DISTRIBUTION AND SEASON-LONG MONITORING OF *PYTHIUM SPECIES* AFFECTING *ZEA MAYS* IN DELAWARE

by

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ABSTRACT

Corn (*Zea mays* L.), is a valuable cash crop for the state of Delaware planted across 71,225 hectares annually with a production value over $121.1 million. In annual disease loss estimates, root rot and death of plants due to *Pythium* infection is one of the top yield-limiting factors in Mid-Atlantic corn production. Across the United States and Ontario, estimates indicate that at least 238 million bushels of corn were lost due to root rots and seedling blights within the four-year span of 2016 to 2019. While multiple organisms can be involved in root rot and seedling blight diseases, *Pythium* is one of the most frequently associated pathogens. This oomycete organism is found worldwide affecting corn almost anywhere wet, waterlogged soils or high humidity growing conditions exist. The genus *Pythium* contains hundreds of species, most of which have been shown to be aggressive plant pathogens. To date, forty-four *Pythium* species have been associated with corn in the United States. Limited research has been conducted to elucidate the distribution of species, fungicide sensitivity ranges, and growth effects of *Pythium* isolates infecting corn in the Mid-Atlantic. In this thesis, surveys were conducted in 2019 and 2020 to molecularly characterize *Pythium* species present across Delaware and screen for fungicide sensitivity and aggressiveness of collected species. On-farm observation trials were established to document season-long effects of early season *Pythium* infection. From the survey, 14 species of *Pythium* were identified among the 367 isolates collected representing three clades. A subset of five species tested were found to be sensitive to mefenoxam, ethaboxam, and picarbutrazox, with

the exception of *Pythium torulosum,* which showed reduced sensitivity to ethaboxam. A larger panel of 12 species were tested for temperature and fungicide interactions, where increasing temperature reduced inhibition of multiple *Pythium* species on fungicide amended media. Season-long tracking of *Pythium* infected plants compared to nonsymptomatic control plants showed significant reductions of stalk flexural rigidity, stalk circumference, root mass, and kernel count. This work serves as a foundation to improve understanding of pathogen biology to better manage disease loss in Mid-Atlantic corn production.

Chapter 1

REVIEW OF *PYTHIUM* SPECIES CAUSING DAMPING-OFF IN CORN

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Review of *Pythium* species Causing Damping-off in Corn

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Abstract:

Seedling blights and root rots caused by *Pythium* species account for almost twenty-five million dollars in annual losses to corn (*Zea mays*) production in the United States and Ontario. Variations in annual rainfall and increasing use of no-till agriculture can favor soilborne pathogens like *Pythium.* To date, forty-four species have been reported as pathogenic to corn in the United States. The average annual corn planting date in the United States has shifted approximately one week earlier in the past decade, exposing young corn plants to longer germination periods of generally cooler temperatures, favoring attack by *Pythium.* Optimal temperatures, aggressiveness, and response to chemical and biological treatment options vary by species. This review consolidates the species of *Pythium* reported as corn pathogens in literature to date. It also provides an

insight into management strategies and discusses variations in fungicide sensitivity observed in corn-related *Pythium* species.

Introduction

Pythium is a large, cosmopolitan, oomycete genus within the phylum Oomycota that accounts for more than 180 species, which display striking intraspecific variations of form, host range, and relative aggressiveness as phytopathogens (Jiang et al 2012; Lévesque et al. 2010, Martin 2000). The genus *Pythium* was originally described by 1800s German botanist and algologist Nathaniel Pringsheim and subsequently moved to a new family (Pythiaceae) by Schröter shortly after (Hendrix and Campbell 1973). *Pythium* was designated as a fungus until genetic research placed the whole Oomycota phylum into a taxonomic group with "uni-flagellate hypochytrids," whose closest genetic relatives are diatoms (phylum Ochrophyta, class Bacillariophyceae) (Lévesque et al. 2010). Taxonomically, *Pythium,* now resides in the domain Eukarya, kingdom Chromista, phylum Oomycota, class Oomycetes, order Pythiales and within the family Pythiaceae (Lévesque et al. 2010). *Pythium* species are subdivided into 11 phylogenetic clades, A through K based on genetic distance in the ITS region (Lévesque and De Cock 2004; Ho 2018; Robideau et al. 2011). Other studies exist such as Martin (2000) and Villa et al. (2006) exploring relationships through COXII, LSU, ITS, or partial β-tubulin gene regions. Although Uzuhashi et al. (2010) suggested a compelling alternative phylogenetic classification system (clades 1-5) based on comparisons of the cytochrome oxidase II and large subunit D1/D2 gene regions, the Lévesque and De Cock A-K system remains pervasively dominant in the literature (Acharya et al. 2017;

Martin 2000; Villa et al. 2006). Probes for qPCR amplification of clades B and F were developed by Acharya et al. 2017 to detect *Pythium* species within corn tissue samples.

Pythium has shiny, translucent, glassy, ("hyaline") hyphae that are externally comprised of cellulose and lack the internal septations common in morphologically similar true fungi (Van der Plaats-Niterink 1981). *Pythium* is commonly found in soils, estuaries, and aquatic environments existing largely as "opportunistic" phytopathogens, which attack their hosts during periods of stress and vulnerability (Lévesque et al. 2010). The majority of phytopathogenic *Pythium* species relevant to agriculture exist in soil. For example, *Pythium graminicola*, a common pathogenic species, is most abundant at soil depths of 7.5-15 cm, while *P. splendens* is commonly found 15-30 cm below the surface (Van der Plaats-Niterink 1981). Most *Pythium* species infect broad ranges of plants with varying aggressiveness, though some are known to parasitize algae, fish, and sometimes mammals (Van der Plaats-Niterink 1981; Pérez et al. 2005; Martin 2000). Some species (*P. periplocum, P. oligandrum,* and *P. acanthicum*) have been reported to parasitize fungi and other common species of *Pythium* (Van der Plaats-Niterink 1981).

Agronomic factors affecting infection of corn by *Pythium*

Diseases caused by *Pythium* in agricultural settings have historically been associated with wet soil conditions, and *Pythium* is perhaps the most damaging soilborne pathogen of corn (*Zea mays*) planted into cool soils (Zhang and Yang 2000). The average annual planting date of corn has shifted earlier over time, exposing young corn plants to generally cooler temperatures and longer germination periods that often

favor pathogen attack (Broders et al. 2007; Matthiesen et al. 2016; Martin and Loper 1999). An example of this earlier planting trend is seen in the state of Delaware where the beginning planting date for corn in 2012 was April 19 and in recent years has shifted to April 11 (NASS and DDA 2013; 2015). Though temperature-related aggressiveness can vary by species, Matthiesen et al. (2016) posit that global climate changes are predicted to increase favorable conditions for soilborne pathogens across the corn belt as temperature and precipitation averages rise. An increase in *Pythium* observed in recent decades has also followed the widespread adoption of reduced or no-till practices (Radmer et al. 2017; Rao 1976; Rojas et al. 2019).

Though many factors are certainly at play, the occurrence of *Pythium* in no-till systems is likely a result of layered crop debris, which serve as an ideal habitat for primary inoculum build-up (Bockus and Shroyer 1998; Rojas et al. 2019). In addition, continuous cropping of the same host within no till systems can further compound inoculum buildup, which can then be moved to new locations by wildlife or humans (Rojas et al. 2019). In some cases, primary inoculum can be vectored by insects as oospores that remain viable in the digestive tracts and frass, which subsequently cause infection after excretion (Hendrix and Campbell 1973; Jiang et al. 2012; Sutton et al. 2006).

Cover crops in rotational cropping systems produce a myriad of positive environmental effects including mitigation of soil erosion and runoff, limiting nutrient leaching, as well as generally improving the physical properties of the soil by supporting the microbiome present (Kaspar and Singer 2011). Much work has been

done and is underway to define the relationship that cover crops, such as winter rye (*Secale cereale*), have with soil-borne pathogens, and how they may act as a "green-bridge" to facilitate pathogen and cash crop interactions (Bakker et al. 2016). No-till and cover crop practices allow for buildup of dead plant tissue matter on the surface of the soil, which supports the natural biology of the soil and subsequent development of plants (Kaspar and Singer 2011). However, Bakker et al. (2016) suggest that the terminated plant material buildup can also act negatively by allowing pathogens to proliferate. Some hemibiotrophic pathogens like *Pythium* are well suited to subsist saprophytically on the dead tissue of the terminated cover crop for weeks until the cash crop is planted and germinates (Bakker et al. 2016). Though other soil-borne pathogens are present, (*Pythium spp., Fusarium spp.* and *Rhizoctonia spp.* often creating multi-genus "species complexes"), *Pythium* should be the main focus for this winter rye and corn pathogen chain (Acharya et al. 2018). In extreme cases, corn plants will perform better following winter fallow than following a winter rye cover crop; and it has been hypothesized that the allelochemical residues left behind by the terminated cover crop can aid the infection of corn by *Pythium spp.* (Acharya et al. 2018; Acharya et al. 2021). Kurtz et al. (2021) demonstrated that corn seedling blight severity by *Pythium spp.* is positively correlated with the proximity of planting to the terminated winter rye plants.

Martin and Loper (1999) offer excellent detailed examples of the species-related pathogenicity levels as related to various soil moistures, temperature, and some references to pH. Optimal temperatures and temperature ranges of species reported on corn are presented in Table 1. Caution is recommended regarding generalized

interpretations of the severity of *Pythium*-induced disease as it relates to temperature, due to the high "intraspecific variation" in many aspects, including hyphal growth, spore germination, and germ tube growth for penetrating the host tissue (Martin and Loper 1999). Van der Plaats-Niterink (1980) notes that the optimum infection temperature may differ in soils compared to *in vitro*. Though activity levels can vary with soil temperature, all phytopathogenic *Pythium spp.* are most aggressive in high humidity and wet soil conditions (Biesbrock 1970; Gold and Stanghellini 1985). Martin and Loper (1999) cite examples of the effects of pH on *Pythium,* including a limitation of isolate recovery in fields with acidic soils below 5.5. Saprophytic capabilities of *Pythium* species, the susceptibility to attack from other organisms, the ability to reproduce, and bioavailability of soil nutrients and minerals that the organism needs to survive can all be affected by pH (Martin and Loper 1999).

