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An improved understanding of Diplodia ear rot in Kentucky corn

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AN IMPROVED UNDERSTANDING OF DIPLODIA EAR ROT IN KENTUCKY CORN

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Agriculture, Food and Environment
at the University of Kentucky

By
Nolan Ryan Anderson
Lexington, Kentucky
Director: Dr. Kiersten Wise, Professor of Plant Pathology
Lexington, Kentucky
2022

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ABSTRACT OF DISSERTATION

AN IMPROVED UNDERSTANDING OF DIPLODIA EAR ROT IN KENTUCKY CORN

Stenocarpella maydis and *Stenocarpella macrospora* are both causal agents of Diplodia ear rot (DER) of corn in the U.S. The current prevalence and distribution of each is unknown worldwide. Signs and symptoms of DER include white mold on and between kernels, bleached husks, and pycnidia visible in the cob pith. The term hidden Diplodia describes when no mycelia or bleached husk is present, but pycnidia are still visible inside the cob. This phenomenon is reported to be caused by late infections of *Stenocarpella* spp. New fungicide nozzle technology has been promoted to increase spray coverage in the lower canopy, which may influence fungicide efficacy against DER. The goals of this research are to 1) understand the species distribution in Kentucky of the two pathogens causing DER, 2) the conditions which influence hidden Diplodia, and 3) the potential for in-canopy fungicide applications to improve control of DER.

To determine the prevalence of causal agents of DER, Kentucky corn fields were surveyed in 2019 and 2020. Ears with signs or symptoms of DER were collected in a specific sampling pattern and taken to the laboratory for analysis. In 2019, 98 fields were surveyed and in 2020, 88 fields, with 133 and 46 symptomatic ears collected each year, respectively. Causal agents were isolated from ears and identified based on conidia morphology. A subset of isolates was used to inoculate corn ears to measure aggressiveness. *S. macrospora* was identified as the cause of 33.8% and 36.9% of the total DER observed in 2019 and 2020, respectively. While *Stenocarpella* sp. isolates differed in aggressiveness ($P<0.0001$), differences were not observed between species ($P=0.8863$). Although *S. maydis* has been described as the main pathogen causing DER, this research indicates the importance of both pathogens in relation to DER.

From 2019 to 2021, the influence of inoculation site and timing of both *Stenocarpella* spp. was assessed under irrigated and dryland conditions. Both species were inoculated at one of four growth-stage timings (V8, R1, R2, R3) or one of four sites on the plant (whorl, silk channel, ear shank, foliar spray). DER severity was significantly greater in R1 inoculation timings compared to R2 and R3 timings. Yield was reduced by an average of 700 kg/ha in treatments inoculated at R1 compared to non-inoculated controls. In the 2020 irrigated trial, whorl inoculation resulted in higher incidence of hidden Diplodia compared to other sites ($P=0.0055$). Results confirm reports that DER can be yield limiting, although there was no evidence to suggest that later infections increase hidden Diplodia.

In 2020 and 2021, trials were established to examine the effect of adding in-canopy drop nozzle or 360 Undercover nozzle fungicide application methods on DER severity, yield, and spray coverage of corn leaves and ears. Two fungicides labeled for suppression of DER were used with each application method. Coverage was assessed via

spray cards in experimental plots. Yield and DER severity were not affected by application method, however DER severity in control plots was low (0-11%). The addition of 360 Undercover nozzles increased spray coverage on the ear (P=0.0139) and ear leaf (P<0.0001) compared to other methods. Although using in-canopy-nozzles did not affect DER severity, the increase in coverage may lead to improved control under higher disease pressure situations.

KEYWORDS: *Stenocarpella maydis, Stenocarpella macrospora,* Diplodia ear rot, hidden Diplodia, fungicide application

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11/10/2022

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AN IMPROVED UNDERSTANDING OF DIPLODIA EAR ROT IN KENTUCKY CORN

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CHAPTER 1. LITERATURE REVIEW

1.1 Corn production

Corn (*Zea mays* L.) has been cultivated for thousands of years (Benz 2001) and debate still occurs about its origin. Several hypotheses exist, one in which teosinite is the ancestor of modern maize (Beadle 1939) and a second in which *Tripsacum* crossed with other plants to form annual teosinte (Eubanks 1995; Mangelsdorf and Reeves 1939). Reports from Mangelsdorf and Reeves 1939, suggested that modern corn is derived from three ancestors, *Zea mays, Zea diploperennis,* and *Tripascum* (Mangelsdorf 1983). More recent reports have suggested that genetic sequences found in modern corn differ from what would be expected if corn was truly a descendant of *Tripsacum* (Bennetzen et al. 2001).

The origin of maize aside, the importance of corn production is undoubted, accounting for 1.1 billion metric tons of grain worldwide in 2020 (FAO 2022). The United States (U.S.) is the world's largest producer of corn amounting to 383 million metric tons of grain in 2021 (USDA 2022a). Corn is used in many industries, but in the U.S., the majority of corn is used for animal feed and ethanol production (Taylor et al. 2006). Due to economics and government policy involving ethanol production (Wallander et al. 2011), corn production by kilograms and hectares harvested compared to other crops has been increasing since the early 2000's (Fausti 2015). Government policies such as the Renewable Fuel Standard and tax credits for ethanol production were aimed to replace a portion of gasoline with ethanol in response to The Clean Air Act which required the addition of oxygenate additives to fuel to combat air pollution (Wallander et al. 2011).

Not only is the U.S. the world's largest corn producer but it is also the world's largest exporter of corn, with the U.S. share at over thirty percent of worldwide exports (USDA 2022b). The export market for U.S. corn in 2021 was valued at over $18 billion USD, with China as the biggest recipient of exported grain, followed by Mexico and Japan (USDA 2022b).

1.2 Diseases of corn

Corn can be infected by numerous different pathogen species causing many different diseases including foliar blights, stalk rots, ear rots, and seedling blights. Foliar diseases are the most economically important diseases of corn. In terms of U.S. corn production, the most important are gray leaf spot (*Cercospora zeae-maydis* Tehon & E.Y. Daniels), southern rust (*Puccinia polysora* Underw.) and a new disease tar spot (*Phyllachora maydis* Maubl.). From 2012 to 2021, gray leaf spot accounted for an estimated loss of 46.4 million metric tons of corn grain and southern rust resulted in an estimated loss of 19.7 million metric tons (Mueller et al. 2021b,2022; Mueller et al. 2020; Mueller et al. 2016). Estimated losses due to tar spot grew from 741 thousand metric tons in 2020 to nearly 14 million metric tons in 2021 (Mueller et al. 2021b,2022).

In terms of grain yield loss, ear rots have been the less destructive than other types of disease in recent years. The three most important ear rot diseases in the U.S. are Fusarium ear rot (FER; *Fusarium verticilliodes* Sacc.), Gibberella ear rot (GER; *Fusarium graminearum* Schwabe) and Diplodia ear rot (DER; *Stenocarpella maydis* (Berk.) Sutton; *Stenocarpella macrospora* (Earle) Sutton). Ear rots accounted for an estimated loss of approximately 38.1 million tons of corn between 2012 and 2021

(Mueller et al. 2021b,2022; Mueller et al. 2020; Mueller et al. 2016). However, yield losses are not the only issue caused by ear rots, as grain that is affected by ear rot diseases can be lightweight and have low test weight (Munkvold and White 2016). Test weight of corn is used as an indicator of grain quality characteristics. In the U.S., the top corn grade, or grade 1, has a test weight of 72 kg/hL (56 pounds per bushel) or greater (USDA 2020). Grain that is of a lower test weight, contains foreign material such as broken kernels, has a moldy odor, or contains mycotoxins may be subject to a reduction in price, called dockage, by processors and grain elevators to farmers (USDA 2020).

Mycotoxins are metabolites produced by fungi that can negatively affect animals and humans when they consume contaminated grain (Munkvold et al. 2019). In 2020 and 2021 an estimated 0.17% and 0.24% of harvested grain was contaminated with mycotoxins (Mueller et al. 2021b,2022). Mycotoxin contamination can result in heavy economic losses in some years. For example, between 2012 and 2019, an estimated 482 million metric tons of grain were contaminated from mycotoxins (Mueller et al. 2020; Mueller et al. 2016). The range in mycotoxin impact from year to year can be extreme, from 7 million metric tons of contaminated grain in 2017 to 157 million metric tons of contaminated grain in 2013 (Mueller et al. 2020). It has been reported that economic losses due to aflatoxin (*Aspergillus* spp.) and fumonisin (*F. verticilliodes*) contamination in corn can be as high as $2 billion USD per year in the U.S. (Mitchell et al. 2016; Munkvold et al. 2019; Wu and Munkvold 2008).

Moldy grain due to ear rot can be problematic due to dockage when selling grain, but it can also cause grain storage issues. During storage fungi in the grain at harvest can continue to grow and affect other kernels, thereby reducing the quality of the grain by

increasing the amount of moldy grain, and/or by increasing the level of mycotoxins in the grain (Christensen and Kaufmann 1965; Holbert and Hoffer 1920). For corn, research has shown that grain moisture levels below fifteen percent restrict fungal growth (Christensen and Kaufmann 1965; Perez et al. 1982; Reed et al. 2007). Studies have indicated that overall water potential and temperature of grain are better predictors of fungal growth potential than grain moisture (Maltini et al. 2003; Mannaa and Kim 2017). However grain moisture has been shown to be an adequate indicator of the potential for fungal growth in storage (Reed et al. 2007), and measuring grain moisture levels is more feasible on a large scale in storage operations such as grain elevators and ports, or in grain storage bins.

1.3 *Stenocarpella* spp.

The *Stenocarpella* genus contains two species, *Stenocarpella maydis* (Berk.) Sutton (Berkely 1847; Sutton and Waterston 1966) and *Stenocarpella macrospora* (Earle) Sutton (Earle 1897). While these pathogens can be found in many regions where corn is cultivated (Casa et al. 2006; Clayton 1927; Earle 1897; Marasas and Van Der Westhuizen 1979; Romero Luna et al. 2016), the only known crop host is corn (Flett 1991). Both species of *Stenocarpella* can infect leaves, stalks, and ears. The *Stenocarpella* spp. are considered facultative saprophytes, as they spend most of their time as parasites but can also grow on dead tissue (Agrios 2005). Both species can cause Diplodia ear rot (Figure 1-1; (Munkvold and White 2016). *Stenocarpella maydis* is also the causal agent of Diplodia stalk rot and *S. macrospora* causes Diplodia leaf streak (Munkvold and White 2016). As pathogens of corn, *S. macrospora* has been shown to be

more aggressive than *S. maydis* on younger stalks and ears of corn plants (Latterell and Rossi 1983). However in culture, *S. macrospora* grows slower and produces fewer pycnidia (Larsh 1938). Through *in vitro* studies, it has been determined that for optimal growth and pycnidiospore production, biotin is needed (Morant et al. 1993). In the same study it was found that *S. maydis* produces more pycnidiospores than *S. macrospora* (Morant et al. 1993). The use of oats or oatmeal agar simulates the natural growth of *Stenocarpella* spp. on corn in culture (Morant et al. 1993; Murphy et al. 1974).

During infection by *S. maydis,* the pathogen produces an appressorium to aid in penetrating plant tissues (Bensch and Van Staden 1992). Within the appressorium, a specialized hyphal structure called a penetration peg can be seen, which is the actual structure that punctures through the plant cell walls (Bensch and Van Staden 1992; Young 1926). Additionally, cell wall degradation is visible at the site of infection by the penetration peg, indicating that *S. maydis* also utilizes cell wall degradation enzymes to aid in the infection process (Bensch and Van Staden 1992). Confirmation of the same infection process by *S. macrospora* has not been reported, however it is assumed it has a similar infection process due to similarities in other aspects of growth, such as conidia production and shape and the two pathogens produce the same signs and symptoms when infecting corn ears.

Identification of these pathogens has historically occurred using serological techniques by isolating the pathogens from infected material and examining the conidia under magnification. The conidia of *S. maydis* (5-8 x 15-34 µm) are smaller than those of *S. macrospora* (13-43 x 95 µm; Figure 1-3; (Anderson et al. 2021; Wilson 1942). More recently, molecular methods utilizing polymerase chain reaction (PCR), were developed

to target the internal transcribed spacer (ITS) region of the fungal genomes to differentiate between the two species in a single reaction (Romero and Wise 2015).

Initial reports have suggested that genetic variability among isolates of *Stenocarpella* spp. genomes is small, however in the same report, the authors stated that the isozyme markers they used may not be suitable for detection of genetic differences due to the conserved nature of the profiles between isolates (Dorrance et al. 1999). In a more recent study, through the use of microsatellite markers, genetic variability was observed between isolates of *S. maydis* (Romero Luna et al. 2017b). Also in Romero Luna et al. 2017b, for the first time mating types genes were discovered in *S. maydis*, indicating the potential for sexual reproduction and recombination between strains (Romero Luna et al. 2017b). These new reports may provide clues as to how the pathogens, and subsequent diseases they cause, spread.

1.3.1 Taxonomy of *Stenocarpella* spp.

The original taxonomy of the *Stenocarpella* spp. was the genus *Diplodia* in the *Botryosphaeriales* order (Sutton 1964). However more recent examinations into asexual reproduction of conidia reclassified the two species into *Stenocarpella* genus based on the shape of conidiogenous cells being more phialidic than the elliptical ones in the genus *Diplodia* (Sutton 1980). Later, based on molecular evidence, the placement of *Stenocarpella* spp. into a separate genus was confirmed because of their segregation into the order *Diaporthales* (Crous et al. 2006).

Domain: Eukaryota

Kingdom: Fungi

Phylum: Ascomycota

Subphylym: Pezizomycotina

Class: Sordariomycetes

Subclass: Sordariomycetidae

Order: Diaporthales

Family: Diaporthaceae

Genus: *Stenocarpella*

Species: *maydis* (Berk) Sutton

Species: *macrospora* (Earle) Sutton

1.3.2 Corn diseases caused by *Stenocarpella* spp. – Diplodia ear rot

Diplodia ear rot (DER) can be caused by both *S. maydis* and *S. macrospora*. In the U.S., *S. maydis* has been reported as the primary pathogen of concern (Larsh 1938; Stevens 1943) while in other parts of the world *S. macrospora* has been reported to be the more prominent pathogen (Renfro and Ullstrup 1976). It has been theorized that a major part of the geographical differences in pathogen prominence is related to the environment (Latterell and Rossi 1983; Munkvold and White 2016; Renfro and Ullstrup 1976). Both species survive in infected plant debris from previous seasons (Eddins 1930) and contain viable conidia for at least a year following plant senescence when left on the surface of the soil (Casa et al. 2003; Flett et al. 1992; Romero Luna et al. 2017a). The surviving pycnidia lay dormant until conditions are favorable for conidia production. Several studies have shown that peak mycelial growth and conidia germination occur between 23° and 30° C (Eddins 1930; Morant et al. 1993; Stevens 1936). *Stenocarpella*

maydis also grows well on corn kernels and can outcompete other pathogens when grain moisture is above 20% (Koehler 1938).

The conidia are released in cirrhi (Anderson et al. 2021; Romero Luna 2016; Zaccaron et al. 2017). These conidia serve as the initial inoculum source to infect corn plants. It is thought that the conidia land on the corn plant and the initial infection begins at the ear shank (Bensch 1995b; Ullstrup 1949). The pathogen then colonizes the ear shank and travels into the ear, causing Diplodia ear rot, which is characterized by a white mold growing on and in between kernels, often starting at the base of the ear (Bensch 1995b) and moving towards the tip. Once entering the cob via the pedicle, *S. maydis* colonizes embryonic tissues, but the pericarp or outside layer of the kernel remains intact (Bensch 1995b; Clayton 1927). In severe cases, the entire corn cob is colonized and the kernels become lightweight and not harvestable.

Occasionally the white mold will not be visible on the outside of the ear, but pycnidia are still visible on the interior of the corn cob (Figure 1-4). This disease expression has been coined hidden Diplodia (Figure 1-4;(Dien et al. 2012; Munkvold and White 2016). It has been stated that this is caused by infections that occur late in the growing season (Munkvold and White 2016), however no research confirming these statements has been reported. Post-harvest, infected material left in the field can overwinter and become an inoculum source for at least a year (Casa et al. 2003; Eddins 1930; Flett et al. 1992; Romero Luna et al. 2017a). Additionally, the fungus can survive in asymptomatic, infected corn seeds, resulting in seed-borne disease, premature death of corn seedlings, and serve as reservoirs for inoculum (Siqueira et al. 2016).

In addition to causing yield losses due to ear infection, *S. maydis* has been associated with the production of the mycotoxins diplodiatoxin (Steyn et al. 1972) and diplonine (Mitchell 1919; Rabie et al. 1985). When affected grain is fed to animals, a condition called diplodiosis can occur, which can result in ataxia, paresis, and paralysis of the afflicted animal (Kellerman et al. 1985; Rabie et al. 1985; Snyman et al. 2011). Negative side effects from feeding animals *Stenocarpella* spp. infected grain such as reduced weight gain, lower egg production, and lower egg weights have been observed in chickens (Blaney et al. 1981; Rabie et al. 1987; Rabie et al. 1985). Inducing diplodiosis by feeding affected grain has also been observed in sheep (Rabie et al. 1985), and cattle (Blaney et al. 1981; Kellerman et al. 1985). Diplodiosis is mainly reported in South Africa (Snyman et al. 2011), parts of South America (Odriozola et al. 2005) and Australia (Blaney et al. 1981), however diplodiatoxin and diplonine have also been isolated from corn ears in Illinois (Rogers et al. 2014).

1.3.3 Corn diseases caused by *Stenocarpella* spp. – Diplodia stalk rot

Diplodia stalk rot (DSR) is caused by *S. maydis,* symptoms of which include a weak stalk, hollow interior of the lower stalk, and stalk discoloration (Munkvold and White 2016). Many stalk rots have similar symptoms, but pycnidia produced by *S. maydis* will be visible emanating from the lower portion of the stalk (Munkvold and White 2016). Stalk rot affects corn production by reducing yields and causing premature drying and stalk breakage, making harvesting ears difficult (Kappelman Jr. and Thompson 1966). From 2012 to 2021, Diplodia stalk rot caused an estimated loss of 3.7 million metric tons of corn in the U.S. and Ontario, Canada (Mueller et al. 2021b,2022;

Mueller et al. 2020; Mueller et al. 2016). The occurrence of Diplodia stalk rot and Diplodia ear rot have been reported to be independent (Thompson et al. 1971); however, both negative and positive correlations were reported, meaning in some instances as DSR increased, DER increased as well. However, the authors also reported the inverse relationship, where DER decreased as DSR increased. These differing correlations suggest that additional factors may be affecting the occurrence of DER when plants are afflicted by DSR.

1.3.4 Corn diseases caused by *Stenocarpella* spp. – Diplodia leaf streak

Diplodia leaf streak is caused by *S. macrospora* and is characterized by cream to brown colored lesions on corn leaves beginning as small oval-shaped lesions, progressing into long, narrow streaks running parallel to the leaf (Figure 1-2; (Anderson et al. 2021; Munkvold and White 2016). As lesions elongate, concentric circles may be visible where the initial infection occurred (Anderson et al. 2021). *Stenocarpella macrospora* can negatively affect the photosynthetic ability of the corn plants by reducing the total amount of chlorophyll within the leaves (Bermúdez-Cardona et al. 2015). Often pycnidia are visible on the surface of the leaf (Figure 1-2), and from the pycnidia, groups of conidia called cirrhi will emanate (Figure 1-5). The infection process can occur without any tropism towards stomata (Bermudez-Cardona et al. 2016), meaning the pathogen can infect anywhere on the leaf. While yield losses due to this disease have not been widely reported, the potential for increasing inoculum levels is theoretically possible. Published reports about Diplodia leaf streak are not as plentiful as the other diseases caused by *Stenocarpella* spp., however it can be found in nearly all

places where corn is grown including the U.S. (Bradley et al. 2010; Munkvold and White 2016). At present there are not reports that link an increase in DLS to an increase in DER, and one report has found that there is no correlation between the two diseases (Grandis et al. 2008).

1.4 *Stenocarpella* spp. as corn pathogens in the U.S.

While *S. maydis* was first reported in 1847 (Berkely 1847), it wasn't until 1909 that it was recognized and described as a corn pathogen (Burrill and Barrett 1909), and *S. macrospora* was isolated from corn ears from Alabama in 1897 (Earle 1897). Since the discovery of both pathogens, *S. maydis* has been noted as the more prominent pathogen in the U.S. (Larsh 1938; Stevens 1943), while *S. macrospora* is more prominent in tropical climates (Casa et al. 2006; Marasas and Van Der Westhuizen 1979; Renfro and Ullstrup 1976; Sutton and Waterston 1966). Early examinations of cultures isolated from infected corn ears in the U.S. have concluded that *S. macrospora* constituted five percent or less of the causal agents (Larsh 1938; Stevens 1943). More recent reports have appeared in the U.S. of *S. macrospora* causing DER in Illinois and Tennessee (Romero Luna et al. 2016). It also has been stated that *S. macrospora* is the less aggressive pathogen of the two species, as mixed inoculations have shown that *S. maydis* outcompetes *S. macrospora* (Hoppe 1936; Kinsel 1937). But the previous studies on causal agents occurred during the late 1930s and early 1940s, which were some of the hottest and driest years on record in the U.S. (NOAA 2022). It is plausible that the timeframe of the collection and subsequent sampling from previous experiments was biased in favor of *S. maydis*, as studies have shown that the optimal temperature for

mycelial growth of *S. maydis* is higher than that of *S. macrospora* (Hoppe 1936; Stevens 1936). Additionally, corn practices have changed dramatically since the 1940s including the adoption of different hybrids, land-use practices, and changes to the way corn is grown, managed, and harvested (Fausti 2015; Kim and Chavas 2003).

