184990	Dalla Lana et al. (2019)
Wisconsin	(BPI 910560) No. MG881846	McCoy et al. (2018)

A draft genome sequence of *P. maydis* (Telenko et al. 2020) was recently published which will provide an important resource for further studies on the origin of *P. maydis* in the U.S., population structure, genetic diversity, and phylogenetic relationships among *Phyllachora* spp. In a recent paper, phylogenetic relationships among species in the order *Phyllachorales* were inferred based on Bayesian analysis incorporating sequence information from five molecular characters: 1) nuclear large subunit ribosomal DNA (nrLSU rDNA); 2) nuclear small subunit ribosomal DNA (nrSSU rDNA); 3) internal transcribed spacer ribosomal DNA (ITS rDNA), and the protein coding genes; 4) DNA-directed RNA polymerase II subunit 2 (RPB2); and 5) Elongation factor 1-alpha (TEF1) (Mardones et al. 2017). It is interesting to note that *P. maydis* showed close similarity to other *Phyllachora* spp. for all five of the molecular regions considered but appeared to be most closely related to *P. graminis* (Mardones et al. 2017).

A study by Hernández-Restrepo et al. (2016) used ITS, Elongation factor 1 alpha (EF1α), RNA polymerase II second largest subunit (RPB2), and small subunit nuclear ribosomal DNA (nrSSU) regions to construct a phylogenetic tree of the *Phyllachorales*, validating the use of these regions and generating sequences that could be adapted for future work with *P. maydis*. To date, there are three phylogenetic trees published with similar loci, but for the *Phyllachora* portion only the ITS gene was used, and species distinctions within the genus still need to be resolved.

A recent re-evaluation of *Monographella* considered four loci for use in taxonomic and phylogenetic studies of this genus. Of the four, the partial beta-tubulin gene region was found to be the most informative, with the RNA polymerase II second largest subunit gene (RPB2) also recommended. The translation elongation factor 1 alpha gene has also been used to differentiate between *Monographella* spp. Unfortunately, no genetic data for *M. maydis* exists in public databases (Hernández-Restrepo et al. 2016).

1.7 Genetic basis of host resistance and breeding for resistance.

Deploying host resistance is potentially both an economical and effective means of managing tar spot. A range of reactions to *P. maydis* have been observed in diverse corn germplasm, indicating that a range of resistance to tar spot exists (Ceballos and Deutsch 1992; Mahuku et al. 2016; Cao et al. 2017). Furthermore, the heritability of tar spot resistance is moderate to high, indicating that breeding to develop resistant populations is possible (Cao et al. 2017).

The genetic architecture of tar spot resistance is complex, but a single large-effect locus for resistance has been consistently detected (Ceballos and Deutsch 1992; Mahuku et al. 2016; Cao et al. 2017). An early study utilizing three segregating bi-parental populations found resistance to symptoms caused by *P. maydis* to be highly heritable and dominant in nature (Ceballos and Deutsch 1992). More recently, a large-effect quantitative trait locus (QTL) located in chromosomal bin 8.03, referred to as qRtsc8-1,/span> was consistently detected across multiple tropical/subtropical populations of corn screened in several locations across Central and South America (Mahuku et al. 2016; Cao et al. 2017). When detected, qRtsc8-1 accounted for 18-43% of the observed phenotypic variation in disease severity (Mahuku et al. 2016; Cao et al. 2017). It is interesting to note that the most significant association identified by Mahuku et al. (2016) in a genome-wide association mapping study was with a leucine-rich repeat receptor-like encoding gene, which would be consistent with a major resistance gene. Several haplotypes were identified in qRtsc8-1 that increased resistance (Mahuku et al. 2016). Together, these results indicate that marker-assisted selection for resistant qRtsc8-1 haplotypes might be an effective strategy for developing tar spot resistant varieties.

1.8 Hybrid reaction and susceptibility to tar spot.

A study by Telenko et al. (2019) evaluated corn hybrid reactions to tar spot during the 2018 U.S. Midwest epidemic. In that study, all hybrids rated were susceptible to tar spot pathogen. Severity of leaf symptoms ranged from minor (1-15%) to severe (40-50%). Data from these hybrid trials demonstrated a range in hybrid susceptibility and reaction to tar spot, where every 1% increase in tar spot severity resulted in an estimated 21.5 to 91.5 kg/ha loss (Telenko et al. 2019).

1.9 Future outlook and challenges.

Tar spot has become a high-profile emerging disease in the U.S. due to its recent identification and spread, documented impact on corn yields, and the threat it poses to corn production. Mottaleb et al. (2019), indicated that tar spot can become established throughout the U.S. Corn Belt. Unfortunately, there is a general lack of information about this pathosystem. For instance, currently there is no evidence of *M. maydis* association with fisheye lesions in the U.S. Hence, future research that surveys a large collection of tar spot-infected corn from different

regions would help test previously established hypotheses and provide critical information to understand this disease and fisheye symptom development. Hypotheses that may explain these observations are that fisheye lesions are a result of *P. maydis* infection alone, and/or specific pathogen x host x environmental conditions result in their development. Alternatively, fisheye lesions may be caused by a different fungus that was incorrectly identified as *M. maydis* in previous studies. This hypothesis is difficult to confirm as no vouchers of *M. maydis* exist from the initial species description and no molecular data exist for *M. maydis* (Hernández-Restrepo et al. 2016). *Monographella* previously were defined as members of the genus *Fusarium*. Could certain local species of *Fusarium* be responsible for fisheye development? Finally, an unidentified organism may be the cause for the development of fisheyes.

The events underlying *P. maydis* emergence in the U.S. are currently unknown. Thus, there is need for investigating the genetic diversity and population structure of *P. maydis*. This information will help determine whether *P. maydis* was an endemic pathogen already present in the U.S. that underwent genetic changes that resulted in the ability to infect corn, or whether *P. maydis* was introduced to the U.S. by movement of people, crop material, or weather systems. Developing effective and long-lasting prevention strategies is key for tar spot management. For that reason, we need to increase the current understanding of pathogen biology and disease epidemiology, which would include a better understanding of changes in disease intensity over time and space. Visual tar spot surveillance methods and diagrammatic scales that partition severity into predetermined stroma or fisheye/necrotic severity classes are available (Hernández and Islas 2015). However, these diagrammatic scales are based on leaf sections rather than the whole leaf; this might present a challenge as symptoms might not be uniform across the leaf blade. Although the development and diversity of tar spot symptoms has not been characterized thoroughly, such work is foundational for disease phenotyping. The information generated is key to developing epidemiological criteria to support breeding tactics (Fernandez-Campos and Gongora-Canul et al. 2020) against this disease. Autonomous aerial vehicles offer an alternative for tar spot phenotyping since they can be equipped with a range of sensors that measure spectral reflectance (Loladze et al. 2019; Mahlein et al. 2016). Several vegetation indices obtained from multispectral and thermal data have been correlated with tar spot severity and losses of grain yield in the absence of fungicide treatment (Loladze et al. 2019). Future studies in this area should

determine whether remote sensing platforms can describe temporal and spatial dynamics of the disease (Gongora-Canul et al. 2020).

Effective management strategies for tar spot are limited and are based on what is known of tar spot in Mexico, Central America, and South America (Kleczewski et al. 2019). Limited field data is available from the U.S. Kleczewski et al. (2019) proposed that management strategies need to target environmental conditions, fungal populations, hybrid genetics and cropping systems associated with each region. Tar spot management strategies have been recommended but remain limited in the U.S. due to the recent appearance of this pathogen. These strategies include (1) avoid highly susceptible hybrids, (2) consider application of fungicides with mixed mode of action at appropriate timing close to the onset of the epidemic, (3) manage irrigation, (4) rotate crops to allow *P. maydis* infected residue to decompose, and (5) remove residue from fields (Kleczewski et al. 2019; Telenko et al. 2019). Though fungicides are available for managing tar spot, the optimum timing and number of applications needed if an early epidemic occurs is not well established. Teams are also working on the development of a reliable protocol for artificial inoculations under controlled environments, and to determine economically sound management options for combatting tar spot. Host resistance will become an important tool for control of tar spot. Little is known about resistance in germplasm adapted to the U.S. and whether previously identified QTLs will be effective against *P. maydis* populations in the U.S. Furthermore, genomic selection is a powerful tool that can take advantage of many small-effect loci to develop resistant lines (Meuwissen et al. 2001; Poland and Rutkowski 2016). Genomic prediction models had moderate-to-high prediction accuracy for tar spot, showing promise that genomic selection may be an effective method to improve tar spot resistance in breeding programs (Cao et al. 2017).

We believe that the development of effective management strategies for this understudied pathogen requires increased understanding of its biology and epidemiology, and developing and deploying rapid diagnostic methods, effective weather-based warning systems, systematic surveillance, and resistant germplasm for regions at risk.

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CHAPTER 2. SMALL BUT SIGNIFICANT GENETIC DIFFERENTIATION AMONG POPULATIONS OF PHYLLACHORA MAYDIS IN MIDWESTERN UNITED STATES REVEALED BY MICROSATELLITE (SSR) MARKERS.

*This chapter will be submitted as a research article in Plant Disease Journal.

2.1 Abstract.

Phyllachora maydis Maubl, the causal pathogen of tar spot of corn (*Zea mays* L.), has recently emerged in the United States and Canada. Studies related to its genetic diversity and population structure are limited and are necessary to improve our understanding of this pathogen's biology, ecology, epidemiology, and evolutionary potential within this region. This study developed and used, 13 microsatellites (SSR markers) to assess the genetic population structure, diversity, gene flow and reproductive mode of 181 *P. maydis* samples across five geographical regions (states) in the Midwest U.S. The polymorphic information content (PIC) of loci ranged from 0.32 to 0.72 per locus, indicating their high utility for assessing the dynamics of *P. maydis* populations. Analysis of molecular variance (AMOVA) detected a low but significant genetic differentiation (F_{ST} = 0.15) among populations, where 85% of the variance resided within populations and only 15% was among populations. *P. maydis* populations are highly diverse (He = 0.55), reproducing sexually (rBard; $p = > 0.001$), with moderate gene flow (Nm = 2.80), but not geographically structured. Cluster analyses based on genetic distances, principal coordinate analysis (PCA), and STRUCTURE algorithm predicted two microsatellite clusters (k = 2) of severe genetic admixture among Midwest populations. Samples did not cluster exclusively by geographical regions; although Indiana samples clustered chiefly together, those from other states were more dispersed indicating admixture among the 181 samples from the five Midwest regions. These 13 highly polymorphic molecular markers could be used for future investigations of this pathogen's population dynamics both within and outside of the U.S.

2.2 Introduction.

Corn (*Zea mays* L.) is the largest staple food crop produced globally, at 1.1 billion metric tons (USDA-NASS 2021). It is an essential source of animal feed, food, fuel, and export in the United States (USDA-NASS 2021) but is susceptible to several diseases incited by many plant pathogens (Wise et al. 2016; Mueller et al. 2020). In 2015, a new foliar disease of corn, tar spot, was detected in the U.S. in Illinois and Indiana but without significant economic impact (Ruhl et al. 2016). However, in 2018, several corn-producing areas in the Midwest experienced their first major epidemic of tar spot in which 4.5 million metric tons (184.9 million bushels) worth $US 658.7 million were lost (Mueller et al. 2020). Tar spot was later detected in Ontario, Canada in 2020 (Tenuta 2020). Tar spot has become one of the most significant and severe diseases of corn in the Midwest and Ontario, Canada in terms of economic and yield losses in recent years (Mottaleb et al. 2019; Mueller et al. 2022). In 2021, 6.0 MMT (235.2 million bushels) of corn yield were lost in the U.S. and Ontario, Canada valued at $US 1,268 MMT (Mueller et al. 2022).

Tar spot is characterized by signs and symptoms of small black fungal fruiting structures (stromata) scattered over the leaf surface and necrotic lesions that likely lead to reduced photosynthetic efficiency, resulting in poor grain fill, reduced silage quality, and poor yields. Corn yield losses of 40-60% can occur in severely affected fields in North America with some fields experiencing complete crop loss if tar spot is not managed (Dittrich et al. 1991; Hock et al. 1995; Pereyda-Hernández et al. 2009; Mottaleb et al. 2019; Telenko et al. 2020).

Tar spot of corn is caused by *Phyllachora maydis* Maubl, an obligate biotrophic fungal pathogen belonging to the order *Phyllachorales* in the class Sordariomycetes (Maublanc 1904; Parbery 1967; Bajet et al. 1994). This order contains approximately 160,000 species known globally, of which 1,226 are currently acknowledged (Cannon 1997; Kirk et al. 2008; Maharachchikumbura et al. 2016; Mardones et al. 2017). Mardones et al. (2017) and Broders et al. (2021) conducted a comprehensive assessment of *P. maydis*, providing evidence that understanding of this species and its genera is limited and requires significant attention. Species found in the order *Phyllachorales* are associated with many graminaceous plants but can also infect dicots and are presumed to be highly host specific but found on hosts across a vast range of habitats. Although the assumptions of host specificity do not always hold for some genera within the *Phyllachorales*, so far corn is the only known host for *P. maydis* (Cannon 1991 and 1997; Cline 2005; Kleczewski et al. 2020; Parbery 1967; Valle-Torres et al. 2020).

Phyllachora maydis was first identified in Mexico during 1904 and was detected in 18 other countries in the Caribbean, Central, and South America during the past century. Since *P. maydis* first detection in the U.S., the disease has spread to at least 14 states in the U.S. and Ontario, Canada, and its range continues to expand every year (https://corn.ipmpipe.org/tarspot/.). *Phyllachora maydis* has two reproductive states; the sexual state produces ascospores and the asexual, conidia. Infection is presumed to occur primarily via the ascospores, which are thought to overwinter in asci in perithecia produced in stromata on corn residue (Groves et al. 2020; Kleczewski et al. 2019). Under warm and humid conditions, ascospores are ejected forcibly from perithecia and can be disseminated up to 1,200 m from the inoculum source by wind or rain to nearby susceptible plants (Kleczewski and Bowman 2020). Infection by ascospores typically becomes visible within 12-15 days (Hock et al. 1995; Carson 1999; Kleczewski et al. 2019; Telenko et al. 2021) as brown-black, raised, semicircular fungal bodies (stromata) on the surfaces of corn foliage that resemble spots of tar. Stromata also may occur on stalks and ear leaf husks. In severe cases, leaf blights, and early senescence may occur, leading to plant death (Carson 1999; Hock et al. 1995; Parbery 1967; Valle-Torres et al. 2020). Tar spot signs and symptoms usually progress from the lower to the upper canopy under favorable conditions (Hock et al. 1989; Bajet et al. 1994), but in the Midwest U.S. and Canada, "top-down" disease progression also has been observed particularly when the pathogen moves into new areas (Valle-Torres et al. 2020).

The conditions that facilitated the emergence and rapid spread of *P. maydis* in the U.S. and Canada are unclear. Valle-Torres et al. (2020) and Broders et al. (2021) hypothesized that *P. maydis* may have emerged in the U.S. due to an introduction from Mexico, Puerto Rico, or other Central American countries through several possible pathways, including the movement of infected plant materials by humans and possible long-distance dispersal of spores by wind, rain, or tropical storms such as hurricanes. Despite the persistence of *P. maydis* in cornfields across the U.S. and Canada, there is limited information on the pathogen's genetic variation, gene flow, and population structure, which may influence disease severity and affect the efficacy of the host resistance component of disease management. One or multiple cycles of sexual reproduction annually may help generate variation in the *P. maydis* population in the U.S. Additionally, recombination may occur via sexual reproduction, leading to high variation and diversity and consequent changes in the population genetic structure in the U.S. Gene flow is an evolutionary factor that substantially defines the population structure of many plant pathogen species

(McDermott and McDonald 1993). Gene flow is the movement of genes into or out of a population (Saltkin 1985; McDermott and McDonald 1993). This can occur in fungi by the movement of spermatia, or migration of individuals and even a diverse population. Gene flow leads to genetic homogeneity among populations in the absence of natural selection and genetic drift, resulting in the mixing of alleles among populations. On the other hand, when gene flow is restricted population divergence via selection and genetic drift will not be mitigated, leading to speciation (Heip et al. 1998). Its impact on the population structure is estimated based on the migration rate (Nm) of individuals (i.e., the number of individuals that would be exchanged between populations per generation to account for the observed population differentiation) (Giraud et al. 2008). An essential factor to consider in devising management strategies against diseases is variation within pathogen populations (McDonald 1995; Grünwald et al. 2017). Detailed investigations of pathogen genetic diversity and population genetic structure in different geographical regions are required, as these reflect the history and the evolutionary potential of the pathogen (McDonald 1997). Furthermore, understanding the variation and genetic diversity within and among regional populations of a pathogen can help locate its possible center of origin (Stukenbrock and McDonald 2008).

The obligately biotrophic nature of *P. maydis* currently makes obtaining pure cultures difficult. However, it is possible to extract DNA directly from stromata embedded in foliage and DNA-based molecular markers can be amplified successfully to make inferences on population genetic parameters. Molecular tools have been handy over the years in uncovering the genetic structure of many plant pathogen populations worldwide (Morgante and Olivier 1993; Dutech et al. 2007; Medini and Hamza 2008; Gautier et al. 2014). Some frequently used tools include Restriction Fragment Length Polymorphisms (RFLPs), Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphisms (AFLPs), and Simple-Sequence Repeats (SSRs) or microsatellite markers. Of these molecular tools, SSR markers are highly favored for genetic analyses in population studies because they are species-specific, multiallelic, reproducible, highly polymorphic, and are easily amplified via polymerase chain reactions (PCR) (Morgante and Olivieri 1993; Winter and Kahl 1995; Dutech et al. 2007; Medini and Hamza 2008; Gautier et al. 2014). SSR markers are cost-effective and provide a more reliable interpretation of the population's genetic diversity (Guichoux et al. 2011). They can help answer many questions in fungal population biology and genetics. For instance, they have helped understand the diversity

levels, sources of variation, pathogen dispersal, reproductive mode, and host selection within several rust pathogen populations such as *Melampsora larici-populina, Puccinia graminis, Piccinia striiformis f.sp. tritici* (Dutech et al. 2007; Barres et al. 2012; Danies et al. 2014; Berlin et al. 2012; Ali et al. 2014).

The goal of this research was to develop a set of polymorphic microsatellite markers that would allow for effective genotyping of *P. maydis* isolates for genetic analyses of its populations within and outside of the U.S., and to use those markers to test the following four hypotheses: 1) that genetic differences exist among *P. maydis* isolates across corn-production areas in the U.S.; 2) that *P. maydis* populations in Midwest U.S. are geographically structured; 3) that there is gene flow; and 4) that there is evidence for sexual recombination among *P. maydis* populations across corn-producing areas in five Midwest states. Testing these hypotheses will provide a baseline about the genetic structure of *P. maydis* populations soon after its introduction into the U.S., its evolutionary potential, and can help inform the development of future management approaches.

2.3 Materials and Methods.

2.3.1 Sample collection procedure.

Corn leaf samples exhibiting tar spot symptoms were collected from five corn-growing states (Illinois, Indiana, Iowa, Michigan, and Wisconsin) in the Midwest U.S. from 2018 to 2020 (Table 2.1 and Figure 2.1). Samples from Indiana that were submitted to the Purdue University Plant and Pest Diagnostic Laboratory in 2015 and 2017 also were included. Additional collection details are presented in Supplementary Table 2.8. All samples were dried by pressing in newspaper and stored at room temperature until they were processed for DNA extraction.

Table 2.1. Sampling information of *Phyllachora maydis* from five Midwestern states.

Region	Isolate ID	No. of samples	No. of counties	Collection year
Iowa (IA)	IA01 - IA37	37	29	2019-2020
Illinois (IL)	IL01 - IL19	19	18	2019-2020
Indiana (IN)	IN01 - IN95	95	50	2015-2020
Michigan (MI)	MI01 - MI10	10	6	2019-2020
Wisconsin (WI)	WI01 - WI20	20	17	2019-2020
Total		181	120	

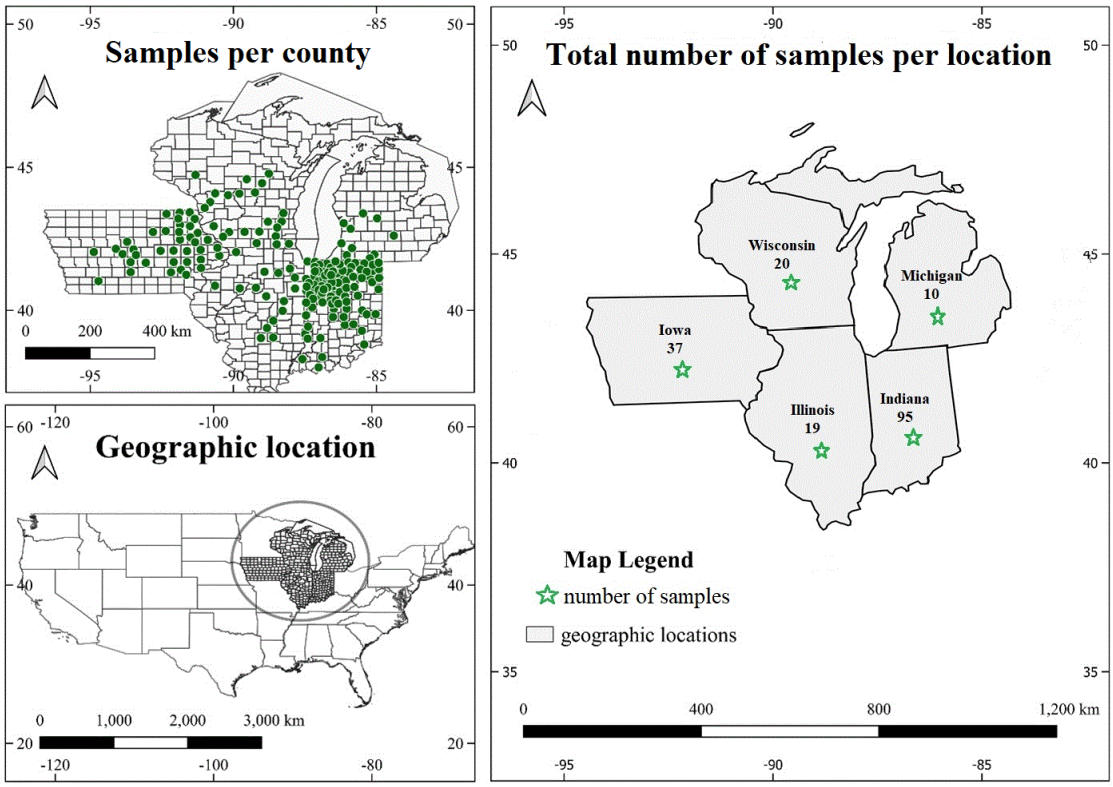

Figure 2.1. Geographic locations of the 181 *Phyllachora maydis* samples obtained from Midwest U.S. Stars with numbers denote the geographic locations (states) and number of samples analyzed, respectively.