Economic losses in corn due to *Pythium*

Pythium species can cause root rots and seedling blights in numerous hosts, including corn. There was an estimated loss of 238 million bushels of corn to various root rots and seedling blights in the United States and Ontario between 2016 and 2019 (Mueller et al. 2020). It is also estimated that in 2012 about 40% of losses incurred by root rots and seedling blights were caused by *Pythium* species (Mueller et al. 2016). This means that in the past four years *Pythium* may be responsible for about 95 million bushels lost. At $3.75 per bushel of corn, the monetary losses total over 357 million dollars (NASS 2020). Rojas et al. (2019) reported corn production costs amounted to "$332 dollars per acre, where corn seed represents 30-40% of costs," and made note

that profit margins depend wholly on “uniform germination” and healthy establishment of plants.

Pythium species associated with corn

To date, forty-four *Pythium* species have been implicated as direct causal agents of seed- and seedling-blight of corn in the United States (Acharya et al. 2018; Broders et al. 2007; Dorrance et al. 2004; Ellis et al. 2012; Matthiesen et al. 2016; Rojas et al. 2019). Recent characterization surveys of *Pythium* in agricultural fields have yielded differing results. Broders et al. (2007) found 11 species associated with corn in Ohio, predominantly *Pythium dissotocum* and *Pythium sylvaticum*. Jiang et al. (2012) completed a survey of *Pythium* in soil samples in Illinois that reported 27 different species, predominantly *Pythium oopapillum* and *Pythium diclinum*. In Michigan, Rojas et al. (2019) recovered 33 species of *Pythium* from corn fields with *Pythium inflatum* and *Pythium sylvaticum* being the dominant species. *Pythium spp.* have been broadly reported in Arizona, California, Colorado, Florida, Hawaii, Iowa, Illinois, Indiana, Kentucky, Louisiana, Maryland, Montana, North Carolina, North Dakota, South Carolina, Tennessee, Texas, and Wisconsin (Table 1.1).

Symptoms of *Pythium* in Corn

The symptoms of *Pythium* infection of corn are similar to infection by the pathogen on other hosts, namely water-soaking, dark discoloration, and complete necrosis of roots and hypocotyls as well as wilting, chlorosis, and necrosis of leaves (Erwin 1957; Johann et al. 1928). Johann et al. (1928) describe initial infection occurring at the rootlet tips, the root cortex rotting followed by colonization of the

vascular elements. Johann et al. (1928) also note that discoloration of the tissue only occurred after secondary organisms invaded the *Pythium*-infected "water-soaked" tissues. Corn plants with severe root infection will often have root cortex sloughing, in which *Pythium* will colonize and degrade the cortex, while leaving the vascular bundles generally intact (Elliott 1943; Rojas et al. 2019). Plants that survive infection between germination until about vegetative growth stage V2 (when two of the lowest leaves show a visible collar) will generally survive, but will be stunted and have limited performance in all aspects as compared to healthy plants (Broders et al. 2007). *Pythium* is also associated with stalk rots in which a one- to two-inch, browning, necrotic patch will occur on lower internodes, often causing lodging (Elliott 1942). There have also been recorded cases in which *Pythium* will infect mature corn plants. In some descriptions, *Pythium*-related stalk rots have been observed on five-foot-tall plants with symptoms that develop quickly during a hot humid period following a rainy period (Biesbrock 1970; Elliott 1942). In cases where environmental conditions are not optimal for pathogenicity, *Pythium* infection will often manifest as seedling soft rot or a "gradual reduction of roots" (Johann et al. 1928).

Infection

Pythium produces motile zoospores that are described as bean-, pear- or kidney-shaped ("reniform") (Middleton 1943; Van der Plaats-Niterink 1981). All parts of the host root (root cap, root hairs, elongation zone, etc.) can be penetrated by the pathogen, which utilizes hyphal penetration pegs, super-fine hyphae, and production of pectinolytic or cellulolytic enzymes to digest the hosts' cuticle and outer cells (Sutton et

al. 2006; Van der Plaats-Niterink 1981). Increased root mucilage production may increase attraction of *Pythium* species to roots (Zheng et al. 2000). The process of zoospore encystment and infection preceding host colonization begins with a zoospore reaching the infection court (host root), losing its flagella, and developing a thick outer coating to adhere to the surface of the host. These steps are followed by development of a germ tube, which becomes a "prepenetration hypha" that directly penetrates the host's "cuticle, natural openings, or wounds" (Kim et al. 1974; Sutton et al. 2006). Appressorial formation as a means of penetration is variable in *Pythium*, often depending on species and environmental conditions such as temperature and moisture (Agnihotri 1969; Sutton et al. 2006). Alternatively, mycelium of *Pythium spp.* that have broken off from the "mother mycelia," can form multiple bulbous appressoria upon contact with the host tissues that individually develop infection pegs that in turn directly penetrate and infect the host (Kim et al. 1974).

Pythium species also produce oospores that act as long-term survival structures that can perpetuate a form of stasis in adverse conditions in some cases for several years (Jiang et al. 2012; Van der Plaats-Niterink 1981). Their hardiness to adversity is exemplified by Hendrix and Campbell (1973) who note that when collections of oospores become established in the soil, it is extremely difficult to eradicate them without using wide spectrum soil fumigants. One source described two different types of oospores: thick-walled oospores with an extended dormant period and thin-walled oospores, which delay germination in waiting for more appropriate conditions (Lumsden 1975). The oospores will either expand to fill the oogonium (plerotic

oospores) or incompletely grow to leave a space between the oogonial and oospore walls (aplerotic oospores); this feature is diagnostic and can help in species identification when spores are encysted in corn roots (Van der Plaats-Niterink 1981). There are also several species of *Pythium* that make chlamydospores in lieu of oospores, although none of these species have yet been implicated in corn disease (Van der Plaats-Niterink 1981).

Pythium infections in corn are commonplace and most often manifest as necrotic root and seed rots that develop into pre- and post-emergence damping-off (Matthiesen et al. 2016). Though the specific anatomical location of root colonization (stele, cortex, etc.) can depend on the host, infection in corn generally manifests as colonization of the cortical tissue of the root, often leaving the vascular bundle intact (Elliot 1943; Sutton et al. 2006). Matthiesen et al. (2016) note that seed rot may primarily cause pre-emergence damping off, while root rot is likely the cause of post-emergence damping-off symptoms; highlighting the disparity in infection location as the cause of specific symptoms. *Pythium*-infected corn plants that do emerge will often have streak-like yellow-brown lesions on the roots and hypocotyls that progress to dark brown, that often girdle the tissue and lead to necrosis (Rao 1978). One-month-old plants undergoing infection will die quickly, whereas older plants wilt for several days before dying (Erwin 1957). Corn yields are reduced simply because crop stands are reduced and thus the number of plants that can reach maturity for harvest (Nafziger 1991).

Chemical Management

Being a soilborne pathogen, *Pythium* is often controlled using fungicides/oomyceticides that are applied as a coating around the seed before planting. Variations in sensitivity to seed treatments have been reported. Acharya et al. (2018) and Schenck et al. (2017) describe the limitations of select seed treatment fungicides for controlling *Pythium* present in corn systems that rely on a winter cereal rye as a cover crop. This limitation holds especially true in members of Clade B *Pythium* (Acharya et al. 2018; Schenck et al. 2017). Matthiesen et al. (2016) describes how ambient temperature can alter the effectiveness of seed treatment oomyceticides, and Rojas et al. (2019) demonstrated that there are differences in sensitivity to seed treatment fungicides among different clades of *Pythium.* Broders et al. (2007) reported variation in sensitivity of *Pythium* species to fungicides and oomyceticides (mefenoxam, azoxystrobin, trifloxystrobin, and captan) which are commonly used in agriculture as corn seed treatments for *Pythium* control. In comparing studies that tested the same fungicides, year to year, results fluctuated. Insensitivity to seed treatment fungicides that target oomycetes in corn-pathogenic *Pythium* species is inconsistent across studies, suggesting, perhaps, that it develops in local populations (Table 1.2) (Broders et al. 2007; Dorrance et al. 2004; Matthiesen et al. 2016; Matthiesen and Robertson 2020; Radmer et al. 2017; Rojas et al. 2019).

Metalaxyl/Mefenoxam

Since its first recorded use in 1984, many instances of metalaxyl resistance and fungicide insensitivity were reported among populations of *Pythium* species infecting

other hosts, in some cases as soon as three years after beginning first treatment. In rare occasions, metalaxyl can stimulate the growth of the pathogen (Dorrance et al. 2004; Lookabaugh 2015; Taylor 2002). Cross-resistance to metalaxyl has been documented from use of other phenylamides such as furalaxyl, ofurace, benalaxyl, cyprofuram, and oxadixyl (Koeller 1991; White et al. 1988). Metalaxyl is therefore highly restricted by the Fungicide Resistance Action Committee, often only purchasable in pre-packed mixtures for very limited applications in field settings, FRAC stating that "phenylamides should only be used on a preventive and not curative or eradicative basis" (Koeller 1991; FRAC 2020). Matthiesen et al. (2016) showed increased and decreased metalaxyl sensitivities in *Pythium* species isolated from corn when screened at various temperatures. Mefenoxam (metalaxyl-M) is the stereoisomer of metalaxyl and has been widely used in agriculture for controlling *Pythium.* Nearly all the studies present in Table 1.2 that incorporated mefenoxam indicate that the species tested showed sensitivity.