During the first half of the 20th century, DER was a disease of major concern in the U.S. (Burrill and Barrett 1909; Larsh 1938). However, research and breeding efforts (Kappelman Jr. and Thompson 1966) to manage the disease appeared to have slowed the incidence and severity as fewer reports of the damage were observed into the latter half of the 20th century. Still, localized epidemics have occurred in parts of the U.S. including Illinois (Bradley 2009), Iowa (Munkvold and Yang 1993) and parts of Ohio (Malvick 2001). These localized epidemics are not unique to the U.S., as they have been observed in other countries such as Nigeria (Olatinwo et al. 1999b), South Africa (Marasas and Van Der Westhuizen 1979), and Brazil (Rossouw et al. 2009). Specific reasons for these epidemics are unclear; however, several factors may be influencing disease incidence and severity, including but not limited to, environmental factors, pathogen adaptations, host resistance, and management practices.

1.5 Management of DER

1.5.1 Management of DER – host resistance

The best and most preferred method of disease management is host resistance (Maloy 2005). Using resistant varieties or hybrids is preferred because they will often offer season-long protection and sometimes alleviate the need for other management tools. Early examination into host resistance to DER found that ears that remain upright

on the stalk, rather than dropping at the end of the season, were more susceptible to DER (Koehler 1959) and that tighter husks around the cob provided some protection from ear rots (Koehler 1959; Rossouw et al. 2002). More recent findings have stated that upright ears may indicate that ears are affected by DER rather than a susceptibility factor, and that they are upright due to being lighter weight (Rossouw et al. 2002). In several studies, host resistance was observed from inbred lines with different genetic lineages, however it was noted that interactions were observed across seasons involving genotype and isolate, meaning in some years certain inbreds were resistant to certain isolates, and in other years, different inbreds were resistant to different isolates (Van Rensburg and Ferreira 1997; Van Rensburg et al. 2003).

There have been mixed results in determining the most appropriate way to artificially inoculate corn to produce sufficient levels of ear rot to screen for resistance but not overwhelm plants and obfuscate the results. Some studies indicate that a conidial suspension sprayed on silks during silking (R1) is preferred (Klapproth and Hawk 1991; Ullstrup 1949), while others found placing infected kernels behind ears where the ear attaches to the stalk was more appropriate (Bensch 1995a; Bensch et al. 1992). It should be noted that these different studies took place in the U.S. (Klapproth and Hawk 1991 and Ullstrup 1949), and in South Africa (Bensch 1995a and Bensch et al, 1992), so differences in environment and local cultural practices may play a role in which inoculation method works best. It has also been noted that earlier infections result in a higher level of disease severity (Bensch et al. 1992; Chambers 1988). Adding to the difficulty of finding host resistance in U.S. populations, many of the female corn parents used pre-2000 were highly susceptible to DER (Dorrance et al. 1998), and naturally

resistant parental lines would most likely come from tropical climates, making adapting other agronomic traits to temperate climates such as the U.S. an additional challenge (Rossouw et al. 2009). Introducing resistance into germplasm is the first step to developing hybrids resistant to DER (Rossouw et al. 2002). No correlation has been shown between host resistance to DER and DLS (Hooker 1956; Thompson et al. 1971). Based on the number of published reports, current breeding efforts in corn appear to be focused on other diseases.

1.5.2 Management of DER – cultural practices

Using cultural practices to manage diseases is a protection or eradication measure, oftentimes used to reduce the amount of the pathogen in the field (Maloy 2005; Sumner et al. 1981). The two most widely used cultural practices for managing field crop diseases are crop rotation and tillage. Crop rotation involves planting and growing different crops in subsequent years that are not hosts to the pathogens one is trying to manage. In some instances, such as take-all root rot (*Geaumannomyces graminis*) in wheat, a short rotation of one to two years away from susceptible hosts can help control the disease (Bockus and Tisserat 2000). However due to the ability of *Stenocarpella* spp. to survive on the surface in residue for over a year (Casa et al. 2003; Flett et al. 1992; Romero Luna et al. 2017a), short crop rotations may not be suitable for eradicating the pathogen from fields. Also, the dispersal of conidia of *Stenocarpella* spp. appears to be limited to localized movements; i.e. less than 120 m from the inoculum source (Casa et al. 2004). Even though complete eradication may not be economically feasible or even

possible, one study has shown that a one year rotation away from corn did significantly reduce the severity of DER (Flett et al. 2001).

Tillage practices may be more suitable for management of DER, as the primary inoculum source is from previously infected residue (Eddins 1930; Flett et al. 1992; Ullstrup 1964). Tillage practices are used to remove pathogen hosts and to reduce residue from previous years (Maloy 2005). In the U.S., the implementation of conservation tillage practices, which are less aggressive tillage methods, have steadily increased since the early 2000s (Claassen et al. 2018). The practices are used to protect soils from erosion due to wind and water (Larson et al. 1978; Ranske 1980). In some Kentucky counties, over half of the hectares farmed are under some form of conservation tillage practice (USDA 2019). However, these practices leave more stubble or residue on the surface of the soil, potentially increasing the likelihood of pathogen carryover in residue from one year to the next. Given the nature of pathogen survival and dispersal of *Stenocarpella* spp., the potential exists for tillage to effectively reduce inoculum and subsequent DER.

Deep tillage has been shown to reduce conidia viability in simulated tillage trials (Romero Luna et al. 2017a) and reduce DER if used every year (Flett et al. 1998). However when repeating simulated trials from Romero Luna et al. 2017a, using commercial tillage equipment, tillage was not effective at reducing DER (Romero Luna 2016). The nature of the discrepancies between reported research on effectiveness of tillage in reducing DER is unknown, however research studies by Flett et al. 1998 and Romero Luna 2016 were performed in different regions of the world where environmental conditions may have played a role in residue breakdown, and subsequent

viability of conidia. It has been theorized that tissue breakdown is a component of inoculum survivability and that differences in the breakdown rates among hybrids may influence the longevity of residue-borne inoculum (Romero Luna et al. 2017a). Using deep tillage practices may help reduce the inoculum levels of *Stenocarpella* spp.; however, the lower economic impact that DER poses may not sway farmers to shift away from practices that are used to enhance other variables in corn production.

1.5.3 Management of DER – chemical control

Fungicide use in corn has been increasing over the last twenty years (USDA 2022c; Wise and Mueller 2012). The use of fungicides for controlling diseases is a means of protecting the plants (Maloy 2005). Fungicides are typically applied to corn in a prophylactic manner which means they are used a protective measure to preempt disease (Mueller et al. 2021a). Fungicides in the quinone outside inhibitor (QoI) chemical class (Fungicide Resistance Action Committee (FRAC) Group 11) move through plants in a translaminar fashion, meaning once absorbed into the leaves they can move to the opposite side of the leaf (Beckerman 2018; Vincelli 2002). Other fungicides such as demethylation inhibitors (DMI; FRAC Group 3) are xylem-mobile and can move upward in the plant from the point of absorption, and succinate dehydrogenase inhibitors (SDHI; FRAC Group 7) are locally systemic with limited mobility away from the contact site (Beckerman 2018). While fungicides are used in corn primarily to control foliar diseases such as gray leaf spot (*Cercospora zeae-maydis*;(Paul et al. 2011), they are also utilized for controlling other disease such as stalk rots (Shelby et al. 2018; Silva et al. 2018), seedling diseases (Mao et al. 1997), and ear rots (Andriolli et al. 2016).

Fungicides are also promoted for plant health benefits in the absence of disease pressure (Wise and Mueller 2012). In the U.S., few fungicides have ear rots as a target disease on their label. Fungicides labeled to control or suppress ear rots include Trivapro (benzovindiflupyr (SDHI) + azoxystrobin (QoI) + propiconazole (DMI); Syngenta, Greensboro, NC), Quilt Xcel (azoxystrobin + propiconazole; Syngenta) for the suppression of Diplodia ear rot, Miravis Neo (pydiflumetofen (SDHI) + azoxystrobin (QoI)+ propiconazole (DMI); (Syngenta) for the suppression of DER and FER, and Proline (prothioconazole (DMI); Bayer, Research Triangle Park, NC) for the control of FER, GER and Aspergillus ear rot.

Fungicides are used and promoted for use in corn at a variety of different growth stages. While many applications around tasseling (VT) are for controlling foliar diseases such as gray leaf spot and southern rust, applications during silking (R1;(Abendroth et al. 2011) and shortly after have been shown to be the most effective at controlling ear rots (Andriolli et al. 2016). Field studies have shown that reduction in Fusarium ear rot severity is possible using prothioconazole (Masiello et al. 2019; Mazzoni et al. 2011) and other DMI fungicides such as tebuconazole (Mazzoni et al. 2011), tetraconazole, and cyproconazole (De Curtis et al. 2011). Gibberella ear rot severity was reduced by using azoxystrobin plus cyproconazole (Andriolli et al. 2016). Diplodia ear rot has been reportedly reduced using benomyl (FRAC Group 1, MBC) and mancozeb (FRAC Group M, multi-site(Marley and Gbenga 2004; Warren and von Qualen 1986) in South America. However, to date, no fungicides registered for use on corn in the U.S. have been shown to reduce DER in the U.S. (Romero Luna and Wise 2015). In this study, field control of DER and *in vitro* sensitivity of *S. maydis* to propiconazole and

prothioconazole was assessed. Fungicide application *in vivo* was not effective at reducing DER severity, but *in vitro* experiments showed the same active ingredients reducing fungal growth (Romero Luna and Wise 2015). There are also conflicting reports about the efficacy of fungicides in controlling Gibberella ear rot at the field level. Where in one study azoxystrobin plus cyproconazole was effective at controlling GER (Andriolli et al. 2016) while other fungicides such as pyraclostrobin (QoI) and azoxystrobin + propiconazole, were not effective at controlling GER (Anderson et al. 2017). While field control of GER has seen different results, *in vitro* control of the pathogen has been reported in several studies (Anderson et al. 2020; Spolti et al. 2014).

1.5.4 Management of DER – harvest and storage

Management of corn ear rots such as DER does not end at harvest. Recommendations for ear rot management during harvest include scouting fields before harvest and if more than 10% of scouted ears have more than ten percent of the ears affected by ear rots, harvest should be completed as soon as possible, and affected grain should be segregated from high-quality grain (Robertson 2009; Wise et al. 2016). Because of potential issues with additional fungal growth and mycotoxin accumulation during storage, considerations for management must be taken into account when storing corn (Fernandez et al. 1985; Holbert and Hoffer 1920). For ear rots of corn, storage of grain above fifteen percent moisture is at risk of fungi spreading throughout the grain (Perez et al. 1982). Additionally, grain that is approximately fifteen percent moisture but stored at temperatures higher than 10° C is also at risk for fungi infecting other grain (Christensen and Kaufmann 1965; Munkvold et al. 2019; Perez et al. 1982; Reed et al.

2007). When DER is present in the field it is recommended that grain be dried below fourteen percent moisture and stored at temperatures below 10° C and if possible at 0° C (Woloshuk and Wise 2008).

1.6 Fungicide application methods in corn

Historically, post-tassel fungicide applications in corn have been applied via aerial methods, utilizing airplanes and helicopters (Mueller et al. 2021a; Smith and Thomson 2003; Wise et al. 2019). However, with advancements in high clearance sprayers, applications using ground driven technology have become more common and more economical. The most commonly used fungicides in field crops move through the plant either translaminarly or are xylem mobile (McGrath 2005; Mueller et al. 2021a). Since movement of the fungicides used in field crops is localized, coverage of the intended target is thought to have a large role in the overall efficacy of fungicide applications (Balardin et al. 2010; Mueller et al. 2021a). For several foliar diseases in other crops, increasing spray coverage has shown to be effective at further improving disease control (Hagan et al. 2017; Moura et al. 2017; Vincelli and Dixon 2007). Additionally, using either ground-driven or aerial applications in corn, fungicide deposition and coverage decreases in the lower canopy compared to the upper canopy (Menechini et al. 2017; Wolf and Bretthauer 2008). Currently, a new type of nozzle body, 360 Undercover® (Figure 1-6; 360 Yield Center, Morton, IL) for ground-driven sprayers is promoted to increase spray coverage within the canopy. The 360 Undercover® nozzle bodies are used within the canopy and have a 4-nozzle arrangement including one horizontal nozzle aimed backwards, one horizontal nozzle aimed

backwards and upwards at a 45° angle, and two nozzles oriented vertically aimed outward from the body in each direction (Figure 1-6). Using 360 Undercover® nozzle bodies is effective at increasing spray coverage up to five times in the mid canopy (around ear height) when compared to conventional overhead-only application types (Penney et al. 2021). Not only do they increase fungicide coverage in corn, but the 360 Undercover® nozzles have increased spray coverage in the lower canopy in soybean (Viggers et al. 2022). Drop nozzle systems, such as the self-made drop nozzle system used in Hagan et. al 2017, were able to reduce foliar disease in cotton (Hagan et al. 2017). Due to potential reduced coverage in the lower canopy combined with the limited mobility of the fungicides used in field crops, fungicide application methods have been theorized to be a potential limiting factor in the control of ear rot diseases (Anderson et al. 2017; Romero Luna and Wise 2015).

1.7 Research objectives

The most recent published report of both *Stenocarpella* spp. populations causing DER in the U.S. was in the 1940s (Larsh 1938; Stevens 1943). Since then, *S. macrospora* has been reported as a causal agent of DER in several U.S. states including Tennessee and Illinois (Romero Luna et al. 2016). The first objective is to determine the relative prevalence of causal agents of DER in Kentucky corn fields. Published reports also state that *S. macrospora* does not grow as well *in vitro* or produce as many pycnidiospores as *S. maydis* (Larsh 1938), however it is said to be the more aggressive pathogen (Latterell and Rossi 1983). The second objective is to determine the relative aggressiveness of isolates of *Stenocarpella* spp. collected from KY corn fields.

It is reported that hidden Diplodia ear rot occurs due to late infection timing (Munkvold and White 2016), however no mention is made of the actual growth stage or if other factors may influence the occurrence. The third objective is to determine if infection point or inoculation timing affect the occurrence of hidden Diplodia.

There have been several reports of fungicide lacking efficacy against ear rots in research trials. Other research also shows that fungicides are effective against the same pathogens *in vitro.* Additionally, fungicide coverage decreases in corn the further down the canopy (Menechini et al. 2017; Wolf and Bretthauer 2008), and some have suggested that the effectiveness of fungicides in controlling ear rots may be limited by application methods (Anderson et al. 2017; Romero Luna and Wise 2015). The fourth objective is to determine if utilizing in-canopy fungicide applications in corn reduces DER and improves spray coverage on the ear leaf and ear.

Figure 1-1 Signs of Diplodia ear rot caused by *Stenocarpella maydis* and *Stenocarpella macrospora*, which include a white mold growing on and in between kernels.

Figure 1-2 Elongated necrotic lesion on a corn leaf with visible pycnidia within the lesions, indicative of Diplodia leaf streak caused by *Stenocarpella macrospora*.

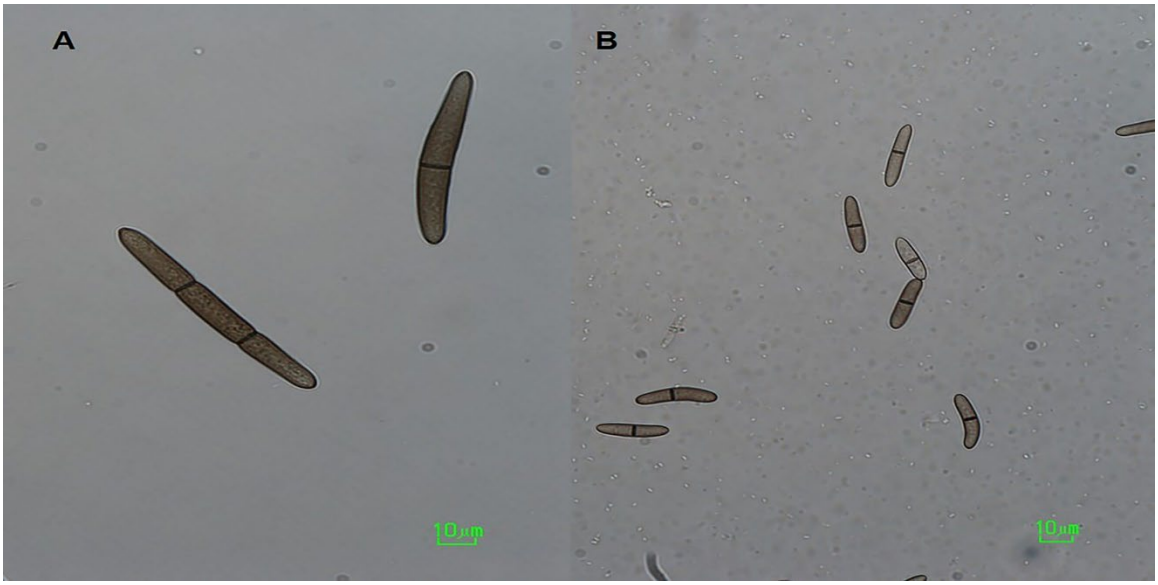

Figure 1-3 Conidia of *Stenocarpella macrospora* (13-43 x 95 µm; A) and *Stenocarpella maydis* (5-8 x 15-34 µm; B) at 400 x magnification.

Figure 1-4 Diplodia ear rot symptoms of hidden Diplodia including discolored kernels on the corn ear (left), and signs of pycnidia of *Stenocarpella* spp. in the cob pith (right)(arrow).

Figure 1-5 Group of conidia called cirrhi emanating from a pycnidium within a lesion of Diplodia leaf streak caused by *Stenocarpella macrospora*.

Figure 1-6 A360 Undercover® nozzle body (360 Yield, Morton, IL) with 4 nozzle arrangements; horizontal and backwards (black cap), horizontal and 45° upwards (orange nozzle tip), and one pointed vertically outward in both directions (orange tips).

CHAPTER 2. FREQUENCY OF *STENOCARPELLA* SPP. CAUSING DIPLODIA EAR ROT IN KENTUCKY CORN

2.1 Abstract

Kentucky corn (*Zea mays* L.) fields were surveyed in 2019 and 2020 for the presence of Diplodia ear rot (DER) to document the prevalence and distribution of the disease and determine frequency of both DER causal agents, *Stenocarpella maydis* (Berk.) Sutton and *Stenocarpella macrospora* (Earle) Sutton. In 2019, 92 fields were surveyed and 125 of 4,600 inspected ears were confirmed to be infected by either causal agent. In 2020, 87 fields were surveyed, and 45 of 4,350 ears examined were confirmed to be infected by either causal agent. Fungi were isolated from corn ears with suspected signs and symptoms of DER, and causal species was identified based on morphology of conidia. In 2019, *S. macrospora* was identified in 33.8% of ears, and 36.9% of ears in 2021, compared to 66.2% in 2019 and 63.1% in 2020 identified as *S. maydis*. Severity of DER did not differ across different geographic regions in Kentucky, and no species was more prevalent than another in any region sampled. However, analysis of spatial distribution of infected ears by pathogen using average nearest neighbor index (NNI) indicated evidence of clustering of DER caused by *S. maydis* and *S. macrospora* in 2019 and by *S. maydis* in 2020. Results from this study emphasize the importance of including both *Stenocarpella* spp. in future research and discussion on DER.

2.2 Introduction

Diplodia ear rot (DER) of corn (*Zea mays* L.) is caused by two different pathogens, *Stenocarpella maydis* (Berk.) Sutton (Berkely 1847; Sutton 1980) and

Stenocarpella macrospora (Earle)(Earle 1897; Sutton 1980). Both pathogens cause identical disease signs and symptoms, which include white mold growing on and between kernels (Figure 2-1), bleaching of the ear husk, discolored and lightweight kernels, and visible pycnidia in the cob pith (Munkvold and White 2016). Identification of both pathogens has typically occurred through microscopic examination of conidia, where the conidia of *S. macrospora* are 6 to 13 x 45 to 95 µm compared to conidia of *S. maydis* which are 5 to 8 x 15 to 34 µm (Anderson et al. 2021). While the signs and symptoms of DER are identical regardless of the causal agent, reports have indicated that *S. macrospora* is more aggressive on vegetative tissue than *S. maydis* (Latterell and Rossi 1983). Both pathogens can also cause additional diseases such as Diplodia stalk rot (DSR) caused by *S. maydis* and Diplodia leaf streak (DLS) caused by *S. macrospora* (Munkvold and White 2016). Primary inoculum is mainly residue-borne (Eddins 1930; Ullstrup 1964), and inoculum from *S. maydis*-infected corn residue has remained viable after a year on the soil surface (Casa et al. 2003; Flett et al. 1992; Romero Luna et al. 2017a). Inoculum movement of *Stenocarpella* spp. appears to be slow, moving a maximum of two meters vertically and one hundred-twenty meters horizontally from the source during a growing season (Casa et al. 2004).

Between 2016 and 2019, it was estimated that nearly 14 million metric tons of corn yield in the U.S. and Ontario, Canada were lost to DER (Mueller et al. 2021b). Although DER is not as annually yield-limiting as other ear rot diseases, it can still have a significant impact. For example, in 2016, losses from DER alone were estimated at 4.4 million metric tons (Mueller et al. 2020). It should be noted that in the reported disease

loss estimates, the causal agents of DER were not identified, and estimates are from DER caused by either pathogen.

In the U.S., *S. maydis* is viewed as the more prevalent and important pathogen, and there is limited survey data indicating prevalence and distribution of *S. macrospora.* From 1937 to 1941, only 3.7 to 5.9% of DER identified in the U.S. was caused by *S. macrospora* (Stevens 1943). While historic reports have suggested *S. macrospora* to be less prevalent in the U.S., it is recognized as a major ear rot pathogen in South America and South Africa (Casa et al. 2006; Marasas and Van Der Westhuizen 1979). *Stenocarpella macrospora* has been reported to be more prevalent in tropical climates (Marasas and Van Der Westhuizen 1979; Sutton and Waterston 1966); however, *S. macrospora* was recently identified as causing DER in Illinois and Tennessee (Romero Luna et al. 2016) after not being associated with DER in the U.S. since 1952 (Roane and Roane 1994).