2.3.2 DNA extraction and identification of fungal samples.

Corn leaf samples with tar spot stromata were placed in a solution containing 2.5% (v/w) commercial bleach (a.i. 5.0% sodium hypochlorite) with one drop of Tween 20 added to every 50 ml of solution (Bio-Rad Laboratories, Hercules, CA) for 10 s to disinfest their surfaces. Samples were then rinsed twice with sterile water, placed on paper towels and air dried. Three to five stromata of *P. maydis* without necrotic halos and in close proximity to each other were excised from each leaf sample using a sterile surgical blade. Care was taken to limit the samples to stromata with the least amount of leaf material possible. The excised stromata were placed in sterile 2-ml microcentrifuge Polymerase Chain Reaction (PCR) tubes for processing. The total genomic DNA (gDNA) from the 181 *P. maydis* samples were recovered using a modified cetyltrimethylammonium bromide-based protocol (CTAB; Healey et al. 2014). A *P. maydis* species-specific conventional PCR assay was used to confirm the identity of all stroma samples before microsatellite analysis. This method can identify *P. maydis* by targeting part of the internal transcribed spacer (ITS1-5.8S-ITS2) region using ITS1F and ITS4 primers (White et al. 1990; Gardes and Bruns 1993). The amplified DNA sequence was analyzed by BLAST against a database generated from a partial assembly of the *P. maydis* genome (GenBank accession number: JAALGG000000000; Telenko et al. 2020). The ITS region was located on a 0.42-Kb contig (GenBank accession number: SUB11131062). The sequence of this contig was used to identify two specific sets of primers for the *P. maydis* conventional PCR assay. This species-specific set of primers (*P. maydis*-specific F and R) was synthesized by Integrated DNA Technologies (Coralville, IA). After confirming *P. maydis* identity, the DNA from each stroma was measured with a Nanodrop 1000 spectrophotometer (Thermofisher Scientific, Waltham, MA), adjusted to a concentration of 0.2 ng/ul, and stored in nuclease-free sterile water at 4 ºC until further molecular analyses.

2.3.3 Microsatellite marker development and primer design.

Primers for thirteen microsatellite loci (Table 2.2) were developed from the draft genome sequence data of *P. maydis* (GenBank accession number: JAALGG000000000). For SSR prediction, the *P. maydis* draft genome sequence was used as input in QDD version 3.1.2b (perlapps/group/bioinformatics/apps/QDD-3.1.2/QDD). This program uses a set of Perl scripts

that integrates the program Primer3 (Koressaar and Remm 2007) for designing primers flanking each SSR following the protocol outlined in Diaz-Valderrama and Aime (2016). The Primer3 parameters were as follows: primer lengths of 18 to 22 base pairs (bp); primer melting point (Tm) of 60 to 62 ºC; and product sizes of 141 to 325 bp. Primer pairs for thirty microsatellite loci containing two- or three-nucleotide motifs were screened initially for polymorphism against ten isolates of *P. maydis* collected from five U.S. states during different years. Among the thirty microsatellite primer pairs tested, thirteen were selected based on their ability to consistently amplify across all ten tested *P. maydis* samples and were polymorphic (Table 2.2).

Table 2.2. Sequences and properties of the 13 microsatellite loci used to analyze five populations of *Phyllachora maydis* in the Midwest U.S.

Primer	Repeat motif	Sequence (5'- 3') [x]	Expected size range (bp)	Annealing temperature (^{0}C)
PM_SSR01	$(AAG)_9$	F: M13-CACAAATCCATTGCGAGCGG R: TAACCTGTGTAGCAGCAGGC	200 - 230	60
PM_SSR02	$(CG)_5$	F: M13-AAATCAATCCACCGCACCCA R: TCGACACACTTCTCTTCGCC	204 - 230	60
PM_SSR03	$(AAC)_5$	F: M13-GAGGCTCCGACGGATACAAC R: CGAGCGAGCTAAAGACGACA	218 - 232	60
PM_SSR04	$(ACC)_7$	F: M13-CGAAGGAGAATCGGCGGAAT R: GCAGTGGGCTTACATGGTGA	240 - 320	60
PM_SSR06	$(AG)_5$	F:M13-CTCTGCTTGATGACCTCGGG R: GTTTGGCCTCGACTACCTCC	270 - 320	61
PM_SSR10	$(ATC)_6$	F: M13-GGATATCGCCAAGGTCGTGG R: CCGAGACCCTCCATTCCTCA	298 - 315	60
PM_SSR12	$(AC)_9$	F: M13-CCGGATGGATGTGCAGTCAT R: CTTACTGTCCCTTGCGGTGG	182 - 310	61
TS_SSR03	$(AGC)_{51}$	F: M13-GTGATCTGGCAGTCCTTGGG R: AAAGTCCAGCCGCCACCTAT	230 - 320	62
TS_SSR05	$(AG)_{44}$	F: M13-CTACTTTGCGCGCGTGAC R: GAGGACACAGTGCCGAGTTT	141 - 235	60
TS_SSR14	$(AG)_{40}$	F: M13-CCAACCAGATCTCACCGTGC R: CAAACAGGGACGCCTAGAGG	210 - 230	60
TS_SSR15	$(AG)_{38}$	F: M13-CCACGCGATTAAGCCACAAG R: CCGAGGGAGGCTTCGATTG	280 - 316	61
TS_SSR18	$(AG)_{35}$	F: M13-CGGACCACAACGTCGATACA R: GGCAACATGGACAACGACAC	256 - 310	60
TS_SSR21	$(AG)_{33}$	F: M13- CGGCACAATGTACGTAGTGG R: CGAGAGCTCTTCCGGTCTTG	209 - 229	61
P. maydis-specific		F: GTGCTCAGAGAGGCCAGTAA R: TGAGAACCCCAGGAGGGATA	623 - 651	60
ITS		1F: CTTGGTCATTTAGAGGAAGTAA 4R: TCCTCCGCTTATTGATATGC	1,130 – 1,160	60

[x] PCR was carried out in a single nested reaction which includes a third primer (M13 universal primer-fluorescent tag of either 6FAM™, NED™, PET®, or VIC® dyes) not listed in the table.

2.3.4 SSR amplification and genotyping.

Microsatellite loci were prepared for fragment analysis via an ABI PRISM® 3730XL genetic analyzer using a modified M13 method outlined by Schuelke (2000) and Diaz-Valderrama and Aime (2016). Each forward SSR primer contained an 18-bp tail added at its 5' end with universal M13 primer (5'-TGTAAAACGACGGCCAGT- 3') which was previously labeled at its 5' end with one of four specific fluorescent dyes (6-FAM®, NED®, PET™ and VIC™; Thermo Fisher Scientific, Waltham, MA, U.S.A). PCR amplifications were carried out in a final volume of 12.5 ul, containing 6.25 ul of Taq 2x Master Mix (New England Biolabs, Ipswich, MA, U.S.A), 0.16 ul of the forward primer with the M13 tail, 0.47 ul of the M13 primer with one of the four fluorescent dyes and 0.63 ul of the reverse primer (all at a concentration of 10 uM), and 5.0 ul of DNA template (0.2 ng/ul). Thermal cycling included 94 °C for 5 min; 30 cycles of 94 °C for 30 s, TM for microsatellite primer for 45 s, 72 °C for 45 s; eight cycles of 94 °C for 30 s, 53 °C for 45 s, 72 °C for 45 s; and 1 cycle of 72 °C for 10 min. PCR products were multiplexed post PCR by physically mixing four aliquots each of PCR products labeled with different fluorescent dyes and sent to CD Genomics (Shirley, NY, U.S.A) for fragment analysis via capillary electrophoresis, which utilizes an ABI PRISM 3730xl automated sequencer. Sequenced samples were returned, and allele size scoring of the fragments was performed using GeneMarker software 3.0.1 (SoftGenetics, State College, PA U.S.A.). Each SSR primer pair was presumed to amplify a single genetic locus and bands of different molecular weights were considered as different alleles. Amplification, sequencing, and genotyping of *P. maydis* samples were replicated three times with the same DNA preparations to ensure allele prediction due to the obligate nature of the pathogen.

2.3.5 Microsatellite diversity indices across populations.

Microsatellite loci summary statistics were calculated using the 'locus_table' function within the R package poppr v 3.0.2 (Kamvar et al. 2014) and GenAlEx software version 6.501 (Peakall and Smouse 2012). Locus diversity indices included: the number of alleles (Na), Nei's (1978) unbiased gene diversity (he), polymorphic information content (PIC), and genetic evenness (E_5) which estimates the uniformity of genotype distribution across populations. $E_5 = 1$ means genotypes occur at equal frequency, regardless of richness (Grünwald et al. 2003).

2.3.6 Population genetic diversity.

Population genetic diversity analyses were estimated from the scored marker data. Genetic diversity measures the richness and abundance of genotypes in a population. It was estimated based on two indices: the Shannon-weiner Index of MLG diversity (I) (Shannon 2001) and Simpson's complement index of MLG diversity (λ) (Simpson 1949) with the R package poppr. Simpson's (1949) measures the probability that two individuals chosen at random from the population will be found to belong to the same group. Additionally, the R package poppr v3.0.2 (Kamvar et al. 2014), was used to identify unique multilocus genotypes (MLGs) and expected MLGs at the smallest sample size (eMLG) based on microsatellite allele sizes. The number of alleles (Na), number of effective alleles, number of private alleles (Pa), genetic evenness (E_5), polymorphic information content (PIC), and Nei's (1978) unbiased gene diversity (he) were estimated with the R package poppr v 3.0.2 and GenAlEx software version 6.501 (Peakall and Smouse 2012). Genetic evenness (E_5) which values ranges from 0 to 1, measures the distribution of genotypic abundances without relying on the number of genotypes in a population (Grünwald et al. 2003).

2.3.7 Linkage disequilibrium.

Linkage disequilibrium test (index of association, I_A and rBarD) (Agapow and Burt 2001) was done using the R package poppr v.3.0.2 with 999 permutations using the 'ia' function to determine if populations are clonal or sexual. In clonally reproducing populations, significant disequilibrium ($\alpha = 0.001$) is expected due to linkage among loci whereas in sexually reproducing populations linkage among loci is not expected (Brown et al. 1980). The null hypothesis tested is that alleles observed at different loci are not linked if populations are sexual, while alleles recombine freely into new genotypes during sexual reproduction.

2.3.8 Population genetic differentiation, gene flow, and structure analysis.

Tests for population genetic differentiation (F_{ST} and *p*- values) over 999 bootstrap replications were performed and gene flow (Nm) was estimated using the same GenAlEx software. An analysis of molecular variance (AMOVA) and estimate of pairwise F statistics (F_{ST}) among the groups were also performed to measure the probable differentiation among different groups

and assessment of gene migration among populations over time (gene flow) using GenAlEx software.

Population structure was further assessed with STRUCTURE version 2.3.4 (Pickard et al. 2000), based on the individual-based Bayesian clustering methods. A continuous series of K values from 1 to 10 were tested in 10 independent runs to deduce the optimal K value for the genotypes using the ΔK method (Evanno et al. 2005). Each run comprised a burn-in length of 100,000 followed by 100,000 MCMC (Markov Chain Monte Carlo) replicates. The most likely values of K were chosen based on ΔK that was computed with Structure Harvester version 0.6.94 (Earl and Von Holdt 2012). The optional alignment of clusters across individual runs for each K was determined using CLUMPP version 1.1.2 (Jakobsson and Rosenberg 2007), which included a greedy algorithm and 10,000 random input orders of 10 independent STRUCTURE runs.

The genetic-structure plot was drawn by Distruct version 1.1 software (Rosenberg 2010). The population structure based on the genetic distance among all sampled individuals was further revealed by principal coordinate analysis (PCoA) using GenAlEx software. Additionally, discriminant analysis of principal components (DAPC) was performed using the find.clusters command of the R package adegenet v 1.3 (Jombart et al. 2010) to identify the genetically differentiated clusters across the *P. maydis* studied populations.

Lastly, a genetic dissimilarity matrix was computed based on the continuous Euclidian dissimilarity index and Nei's standard genetic distance (DST, corrected) (Nei 1972) over 1,000 bootstrapped replications based on the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) trees were generated 'genpop' with R package adegenet v1.3.

2.4 Results.

2.4.1 Microsatellite polymorphism and gene diversity.

A total of 181 *P. maydis* samples were genotyped using 13 polymorphic SSR markers (Table 2.3). Among the entire population 85 alleles were recovered and the number of alleles per locus (Na) varied from 3 to 13 with an average of 6.5. The highest number of alleles (Na = 13) was detected at locus TS_SSR15 (Table 2.3). For the entire studied population, gene diversity (He) (equivalent to the expected heterozygosity in a diploid) among loci ranged from 0.35 (PM_SSR06) to 0.76 (TS_SSR15) with an average of 0.55 (Table 2.3). Genetic evenness (E_5) (distribution of

genotypic abundance) of markers ranged from 0.55 (PM_SSR06) to 0.88 (TS_SSR03) with an average of 0.70 (Table 2.3). Informativeness of individual loci as measured by their polymorphic information content (PIC) ranged from 0.32 (PM_SSR06) to a maximum of 0.72 (TS_SSR15) with a mean PIC value of 0.50 (Table 2.3).

Table 2.3. Summary statistics of 181 samples of *Phyllachora maydis* for each of the 13 analyzed microsatellite loci.

Locus	Na	He	E_5	PIC
PM_SSR01	7	0.73	0.79	0.69
PM_SSR02	9	0.73	0.81	0.69
PM_SSR03	5	0.50	0.75	0.42
PM_SSR04	7	0.62	0.74	0.54
PM_SSR06	4	0.35	0.55	0.32
PM_SSR10	5	0.55	0.81	0.45
PM_SSR12	6	0.52	0.60	0.47
TS_SSR03	5	0.52	0.88	0.41
TS_SSR05	3	0.49	0.72	0.43
TS_SSR14	8	0.57	0.63	0.52
TS_SSR15	13	0.76	0.65	0.72
TS_SSR18	8	0.48	0.60	0.45
TS_SSR21	5	0.38	0.54	0.35
Mean	6.5	0.55	0.70	0.50

Na = number of observed alleles; He = Nei's (1978) unbiased gene diversity; E_5 = population evenness estimating uniform genotype distribution, E_5 = 1 means genotypes occur at equal frequency, regardless of richness (Grünwald et al. 2003). PIC = Polymorphic information content.

2.4.2 Population genetic diversity.

Table 2.4 summarizes the genetic diversity estimates over all loci within each regional population. All samples were unique and hence 181 unique multilocus genotypes were recovered using the R package poppr v3.02 (Table 2.4). A genotype accumulation curve determined that 100% of the unique multilocus genotypes (MLGs) could be detected with eleven or twelve microsatellite markers (Figure 2.2). Genetic diversity estimates across regional populations had a mean number

of alleles (Na) of 3.7 with a range of 2.7 to 5.5, the mean number of effective alleles was 2.1 (range 1.9 to 2.4), and the frequency of private alleles (Pa) or those that were unique to a single population was 0.45 (range 0.15 to 1.31). All indices of genotypic diversity indicated high diversity: Shannon-Weiner's index (I), a measure of population richness (biodiversity), ranged from 0.82 to 1.03 with a mean diversity index of 0.84; and Simpson's complement index (λ) ranged from 0.90 to 0.99 with mean index of 0.99 (Table 2.4). Likewise, Nei's unbiased gene diversity (He) was high for all populations and ranged from 0.41 to 0.53 (Table 2.4). The abundance of genotypic diversity (E_5 = 1) was evenly distributed in all populations (Table 2.4). The Indiana population had the highest Na, Ne, Pa, I, λ, and He values, whereas the Michigan population had the lowest values for all statistics (Table 2.4). The average percentage of polymorphic loci (%PPL) per population was 94% with a range from 85% (Michigan) to 100% (Illinois and Indiana). The percentage of polymorphic loci for both Iowa and Wisconsin were 92% (Table 2.4).

Table 2.4. Genetic diversity indices across regional populations averaged over the 13 SSR loci.

Population	N	MLG	eMLG	Na	Ne	Pa	I	λ	He	E_5	%PPL
Iowa	37	37	10	3.5	2.2	0.39	0.82	0.97	0.46	1.00	92
Illinois	19	19	10	3.2	2.0	0.15	0.82	0.95	0.49	1.00	100
Indiana	95	95	10	5.5	2.4	1.31	1.03	0.99	0.53	1.00	100
Michigan	10	10	10	2.7	1.9	0.15	0.68	0.90	0.41	1.00	85
Wisconsin	20	20	10	3.6	2.0	0.23	0.82	0.95	0.46	1.00	92
Mean	-	-	-	3.7	2.1	0.45	0.84	0.99	0.55	1.00	94

N = number of samples tested; MLG = number of multilocus genotypes observed; eMLG = number of expected MLGs at the smallest sample size based on rarefaction; Na = Number of alleles per locus; Ne = Number of effective alleles per locus; Pa = number of private alleles (i.e., the number of alleles unique to a single population); I = Shannon-Weiner index of MLG diversity (Shannon 2001); Simpson's complement index of MLG diversity (Simpson 1949); He = Nei's unbiased genetic diversity; E_5 = genetic evenness and %PPL = the percentage of polymorphic loci.

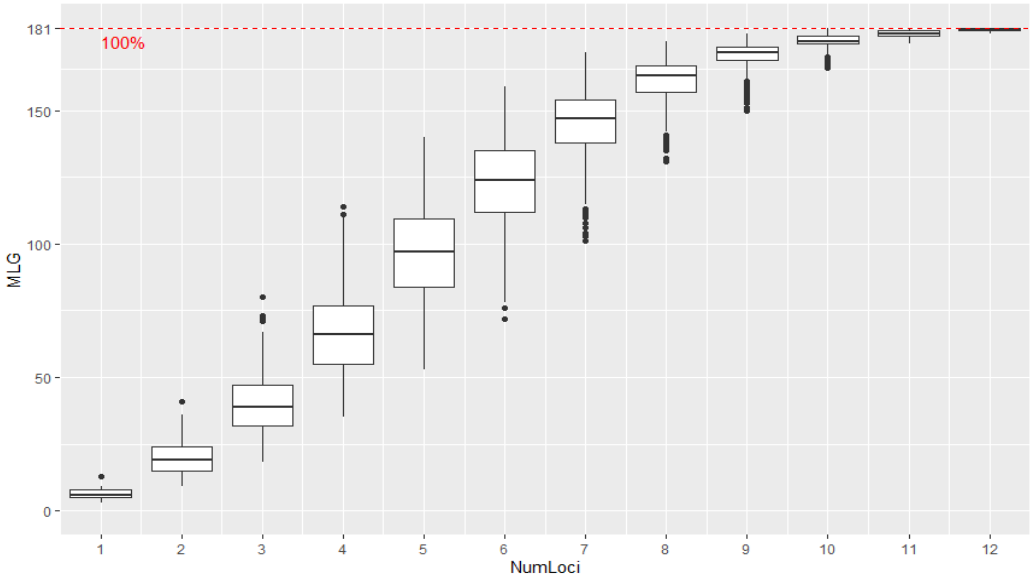

Figure 2.2. Genotype accumulation curve. Unique multilocus genotypes (MLG) of *Phyllachora maydis* detected as the number of microsatellite loci (NumLoci) sampled. When 11 or 12 microsatellite loci are used in genotyping, 100% of the unique multilocus genotypes can be detected. The 100% unique multilocus genotype level is indicated by the red dashed line.

2.4.3 Linkage disequilibrium (LD).

The test for linkage disequilibrium (LD) using the microsatellite data of the 181 samples by the index of association (I_A) and the standard index of association (rBarD) using 999 permutations at *P*-value = 0.001, showed evidence that all regional populations are reproducing sexually (Table 2.5). The null hypothesis is failed to be rejected of no linkage among loci since *P*-value in all populations were greater than 0.001.

Table 2.5. Linkage disequilibrium (LD) based on index of association test (I_A and rBarD) across the five regional populations.

Populations	**I_A**	**p.I_A**	**rBarD**	**p.BarD**
Iowa	0.069	0.203	0.006	0.203
Illinois	0.077	0.267	0.006	0.268
Indiana	0.180	0.050	0.015	0.050
Michigan	0.018	0.410	0.002	0.411
Wisconsin	0.224	0.083	0.020	0.087

I_A = index of association; p.I_A = *P*-value for I_A; rBarD = standardized index of association; p.BarD = *P*-value for p.rD. H0 = no linkage disequilibrium (LD), therefore *P*-value greater than 0.001 indicates populations that are not in LD, which could be an account of random mating.

2.4.4 Population genetic differentiation, gene flow, and structure analysis.

Analysis of molecular variance (AMOVA) based on the F statistics was estimated with and without grouping populations according to their geographical locations. AMOVA showed that 85% of the total variation was due to within-population variation and only 15% was accounted for by genetic divergence among populations (Table 2.6). This result was further validated by the principal coordinate analysis (PCoA) (Figure 2.3), where the first three axes explained 26.8% of the total variation, with each of the coordinates (1, 2 and 3) accounting for 11.7%, 7.9%, and 7.2% of the variation, respectively.

The overall genetic differentiation among populations (F_{ST} = 0.15, P = 0.001) was relatively low but significant at $\alpha = 0.05$. Similarly, pairwise F_{ST} values of genetic distances among all populations were statistically significant (P = <0.01) (Table 2.7). Among all populations, the average estimated gene flow, Nm, was 2.80. Discriminate analysis of principal component showed that *P. maydis* populations in the Midwest are not geographically structured based on collection origins (regions), but instead, saw an intermixing of *P. maydis* samples from Illinois, Indiana, and Wisconsin and then another intermixing of samples from Iowa and Michigan (Figure 2.4). A cluster dendrogram generated using the Unweighted Pair Group Method with Arithmetic mean based on nei_distance bootstrapped grouped the five regional populations into two clusters (1 and 2) that did not correspond with geography (Figure 2.5). Cluster C1 was composed of populations Illinois, Indiana, and Wisconsin and cluster C2 was composed of populations Iowa and Michigan (Figure 2.5).

Finally, Bayesian clustering of the 181 isolates with STRUCTURE software v 2.3.4 revealed delta K (ΔK) values reached a sharp peak at K = 2 (Figure 2.6a), confirming that the 181 samples evaluated in this study could be most likely clustered into two subpopulations. The result (bar plot) also detected a greater degree of genetic admixture between the two subpopulations with no clear geographic origin-based structuring among the five geographical regions from which *P. maydis* was collected in the Midwest (Figure 2.6b).

Table 2.6. Analysis of molecular variance (AMOVA) showing genetic variation within and among *Phyllachora maydis* populations in the Midwest U.S.

Source	Df	SS	MS	Est. Var.	% Var.	F_{ST} (*P*-value)
Among Populations	4	157.36	39.34	0.61	15	0.15 (0.001)
Within Populations	357	1221.56	3.42	3.42	85	
Total	361	1378.93		4.03	100	

Df = Degrees of freedom; SS = Sum of squares; MS = Mean sum of squares; Est. Var = Estimated variance; % = percentage of variance, F_{ST} = genetic differentiation statistic.

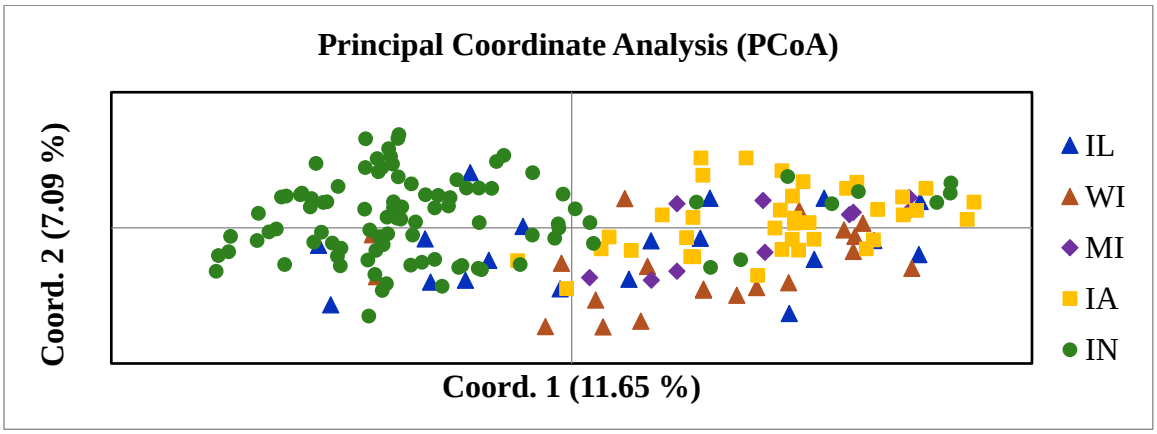

Figure 2.3. Principal coordinate analysis (PCoA) showing the clustering of 181 *Phyllachora maydis* samples as revealed by 13 microsatellite loci. Samples coded with the same color and shape belong to the same population. PCoA explained 26.8% of the total variation and the first three axes (1, 2, and 3) accounted for 11.7%, 7.9% and 7.2%, respectively. Population abbreviations are IA = Iowa, IL = Illinois, IN = Indiana, MI = Michigan, and WI = Wisconsin.