Ethaboxam

Ethaboxam is a broadly used fungicide that targets oomycetes and acts as a microtubule destabilizer (Noel et al. 2019). Matthiesen et al. (2016) reported that temperature changes resulted in variation of ethaboxam sensitivity of *Pythium* isolated from corn. In one study, for example, the effective concentration of ethaboxam to limit 50% of colony growth (EC50) on *Pythium torulosum* was shown to be higher at the lower temperature tested 13°C, whereas the opposite held true for *Pythium sylvaticum*

which performed more aggressively against ethaboxam in higher temperatures (18°C-23°C) (Matthiesen et al. 2016).

Strobilurins

Strobilurins are quinone outside inhibitors (QoI), which interrupt the electron transport chain in the production of ATP, that are generally applied as broad-spectrum controls for fungi with some incidental action against oomycetes (Wood and Hollomon 2003). Broders et al. (2007) note some soilborne organisms like *Pythium* may simultaneously express two alternate respiratory pathways *in vitro*, thus causing them to appear as though they are resistant to strobilurins and can require the addition of SHAM (salicylhydroxamic acid) to inhibit the alternate respiratory pathway in order to measure true reductions of sensitivity by the strobilurin fungicide to the target pathway (Broders et al. 2007, Wood and Hollomon 2003). Each of the studies reviewed in Table 1.2 that included strobilurins azoxystrobin, trifloxystrobin, and/or pyraclostrobin incorporated SHAM to accurately simulate organism conditions in soil to measure efficacy. The studies included seem to indicate a higher incidence of insensitivity to trifloxystrobin in the species tested.

Captan

Captan is a broad-spectrum fungicide with some activity on oomycetes (Broders et al. 2007). Studies have been performed and show mixed results of captan's efficacy on oomycetes based on temperature (Matthiesen et al. 2016). *Pythium oopapillum* and *P. sylvaticum* from corn showed reduced sensitivity to captan at higher growth temperatures whereas *P. torulosum* that was isolated from corn in Iowa showed

increased sensitivity at higher growth temperatures (Matthiesen et al. 2016). Mixed results regarding temperature were reported in *P. sylvaticum* (Matthiesen and Robertson 2021).

Thiram

Thiram is a multi-use product that shows some incidental control of oomycetes among many other organisms (ChEBI: 9495). Studies of populations from numerous hosts show sensitivity of *Pythium debaryanum* and *P. oopapillum* to thiram (Matthiesen et al. 2016; Wavare et al. 2016). Temperature was suggested to affect the performance of thiram as a control of *Pythium* species pathogenic to corn and soybean in Iowa. Higher temperatures decreased the sensitivity of *Pythium oopapillum,* increased the sensitivity of *P. torulosum,* and unpredictably affected thiram's activity against *P. sylvaticum* (Matthiesen et al. 2016; Matthiesen and Robertson 2021).

Biological Controls

Van der Plaats-Niterink (1981) describes biological remedies to *Pythium* including observed antagonistic interactions with: Actinomycetes, the oomycete *Aphanomyces,* bacteria, and other fungi including *Trichoderma, Rhizoctonia, Aspergillus, Penicillium,* and *Laetisaria* on a variety of species of *Pythium*. Bacterial control agents include the introduction of fluorescent pseudomonads, which showed success when introduced as a seed treatment on sweet corn with varying effectiveness at different temperatures (Callan et al. 1990; Callan et al. 1991). Some studies with non-pathogenic strains of gram-negative bacteria *Burkholderia cepacia*, *Trichoderma spp.,*

and *Gliocladium spp.* showed a reduction of *Pythium*-related infections (Mao et al. 1997; Martin and Loper 1999).

Other experimental biological controls of note include the introduction of aggressive, non-pathogenic, primary saprophytes such as *Pythium oligandrum* or *P. nunn,* which can control the niche and effectively deprive other pathogenic *Pythium* species from access to nutrients from debris in a field (Martin and Loper 1999). Martin and Loper (1999) describe some of the known suppression mechanisms that biological controls employ including germ tube or hyphal growth limitation, or interference through the consumption of the chemical signals from seeds and roots that *Pythium* species rely upon for host recognition.

Cultural Control

Hendrix and Campbell (1973) imply an interesting perspective on selection of crops for rotation that allow for better airflow, drainage, or soil-sunlight exposure, which can all contribute to decreasing environmental favorability by the pathogen, thus reducing infection rates. Tiling to improve the drainage of a field can help mitigate the development of waterlogged soil conditions (Sloan et al. 2017). Matthiesen et al. (2016) offer compelling information regarding the mixed results of planting date adjustment, summarizing that it would be an effective means of control in fields containing *P. torulosum* and *P. sylvaticum,* but would not be nearly as effective against *P. oopapillum* or *P. lutarium*. Acharya et al. (2017) notes a negative correlation between time between rye cover crop termination and *Pythium*-related disease incidence in corn fields.

Acharya et al. (2017) also discuss the proximal relationship of terminated rye cover crop and *Pythium* disease incidence and present results that suggest that using precision agricultural techniques, cover crops could be planted and strip-tilled between the rows where corn is planted and the physical distance would help limit the proliferation and bridging of disease.

Conclusions

As no-till practices and extreme weather such as high-volume rain events and temperature fluctuations progress, *Pythium* will inevitably continue to negatively affect corn yields. Bakker et al. (2016) suggest that limiting transmission of primary inoculum between cover crops and cash crops will depend upon finding and developing cover crops that are not sensitive to *Pythium* infection and can help reduce primary inoculum build-up, however due to the large host range of *Pythium,* this may prove unlikely. Throughout the literature, there are suggestions of the effects of pH on *Pythium* mostly pertaining to initiation of reproductive or colonizing structures, little work has been done to elucidate the optimal pH of each species and to address the possible agricultural applications of this information, although the practicality of controlling pH in large scale agricultural settings is a major limiting factor. There is much variation between species in their sensitivity to active ingredients that target oomycetes and pathogenicity, while the symptoms of infected plants remain relatively constant in their appearance across different species of *Pythium*. Nearly all labelled chemicals have reduced efficacy on one or multiple species of *Pythium.* Maximizing yields in corn in the future will depend upon developing new research areas as well as responsibly utilizing the

information present to implement cultural controls (soil drainage, cover crop selection), biological controls (introducing and supporting pre-existing *Pythium* antagonists) and taking care to limit the spread of inoculum to susceptible crops.

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Table 1.1 *Pythium* species reported on corn in the United States

***Pythium* species**	**Clade[z]**	**State(s) reporting *Pythium* in corn[y]**	***Pythium* growth temperatures (optimal, min, max)[x]**	**Additional References**
P. acanthicum Drechsler	D	IA	25-30°C, 5°C, 38°C	
P. adhaerens Sparrow	A	US	N/A	Uzuhashi et al. 2015
P. amasculinum Yü	D	IA	N/A	
P. angustatum Sparrow	B	US	25°C, 5°C, 35°C	
P. aphanidermatum (Edson) Fitzp. (syn. *P. butleri*)	A	CA, FL, IA, IN, KY, MO, SD, TX, VA,	35-40°C, 10°C, 40°C	
P. aristosporum Vanterpool	B	MI	25°C, 5°C, 30°C	
P. arrhenomanes Drechsler	B	HI, IA, IL, KY, MD, MN, MO, OH, PA, SD, TN, WI	25-30°C, 5°C, 35°C	
P. attrantheridium Allain-Boulé and Lévesque	F	OH	20°C, 5°C, 30°C[x]	Allain-Boulé, et al. 2004
P. sp balticum	N/A	IA	N/A	Rojas et al. 2019
P. catenulatum VD Matthews	B	OH	30°C-35°C, 10°C, 40°C	

P. debaryanum Hesse	F	CT, FL, HI, IA, MN, MO, MT, ND, OK, SD, WI, WY	28°C, 1°C, 37°C	Thomson et al. 1971
P. dissotocum Drechsler	B	OH	28°C, 1°C, 34°C 20-25°C, 5°C, 35°C	Middleton 1943
P. graminicola Subramaniam	B	CA, CO, GA, HI, IA, IL, KY, MN, MO, ND, OH, SD, WI	30°C, 5°C, 38°C	
P. heterothallicum Campbell & Hendrix	I	MI	25°C, 5°C, 35°C	
P. hydnosporum (Mont.) Schröter	D	MI	28°C, 4°C, 35°C	
P. hypogynum Middleton	E	IA	31-34°C, 1°C, 37°C	
P. inflatum Matthews	B	OH	30°C, 5°C, 37°C	
P. irregulare Buisman	F	IA, OH, WI	30°C, 1°C, 35°C	
P. longandrum Paul	E	MI	25°C	Paul 2001
P. lutarium Ali-Shtayeh	B	AR, IA IL, IN, KS, MI, ND, NE	25°C, 5°C, 42°C	Matthiesen et al. 2020; Uzuhashi et al. 2015
P. nodosum Paul	J	MI	20-25°C	Paul et al. 1998
P. oligandrum Drechsler	D	MI	30°C, 7°C, 37°C	

Pythium oopapillum Bala et al.	B	AR, IA, IL, IN, KS, MI, MN, ND, NE, SD	30°C, 5°C, 30°C	Bala et al. 2010; Matthiesen et al. 2020; Rojas et al. 2017
P. orthogonon Ahrens	J	MI	30°C, 15°C, 40°C	
P. pachycaule Ali-Shtayeh	B	MI	N/A	Rojas et al. 2019
P. paroecandrum Drechsler	F	IA, WI	25°C, 5°C, 35°C	
P. periilum Drechsler	B	MI	30°C, 10°C, 40°C	
P. periplocum Drechsler	D	MI	30°C, 5-10°C, 40°C	
P. perplexum Kouyeas & Theohari	J	MI	22°C, 4°C, 32°C	
P. pleroticum T. Ito	E	MI	N/A	Rojas et al. 2019
P. pulchrum Minden	N/A	WI	N/A	Minden 1916
P. rostratifingens de Cock & Lévesque	E	MI	27°C, 3°C, 33-36°C	Choudhary et al. 2016; De Cock and Lévesque 2004
P. rostratum Butler	E	HI, IA, WI	25°C, 5°C, 35°C	Butler 1907
P. schmitthenneri Ellis et al.	E	OH	21°C, 4°C, 32°C	Ellis et al. 2012
P. selbyi Ellis et al.	E	OH	21°C, 4°C, 32°C	Ellis et al. 2012
P. spinosum Sawada	F	IA	25°C, 5°C, 35°C	Robertson et al. 2016;
P. splendens Braun	I	HI, IA	25°C, 5°C, 34°C	

P. sylvaticum Campbell and Hendrix	F	AR, IA, IL, IN, OH, KS, MI, MN, ND, NE, SD, WI	25°C, 5°C, 35-40°C	Matthiesen and Robertson 2020
P. tardicresens Vanterpool	B	MI	25°C, 7°C, 28°C	Robideau et al. 2011
P. torulosum Coker and Patterson	B	AR, IA, IL, IN, OH, KS, MI, MN, ND, NE, WI	25°C, 5°C, 30°C	Matthiesen and Robertson 2020
P. ultimum Trow	I	MA, ND, SD, WI	25-30°C, 5°C, 35-40°C	
P. ultimum Trow *var. ultimum*	I	OH	25-30°C, 5°C, 35-40°C	
P. ultimum Trow var. *sporangiiferum*	I	OH	25°C, 5°C, 37°C - 40°C	
P. vexans de Bary	K	WI	30°C, 5°C, 35°C	

[z] Clade assignments for *Pythium* species as presented in Lévesque and de Cock (2004).