Currently, management of DER does not differ depending on causal agent. Host resistance is typically the most reliable method of disease management (Maloy 2005). However, resistance to DER appears to be very complex with hybrid differences being interconnected with environmental conditions (Van Rensburg and Ferreira 1997). Non-additive genes appear to play a major role in hybrid resistance to DER (Olatinwo et al. 1999a) and dominant genes are the predominant form of resistance (Wiser et al. 1960), suggesting that hybridization with resistant hyrbids could increase DER resistance . However in the U.S., many of the female inbred lines used as parents to commercial hybrids are susceptible to DER (Dorrance et al. 1998). Crop rotation away from corn and deep tillage using moldboard plows have been shown to effectively reduce inoculum

pressure and subsequent DER (Flett et al. 2001; Flett and Wehner 1991; Romero Luna et al. 2017a). However in the U.S., tillage practices have shifted since the 2000s to more conservation and no-till practices (Claassen et al. 2018) due to benefits in reduction of soil erosion due to wind and water (Larson et al. 1978).

Because of both pathogens' ability to remain viable in surface residue for over a year, the increased use of conservation and no-till practices in the U.S., and the increased corn hectares in the U.S. since the first published reports of *Stenocarpella* spp. populations causing DER in the U.S., it is suspected that the prevalence of each *Stenocarpella* spp. has changed since the 1940s. To our knowledge, there have been no recent efforts to categorize or monitor the population of *Stenocarpella* spp. causing DER in the U.S., and limited research exists that has examined differences in DER severity by pathogen. The objectives of this study are to (1) determine the prevalence of *S. maydis* and *S. macrospora* causing DER in Kentucky and (2) determine if *S. macrospora* is more aggressive than *S. maydis* on corn ears.

2.3 Materials and methods

2.3.1 Survey methodology

Fields were sampled across Kentucky in 2019 and 2020. Kentucky has five distinct physiographic regions (UK-UKGS 2012) with the majority of grain production in the western part of the state. The five regions in Kentucky are the Eastern KY Coalfield, Bluegrass, Jackson Purchase, Pennyrile and Western KY Coalfield, with much of the grain production focused in the Mississippi Embayment (Jackson Purchase),

Mississippian Plateaus (Pennyrile) and Western KY Coalfield areas (Figure 2-2; (KGS 2012).

2.3.2 Sampling

Sampled fields from both years of the survey were chosen based on the amount of harvested corn hectares per county in Kentucky. Using USDA-NASS statistics (USDA 2022b), for every 4,046 hectares of harvested corn within a county, one field was sampled. No two sampled fields were closer than 3.2 km of each other. In total, 92 fields encompassing 31 counties were sampled in 2019 (Figure 2-3), and 87 fields encompassing 27 counties were sampled in 2020 (Figure 2-4). Locations of sampled fields can be found in Appendix 1. Sampling occurred during growth stages late dough through physiological maturity (R4-R6;(Abendroth et al. 2011). At each field, 5 points were sampled in a "W" pattern, with each point at least 30 m apart, increasing in distance to over 100 m in larger fields. At each sampling point, husks of 10 ears were removed and each individual ear was examined for symptoms and signs of DER, for a total of 50 ears per field. In total, 4,600 ears were examined for DER in 2019 and 4,350 ears were examined in 2020. If DER was present, disease severity of each ear with symptoms or signs was rated on a 0-100% scale, with 0% being no visible disease present and 100% being the entire ear showing evidence of DER. Where DER was present, ears were collected for species identification. Global position system (GPS) location, previous crop (corn or soybean), and residue coverage of the soil (0-100%) were also measured for each field (Appendix 1).

2.3.3 Isolation and identification of causal organisms

Stenocarpella spp. were isolated from collected ears by removing 8 to 10 kernels from each with signs or symptoms, with each ear processed separately. Kernels were placed in a diluted bleach solution (0.525 % NaHClO) for 2 minutes, followed by a rinse in sterile water for 2 minutes. Surface-sterilized kernels were dried on sterile paper towels then plated onto oatmeal agar (BD, Franklin Lakes, NJ) at 3 to 4 kernels per plate, with each plate only containing kernels from one ear. Incubation of plated kernels occurred at 25° C for 5 days under a 12 hr light/dark cycle. After incubation, all kernels that had fungal growth were examined under a microscope for the presence of conidia. A 50 to 100 μL drop of sterile water was placed on each culture emanating from a kernel, and a sterile inoculating loop was used to gently scrape the culture where the water was placed and transferred to a microscope slide. Species were determined by size of conidia, as *S. macrospora* conidia are larger (13 x 43-95 mm) than *S. maydis* conidia (Anderson et al. 2021). Ears that showed signs or symptoms of DER but no *Stenocarpella* spp. was positively isolated or identified were removed from future analysis.

After species identification, conidia were removed and placed on a 3% water agar (BD, Franklin Lakes, NJ) plate. One colony per ear was selected for use in future experiments. Only one colony per ear was needed because there were no instances in either year where both *Stenocarpella* species were isolated from a single ear. The water agar plates were incubated at 25° C for 18 to 24 hrs. Single conidia were selected from water agar plates with the use of a stereo microscope and sterile needle, and four single conidia were placed on a single oatmeal agar plate and incubated at 25° C for 5 days. After 5 days, a single colony was sub-cultured onto a new oatmeal agar plate. Isolates

were then stored for later use by growing cultures for 7 to 10 days at room temperature under 12 hr light dark cycle. Popcorn kernels were soaked in water overnight, and the water drained the following day and autoclaved. Sterile popcorn kernels were placed on a new oatmeal agar plate with a 5 mm fungal plug and incubated at 25° C under a 12 hr light dark cycle. After 10-14 days colonized kernels were collected from the plates, dried overnight, and stored in cryotubes at -80° C for future use.

2.3.4 Aggressiveness of *S. maydis* and *S. macrospora* on corn ears

A selection of isolates was chosen from the 2019 and 2020 isolate collection for inclusion in aggressiveness experiments. Thirteen isolates of *S. maydis* and 4 isolates of *S. macrospora* from 2019, and 12 isolates of *S. maydis* and 5 isolates of *S. macrospora* from 2020 were tested. Selected isolates were removed from long-term storage and plated on fresh oatmeal agar plates. After incubating for 14 days at room temperature under 12 hr light dark cycle, plates were washed with sterile water to release conidia. Collected conidia were placed in 50 mL conical tubes ensuring each isolate was in a separate tube. Each conidial suspension was quantified using a hemocytometer and diluted to 5,000 conidia per mL.

Corn ears of Pioneer hybrid P1555 CHR (Pioneer, Johnston, IA), were collected at early-mid milk stage (R3;(Abendroth et al. 2011) and stored in husks for no longer than 10 days at 4° C for testing. Pioneer hybrid 1555 CHR had a company rating of five for DER resistance on a scale of one to nine, with one being poor and nine being excellent. Ears were husked and those showing signs or symptoms of disease or injury

were discarded. The remaining ears were placed in a diluted bleach (0.525 % NaHClO) bath for 5 minutes followed by a bath in sterile water for 5 minutes.

Using a modified version of pervious reported protocol (Dolezal et al. 2013), a single sterilized needle was dipped into the conidial suspension and inserted into the base row of kernels on each of 3 cobs (replicates) per isolate. The needle was dipped into the suspension after each inoculated kernel. All three inoculated cobs were placed into a sterile humid chamber and sealed. Three ears were inoculated with water only to serve as a control. Bags of inoculated ears were placed in a growth chamber (AR-22L, Percival Scientific, Perry, IA) in a complete random design under a 12 hr light/dark cycle, held at 29° C during the light cycle and 24° C during the dark cycle. Following incubation for 12 days, all ears were rated for DER signs or symptoms. Visual signs of white mold and symptoms of discolored kernels (Figure 2-5) were measured in cm from the base of the cob to where symptoms/signs ended. All cobs were also split lengthwise, and measurements (cm) of DER symptoms and signs were collected from the base of the cob to the end of visible symptoms (Figure 2-5).

2.3.5 Data analysis

Causal agent species, DER incidence, and DER severity data from the field-level survey were organized by the different physiographic regions within Kentucky and analyzed using chi-square analysis (PROC FREQ) in SAS V9.4 (SAS Institute, Cary, NC). This analysis was performed to determine if DER incidence and severity varied by location across regions in Kentucky. Pairwise comparisons were performed (PROC TTEST) to determine if the causal agent differed across the sampled regions and if causal

species impacted DER severity. Using GPS coordinates, nearest neighbor index (NNI) was estimated to determine if species locations were spatially related using the SpatialEco package in RStudio (2021.09.1). A NNI analysis was performed on two sets of data, one including all ears confirmed to have DER to test if infection of one ear was linked to infection of other ears, and a second using a maximum of one ear per species per field to test if any level of infection of a field was linked to the infection of a different field. These analyses aimed to help quantify relationships between pathogens and disease spread.

Measurement data of DER signs and symptoms from laboratory aggressiveness experiments were subjected to analysis of variance using PROC GLIMMIX in SAS V9.4 to determine if DER severity differed by isolate, and pairwise comparisons (PROC TTEST) were performed to determine if causal species influenced DER severity.

2.4 Distribution of *Stenocarpella* species across Kentucky

In 2019, 128 of the 4,600 inspected ears had signs or symptoms of DER. Severity of DER, measured as a percent of ear with visible signs and symptoms in these ears ranged from 1-100%. Combining DER severity ratings from all ears examined, including those with no disease present, the overall mean severity in 2019 was 8.7%. Diplodia ear rot was observed in 58 of the 92 fields, or 63% of sampled fields. Based on conidial morphology in 2019, 81 sampled ears were found to have DER caused by *S. maydis,* and 44 ears with DER were caused by *S. macrospora*, and 3 were undetermined. Mean severity of DER in 2019 on ears infected with *S. maydis* was 54%, while mean severity on ears with DER caused by *S. macrospora* was 69%.

In 2020, incidence of DER was much lower than in 2019. Only 46 of 4,350 inspected ears had signs of DER, and DER was observed in 25 of the 88 fields, representing 28.4% of sampled fields. Combining DER severity ratings from all ears examined, including those with no disease present, overall mean severity was 3.5% in 2020. Based on conidial morphology in 2020, 28 sampled ears with DER were confirmed to be infected by *S. maydis,* while 17 ears were found to have DER caused by *S. macrospora,* and the causal agent of one ear with signs and symptoms of DER was undetermined. Mean severity of DER in 2020 on ears infected with *S. maydis* was 55% and by *S. macrospora* was 70%.

Chi-square analysis indicated that DER incidence and severity did not vary by geographic region in KY (Table 2-1). Thus, all data were combined for future analyses.

In the analysis of all ears with confirmed DER, NNI indicated clustering of samples infected by *S. macrospora* in 2019 and *S. maydis* in 2019 and 2020 (Table 2-2). When analyzing a maximum of one ear of DER caused by each pathogen per field, distribution of both pathogens in both years favors random or dispersed distribution (Table 2-3).

2.5 Influence of causal agent on DER severity and incidence

Using a chi-square analysis, the frequency of *Stenocarpella* species detected was not different across geographic regions (Table 2-4), therefore geographic region was not included as a factor in further analysis. In 2019, ears infected by *S. macrospora* had greater disease severity than those infected by *S. maydis* (Table 2-5), however in 2020 no differences were observed between species for disease severity (Table 2-5).

In vitro experiments showed that aggressiveness on corn ears inoculated with *S. maydis* or *S. macrospora* differed by isolate on both the outside of the ear ($P < 0.0001$) and inside of the ear ($P < 0.0001$), however no differences in aggressiveness between *Stenocarpella* species were observed (Table 2-6).

2.6 Discussion

This research provides evidence that, in Kentucky, DER is annually caused by both *S. maydis* and *S. macrospora,* and *S. macrospora* is a more prevalent pathogen in the state than previous literature suggests. Previous research suggested that less than 6% of DER is caused by *S. macrospora* in southern states (Larsh 1938; Stevens 1943), while we estimated that approximately 33% of ears with DER were infected by *S. macrospora* in both years of our study.

In tropical climates with high precipitation and humidity, *S. macrospora* is more commonly associated with DER (Latterell and Rossi 1983; Renfro and Ullstrup 1976). Given that the previous studies on prevalence (Larsh 1938; Stevens 1943) occurred during the driest 5-year period recorded in Kentucky (Runkle et al. 2022) and our study occurred during years with above average precipitation (Runkle et al. 2022), differences in environmental conditions may have played a role in the discrepancy between studies. Additionally, both pathogens cause identical symptoms and signs on corn ears, and to our knowledge, there is no national monitoring program to determine the causal agent of DER, which means that DER caused by *S. macrospora* may be more widespread across the U.S. than originally known. The causal agent of Diplodia leaf streak (DLS), *S. macrospora,* has been reported in several U.S. states in recent years (Anderson et al.

2021; Bradley et al. 2010). While the relationship between DLS and DER caused by *S. macrospora* was not examined in this study, it should be examined in subsequent studies to determine if an increase in foliar inoculum is contributing to an increase in DER caused by *S. macrospora*.

In this study, we identified ears that had signs and symptoms of DER caused by each species; however, no ears were found to have been infected by both pathogens. Co-infection of corn ears has been observed with other ear rot pathogens including multiple *Fusarium* spp. (Logrieco et al. 2002) but it has not been reported for *Stenocarpella* spp. Competition across and between fungal species causing ear rot has been observed in other corn ear rot pathogens including *Fusarium* spp. (Ferrigo et al. 2020), and *Aspergillus* spp. (Mehl and Cotty 2013; Mehl et al. 2012; Probst et al. 2011). Based on previous research suggesting *S. macrospora* is more aggressive than *S. maydis,* one would suspect that *S. macrospora* would outcompete *S. maydis* on an ear (Latterell and Rossi 1983). However, the lower production of pycnidia and additional nutrient requirements of *S. macrospora* (Marasas and Van Der Westhuizen 1979) may explain why it is less prevalent than *S. maydis*. In Hoppe 1936, aversion was observed *in vitro* between cultures of different *Stenocarpella* species and between some cultures of the same species, and when co-inoculated in corn ears, only re-isolations of *S. maydis* were successful (Hoppe 1936). Even though *S. macrospora* has been shown to be the more aggressive pathogen in limited studies, in our study there were no differences in aggressiveness observed between species, indicating that other factors likely contribute to why it is less prevalent than *S. maydis* on corn ears.

Kentucky has distinct geographical areas of corn production with different environmental and production aspects. While our initial thought was that these distinct geographic regions in KY may have different compositions of species that influence DER, that proved not to be the case. In performing the chi-square analysis, we determined that the species distribution of *Stenocarpella* spp. causing DER was due to random sampling and did not have a region-based correlation (Table 2-4). It has been suggested that DER prevalence varies by region (Marasas and Van Der Westhuizen 1979) although this may be more due to climate than geography or cultural practices. We also found no connection between DER severity and the cultural practices of crop rotation and tillage type (data not shown). This contrasts with previous reports that deep tillage and crop rotation can reduce DER severity (Flett et al. 1998,2001). Even though Kentucky does have different regions where different cultural practices are implemented, it is possible that the differences in those practices are not great enough to affect DER severity. Flett et al. 1998 summarized that moldboard plowing each season was the only effective tillage practice to reduce DER severity. There is almost no moldboard plowing used in Kentucky, as no-till or conservation tillage practices are twice as common as intensive tillage practices (USDA 2019). Conversely, studies on rotation systems such as Flett et al. 2001 summarized that crop rotation to wheat (*Triticum aestivum*), soybean (*Glycine max*) and peanut (*Arachis hypogaea*) had the greatest effect on reducing DER severity. This would be a similar rotation schedule in Kentucky of rotating between corn and soybeans or corn/wheat/soybean in subsequent years. Environmental differences could affect the amount of corn residue that remains each season and a climate where milder winters occur, such as South Africa and tropical regions, could increase the rate of

residue breakdown. Additionally, during experiments on *Stenocarpella* spp. survival on residue in the U.S., it was suggested that differing decomposition rates of corn hybrids may influence the survival of sources of inoculum in subsequent seasons (Romero Luna et al. 2017a). Positive identification of DER and causal agents could be skewed in this study towards more susceptible hybrids and to areas where residue that decomposes more slowly were used since information about hybrid decomposition or hybrid resistance from surveyed fields was not collected in this study.

Cultural practices have also changed since the publication of the initial survey on prevalence of *S. macrospora* in corn, including the use of improved, modern hybrids with different disease resistance, less invasive tillage practices, and earlier harvesting practices. Management of DER through cultural practices has been difficult to assess as tillage and rotations have shown to be effective at reducing DER in some instances (Flett et al. 1998,2001) but not others (Romero Luna 2012). Chemical management of DER has also been inconsistent in the U.S., with fungicides effective *in vitro* but not *in vivo* (Romero Luna 2012). Also, hybrid resistance may only provide consistent management of DER in cases of intermediate disease pressure (Flett and McLaren 1994). The genetic diversity of *S. maydis* is known to be high (Fedrigo et al. 2016; Romero Luna et al. 2017b) across different regions and within regions, and virulence differs by *Stenocarpella* species (Kappelman Jr et al. 1965; Maxwell and Thompson 1974). This complex nature of resistance and isolate interactions may influence variations in effectiveness of management strategies.

Other researchers have found success using average nearest neighbor index (NNI) as a tool to determine spatial distribution patterns, which can help determine how

pathogen populations are related or influence disease severity over geographic areas (Blank et al. 2019; Falcón-Brindis et al. 2021). Nearest neighbor index values less than one indicated clustering, numbers greater than one up to a maximum of 2.15 indicate normal dispersal, while numbers near one indicate random distribution. Negative z-scores from NNI analysis indicate clustering while a positive z-score indicates dispersion. Using NNI on all ears with a confirmed causal agent, we observed that for both species in 2019 and for *S. maydis* in 2020, locations with confirmed disease appear to be clustered since NNI numbers were less than one with negative z-scores (Table 2-2). This grouping of positive samples, or clustering, is not unexpected as it has been hypothesized that the dispersal area of *Stenocarpella sp.* conidia may be relatively small (Casa et al. 2004). Alternatively, when removing duplicate ears caused by the same species found in the same field from the analysis, the results are reversed and both species in both years appear to be randomly distributed with values greater than one and positive z-scores (Table 2-3). This would indicate that infections across different fields are not spatially related but that it would be more common to find multiple ears infected by the same species within each field. Looking at both NNI analyses, this study would support earlier reports about the limited movement of *Stenocarpella* spp. inoculum from the original source (Casa et al. 2004). Additionally, the failure to see differences in causal agent across different regions in KY would suggest that the causal agent of individual ears may be influenced heavily by pathogen dispersal rather than other agricultural practices.

This research highlights the importance of studying both pathogens that cause DER when developing management strategies, specifically within Kentucky. This study

demonstrates that *S. macrospora* causes DER more widely than previously observed; however, the survey was limited to Kentucky and not necessarily representative of the entire corn growing area within the U.S. More research is needed to assess *Stenocarpella* populations in other regions and determine if these results are consistent throughout the U.S., or if regional differences influence pathogen prevalence and disease severity. Knowing the prevalence of *Stenocarpella* species causing DER may aid in improving DER management since previous research and hybrid selection in the U.S. has focused on *Stenocarpella maydis*.

Figure 2-1 Signs and symptoms of Diplodia ear rot caused by *Stenocarpella maydis* or *Stenocarpella macrospora*, including a bleached husk and white mold growing on and between corn kernels.

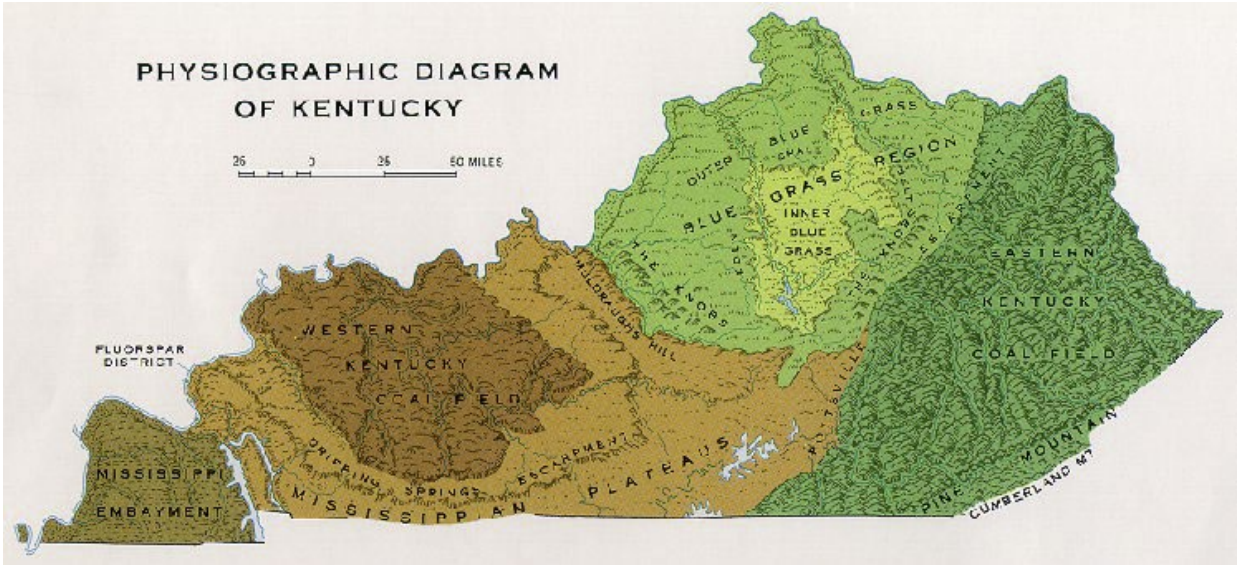

Figure 2-2 Physiographic diagram of geographic areas within Kentucky. https://www.uky.edu/KGS/geoky/physiographic.html

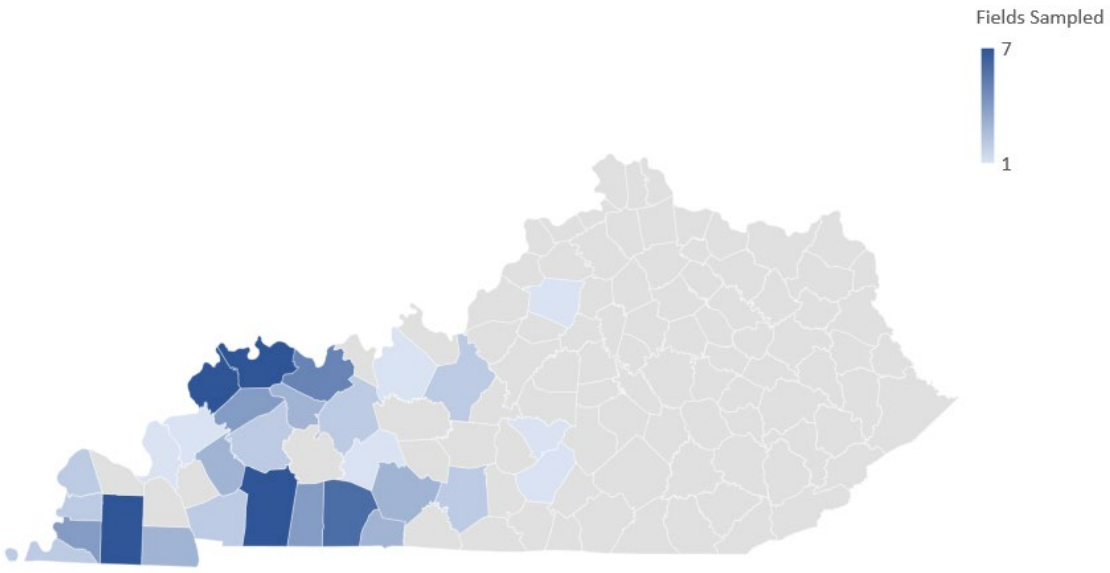

Figure 2-3 Counties in Kentucky where fields were examined in 2019 for the presence of Diplodia ear rot caused by *Stenocarpella maydis* or *Stenocarpella macrospora*. Darker shading indicates a higher number of fields sampled per county.