Table 2.7. Population genetic differentiation measured by F_{ST} (below the diagonal) in pairwise comparisons among the five populations of *Phyllachora maydis* with p values (above the diagonal).

F_{ST}/*P*-value	IL	WI	MI	IA	IN
IL	---	0.001	0.001	0.001	0.001
WI	0.08	---	0.001	0.001	0.001
MI	0.18	0.12	---	0.001	0.001
IA	0.16	0.12	0.12	---	0.001
IN	0.13	0.15	0.17	0.16	---

IA = Iowa, IL = Illinois, IN = Indiana, MI = Michigan and WI = Wisconsin.

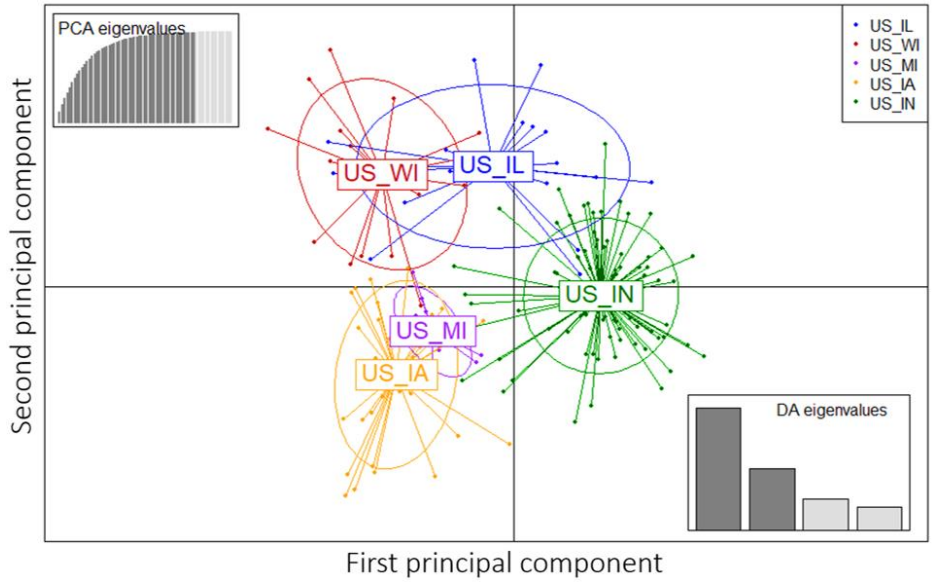

Figure 2.4. Discriminate analysis of principal component of five *Phyllachora maydis* populations in the Midwest. 1000 replicates for 181 *Phyllachora maydis* samples from the five populations in the Midwest U.S formed clusters of severe admixtures. Each color represents a population: yellow = Iowa (US_IA); blue = Illinois (US_IL); green = Indiana (IN); purple = Michigan (US_MI) and orange = Wisconsin (US_WI).

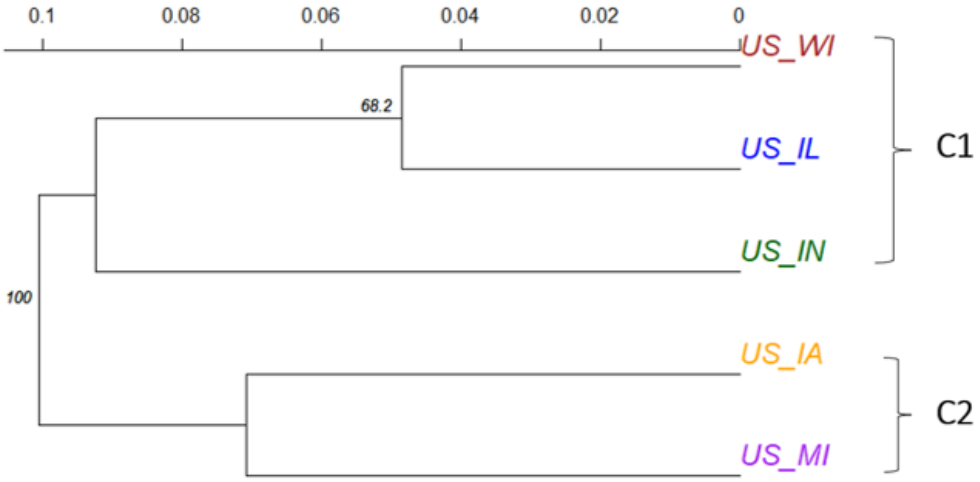

Figure 2.5. Unweighted pair-group method with arithmetic mean dendrogram showing the genetic relationships among the five populations of *Phyllachora maydis* from the Midwest based on Nei's (1972) genetic distance over 1,000 replicates. *P. maydis* samples formed two clusters C1 (Illinois, Indiana, and Wisconsin) and C2 (Iowa and Michigan). Numbers above branches represent percentage of bootstrap values, and values less than 60% were not indicated.

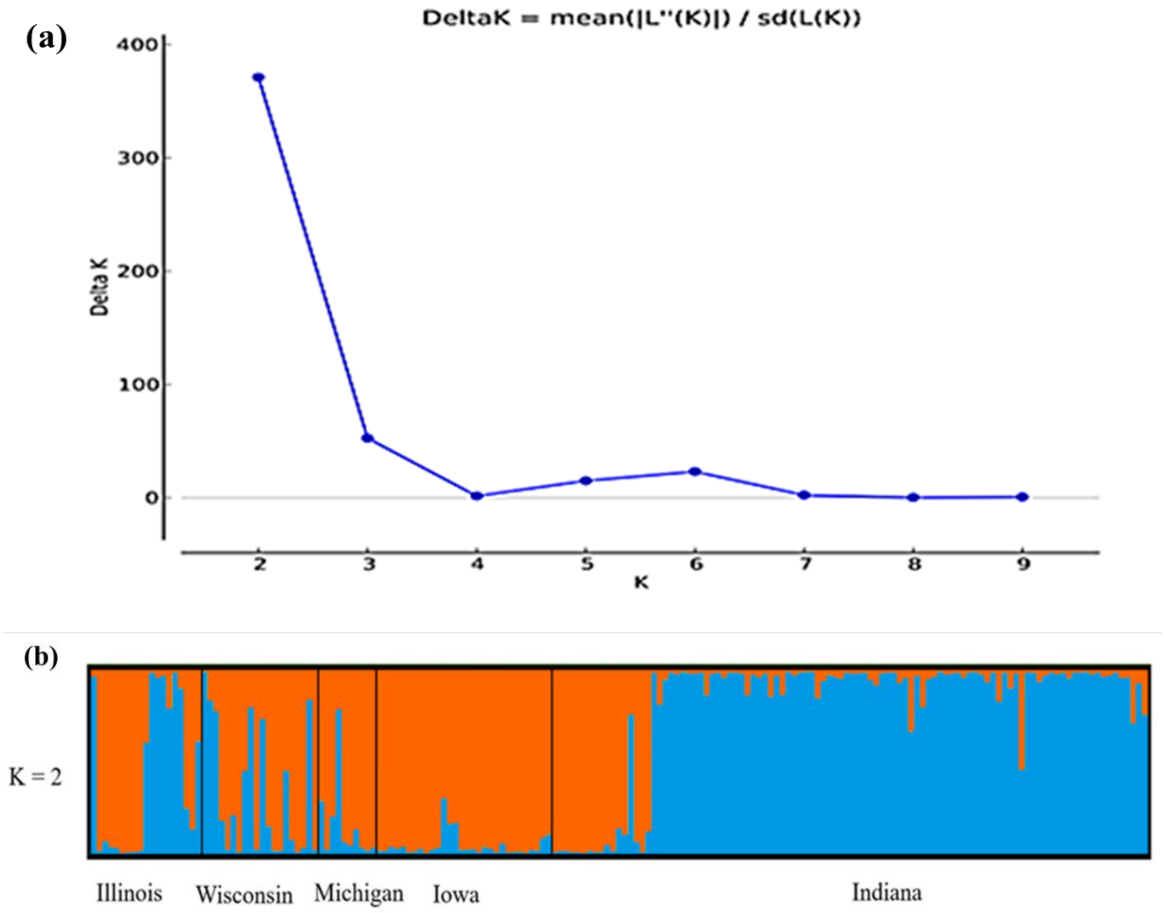

Figure 2.6. Population structure of 181 samples of *Phyllachora maydis* representing five populations in the Midwest U.S. (a) Best delta K value estimated using the method of Evano et al. (2005); and (b) Graph of the estimated population structure for K = 2 designated by Structure Harvester. The different (orange and blue) colors represent genetic groups or subpopulations. The x-axis represents each *P. maydis* sample shown by a vertical line fragmented into K colored sections with length proportional to each of the K inferred clusters and the y-axis represents the proportion of ancestry to each cluster.

2.5 Discussion.

This is the first analysis of the genetic structure, variation, diversity, mode of reproduction, and gene flow of *Phyllachora maydis* populations using SSR markers. Knowledge on the population structure, genetic variation, diversity, mode of reproduction, and gene flow are essential to understanding how pathogen populations spread and overcome host resistance (McDonald and Linde 2002; Morris et al. 2014). Rampersad et al. (2013) emphasized that areas of high biodiversity may serve as sources for the emergence of new genotypes with novel biological characteristics and consequent changes in pathogen resistance to chemical compounds and increased fitness within their populations.

In this analysis, 13 highly polymorphic SSR markers were developed and used to assess the population structure, genetic variation, diversity, mode of reproduction and gene flow of *P. maydis* samples from five corn-growing states in the Midwest U.S. The polymorphisms detected by these SSR markers ranged from reasonably informative (>0.25 PIC <0.50) to highly informative (PIC >0.50). These SSR loci displayed allelic diversity among *P. maydis* samples, from 3 to 13 alleles per locus, where the PIC values ranged from 0.32 to 0.72. The PIC provides an estimate of the discriminatory power of a locus by considering the number and relative frequencies of the alleles (Marulanda et al. 2014). All 13 loci displayed differences for numbers of effective and private alleles, or those unique to a specific geographical area and are useful in comparing diversity between species or populations (Mahmodi et al. 2014). The Indiana population contained the highest number of private alleles compared to samples from the other states. Although this is certainly due at least in part to its larger sample size, the very large difference between the mean number of private alleles per locus in Indiana (1.31) versus the highest in any other state of only 0.39 in Iowa may reflect a biological difference. Possible explanations for this difference may be that the Indiana population received higher diversity from a possible donor population and/or that is has existed longer so has had more time to accumulate new alleles. However, this would require a comparison analysis of *P. maydis* to the other species of *Phyllachora* to rule out the possibility of a host shift and allele accumulation over time. Another possible explanation is the potential that Indiana population is being a continuous receptor of *P. maydis* populations from an introduction/establishment pathway. Since we cannot discard that *P. maydis* might have been introduced from source(s) outside the U.S., a quantitative introduction pathway risk analysis is required. Such assessment could benefit in the understanding of possible

routes of entry and establishment into the U.S. With such quantitative analysis we could, for example, estimate the risk that good imports or weather events poses as entry, for establishment, and outbreak events (Cruz 2013). Additional sampling from other locations is required to test these hypotheses.

Results from AMOVA indicated that the highest percentage of variation (85%) was within populations of *P. maydis* and that gene diversity within the Midwest populations was high (He = 0.55). This high within-population diversity may be attributed to the recent emergence or introduction of the fungus to northern North America. Higher genetic diversity was observed in the Indiana (He = 0.53) and Illinois (He = 0.49) populations compared to those from other states, possibly due to the initial introduction and therefore detection of *P. maydis* in the U.S. in Indiana and Illinois during 2015. From a quantitative introduction pathway point of view, results might indicate that these States might be significant receptors (founder or potentially new arrivals). However, more research is needed to address this hypothesis. Nevertheless, the higher diversity in the Indiana population, compared those in the other regions, may also be attributed to the larger number of assessed samples, thus revealing more genetic information of the population or the possibly of ongoing arrivals of the pathogen in the U.S. Lower genetic diversities were observed for Iowa (He = 0.46), Wisconsin (He = 0.46), and Michigan (He = 0.41) populations, which could be associated with the later introduction or emergence of the pathogen into these areas. The amount of gene diversity within a population may also be a function of population size where older populations have maintained higher levels of gene diversity compared to a recently colonized habitat (Rampersad et al. 2013). Differences in diversity levels across *P. maydis* populations in the Midwest U.S. may also be due to environmental conditions, geography, or corn genetics. Bennett et al. (2005) and Marulanda et al. (2014), both indicated that gene flow, sexual and asexual recombination could generate and affect genetic diversity within pathogen populations. This study shows sexual recombination/reproduction evidence in all the regional populations based on evidence of no linkage among loci (I_A and rBarD: $p > 0.001$). New genotypes can emerge during sexual reproduction and pathogens with active sexual cycles pose more significant risks (McDonald and Linde 2002). The existence of sexual recombination may give rise to new genotypes with advantageous allele combinations, which are potentially more adaptable to overcome host resistance, thus spreading it across populations for generations (Milgroom 1996). Nevertheless, clonally reproducing fungi may show as many alleles as those that undergo

recombination, suggesting that gene diversity may not always be influenced by the reproduction mode of pathogens (McDonald 1997). Larger samples from the other states are needed to distinguish among these possibilities and to test whether the higher diversities in Indiana and Illinois reflect current and ongoing potential introductions or are due to sampling effects.

Analyses of *P. maydis* populations in the Midwest U.S. showed that *P. maydis* samples from the five sampled states were related/genetically alike based on their genetic identity and there was low but statistically significant genetic differentiation ($F_{ST} = 0.15$, $P = 0.001$) among Midwest populations. Further, results from principal coordinate, discriminate component and cluster analyses showed that *P. maydis* populations within the Midwest are not geographically structured, instead populations were of a greater degree of genetic admixtures with moderate gene flow (Nm = 2.80), also referred to as the migration rate, between *P. maydis* populations in this analysis. The relatively low degree of genetic differentiation and moderate gene flow observed among the *P. maydis* populations examined in this analysis may be due to inoculum dispersal over long distances even from locations outside of the U.S. This may have allowed the pathogen to spread among corn production areas in the Midwest U.S. since ascospores of *P. maydis* can be disseminated up to 1,200 m from the inoculum source (Kleczewiski et al. 2021).

The exact mode of spread of *P. maydis* throughout the Midwest U.S. Corn Belt is not fully understood. However, the two most likely hypotheses include long-distance aerial dissemination of spores, or movement of infested corn material from one location to the next, presumably through human activities but also possibly via climatic systems such as high wind events including derechos and tornados (Valle-Torres et al. 2020; Broders et al. 2021). Dissemination of infested corn material may have resulted in migration and gene flow between populations resulting in the genetic signal of admixture among samples from the different geographical origins. Gene flow is promoted by several activities such as movement or exchange of infested plant material, and long-distance dispersal of spores (McDermott and McDonald 1993; Milgroom 1996; McDonald 1997; McDonald and Linde 2002; Milgroom et al. 2003). Kawecki (2004) indicated that divergence between pathogen populations could occur within local environments because of adaptation and genetic drift that increase relative fitness in different niches. When Nm > 1 migration will be sufficient to reduce genetic differentiation among populations (McDermott and McDonald 1993; Wright 1951).

STRUCTURE and population genetic analyses based on Nei's (1972) genetic distance supported the subdivision of *P. maydis* samples into two clusters, suggesting the possibility of polymorphism within the species. These results support the findings of Broders et al. (2021) that revealed variation and possibly multiple species related to *P. maydis* in northern North America. Identification of population subdivision within a particular geographic area could be associated with variations in the agro-ecosystems, such as sources of inoculum and host or tissue specificity (Milgroom and Peever 2003). The present analysis of *P. maydis* samples from five corn-producing states in the Midwest U.S. revealed higher than expected genetic diversity for a founder population and small but significant genetic differentiation among populations when assessed using 13 newly developed SSR markers. Analyses of additional samples with these molecular markers are needed to further assess populations from other affected regions within and outside the U.S. to gain a better understanding of the population genetics and dynamics of *P. maydis* in its source and founder populations.

The 13 highly polymorphic SSR markers identified in this study helped in understanding the current population structure, genetic variation, genetic diversity, reproductive mode, and gene flow of *P. maydis* in the Midwest U.S. The observed genetic diversity in all five populations was moderate to high and genetic variation among populations was low but significant, suggesting inter-state dispersal of inoculum. The information generated in this study could be useful in understanding the biology and spread of *P. maydis* in the Midwest. This information provides a background that could aid in future studies on disease epidemiology, host-pathogen interactions, and help guide the development of disease management strategies. These 13 SSR markers could be useful for characterizing *P. maydis* samples from within the U.S. and other countries. They could further be useful taxonomic and phylogenic studies, functional genomics, genome mapping, gene tagging and quantitative trait linkage (QTL) analysis, hybrid testing and hybridization, and marker assisted selection (MAS) breeding studies, pertaining to this pathogen and disease.

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2.7 Supplementary materials.

Supplementary Table 2.8. Detailed information about samples used in this analysis.

Collection ID	Sample ID	State	County	Year collected
191232	IA01	Iowa	Cedar	2019
191668	IA02	Iowa	Fayette	2019
191249	IA03	Iowa	Tama	2019
191467	IA04	Iowa	Delaware	2019
191788	IA05	Iowa	Linn	2019
191717	IA06	Iowa	Warren	2019
191811	IA07	Iowa	Keokuk	2019
191674	IA08	Iowa	Carrol	2019
190738	IA09	Iowa	Iowa	2019
190719	IA10	Iowa	Buchanan	2019
191359	IA11	Iowa	Jackson	2019
191468	IA12	Iowa	Clinton	2019
191536	IA13	Iowa	Allamakee	2019

DT9200	IA14	Iowa	Johnson	2019
DT9215	IA15	Iowa	Jones	2019
DT17	IA16	Iowa	Boone	2019
DT14	IA17	Iowa	Jasper	2019
DT15	IA18	Iowa	Washington	2019
DT9167	IA19	Iowa	Bremer	2019
DT7	IA20	Iowa	Winneshiek	2019
DT9143	IA21	Iowa	Benton	2019
DT2	IA22	Iowa	Hamilton	2019
DT91696	IA23	Iowa	Story	2019
DT9185	IA24	Iowa	Clayton	2019
DT11	IA25	Iowa	Butler	2019
DT9187	IA26	Iowa	Muscatine	2020
DT9326	IA27	Iowa	Story	2020
DT9327	IA28	Iowa	Washington	2020
DT9325	IA29	Iowa	Polk	2020
DT9305	IA30	Iowa	Boone	2020
DT9332	IA31	Iowa	Winneshiek	2020
DT9320	IA32	Iowa	Howard	2020
DT9316	IA33	Iowa	Fayette	2020
DT9310	IA34	Iowa	Dubuque	2020
DT9300	IA35	Iowa	Allamakee	2020
DT9307	IA36	Iowa	Clayton	2020
DT9311	IA37	Iowa	Delaware	2020
191611	IL01	Illinois	Peoria	2019
191817	IL02	Illinois	La Salle	2019
191688	IL03	Illinois	Will	2019
191689	IL04	Illinois	Kankakee	2019
191980	IL05	Illinois	Shelby	2019
191731	IL06	Illinois	Winnebago	2019

191981	IL07	Illinois	Effingham	2019
191982	IL08	Illinois	Iroquois	2019
191983	IL09	Illinois	Fayette	2019
191042	IL10	Illinois	Carrol	2019
191984	IL11	Illinois	Moultrie	2019
191821	IL12	Illinois	Woodford	2019
191822	IL13	Illinois	Champaign	2019
191823	IL14	Illinois	Lake	2019
191824	IL15	Illinois	Mclean	2019
191825	IL16	Illinois	McHenry	2019
191826	IL17	Illinois	Champaign	2020
191827	IL18	Illinois	Grundy	2020
191828	IL19	Illinois	Warren	2020
75	IN01	Indiana	Miami	2019
11	IN02	Indiana	Lagrange	2019
143	IN03	Indiana	Noble	2019
25	IN04	Indiana	Jefferson	2019
128	IN05	Indiana	Jasper	2019
125	IN06	Indiana	Pulaski	2019
133	IN07	Indiana	Fulton	2019
123	IN08	Indiana	White	2019
121	IN09	Indiana	Carrol	2019
64	IN10	Indiana	Porter	2019
127	IN11	Indiana	Starke	2019
146	IN12	Indiana	Marion	2019
141	IN13	Indiana	DeKalb	2019
4	IN14	Indiana	Saint Joseph	2019
7	IN15	Indiana	Elkhart	2019
139	IN16	Indiana	Allen	2019
144	IN17	Indiana	Marshall	2019

135	IN18	Indiana	Kosciusko	2019
130	IN19	Indiana	Cass	2019
68	IN20	Indiana	Newton	2019
130	IN21	Indiana	Benton	2019
68	IN22	Indiana	Boone	2019
70	IN23	Indiana	Clinton	2019
24	IN24	Indiana	Johnson	2019
136	IN25	Indiana	Whitely	2019
49	IN26	Indiana	Shelby	2019
12	IN27	Indiana	Steuben	2019
40	IN28	Indiana	Sullivan	2019
61	IN29	Indiana	La Porte	2019
67	IN30	Indiana	Lake	2019
62	IN31	Indiana	Henry	2019
66	IN32	Indiana	Warren	2019
108	IN33	Indiana	Hamilton	2019
17	IN34	Indiana	Tippecanoe	2019
103	IN35	Indiana	Greene	2019
90	IN36	Indiana	Pulaski	2020
20301	IN37	Indiana	Porter	2020
20058	IN38	Indiana	Vermillion	2020
20062	IN39	Indiana	Clinton	2020
20632	IN40	Indiana	Lake	2020
20634	IN41	Indiana	Warren	2020
20320	IN42	Indiana	Steuben	2020
20067	IN43	Indiana	Hendricks	2020
20315	IN44	Indiana	Elkhart	2020
20629	IN45	Indiana	Newton	2020
20317	IN46	Indiana	DeKalb	2020
20040	IN47	Indiana	Tippecanoe	2020