[y] *Pythium* species pathogenic to corn and their distribution in the US as reported by the United States Department of Agriculture, Agricultural Research Service Fungal Database. Locations identified in recent surveys are specified in the additional references column.

[x] Information on cardinal temperatures of optimal, minimum, and maximum growth collected from Van der Plaats-Niterink (1981) except where noted in additional references column. N/A indicates that the cardinal temperature is not available in the literature at time of publication.

Table 1.2 Ranges of sensitivity observed during *in vitro* fungicide efficacy screening of *Pythium* species pathogenic to corn.

Species	Fungicides[z]							
	Metalaxyl	Mefenoxam	Ethaboxam	Captan	Thiram	Azoxystrobin	Trifloxystrobin	Pyraclostrobin
P. acanthicum		HS[5]	MS[5]					
P. amasculinum		HS[5]	MS[5]					
P. arrhenomanes		HS[5]	HS[5]					
P. attrantheridium		MS[2], HS[5]	MS[5]	MS[2]		MS[2]	HI[2]	
P. catenulatum	MI[1]							
P. dissotocum		MI[2], MS[5]	MS[5]	MI[2]		MS[2]	MI[2]	
P. graminicola		MI[2]		MI[2]		HS[2]	MI[2]	
P. heterothallicum		HS[5]	MS[5]					
P. inflatum		MS[2], MS[5]	MS[5]	MI[2]		HS[2]	MS[2]	
P. irregulare	MS[1]	MS[2], MS[4], MS[5]	HS[4], MS[5]	MS[2]		MI[2], MI[4]	HI[2], HI[4]	MI[4]

P. lutarium	MS[3], MI[6]	MI[5]	HS[3], MI[5], MS[6]	MS[3], MS[6]	MS[3], MS[6]	MI[3], HI[6]	MI[3]	HI[3]
P. oopapillum	MS[3], MS	MS[4], MS[5]	HS[3], MS[4], MS[5], MS[6]	HS[3], MS[6]	HS[3], MS[6]	MI[3], MS[4], MI[6]	MI[3], MS[4]	HI[3], MS[4]
P. orthogonon		MS[5]	MS[5]					
P. paroecandrum	MS[1]							
P. periilum		HS[5]	MS[5]					
P. perplexum		MS[5]	MS[5]					
P. rostratifingens		HS[5]	HI[5]					
P. splendens	MI[1]							
P. sylvaticum	MI[3], HI[6]	MS[2], HS[4], MS[5]	HS[3], HS[4], MS[5], MS[6]	MS[2], HS[3], MI[6]	MS[3], MI	MI[2], MI[3], MS[4], HI[6]	HI[2], HI[3], HI[4]	HI[3], MS[4]
P. tardicrescens		MS[5]	MS[5]					
P. torulosum	MI[1], MI[3], MS[6]	MS[2], HS[5]	MS[3], HI[5], MI[6]	MI[2], MS[3], MS[6]	MS[3], MS[6]	HS[2], MS[3], MI[6]	HS[2], MI[3]	HI[3]
P. ultimum		MS[2], MS[5]	MS[5]	MS[2]		MI[2]	HI[2]	
P. ultimum var. *ultimum*		HS[4], HI[5]	MS[4], MS[5]			MS[4]	HI[4]	MS[4]

P. ultimum var. *sporangiiferum*	MS[4]	AS[4]		MS[4]	HI[4]	MS[4]

[z] Sensitivity rating based on EC50 values (HS) highly sensitive, (MS) medium sensitive, (MI) medium insensitive, and (HI) highly insensitive. (1) Dorrance et al. 2004, (2) Broders et al. 2007, (3) Matthiesen et al. 2016, (4) Radmer et al. 2017, (5) Rojas et al. 2019, and (6) Matthiesen and Robertson 2020.

Chapter 2

CHARACTERIZATION OF *PYTHIUM* SPECIES ASSOCIATED WITH CORN IN DELAWARE

Abstract

Corn (*Zea mays* L.) is the top grain crop by hectares grown in the state of Delaware. Cool, wet spring conditions and a shift in the average planting date have contributed to a rise of *Pythium*-related pre- and post-emergence damping-off of corn. To date, forty-four species of *Pythium* have been reported in association with root and seed rot in corn in the United States, however, no characterization studies have been performed in Delaware. Symptomatic seedling corn plants displaying wilting and root lesions were collected from 33 fields across Delaware in 2019 and 2020. Root tissues from infected plants were sectioned, surface disinfested, and plated onto pimaricin-ampicillin-rifamycin-pentachloronitrobenzene (PARP) selective medium. Pure culture DNA was extracted and amplified at three gene regions (ITS4, COXII, and Partial β-Tubulin) for identification by molecular sequencing. In total, 14 species were recovered: *P. arrhenomanes, P. attrantheridium, P. catenulatum, P. coloratum, P. graminicola, P. inflatum, P. irregulare, P. oligandrum, P. periilum, P. periplocum, P.spinosum, P. sylvaticum, P. tardicrescens,* and *P. torulosum*. This is the first report of *P. coloratum* being pathogenic on corn in the United States.

Select species were subjected to *in vitro* fungicide screening to calculate EC50 values for seed treatment active ingredients mefenoxam, ethaboxam, and picarbutrazox. All species tested showed sensitivity to mefenoxam and picarbutrazox. All species tested on ethaboxam showed sensitivity with the exception of *P. torulosum,* which showed decreased sensitivity. Using generated EC50 values, 12 species from the survey were screened in temperature controlled *in vitro* assays for percent inhibition on Petri dishes amended with a single concentration of mefenoxam, ethaboxam, or picarbutrazox at 18ºC, 21ºC, and 25ºC. As temperatures increased, percent inhibition was significantly reduced in 10 species on plates amended with mefenoxam, five species on plates amended with ethaboxam, and 10 species on plates amended with picarbutrazox. The same 12 species were used in greenhouse panels to screen isolate aggressiveness on field and sweet corn lines. Significant reduction of emergence was observed due to *P. inflatum,* but no significant differences were observed among species for plant height, shoot weight, and root weight.

Introduction

One of the most damaging diseases of corn (*Zea mays* L.) in the Mid-Atlantic region (MAR) of the United States is pre- and post-emergence damping off caused by multiple species in the genus *Pythium.* Infection of corn by *Pythium* will manifest as a slight discoloration with the appearance of yellow or brown lesions, which can expand and allow the entry of secondary pathogenic

organisms. This often leads to complete necrosis of the roots and hypocotyls, resulting in wilting and eventual death of the aboveground plant parts (Figure 2.1) (Erwin 1957; Johann et al. 1928). Plants that become infected while emerging will often decline very quickly, while plants that acquire infection later will demonstrate wilting and chlorosis for a longer period before dying completely (Erwin 1957). Colonization of ungerminated corn seeds can result in seed rots and a complete lack of emergence known as pre-emergence damping-off (Matthiesen et al. 2016). Corn plants that survive infection past the point where the two lowest leaves bear visible collars (V2) will often survive but with slower development and stunting (Broders et al. 2007).

Cool wet soils can delay corn emergence and favor infection by *Pythium* species, causing pre- and post-emergence damping-off with devastating effects to yield potential (Zhang and Yang 2000). In Delaware, the average planting date of corn shifted from April 19 in pre 2012 to April 11 since 2015. Differences in soil temperature can increase the amount of time needed for germination and emergence, and this shift has correlated with elevated occurrence of *Pythium* in recent wet springs. Slower germination can also allow for the breakdown of seed applied fungicide treatments intended for *Pythium* control as seeds sit longer in the soil before they are actively growing and setting additional roots (Broders et al. 2007). These factors, in conjunction with the widespread incorporation of no-till practices that allow debris-layer to buildup

and serve as a bridge for primary inoculum, work in tandem to challenge successful corn stand establishment (Radmer et al. 2017; Rao 1976; Rojas et al. 2019).

Species of *Pythium* have differing minima, maxima, and optima regarding ambient temperatures at which they grow (Van der Plaats-Niterink 1980). Corn-pathogenic species will often not grow at temperatures below 3°C or above 40°C (some showing significant slowing at 30°C), with optimal ranges most often around 20°C-25°C; a notable exception is *Pythium aphanidermatum* (optimal range 35°C-40°C) e.g. (Van der Plaats-Niterink 1980). Temperature can also influence efficacy of seed applied fungicides with individual species having varying responses (Matthiesen et al. 2016).