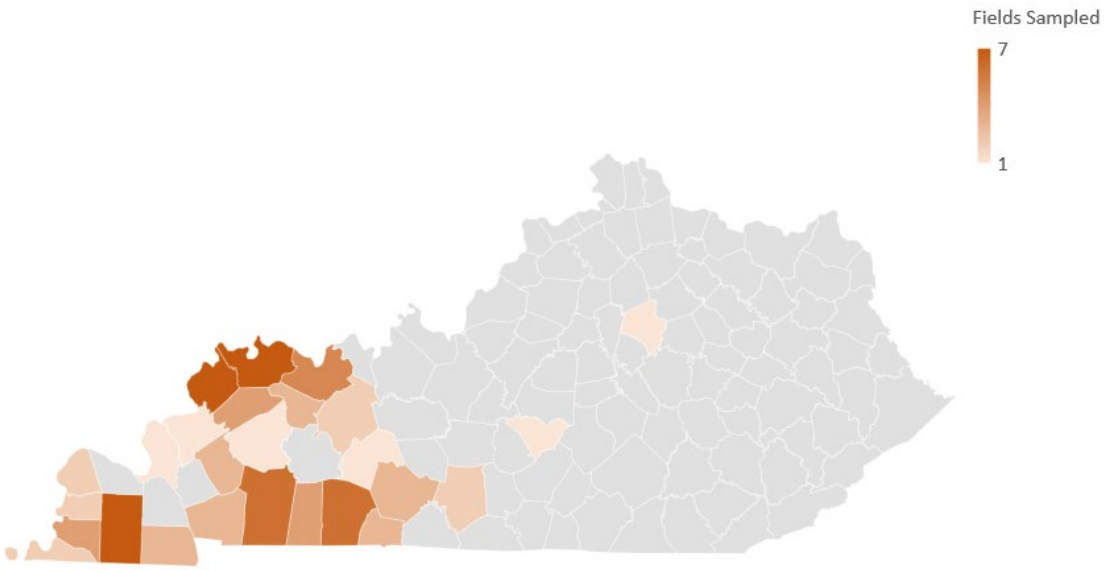

Figure 2-4 Counties in Kentucky where fields were examined in 2020 for the presence of Diplodia ear rot caused by *Stenocarpella maydis* or *Stenocarpella macrospora*. Darker shading indicates a higher number of fields sampled per county.

Figure 2-5 Corn ear with white mold growing on and in between kernels, a sign of Diplodia ear rot (A) and symptoms of discolored cob pith (B) following infection by *Stenocarpella macrospora* on the same ear.

Table 2-1 Field incidence and overall severity by geographic region in Kentucky in 2019 and 2020 of Diplodia ear rot caused by *Stenocarpella maydis* or *Stenocarpella macrospora* analyzed using chi-square analysis.

	2019		2020	
Region	Incidence (%)	Severity (%)	Incidence (%)	Severity (%)
Bluegrass	0.0	0.0	-	-
Jackson Purchase	70.0	5.7	35.0	3.0
Pennyrile	59.4	9.5	24.2	2.4
W KY Coalfield	71.8	9.8	41.3	4.9
P-value	0.3471	0.4701	0.3340	0.2650

Table 2-2 Nearest neighbor index (NNI) analysis of all ears of Diplodia ear rot caused by either *Stenocarpella maydis* or *Stenocarpella macrospora* from 2019 and 2020 Kentucky corn fields.

	2019		2020	
	S. maydis	*S. macrospora*	*S. maydis*	*S. macrospora*
NNI	0.4435	0.3136	0.5416	0.9999
Z-score	-9.7571	-8.8083	-4.6390	-0.0011
P-value	<0.0001	<0.0001	<0.0001	0.9909

Table 2-3 Nearest neighbor index (NNI) analysis of fields with at least one confirmed ear of Diplodia ear rot caused by *Stenocarpella maydis* or *Stenocarpella macrospora* from 2019 and 2020 in Kentucky corn fields.

	2019		2020	
	S. maydis	*S. macrospora*	*S. maydis*	*S. macrospora*
NNI	1.0193	0.9618	1.4653	1.7690
Z-score	0.2526	-0.3728	3.8800	5.3040
P-value	0.8006	0.7093	0.0001	<0.0001

Table 2-4 Frequency of ears with Diplodia ear rot caused by *Stenocarpella maydis* and *S. macrospora* by geographic region in Kentucky in 2019 and 2020.

	2019		2020	
Region	*S. maydis (%)*	*S. macrospora* (%)	*S. maydis (%)*	*S. macrospora* (%)
Bluegrass	.	.	100.0	0.0
Jackson Purchase	53.9	46.2	75.0	25.0
Pennyrile	71.4	28.6	33.3	66.7
W KY Coalfield	65.6	34.4	60.9	39.1
P-value	0.3335		0.0784	

Table 2-5 Disease severity of Diplodia ear rot (DER) on a 1 to 100% scale by causal agent from ears confirmed infected with either *Stenocarpella maydis* or *Stenocarpella macrospora* from Kentucky corn fields in 2019 and 2020.

	2019	2020
	DER severity (%)	DER severity (%)
S. maydis	54.0 B	55.2
S. macrospora	69.1 A	70.0
P-value	0.0245	0.1730

Table 2-6 Analysis of sign and symptom distance (cm) on the outside and inside of corn ears inoculated with *Stenocarpella maydis* or *Stenocarpella macrospora* isolates collected from Kentucky in 2019 and 2020.

Species	Outside ear (cm)	Inside ear (cm)
S. maydis	106.2	99.0
S. macrospora	95.7	87.3
P-value	0.1576	0.1273

CHAPTER 3. EFFECT OF TIMING AND SITE OF INOCULATION BY *STENOCARPELLA MAYDIS* AND *STENOCARPELLA MACROSPORA* ON DIPLODIA EAR ROT OF CORN

3.1 Abstract

Stenocarpella maydis (Berk.) Sutton and *Stenocarpella macrospora* (Earle) Sutton are both causal agents of Diplodia ear rot (DER) of corn in the United States. Typical signs and symptoms of DER include white mold on and between kernels, bleached husks, and pycnidia in the cob pith. Hidden Diplodia describes a phenomenon where no visible ear mold or bleached husk is observed but, pycnidia are still visible on the inside of the cob. This phenomenon can result in misdiagnosis of DER while scouting and is not well understood. Field experiments were established to examine how inoculation timing and site of infection influenced DER severity and the incidence of hidden Diplodia. Over three years, DER severity, hidden Diplodia incidence, and yield were measured in plots inoculated by each of the causal agents of DER, at one of four growth stages (V8, R1, R2, R3) and one of four locations on the plant (whorl, silk channel, ear shank, foliar). Experiments were conducted under dryland and irrigated conditions in 2019 and 2020, and under irrigated conditions in 2021. Treatments inoculated by either pathogen at R1 resulted in higher DER than treatments where inoculation occurred at R3 in all years. Yield was reduced in treatments receiving inoculation at R1 compared to the non-inoculated treatment in 2019 dryland (P = 0.0076), 2020 irrigated (P = 0.0008), 2020 dryland (P < 0.0001), and in 2021 (P = 0.0032) experiments. In the 2019 dryland experiment, treatments with inoculation at R3

resulted in a higher incidence of hidden Diplodia than non-inoculated treatments ($P = 0.0293$). In one year (2019), treatments inoculated with *S. macrospora* resulted in a 17.5% incidence of hidden Diplodia, compared to 2.5% hidden Diplodia in treatments inoculated with *S. maydis* ($P < 0.0001$).

3.2 Introduction

Diplodia ear rot (DER) of corn (*Zea mays* L.) is caused by two pathogens, *Stenocarpella maydis* (Berk.) Sutton and *Stenocarpella macrospora* (Earle) Sutton (Sutton 1980). Symptoms include low kernel weight, discolored kernels, and bleached husks. Signs of the pathogen include white mold growing in between and on kernels typically starting at the base of the ear, and visible pycnidia in the cob pith (Munkvold and White 2016). Occasionally no external signs will be present on the ear and the only visible symptoms are discolored kernels and light-weight ears (Figure 3-1a). However, these ears will have pycnidia visible on the inside of the ear meaning that the ear is still affected by DER (Figure 3-1b). This phenomenon is referred to as hidden Diplodia (CPN 2022; Rossouw et al. 2002) and is not well understood.

Estimated losses in the U.S. and Ontario, Canada due to DER rank as the second most destructive ear rot of corn behind Fusarium ear rot, with estimated losses totaling nearly 7 million tons for DER (Mueller et al. 2020). Losses due to ear rots are not limited to a reduction in yield only, as ear rots such as DER can result in moldy grain (Eddins 1930). Grain that has DER can be lightweight and contain broken kernels (Munkvold and White 2016; Woloshuk and Wise 2008)

thus reducing test weight (Dien et al. 2012), and emit a moldy odor. Low test weight and grain with odors are subject to dockage at sale (USDA 2020), reducing the value of the crop. Additionally, storing grain affected by DER can result in spread and infection of other kernels in the stored grain (Holbert and Hoffer 1920; Koehler 1938). To combat issues with storing grain that is affected by DER, farmers are encouraged to harvest the crop as quickly as possible and dry grain to less than 15% grain moisture to help reduce the spread of DER in storage (Woloshuk and Wise 2008).

Diplodia ear rot pathogens *S. maydis* and *S. macrospora* overwinter in residue from previous seasons (Eddins 1930; Ullstrup 1964), and it has been concluded in the U.S. that pycnidia can produce viable conidia for up to 17 months when left on the soil surface (Romero Luna et al. 2017a). Other studies conducted in Brazil (Casa et al. 2003) and South Africa (Flett et al. 1992), have also shown that pycnidia produce viable conidia from infested residue that remained on the soil surface for 11 months. Pycnidia of these two species will lay dormant until conditions are appropriate. Studies under controlled conditions have shown that conidial germination occurs within 16 hours at 28° to 32° C (Eddins 1930) and that optimal growth occurs between 18° and 24° C (Morant et al. 1993).

Timing and location of initial infection have been shown to affect DER severity. Experiments that isolated *S. maydis* from ear parts, including the shank, concluded that initial infection occurs at the base of the ear where the shank meets the stalk and then moves up towards and into the ear (Bensch 1995b; Bensch et al. 1992) and in severe cases encompasses the entire ear and husk (Eddins 1930; Munkvold and White 2016). A newer study by Xia et. al 2011, showed that infection progresses though the husk of the

ear before entering corn kernels and that the pathogen congregates within the endosperm of the kernels (Xia et al. 2011). Additionally, Xia et al. 2011 observed degraded cell wall tissue near infection sites prompting the theory that *S. maydis* utilizes cell wall-degrading enzymes, confirming earlier reports of cellulose degradation within infection sites (BeMiller et al. 1969). Multiple studies in South Africa have reported that when inoculations occur near the base or the ear, the resulting DER severity is greater than when inoculation occurs lower on the stalk or on the silks of the ear (Bensch 1995a; Bensch et al. 1992). However, in the U.S., injecting ears with *S. maydis* resulted in higher DER than inoculations occurring at the ear sheath or to the silks (Klapproth and Hawk 1991). Chambers (1988) found that DER severity was greatest when inoculations occurred at mid-silk and decreased at each inoculation timing past mid-silk. Similarly, germplasm resistance screening studies in South Africa determined that inoculation during the silking period resulted in higher disease severity than inoculations that occurred after silking (Bensch 1995a; Bensch et al. 1992).

Although many of these previous studies aiming to determine the effect of timing and location of initial inoculum on DER included multiple inoculation locations and multiple timings to optimize germplasm screening for DER, none have reported on the prevalence of hidden Diplodia. In the case of hidden Diplodia, the signs of the pathogen are often obscured or not easily visible (Dien et al. 2012). The impact of the disease may not be noticed until harvest, when grain with poor yield and test weight are observed. If undetected, hidden Diplodia could lead to grain quality issues that can be exacerbated if grain is not stored

correctly. In some descriptions of DER symptoms, it is suggested that hidden Diplodia occurs when *Stenocarpella* spp. infect ears after silking (R1; (Munkvold and White 2016). Hidden Diplodia is also problematic in seed corn, as planting infected seeds can cause seedling disease, resulting in weakened seedlings or pre-emergence seedling death (Siqueira et al. 2014).

A better understanding of how infection timing and location influence DER development is needed to help accurately quantify disease impact and aid in disease management. Understanding the conditions that result in hidden Diplodia can also aid in diagnosis of DER. The objectives of this study are to 1) determine if inoculation timing by either *S. maydis* or *S. macrospora* at growth stages R1 (silking), R2 (blister), or R3 (milk) influence the occurrence of hidden Diplodia, and 2) determine if inoculation by either *S. maydis* or *S. macrospora* at the ear shank, ear silk, or corn whorl at eight leaf collar stage (V8), affect the incidence of hidden Diplodia.

3.3 Materials and methods

3.3.1 Field trial establishment

Field trials were conducted over three years (2019-2021) at the University of Kentucky Research and Education Center in Princeton, KY. In 2019 and 2020, experiments were established under irrigated and dryland conditions to examine the effects of inoculation timing and infection point on DER signs and symptoms. In 2021, trials were conducted only under irrigation to examine the effects of inoculation timing on DER signs and symptoms. Trials were planted on April 24 in 2019, May 11 in 2020, and May 12 in 2021. All trials in all years were planted with Pioneer hybrid 1555 CHR

at a population of 79,000 seeds per hectare. Pioneer hybrid 1555CHR has a company rating of five for DER resistance on a scale of one to nine with one being poor and nine being excellent. All trials were managed according to local recommendations including pre-plant fertilizer application of 224 kg of nitrogen per hectare, pre- plant application of glyphosate at 189 g per hectare + ammonium sulfate at 380 g per hectare, pre-emergence herbicide application of glyphosate at 189 g per hectare, 138 g of atrazine per hectare, 8 g bicyclopyrone per hectare, 33 g mesotrione per hectare, 295 g S-metolachlor per hectare, and ammonium sulfate added at 380 g per hectare. A post-emergence herbicide application of glyphosate at 189 g per hectare and ammonium sulfate at 380 g per hectare occurred at the four-leaf collar growth stage (V4) each year.

In both years, an experiment was established to test the effect of inoculation timing of *S. maydis* and *S. macrospora* on DER severity and hidden Diplodia occurrence. Separately in each year, a different experiment tested the impact of *S. maydis* and *S. macrospora* infection point on DER severity and hidden Diplodia incidence. Irrigated trials received at least 2.6 cm of water per week which was a combination of lateral overhead irrigation and natural rainfall. All trials were arranged in a randomized complete block design with plots consisting of 4 rows on 76 cm spacing with a length of 9.1 m. Each treatment was replicated four times.

3.3.2 Impact of *S. maydis* and *S. macrospora* infection point on hidden Diplodia

Experimental treatments in 2019 and 2020 to test the impact of plant infection point on DER consisted of a non-inoculated control, *S. maydis* inoculated into the whorl at the eight-leaf collar stage (V8), *S. maydis* applied as a conidial suspension to the silks at silking (R1), *S. maydis* injected into the silk channel at R1, *S. maydis* injected into the ear shank at R1, *S. macrospora* whorl inoculation at the eight-leaf collar stage (V8), *S. macrospora* applied as a conidial suspension to the silks at silking (R1), *S. macrospora* injected into the silk channel at R1, and *S. macrospora* injected into the ear shank at R1.

3.3.3 Impact of *S. maydis* and *S. macrospora* inoculation timing on hidden Diplodia

Experimental treatments in 2019 and 2020 to test the impact of inoculation timing on DER development consisted of a non-inoculated control, *S. maydis* injected into the ear shank at R1, *S. maydis* injected into the ear shank at blister stage (R2), *S. maydis* injected into the ear shank at milk stage (R3), *S. macrospora* injected into the ear shank at R1, *S. macrospora* injected into the ear shank at R2, and *S. macrospora* injected into the ear shank at R3.

The experiment in 2021 to test the impact of inoculation timing on DER development consisted of a non-inoculated control, *S. maydis* whorl inoculation at eight-leaf collar stage (V8), *S. maydis* injected into the ear shank at R1, *S. maydis* injected into the ear shank R2, *S. maydis* injected into the ear shank injection R3, *S. macrospora* whorl inoculation at V8, *S. macrospora* injected into the ear shank at R1, *S. macrospora* injected into the ear shank at R2, and *S. macrospora* injected into the ear shank at R3.

3.3.4 Inoculum production and preparation

Inoculum for all trials was produced using two isolates in pure culture of *S. maydis* and two isolates in pure culture of *S. macrospora.* All isolates were originally collected in Kentucky in 2018. In order to produce pycnidia, isolates were plated onto oatmeal agar (BD, Franklin Lakes, NJ; (Morant et al. 1993) and incubated at 74°C on a 12-hr diurnal cycle of light and dark. Experiments in 2019 and 2020 testing infection point utilized dry infested sorghum and liquid conidial suspensions, while experiments testing inoculation timing used liquid conidial spore suspension only. In 2021, both conidial suspension and infested grain sorghum (*Sorghum bicolor*) inoculum types were used in experiments to determine if inoculation timing influenced hidden Diplodia.

Inoculum for whorl inoculations was made by infesting sterilized sorghum and placing 5 g of dried inoculum in the whorl of each corn plant in the center two rows of each treatment (Romero Luna and Wise 2015). Approximately 10 mL of water was added to each whorl immediately following inoculation. Grain sorghum seeds were prepared by soaking in water for 24 hrs, draining the excess water, then placing 2.5 kg of seeds into spore bags (MycoSupply, Pittsburgh, PA). The spore bags were then autoclaved twice for 60 mins at 121° C and 15 PSI, ensuring to mix the seeds between autoclave cycles. and allowed to cool overnight. One plate of each isolate was placed into separate bags and resealed and allowed to grow for 3 weeks at room temperature under 12 hr light dark cycle. After fully colonized, typically taking 10-14 days, grain sorghum seeds were dried and stored in a cool, dry area until use.

Ear and shank injections were performed using a previously described method of ear injection using an auto-filing syringe and hydration backpack (Anderson et al. 2016). Five mL of a conidial suspension (1,000 conidia/mL) was injected into each respective ear and ear shank in specified treatments. Conidial suspensions were prepared by growing the same isolates used for the whorl inoculation inoculum production on oatmeal agar for two weeks. After two weeks, pycnidia were visible on the surface of the plates. Each plate was flooded with 5 mL of sterile water, allowed to sit for 5 minutes, and then gently scraped using a glass rod. Conidial suspensions were kept separate by isolate, and spore concentrations were quantified using a hemocytometer. Mixtures of each species were made from the two isolates of the same species to ensure a 1:1 ratio of conidia in the final mixture at 1,000 conidia per mL. The conidial mixtures were created the same day that inoculations occurred.

Foliar application of the conidial suspension was performed using a CO_2 backpack sprayer (R&D Sprayers, Opelousas, LA). A ninety-degree Teejet dual adapter with XR8003 tips (TeeJet, Glendale Heights, IL) tips was attached to a single nozzle handheld boom. Each treatment was sprayed with 600 mL of the appropriate conidial suspension. Conidial suspensions were prepared in the same manner as the inoculum for ear and shank injections. The concentration of the suspension was 3,000 conidia/mL, resulting in 1.8×10^6 conidia applied to each plot. All inoculations were performed in the evenings after 7 pm Central Daylight Time.

3.3.5 Assessments of Diplodia ear rot

Diplodia ear rot ratings were performed when corn reached physiological maturity (R6). Ratings for all trials consisted of removing the husks from 5 plants per plot and rating for the presence of DER signs and symptoms on the outside of the ear. Disease severity ratings were collected on a percentage of ear with visible signs and symptoms scale (1-100%) when visible signs and symptoms were observed. The incidence of hidden Diplodia was noted when signs were observed. Plots were harvested using a Wintersteiger Delta plot combine (Reid im Innkreis, Austria), with test weight, plot total weight, and moisture values collected for each plot with an attached grain gauge (H2, Harvestmaster, Logan, UT). Plot weights used to calculate grain yields were adjusted to kilograms per hectare and adjusted to 15.5% moisture.

3.3.6 Statistical analysis

Pairwise comparison (PROC TTEST) was performed using SAS v9.4 (SAS Institute, Cary, NC) to determine if trials from 2019 and 2020 could be combined into a single analysis. Disease assessment data were subjected to analysis of variance (PROC GLIMMIX) using SAS v9.4 to determine if infection point or inoculation timing influenced DER severity, hidden Diplodia incidence, test weight, and yield. Experiments on inoculation timing and infection point examined different aspects of infection and different techniques were used for inoculation with *Stenocarpella* spp. therefore analysis of all variables between experiments were performed separately.