20005	IN48	Indiana	White	2020
20305	IN49	Indiana	Saint Joseph	2020
20311	IN50	Indiana	Starke	2020
20340	IN51	Indiana	Henry	2020
20303	IN52	Indiana	La Porte	2020
20338	IN53	Indiana	Wayne	2020
20510	IN54	Indiana	Wabash	2020
20053	IN55	Indiana	Decatur	2020
20071	IN56	Indiana	Sullivan	2020
20069	IN57	Indiana	Vigo	2020
20636	IN58	Indiana	Fountain	2020
20016	IN59	Indiana	Adams	2020
20508	IN60	Indiana	Jasper	2020
20627	IN61	Indiana	Benton	2020
20615	IN62	Indiana	Spenser	2020
20613	IN63	Indiana	Dubois	2020
20402	IN64	Indiana	Grant	2020
20027	IN65	Indiana	Gibson	2020
20401	IN66	Indiana	Howard	2020
15-01407	IN67	Indiana	Allen	2015
15-01529	IN68	Indiana	Tipton	2015
15-01368	IN69	Indiana	Cass	2015
15-01444	IN70	Indiana	Carrol	2015
15-01544	IN71	Indiana	Clinton	2015
15-01587	IN72	Indiana	Fulton	2015
17-01904	IN73	Indiana	Fulton	2017
17-02018	IN74	Indiana	Porter	2017
17-02015	IN75	Indiana	Pulaski	2017
17-01816	IN76	Indiana	Starke	2017
17-02017	IN77	Indiana	White	2017

18-01892	IN78	Indiana	Clinton	2018
18-01889	IN79	Indiana	Benton	2018
18-01876	IN80	Indiana	Elkhart	2018
18-01919	IN81	Indiana	Allen	2018
18-01926	IN82	Indiana	White	2018
18-01878	IN83	Indiana	Marshall	2018
18-01838	IN84	Indiana	White	2018
18-01867	IN85	Indiana	Fulton	2018
18-01891	IN86	Indiana	Carrol	2018
18-01888	IN87	Indiana	Newton	2018
18-01671	IN88	Indiana	La Porte	2018
18-01886	IN89	Indiana	Starke	2018
18-01890	IN90	Indiana	Cass	2018
18-01887	IN91	Indiana	Pulaski	2018
18-01744	IN92	Indiana	Porter	2018
18-01959	IN93	Indiana	Noble	2018
18-01758	IN94	Indiana	Jasper	2018
18-01877	IN95	Indiana	Kosciusko	2018
191416	MI01	Michigan	Cass	2019
190701	MI02	Michigan	Branch	2019
191017	MI03	Michigan	Ingham	2019
190702	MI04	Michigan	Ottawa	2019
190703	MI05	Michigan	Montcalm	2019
190704	MI06	Michigan	Van Buren	2019
190705	MI07	Michigan	Montcalm	2020
190706	MI08	Michigan	Branch	2020
190707	MI09	Michigan	Ottawa	2020
190708	MI10	Michigan	Van Buren	2020
191875	WI01	Wisconsin	Monroe	2019
191808	WI02	Wisconsin	Waupaca	2019

191459	WI03	Wisconsin	Lafayette	2019
191589	WI04	Wisconsin	Shawano	2019
191756	WI05	Wisconsin	Waushara	2019
191119	WI06	Wisconsin	Walworth	2019
191120	WI07	Wisconsin	Waukesha	2019
191121	WI08	Wisconsin	Portage	2019
191122	WI09	Wisconsin	Washington	2019
191123	WI10	Wisconsin	Vernon	2019
191124	WI11	Wisconsin	Rock	2019
191125	WI12	Wisconsin	Waushara	2019
191126	WI13	Wisconsin	Green	2019
191127	WI14	Wisconsin	Juneau	2019
191128	WI15	Wisconsin	Jefferson	2019
191129	WI16	Wisconsin	Grant	2019
912020	WI17	Wisconsin	Adams	2020
912021	WI18	Wisconsin	Monroe	2020
912022	WI19	Wisconsin	Eau Claire	2020
912023	WI20	Wisconsin	Lafayette	2020

CHAPTER 3. NET RETURNS OF FOLIAR FUNGICIDES AND FUNGICIDE TIMING FOR MANAGING TAR SPOT OF CORN IN INDIANA, U.S.A.

*This chapter will be submitted as a research article in Plant Disease Journal.

3.1 Abstract.

Tar spot, caused by *Phyllachora maydis* Maubl., has emerged as a yield limiting foliar disease of corn (*Zea mays* L.) in Indiana. The net return of foliar fungicides and application timing for managing tar spot has never been studied. Field experiments were conducted in Indiana to assess fungicide efficacy and timing in reducing tar spot severity, increasing greenness, impact on yield, and net returns under high and low disease pressure. All fungicides evaluated increased canopy greenness, but only prothioconazole + trifloxystrobin + fluopyram (Delaro Complete), metconazole + pyraclostrobin (Headline AMP), cyproconazole + picoxystrobin (Aproach Prima), and mefentrifluconazole + pyraclostrobin (Veltyma) reduced disease severity, protected yield, and resulted in significantly higher net returns when compared to the nontreated control.

Additionally, it was found that timing of propiconazole + benoindiflupyr + azoxystrobin (Trivapro) application at the tassel/silk (VT/R1) to dough (R4) corn growth stages resulted in significant yield increases and profitability over no fungicide application. The yield response of foliar fungicides and application timing was 2.1 to 6.3 times greater when high disease pressure relative to low disease pressure. This study demonstrates that foliar fungicides and appropriately timed fungicide applications can be used to profitably manage tar spot in Indiana, especially under high disease pressure where expected net returns from foliar fungicides was $68.9/ha higher and application timing was $183.3/ha higher compared to when applied under low disease pressure. The probability of a fungicide application breaking even is 50% greater when disease pressure is high compared to when low disease pressure.

3.2 Introduction.

Indiana ranks fifth in the United States for corn production with an estimated 24.9 million metric tons [15.0 billion bushels] in annual production (USDA-NASS 2021). The climate in Indiana is suitable for the development of many corn diseases (Wise et al. 2016). In 2018, Indiana experienced the first yield-reducing epidemic of tar spot of corn, a disease that lessens the quality and quantity of corn grain and silage (Telenko et al. 2019a). Tar spot of corn is caused by a foliar pathogen, *Phyllachora maydis* Maubl., and has become an economically important and prevalent disease in the Midwest U.S. (Maublanc 1904; Valle-Torres et al. 2020: Mueller et al. 2022). The disease was initially detected in the U.S. in 2015 in Indiana and Illinois (only 7 counties in Indiana confirmed this disease in 2015). Tar spot has now been detected in 14 states and Ontario, Canada, with predictions of spreading throughout Midwest U.S. (Mottaleb et al. 2019; Athey 2020; Malvick et al. 2020; Tenuta, 2020; Valle-Torres et al. 2020; Collins et al. 2021; Jackson-Ziems 2021, Wise 2021; Pandey et al. 2022). As of September 13, 2021, tar spot has been confirmed in 82 of Indiana's 92 counties (https://corn.ipmpipe.org/tarspot/). Localized epidemics have been severe in several northern and a few southwestern Indiana counties (Telenko 2021 *personal communication*).

In 2021, corn yield loss due to tar spot in the Unites States amounted to 5.9 million metric tons (231.3 million bushels) (Mueller et al. 2022). In Indiana tar spot led to a 4.0% loss in corn production in 2021 valued at $US 253.5 million (Crop Protection Network 2022). Environmental models predicted $US 231.6 million revenue loss for every 1% (1.5 MMT) of corn yield loss due to tar spot in the U.S (Mottaleb et al. 2019), where the rate of yield loss is dependent on the environmental conditions favoring disease development, severity of epidemics, genotype susceptibility level, and the corn growth stage (Hock et al. 1989; Pereyda-Hernández et al. 2009).

Symptoms of tar spot include signs of black-brown, semi-circular, raised fungal fruiting bodies (stromata) embedded in the surfaces of leaves, stems, and husks of ears on plants of any age (Parbery 1967; Valle-Torres et al. 2020; Telenko et al. 2021). As tar spot progresses over time in heavily infested fields, green foliage becomes blighted and senesces leading to death of the plant (Lui 1973; Valle-Torres et al. 2020). Infection is initiated by the pathogen's ascospores, which under optimum conditions, may reduce the photosynthetic feature of leaves, grain filling, quality and quantity of grains, and silage production (Hock et al. 1989; Ceballos and Dutsch 1992).

Infested corn residue is the primary inoculum source that hosts overwintering ascospores (Kleczewski et al. 2019; Groves et al. 2020).

Currently recommended management strategies for tar spot in the U.S. include planting moderately tolerant hybrids, application of foliar fungicides, crop rotation and tillage for residue management (Kleczewski et al. 2019; Telenko et al. 2019; Wise et al. 2019; Corn Disease Working Group 2020; Da Silva et al. 2021b; Telenko et al. 2020, 2022). Fungicide protection is a necessary tactic in plant disease management to reduce disease damage, increase crop production and net return (Paul et al. 2011; Bradley 2012; Wise et al. 2019). There has been a significant increase in the usage of fungicides in hybrid corn production in the U.S. due to the benefits of increased yields in the presence of disease, delayed leaf senescence, and decreased lodging at harvest (Wise and Mueller 2011; Tedford et al. 2017). Certain fungicides have also been shown to increase yield, even in the absence of disease, such as those in the quinone inside inhibitors (QoI) group (Zhang et al. 2010). However, fungicide applications may not always be economically beneficial (Bartlett et al. 2002; Venancio et al. 2003; Paul et al. 2011). Applying a foliar fungicide to corn is usually based on the crop developmental stage, environmental factors, the susceptibility of the host, disease severity, and crop price (Ward et al. 1997; Nelson and Meinhardt 2011; Mueller et al. 2021).

On average, fungicides can provide protective disease coverage for 14 to 21 days following application (Mueller et al. 2013). Tar spot has a latent period of 12 to15 days for noticeable signs and symptoms (Hock et al. 1995; Carson 1999; Telenko et al. 2020). Hence, a well-timed and informed fungicide application program will be most important in reducing tar spot severity, increasing yields, and achieving a positive return on investment. For many foliar corn diseases, the current recommendation for fungicide application is at the anthesis-crop development stages, tassel (VT) to silk (R1) or up to milk (R3) (Abendroth et al. 2011; Mueller et al. 2021). Fungicide application at the VT to R1 or up to R3 may be economically beneficial, but is dependent on the foliar disease, hybrid, and environmental conditions (Tedford et al. 201; Paul et al. 2011; Mueller et al. 2021). Yield loss in a crop is most prominent at tasseling through early grain fill and less prominent as the crop approaches maturity (Mueller et al. 2021). Late vegetative and early reproductive fungicide application targets foliar disease management, grain productivity, and is most likely to gain a positive economic return when conditions are favorable for disease development (Tedford et al. 2017; Paul et al. 2011; Mueller et al. 2021).

A study by Wise et al. (2019), evaluated corn yield response to foliar fungicides in the U.S. and Ontario, Canada. They concluded that foliar fungicides could significantly increase corn grain yields, but growers needed to focus on applications at the tassel (VT) growth stage to ensure the likelihood of a positive return on investment. Notably, a positive return to fungicide investment is most likely to occur in locations and years when disease incidence and severity levels are high and a negative economic return in years when severity levels are low (Ransom and McMullen 2008; Wiik and Rosenqvist 2010; Wegulo et al. 2011, Edwards et al. 2012a). The economic return to fungicide treatments is also subject to the treatment cost, treatment application method, and commodity price.

Information regarding fungicide efficacy, application timing, and the economic return associated with fungicide programs is needed to help growers make more informed tar spot management decisions. There are few published field studies on the efficacy and timing of fungicides to manage tar spot in the Indiana and the Midwest (Da Silva et al. 2020a,b; 2021b; Ross et al. 2020a,b, 2021a,b; Telenko et al. 2019b,c, 2020a-c, 2021a,b, 2022; Waibel et al. 2021a,b). To our knowledge, the net return of hybrid corn under fungicide programs at different stages of disease progression and under different disease condition levels has not been studied quantitatively in Indiana. There are no thresholds established for fungicide applications based on tar spot severity levels in the U.S., making it difficult to recommend to corn growers when to apply fungicides. A study by Wise et al. (2019), showed that different fungicide classes and application timings have been beneficial for managing disease and increasing producer returns. Likewise, Telenko et al. (2022) showed that a two- and three-mode-of-action fungicide are more efficacious and beneficial in reducing tar spot severity; but a three mode-of action is the best for disease reduction and protecting yields. Nevertheless, the question remains which of these fungicides available for tar spot management produces the highest net return for Indiana corn growers.

The goal of this study was to conduct small-plot field trials and evaluate fungicide efficacy and timing for tar spot management in Indiana. This study was designed to test the following hypothesis: that appropriate selection and strategic timing fungicide applications can significantly reduce tar spot severity, protect yield, and increase the probability of positive net return to Indiana growers. The objectives were to assess the effects of foliar fungicides and fungicide timing on tar spot severity and green canopy of corn. Additionally, to estimate the yield response, expected net return and probability of recovering fungicide program cost under high and low tar spot disease

conditions. Results from this study will aid corn growers with the information necessary to manage tar spot of corn economically. Results will also serve as a foundation for stochastic methods to quantify and forecast losses associated with tar spot of corn.

3.3 Materials and Methods.

3.3.1 Study locations.

During the 2019, 2020, and 2021 growing seasons, two types of field experiments, fungicide efficacy and fungicide timing, for tar spot were established at two of Purdue University's research centers: Pinney Purdue Agricultural Center (PPAC) at Wanatah, LaPorte, IN (coordinates: 41°27'20.15"N, 86°56'36.66"W) and the Agronomy Center for Research and Education (ACRE), at West Lafayette, Tippecanoe County, IN (Coordinates: 40°29'33.28"N, 87°0'11.14"W). Wanatah is in Northern Indiana whereas West Lafayette is in Central Indiana, both of differing climatic conditions and history of tar spot. Detailed trial information is presented in Table 3.1. In 2021, these experiments were not carried out at the West Lafayette location due to extremely low tar spot disease incidence in previous years.

3.3.2 Experiment design.

Each experiment type was laid out in a randomized complete block design with fifteen and eleven treatments in four replications for fungicide efficacy and application timing experiments, respectively. Field plots were 3.0 m wide and 9.1 m long and consisted of four rows 76.2 cm apart. The two center rows of each four-row plot were used for all data collection in each trial. All fields were previously established with corn and standard agronomic practices for corn production in Indiana were followed. Corn hybrid W2585SSRIB was planted at a density of 13,759 seeds per hectare (34,000 seeds per acre) for all trials. Irrigation was supplemented weekly in the fungicide efficacy trials to encourage disease in years when natural precipitation did not reach 25.4 mm at Wanatah, IN. All fungicide treatments used contained a non-ionic surfactant (Preference) at a rate of 0.25% v/v and were registered for use on corn in Indiana. Fungicide treatments were applied using either a CO_2 backpack sprayer or a Lee self-propelled sprayer equipped with a 3.0 m boom, fitted with six TJ-VS 8002 nozzles spaced 0.5 m apart delivering fungicides at 3.6-mph with 140.3 L/ha at 275.8 kPa.

Table 3.1. Detailed information of field experiments conducted in Indiana for tar spot of corn during 2019, 2020, and 2021.

Site and year	Planting date	Irrigation (Y/N)	Fungicide application date (Growth stage [z])	Date of 1st tar spot detection	Harvest date
Fungicide efficacy experiments					
Wanatah 2019	8 Jun	Y	8 Aug (VT/R1)	2 Aug	28 Oct
Wanatah 2020	9 Jun	Y	7 Aug (VT/R1)	28 Jul	6 Nov
Wanatah 2021	27 May	Y	6 Aug (VT/R1)	9 Jul	4 Nov
West Lafayette 2019	4 Jun	N	4 Aug (VT/R1)	Not detected	15 Oct
West Lafayette 2020	25 May	N	25 Jul (VT/R1)	10 Aug	18 Oct
Application timing experiments					
Wanatah 2019	8 Jun	N	8 Jul (V7), 15 Jul (V9), 19 Jul (V10), 7 Aug (VT/R1), and 23 Aug (R2)	2 Aug	28 Oct
Wanatah 2020	8 Jun	N	14 Jul (V8), 20 Jul (V10), 7 Aug (VT/R1), 21 Aug (R2), 2 Sep (R3), 11 Sep (R4), and 23 Sep (R5)	4 Aug	4 Nov
Wanatah 2021	27 May	N	23 Jul (V8), 2 Aug (V10), 6 Aug (VT/R1), 20 Aug (R2), 30 Aug (R3), 10 Aug (R4), and 16 Aug (R5)	9 Jul	4 Nov
West Lafayette 2019	4 Jun	N	5 Jul (V6), 11 Jul (V8), 17 Jul (V10), 4 Aug (VT/R1), and 16 Aug (R2)	Not detected	15 Oct
West Lafayette 2020	25 May	N	1 Jul (V8), 13 Jul (V11), 25 Jul (VT/R1), 9 Aug (R2), 18 Aug (R3), 25 Aug (R4), and 9 Sep (R5)	15 Sep	18 Oct

[z] All fungicides were applied at the tassel/silk (VT/R1) corn growth stage for fungicide efficacy experiments and at the six-leaf (V6) or seven-leaf (V7), eight-leaf (8-leaf) or nine-leaf (V9), ten-leaf (V10) or eleven-leaf (V11), tassel-silk (VT/R1), blister (R2), milk (R3), dough (R4), and dent (R5) or a double application at the V6-V8 fb VT/R1 corn growth stages for fungicide timing experiments.

3.3.3 Fungicide efficacy trials.

Field plots were designed to evaluate the efficacy and net return of fourteen fungicide treatments as compared to a nontreated control. The fourteen fungicide treatments included various active ingredients from different chemical groups, quinone outside inhibitors (QoIs, strobilurins), C-14 de-methylation inhibitors (DMIs, azoles), and succinate dehydrogenase inhibitors (SDHIs, carboxamides) in combinations (Table 3.2) (Fungicide Resistance Action Committee, 2021). The fungicide treatments were propiconazole (Tilt, Syngenta Crop Protection, Greensboro, NC), prothioconazole (Proline, Bayer Crop Science, St. Louis, MO), pyraclostrobin (Headline, BASF Corporation, Research Triangle Park, NC), flutriafol + bixafen (Lucento, FMC Corporation, Philadelphia, PA), flutriafol + azoxystrobin (Topguard, FMC Corporation, Philadelphia, PA), azoxystrobin + propiconazole (Quilt Xcel, Syngenta Crop Protection, Greensboro, NC), cyproconazole + picoxystrobin (Aproach Prima, Corteva Agriscience, Wilmington, DE), mefentrifluconazole + pyraclostrobin (Veltyma, BASF Corporation, Research Triangle Park, NC), metconazole + pyraclostrobin (Headline AMP, BASF Corporation, Research Triangle Park, NC), prothioconazole + trifloxystrobin (Delaro, Bayer Crop Science, St. Louis, MO), mefentrifluconazole + fluxapyroxad + pyraclostrobin (Revytek, BASF Corporation, Research Triangle Park, NC), propiconazole + benoindiflupyr + azoxystrobin (Trivapro, Syngenta Crop Protection, Greensboro, NC), prothioconazole + trifloxystrobin + fluopyram (Delaro Complete, Bayer Crop Science, St. Louis, MO), and propiconazole + pydiflumetofen + azoxystrobin (Miravis Neo, Syngenta Crop Protection, Greensboro, NC). All fungicide treatments were applied at the tassel-silk (VT/R1) corn growth stage. Manufacturer's recommended dosages for each fungicide treatment were followed. See Table 3.2 for details on percent active ingredient, FRAC code, application rate, cost of fungicide program for ground and aerial method, and year used.

3.3.4 Fungicide timing trials.

Field plots were designed to assess the net return of the mixed mode of action fungicide propiconazole + benoindiflupyr + azoxystrobin (Trivapro, Syngenta Crop Protection, Greensboro, NC) applied at different growth stages of corn during tar spot disease progression (Table 3.2). This experiment included ten treatments, of which one was a nontreated control for comparison purposes. The other nine treatments were a single application of propiconazole + benoindiflupyr

+ azoxystrobin at 0.96 L/ha applied at the six/seven-leaf stage (V6/V7), eight/nine-leaf stage (V8/V9), ten/eleven-leaf stage (V10/V11), tassel-silk (VT/R1), blister (R2), milk (R3), dough (R4), or dent (R5) growth stages; or a two-application program applied at V6 to V8 followed by (fb) a tassel-silk application (V6-V8 fb VT/R1). See Table 3.2. for details on percent active ingredient, FRAC code, application rate, cost of fungicide program, and year used.

Table 3.2. Summary information about the fungicide treatments used in this study.

FRAC code [x]	Active ingredients (%)	Product name and manufacturer	Application rate (L/ha)	Average fungicide program cost [y]		Year evaluated
				Ground ($/ha)	Aerial ($/ha)	
3	propiconazole (41.80%)	Tilt 3.6 EC, Syngenta Crop Protection, Greensboro, NC	0.28	31.48	37.48	2020 2021
3	prothioconazole (41.00%)	Proline 480 SC, Bayer Crop Science, St. Louis, MO	0.40	17.06	23.06	2019
11	pyraclostrobin (23.60%)	Headline 2.09 SC, BASF Corporation, Research Triangle Park, NC	0.42	23.17	29.17	2019 2020 2021
3 + 7	flutriafol (26.47%) + bixafen (15.55%)	Lucento, FMC Corporation, Philadelphia, PA	0.35	27.45	33.45	2020 2021
3 + 11	flutriafol (18.63%) + azoxystrobin (25.30%)	Topguard EQ, FMC Corporation, Philadelphia, PA	0.49	31.21	37.21	2019
3 + 11	azoxystrobin (13.50%) + propiconazole (11.70%)	Quilt Xcel 2.2 SE, Syngenta Crop Protection, Greensboro, NC	0.98	28.98	34.98	2019
3 + 11	cyproconazole (7.17%) + picoxystrobin (17.94%)	Aproach Prima 2.34 SC, Corteva Agriscience, Wilmington, DE	0.48	22.36	28.36	2019 2020 2021

Table 3.2 continued

3 + 11	mefentrifluconazole (17.56%) + pyraclostrobin (17.56%)	Veltyma, BASF Corporation, Research Triangle Park, NC	0.49	28.64	34.64	2019 2020 2021
3 + 11	metconazole (5.14%) + pyraclostrobin (13.64%)	Headline AMP 1.68 SC, BASF Corporation, Research Triangle Park, NC	1.02 in 2019 0.73 in 2020 0.73 in 2021	23.17	29.17	2019 2020 2021
3 + 11	prothioconazole (16.0%) + trifloxystrobin (13.70%)	Delaro 325 SC, Bayer Crop Science, St. Louis, MO	0.56	40.67	46.67	2019 2020 2021
3 + 7 + 11	mefentrifluconazole (11.61%) + fluxapyroxad (7.74%) + pyraclostrobin (15.49%)	Revytek, BASF Corporation, Research Triangle Park, NC	0.56	30.91	36.91	2020 2021
3 + 7 + 11	propiconazole (11.90) + benoindiflupyr (2.90%) + azoxystrobin (10.50%)	Trivapro 2.21 SE, Syngenta Crop Protection, Greensboro, NC	0.96	29.53	35.53	2019 2020 2021
3 + 7 + 11	prothioconazole (14.90%) + trifloxystrobin (13.10%) + fluopyram (10.90%)	Delaro Complete 3.83 SC, Bayer Crop Science, St. Louis, MO	0.56	27.52	33.52	2021
3 + 7 + 11	propiconazole (11.60%) + pydiflumetofen (7.00%) + azoxystrobin (9.30%)	Miravis Neo 2.5 SE, Syngenta Crop Protection, Greensboro, NC	0.96	31.48	37.48	2019 2020 2021

[x] FRAC codes are designated by the Fungicide Resistance Action Committee as a system to identify the active ingredient mode of action and resistance risk (FRAC code list 2021; http://www.frac.info/). Class; 3=Sterol biosynthesis inhibitor: C-14 de-methylation inhibitors (DMI) or azoles fungicides; 7=Inhibitor of respiration in complex II at SDH: succinate dehydrogenase inhibitors (SDHI) or carboxamide fungicides; 11=inhibitor of respiration in complex III at QoI: quinone outside inhibitors (QoI) or strobilurins fungicides. [y] Includes the product cost and product application cost obtained from representatives of agricultural-based companies manufacturing and taking the average across the three years.