Chemical mitigation strategies for *Pythium* in corn are primarily seed treatments that are applied to seed before planting. Some of the most commonly used chemicals include metalaxyl and metalaxyl-M (mefenoxam); ethaboxam; strobilurins like azoxystrobin, trifloxystrobin, pyraclostrobin; phthalamides like Captan or Thiram; or more recently picarbutrazox. Seed treatment products with efficacy against *Pythium* are rarely sold individually, and more commonly are applied to seed in combination with other fungicide and insecticide active ingredients (Broders et al. 2007; Matthiesen et al. 2016; Koeller 1991; Lévesque et al. 2010). Studies performed to gauge the efficacies of active ingredients targeting *Pythium* management have yielded mixed results, even in comparisons

bearing similar sets of species. These studies have identified how species composition and temperature can greatly impact the level of control observed *in vitro* (Dorrance et al. 2004; Broders et al. 2007; Matthiesen et al. 2016; Radmer et al. 2017; Rojas et al. 2019; Matthiesen and Robertson 2020).

To date, forty-four species of *Pythium* have been implicated with pre- and post-emergence damping-off of corn (Bickel and Koehler 2021; Acharya et al. 2018; Broders et al. 2007; Dorrance et al. 2004; Ellis et al. 2012; Matthiesen et al. 2016; Rojas et al. 2019). In recent surveys, species distribution and abundance appear to vary based on geographic region. Two surveys in Ohio and one in Michigan recovered 5, 11, and 33 different species, respectively (Broders et al. 2007; Dorrance et al. 2004; Rojas et al. 2019). While numerous species have been identified, the most common to each study were *P. dissotocum, P. sylvaticum,* and *P. inflatum*, respectively (Broders et al. 2007; Jiang et al. 2012; Rojas et al. 2019). The USDA-ARS reports *Pythium* species affecting corn in Arizona, California, Colorado, Florida, Hawaii, Iowa, Illinois, Indiana, Kentucky, Louisiana, Maryland, Montana, North Carolina, North Dakota, South Carolina, Tennessee, Texas, and Wisconsin. No characterization studies of *Pythium* infecting corn have been conducted in the state of Delaware.

The objectives of this study were to (i) identify the species of *Pythium* present in Delaware that are associated with corn pre- and post-emergence damping-off, (ii) screen sensitivities of Delaware isolated *Pythium* species to

seed treatment active ingredients mefenoxam, ethaboxam, and picarbutrazox, and (iii) assess species pathogenicity under different temperatures in controlled environments.

Materials & Methods

*Isolate Collection and Identification***.** Corn seedlings showing symptoms of *Pythium* infection were collected in 33 Delaware fields associated with wet, waterlogged conditions that reported damping-off during the spring of 2019 or 2020. Plants were brought into the lab and washed with tap water to remove excess soil. Symptomatic roots and hypocotyls showing brown lesions and necrosis were sectioned from the plants and 5 mm tissue pieces were surface disinfested in 0.825% sodium hypochlorite solution for 30 s and rinsed in deionized (DI) water for 30 s. The disinfested root and hypocotyl segments were plated onto a PARP selective medium (V8 agar (20g/l Bacto agar (Fisher Scientific), 200 ml/l clarified V8 juice) amended with pimaricin, ampicillin, rifampicin, and PCNB [Terraclor 75% WP, Southern Ag Insecticides, Inc.]) (Jeffers and Martin 1986) and left to incubate at 23°C for five to seven days. Isolates showing glassy, hyaline hyphae were repetitively sub-cultured onto PARP medium plates to achieve pure culture.

For DNA extractions, three 6.75 mm plugs from each pure culture were transferred into 100 x 15 mm Petri dishes containing 20 ml of potato-dextrose broth (PDB) (Difco). Plates were left at 23°C until fully colonized. Isolates

showing no contamination were vacuum-aspirated through autoclave-sterilized filter paper in a Buchner funnel. Dried tissue was separated into 1.5 ml microcentrifuge tubes and stored at -2°C for DNA extraction.

Genomic DNA from dried, frozen tissue was extracted by macerating tissue with 400 µl of cell lysis solution (Qiagen, Hilden, Germany) using autoclave-sterilized plastic pestles. Following the addition of 3 µl Puregene proteinase K (Qiagen) to each sample, tubes were vortexed for 20 s, and incubated at 55°C for 1 h. The supernatant of microcentrifuged samples were treated with RNase (3ul; Qiagen) and incubated at 37°C for 30 min followed by the addition of 200 µl of protein precipitation solution (Qiagen) and 1 h incubation on ice. Tubes were then centrifuged for 5 min and supernatant was pipetted into microcentrifuge tubes containing 400 µl isopropanol to precipitate DNA. Tubes were centrifuged for 3 min, the supernatant was poured off, and the DNA pellet was washed with 200 µl ethanol. After centrifuging, supernatant was poured off and samples were allowed to dry on heating block before being rehydrated with 50µl of sterile, nuclease-free water. DNA concentration and purity were measured by spectrophotometry and standardized to a concentration of 20 ng ml^{-1} before storage at -2°C.

For each isolate, three loci: partial beta tubulin (*BT*), cytochrome c oxidase subunit II (COII), and nuclear rDNA internal transcribed spacer (ITS1-5.8S-ITS2 =ITS) were amplified with polymerase chain reaction (PCR) (Villa et

al. 2006; Martin 2000). PCR reactions were 50 µl and consisted of 10 X DreamTaq Master Mix, 10 µM of each forward and reverse primer: ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3'); BT5 (5′-GTATCATGTGCACGTACTCGG-3′) and BT6 (5′-CAAGAAAGCCTTACGACGGA-3′); and FM66 (5'-TAGGATTTCAAGATCCTGC-3') and FM58 (5'-CCACAAATTTCACTACATTGA-3'), and 20 ng of genomic DNA. Thermal cycling parameters were as follows: 94°C for 5 min followed by 35 cycles of DNA denaturation for 45 s at 94°C; primer annealing for 30 s at 53°C; and primer extension for 90 s at 72°C. A final extension step of 72°C for 10 min completed the cycle.

Amplification was verified using gel electrophoresis of 90 V for 40 min. Aliquots of amplified products were cleaned with ExoSAP-IT (USB products Affymetrix, Inc. ThermoFisher Scientific, Walthan Massachusetts). Samples were sequenced at Eurofins Genomics (Louisville, KY). Analysis of sequence data was completed with CLC Main Workbench 20.0.4 (CLC Bio: a Qiagen Company, Germany) and copied into the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) for comparison against published, identified specimens. Generated sequences of the ITS region were deposited in GenBank (Table 2.1).

In Vitro Fungicide Sensitivity Screening. Amended media Petri dish assays were established to determine the EC50 (concentration of fungicide needed to inhibit mycelial growth by 50%) of *Pythium* isolates to three fungicides with activity against *Pythium.* The active ingredients screened included mefenoxam (ApronXL®, Syngenta Crop Protection Inc., Greensboro NC), ethaboxam (Elumin®, Valent BioSciences LLC), and picarbutrazox (Vayantis®, Syngenta Crop Protection Inc., Greensboro NC). Mefenoxam belongs to the PhenlyAmides group (Fungicide Resistance Action Committee [FRAC] code 4). Ethaboxam resides in the thiazole carboxamide group (FRAC 22) and picarbutrazox is a new product in the tetrazolyloxime group with an unknown mode of action (FRAC U17). Being a new product, preliminary picarbutrazox trials were conducted to identify the concentration range for EC50 screening (unpublished). For in vitro screening, 10% V8 agar with 20 g Bacto Agar (BD Difco) was amended with each fungicide upon cooling to 55°C. For mefenoxam and ethaboxam, fungicide concentrations were 0.01, 0.1, 1.0, or 10 ug ml^{-1}. Concentrations for picarbutrazox were 0.00001, 0.0001, 0.001, or 0.01 ug ml^{-1}.

Selected isolates were transferred to fresh V8 agar plates and grown for 4 d before an 8 mm plug was transferred from the colony edge and placed in the center of each 60 x 15 mm Petri plate containing 8 mL of cooled amended media. A set of non-amended plates were used as the control baseline. Cultures were incubated for 48 hours in low light at 21°C and colony diameter was

measured with a digital caliper. Each treatment consisted of three replicate dishes per concentration for each isolate. Tests with ethaboxam and picarbutrazox were conducted twice, and mefenoxam three times. The diameter of the mycelial plug was subtracted, and percent growth inhibition was calculated using the formula $\% \, Inhibition = \left(\frac{diameter \, of \, control - diameter \, of \, treatment}{diameter \, of \, control} \right) * 100$. Data from the replicated experiments for each active ingredient were combined and sorted by species. EC50 values were calculated for each species by linear regression of the percent inhibition on log10-transformed fungicide concentration in SAS 9.4 (SAS Institute, Cary, North Carolina).

In vitro Temperature Controlled Assays. Using the generated EC50 values, a subset of isolates collected throughout the trial were screened for sensitivity to mefenoxam, ethaboxam, and picarbutrazox at 18, 20, and 25° C. Medium was prepared as described above and an 8 mm diam plug of each isolate was transferred to the center of a 60 x 15 mm Petri plate. Each treatment consisted of three replicate dishes, and each fungicide test was conducted twice. Isolates were incubated in the dark for 48 h at 60% relative humidity conditions in chambers set at 18°C and 25°C (Conviron E7/2 Reach-In Growth Chamber) and in room temperature conditions (21°C). The diameter of the mycelial plug was subtracted, and percent growth inhibition was calculated using the formula described above.

Data was analyzed for normality, trial effects, and temperature effects. If no trial effects were present, trials were combined for analysis and replicated experiments for each fungicide were subjected to mixed-model analysis of variance (ANOVA) using PROC GLIMMIX in SAS 9.4 (SAS Institute, Cary, North Carolina). Temperature, species group, and interaction of temperature and species group were treated as fixed effects, while trial, replication, and overall error term were treated as random effects. Fixed effects were tested for significance at $\alpha=0.05$, and significant differences were separated using paired *t* tests.