3.4 Inoculation timing effect on DER severity, hidden Diplodia incidence, yield, and test weight

Statistical analysis showed a significant effect of year on yield and DER severity in 2019 and 2020, therefore results from each year were analyzed separately. In 2020, irrigated trials were analyzed separately from those under dryland conditions because significant interactions were observed between dryland or irrigated conditions and inoculation timing ($P < 0.0001$).

Severity of DER in 2019 ranged from no disease signs or symptoms (0%) to the entire ear infested and colonized (100%). In 2019, inoculations occurring at R1 resulted in a 200% increase in DER severity compared to all other treatments, and inoculations at R2 resulted in higher DER severity than the non-inoculated treatment (Figure 3-2a). The range of DER severity in 2020 in both irrigated and dryland experiments was 0-100% of the ear. In dryland experiments in 2020, inoculations at R2 resulted in at least a 200% increase in DER severity compared to all other treatments (Figure 3-2b). In irrigated experiments in 2020, inoculations at R1 and R2 resulted in at least a 17.4% increase over inoculations that occurred at R3 and a 65.5% increase over the non-inoculated controls (Figure 3-2c).

The range of DER severity in 2021 experiments was no disease (0%) to full ear colonized with signs of the pathogen (100%). In 2021, inoculations at V8 resulted in twice as much DER compared to all other treatments (Figure 3-2d). Inoculations at R1 resulted in higher DER severity than inoculations at R2, R3, and the non-inoculated control (Figure 3-2d).

Incidence of hidden Diplodia was scored on a positive (1) or negative (0) scale, and all trials in all years contained at least one positive ear of hidden Diplodia. Pair-wise comparisons showed no difference in the incidence of hidden Diplodia between 2019 and 2020 nor between irrigation conditions, therefore, data from 2019 and 2020 were combined into a single analysis. Of the 560 ears examined in 2019 and 2020 combined, 46 had signs of hidden Diplodia. Timing of inoculation in 2019 and 2020 had no effect on the incidence of hidden Diplodia ($P = 0.1942$; Table 3-1). In 2021, of the 180 ears examined for DER, 27 ears had signs of hidden Diplodia. Treatments inoculated at R1 had higher incidence of hidden Diplodia compared to treatments with inoculations at R2, R3, and non-inoculated controls ($P = 0.0094$; Table 3-1).

No differences in yield were observed in 2019 between irrigated and dryland trials, therefore both irrigated and dryland experiments in 2019 were combined into one analysis. Inoculation timing did not affect yield in 2019 ($P = 0.0827$; Figure 3-3a). Cropping system (dryland vs. irrigated) affected yield in 2020; therefore, results were analyzed separately ($P = 0.0276$). Under dryland conditions in 2020, inoculation at R2 resulted in a 1,300 kg/ha yield reduction compared to all other inoculation timings ($P = 0.0004$; Figure 3-3b), whereas under irrigated conditions, inoculation timings of R1 and R2 resulted in a yield reduction of at least 1,200 kg/ha compared to non-inoculated controls ($P = 0.0361$; Figure 3-3c). In 2021, treatments inoculated at V8 and at R1 resulted in a 1,500 kg/ha yield reduction compared to inoculations at R2, R3 and non-inoculated controls ($P < 0.0001$; Figure 3-3d).

Inoculation timing did not affect test weight in 2019 experiments ($P = 0.0778$; Figure 3-4a). Significant interactions in 2020 were observed between irrigation conditions, timing of inoculations, and pathogen species when analyzing for test weight and experiments in 2020 were separated into irrigated and dryland conditions for further analysis. In 2020, inoculation at R2 resulted in reduced test weight of five kg/hL compared to all other inoculation treatments under dryland conditions ($P = 0.0021$; Figure 3-4b). Under irrigated conditions, inoculation at R1 and R2 resulted in a ten percent reduction in test weight compared to non-inoculated controls ($P = 0.0012$; Figure 3-4c). In 2021, inoculation at V8 and R1 reduced test weight by up to six kg/hL compared to inoculations at R2, R3, and non-inoculated controls ($P < 0.0001$; Figure 3-4d).

Significant interactions were observed between year of trial and pathogen species on severity of DER ($P = 0.0003$) and the incidence of hidden Diplodia ($P = 0.0038$), therefore, all years were analyzed separately. In 2019, inoculation by either *S. maydis* or *S. macrospora* increased DER severity ($P = 0.0016$), and both pathogens caused equal amounts of DER (Table 3-2). In 2020, *S. macrospora* inoculation resulted in higher levels of DER severity compared to inoculations with *S. maydis* (Table 3-2). Inoculation with either pathogen resulted in greater disease than non-inoculated controls ($P < 0.0001$; Table 3-2). In 2021, inoculations with *S. maydis* resulted in nearly twice as much DER than inoculations with *S. macrospora* and non-inoculated controls ($P = 0.0016$; Table 3-2). The incidence of hidden Diplodia was five times higher in 2019 in treatments inoculated with *S. macrospora* than treatments inoculated with *S. maydis* and the non-

inoculated treatment ($P < 0.0001$; Table 3-2). Incidence of hidden Diplodia was not affected in 2020 or 2021 by either pathogen (Table 3-2).

3.5 Influence of infection point on DER severity, incidence of hidden Diplodia, yield, and test weight

Diplodia ear rot severity varied between years with severity ranging from no disease signs or symptoms (0%) to the entire ear colonized (100%) in treatments. Diplodia ear rot severity was different between years ($P = 0.0003$), therefore each year was analyzed separately for disease parameters. Yield did not differ across years ($P = 0.10240$), so both years were combined into one analysis for agronomic variables. Irrigation did not affect DER severity in 2019 ($P = 0.1143$) but in 2020, irrigated plots had greater DER severity than dryland plots, 34.2% and 21.3%, respectively ($P = 0.0051$). Experiments in 2019 were combined into a single analysis for DER severity and hidden Diplodia incidence, while in 2020, experiments were analyzed separately by irrigated and dryland conditions. In 2019, shank injections resulted in complete colonization of the ear (100% severity) and whorl inoculations resulted in 41.8% DER severity, both of which resulted in greater DER than foliar inoculations and non-inoculated controls ($P < 0.0001$; Figure 3-5a). In 2020, infection point did not affect DER severity under dryland conditions ($P = 0.1732$; Figure 3-5b), but in irrigated experiments, shank inoculations resulted in three times more DER than non-inoculated controls ($P < 0.0001$; Figure 3-5c). The incidence of hidden Diplodia was not affected by infection point in 2019 ($P = 0.1362$) or in the 2020 irrigated trial ($P = 0.5957$;

Figure 3-6a; 3-6c). However, whorl inoculation resulted in increased incidence of hidden Diplodia under 2020 dryland conditions compared to all other infection points (P = 0.0136; Figure 3-6b).

While year did not affect corn yield (P = 0.1024), irrigation conditions affected yield with irrigated plots having a higher yield than dryland plots, 9,725 kg/ha and 8,261 kg/ha respectively (P = 0.0055). Therefore, agronomic variables were analyzed by irrigated and dryland conditions. Under dryland conditions, shank inoculations resulted in a 50% yield reduction compared to all other inoculation treatments. Whorl inoculations resulted in decreased yield compared to foliar inoculations and non-inoculated controls (P < 0.0001; Figure 3-7a). Under irrigated conditions, shank inoculations reduced yield by over 3,000 kg/ha (approximately 27% decrease) compared to non-inoculated controls (P < 0.0001; Figure 3-7b). Test weight under dryland conditions was lower in treatments that received shank inoculations compared to all other treatments (P < 0.0001; Figure 3-7c). Under irrigated conditions, shank inoculations resulted in reduced test weight of corn compared to all other infection points tested (P < 0.0001; Figure 3-7d).

An interaction between year and pathogen species of inoculation was observed for the incidence of hidden Diplodia (P = 0.0216), therefore, each year was analyzed separately. In 2019, inoculation with either *S. maydis* or *S. macrospora* increased DER severity compared to non-inoculated controls (Table 3-3); however, the incidence of hidden Diplodia was not affected by which species was used in inoculations (Table 3-3). In 2020, irrigation influenced the severity of DER (P = 0.0218), therefore, the irrigated and dryland experiments were analyzed separately. Severity of DER was higher in all

inoculated treatments, regardless of species, compared to non-inoculated controls ($P < 0.0001$; Table 3-3). Under dryland conditions in 2020, DER severity was similar regardless of pathogen species ($P = 0.7783$) and irrigated conditions ($P = 0.2304$; Table 3-3). The incidence of hidden Diplodia in infection point experiments was not affected by pathogen species any year (Table 3-3).

3.6 Discussion

This research provides multi-year, multi-environmental results describing how inoculation timing and infection point of both *Stenocarpella* species influence Diplodia ear rot severity in corn. In our studies, DER was more severe in treatments with earlier inoculation timings, which is similar to previous experiments where inoculation around R1 resulted in greater DER than inoculation timings after silking (Bensch 1995a; Bensch et al. 1992; Chambers 1988). While previous experiments focused on *S. maydis* inoculation, the experiments presented here included both causal agents of DER, providing additional insight into the impact of inoculation timing of *S. macrospora* on DER severity. To our knowledge, this is the first study examining the effects of inoculation timing and infection point on *S. macrospora*.

Our experiments also attempted to better understand the phenomenon known as hidden Diplodia, which has been reported, but it is not well understood what conditions lead to this specific disease expression. In our trials, incidence of hidden Diplodia was greater in treatments that received inoculations at R1 in 2021 (Table 3-1), and through whorl inoculations at V8 (Figure 3-6b). Past reports

indicate that hidden Diplodia likely occurs due to later infection timings (Munkvold and White 2016). In our trials, a significant increase in hidden Diplodia was associated with earlier inoculation timings. However, in this study, overall DER severity was also greater at the earlier inoculation timings, suggesting that inoculation timing may not be the only factor affecting the occurrence of hidden Diplodia. In the 2019 inoculation timing trials, treatments inoculated with *S. macrospora* had a greater incidence of hidden Diplodia than treatments inoculated with *S. maydis* (Table 3-1). This could indicate that *S. macrospora* is more prone to cause hidden Diplodia compared to *S. maydis*. However, we used a limited number of isolates in this experiment, and these results were not observed in each year of the experiment, so more research would be needed to determine if *Stenocarpella* species influences the occurrence of hidden Diplodia.

Despite not being able to definitively prove the cause of hidden Diplodia, we did learn more information about conditions favoring infection by different *Stenocarpella* species. In infection point trials, both pathogens caused similar amounts of disease (Table 3-3). However, in inoculation timing experiments, *S. macrospora* resulted in higher levels of DER in 2020, but the reverse was observed in 2021 when *S. maydis* caused higher levels of DER (Table 3-2). It has been reported that *S. macrospora* is more aggressive of the two pathogens on younger tissue (vegetative stages;(Latterell and Rossi 1983), but there is limited information comparing aggressiveness of species on older plants. The growth of *S. maydis* in culture is more prolific than *S. macrospora* (Morant et al. 1993) and *S. maydis* appears to grow at warmer temperatures than *S. macrospora* (Stevens 1936). This would imply that in warm years *S. maydis* would more effectively colonize ears compared to *S. macrospora*. In our experiments, average temperatures

during reproductive growth stages of corn in June and July were slightly greater in 2020 compared to 2019 and 2021 (Table 3-4), however treatments with *S. macrospora* resulted in greater DER in 2020 when compared to *S. maydis*. However, temperatures were not taken at ear height in our experiments, and it is possible the conditions inside the corn canopy favored one pathogen over the other in 2020 and 2021. It is also important to note that in tropical climates *S. macrospora* is a more prevalent pathogen (Renfro and Ullstrup 1976), indicating that the disease is favored by warm temperatures.

Temperature is not the only important environmental factor in disease development. Moisture, especially in the corn ear, can affect pathogen growth (Koehler 1938). Between June and August, both 2020 and 2021 had similar overall amounts of precipitation; however, 2020 experienced more rain events. The additional rain events, in addition to our irrigation program may have been more favorable for *S. macrospora* growth.

Silk channel inoculation at R1 was included in preliminary experiments but removed from final experimental analysis as the resulting DER severity was near 100% and harvestable grain was near zero. Previous research has shown that earlier inoculations can cause near complete kernel loss, however this study used a colonized toothpick method for inoculation (Chambers 1988). In studies with ear injection inoculations, incidence was greatest with injection methods, however complete ear losses were not reported (Klapproth and Hawk 1991). In our study applying liquid inoculum as a foliar treatment was not effective in inducing symptoms. This was surprising, as it has been effective in previous research and

indicates this may more closely resemble natural infection (Klapproth and Hawk 1991; Ullstrup 1949). While care was taken to ensure proper conditions for infection, such as inoculating late in the day, and in the case of irrigated trials, applying water the following day, disease severity was not increased by spray inoculation compared to non-inoculated controls (Figure 3-5a; 3-5b; 3-5c).

As only one hybrid was used in testing, albeit one that was moderately susceptible to DER, experiments testing inoculation timing on hybrids with differing levels of susceptibility may help determine the role of hybrid resistance on expression or occurrence of hidden Diplodia. These experiments were performed to elucidate if the cause of hidden Diplodia was in part due to inoculation timing or infection point. Conclusive evidence to determine the cause of hidden Diplodia was not evident based on our results. However, these trials provide some insight into the potential for *S. macrospora* to cause more hidden Diplodia under certain conditions and theorize that the occurrence of these symptoms may be more complex than infection timing alone and involve other factors including weather and hybrid resistance.

Inoculation timing and infection point of *S. macrospora* and *S. maydis* also impacted grain yield and test weight. Generally, the earlier inoculations, which resulted in higher DER severity also resulted in less harvestable grain (Figure 3-3c; 3-3d) and lower test weight (Figure 3-4c; 3-4d). This was expected as previous research indicates that severe ear rot infections cause can large yield losses, not only for DER (Chambers 1988), but also other ear rot diseases such as Fusarium ear rot (Presello et al. 2008). While yield losses are important, the impact of DER on test weight of harvested corn is also important. In the U.S., corn with a test weight below 72 kg/hL may be subject to

dockage or a reduced price when selling the grain (USDA 2020). Curtailing losses of yield and combating low test weights in corn are an important consideration in any ear rot management strategy.

These experiments provide a more recent example of the potential for *S. maydis* and *S. macrospora* to cause DER and subsequent yield and test weight losses in corn in the U.S., and that the timing of infection plays an important role in subsequent yield loss and test weight reduction. While in-season management strategies may be limited, information about diseases causing significant yield losses is still vital to corn production, as these pathogens not only possess the ability to reduce yield, but also reduce grain quality through test weight reduction and post-harvest storage issues.

Figure 3-1 Symptoms of discolored kernels of hidden Diplodia (A) on corn ear in Kentucky, and signs of *Stenocarpella* spp. pycnidia visible in pith of the cob (B).

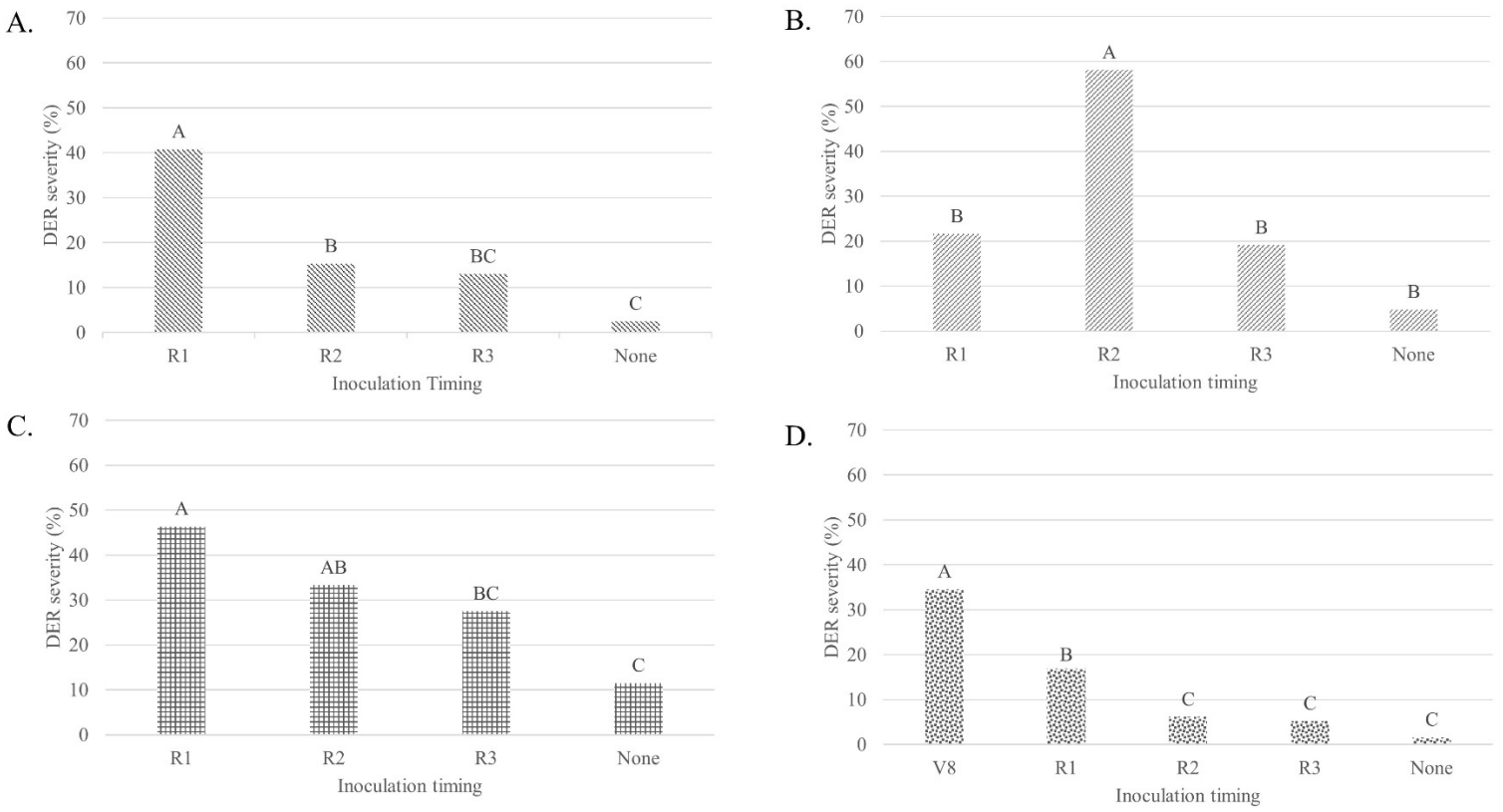

Figure 3-2 Effect of inoculation timing with *Stenocarpella maydis* and *Stenocarpella macrospora* on Diplodia ear rot (DER) severity (0-100%) in field experiments conducted in KY under irrigated and dryland conditions in 2019 (A), dryland conditions in 2020 (B), irrigated conditions in 2020 (C), and irrigated conditions in 2021 (D). Values with different letters are significantly different (P = 0.05).

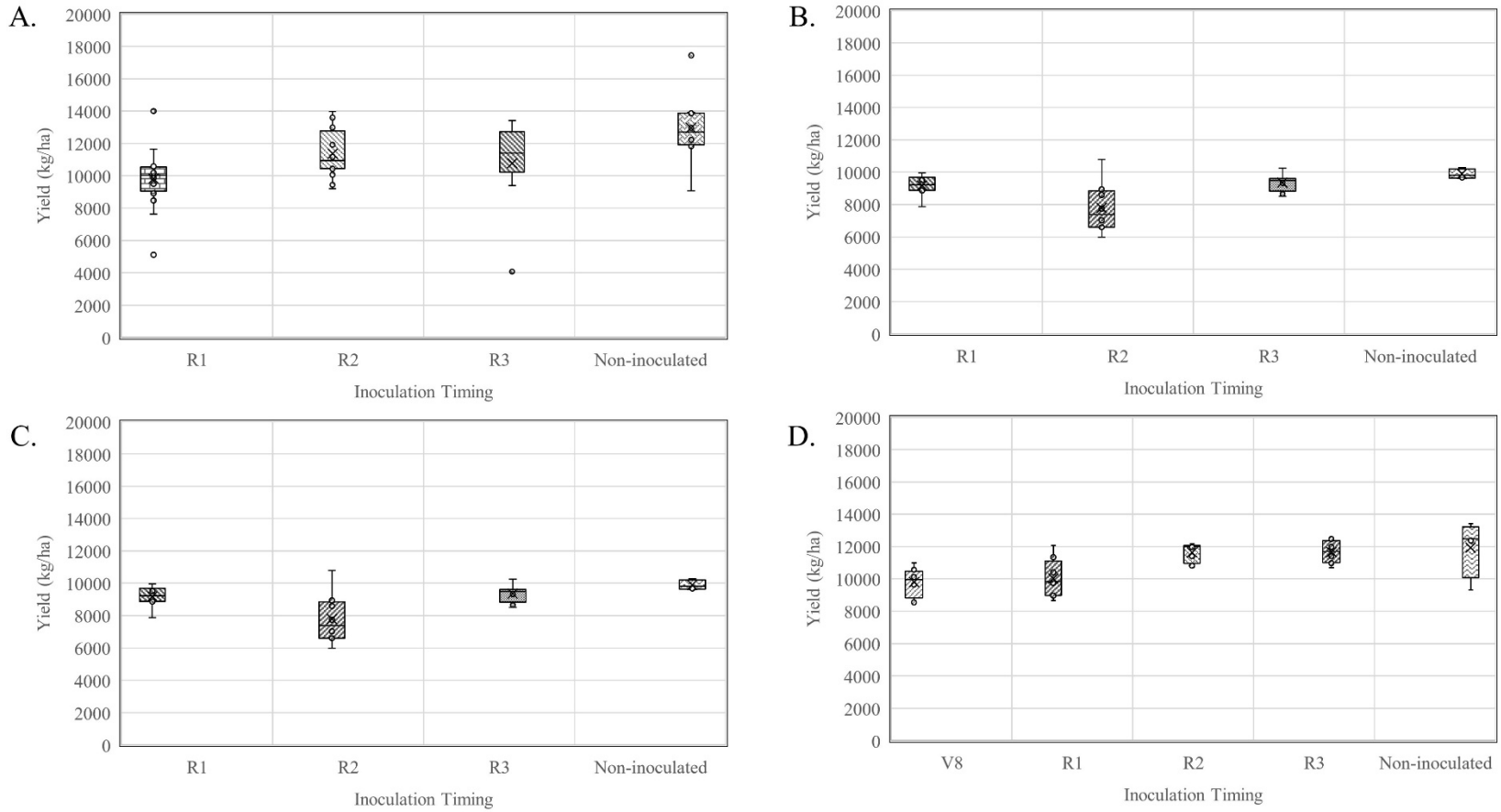

Figure 3-3 Effect of inoculation timing with *Stenocarpella maydis* and *Stenocarpella macrospora* on corn yield in kilograms per hectare (kg/ha) in field experiments in KY under dryland and irrigated conditions in 2019 (A), dryland conditions in 2020 (B), irrigated conditions in 2020 (C), and irrigated conditions in 2021 (D).