3.3.5 Weather conditions.

Average monthly mean air temperatures(°C), precipitation (mm), and relative humidity (%) were obtained from the weather station located near the respective experimental sites (Purdue Mesonet stations, https://ag.purdue.edu/indiana-state-climate/) (Table 3.3). Twenty-year weather data summaries were also obtained from the Indiana State Climate Office as a standard for normal weather conditions and were used for comparison. The months of June to October were selected and compared to this 20-year average for mean air temperatures, precipitation, and relative humidity (Table 3.3).

Table 3.3. Average monthly mean air temperature (°C), total precipitation (mm), and relative humidity (%) from June to October 2019 to 2021 obtained from the weather recording stations near each research location.

Months	Mean air temperature[z] (°C)				Total precipitation[z] (mm)				Relative humidity[z] (%)			
	2019	**2020**	**2021**	**20-year average**	**2019**	**2020**	**2021**	**20-year average**	**2019**	**2020**	**2021**	**20-year average**
						WANATAH, IN						
Jun	20.2	21.9	22.0	21.2	69.5	61.0	143.0	71.2	73.8	65.4	70.9	72.1
Jul	21.9	23.1	22.7	22.3	25.8	76.8*[y]	75.9	75.7	76.1	76.9	81.2	75.8
Aug	22.3	21.0	22.1	21.0	33.4	40.2*	102.4	89.0	76.7	76.2	79.3	80.4
Sep	19.1	17.2	19.2	18.4	90.9	42.7*	40.2*	57.9	78.6	74.4	69.3	75.8
Oct	11.2	9.4	17.4	11.3	70.2	73.3	159.5	82.2	70.4	71.9	81.4	73.9
						WEST LAFAYETTE, IN						
Jun	21.2	25.4	-	22.1	128.3	73.2	-[x]	86.8	70.2	69.2	-	70.3
Jul	24.5	26.3	-	24.2	78.2	71.2	-	78.9	74.0	74.6	-	74.8
Aug	22.7	23.2	-	23.1	67.6	53.4	-	62.1	73.9	75.3	-	76.2
Sep	21.2	18.9	-	19.2	58.4	56.8	-	59.2	74.0	72.6	-	73.3
Oct	12.4	12.2	-	13.4	76.2	74.1	-	69.8	71.1	70.0	-	72.3

[z] Data courtesy of Indiana State Climate Office. https://ag.purdue.edu/indiana-state-climate/. Taken from Purdue Mesonet stations at the Pinney Purdue Agricultural Center (PPAC), Wanatah IN and Purdue Agronomy Center for Research and Education (ACRE), West Lafayette IN.

[y] '*' = 25.4 mm irrigation water was supplemented weekly when natural rainfall did not meet 25.4 mm or higher. 76-, 102-, and 51-mm irrigation was supplemented in 2020 for the months of Jul, Aug, and Sep, respectively. In Sept 2021, 51 mm irrigation water was supplemented.

[x] '-'= experiment was not conducted in that particular year.

3.3.6 Disease severity assessments.

The severity of tar spot was assessed weekly from the first detection of tar spot in each trial to corn growth stage dent (R5) or physiological maturity (R6). The disease severity assessment included two variables: the percent tar spot stroma and percent tar spot foliar symptoms in the canopy based on a standardized rating scale for tar spot (Telenko et al. 2021a). An intra-rater reliability test was performed before data collection to reduce data biases and to ensure some level data consistency. Percent tar spot stroma was rated by visually assessing the leaf area (0-100%) covered with fungal stroma, whereas the percent tar spot foliar symptoms assessed the amount of leaf area (0-100%) that exhibited chlorotic and necrotic symptoms. Five plants per plot (subsamples) were randomly selected, and disease severity was rated on three leaves: the ear leaf (EL), ear leaf minus two leaves (EL-2), and ear leaf plus two leaves (EL+2). These three leaves were then averaged for a single value of disease severity estimate per every experimental unit (plant), and experimental units were average to represent a single value per plot. Only the final tar spot severity ratings recorded at R5 or R6 for each site-year were used for disease analysis between locations and years.

3.3.7 Green canopy of corn and yield assessment.

The two center rows of each plot were harvested using a small plot combine (Kincaid 8XP). Yields were standardized to 15.5% moisture prior to analysis. Changes in yield due to fungicide application compared to the nontreated plots were calculated using the formula Yield Increase (Ydiff) = Yf-Yc, where Yf is the yield due to fungicide application and Yc is yield of the nontreated control. Ydiff(s) were used in the economic analysis.

3.3.8 Statistical Analysis.

Data analyses were performed in SAS 9.4 (SAS Institute, Cary, NC). Analysis of variance (ANOVA) was performed using a generalized linear mixed model procedure fitted within PROC GLIMMIX. In this model, fungicide treatment was treated as the only fixed effect. To determine which fungicide treatments (products and application timing) had the highest efficacy in managing tar spot, offered yield protection and net return large enough to recover the cost of investment, site-years (location x year) were treated as a form of replication. The random effect accounted for

the heterogeneity of variance among, which included an intercept along with replicate as the subject effect and site-year as the group effect. For all analyses, a normal distribution was used with Kenward-Rogers degrees of freedom as defined as the ddfm = kr option in the model statement to account for missing observations (Littell et al. 2006). Exploratory analysis showed normal residuals for disease severity values; hence these data were analyzed without transformation. Treatment least-square means (lsmeans) were obtained using lsmeans statement with a Tukey-Kramer adjustment at a 5% level of significance ($\alpha = 0.05$) (Piepho 2012).

3.3.9 Economic Analysis.

Due to the recent emergence of tar spot in the Midwest U.S., no threshold for severity and yield loss is established for tar spot of corn. To assess the yield response and expected net return from foliar fungicides and fungicide application timing under high and low tar spot disease conditions, site-years were grouped into two baseline disease severity categories determined by the percent disease severity in nontreated control plots at a 5-percent cutoff point. Paul et al. (2011) proved that this 5-percent cutoff helped to justify the significance of success in using fungicides for managing gray leaf spot. In our study, the two baseline disease severity categories used were i) High tar spot disease severity condition (TS high), where tar spot severity in the nontreated control plots were greater than 5% severity and ii) Low tar spot disease severity condition (TS low) where tar spot severity in the nontreated control plots were less than 5% severity. In our study average tar spot severity in the nontreated control plot for site years at the Wanatah location was greater than 25% (Table 3.4), hence these site-years were analyzed as TS high. For site-years at the West Lafayette location, tar spot severity in the nontreated control plots were less than 1% (Table 3.4), so these site-years were analyzed as TS low.

Table 3.4. Average tar spot severity in the nontreated controls at each site-year for field experiments conducted in Indiana.

Fungicide Efficacy Trials [z]		
Site-years	**Severity of tar spot stroma (%)** [x]	**Severity of tar spot foliar symptoms (%)** [w]
Wanatah 2019	29.6	41.8
Wanatah 2020	30.7	75.3
Wanatah 2021	33.0	100.0
West Lafayette 2019	0.0	0.0
West Lafayette 2020	0.1	0.0
Fungicide Timing Trials [y]		
Wanatah 2019	27.1	69.5
Wanatah 2020	29.2	55.9
Wanatah 2021	35.5	92.3
West Lafayette 2019	0.0	0.0
West Lafayette 2020	0.3	0.0

[z] Fourteen foliar fungicides and a nontreated control were compared for efficacy in reducing tar spot severity.

[y] Ten fungicide application timings and a nontreated control were compared for effectiveness in reducing tar spot severity.

[x] Severity of tar spot stroma was assessed visually by evaluating the percentage leaf area (0-100%) covered with fungal stroma on the ear leaf, and ear leaf ± 2 leaves on five plants per plot at dent (R5) or maturity (R6) corn growth stage. Values were averaged before analysis.

[w] Severity of tar spot foliar symptoms was assessed visually by evaluating the percentage leaf area (0-100%) covered with chlorotic-necrotic lesions on the ear, and ear leaf ± 2 leaves on five plants per plot at R5 or R6 corn growth stage. Values were averaged before analysis. Values are least-square means from each trial.

A partial budgeting approach was used to calculate the expected net return (NR) of each fungicide treatment focusing only on the revenues and costs that are changed with the grower's decision to apply a foliar fungicide. This is not the same as the net return inclusive of all revenues and expenses faced by the grower. Net returns are calculated in dollars per hectare ($/ha) using the following modified equation originally developed by Munkvold et al. (2001),

$$NR = P * Ydiff - N (F_{pc}),$$

where P is the corn price ($/kg), Ydiff is the change in yield due to fungicide application as compared to the nontreated control (kg/ha), N is the number of fungicide applications, and F_{pc} is the cost of the fungicide program ($/ha), which sums the product cost and application cost. Both ground and aerial methods for fungicide application were assessed. The costs of each fungicide program used in this study are listed in Table 3.2. Fungicide program costs were obtained from representatives of agricultural-based companies/industries manufacturing these products and by inquiring from corn growers and those who carry out spraying operations and taking the average across the three years. An average corn price of $0.17 per kilogram was used in this study which was obtained from data provided by the USDA National Agricultural Statistics Service (USDA-NASS 2021) for the last five years, 2017 to 2021.

3.3.10 Economic risk analysis

Costs associated with fungicide programs are subject to variability due to market fluctuations and the differences associated with the efficacy of generic fungicides (Corn Disease Working Group 2020). Corn growers may want to predict how a fungicide program may perform in future growing seasons or may be interested in estimating the financial risk associated with a fungicide program if used when it is not warranted. Hence, the probability of recovering the investment of each fungicide program is estimated as outlined by Munkvold et al. (2001), Bruin et al. (2010), and Bestor (2011). A range of fungicide program costs of $15 to $60/ha and corn prices of $0.13, $0.17, and $0.21/kg ($3.20, $4.20 and $5.20/bu.) were used to calculate the probability of breaking even on fungicide program cost (designated as ProbFC). These corn prices represent the near average of the last five years and price levels above and below the current levels to understand how the net return probabilities change as prices fluctuate.

Following a modified equation by Munkvold et al. (2001) and Bestor (2011), the probability of breaking even on fungicide program cost (*ProbFC*) of each fungicide program was calculated as $ProbFC = \Phi\left[\frac{Ydiff-\beta_0}{s}\right]$,

where Φ is the cumulative standard-normal distribution function and s is the estimated among-replication stand+ard deviation, Ydiff is the yield effect due to fungicide treatment, and β_0 is the yield difference needed to offset the cost of fungicide treatment (kg/ha), as $\beta_0 = \frac{Fpc}{P}$. Least significant differences (LSD) at α = 0.05 was used to separate mean of treatment net returns.

3.4 Results.

3.4.1 Average monthly weather conditions.

Average monthly air temperatures, precipitation and relative humidity profiles differed each year at each research location. These differences in weather conditions are presented in Table 3.3. The average monthly mean air temperatures across site years (location x year) for the period of June to October ranged from 9.4 to 26.3 °C where the 20-year average (normal) ranged from 11.3 to 24.2 °C (Table 3.3). Mean air temperature was 6.1 °C, 2.0 °C, and 3.3 °C above normal (20-year) air temperature in October 2021 at Wanatah, in September 2019 at West Lafayette, and in June 2021 at West Lafayette, respectively (Table 3.3). Of the two locations, the average monthly mean air temperature was 0.8 °C warmer at Wanatah when compared to West Lafayette (Table 3.3).

The average monthly total precipitation across site-years ranged from 25.8 to 159.5 mm whereas the total 20-year average (normal) precipitation ranged from 57.2 to 89.0 mm (Table 3.3). Monthly precipitation was 33.0 mm, 71.8 mm, 13.4 mm, and 77.3 mm higher than the 20-year average monthly total precipitation at Wanatah, IN in September 2019, June 2021, August 2021, and October 2021, respectively. At West Lafayette, IN, the average monthly total precipitation was 41.5 mm, 5.5 mm, 6.4 mm and 4.3 mm higher than the 20-year average precipitation for June 2019, August 2019, October 2019, and October 2020, respectively (Table 3.3). Average monthly total precipitation was 195.5 mm higher at Wanatah, IN when compared to West Lafayette, IN (Table 3.3).

The average monthly relative humidity across site-years ranged from 65.4 to 81.4% across site years where the 20-year average relative humidity ranged from 72.1 to 80.4% (Table 3.3). Average monthly relative humidity was 3.8%, 2.8%, 4.2%, 5.4%, and 7.5% higher than the 20-year average (normal) relative humidity at Wanatah, IN, August 2019, September 2019, August 2020, July 2021, and October 2021, respectively (Table 3.3).

Overall, site-year at Wanatah 2021 was characterized by extreme wetness based on the higher amounts of precipitation (71.8 mm, 13.4 mm, and 7.7 mm) recorded for June, August, and October, respectively, when compared to the 20-year monthly totals (Table 3.3).

3.4.2 Effect of foliar fungicides on tar spot severity and green canopy of corn.

Tar spot severity assessed the percent tar spot stroma and percent tar spot foliar symptoms (necrosis/chlorosis) in the canopy as measured by the mean of severity taken from the ear leaf and ear leaf ± two leaves at R5 (dent) or R6 (maturity). Tar spot severity varied across site-years and ranged from 0.0 to 33.0% for severity of stroma and 0.0 to 100% for severity of foliar symptoms in the nontreated control (Table 3.4). Severity of tar spot stroma in the canopy was significantly reduced by 7.3% to 10.4% over the nontreated control by propiconazole + pydiflumetofen + azoxystrobin (Miravis Neo), cyproconazole + picoxystrobin (Approach Prima), prothioconazole + trifloxystrobin + fluopyram (Delaro Complete), prothioconazole + trifloxystrobin (Delaro), mefentrifluconazole + pyraclostrobin (Veltyma), pyraclostrobin (Headline), metconazole + pyraclostrobin (Headline AMP), and mefentrifluconazole + fluxapyroxad + pyraclostrobin (Revytek) (Figure 3.1). Despite this significance, no statistical differences were observed among all fungicides evaluated in reducing the severity of tar spot stroma in the canopy (Figure 3.1).

All fungicides significantly reduced the severity of tar spot foliar symptoms by 13.8% to 18.1% over the nontreated control except for propiconazole (Tilt), prothioconazole (Proline), flutriafol + bixafen (Lucento), propiconazole + benoindiflupyr + azoxystrobin (Trivapro), azoxystrobin + propiconazole (Quilt Xcel), and prothioconazole + trifloxystrobin + fluopyram (Delaro Complete) (Figure 3.2). No statistical differences were observed among fungicides in reducing tar spot foliar symptoms (Figure 3.2).

All fungicides significantly increased canopy greenness by 9.2% to 18.3% over the nontreated control (Figure 3.3). Green canopy of corn was significantly greener by 8.9% with mefentrifluconazole + pyraclostrobin when compared to propiconazole (Figure 3.3).

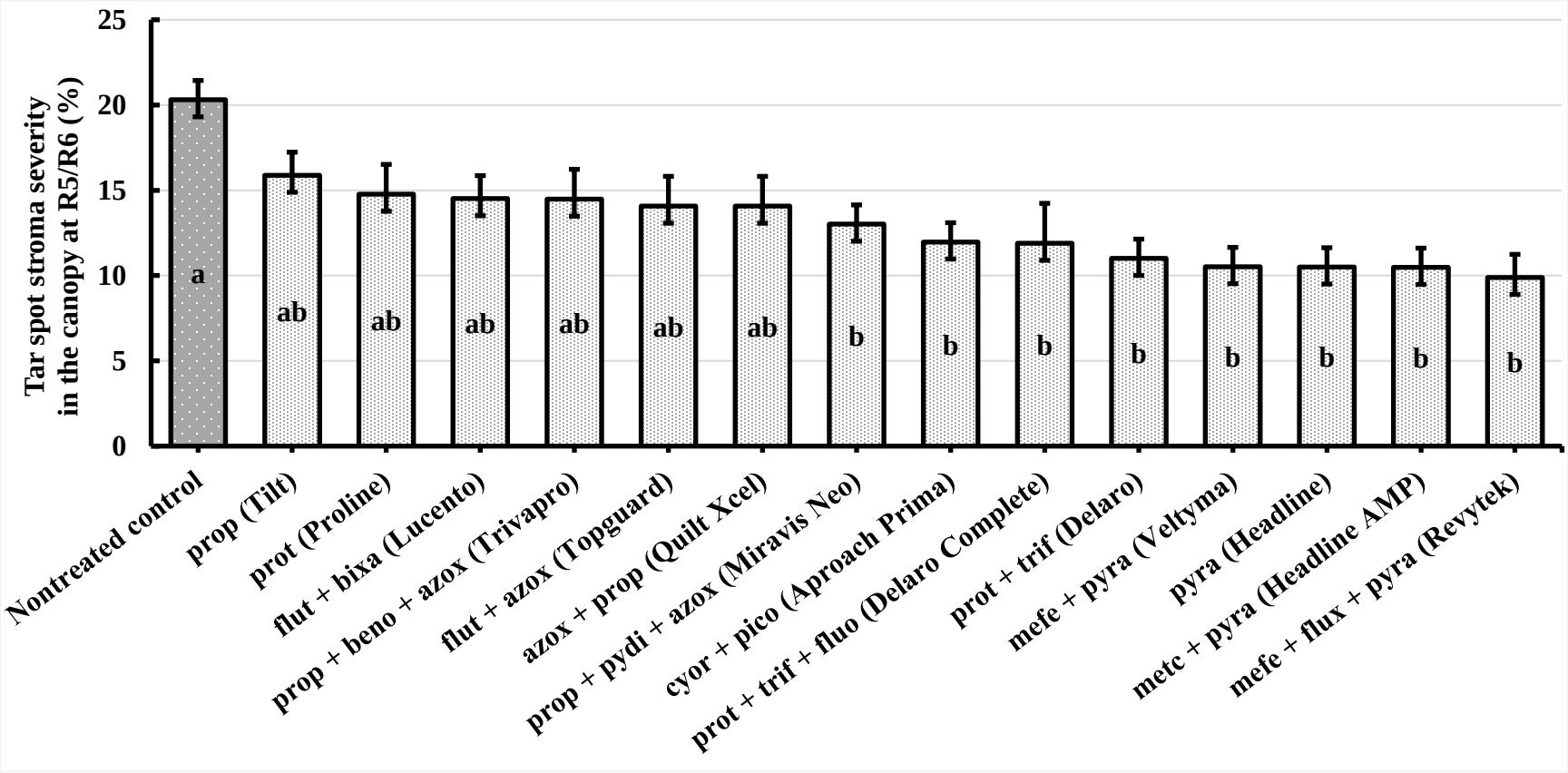

Figure 3.1.Effect of foliar fungicides on tar spot stroma severity in the canopy at dent or maturity (R5/R6) growth stages in Indiana. Fungicide treatments (active ingredients) were azox = azoxystrobin, beno = benoindiflupyr, bixa = bixafen, cyor = cyoroconazole, fluo = fluopyram, flut = flutrifol, flux = fluxapyroxad, mefe = mefentrifluconazole, metc = metconazole, pico = picoxystrobin, prop = propiconazole, prot = prothioconzole, pydi = pydiflumetofen, pyra = pyraclostrobin, trif = trifloxystrobin. Severity of tar spot stroma was assessed visually by evaluating the percentage leaf area (0-100%) covered with fungal stroma on the ear leaf, and ear leaf ± 2 leaves on five plants per plot at dent (R5) or maturity (R6) corn growth stage. Values were averaged before analysis. Least squares means are the averages from trials conducted at two locations (Wanatah and West Lafayette) from 2019 to 2021 in Indiana representing five site-years (p = 0.01). Values with different letters are significantly different based on least-square means test (α = 0.05).

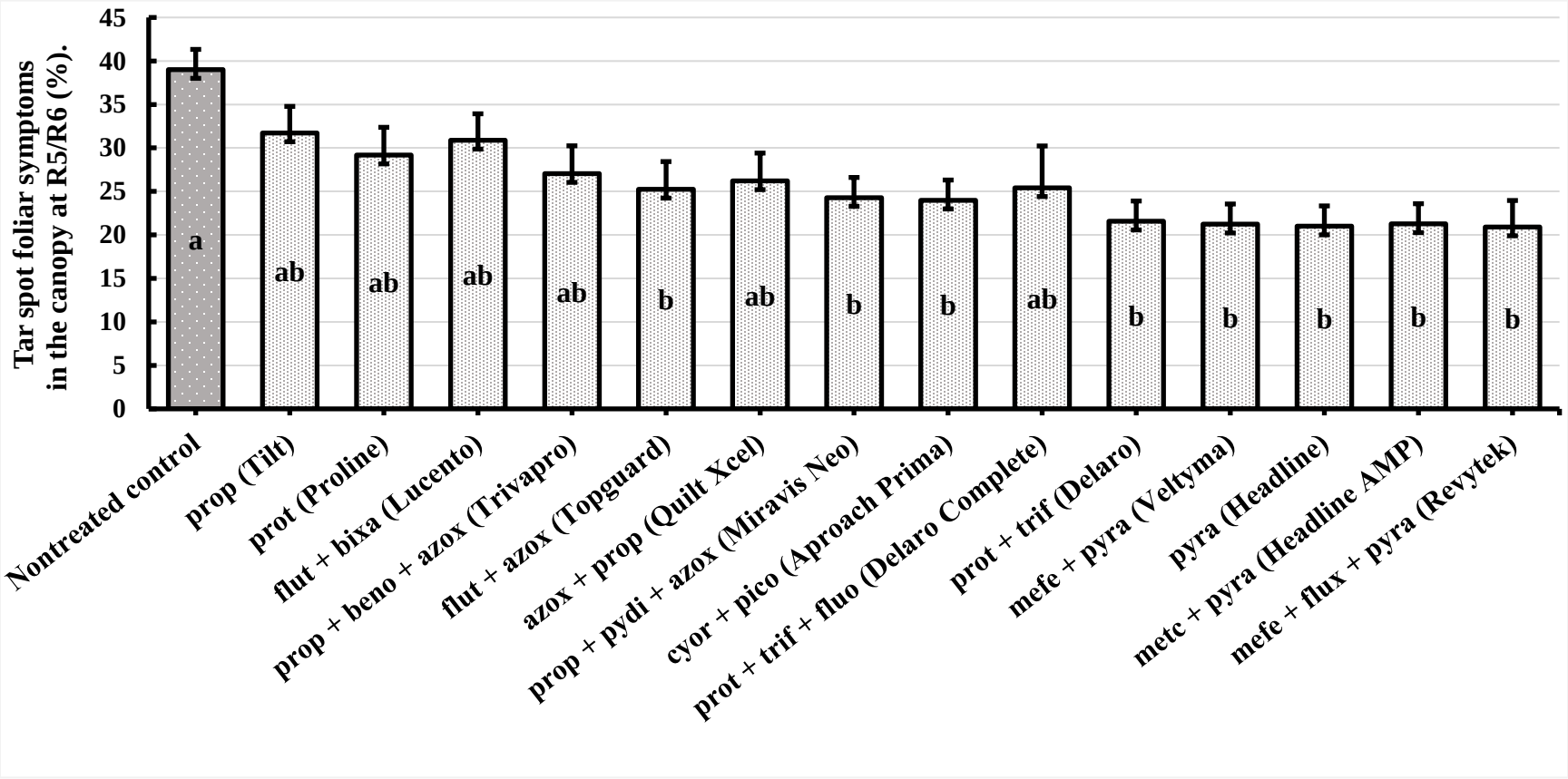

Figure 3.2. Effect of foliar fungicides on tar spot foliar symptoms in the canopy at dent or maturity (R5/R6) in Indiana. Fungicide treatments (active ingredients) were azox = azoxystrobin, beno = benoindiflupyr, bixa = bixafen, cyor = cyoroconazole, fluo = fluopyram, flut = flutrifol, flux = fluxapyroxad, mefe = mefentrifluconazole, metc = metconazole, pico = picoxystrobin, prop = propiconazole, prot = prothioconzole, pydi = pydiflumetofen, pyra = pyraclostrobin, trif = trifloxystrobin. Severity of tar spot foliar symptoms was assessed visually by evaluating the percentage leaf area (0-100%) covered with chlorotic-necrotic lesions on the ear, and ear leaf ± 2 leaves on five plants per plot at R5 or R6 corn growth stage. Values were averaged before analysis. Least squares means are the averages from trials conducted at two locations (Wanatah and West Lafayette) from 2019 to 2021 in Indiana representing five site-years (p = 0.001). Values with different letters are significantly different based on least-square means test (α = 0.05).