Greenhouse Panels. Greenhouse trials were established to assess isolate aggressiveness. Inoculum was prepared by combining 25 g of long grain white rice and 18 ml of deionized H_2O into 250 ml Erlenmeyer flasks, which were autoclaved for 1 h on two separate days. Cooled rice flasks were each inoculated with three 6.75 mm diam plugs from each selected isolate. Flasks were incubated for five days at 23°C and agitated daily for even colonization of rice granules. In the greenhouse, 10.16 cm square plastic pots were filled with 200g of ProMix BK55 growing substrate without mycorrhizae (ProMix Quakertown, PA) and watered in with 400 ml of tap water. For each isolate, four pots of plain, non-treated NK9610-5112 field corn seed, and 2 pots of Aspire sweetcorn hybrid seeds treated with mefenoxam, captan, carboxin thiamethoxam, difenoconazole, and thiram safecoat ® were set up in plastic trays with no

drainage holes. Runoff water from watering in was allowed to collect at the bottom of the tray and saturated conditions were maintained throughout the trial. Within each pot, a single corn seed was planted into the center at a depth of 5 cm. Pots were then inoculated by incorporating 0.6 g of the colonized rice inoculum into the upper 2.5 cm of the surface of each pot. Gloves and tools were sterilized between each isolate tray. Two control trays were established by adding sterile rice. Plants were maintained in the greenhouse at an average temperature of 24.5°C and 57% relative humidity. Supplemental lighting was provided from 7AM-9PM when outside light radiation levels were below 600 W/m^2 (watts per square meter).

At 18 d, plants were assessed for differences in plant germination and emergence, height, root weight, and fresh top weight. Germination and emergence were verified by checking any pots without a plant for the presence of a seed that failed to germinate or a dead seedling. Plant height was measured from the crown of each plant to the tip of the most expanded leaf. Corn stalks were cut at the soil line for fresh top weight. Remaining root crowns were swirled in water to remove soil debris, dried on paper towels, and weighed. The entire trial was repeated twice for a total of three runs. Symptomatic tissue was plated onto selective media to complete Koch's postulates.

Germination data was analyzed for normality and trial effects. If no trial effects were present, trials were combined for analysis. Data was subjected to

mixed-model analysis of variance (ANOVA), means were separated using Tukey's HSD in JMP (SAS Institute, Cary, North Carolina). For plant emergence, root weight, and plant height trials were normalized against the average non-inoculated control plants of each trial to account for seasonal variation among trials. Ratios and standard deviation were calculated in Excel (Microsoft Corporation, Redmond, WA) and plotted with ggplot2 in R (Wickham 2016; R Core Team 2020).

Results

Isolate Collection and Identification. Symptomatic corn seedlings were collected throughout the state of Delaware from 12 locations in 2019 and 21 locations in 2020. In total, 367 isolates were collected (Table 2.1). In 2019, 108 isolates were recovered from 12 locations throughout the state of Delaware representing 8 different species (*P. arrhenomanes, P. coloratum, P. graminicola, P. inflatum, P. spinosum, P. sylvaticum, P. tardicrescens,* and *P. torulosum*). In 2020, 259 isolates were recovered representing 14 different species (*P. arrhenomanes, P. attrantheridium, P. catenulatum, P. coloratum, P. graminicola, P. inflatum, P. irregulare, P. oligandrum, P. periilum, P. periplocum, P. spinosum, P. sylvaticum, P. tardicrescens,* and *P. torulosum*). Combined across both years, 14 different species were identified in association with corn disease in Delaware. The dominant species was *P. graminicola* accounting for 68.9% of the collection in 2019 and 26.4 % in 2020. Identified

species were grouped into 3 clades based on analysis of the ITS region, predominantly Clade B with members of Clade D and Clade F as well (Figure 2.2).

In Vitro Fungicide Sensitivity Screening. A subset of 25 isolates representing the five most commonly identified species from the survey (*P. graminicola, P. sylvaticum, P. attrantheridium, P. torulosum,* and *P. inflatum*) were selected for *in vitro* fungicide screening to estimate the effective concentration to limit 50% of colony growth Selected isolates included 10 from the 2019 collection and 15 from the 2020 collection. Percent inhibition was determined at each fungicide concentration to generate EC50 values (Figure 2.3). Data from ethaboxam and picarbutrazox required a logarithmic and Box-Cox (lambda 5, 2.065, respectively) transformations to fit normal distribution. EC50 values of mefenoxam ranged from 0.02-0.14 ug ml^{-1} (Table 2.2). For ethaboxam, four of the five species ranged from 0.08 to 0.21 ug ml^{-1}, but *P. torulosum* had an EC50 >10 ug ml^{-1}. Isolates were most sensitive to picarbutrazox with EC50 values ranging from 0.0003 – 0.001 ug ml^{-1}.

Based on the calculated EC50 values, a single concentration of 0.01 ug ml^{-1} for mefenoxam, 0.01 ug ml^{-1} for ethaboxam, or 0.0001 ug ml^{-1} for picarbutrazox was used to determine the differences in sensitivity among thirty-two isolates at the three temperatures. Selected isolates included twelve species: *P. arrhenomanes, P. attrantheridium, P. catenulatum, P. coloratum, P.*

graminicola, P inflatum, P. irregulare, P. periilum, P. spinosum, P. sylvaticum, P. tardicrescens, and *P. torulosum* (Figure 2.4).

In mefenoxam amended plates, *P. attrantheridium* (Figure 2.4), *P. catenulatum, P. coloratum, P. graminicola, P. irregulare, P. periilum, P. spinosum, P. sylvaticum, P. tardicrescens,* and *P. torulosum* all had lower percent inhibition values as ambient temperature increased (Table 2.3). This pattern did not hold true for *P. arrhenomanes* or *P. inflatum.* For species grown on media amended with ethaboxam at 18°C, 21°C, and 25°C, significantly reduced inhibition was seen in *P. coloratum, P. graminicola, P. inflatum, P. sylvaticum,* and *P. tardicresens* between temperatures of 18°C and 25°C. Temperature caused no significant difference in percent inhibition of *P. arrhenomanes, P. attrantheridium* (Figure 2.4)*, P. catenulatum, P. irregulare, P. periilum, P. spinosum,* or *P. torulosum* on ethaboxam amended media. In samples tested against picarbutrazox, temperature was unrelated to inhibition in *P. inflatum, P. irregulare,* and *P. torulosum.* All other species tested demonstrated significant reduced average inhibition between 18°C and 25°C on picarbutrazox treated plates.

In mefenoxam-amended plates at 18°C, the highest percent inhibition was observed in *P. catenulatum* (76.1%) and *P. torulosum* (73.3%) (Table 2.3). At 21°C, *P. torulosum* (69.9%) and *P. catenulatum* (63.1%) continued to have the highest percent inhibition values. The species least sensitive to mefenoxam at

21°C was *P. irregulare.* Among all species, *P. irregulare, P. spinosum, P. graminicola, P.coloratum, P. tardicrescens, P. periilum, and P. inflatum* had the lowest percent inhibition values on mefenoxam amended media. In all cases, the least percent inhibition was observed at 25°C.

Ethaboxam treated plates had the highest percent inhibition for *P. arrhenomanes* and *P. catenulatum* when incubated at temperatures of 18°C and 25°C (Table 2.3). Of all the species tested at 21°C*, P. catenulatum* was inhibited the most by ethaboxam. At both 18°C and 21°C, the species *P. spinosum* and *P. torulosum* were found to be least inhibited by ethaboxam. Isolates of *P. irregulare, P. spinosum, P. sylvaticum,* and *P. torulosum* demonstrated the least inhibition to ethaboxam at 25°C.

In the picarbutrazox trial at 18°C, *P. graminicola, P. inflatum,* and *P. periilum* were inhibited the most and *P. sylvaticum* had significantly less inhibition (Table 2.3). At 21°C, on picarbutrazox media, *P. graminicola, P. inflatum, P. periilum,* and *P. tardicrescens* had the highest percent inhibition and *P. catenulatum, P. spinosum,* and *P. sylvaticum* had the lowest percent inhibition. At 25°C, picarbutrazox inhibited *P. tardicrescens* significantly more than other species and *P. sylvaticum* significantly less than the other species.

Greenhouse Panels. Twelve species *P. sylvaticum, P. irregulare, P. inflatum, P. coloratum, P. spinosum, P. arrhenomanes, P. graminicola, P. attrantheridium, P. torulosum, P. tardicrescens, P. catenulatum,* and *P. periilum* were assessed

for isolate aggressiveness in greenhouse panels (Figure 2.5). Only one species, *P. inflatum,* was found to have a significantly lower germination rate of field corn than the control (Table 2.4). There was no significant difference observed in germination rates of sweet corn plants inoculated with *Pythium* species as compared to the uninoculated control (Table 2.5). Any plants that did not germinate or that were damaged by rodents (trial 2 only) were removed from the data set for analysis of root weight and plant height. Values from within each trial were normalized for average control values (Figure 2.6) and no significant difference were observed in root weight or plant height of field or sweet corn.

Discussion

Previous surveys of *Pythium* conducted in the mid-west United States have identified numerous species pathogenic to corn. This survey is the first comprehensive analysis of *Pythium* species distribution on corn in the MAR. Of the 14 species identified, *P. coloratum* has not been previously reported as pathogenic to corn in the United States. Koch's postulates were conducted to confirm pathogenicity. The species identified in this survey shared some overlap with similar characterization studies by Broders et al. (2007), Dorrance et al. (2004), Jiang et al. (2012), and Rojas et al (2019) that also isolated *P. attrantheridium, P. graminicola, P. inflatum, P. sylvaticum, P. torulosum, P. catenulatum, P. periilum, P. oligandrum,* and *P. arrhenomanes*. Although composition shared similarities, there were differences in dominant species

identified. Notably *P. graminicola* represented more than 50% of the MAR collection, but only about 4% of the 105 corn-related isolates recovered in Broders et al. (2007), where *P. dissotocum* represented the majority at over 25%. In stark contrast to the current MAR study, other corn-related *Pythium* surveys performed by Dorrance et al. (2004) and Rojas et al. (2019), as well as soil-surveys from fields on corn-soybean rotation performed by Jiang et al. (2012), all recovered no isolates of *P. graminicola,* but instead found majorities of *P. splendens, P. inflatum,* and *P. oopapillum*, respectively.