Table 3-1 Effect of inoculation timing with *Stenocarpella maydis* and *Stenocarpella macrospora* on hidden Diplodia incidence expressed as percentage of ears with symptoms (0-100%) in 2019 and 2020 combined, and 2021. Values with different letters are significantly different ($P = 0.05$).

	Hidden Diplodia incidence (%)	
Inoculation timing	2019 & 2020	2021
V8[+]	n/a[++]	20.0 AB
R1[+]	6.9	30.0 A
R2[+]	6.3	7.5 B
R3[+]	11.3	7.5 B
None	8.8	5.0 B
P - value	0.1942	0.0094

+ Inoculations were performed at different corn growth stages including eight leaf collar (V8), silking (R1), blister (R2) and milk (R3;(Abendroth et al. 2011).

++ Inoculations at V8 were not performed in 2019 or 2020.

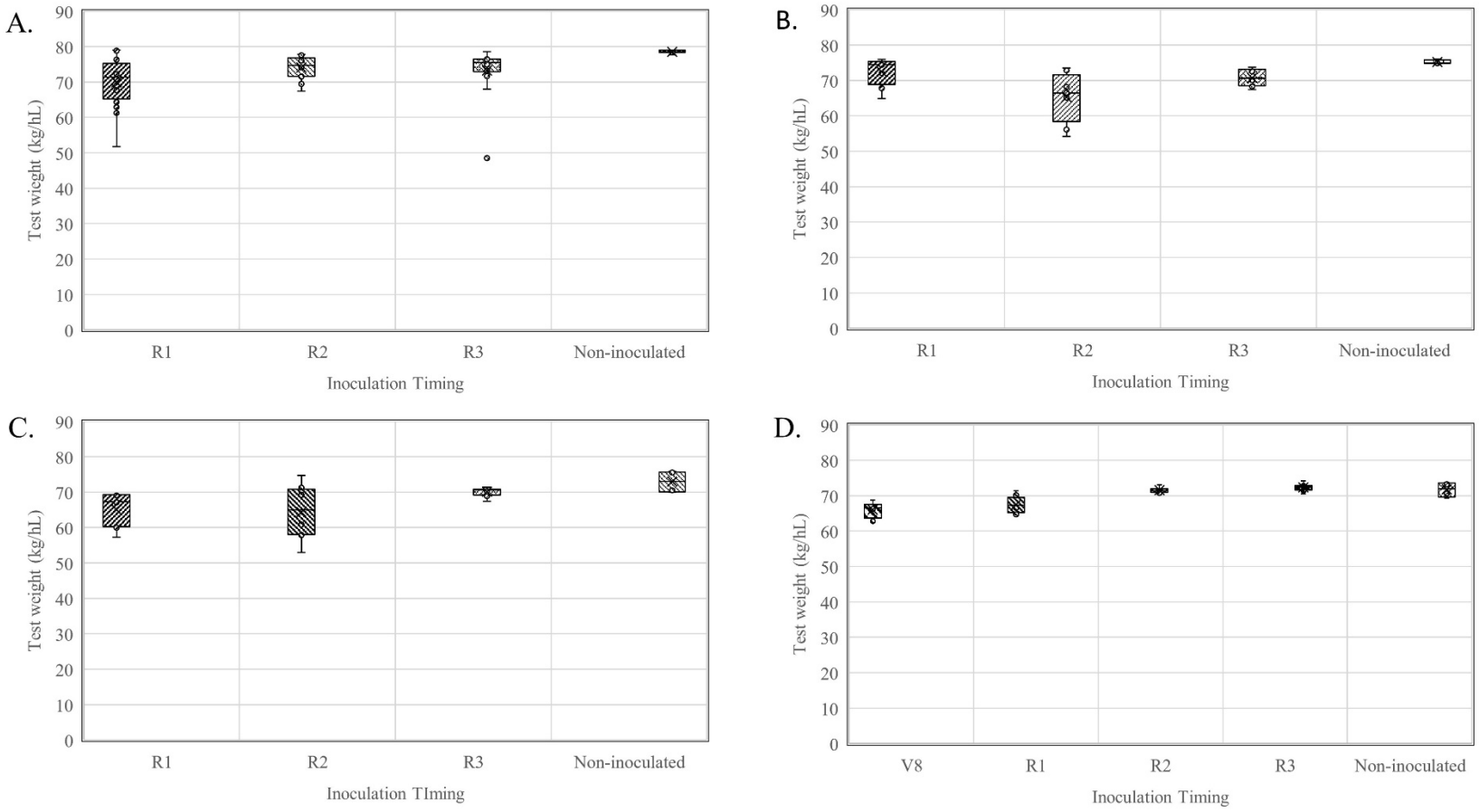

Figure 3-4 Effect of inoculation timing with *Stenocarpella maydis* and *Stenocarpella macrospora* on corn test weight in kilogram per hectoliter (kg/hL) in field experiments in Princeton KY under dryland and irrigated conditions in 2019 (A), dryland conditions in 2020 (B), irrigated conditions in 2020 (C), and irrigated conditions in 2021 (D).

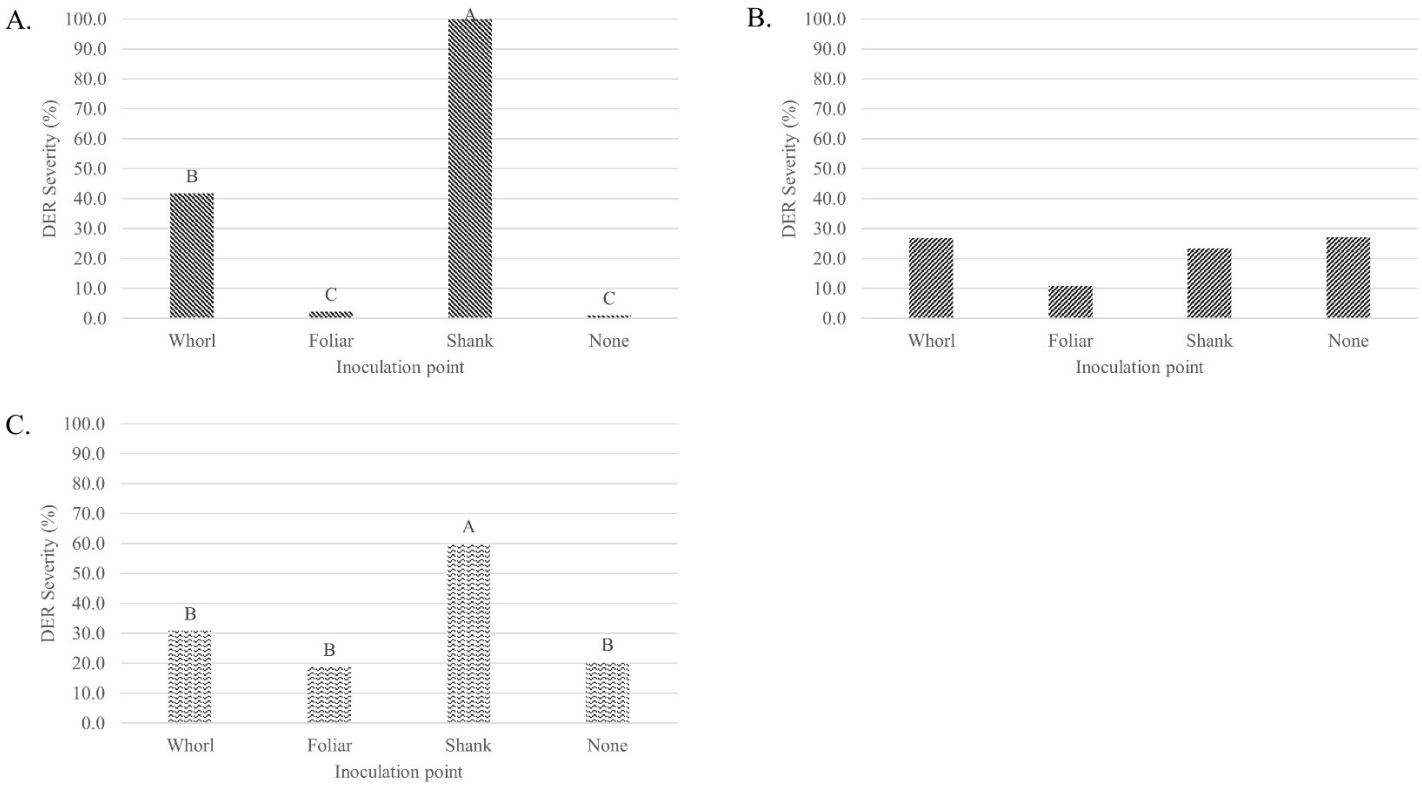

Figure 3-5 Effect of corn infection point with *Stenocarpella maydis* and *Stenocarpella macrospora* on DER severity (0-100%) in KY in 2019 under irrigated and dryland conditions (A), in 2020 under dryland conditions (B), and in 2020 under irrigated conditions (C). Values with different letters are significantly different ($P = 0.05$).

Table 3-2 Effect of corn ear shank inoculations at eight leaf-collar, silking, blister, and milk growth stages with *Stenocarpella maydis* or *Stenocarpella macrospora* on the severity of Diplodia ear rot (DER; 0-100%) and the incidence of hidden Diplodia (0-100%) in Kentucky in 2019, 2020, and 2021. Values with different letters are significantly different ($P = 0.05$).

	DER severity (%)			Hidden Diplodia Incidence (%)		
Species	2019	2020	2021	2019	2020	2021
S. maydis	22.5 A	25.5 B	20.4 A	2.5 B	5.8	16.3
S. macrospora	11.9 A	43.2 A	11.0 B	17.5 A	6.7	16.3
Non-inoculated	2.5 B	8.1 C	1.6 B	0.0 B	17.5	5
P - value	<0.0001	<0.0001	0.0069	<0.0001	0.8082	0.4177

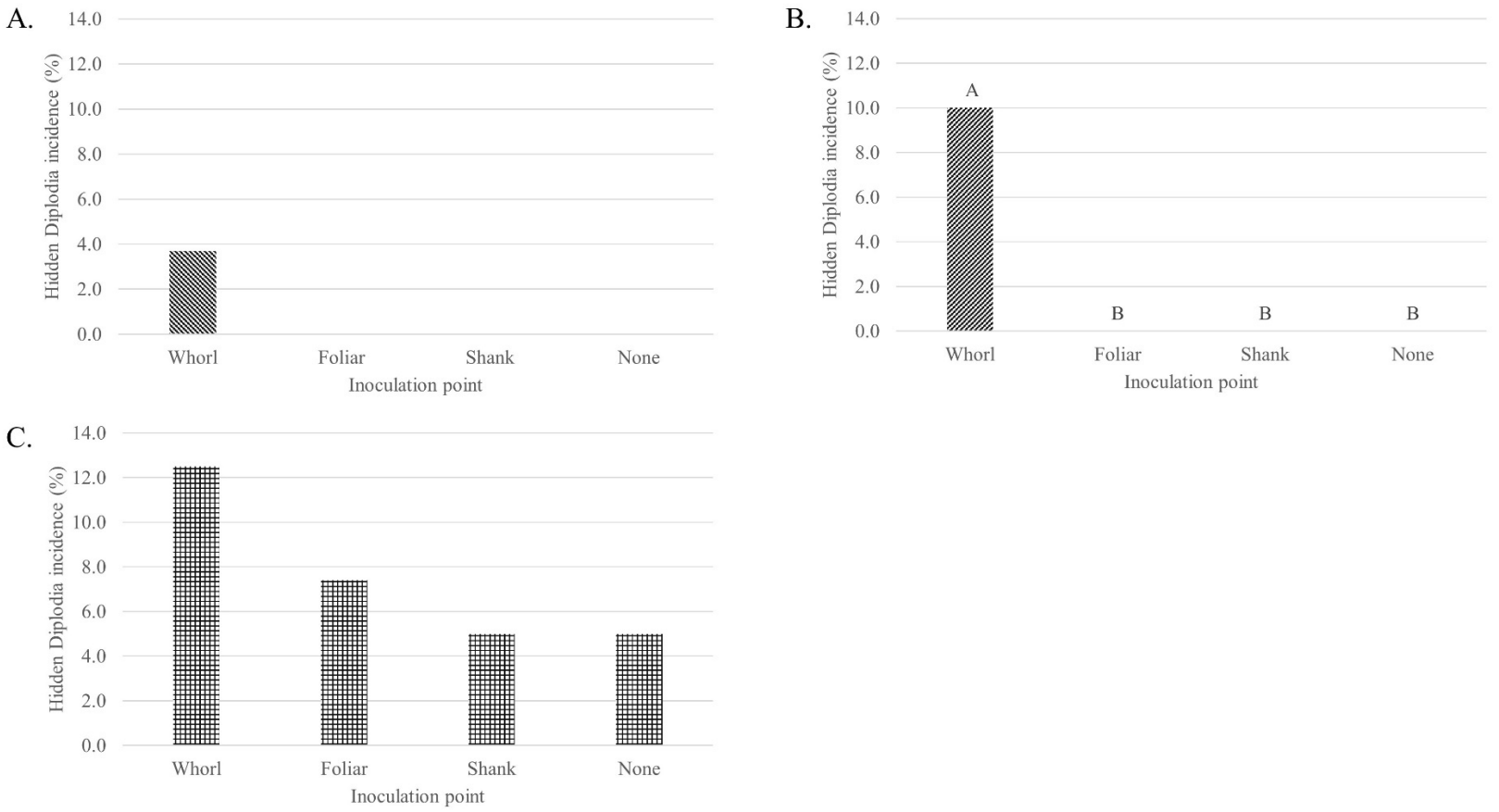

Figure 3-6 Effect of corn infection point with *Stenocarpella maydis* and *Stenocarpella macrospora* on incidence of hidden Diplodia (0-100%) in KY in 2019 under irrigated and dryland conditions (A), in 2020 under dryland conditions (B), and in 2020 under irrigated conditions (C). Values with different letters are significantly different ($P = 0.05$).

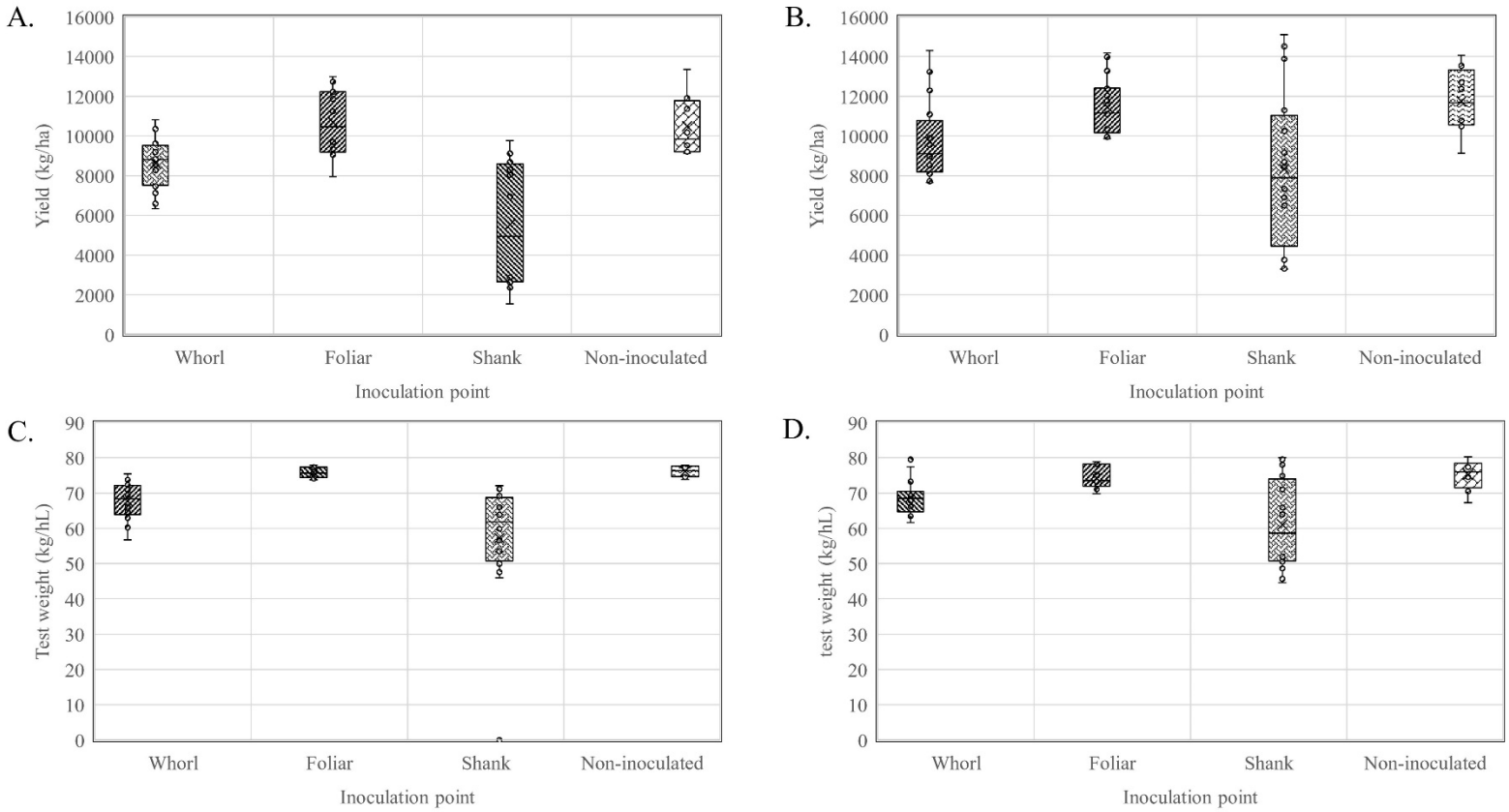

Figure 3-7 Effect of corn infection point with *Stenocarpella maydis* and *Stenocarpella macrospora* on corn yield in kilograms per hectare (kg/ha) in KY during the 2019 and 2020 growing season under dryland (A) and irrigated (B) conditions and the effect on test weight of corn in kilograms per hectoliter (kg/hL) under dryland conditions (C) and irrigated conditions (D).

Table 3-3 Effect of corn whorl, ear shank, and silk spray inoculations with *Stenocarpella maydis* and *Stenocarpella macrospora* combined for analysis on Diplodia ear rot (DER) severity (0-100%) and the incidence of hidden Diplodia in Kentucky under dryland and irrigated conditions in 2019 and 2020. Values with different letters are significantly different ($P = 0.05$).

	DER severity (%)			Hidden Diplodia Incidence (%)		
Species	2019	2020 dryland	2020 irrigated	2019	2020 dryland	2020 irrigated
S. maydis	44.9 A	28.0	34.4	1.8	8.3	11.7
S. macrospora	45.5 A	28.7	38.5	1.7	3.3	5.0
Non-inoculated	1.9 B	23.8	20.5	0.0	2.5	5.0
P - value	<0.0001	0.7783	0.2304	0.6246	0.1517	0.3445

Table 3-4 Summary of average high, average mean, average low temperatures in degrees Celsius (° C) for the months of June, July and August during the 2019, 2020, 2021 growing season as recorded in Princeton, KY. Total number of days above 32° C, days with more than 0.25 centimeter (cm) precipitation and total precipitation (cm), during the months of June, July and August during the 2019, 2020, 2021 growing season as recorded in Princeton, KY.

	June 2019	July 2019	August 2019	June 2020	July 2020	August 2020	June 2021	July 2021	August 2021
Average high (° C)	28.3	30.2	30.0	29.2	31.1	28.8	28.8	29.7	30.0
Average mean (° C)	22.9	25.2	24.3	23.3	26.1	23.9	23.8	24.8	25.0
Average low (° C)	17.6	20.1	18.7	17.4	17.3	19.1	18.8	19.9	20.0
Days above 32° C	2	4	7	1	8	1	2	3	6
Days above 0.25 cm rain	8	10	5	8	6	10	5	7	6
Total precipitation (cm)	10.9	7.9	16.0	13.0	17.5	9.7	17.5	17.8	7.9

CHAPTER 4. EFFECT OF IN-CANOPY FOLIAR FUNGICDE APPLICATION METHODS ON DIPLODIA EAR ROT OF CORN

4.1 Abstract

Foliar fungicides are available to suppress Diplodia ear rot (DER), caused by *Stenocarpella maydis* (Berk.) Sutton and *Stenocarpella macrospora* (Earle) Sutton, in corn, but previous research has indicated limited efficacy against the disease. Experiments were conducted in 2020 under dryland conditions and in 2021 under irrigated conditions to examine the effect of ground-driven fungicide nozzle application technology on DER severity, corn yield, and spray coverage on the ear leaf and ear of corn plants. Application methods included over-canopy nozzles, over-canopy + drop nozzles, and over-canopy + 360 Undercover® nozzles. Within each application method, treatments consisted of a non-inoculated control, or were inoculated with a conidial suspension of *S. maydis.* The fungicides benzovindiflupyr + azoxystrobin + propiconazole and pydiflumetofen + azoxystrobin + propiconazole were applied with each method to measure efficacy against DER. Fungicide spray coverage on corn leaves and ears was assessed for each application method. In both years, neither fungicide application nor application method reduced DER severity. However, disease severity in the non-treated control was low, in 2021 overall DER severity was 0.5% based on a 0-100% scale. No fungicide applications increased yield compared to the non-treated control. The use of over-canopy + 360 Undercover® nozzles increased spray coverage on the ear leaf ($P = 0.0139$) and the ear ($P < 0.0001$) compared to both over-canopy and over-canopy + drop nozzle types.