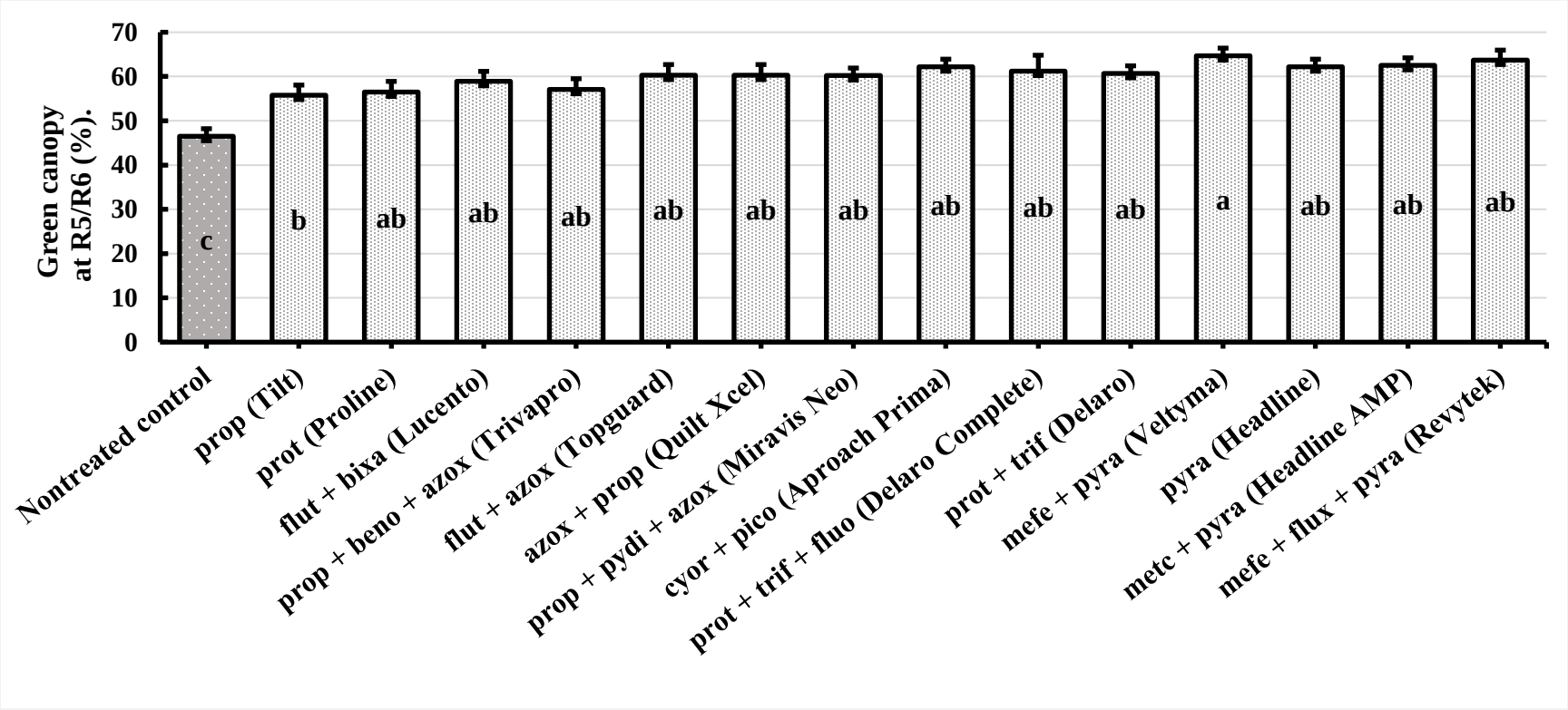

Figure 3.3. Effect of foliar fungicides on green canopy of corn at dent or maturity (R5/R6) in Indiana. Fungicide treatments (active ingredients) were azox = azoxystrobin, beno = benoindiflupyr, bixa = bixafen, cyor = cyoroconazole, fluo = fluopyram, flut = flutrifol, flux = fluxapyroxad, mefe = mefentrifluconazole, metc = metconazole, pico = picoxystrobin, prop = propiconazole, prot = prothioconzole, pydi = pydiflumetofen, pyra = pyraclostrobin, trif = trifloxystrobin. Percent green canopy was determined by visually assessing the amount of whole plant canopy (0-100%) that remained green at R5 or R6 corn growth stage. Least squares means are the averages from trials conducted at two locations (Wanatah and West Lafayette) from 2019 to 2021 in Indiana representing five site-years ($p = 0.01$). Values with different letters are significantly different based on least-square means test ($\alpha = 0.05$).

3.4.3 Effect of fungicide timing on tar spot severity and green canopy of corn.

Tar spot severity in the canopy varied across site-years and ranged from 0.0 to 35.5% for tar spot stroma and 0.0 to 92.3% for tar spot foliar symptoms in the nontreated control (Table 3.3). Propiconazole + benoindiflupyr + azoxystrobin (Trivapro) applied at the blister (R2) and milk (R3) corn growth stages significantly lowered tar spot stroma severity in the canopy by 9.0% and 8.3% over the nontreated control plots, respectively (Figure 3.4). However, the applications made at the R2 and R3 corn growth stages were not statistically different from application made at the tassel-silk (VT/R1), six-eight-leaf stage followed by (fb) tassel-silk (V6-V8 fb VT/R1), dough (R4), and dent (R5) corn growth stages (Figure 3.4).

Severity of tar spot foliar symptoms was significantly reduced over the nontreated control by 26.2% and 38.3% with applications made at the R2 and R3 corn growth stages respectively; but these were not statistically different from application made at the V10/V11, VT/R1, V6-V8 fb VT/R1, R4, and R5 corn growth stages (Figure 3.5).

Green canopy of corn ranged from 48.7% to 72.2% when timing applications at different corn growth stages (Figure 3.6). Only applications made at the R2 and R3 corn growth stages significantly increase green canopy of corn over the nontreated control, but these were not statistically different from applications made at the V8, VT/R1, V6 fb VT/R1, V8 fb VT/R1, R4 and R5 corn growth stages (Figure 3.6).

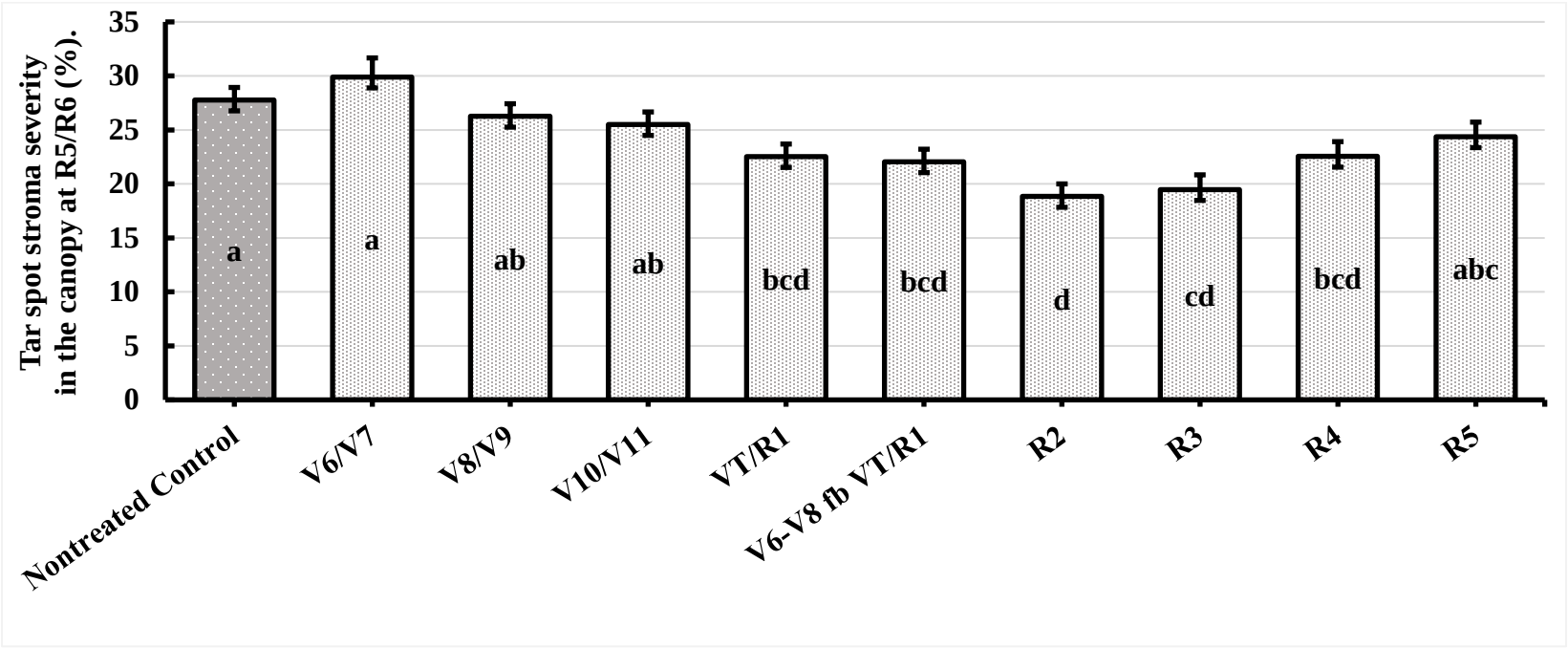

Figure 3.4. Effect of timing propiconazole + benoindiflupyr + azoxystrobin (Trivapro) application at 0.96 L/ha on tar spot stroma severity at dent or maturity (R5/R6) in Indiana. Single application program at V6/V7 = six/seven-leaf stage, V8/V9 = eight/nine-leaf stage, V10/V11 = ten/eleven-leaf stage, VT/R1 = tassel-silk, R2 = blister, R3 = milk, R4 = dough, R5 = dent growth stages and a two-application program at V6 to V8 followed by (fb) a tassel-silk application (V6-V8 fb VT/R1). Severity of tar spot stroma was assessed visually by evaluating the percentage leaf area (0-100%) covered with fungal stroma on the ear leaf, and ear leaf ± 2 leaves on five plants per plot at dent (R5) or maturity (R6) corn growth stage. Values were averaged before analysis. Least squares means are the averages from trials conducted at two locations (Wanatah and West Lafayette) from 2019 to 2021 in Indiana representing five site-years (p = 0.01). Values with different letters are significantly different based on least-square means test (α = 0.05).

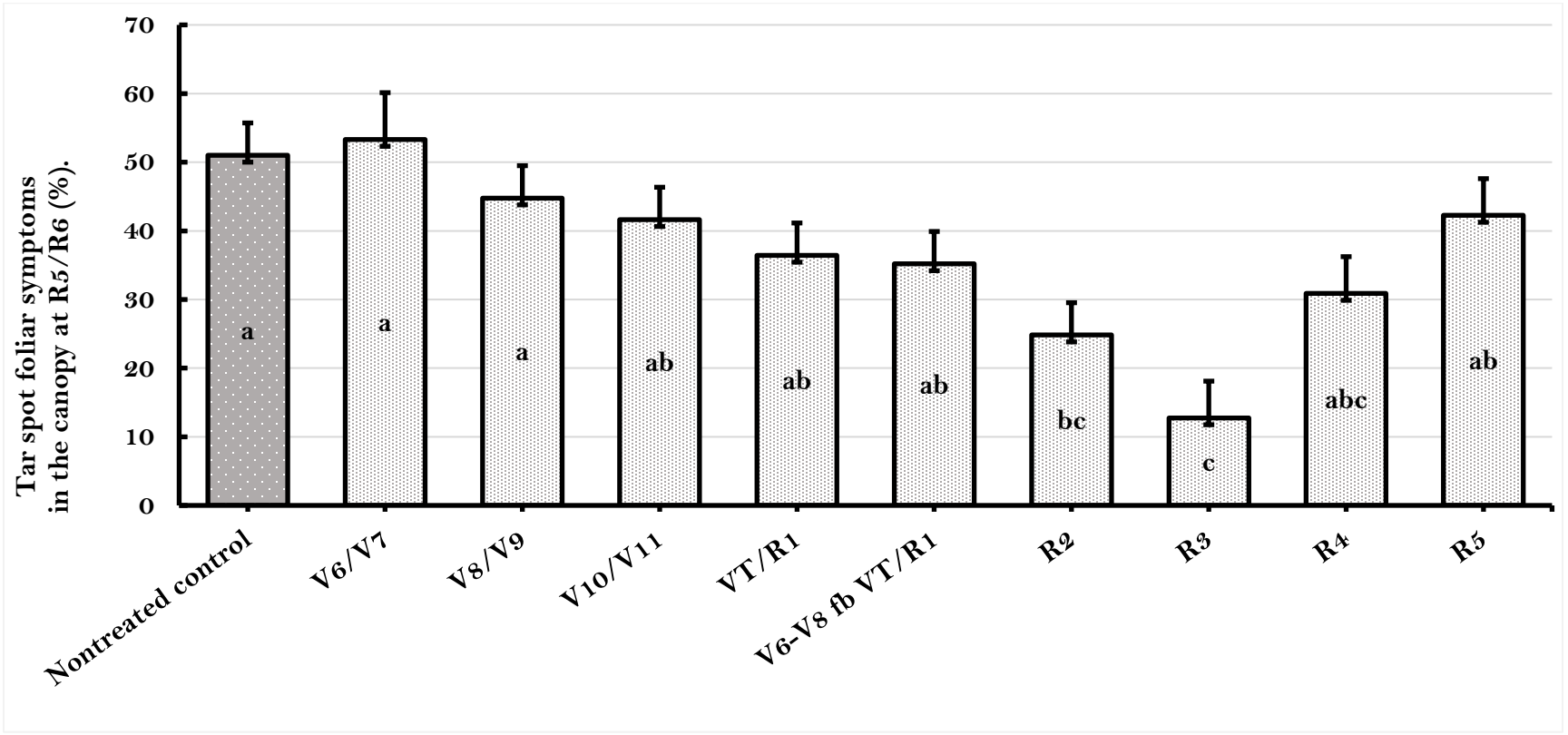

Figure 3.5. Effect of timing propiconazole + benoindiflupyr + azoxystrobin (Trivapro) application at 0.96 L/ha on severity of tar spot foliar symptoms at dent or maturity (R5/R6) in Indiana. Single application program at V6/V7 = six/seven-leaf stage, V8/V9 = eight/nine-leaf stage, V10/V11 = ten/eleven-leaf stage, VT/R1 = tassel-silk, R2 = blister, R3 = milk, R4 = dough, R5 = dent growth stages and a two-application program at V6 to V8 followed by (fb) a tassel-silk application (V6-V8 fb VT/R1). Severity of tar spot foliar symptoms was assessed visually by evaluating the percentage leaf area (0-100%) covered with chlorotic-necrotic lesions on the ear, and ear leaf ± 2 leaves on five plants per plot at R5 or R6 corn growth stage. Values were averaged before analysis. Least squares means are the averages from trials conducted at two locations (Wanatah and West Lafayette) from 2019 to 2021 in Indiana representing five site-years (p = 0.01). Values with different letters are significantly different based on least square means test (α = 0.05).

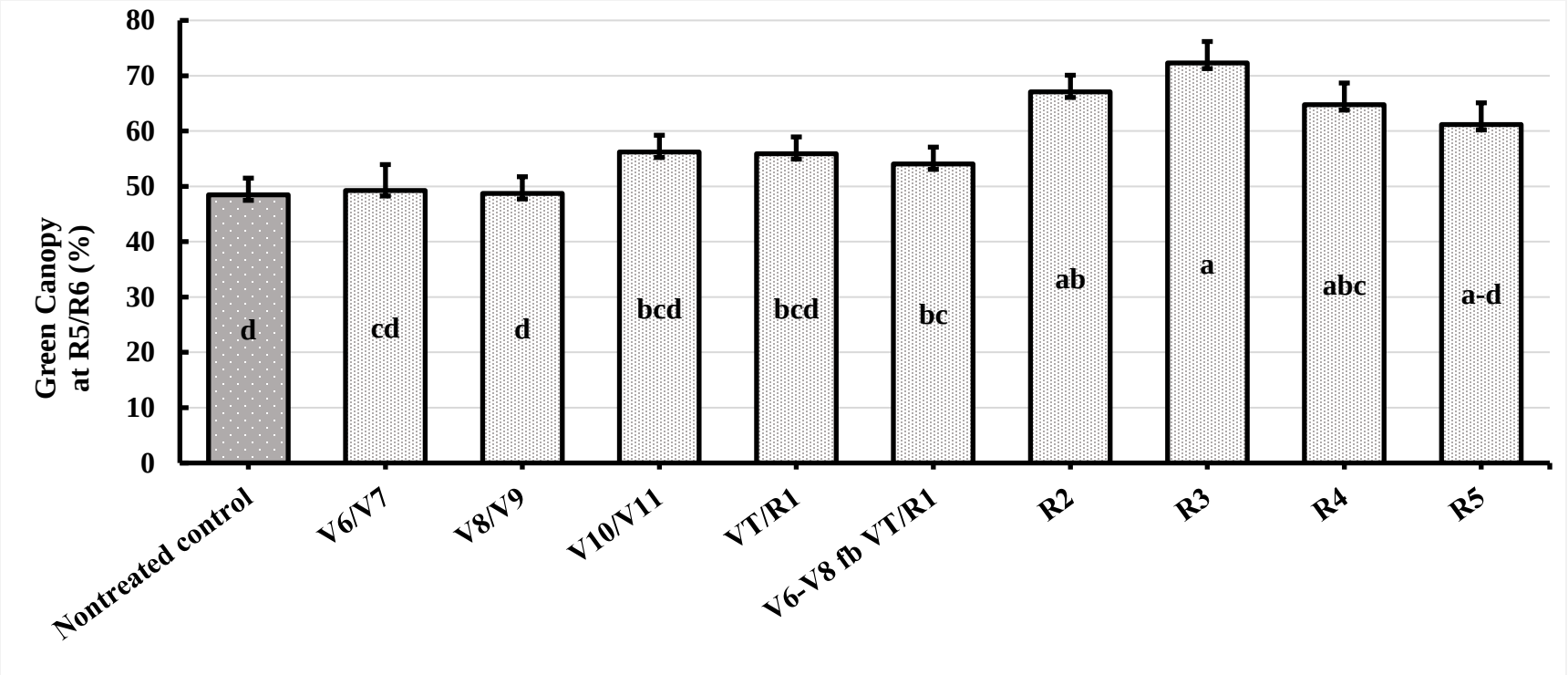

Figure 3.6. Effect of timing propiconazole + benoindiflupyr + azoxystrobin (Trivapro) application at 0.96 L/ha on green canopy of corn at dent or maturity (R5/R6) in Indiana. Single application program at V6/V7 = six/seven-leaf stage, V8/V9 = eight/nine-leaf stage, V10/V11 = ten/eleven-leaf stage, VT/R1 = tassel-silk, R2 = blister, R3 = milk, R4 = dough, R5 = dent growth stages and a two-application program at V6 to V8 followed by (fb) a tassel-silk application (V6-V8 fb VT/R1). Percent green canopy was determined by visually assessing the amount of whole plant canopy (0-100%) that remained green at R5 or R6 corn growth stage. Least squares means are the averages from trials conducted at two locations (Wanatah and West Lafayette) in Indiana representing five site-years ($p = 0.01$). Values with different letters are significantly different based on least square means test ($\alpha = 0.05$).

3.4.4 Yield response and expected net return from foliar fungicides under high and low tar spot disease severity conditions.

Site-years at Wanatah IN were analyzed as TS high and site-years at West Lafayette IN were analyzed as TS low (Table 3.3), to assess the yield response and net return of foliar fungicides. Yield was significantly increased over the nontreated control when TS high by the prothioconazole + trifloxystrobin + fluopyram (Delaro Complete), metconazole + pyraclostrobin (Headline AMP), cyproconazole + picoxystrobin (Aproach Prima), mefentrifluconazole + pyraclostrobin (Veltyma), mefentrifluconazole + fluxapyroxad + pyraclostrobin (Revytek), propiconazole (Tilt), and pyraclostrobin (Headline) (Table 3.2). No significant yield differences were observed across fungicides when TS low (Table 3.2). Across fungicides, yield response ranged from -73.1 to 1176.8 kg/ha with an average yield increase of 597.2 kg/ha when TS high and -494.6 to 699.2 kg/ha with an average yield increase of 192.0 kg/ha when TS low (Table 3.5). On average, the yield increases associated with fungicide applications were 405.1 kg/ha higher when disease severity was high relative to when disease severity was low (Table 3.5).

In this study, 85.7% of the fungicides evaluated resulted in a positive net return when TS high relative to 53.8% when TS low using either a ground or aerial method (Table 3.5). The expected net returns resulting from foliar fungicides were significantly higher with application of prothioconazole + trifloxystrobin + fluopyram (Delaro Complete), metconazole + pyraclostrobin (Headline AMP), cyproconazole + picoxystrobin (Aproach Prima), and mefentrifluconazole + pyraclostrobin (Veltyma) when TS high using a ground of aerial application method (Table 3.5). No significant differences were observed across fungicides for net return when TS low (Table 3.5). The net return from foliar fungicides applied using a ground method saw expected net returns ranging from -$29.5 to $172.5/ha with a mean net return of $73.0/ha when TS high and net returns ranging from -$115.6 to $90.2/ha with a mean net return of $4.0/ha when TS low (Table 3.5). Likewise, the expected net return from foliar fungicides applied using an aerial method saw net returns ranging from -$39.5 to $162.5/ha with a mean of $63.0/ha when TS high and net returns ranging -$125.6 to $80.2/ha with an expected net return of -$6.0/ha when TS low (Table 3.5). On average, using a ground or aerial application method, the expected net return from fungicides was 10.5 times ($68.9/ha) and 18.5 times ($68.9/ha) higher when disease severity was high relative to when disease severity was low, respectively (Table 3.5).

Table 3.5. Yield response and net return from foliar fungicide programs for ground and aerial application methods per disease condition groups Tar spot high (TS high) vs. Tar spot low (TS low) in Indiana.