These differences in species composition and prevalence in comparison to similar studies from other regions could be attributed to numerous conditional factors present in Delaware, including sandier soils as compared to richer, loam-filled soils of the Midwest; also the differences in average temperature where low extremes are not as common in Delaware as they are in parts of the Midwest. Similarities, however do exist in the possible multi-host crop rotation conditions that are often common in Delaware and the Midwest, in which *Pythium* species can infect common cover crops like rye as well as the cash crops seen the average corn-soybean-wheat rotation.

As compared to other species of corn-pathogenic *Pythium, P. graminicola* grows optimally at 30°C, which is slightly above the average optimum of about 26°C. Species *P. splendens* and *P. oopapillum,* which were the most commonly found species from other surveys in the United States both

have lower growth temperature thresholds than *P. graminicola.* The optimum, minimum, and maximum of *P. graminicola* has been observed to be 30ºC, 5ºC, and 38ºC. Samples of *P. inflatum* were shown to have a similar range at 30ºC, 5ºC, 37ºC. The most common species found by Dorrance et al. (2004), *P. splendens* (25ºC, 5ºC, 34ºC), has a similar optimum to *P. graminicola,* but a slightly lower high temperature tolerance. The most common corn-pathogenic species found in the soil survey of corn-soybean fields by Jiang et al. (2012), *P. oopapillum* (30ºC, 5ºC, 30ºC), had a much lower high-temperature threshold than *P. graminicola*.

The isolates tested in the EC50 determination experiments for mefenoxam, ethaboxam, and picarbutrazox represented five of the most commonly recovered species in the survey at the time of testing (Table 2.2). While all species were inhibited by picarbutrazox, *P. sylvaticum* had the highest EC50 value. Within mefenoxam screening, *P. inflatum* had the highest EC50 value. In a study performed by Rojas et al. (2019), *P. ultimum* var. *ultimum* had the highest EC50 for mefenoxam at >100 ug ml^{-1}, while isolates of *P. inflatum* had average EC50 values of 0.638 ug ml^{-1} mefenoxam. This is similar to our study in which EC50 values for of mefenoxam in *P. inflatum* was 0.14ug ml^{-1}. Broders et al. (2007) found higher inhibition of *P. inflatum* compared to *P. graminicola* on 5 ug ml^{-1} mefenoxam, in which growth was limited to 80% and 70% of their respective, untreated controls. This relationship is reversed in our

study where *P. inflatum* percent inhibition tracks lower than *P. graminicola,* particularly at 18°C (Table 2.3).

The most notable result of EC50 trials was the greater than 10 ug ml^{-1} response of *P. torulosum* isolates to ethaboxam. Rojas et al. (2019) found a similar effect in samples of *P.* aff. *torulosum* which exhibited EC50 estimations greater than 100 ppm *in vitro.* Radmer et al. (2017) found that samples of *P. acrogynum* (not yet reported on corn) were the least inhibited by ethaboxam. Matthiesen et al. (2016) found that samples of *P. torulosum* on ethaboxam amended media did not grow significantly differently than the other three species tested. The results of the EC50 comparisons with this trial and the similarity of insensitivity in *P. torulosum* seen in Broders et al. (2007) point to the possibility that the species *P. torulosum* may not be as effectively managed by seed treatment products compared to other species. .

In the temperature trials on mefenoxam amended media, there was no significant change in percent inhibition of species *P. arrhenomanes, P. inflatum,* or *P. periilum* (Table 2.3). In all other species, there was a pattern that emerged for 10 of the 12 species that as temperature increased mefenoxam was significantly less efficacious. In isolates of *P. sylvaticum,* there was a significant reduction in inhibition at temperatures above 18ºC. Samples of *P. catenulatum, P. coloratum,* and *P. torulosum* showed significant reductions of inhibition above 21ºC. Isolates of *P. attrantheridium, P. irregulare, P. spinosum,* and *P.*

tardicrescens showed significant reductions in mefenoxam efficacy at 25ºC compared to 18ºC. Samples of *P. graminicola* showed significant decline in inhibition at each temperature increase. Matthiesen et al. (2016) tested four species against metalaxyl and found that *P. lutarium* and *P. sylvaticum* demonstrated a similar pattern in which fungicide efficacy of colony inhibition decreased as temperature increased. This same pattern did not hold true when *P. oopapillum* and *P. torulosum* were tested at three temperatures on metalaxyl (Matthiesen et al. 2016).

Reduced inhibition was observed in 5 of the 12 species tested on ethaboxam as the temperature increased. The isolates of *P. catenulatum* that were tested in this experiment proved to be highly sensitive to ethaboxam, showing nearly 100% inhibition, whereas *P. torulosum* had an EC50 >10 ppm coinciding with nearly 0% inhibition. This range of response highlights the value of understanding isolate composition in relation to management decisions. Isolates of *P. arrhenomanes, P. attrantheridium, P. catenulatum, P. irregulare, P. periilum, P. spinosum,* and *P. torulosum* showed no significant difference in ethaboxam efficacy of inhibition as temperatures changed. Samples of *P. coloratum, P. graminicola, P. inflatum, P. sylvaticum,* and *P. tardicrescens* tested on ethaboxam all showed significant reductions in colony inhibition as temperatures increased between 18ºC and 25ºC.

In the picarbutrazox screening, when temperatures increased, percent inhibition values decreased significantly in 10 of the 12 species. Above temperatures of 18ºC there was a reduction in inhibition of *P. tardicrescens* by picarbutrazox. At temperatures greater than 21ºC there was a significant drop in the efficacy of colony inhibition in isolates of *P. arrhenomanes, P. attrantheridium, P. catenulatum, P. graminicola, P. irregulare, P. periilum, P, spinosum,* and *P. sylvaticum* This pattern was not significant in isolates of *P. inflatum* or *P. torulosum.*

Mefenoxam and the stereoisomer metalaxyl are functionally similar and both inhibit the growth of *Pythium* by interrupting RNA synthesis, effectively impeding the cell's ability to translate information arriving from the nucleus to be turned into proteins (Koeller 1991; Lévesque et al. 2010). The Fungicide Resistance Action Committee (FRAC) states that phenylamides like mefenoxam and metalaxyl are high risk for the development of resistance in target organisms (FRAC 2021b). Cross-resistance to mefenoxam and metalaxyl has been documented from use of other phenylamides such as furalaxyl, ofurace, benalaxyl, cyprofuram, and oxadixyl (Koeller 1991; White et al. 1988). Resistance to mefenoxam has been reported in a number of *Pythium* species, but to date no resistance has been reported within corn production.

Ethaboxam is a microtubule destabilizer that affects β-tubulin in mitosis causing problems with cellular structure during division, thus inhibiting growth

(FRAC 2021b). It is classified as medium to low risk for resistance development potential and can potentially have cross resistance with zoxamide (FRAC 2021b).

Picarbutrazox is an oomycete targeted fungicide with a new and yet undetermined mode of action (FRAC 2021c). Since resistance to mefenoxam has been observed on *Pythium* species in other cropping systems, the ability to rotate with additional modes of action strengthens integrated management strategies. No cross resistance to PhenylAmides like mefenoxam or other fungicides have been found. The first commercial product containing picarbutrazox was released in 2021, so all isolates screened in the current trial had no prior exposure to picarbutrazox. The EC50 data generated in this trial will serve as a baseline to track continued performance of picarbutrazox as it enters the market and is used more widely within corn production.

In recent seasons, corn in Delaware fields has averaged 100 Growing Degree Days (GDD) to emerge (Miller 2020). The GDD calculation is affected by both the minimum and maximum temperature recorded each day. Cooler temperatures earlier in the season require more days to meet the threshold GDD for germination. Though the increased days to meet GDD in cooler conditions result in the seed being in the soil longer, the fungicides screened in this study should also be functioning at highest efficacy at early season soil temperatures.

This caveat, however, must be balanced with field history, environmental conditions at the time of planting, and field history.

In the greenhouse panels, *P. inflatum* was the only species to significantly impact germination, (Table 2.4). Rojas et al. (2019) performed a similar study in which corn plants were inoculated with various *Pythium* species and the metrics of plant height and weight were used to make inferences about aggressiveness. In the Rojas trial, *P. inflatum* caused a significant reduction ($p < 0.05$) in both metrics. Radmer et al. (2017) presents their aggressiveness data for *Pythium* panels in terms of ratios of treated root mass growth compared to uninoculated controls. They found a 1.4 to 1 ratio of untreated to treated root mass, which reduced significantly ($p < 0.05$) at increased temperatures (Radmer et al 2017). Broders et al. (2007) report that *Pythium inflatum* caused lesions on the roots, but no significant difference in pathogenicity based on germination rates and symptomology. Seasonal variations were observed among trials with corn growing better as day length increased and greenhouse conditions became warmer. Future trials could include inoculation optimization to maximize post-emergence symptoms in inoculated plants for a more robust comparison of species effects.

The outcome that multiple species of *Pythium* were identified as causal agents of corn damping-off in the current Delaware study is consistent with results generated in other regions of the United States. Unlike other studies,

Pythium graminicola was the dominant isolate recovered and one new species, *P. coloratum,* was reported for the first time in corn. *In vitro* trials for EC50 values indicated varying percent inhibition among species. In controlled temperature trials, increasing temperatures reduced the percent inhibition across most species in all active ingredients tested. The results of this data have implications when considering optimal planting date and impact of environmental conditions on fungicide response and level of disease observed.

This study provides baseline data that can be expanded in future trials. Future greenhouse aggressiveness trials that incorporate seed applied fungicide treatments for comparisons of different *Pythium* species' aggressiveness versus non-treated seeds could be an avenue of exploration. On-farm field studies aimed at improved understanding of planting date, seed treatment, and *Pythium* species composition interactions may also provide useful information to improve management. As planting dates trend earlier and extend germination times, emerging seedlings may lose the efficacy of the seed applied fungicide before they are large enough to survive infection. Therefore, the quantification of the speed of chemical degradation and decrease of efficacy of the seed treatment fungicides in the soil in relationship to individual species of *Pythium* may also be of value.