4.2 Introduction

Corn (*Zea mays* L.) production in the U.S. in 2022 was approximately 36 million hectares (USDA 2022a), making it one of the most cultivated crops in the U.S. Fungicide use in corn has been increasing from approximately 9% of corn hectares sprayed with foliar fungicide in 2010 to 19% in 2021 (USDA 2022c). Fungicides are commonly used to manage diseases causing yield and grain quality losses, including foliar diseases such as gray leaf spot (*Cercospora zeae-maydis* Tehon & E.Y. Daniels) and southern rust (*Puccinia polysora* Underw.). Fungicides are also available for ear rot management, including Fusarium ear rot (*Fusarium verticilliodes* Sacc.), Gibberella ear rot (*Fusarium graminearum* Schwabe) and Diplodia ear rot (*Stenocarpella maydis* Berk. Sutton or *Stenocarpella macrospora* Earle Sutton). Between 2016 and 2019 Diplodia ear rot (DER) accounted for nearly 7 million metric tons of estimated yield losses in the U.S. and Ontario, Canada (Mueller et al. 2020).

While foliar fungicide applications on corn occur throughout most of the growing season, the most common application timing is tasseling (VT) to silking (R1; (Abendroth et al. 2011; Wise et al. 2019), which often happen simultaneously. A fungicide application at VT/R1 has been shown to be the most economically effective application timing in corn production, when compared to earlier vegetative stage applications (Bradley et al. 2020; Wise et al. 2019). Even though fungicide applications are more profitable during the late vegetative to early reproductive stages, applications during early vegetative stages (V4-V10) are still marketed to farmers not only for disease control, but for physiological benefits (Wise and Mueller 2012). Foliar fungicide

application on tasseling or post-tassel corn primarily occurs through aerial methods (Mueller et al. 2021a; Smith and Thomson 2003), which can increase costs compared to applications using ground-driven equipment. However, with advancements in ground-driven high-clearance sprayer technology, more applications are occurring with this type of spray equipment.

Ground-driven spray equipment gives farmers more flexibility in application timing and product selection. This equipment also allows for the use of newer fungicide application methods such as drop nozzles and in-canopy nozzles that have been developed and promoted to increase spray coverage within the corn canopy. One such example of in-canopy nozzles is the 360 Undercover® (360 Yield, Morton, IL) nozzles which are a nozzle body that has four different nozzle positions, one aimed horizontally backwards, one horizontally aimed 45° upwards and backwards, and one each aimed vertically pointing outward in each direction (Figure 2). Hagen et al. 2017 showed that the use of drop nozzles that spray into the cotton canopy had the potential to reduce target spot of cotton (*Corynespora cassicola* (Berk. & M. A. Curtis) C. T. Wei;(Hagan et al. 2017). Research has also shown that 360 Undercover® nozzles can increase spray coverage in corn within the canopy up to five times over traditional over-canopy only methods (Penney et al. 2021).

For most fungicides used in field crops, movement within plants is either translaminar or acropetal, and not truly systemic (Mueller et al. 2021a), highlighting the need for increased spray coverage to improve the chances of fungicide efficacy. Research has shown that fungicide applications occurring above the corn canopy have decreased spray coverage lower in the canopy (Menechini et al. 2017; Penney et al.

2021; Wolf and Bretthauer 2008). This is important because the target site for fungicides used for ear rot management would be the ear or ear shank, which are in the middle of the canopy. Fungicides need to land on or very near the target site to be effective, (Balardin et al. 2010), meaning that traditional over-canopy fungicide applications may not provide the spray coverage needed at the target site specific for ear rot management.

In the U.S., few fungicides have ear rots as target diseases on their labels. These products include Trivapro (benzovindiflupyr, Fungicide Resistance Action Committee (FRAC) Group 7, succinate dehydrogenase inhibitor (SDHI) + azoxystrobin, FRAC Group 11, quinone outside inhibitor (QoI) + propiconazole, FRAC Group 3, demethylation inhibitor (DMI; Syngenta, Greensboro, NC), Quilt Xcel (azoxystrobin + propiconazole; Syngenta) and Miravis Neo (pydiflumetofen; FRAC Group 7 (SHDI) + azoxystrobin + propiconazole; Syngenta) for suppression of Diplodia ear rot and Proline (prothioconazole, FRAC Group 3, DMI; Bayer, Research Triangle Park, NC) for the control of Fusarium ear rot (*Fusarium verticilliodes* (Sacc.) Nierenberg), Gibberella ear rot (*Fusarium graminearum* Schwabe) and Aspergillus ear rot (*Aspergillus flavus* Link). Field level control of DER has been achieved using benomyl (FRAC Group 1, methyl benzimidazole carbamate (MBC), and mancozeb (FRAC Group M, multi-site;(Marley and Gbenga 2004; Warren and von Qualen 1986), however there has been limited success in the U.S. using labelled products to suppress DER (Romero Luna and Wise 2015). Although Romero Luna and Wise 2015 reported that field control of DER using Quilt Xcel was not observed, but their *in vitro* studies showed that the active ingredients azoxystrobin and propiconazole did reduce fungal growth compared to a no fungicide control. The discrepancy in efficacy between *in vivo* and *in vitro* experiments raises

important questions about the efficacy of field applications. It has been suggested in previous studies evaulating fungicide efficacy for ear rot control that due to the limited systemic movement of fungicide in the plants, application methods may be a limiting factor in successful control of ear rots (Anderson et al. 2017; Romero Luna and Wise 2015).

The objectives of this research are to 1) determine if using in-canopy fungicide reduces DER severity compared to over-canopy applications, and 2) determine if in-canopy applications increase fungicide coverage on the ear and ear leaf compared to over-canopy applications.

4.3 Materials and methods

4.3.1 Field trial establishment

Field trials were conducted in 2020 and 2021 at the University of Kentucky Research and Education Center in Princeton, KY. The trial was conducted under dryland conditions in 2020 and under irrigation in 2021. Trials were planted on May 11, 2020 and May 12, 2021 with corn hybrid P1555 CHR (Pioneer, Johnston, IA) at a population of 79,073 seeds per hectare. Corn hybrid P1555 CHR was rated a five on a one to nine scale of resistance to DER, with one being poor and nine being excellent. Trials were arranged in a randomized complete block design with plots consisting of 4 rows on 76 cm spacing with a length of 9.1 m. Each treatment was replicated four times. In 2021, lateral overhead irrigation was applied to ensure a total of at least 2.5 cm of water occurred within the trial every week. The total was composed of naturally occurring rain and irrigation. Local agronomic practices were followed in trials in both years including

no tillage, fertilizer application of 36.7 kg of nitrogen per hectare, 11.1 kg of phosphorous per hectare, and 9.3 kg potassium per hectare. Weed management consisted of a spring burndown on April 7, 2020, and March 23, 2021, using glyphosate at 189 g per hectare and ammonium sulfate added at 380 g per hectare. Pre-emergence herbicide applications occurred on May 1, 2020, and May 11, 2021, using glyphosate at 189 g per hectare, 138 g of atrazine per hectare, 8 g bicyclopyrone per hectare, 33 g mesotrione per hectare, 295 g S-metolachlor per hectare, and ammonium sulfate added at 380 g per hectare. Postemergence applications of glyphosate at 189 g per hectare and ammonium sulfate added at 380 g per hectare were applied each year between the 3 and 4 leaf collar growth stages (V3-V4;(Abendroth et al. 2011).

4.3.2 Fungicide application treatments

Experimental treatments in both years consisted of four fungicide application methods; non-treated, over-canopy foliar nozzles (XR8002, TeeJet, Glendale Heights, IL), over-canopy foliar nozzles (XR8001, Teejet) + drop nozzles (61 cm long hose drops with XR80015 tips, TeeJet), and over-canopy (XR80015) + 360 Undercover® nozzles (XR8001). Three fungicide treatments were applied at labeled rates within each application method: non-treated, pydiflumetofen 115.6 g/ha + azoxystrobin 152.4 g/ha + propiconazole 190.9 g/ha (Miravis Neo), and benzovindiflupyr 45.9 g/ha + azoxystrobin 168.9 g/ha + propiconazole 190.9 g/ha (Trivapro). Fungicide applications were applied using a LeeAgra Avenger high clearance ground-driven sprayer (LeeAgra, Lubbock, TX). Nozzle spacing for over-canopy applications was 45.7 cm, while in-canopy applications of drop nozzle and 360 Undercover® nozzles occurred between rows (76

cm). For the 360 Undercover® nozzle bodies, based on manufacturer's recommendations, three of the four nozzles were used in this experiment; two of which are directed outward from the middle of the row pointing in each direction and a third pointed back and slightly upwards (Figure 4-1). The ground clearance of the over-canopy boom was 2.75 m; drop nozzles were attached directly to the boom, and 360 Undercover® nozzles were adjusted so that nozzle spray was directed at ear height. Carrier volume for all fungicide applications was 187.1 L/ha. Treatments that included both over-canopy and in-canopy applications were calibrated so the over-canopy and in-canopy sections sprayed equal amounts of liquid.

4.3.3 Spray coverage measurements

Spray coverage of each application method was assessed by adding Tracer hot pink foam colorant (Precision Laboratories. Waukegan, IL) to the fungicide spray mixture at a rate of 0.2% v/v. Kromekote paper (CTI Paper, Sun Prairie, WI) was cut into 7.6 cm x 43 cm strips and attached using paper clips on the ear leaf of three plants per plot in all four replications of each treatment. Kromekote paper was also cut into 7.6 cm x 15.2 cm strips and attached using paper clips around three ears per plot in all four replications of each treatment receiving dye. As ears are newly emerging around R1, spray cards covered approximately 80% or more of each ear. Dry cards were collected one hour after application.

4.3.4 Inoculation treatments and inoculum preparation

Two inoculation treatments occurred within all application methods: non-inoculated, and foliar application of 1.8 million conidia of *S. maydis* per plot. Inoculum was generated by plating two pure cultures of *S. maydis* derived from single-spore isolates collected from Kentucky in 2018 onto plates of oatmeal agar (BD, Franklin Lakes, NJ). After incubating for 14 days at 25° C on a 12 hr light/day cycle, pycnidia were visible on the plates. Each plate was flooded with 10 mL of sterile water, allowed to sit for 5 minutes, and then gently scraped using a glass rod. The conidia were collected into a 250 mL beaker, keeping each isolate separate. The resulting conidial suspensions were quantified using a hemocytometer. A mixture with a final concentration of 6,000 conidia per mL was made from the two isolates to ensure a ratio of 1:1. The adjusted spore concentration was applied to designated plots 24 hours after fungicide application using a CO_2 backpack sprayer (R&D Sprayers, Opelousas, LA) with a split nozzle pointed towards each row from the center of the plot. The boom was held at ear height.

4.3.5 Data collection and statistical analysis

Disease assessments were performed at growth stage R5 (dent) by peeling the husks from five arbitrarily selected ears in each plot and scoring DER severity on a percentage scale with 0% being no visible signs or symptoms and 100% being the entire ear colonized. All plots were harvested using a plot combine (Wintersteiger, Reid im Innkreis, Austria) and plot yield, moisture and test weight were collected. Kromekote spray cards were scanned into digital images and spray coverage was assessed using ImageJ (NIH, Bethesda. MD). A selection of spray cards was chosen to create parameters

within ImageJ that was the used for all cards to assess spray coverage as percent of card covered (0-100%).

Pairwise comparisons (PROC TTEST) using SAS v9.4 (SAS Institute, Cary, NC) were performed to determine if differences in DER severity and yield were affected by year of experiment. Analysis of variance (PROC GLIMMIX) in SAS v9.4 was performed to determine if application method, or fungicide product affected DER severity or yield. Analysis of variance (PROC GLIMMIX) in SAS v9.4 was also performed to determine if application method affected spray coverage on the ear and ear leaf.

4.4 Effect of in-canopy fungicide applications on DER severity and corn yield

Significant differences in DER were observed between 2020 and 2021 (P = 0.0002), therefore, each year was analyzed separately. Inoculation with *S. maydis* did not significantly increase DER severity (P = 0.1552), so inoculation was not included as a factor in analysis. In 2020, DER severity on individual ears ranged from 0% to 30%, and in 2021 DER severity on individual ears ranged from 0% to 80%. In both 2020 and 2021, neither application method, nor fungicide product had an effect on DER severity (Table 4-1; 4-2).

Significant differences in yield were observed between 2020 and 2021 (P < 0.0001), therefore, each year was analyzed separately due to different irrigation conditions. Inoculation with *S. maydis* did not significantly affect yield (P = 0.1075), therefore, inoculation type was not included as a factor in analysis. Yield on a per plot basis in 2020 ranged from 8,469 kg/ha to 12,369 kg/ha and from 10,503 kg/ha to 15,503

kg/ha in 2021. In both years, neither application method nor fungicide product had a significant effect on yield (Table 4-1; 4-2).

4.5 Effect of in-canopy fungicide applications on spray coverage on the ear and ear leaf

Spray coverage data from both years were combined into a single analysis. Spray coverage on the ear leaf ranged from 1.0 to 49.3% for over-canopy treatments, from 0 to 48.6% for over-canopy + drop nozzle treatments, and from 13.0 to 58.4% for over-canopy + 360 Undercover® treatments. Spray coverage on the ear ranged from 0 to 9.3 % for over-canopy treatments, from 0.9 to 68.3 % for over-canopy + drop nozzle treatments, and from 3.8 to 42.1 % for over-canopy + 360 Undercover® treatments. The over-canopy + 360 Undercover® nozzles increased spray coverage on the ear leaf compared to over-canopy and over-canopy + drop nozzle methods ($P = 0.0141$; Figure 4-2a). Likewise, the greatest amount of spray deposition on ears was observed with over-canopy + 360 Undercover® nozzles compared to over-canopy + drop nozzle, both of which had improved spray coverage compared to over-canopy applications ($P < 0.0001$; Figure 4-2b).

4.6 Discussion

Fungicide coverage is an important component of efficacy because mobility of fungicides used on field crops is limited to acropetal, or upwards and outwards from the point of contact, and translaminar, or from side of a leaf to another (Mueller et al. 2021a). Increased spray coverage increases the likelihood of a successful fungicide application. Previous research on the use of 360 Undercover® nozzles has shown that this

method can increase spray coverage on lower leaves compared to traditional over-canopy nozzles and aerial methods (Penney et al. 2021). Our aim was to focus on ear rot diseases, specifically DER, given the different spatial orientation of leaves (horizontal) to ears (vertical) and examine how differences in spray coverage on the two plant parts might account for the discrepancy in efficacy seen between *in vivo* and *in vitro* studies on corn ear rot pathogens. In our study, spray coverage on the ear leaf was increased by 44%, and by 550% on the ear, when over-canopy + 360 Undercover® nozzles were used compared to over-canopy nozzles alone (Figure 4-2a).

It should be noted that the range of spray coverage within a plot or from plant-to-plant was variable. For example, the greatest amount of spray deposition observed on an individual ear was 68.3% from the over-canopy + drop nozzle. However, mean spray deposition for all over-canopy + drop nozzle cards was lower than the mean spray deposition observed for over-canopy + 360 Undercover® nozzles (Figure 4-1). This raises important questions on the uniformity of spray applications on post-tassel corn and the number of plants receiving adequate spray coverage. The uniformity of spray application using in-canopy methods could be a limiting factor in ear rot control.

The utility of in-canopy fungicide application is not unique for corn as 360 Undercover® nozzles have shown to increase spray coverage in soybean (Viggers et al. 2022) and drop nozzles have shown to decrease foliar disease in cotton compared to over-canopy nozzles (Hagan et al. 2017). Our study used Teejet XR-style nozzles to maintain a fine droplet size for all applications, and sprayer speed was limited to less than 6 km/h by equipment capabilities of the high-clearance sprayer available. Fine droplet sizes (40-100 μm) have been shown to provide increased spray coverage over

larger droplet sizes (Bateman et al. 2019) in corn systems using herbicides (Feng et al. 2003) and provide increased spray deposition in other crops such as wheat (Halley et al. 2008). In other cropping systems such as wheat, faster sprayer speeds reduced coverage (Sreš et al. 2015; White et al. 2022) and using dual-fan or turbo nozzles resulted in better deposition on vertically oriented wheat heads (Lehoczki-Krsjak et al. 2015). While the orientation of the 360 Undercover® nozzles (Figure 4-1) should alleviate issues with nozzle selection and orientation, coverage on ears using drop nozzles may be limited by orientation of the nozzle tip.

It should also be noted that this study focused on fungicide coverage on the ear leaf and ear and did not measure spray coverage in the upper canopy. Penny et al. 2021 reported that spray coverage in the upper canopy was reduced when using the 360 Undercover® compared to over-canopy nozzles alone, but, this did not result in differences in control of gray leaf spot in the same study (Penney et al. 2021). Further research on the uniformity of spray deposition with in-canopy fungicide applications could aid with spray optimization and potentially improve disease control. Additionally, investigating reduced coverage in the upper canopy may be important for management of southern rust of corn (*Puccinia polysora* Underw.), an economically important disease in Kentucky. Future work would be needed to assess if the reduction in coverage on the upper canopy from use of 360 Undercover® nozzles has a detrimental effect on control of foliar diseases.

It is difficult to draw conclusions on the effectiveness of fungicide applications in reducing DER in our study due to the low disease pressure observed. In both years, disease pressure was low, with under 2% DER severity observed in non-fungicide-

treated plots. Not only was DER pressure low, but foliar diseases such as gray leaf spot were also observed at low severity in both years on the ear leaves and upper canopy. Low DER severity may obfuscate the true effect that improved spray deposition and application method may have provided on reducing DER. These results are similar to previous observations of fungicide efficacy in other ear rot pathosystems. In experiments where severity of Gibberella ear rot was below 20% in non-treated controls, fungicide applications did not reduce ear rot severity (Anderson et al. 2017; Reed et al. 2021), but in cases where disease severity of Gibberella ear was over 20% in non-treated controls, applications of azoxystrobin + cyproconazole + carbendazim and applications of prothioconazole reduced disease severity compared to non-treated controls (Andriolli et al. 2016).

Despite inoculating treatments to increase DER pressure in the trial, we had limited DER develop in the trials. While previous research has suggested the best way to ensure high disease pressure is by inoculating ears directly (Bensch et al. 1992; Klapproth and Hawk 1991), we felt due to the limited systemic mobility of fungicides and the prophylactic nature of fungicide applications, direct silk channel inoculation could introduce inoculum into the ear, bypassing areas of the ear where fungicide residue would be located. Also, a foliar application of inoculum would more accurately depict a natural infection of *S. maydis* (Klapproth and Hawk 1991; Ullstrup 1949), as opposed to artificial wounding and injection.

In 2021, even irrigation throughout the year to maintain moisture and humidity within the canopy did not increase disease pressure sufficiently to cause disease. It has been suggested that DER severity can increase due to late season (R2 and later) heavy

rains (Romero Luna and Wise 2015; Van Rensburg and Ferreira 1997). While irrigation has been used to increase disease pressure of other ear rots such as Gibberella ear rot (Schaafsma et al. 1997), the amount and timing of our irrigations may not have been adequate or optimized for DER. Irrigation in this study was performed so that every week 2.5 cm of precipitation was applied between the combination of rainfall and irrigation as well as ensuring precipitation whether natural or through irrigation was applied to plots the day following inoculation. In both years, irrigation was used more in August than June and July because of less rainfall (Table 4-3), limiting the number of weeks that precipitation would have exceeded 2.5 cm. The effect of in-canopy moisture and temperature on DER severity is not known, therefore, additional research is needed to optimize conditions for disease development. Under higher disease pressure conditions, we hypothesize that the relationship between fungicide coverage on the ear and the impact on DER severity may have been clearer.

Yield effects were also examined, as DER caused by *S. maydis* has been shown to decrease yield in the U.S. (Mueller et al. 2021b,2022; Mueller et al. 2020; Mueller et al. 2016; Romero Luna and Wise 2015). Previous research has shown that a yield response to fungicide application under low disease pressure conditions can be inconsistent (Mallowa et al. 2015; Wise et al. 2019), so it is not surprising given the low overall disease pressure in our studies that no increase in yield was observed due to fungicide application (Table 4-1).

This experiment demonstrates the utility in using in-canopy fungicide applications to improve fungicide spray coverage on the ear leaf and ear of corn. While positive effects on yield and a reduction in DER severity were not observed, an increase in spray

coverage may increase the likelihood of improved disease control in areas or years with high disease pressure. The fungicide application methods described in this paper, however, may not be economically feasible for many corn farmers. At the time of this publication, the cost of a 360 Undercover® nozzle unit was approximately $500 per nozzle unit, which includes the nozzle body, hanging bracket and hoses. This would mean that to outfit a sprayer with a width of 29.3 m (38 rows of corn on 76 cm spacing), it would cost approximately $20,000. It is important to consider the financial investment when making any management decisions like adding equipment for fungicide applications. Future work is needed to improve overall efficacy of in-canopy fungicide applications in corn and to better understand the economic impacts of employing these methods.

Figure 4-1 Photo of 360 Undercover® nozzle body (360 Yield, Morton, IL), with a four nozzle arrangement, one horizontally aimed backwards (capped), one horizontally aimed upwards and backwards (Teejet XR8001 tip), and two aimed vertically and outward on each side (Teejet XR8001 tip).

Table 4-1 Effect of over-canopy, over-canopy + drop nozzle, and over-canopy + 360 Undercover® fungicide applications in Kentucky corn during the 2020 and 2021 growing seasons on Diplodia ear rot (DER) severity measured in percentage from 0-100%, and yield measured in kilograms per hectare (kg/ha). Data are combined from treatments of non-inoculated and inoculation by spraying silks at silking with a conidial suspension of *Stenocarpella maydis*.