Treatments[z]	FRAC[y]	Yield increase[x] (kg/ha)		Ground net return[w] ($/ha)		Aerial net return[w] ($/ha)	
Active ingredient	**code**	**TS high [v]**	**TS low [u]**	**TS high**	**TS low**	**TS high**	**TS low**
prothioconazole + trifloxystrobin + fluopyram	3+7+11	1176.8 a[t]	-[s]	172.5 a	-	162.5 a	-
metconazole + pyraclostrobin	3+11	898.8 ab	556.5	123.6 ab	65.5	113.6 ab	55.5
cyproconazole + picoxystrobin	3+11	872.1 ab	2.8	125.9 ab	-21.9	115.9 ab	-31.9
mefentrifluconazole + pyraclostrobin	3+11	858.5 ab	699.2	117.3 ab	90.2	107.3 ab	80.2
mefentrifluconazole + fluxapyroxad + pyraclostrobin	3+7+11	785.7 abc	174.8	102.7 abc	-1.2	92.7 abc	-11.2
propiconazole	3	680.8 abc	-53.0	84.3 abc	-40.5	74.3 abc	-50.5
pyraclostrobin	11	606.7 abc	257.7	80.0 abc	20.7	70.0 abc	10.7
propiconazole + pydiflumetofen + azoxystrobin	3+7+11	571.4 a-d	-494.6	65.6 abc	-115.6	55.6 abc	-125.6
prothioconazole + trifloxystrobin	3+11	566.5 a-d	-221.2	55.6 abc	-78.3	45.6 abc	-88.3
propiconazole + benoindiflupyr + azoxystrobin	3+7+11	511.9 a-d	113.2	57.5 abc	-10.3	47.5 abc	-20.3
azoxystrobin + propiconazole	3+11	425.5 a-d	501.7	43.4 abc	56.3	33.4 abc	46.3
flutriafol + bixafen	3+7	355.4 a-d	352.8	32.9 abc	32.6	22.9 abc	22.6
flutriafol + azoxystrobin	3+11	123.2 bcd	376.2	-10.3 bc	32.8	-20.3 bc	22.8
Prothioconazole	3	-73.1 cd	230.2	-29.5 c	22.1	-39.5 c	12.1
Nontreated control	-	0.0 d	0.0	0.0 c	0.0	0.0 c	0.0
Average		**597.2**	**192.0**	**73.0**	**4.0**	**63.0**	**- 6.0**

[z] All fungicides were applied at the tassel-silk (VT/R1) corn growth stages.

[y] 3 = Sterol biosynthesis inhibitor: C-14 de-methylation inhibitors (DMI) or azoles fungicides; 7 = Inhibitor of respiration in complex II at SDH: succinate dehydrogenase inhibitors (SDHI) or carboxamide fungicides; 11 = inhibitor of respiration in complex III at QoI: quinone outside inhibitors (QoI) or strobilurins fungicides.

[x] Yield acquired due to fungicide treatment.

[w] Difference in yield between treated and nontreated plots times the price of corn minus the cost of fungicide program for ground or aerial application method. [v] Tar spot severity in nontreated plots was ≥ 5 % = TS high. Site-years at Wanatah IN were grouped here.

[u] Tar spot severity in the nontreated plots was < 5 % = TS low. Site-years at West Lafayette IN were grouped here.

[t] Values with different letters are significantly different based on least significant differences test ($\alpha = 0.05$).

[s] '-'Fungicide treatment was not assessed under that disease condition group.

3.4.5 Yield response and expected net return from fungicide timing under high and low tar spot disease severity conditions.

Timing propiconazole + benoindiflupyr + azoxystrobin (Trivapro) at 0.96 L/ha applied at the R2, R3, VT/R1, R4, and V6-V8 fb VT/R1 corn growth stages significantly increased yield over the nontreated control when TS high (Table 3.6). However, under TS low, timing applications did not result in any significant differences at any corn growth stage (Table 3.6). Across the application timings evaluated, yield increase ranged from 30.2 to 1398.7.kg/ha with an average yield increase of 894.6 kg/ha when TS high and ranged from -752.0 to 336.8 kg/ha with an average of -183.8 kg/ha when TS low (Table 3.6). On an average, the yield increase was 1078.3 kg/ha higher when disease severity was high relative to when disease was low (Table 3.6).

Propiconazole + benoindiflupyr + azoxystrobin applied at the R2, R3, VT/R1, and R4 corn growth stages resulted in significantly higher net returns when compared to the nontreated control when TS high using a ground application method (Table 3.6). No significant differences were observed across application timing for net return when TS low using a ground application method (Table 3.6). The net return from timing propiconazole + benoindiflupyr + azoxystrobin, applications at different corn growth stages using a ground method saw net return ranging from -$24.4 to $208.3/ha with an average net return of $119.3/ha when TS high and -$157.4 to $27.7/ha with an average net return of $64.1/ha when TS low (Table 3.6).

With an aerial application method, timing propiconazole + benoindiflupyr + azoxystrobin saw significantly higher net return with applications made at the R2, R3, and VT/R1 corn growth stages compared to the nontreated control when TS high (Table 3.6). No significant differences were observed across application timing for net return when TS low using an aerial application method (Table 3.6). The expected net returns from an aerial method when timing applications saw net returns ranging from $34.4 to $198.3/ha with average net return of $108.2/ha when TS high and -$167.4/ha to $17.7/ha with an average net return of -$75.2/ha when TS low (Table 3.6).

On average, the expected net return from timing propiconazole + benoindiflupyr + azoxystrobin using either a ground or aerial method was $183.3/ha higher when disease severity is high relative to when disease severity is low (Table 3.6).

Table 3.6. Average yield response and net return from propiconazole + benoindiflupyr + azoxystrobin (Trivapro) application at different corn growth stages for ground and aerial application methods per disease condition groups Tar spot high (TS high) vs. Tar spot low (TS low) at five site-years Indiana.

Treatments[z] Application timing	**Yield increase[y] (kg/ha)**		**Ground net return[x] ($/ha)**		**Aerial net return[x] ($/ha)**	
	TS high[w]	**TS low[v]**	**TS high**	**TS low**	**TS high**	**TS low**
Blister (R2)	1398.7 a[u]	-131.7	208.3 a	-51.9	198.3 a	-61.9
Milk (R3)	1340.7 ab	-346.9	198.4 ab	-88.5	188.4 ab	-98.5
Tassel/silk (VT/R1)	1265.5 a	-44.7	185.6 a	-37.2	175.6 a	-47.2
Dough (R4)	1151.4 abc	-292.1	166.2 abc	-79.2	156.2 abc	-89.2
Six/eight leaf stage fb tassel/silk (V6/V8 fb VT/R1)	1025.8 abc	-78.7	115.3 a-d	-72.4	95.3 abc	-92.4
Ten/eleven leaf stage (V10/V11)	814.9 a-d	-292.3	109.0 a-d	-79.2	99.0 abc	-89.2
Dent (R5)	635.4 a-d	-752.0	78.5 a-d	-157.4	68.5 abc	-167.4
Eight/nine leaf stage (V8/V9)	388.5 bcd	-52.3	36.5 bcd	-38.5	26.5 bc	-48.5
Six/seven leaf stage (V6/V7)	30.2 cd	336.8	-24.4 cd	27.7	-34.4 c	17.7
Nontreated control	0.0 d	0.0	0.0 d	0.0	0.0 c	0.0
Average	**894.6**	**-183.8**	**119.3**	**-64.1**	**108.2**	**-75.2**

[z] All timings received propiconazole + benoindiflupyr + azoxystrobin (Trivapro) at 0.96 L/ha.

[y] Yield acquired due to fungicide treatment.

[x] Difference in yield between treated and nontreated plots times the price of corn minus the cost of fungicide program for ground or aerial application method.

[w] Tar spot severity in nontreated plots was ≥ 5 % = TS high. Site-years at Wanatah IN were grouped here.

[v] Tar spot severity in the nontreated plots was < 5 % = TS low. Site-years at West Lafayette IN were grouped here.

[u] Values with different letters are significantly different based on least significant differences test (α = 0.05).

3.4.6 Prediction and risk analysis.

The probability of recovering fungicide program costs were evaluated for the foliar fungicides and fungicide timings that significantly lowered tar spot severity, or increased and protected yields, or resulted in a positive net return over the nontreated controls in both high and low tar spot disease conditions. The probabilities are presented in Figures 3.7 and 3.8. Based on the severity data, yield response and economic analysis, prediction and risk analysis were carried out for fungicide programs that included the products, cyproconazole + picoxystrobin (Aproach Prima), prothioconazole + trifloxystrobin (Delaro), prothioconazole + trifloxystrobin + fluopyram (Delaro Complete), metconazole + pyraclostrobin (Headline AMP), pyraclostrobin (Headline), propiconazole + pydiflumetofen + azoxystrobin (Miravis Neo), mefentrifluconazole + fluxapyroxad + pyraclostrobin (Revytek), and mefentrifluconazole + pyraclostrobin (Veltyma) (Figure 3.7). Additionally, prediction and risk analysis were carried out for programs that were a single application of propiconazole + benoindiflupyr + azoxystrobin (Trivapro) at the tassel/silk (VT/R1), blister (R2), Milk (R3), dough (R4) and two applications at the six-to-eight-leaf stage fb tassel-silk (V6-V8 fb VT/R1) corn growth stages were evaluated (Figure 3.8).

For all fungicide programs, the probability of at least recovering fungicide investment (breaking even) increased with increasing corn price at a given fungicide program cost and decreased with increasing fungicide program cost at a given corn price (Figure 3.7 and 3.8). These probabilities varied across fungicide programs in both types of experiments but overall, the probability of breaking even were consistently higher when TS high relative to when TS low for different price-cost combinations (Figures 3.7 and 3.8). The probability of breaking even ranged from 16.0 to 98.0% across fungicides evaluated when TS high and ranged 1.0 to 84.0% when TS low for different price-cost combinations (Figure 3.7). In most cases, the probability of breaking even when TS low was below 50% for all fungicide programs except for metconazole + pyraclostrobin (Headline AMP), and mefentrifluconazole + pyraclostrobin (Veltyma) programs, which were above 50.0% under TS low when program costs were below $45.00/ha at any given corn price (Figure 3.7).

The probability of breaking even with propiconazole + benoindiflupyr + azoxystrobin (Trivapro) at different application timings ranged from 49.0 to 93.0% when TS high and 7.0 to 68.0% when TS low (Figure 3.8). The breaking even probability was above 50.0% for all

application timings evaluated when TS high and below 50.0% when TS low (Figure 3.8). In all cases, these probabilities were below 50% under TS low (Figure 3.8).

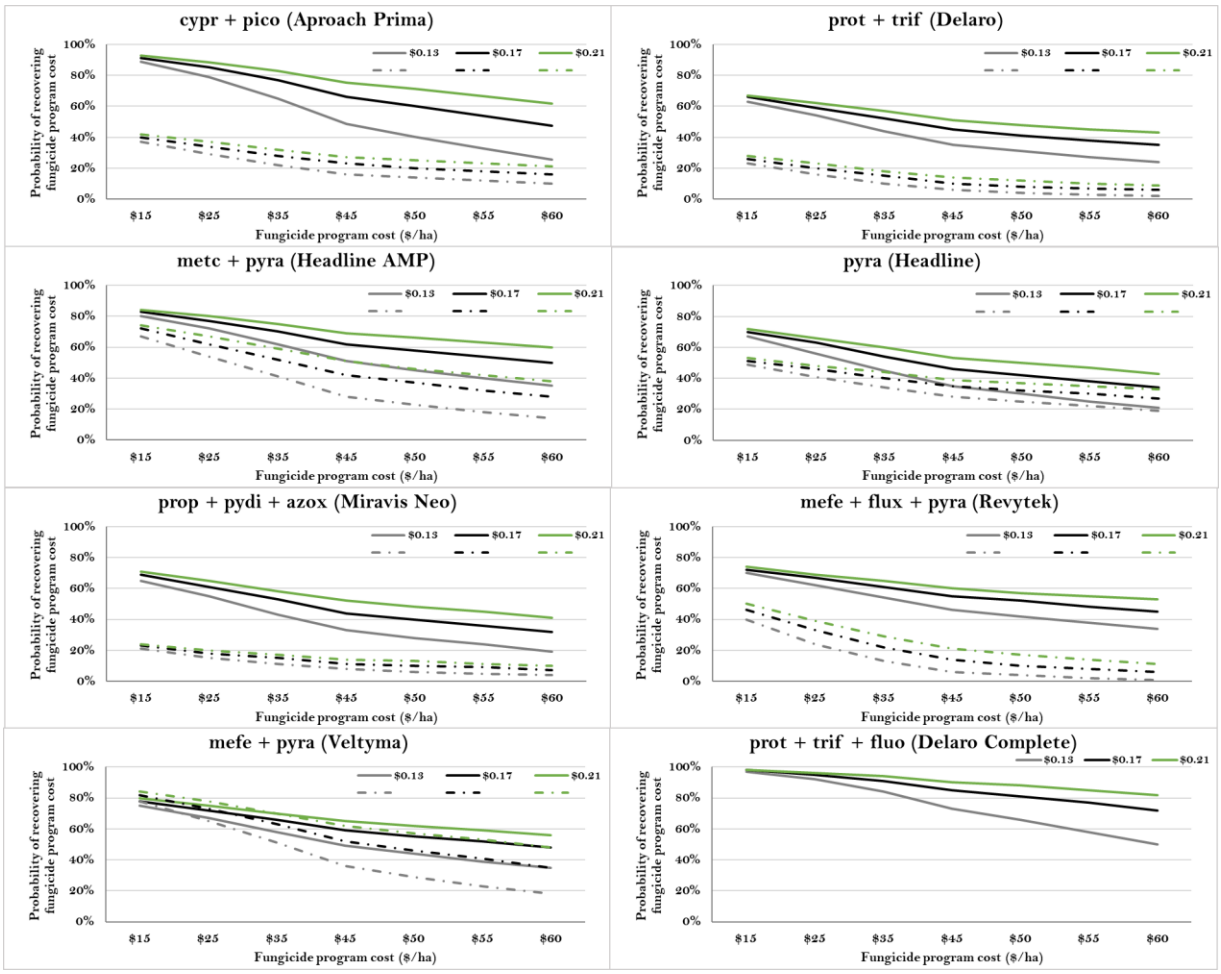

Figure 3.7. Probability of recovering fungicide cost for a range of corn market prices in $/kg, and fungicide costs in $/ha estimated for eight fungicide programs applied under high (solid lines) and broken lines) tar spot disease conditions based on estimated yield differences and between-trials standard deviation. Fungicide active ingredients were: azox = azoxystrobin, beno = benoindiflupyr, bixa = bixafen, cyor = cyoroconazole, fluo = fluopyram, flut = flutrifol, flux = fluxapyroxad, mefe = mefentrifluconazole, metc = metconazole, pico = picoxystrobin, prop = propiconazole, prot = prothioconzole, pydi = pydiflumetofen, pyra = pyraclostrobin, trif = trifloxystrobin.

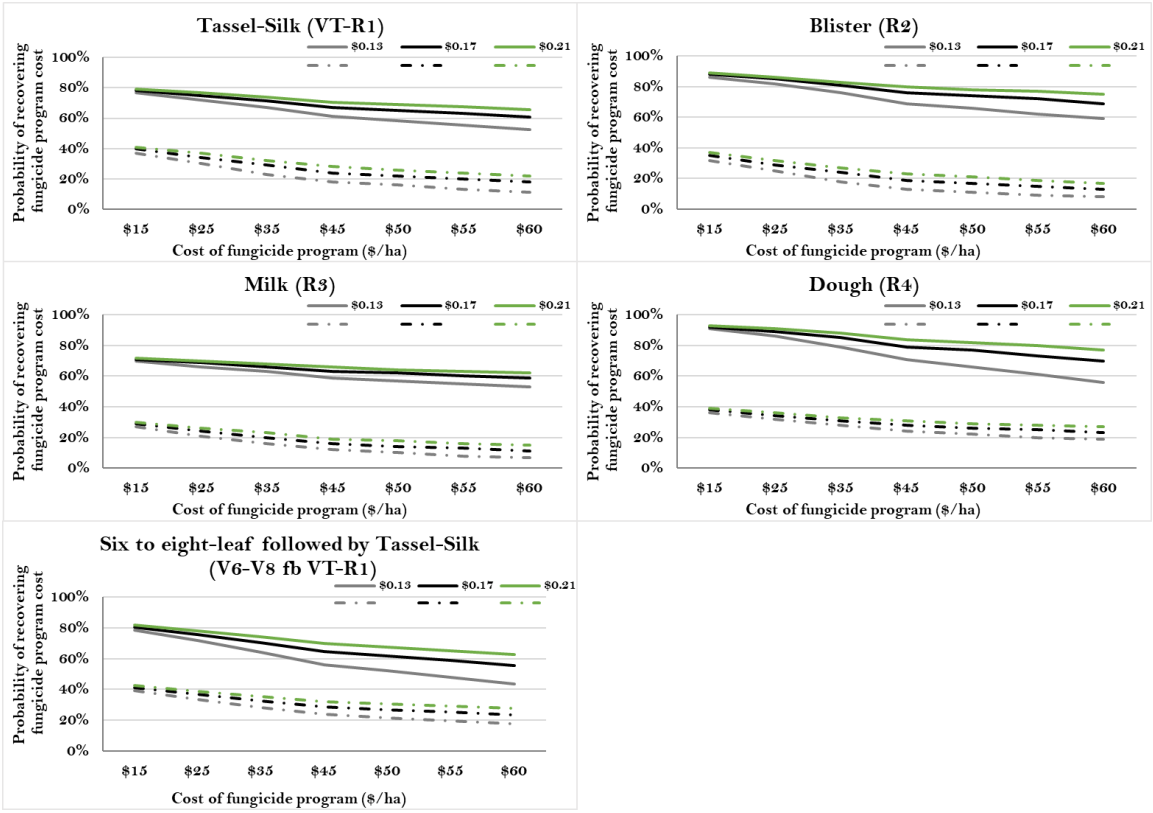

Figure 3.8. Probability of recovering fungicide cost for a range of corn market prices in $/kg, and fungicide costs in $/ha estimated for propiconazole + benoindiflupyr + azoxystrobin (Trivapro) at 0.96 L/ha applied at 5 different timings (corn growth stages) under high (solid lines) and low (broken lines) tar spot disease conditions based on estimated yield differences and between-trials standard deviation.

3.5 Discussion.

Fungicide use has increased considerably in corn production in the United States, even under low disease severity conditions. Some scientists have even been confronted with whether it is economical for growers to use fungicides for benefits beyond disease control (Munkvold et al. 2008; Paul et al. 2011; Wise and Mueller 2011; Mallowa et al. 2015; Mueller et al. 2019). Fungicides are the most effect management tools that are currently available for tar spot. Due to the severe economic impact tar spot has on corn, corn growers are greatly interested in being able to appropriately select and time fungicide applications to reduce disease severity, increase yields and maximize profits.

We evaluated the effect of foliar fungicides and fungicide timing on tar spot management in Indiana and the yield response and net return resulting from their use on corn. Final disease severity in the nontreated control plots in three (Wanatah 2019, 2020, and 2021) of the five site-years was relatively high and may have been strongly influenced by the environment (more monthly precipitation) and the earlier onset of the disease in these trials. Fungicides vary in efficacy when protecting crops from diseases (Wise et al. 2021). Most of the fungicides evaluated in this study reduced tar spot severity and increased green canopy of corn. These results are consistent with those reported by Ross et al. (2020a, 2021a) and Telenko et al. (2022), who showed that the severity of tar spot was reduced by all fungicides evaluated in 2019, 2020, and 2021. Compared with the nontreated control, some of the fungicides evaluated in our study did not significantly reduced tar spot severity. This suggest that the efficacy of a fungicide depends on appropriately timing the application and may also be influenced by the amount of disease present in the field (Coulter 2010; Wise and Mueller 2011). In our study, a single application of propiconazole + benoindiflupyr + azoxystrobin (Trivapro) made at the R2 or R3 corn growth stages was the most effective in lowering tar spot severity and increasing green canopy of corn. Notably, this application window could vary with the use of other fungicide products, different locations, and different disease pressure.

Though most of the fungicides and fungicide timing evaluated in our study significantly reduced tar spot severity and increased green canopy of corn, the reduction in disease did not consistently translate into significant yield benefits. For example, prothioconazole + trifloxystrobin (Delaro) and propiconazole + pydiflumetofen + azoxystrobin (Miravis Neo) significantly reduced tar spot severity over the nontreated control but did not result in significant

yield increase when TS high or TS low when compared to the nontreated control. Vice versa, some application timings which did not significantly reduced tar spot severity and recorded a high disease severity value at the final disease data collection but saw significantly yield increases. For example, propiconazole + benoindiflupyr + azoxystrobin (Trivapro) applications made by the VT/R1, V6-V8 fb VT/R1, and R4 corn growth stages did not significantly reduced tar spot severity over the nontreated control when TS high, but these applications resulted in significant yield increases under a high disease condition. Two explanations could be due to disease continuing to infest corn leaves later in the growing season after the fungicide activity window has ended (VT/R1 or V6-V8 fb VT/R1) or due to late disease onset (R4) and therefore not impacting grain fill. Late-season disease development occurring closer to R4 and R5 would have less impact on grain fill and yield response, resulting in high foliar disease severity values at the end of the season (Pau et al. 2011). Overall, the yield response of foliar fungicides and application timing in our study was 2.1 to 6.3 times higher (405.1 to 1094.6 kg/ha) when disease severity was high compared to when disease severity was low.

Nevertheless, it is important to determine if yield protection is enough to offset fungicide costs, and what is the expected net return on investment under high and low tar spot disease conditions? Profits from fungicide use are most common when disease severity is high (Paul et al. 2011). Under low disease severity, fungicides application is not likely to be profitable and thus may have little direct impact on enhancing crop production (Johnson 1987; Paul et al. 2011; Mallowa et al. 2015, Wise et al. 2019). Results from our study demonstrate that foliar fungicides and appropriately timed fungicide applications can be used profitably to manage tar spot of corn in Indiana, but profitability is more likely when high disease conditions relative to low disease conditions. These results are consistent with other studies assessing the economical use of fungicides on corn (Johnson 1987; Tedford et al. 2017; Wise et al. 2019). Nevertheless, the expected net returns in this study are based on a deterministic model method and thus stochastic model methods could be use in the future to forecast the variations of prices and returns on in real-time. Overall, the net return from foliar fungicides was $68.9/ha higher and application timing was $183.3/ha higher using either a ground or aerial method when high tar spot disease pressure occurred compared to low disease pressure.

The breaking even probability varied across fungicide programs. For all fungicide programs, the probability of breaking even increased with increasing corn price at a given

fungicide program cost and decreased with increasing fungicide program cost at a given corn price. The probability of breaking even fungicide programs was consistently higher, above 50%, when high tar spot disease pressure occurred than with low disease pressure for different price-cost combinations. This study is the first economic analysis of net return and yield response of foliar fungicides and fungicide application timing on hybrid corn in Indiana under high and low tar spot disease conditions. Form our analysis only four fungicides were capable of expected net return significantly higher than the nontreated control. These fungicides were prothioconazole + trifloxystrobin + fluopyram (Delaro Complete), metconazole + pyraclostrobin (Headline AMP), cyproconazole + picoxystrobin (Aproach Prima), and mefentrifluconazole + pyraclostrobin (Veltyma). Additionally, one application made at the R2, R3, R4, and VT/R1 resulted in significantly higher net return when compared to the nontreated control when using propiconazole + benoindiflupyr + azoxystrobin (Trivapro). It is important to know that these results may change with different fungicide products, locations, or years. Hence future studies are needed to determine the efficacy of new products, risk for resistance development and optimum timing of each product to help Indiana corn growers. This study demonstrates that foliar fungicides and appropriately timing fungicide applications can increase yield, green canopy of corn and profits, but profitably is most likely when high disease severity conditions exist.

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CHAPTER 4. AN INTEGRATED MANAGEMENT STRATEGY FOR TAR SPOT OF CORN IN INDIANA.

*This chapter will be submitted as a research article in Plant Health Progress Journal.

4.1 Abstract.

Tar spot is a major foliar disease of corn in Indiana which causes significant yield loss in heavily infested fields. To protect corn grain yields in Indiana, identifying integrated management strategies has become a top priority. In this study, we assessed the integration of tillage, hybrid, and fungicide on reducing tar spot severity and protecting yields. Results from our study showed that tillage did not impact tar spot management in Indiana, and this was speculated to be caused by interplot interference by aerial movement of ascospores which our field experimental design did not accommodate for. All other main effects of hybrid and fungicide, and their interactions affected tar spot severity and green canopy. No significant effect of hybrid or fungicide was found on yield. The overall results of this study showed that hybrids reacted differently to tar spot in the absent and presence of a fungicide. Without a fungicide application, hybrids with moderate resistance to tar spot, alone are capable of significantly reducing disease severity by 54.5% to 62.5% over a susceptible hybrid. These hybrids are also capable of increasing green canopy over a susceptible hybrid. Consequently, the addition of a fungicide to a susceptible hybrid can further reduce disease severity and increase green canopy, but not to hybrids with moderate resistance to tar spot. With or without fungicides, hybrids did not differ significantly from each other suggesting that their incorporation into an integrated management strategy will require knowledge of their genetic background and yield potential. To successfully evaluate tillage effect on tar spot management in Indiana would require further separation of the treatment plots in experimental design. Also, to ensure all aspect of the disease triangle is considered.

4.2 Introduction.

Tar spot of corn caused by *Phyllachora maydis* Maubl is now a prevalent and important foliar disease in the Midwest United States (Mueller et al. 2020). It was first reported in seven counties of Indiana in 2015, but without significant economic impact (Bissonnette 2015; Ruhl et al. 2016). However, in 2018, Indiana experienced the first epidemic of tar spot which resulted in a total yield loss of 3.3 million bushels (0.8 million metric tons) with monetary loss equivalent to US$ 120.2 million (Crop protection Network 2022). Tar spot has become severe in the years since 2018 where it has now spread to 82 of Indiana's 92 counties (https://corn.ipmpipe.org/tarspot/.; Telenko et al. 2020). In addition, for the 2021 corn cropping season, total yield loss in Indiana was approximately 46.5 million bushels (4.0 % of the total corn production) valued at $US 253.5 million (Corn Protection Network 2022).

Phyllachora maydis favors extended leaf wetness of > 7 hours, high relative humidity of > 75%, with optimum temperatures of 16- 21 °C (Valle-Torres et al. 2020). Corn is the only known host for *P. maydis* where infections are caused by the sexual ascospores at any corn growth stage (Cline 2005; Kleczewski et al. 2019; Groves et al. 2020). Signs and symptoms of *P. maydis* infection become visible after 12- 15 days post-infection (Hock et al. 1995; Valle-Torres et al. 2020). Corn residue hosts overwintering ascospores in fungal fruiting bodies (stromata) and contributes to the primary source of inoculum responsible for driving epidemics (Kleczewski et al. 2019; Groves et al. 2020). This may pose a greater issue in the next cropping cycle, especially in conservation tillage systems where the residue remains on the soil surface. Pathogen survival on crop residue in several field crops (corn, soybean, and wheat) was thought to be responsible for the initiation and severity of a few plant disease epidemics (Latterrel and Rossi 1983; Nutter et al. 1993; Grau et al. 2004; Mengistu et al. 2014). For example, *Cercospora sojina* survival in crop residue was responsible for the initiation and development of a frogeye leaf spot epidemic of soybean in southern states. The severity of the epidemic was greater in the no-till fields as opposed to tilled fields (Grau et al. 2004; Mengistu et al. 2014). Past studies have concluded that the widespread adoption of conservation tillage systems combined with continuous corn and large amounts of surface residue are responsible for the prevalence and severity of grey leaf spot throughout the U.S Corn Belt (Latterrel and Rossi 1983; Nutter et al. 1993). More recently, it was found that conservation tillage did not affect the severity of frogeye leaf spot (Mengistu et al. 2018). Variations in these results over the years may be attributed to the differences in environments and

other factors pertaining to the field research experimental design (Mengistu et al. 2014). Therefore, need for further evaluation of a tillage effect on disease initiation and development is necessary (Mengistu et al. 2018).

Data from the Agricultural Resources Management Survey on the production practices of corn, cotton, soybean, and wheat producers show that roughly 51 percent used strip-till at least once over a four-year period and twenty-one percent used strip-till in every year during the same four-year period (Claassen et al. 2018). A strip-till system is a conservation or reduced field tillage system that combines no-till and conventional tillage to produce row crops. In this system, greater than 30 % of crop residue is left on the soil surface after harvest in an effect to reduce soil erosion caused by wind and water runoff (Wade et al. 2015). Historical practice of reducing crop residue by tillage has been promoted to reduce debris-borne plant diseases and hence strip tillage may become problematic (Boosalis et al. 1981). To our knowledge, there are no published studies on the effect of tillage on managing the development of tar spot epidemics in corn production in the U.S. and this research would be the first to explore this strategy.

Management of tar spot in the U.S. is primarily by planting less susceptible hybrids, using foliar fungicides, crop rotation, irrigation, and residue management (Kleczewski et al. 2019; Telenko et al. 2019; Valle-Torres et al. 2020; Da Silva et al. 2021). Presently, all corn hybrids evaluated in Indiana have shown varying levels of susceptibility and tolerance to tar spot (Telenko et al. 2019). There is no hybrid available in the U.S. that has been shown to be fully resistant to this disease. Fungicides have become an important tool for tar spot management programs in the Midwest U.S. where several combinations of quinone outside inhibitors (QoIs), demethylation inhibitors (DMIs), and succinate dehydrogenase inhibitors (SDHIs) are registered to manage foliar diseases of corn (Telenko et al. 2022; Corn Disease Working Group 2022). The efficacy of these fungicides depends on timing of application, host growth stage, level of host resistance, and environmental conditions. Furthermore, yield benefits can be achieved if these registered fungicides are judiciously applied at the time of disease onset.

Multiple applications and combinations of different chemistries may be necessary when the environment is conducive for disease development. However, fungicides pose the risk of resistance since repeated large-scale use of fungicides with similar mode of action places selection pressure on the pathogen population (Gisi et al. 2002; Avenot et al. 2010). Therefore, tar spot management cannot rely solely on fungicides since they are a proven short-term disease

management strategy. A long-term disease management strategy for tar spot requires the integrated use of partially resistant hybrids, tillage practices, crop rotation with a non-host and chemical control. We hypothesized that depending on corn hybrid and the disease management approach (tillage and fungicide), may influence tar spot and grain yield. The objective of this study was to assess the impact of tillage, hybrid, and fungicide integration on tar spot severity, green canopy, and corn yield in Indiana.

4.3 Materials and Methods.

4.3.1 Study location.

A field study was conducted for three years from 2019, 2020, and 2021 at the Pinney Purdue Agricultural Center, Wanatah IN (coordinates: 41°27'20.15"N, 86°56'36.66"W). Corn hybrids were planted at a rate of 6 seeds/meter using a Kincaid plot planter on 6 June in 2019, 6 June in 2020 and on 26 May in 2021. Monthly average for total precipitation (mm), average relative humidity (%) and air temperature (ºC) for the months of May to October were obtained from the Purdue Mesonet Data Hub website of the Indiana State Climate Office (https://ag.purdue.edu/indiana-state-climate/, assessed on 16 November, 2021) near the study location each year. These averages were compared to a 20-year average for the same weather parameters listed above. Twenty-year weather data summaries were obtained from the Indiana State Climate Office and were used as a standard for normal weather. Irrigation was supplemented in the trial by over-head irrigation at one inch (25.4 milliliters) of water weekly in addition to natural precipitation. Irrigation water was not supplemented when precipitation reached 25.4 mm or higher during that week. Weekly supplemented irrigation was not included in the calculation for the total precipitation but was added in a table footnote (Table 4.1).

Table 4.1. Average monthly mean air temperature (^{0}C), total precipitation (mm), and relative humidity (%) from June to October 2019, 2020, and 2021 obtained from the weather recording station near the research location.

Month	Mean air temperature[z] (°C)				Total precipitation[z] (mm)				Relative humidity[z] (%)			
	2019	2020	2021	20-year average	2019	2020	2021	20-year average	2019	2020	2021	20-year average
May	21.9	21.2	19.8	21.0	85.2	289.6	287.0	92.3	73.6	75.5	75.0	74.5
Jun	20.2	21.9	22.0	21.2	69.5	61.0	143.0	71.2	73.8	65.4	70.9	72.1
Jul	21.9	23.1	22.7	22.3	25.8	76.8*[w]	75.9	75.7	76.1	76.9	81.2	75.8
Aug	22.3	21.0	22.1	21.0	33.4	40.2*	102.4	89.0	76.7	76.2	79.3	80.4
Sep	19.1	17.2	19.2	18.4	90.9	42.7*	40.2*	57.9	78.6	74.4	69.3	75.8
Oct	11.2	9.4	17.4	11.3	70.2	73.3	159.5	82.2	70.4	71.9	81.4	73.9

[z] Data courtesy of Indiana State Climate Office. https://ag.purdue.edu/indiana-state-climate/. Taken from Purdue Mesonet stations at the Pinney Purdue Agricultural Center (PPAC), Wanatah IN.

[w]* 25.4 mm irrigation water was supplemented weekly when natural rainfall did not meet 25.4 mm or higher. 76-, 102-, and 51-mm irrigation was supplemented in 2020 for the months of Jul, Aug, and Sep, respectively. 51 mm irrigation water was supplemented in Sep of 2021.

4.3.2 Field experiment design and treatments.

The experimental design was a randomized split-plot arrangement of tillage (2 levels) and hybrid-by-fungicide options (6 levels) with four replications. The main plots consisted of either strip or conventional tillage, applied as a strip across the trial. The subplots consisted of the three hybrids: tar spot susceptible (S0), tar spot moderately resistant 1 (R1) and tar spot moderately resistant 2 (R2) by two fungicide options (non-treated control and treated). Fungicide treatment was 11.9% propiconazole + 2.9% benoindiflupyr + 10.5% azoxystrobin [Trivapro, Syngenta Crop Protection, Greensboro, NC applied at 1.0 L/ha with a non-ionic surfactant [Preference] at 0.25% v/v at tassel/silk (VT/R1)). Tillage strips were chisel plowed on 14 November 2018, 25 November 2019, and 10 November 2020 and cultivated on 6 June 2019, 24 May 2020, and 26 May 2021. Each subplot was of four rows spaced 76.2 cm apart and 9.1 m long. The two center rows of each four-row plot were used for data collection and yield.

Fungicide applications were made on 9 August 2019, 7 August 2020, and 6 August 2021 at the tassel/silk (VT/R1) crop developmental stage using a Lee self-propelled sprayer equipped with a 3.0-m boom, fitted with six TJ-VS 8002 nozzles spaced 20-inch apart at 3.6 mph. The sprayer was calibrated for an output of 140.3 L/ha and 275.8 kPa. Research plots were managed according to the standard practices for grain corn production in Indiana (https://ag.purdue.edu/agry/dtc/Pages/CSFG.aspx).

4.3.3 Disease severity assessments.

Severity of tar spot was assessed weekly from the first detection of tar spot in each trial to corn growth stage dent (R5) or physiological maturity (R6). Assessment of disease severity included two variables: the percent tar spot stroma and percent tar spot foliar symptoms in the canopy based on a standardized rating scale for tar spot (Telenko et al. 2021a). An intra-rater reliability test was performed before data collection to reduce data biases and to ensure some level of data consistency. Percent tar spot stroma was rated by visually assessing the leaf area (0-100%) covered with fungal stroma whereas the percent tar spot foliar symptoms assessed the amount of leaf area (0-100%) that exhibited chlorotic and necrotic symptoms. Five plants per plot (subsamples) were randomly selected and disease severity was rated on the ear leaf (EL). The five leaves were then averaged for a single value of disease severity estimate per plot. These values

were then used to calculate area under the disease progress curve (AUDPC) using the trapezoidal integration method proposed by Madden et al. (2007) for disease accumulated on the ear leaf over time. AUDPCs were standardized (sAUDPC) by dividing AUDPC by the total length of the disease assessment period to make direct comparisons among tar spot epidemics over time, across hybrids and years.

4.3.4 Green canopy of corn and yield assessment.

At the R5 or R6 corn growth stage, percent green canopy was recorded per each plot. Percent green canopy was determined by visually assessing the amount of whole plant canopy (0-100%), that remained green. The two center rows of each plot were harvested on 25 October 2019, 14 November 2020 and 3 November 2021 using a small plot combine (Kincaid 8XP). Yields were standardized to 15.5% moisture prior to analysis. Percent yield protected was calculated as [(treated-nontreated)/treated] x 100.

4.3.5 Statistical analysis.

Analysis of variance (ANOVA) was performed for all variables using a general linear mixed model with the PROC GLIMMIX procedure in SAS v. 9.4 (SAS Institute, Cary, NC) (Littell et al. 2006). Fixed factor effects were tillage, and hybrid-by-fungicide treatment, and the 2-way interaction (tillage x hybrid-by-fungicide). Randoms effects were replication and year. Disease severity ratings (sAUDPC), green canopy and yield data were combined over years and a final ANOVA treated years as a repeated measure type of sub-subunit. Least-square means (lsmeans) of the treatments were computed and compared ($\alpha = 0.05$). All pairwise differences among lsmeans were compared only if the F test was significant ($P \leq 0.05$) (Piepho 2012).

4.4 Results.

4.4.1 Average monthly weather conditions.

Average monthly air temperatures, precipitation and relative humidity profiles differed each year. These differences in weather conditions are presented in Table 4.1. The average monthly mean air temperatures across the three years for the period of May to October ranged from 9.4 to

22.7 °C where the 20-year average (normal temperatures) ranged from 11.3 to 23.3 °C (Table 4.1). Mean air temperature was 3.3 °C above normal (20-year) air temperature at in October 2021 (Table 4.1).

The average monthly total precipitation across for May to October from 2019 to 2021ranged from 25.8 to 289.6 mm whereas the total 20-year average (normal) precipitation ranged from 57.2 to 93.2 mm (Table 4.1). Monthly precipitation was 33.0 mm, 197.3 mm, 194.7 mm, 71.8 mm, 13.4 mm, and 77.3 mm higher than the 20-year average monthly total precipitation in September 2019, May 2020, May 2021, June 2021, August 2021, and October 2021, respectively (Table 4.1).

The average monthly relative humidity ranged from 65.4 to 81.4% where the 20-year average relative humidity ranged from 72.1 to 80.4% for the period of May to June during 2019 to 2021 (Table 4.1). Average monthly relative humidity was 3.8%, 2.8%, 4.2%, 5.4%, and 7.5% higher than the 20-year average (normal) relative humidity in August 2019, September 2019, August 2020, July 2021, and October 2021, respectively (Table 4.1).

Overall, the weather condition of our field location was characterized by warm temperatures, extreme wetness, and optimum relative humidity favoring the development of tar spot.

4.4.2 Effect of tillage by hybrid by fungicide on tar spot severity, green canopy, and yield.

Tar spot severity, which rated the severity of stroma and foliar symptoms on the ear leaf was analyzed as the standardized area under the disease progress curve (sAUDPC) for disease accumulated over time. Results from ANOVA showed no significant effects of tillage or its interaction with hybrid or fungicide on tar spot severity or green canopy of corn (Table 4.2). A significant (p = 0.01) interaction effect was observed for hybrid by fungicide treatment on tar spot severity. In the absence of fungicide application, tar spot severity was significantly lower in the two moderately resistant hybrids: R1 (sAUDPC = 3.3 and 2.6) and R2 (sAUDPC = 4.0 and 3.1) when compared to the tar spot susceptible hybrid S0 (sAUDPC = 9.2 and 11.0) stroma and foliar symptoms accumulation, respectively (Table 4.2). No statistical differences for tar spot severity were observed between the moderately resistant hybrid R1 and R2 (Table 4.2). With the application of the fungicide benzovindiflupyr + azoxystrobin + propiconazole (Trivapro) on hybrids, accumulation of tar spot severity was reduced in all hybrids. However, this reduction was only significant (p = 0.01) in the susceptible S0 hybrid when compared to its nontreated control but not in the moderately resistant R1 and R2 hybrids (Table 4.2).

In the absence of fungicide application, corn canopy was significantly (p = 0.01) greener in the moderately resistant R1 (51.1%) and R2 (48.1%) hybrids when compared to the susceptible S0 (13.0%) hybrid (Table 4.2). No statistical differences in percent green canopy of corn were observed between the moderately resistant R1 and R2 hybrids (Table 4.2). With benzovindiflupyr + azoxystrobin + propiconazole application on the hybrids, corn canopy was greener when compared to their nontreated control. The susceptible S0 hybrid showed a statistically significant (p = 0.01) greener canopy over its nontreated control when compared to the other hybrids and their nontreated controls (Table 4.2).

No significant effect was observed for tillage as a main effect or its interaction with hybrid or fungicide on yield (Table 4.2). No interaction effect of hybrid by fungicide on corn grain yield was observed (Table 4.2). No significant differences in yields were observed among hybrids treated or not treated with benzovindiflupyr + azoxystrobin + propiconazole fungicide (Table 4.2). However, there were always higher yields in the moderately resistant R1 (12,301.0 kg/ha) and R2 (11,858.0 kg/ha) hybrids when compared to the susceptible S0 (11,552.0 kg/ha) hybrid (Table 3.2). Additionally, with the application of benzovindiflupyr + azoxystrobin + propiconazole fungicide, even though not significant, yields were increase in all hybrids when compared to their nontreated controls (Table 4.2). Fungicide application increased corn yield by 7.9% in the susceptible S0 hybrid, 4.2% and 4.7% in the moderately resistant R1 and R2 hybrids, respectively (Table 4.2).

Table 4.2. Least-square means summaries for tillage by hybrid by fungicide field trials conducted in Indiana 2019, 2020, and 2021.

Treatment[z]	Tar spot stroma [x] (sAUDPC)	Tar spot foliar symptoms [w] (sAUDPC)	Green canopy [v] (%)	Yield kg/ha (yield protection %)[u]
Tillage				
Strip	4.8	4.4	44.9	12265
Conventional	4.6	4.5	42.9	12204
Hybrid by Fungicide[y]				
S0, Nontreated	9.2 a[t]	11.0 a	13.0 d	11552
S0, benzovindiflupyr + azoxystrobin + propiconazole	6.2 b	5.6 b	32.0 c	12462 (7.9%)
R1, Nontreated	3.3 cd	2.6 c	51.5 b	12301
R1, benzovindiflupyr + azoxystrobin + propiconazole	2.5 d	1.9 c	62.3 a	12820 (4.2%)
R2, Nontreated	4.0 c	3.1 c	48.1 b	11858
R2, benzovindiflupyr + azoxystrobin + propiconazole	3.0 cd	2.4 c	56.3 ab	12414 (4.7%)
P-value tillage	0.47	0.89	0.52	0.82
P-value hybrid	<0.01	<0.01	<0.01	0.22
P-value fungicide	<0.01	<0.01	<0.01	0.02
P-value hybrid by fungicide	0.02	<0.01	<0.01	0.81
P-value tillage by hybrid by fungicide	0.88	0.98	0.80	0.84

[z] Hybrids used in study were the S0 - tar spot susceptible, R1- tar spot moderately resistant 1, and R2- tar spot moderately resistant 2.

[y] benzovindiflupyr + azoxystrobin + propiconazole (Trivapro), 0.96 L/ha was applied at VT/R1 corn growth stage, Nontreated = No fungicide was applied.

[x] Assessed visually by evaluating the percentage leaf area (0-100%) covered with fungal stroma on the ear leaf (EL) on five plants per plot. Values were averaged before analysis. sAUDPC – standardized area under the disease progress curve was calculated by dividing AUDPC by total length of the disease assessment period.

[w] Assessed visually by evaluating the percentage leaf area (0-100%) covered with chlorotic-necrotic lesions on the ear leaf (EL) on five plants per plot. Values were averaged before analysis

[v] At dent or maturity (R5/R6) corn growth stage the percentage of whole plot canopy (0-100%) that remained green (green canopy) was assessed for each plot.

[u] Percent yield protected was calculated as [(treated-nontreated)/treated] x 100.

[t] Values with different letters are significantly different based on a least-square means test ($\alpha = 0.05$) and indicate pairwise comparisons between nontreated and treated means within hybrids.

4.5 Discussion.

This research was conducted to assess the integration of tillage, hybrid, and fungicide for management of tar spot of corn in Indiana. The excess moisture from natural precipitation (587.5 mm) and the high relative humidity (>75%) from May to October provided favorable conditions for tar spot development particularly in 2020 and 2021. In this study, the overall effect of tillage or its interaction with hybrid and fungicide did not reduce tar spot severity, increase green canopy of corn, or yield in Indiana. We predicted that tar spot severity would be significantly reduced by conventional tillage when compared to strip tillage. This prediction was made since strip tillage tends to leave more than 30% of crop residue on the soil surface (Boosalis et al. 1981; Sumner et al. 1981), which can host *P. maydis* overwintering spores as inoculum (Kleczewski et al. 2019; Groves et al 2020). However, this prediction failed based on our results. We speculate interplot interference occurred since tillage treatments were not sufficiently spaced to account for aerial movement of ascospores. A study by Kleczewski et al. (2020) showed that *P. maydis* ascospores can be dispersed by wind or water to at least 560 m - 1,249 m from the source of inoculum in the U.S. Therefore, re-evaluation of study design is needed to better analyze the effect of different tillage options on tar spot severity in Indiana. We proposed that for future studies evaluating tillage treatments, spatially separated locations be used to limit interplot interference by aerial movement of ascospores.

The hybrids evaluated in this study, reacted differently to tar spot in that they had different levels of susceptibility or tolerance to the disease. In the absence of a fungicide, the moderately resistant hybrids evaluated significantly reduced tar spot severity by 3.1 to 4.2 times over the susceptible hybrid. This result is consistent with data published by Telenko et al. (2019), which concluded that hybrid have varying levels of susceptibility to tar spot in Indiana. Additionally, the moderately resistant hybrids significantly increased green canopy of corn over the susceptible hybrid, but no statistical differences were observed among hybrids for yield. The adding of a single fungicide at the VT/R1 corn growth stage resulted in significantly reductions in tar spot severity in the susceptible hybrid but not in the moderately resistant hybrids. Likewise, a fungicide application significantly increased green canopy of corn in the susceptible hybrid but not in the moderately resistant hybrids. No statistical differences were observed for any of the hybrids for yield.

Results from our study suggests that even though the addition of a fungicide on resistant hybrids can reduce tar spot severity and increase green canopy, this added fungicide may not be necessary for those hybrids with moderate resistance to tar spot resistance since these hybrids can result in lower disease. This is the first integrated management assessment for tar spot of corn in Indiana. It is likely that tar spot will persist in the Midwest for the foreseeable future and current disease management practices (fungicides) will need to include a more integrated disease strategy that incorporates cultural practices (rotation), resistance, prediction tools, and fungicides to protect yield and increase profitability. Future work will require field designs that would have the capacity to evaluate the integration of several management practices (tillage, hybrids, fungicides, irrigation, prediction tools etc.).

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