	DER severity (%)		Yield (kg/ha)	
Application method	2020	2021	2020	2021
Non-treated	1.4	2.5	9,864	12,059
Over-canopy	3.5	1.0	9,964	12,481
Over-canopy + drop nozzle	2.8	0.8	10,165	12,971
Over-canopy + 360 Undercover	2.7	0.0	10,273	12,409
P - value	0.5629	0.6472	0.5197	0.1844

Table 4-2 Effect of pydiflumetofen + azoxystrobin + propiconazole (Miravis Neo, Syngenta, Greensboro, NC) and benzovindiflupyr + azoxystrobin + propiconazole (Trivapro, Syngenta) fungicide applications in Kentucky corn during the 2020 and 2021 growing seasons on Diplodia ear rot (DER) severity measured in percentage from 0-100%, and yield measure in kilograms per hectare (kg/ha).

	DER severity (%)		Yield (kg/ha)	
Treatment	2020	2021	2020	2021
Non-treated	1.4	2.5	9,864	12,059
Pydiflumetofen + azoxystrobin + propiconazole	1.9	0.8	10,062	12,577
Benzovindiflupyr + azoxystrobin + propiconazole	3.1	0.4	10,206	12,663
P - value	0.6454	0.7145	0.5236	0.7481

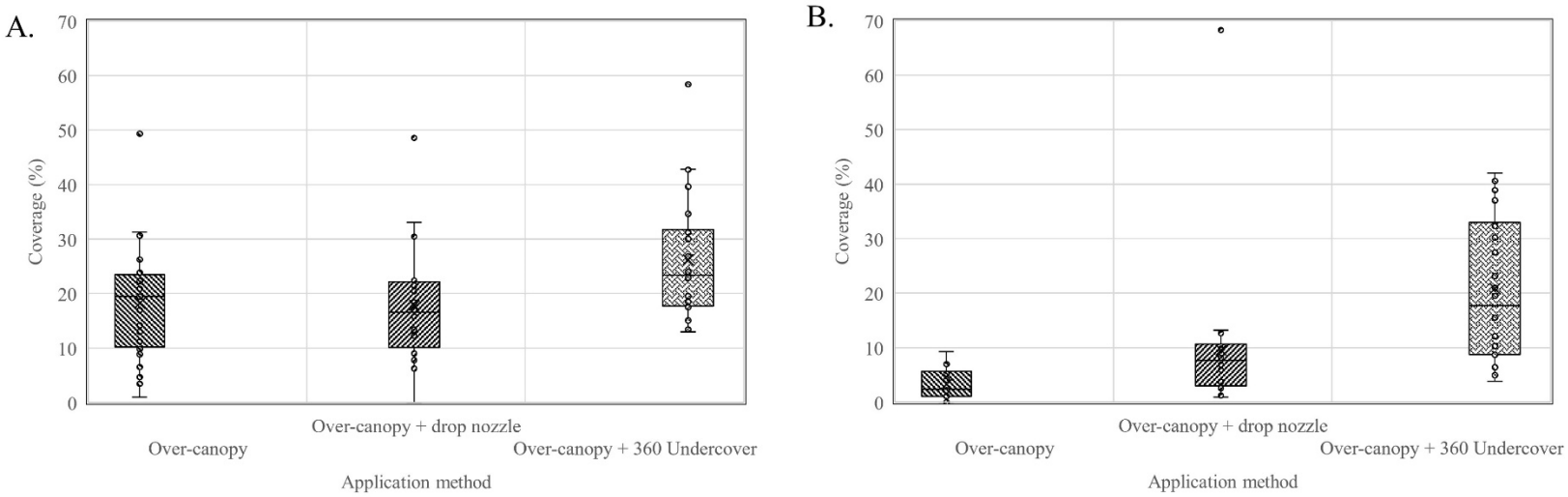

Figure 4-2 Analysis of spray coverage of over-canopy, over-canopy + drop nozzle, and over-canopy + 360 Undercover® nozzle application methods on corn ear leaves (A) and corn ears (B) in Kentucky during the 2020 and 2021 growing seasons.

Table 4-3 Summary of average high, average mean, average low temperatures in degrees Celsius (° C) for the months of June, July and August during the 2020 and 2021 growing seasons as recorded in Princeton, KY along with the total number of days above 32° C, days with more than 0.25 centimeter (cm) precipitation and total precipitation (cm), during the months of June, July and August during the 2019, 2020, 2021 growing seasons as recorded in Princeton, KY.

	Jun-20	Jul-20	Aug-20	Jun-21	Jul-21	Aug-21
Average high (° C)	29.2	31.1	28.8	28.8	29.7	30.0
Average mean (° C)	23.3	26.1	23.9	23.8	24.8	25.0
Average low (° C)	17.4	17.3	19.1	18.8	19.9	20.0
Days above 32° C	1	8	1	2	3	6
Days above 0.25 cm rain	8	6	10	5	7	6
Total precipitation (cm)	13.0	17.5	9.7	17.5	17.8	7.9

APPENDIX 1. KENTUCKY CORN SURVEY LOCATIONS

Year	County	Region	Previous crop	Residue (%)	Latitude	Longitude
2019	Adair	Pennyrile	soybean	2	37.15332	-85.30151
2019	Ballard	Jackson Purchase	wheat	25	37.08209	-89.00416
2019	Ballard	Jackson Purchase	soybean	5	37.08080	-88.90241
2019	Barren	W KY Coalfield	soybean	5	37.09798	-85.97415
2019	Barren	W KY Coalfield	soybean	15	36.98102	-86.03782
2019	Breckenridge	Pennyroyal	soybean	40	37.67642	-86.54804
2019	Butler	W KY Coalfield	soybean	5	37.21860	-86.68082
2019	Caldwell	Pennyrile	corn	30	37.00703	-87.77726
2019	Caldwell	Pennyrile	corn	60	37.02252	-87.81914
2019	Caldwell	Pennyrile	soybean	5	37.09684	-87.87506
2019	Calloway	Jackson Purchase	wheat	60	36.68446	-88.39520
2019	Calloway	Jackson Purchase	soybean	40	36.62002	-88.37913
2019	Calloway	Jackson Purchase	wheat	50	36.60535	-88.44323
2019	Carlisle	Jackson Purchase	soybean	1	36.80571	-88.81421
2019	Carlisle	Jackson Purchase	soybean	35	36.81197	-89.01226
2019	Christian	Pennyrile	soybean	10	36.84276	-87.64257
2019	Christian	Pennyrile	soybean	30	36.72776	-87.37515
2019	Christian	Pennyrile	soybean	40	36.73807	-87.41900
2019	Christian	Pennyrile	wheat	15	36.77127	-87.51579
2019	Christian	Pennyrile	wheat	30	36.74035	-87.57004
2019	Christian	Pennyrile	soybean	10	36.80568	-87.63098
2019	Christian	Pennyrile	soybean	40	36.88151	-87.65131
2019	Crittenden	Pennyrile	soybean	5	37.25616	-88.09363
2019	Daviess	W KY Coalfield	soybean	10	37.72223	-87.13947
2019	Daviess	W KY Coalfield	soybean	20	37.72965	-87.18509
2019	Daviess	W KY Coalfield	soybean	15	37.73782	-87.30076
2019	Daviess	W KY Coalfield	soybean	15	37.72046	-87.32944
2019	Daviess	W KY Coalfield	soybean	25	37.70229	-87.39032
2019	Fulton	Jackson Purchase	soybean	5	36.53979	-89.04570
2019	Fulton	Jackson Purchase	soybean	10	36.55716	-89.13796
2019	Graves	Jackson Purchase	corn	10	36.89646	-88.68750
2019	Graves	Jackson Purchase	soybean	20	36.57182	-88.59231
2019	Graves	Jackson Purchase	soybean	10	36.61975	-88.59348
2019	Graves	Jackson Purchase	wheat	15	36.60582	-88.63651
2019	Graves	Jackson Purchase	wheat	50	36.85559	-88.74652
2019	Graves	Jackson Purchase	corn	45	36.68090	-88.77611
2019	Graves	Jackson Purchase	soybean	10	36.63881	-88.68454
2019	Hardin	Pennyrile	soybean	40	37.74109	-85.80662

2019	Hardin	Pennyrile	soybean	40	37.77530	-86.05703
2019	Henderson	W KY Coalfield	soybean	10	37.67237	-87.57194
2019	Henderson	W KY Coalfield	corn	25	37.71552	-87.58192
2019	Henderson	W KY Coalfield	soybean	25	37.78177	-87.59218
2019	Henderson	W KY Coalfield	corn	30	37.79117	-87.71467
2019	Henderson	W KY Coalfield	soybean	5	37.70788	-87.69750
2019	Henderson	W KY Coalfield	soybean	5	37.78840	-87.48693
2019	Henderson	W KY Coalfield	soybean	10	37.73354	-87.47768
2019	Hickman	Jackson Purchase	corn	30	36.69480	-88.99963
2019	Hickman	Jackson Purchase	soybean	5	36.71843	-88.87146
2019	Hickman	Jackson Purchase	soybean	5	36.62794	-88.87339
2019	Hickman	Jackson Purchase	soybean	20	36.65847	-88.85708
2019	Hopkins	W KY Coalfield	soybean	15	37.35061	-87.54437
2019	Hopkins	W KY Coalfield	soybean	10	37.50903	-87.36992
2019	Livingston	W KY Coalfield	soybean	5	37.19460	-88.24059
2019	Logan	Pennyrile	soybean	5	36.77702	-86.76769
2019	Logan	Pennyrile	soybean	20	36.72283	-86.78206
2019	Logan	Pennyrile	soybean	20	36.69474	-86.92810
2019	Logan	Pennyrile	wheat	25	36.74940	-86.89471
2019	Logan	Pennyrile	soybean	30	36.75296	-86.96465
2019	Logan	Pennyrile	soybean	15	36.77115	-87.00790
2019	Mclean	W KY Coalfield	soybean	5	37.44405	-87.19694
2019	Mclean	W KY Coalfield	soybean	30	37.53482	-87.31565
2019	Mclean	W KY Coalfield	corn	5	37.52009	-87.26506
2019	Meade	Pennyrile	soybean	40	37.89006	-86.14836
2019	Muehlenberg	W KY Coalfield	soybean	5	37.25003	-87.35349
2019	Muehlenberg	W KY Coalfield	soybean	5	37.34890	-87.06321
2019	Ohio	W KY Coalfield	soybean	5	37.43411	-86.86011
2019	Ohio	W KY Coalfield	soybean	30	37.50172	-86.99571
2019	Shelby	Bluegrass	soybean	5	38.20464	-85.25079
2019	Simpson	Pennyrile	soybean	30	36.68641	-86.61930
2019	Simpson	Pennyrile	soybean	5	36.68674	-86.48412
2019	Simpson	Pennyrile	soybean	5	36.73606	-86.64297
2019	Taylor	Pennyrile	soybean	10	37.25240	-85.36048
2019	Todd	Pennyrile	soybean	20	36.80414	-87.09360
2019	Todd	Pennyrile	soybean	20	36.79289	-87.14455
2019	Todd	Pennyrile	wheat	20	36.72966	-87.19181
2019	Todd	Pennyrile	corn	50	36.71866	-87.24448
2019	Trigg	Pennyrile	wheat	45	36.84037	-87.71226
2019	Trigg	Pennyrile	wheat	10	36.73506	-87.69597
2019	Union	W KY Coalfield	corn	15	37.57712	-87.86329
2019	Union	W KY Coalfield	soybean	15	37.63451	-87.82270
2019	Union	W KY Coalfield	corn	10	37.68803	-87.79623

2019	Union	W KY Coalfield	soybean	15	37.73841	-87.92358
2019	Union	W KY Coalfield	corn	20	37.68626	-87.96194
2019	Union	W KY Coalfield	corn	2	37.65591	-88.05537
2019	Union	W KY Coalfield	soybean	10	37.57702	-88.04665
2019	Warren	W KY Coalfield	soybean	10	36.97550	-86.22270
2019	Warren	W KY Coalfield	soybean	5	37.00306	-86.24871
2019	Warren	W KY Coalfield	soybean	25	37.04672	-86.25687
2019	Webster	W KY Coalfield	soybean	25	37.51641	-87.50506
2019	Webster	W KY Coalfield	soybean	10	37.57947	-87.55576
2019	Webster	W KY Coalfield	soybean	1	37.60952	-87.59158
2019	Webster	W KY Coalfield	soybean	25	37.61282	-87.65353
2020	Ballard	Jackson Purchase	soybean	40	37.03600	-88.97140
2020	Ballard	Jackson Purchase	corn	60	37.06769	-88.98503
2020	Barren	Pennyrile	soybean	15	36.99734	-85.97161
2020	Barren	Pennyrile	soybean	20	36.94848	-85.84657
2020	Butler	W KY Coalfield	soybean	10	37.19021	-86.70401
2020	Caldwell	Pennyrile	soybean	15	37.07638	-87.83209
2020	Caldwell	Pennyrile	soybean	20	37.16970	-87.86910
2020	Caldwell	Pennyrile	soybean	20	37.14468	-87.97188
2020	Calloway	Jackson Purchase	soybean	40	36.53435	-88.42500
2020	Calloway	Jackson Purchase	soybean	10	36.58002	-88.43844
2020	Calloway	Jackson Purchase	soybean	10	36.65135	-88.39543
2020	Carlisle	Jackson Purchase	soybean	15	36.79721	-88.86201
2020	Carlisle	Jackson Purchase	soybean	40	36.90840	-88.91210
2020	Christian	Pennyrile	soybean	15	36.87256	-87.61159
2020	Christian	Pennyrile	soybean	70	36.78462	-87.58674
2020	Christian	Pennyrile	soybean	30	36.77465	-87.52438
2020	Christian	Pennyrile	soybean	10	36.77080	-87.41419
2020	Christian	Pennyrile	soybean	40	37.02042	-87.71117
2020	Christian	Pennyrile	soybean	20	36.89605	-87.50957
2020	Crittenden	Pennyrile	soybean	15	37.27780	-88.08120
2020	Daviess	W KY Coalfield	soybean	20	37.67893	-87.06314
2020	Daviess	W KY Coalfield	soybean	20	37.71720	-87.11270
2020	Daviess	W KY Coalfield	soybean	10	37.75780	-87.16220
2020	Daviess	W KY Coalfield	soybean	10	37.75650	-87.21610
2020	Daviess	W KY Coalfield	soybean	10	37.71894	-87.32773
2020	Fayette	Bluegrass	corn	40	38.12603	-84.49689
2020	Fulton	Jackson Purchase	soybean	5	36.51818	-88.94521
2020	Fulton	Jackson Purchase	soybean	5	36.54037	-89.13787
2020	Graves	Jackson Purchase	soybean	20	36.58926	-88.51772
2020	Graves	Jackson Purchase	corn	20	36.64402	-88.59083
2020	Graves	Jackson Purchase	soybean	10	36.66150	-88.61064
2020	Graves	Jackson Purchase	soybean	5	36.60770	-88.69540

2020	Graves	Jackson Purchase	corn	10	36.56210	-88.72211
2020	Graves	Jackson Purchase	corn	20	36.56107	-88.81228
2020	Graves	Jackson Purchase	soybean	5	36.87736	-88.68415
2020	Henderson	W KY Coalfield	soybean	15	37.77880	-87.38157
2020	Henderson	W KY Coalfield	soybean	10	37.79973	-87.43983
2020	Henderson	W KY Coalfield	soybean	10	37.79826	-87.49310
2020	Henderson	W KY Coalfield	soybean	15	37.72898	-87.47358
2020	Henderson	W KY Coalfield	corn	40	37.68459	-87.63347
2020	Henderson	W KY Coalfield	soybean	5	37.70300	-87.69740
2020	Henderson	W KY Coalfield	soybean	10	37.76767	-87.73390
2020	Hickman	Jackson Purchase	soybean	15	36.65068	-89.04356
2020	Hickman	Jackson Purchase	soybean	70	36.69396	-88.88057
2020	Hickman	Jackson Purchase	soybean	10	36.58763	-88.86948
2020	Hickman	Jackson Purchase	corn	5	36.69659	-88.87069
2020	Hopkins	W KY Coalfield	soybean	15	37.38279	-87.33394
2020	Livingston	Pennyrile	corn	25	37.19030	-88.24078
2020	Logan	Pennyrile	soybean	30	36.65595	-87.03166
2020	Logan	Pennyrile	soybean	60	36.70149	-86.76128
2020	Logan	Pennyrile	soybean	50	36.66844	-86.83030
2020	Logan	Pennyrile	soybean	5	36.73184	-86.99128
2020	Logan	Pennyrile	soybean	90	36.79518	-86.95236
2020	Logan	Pennyrile	soybean	25	36.82384	-86.99669
2020	Mclean	W KY Coalfield	soybean	20	37.60715	-87.28536
2020	Mclean	W KY Coalfield	soybean	15	37.56487	-87.27994
2020	Mclean	W KY Coalfield	soybean	20	37.45920	-87.27798
2020	Muehl	W KY Coalfield	soybean	10	37.40972	-87.12644
2020	Muehl	W KY Coalfield	soybean	10	37.34076	-87.20868
2020	Muehl	W KY Coalfield	soybean	15	37.20583	-87.21214
2020	Ohio	W KY Coalfield	corn	20	37.33450	-86.76240
2020	Ohio	W KY Coalfield	soybean	20	37.42862	-86.92897
2020	Simpson	Pennyrile	soybean	30	36.73718	-86.50924
2020	Simpson	Pennyrile	soybean	30	36.70906	-86.62753
2020	Simpson	Pennyrile	soybean	80	36.72038	-86.69590
2020	Taylor	Pennyrile	soybean	10	37.33148	-85.37948
2020	Todd	Pennyrile	soybean	25	36.70078	-87.07161
2020	Todd	Pennyrile	soybean	10	36.73226	-87.25408
2020	Todd	Pennyrile	soybean	10	36.69720	-87.18702
2020	Todd	Pennyrile	soybean	0	36.88274	-87.19172
2020	Trigg	Pennyrile	soybean	70	36.78465	-87.75874
2020	Trigg	Pennyrile	soybean	35	36.92843	-87.77035
2020	Trigg	Pennyrile	soybean	5	36.95067	-87.89259
2020	Union	W KY Coalfield	soybean	5	37.67770	-87.76200
2020	Union	W KY Coalfield	soybean	5	37.63921	-87.84344

2020	Union	W KY Coalfield	soybean	10	37.68161	-87.89586
2020	Union	W KY Coalfield	soybean	15	37.70217	-87.89570
2020	Union	W KY Coalfield	soybean	20	37.65538	-87.96693
2020	Union	W KY Coalfield	soybean	25	37.62379	-88.05873
2020	Union	W KY Coalfield	soybean	20	37.57839	-88.03158
2020	Warren	Pennyrile	soybean	20	36.85452	-86.46536
2020	Warren	Pennyrile	soybean	10	36.86115	-86.48017
2020	Warren	Pennyrile	soybean	5	37.02263	-86.74619
2020	Webster	W KY Coalfield	soybean	10	37.55687	-87.68024
2020	Webster	W KY Coalfield	corn	25	37.54486	-87.72087
2020	Webster	W KY Coalfield	soybean	10	37.55174	-87.70545
2020	Webster	W KY Coalfield	soybean	15	37.51865	-87.76114

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VITA

EDUCATION

University of Kentucky

Lexington, KY December 2022 (expected)

Ph. D. Plant Pathology – College of Agriculture, Food and Environment

Dissertation: An improved understanding of Diplodia ear rot in Kentucky corn

Purdue University

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M.S. Plant Pathology - College of Agriculture

Thesis: Effect of soybean vein necrosis on soybean yield and seed quality, and symptom expression on soybean and alternative hosts

North Dakota State University

Fargo, ND December 2010

B.S. Biotechnology - College of Science and Mathematics

PROFESSIONAL EXPERIENCE

Scientist I: University of Kentucky-Department of Plant Pathology, Princeton, KY, October 2017 – Current

Laboratory Research Associate: Purdue University-Department of Botany and Plant Pathology, West Lafayette, IN, July 2015-October 2017

Laboratory Research Assistant: Purdue University-Department of Botany and Plant Pathology, West Lafayette, IN, March 2012-July 2015

Plant Pathology Microbiologist: North Dakota State University-Department of Plant Pathology, Fargo, ND, November 2011-March 2012

Plant Pathology Research Assistant: North Dakota State University-Department of Plant Pathology, Fargo, ND, January 2006-November 2011

Undergraduate White Wheat Research Assistant: North Dakota State University-Department of Plant Sciences , Fargo, ND, December 2004-October 2005

PROFESSIONAL PRESENTATIONS

Anderson, N.R. and Wise, K. A. 2022. Evaluation of the *Stenocarpella* sp. population causing Diplodia ear rot on corn in Kentucky. American Phytopathological Society North Central Division Meeting. Lincoln, NE.

Anderson, N. R. and Wise K. A. 2021. Frequency of *Stenocarpella macrospora* causing Diplodia ear rot in Kentucky. Corn Disease Working Group Meeting – Virtual.

Anderson, Nolan R., Irizarry, M. D., Bloomingdale, C. A., Smith, D. L., Bradley, C. A., Delaney, D. P., Kleczewski, N. M., Sikora, E. J., Mueller, D. S., and Wise, K. A. 2018. Is soybean vein necrosis virus a threat to soybean production? Southern Soybean Disease Working Group Meeting. Pensacola, FL.

Anderson, N. R., and Wise K. A. 2017. Lessons in triazole fungicide sensitivity testing in *Fusarium graminearum*. U.S. Wheat and Barley Scab Initiative Annual Forum. Milwaukee, WI.

AWARDS

North Central American Phytopathological Society Graduate Student Travel Award. 2021

Southern Soybean Disease Working Group Graduate Student Oral Competition-First Place. 2018

North Central American Phytopathological Society Graduate Student Travel Award. 2017

Department of Botany & Plant Pathology, Purdue University Outstanding M.S. student. 2016

North Central American Phytopathological Society Graduate Student Poster Session-First Place. 2016

North Central American Phytopathological Society Graduate Student Travel Award. 2016

REFEREED PUBLICATIONS

Anderson, N. R., Bradley, C. A., and Wise, K. A. 2021. Diplodia leaf streak of corn: a diagnostic guide. Plant Health Prog. DOI:10.1094/PHP-01-21-0002-DG

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ABSTRACTS PRESENTED AT CONFERENCES

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