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Table 2.1 Genbank accessions and species identification of *Pythium* species isolated in 2019 and 2020

Isolate No.	Species	Collection Year	Clade	#Accession
CP1901	*Pythium sylvaticum*	2019	F	MZ540448[z]
CP1902	*Pythium dissotocum*	2019	B	MZ540449
CP1903	*Pythium sylvaticum*	2019	F	MZ540450
CP1904	*Pythium sylvaticum*	2019	F	MZ540451
CP1905	*Pythium coloratum*	2019	B	MZ540452
CP1906	*Pythium graminicola*	2019	B	MZ540453
CP1908	*Pythium dissotocum*	2019	B	MZ540454
CP1909	*Pythium torulosum*	2019	B	MZ540455
CP1910	*Pythium torulosum*	2019	B	MZ540456
CP1911	*Pythium graminicola*	2019	B	MZ540457
CP1912	*Pythium graminicola*	2019	B	MZ540458
CP1913	*Pythium graminicola*	2019	B	MZ540459
CP1914	*Pythium graminicola*	2019	B	MZ540460
CP1915	*Pythium graminicola*	2019	B	MZ540461
CP1916	*Pythium graminicola*	2019	B	MZ540462
CP1917	*Pythium graminicola*	2019	B	MZ540463
CP1918	*Pythium aristosporum*	2019	B	MZ540464
CP1919	*Pythium arrhenomanes*	2019	B	MZ540465
CP1920	*Pythium arrhenomanes*	2019	B	MZ540466
CP1921	*Pythium graminicola*	2019	B	MZ540467
CP1922	*Pythium arrhenomanes*	2019	B	MZ540468
CP1923	*Pythium arrhenomanes*	2019	B	MZ540469
CP1924	*Pythium tardicrescens*	2019	B	MZ540470
CP1925	*Pythium tardicrescens*	2019	B	MZ540471
CP1927	*Pythium inflatum*	2019	B	MZ540472
CP1928	*Pythium torulosum*	2019	B	MZ540473
CP1929	*Pythium graminicola*	2019	B	MZ540474

CP1930	*Pythium graminicola*	2019	B	MZ540475
CP1931	*Pythium inflatum*	2019	B	MZ540476
CP1932	*Pythium inflatum*	2019	B	MZ540477
CP1933	*Pythium graminicola*	2019	B	MZ540478
CP1934	*Pythium inflatum*	2019	B	MZ540479
CP1935	*Pythium inflatum*	2019	B	MZ540480
CP1936	*Pythium inflatum*	2019	B	MZ540481
CP1937	*Pythium periilum*	2019	B	MZ540482
CP1938	*Pythium aristosporum*	2019	B	MZ540483
CP1939	*Pythium aristosporum*	2019	B	MZ540484
CP1940	*Pythium torrulosum*	2019	B	MZ540485
CP1941	*Pythium arrhenomanes*	2019	B	MZ540486
CP1942	*Pythium graminicola*	2019	B	MZ540487
CP1943	*Pythium graminicola*	2019	B	MZ540488
CP1944	*Pythium arrhenomanes*	2019	B	MZ540489
CP1945	*Pythium graminicola*	2019	B	MZ540490
CP1946	*Pythium coloratum*	2019	B	MZ540491
CP1947	*Pythium graminicola*	2019	B	MZ540492
CP1948	*Pythium graminicola*	2019	B	MZ540493
CP1949	*Pythium graminicola*	2019	B	MZ540494
CP1950	*Pythium periplocum*	2019	D	MZ540495
CP1951	*Pythium graminicola*	2019	B	MZ540496
CP1952	*Pythium graminicola*	2019	B	MZ540497
CP1953	*Pythium graminicola*	2019	B	MZ540498
CP1954	*Pythium graminicola*	2019	B	MZ540499
CP1955	*Pythium graminicola*	2019	B	MZ540500
CP1956	*Pythium graminicola*	2019	B	MZ540501
CP1957	*Pythium graminicola*	2019	B	MZ540502
CP1958	*Pythium graminicola*	2019	B	MZ540503
CP1959	*Pythium graminicola*	2019	B	MZ540504

CP1960	*Pythium graminicola*	2019	B	MZ540505
CP1961	*Pythium tardicrescens*	2019	B	MZ540506
CP1962	*Pythium graminicola*	2019	B	MZ540507
CP1963	*Pythium graminicola*	2019	B	MZ540508
CP1964	*Pythium spinosum*	2019	F	MZ540509
CP1965	*Pythium graminicola*	2019	B	MZ540510
CP1966	*Pythium spinosum*	2019	F	MZ540511
CP1967	*Pythium graminicola*	2019	B	MZ540512
CP1968	*Pythium graminicola*	2019	B	MZ540513
CP1969	*Pythium graminicola*	2019	B	MZ540514
CP1970	*Pythium graminicola*	2019	B	MZ540515
CP1971	*Pythium graminicola*	2019	B	MZ540516
CP1972	*Pythium graminicola*	2019	B	MZ540517
CP1973	*Pythium graminicola*	2019	B	MZ540518
CP1974	*Pythium spinosum*	2019	F	MZ540519
CP1975	*Pythium graminicola*	2019	B	MZ540520
CP1976	*Pythium graminicola*	2019	B	MZ540521
CP1977	*Pythium graminicola*	2019	B	MZ540522
CP1978	*Pythium graminicola*	2019	B	MZ540523
CP1979	*Pythium graminicola*	2019	B	MZ540524
CP1980	*Pythium graminicola*	2019	B	MZ540525
CP1981	*Pythium graminicola*	2019	B	MZ540526
CP1982	*Pythium graminicola*	2019	B	MZ540527
CP1983	*Pythium graminicola*	2019	B	MZ540528
CP1984	*Pythium sylvaticum*	2019	F	MZ540529
CP1985	*Pythium spinosum*	2019	F	MZ540530
CP1986	*Pythium graminicola*	2019	B	MZ540531
CP1987	*Pythium graminicola*	2019	B	MZ540532
CP1988	*Pythium graminicola*	2019	B	MZ540533
CP1989	*Pythium graminicola*	2019	B	MZ540534

CP1990	*Pythium graminicola*	2019	B	MZ540535
CP2001	*Pythium graminicola*	2020	B	MZ540536
CP2002	*Pythium graminicola*	2020	B	MZ540537
CP2003	*Pythium graminicola*	2020	B	MZ540538
CP2004	*Pythium graminicola*	2020	B	MZ540539
CP2005	*Pythium graminicola*	2020	B	MZ540540
CP2006	*Pythium graminicola*	2020	B	MZ540541
CP2007	*Pythium graminicola*	2020	B	MZ540542
CP2008	*Pythium graminicola*	2020	B	MZ540543
CP2009	*Pythium torulosum*	2020	B	MZ540544
CP2010	*Pythium attrantheridium*	2020	F	MZ540545
CP2011	*Pythium attrantheridium*	2020	F	MZ540546
CP2012	*Pythium oligandrum*	2020	D	MZ540547
CP2013	*Pythium graminicola*	2020	B	MZ540548
CP2014	*Pythium graminicola*	2020	B	MZ540549
CP2015	*Pythium graminicola*	2020	B	MZ540550
CP2016	*Pythium spinosum*	2020	F	MZ540551
CP2017	*Pythium coloratum*	2020	B	MZ540552
CP2018	*Pythium spinosum*	2020	F	MZ540553
CP2019	*Pythium spinosum*	2020	F	MZ540554
CP2020	*Pythium graminicola*	2020	B	MZ540555
CP2021	*Pythium graminicola*	2020	B	MZ540556
CP2022	*Pythium graminicola*	2020	B	MZ540557
CP2023	*Pythium graminicola*	2020	B	MZ540558
CP2024	*Pythium graminicola*	2020	B	MZ540559
CP2025	*Pythium graminicola*	2020	B	MZ540560
CP2026	*Pythium graminicola*	2020	B	MZ540561
CP2027	*Pythium torulosum*	2020	B	MZ540562
CP2028	*Pythium graminicola*	2020	B	MZ540563
CP2029	*Pythium torulosum*	2020	B	MZ540564

CP2030	*Pythium torulosum*	2020	B	MZ540565
CP2031	*Pythium graminicola*	2020	B	MZ540566
CP2032	*Pythium torulosum*	2020	B	MZ540567
CP2033	*Pythium graminicola*	2020	B	MZ540568
CP2034	*Pythium torulosum*	2020	B	MZ540569
CP2035	*Pythium torulosum*	2020	B	MZ540570
CP2036	*Pythium graminicola*	2020	B	MZ540571
CP2037	*Pythium torulosum*	2020	B	MZ540572
CP2038	*Pythium graminicola*	2020	B	MZ540573
CP2039	*Pythium torulosum*	2020	B	MZ540574
CP2040	*Pythium graminicola*	2020	B	MZ540575
CP2041	*Pythium graminicola*	2020	B	MZ540576
CP2042	*Pythium graminicola*	2020	B	MZ540577
CP2043	*Pythium tardicrescens*	2020	B	MZ540578
CP2044	*Pythium irregulare*	2020	B	MZ540579
CP2045	*Pythium graminicola*	2020	B	MZ540580
CP2046	*Pythium graminicola*	2020	B	MZ540581
CP2047	*Pythium graminicola*	2020	B	MZ540582
CP2048	*Pythium graminicola*	2020	B	MZ540583
CP2049	*Pythium attratheridium*	2020	F	MZ540584
CP2050	*Pythium graminicola*	2020	B	MZ540585
CP2051	*Pythium graminicola*	2020	B	MZ540586
CP2052	*Pythium coloratum*	2020	B	MZ540587
CP2053	*Pythium graminicola*	2020	B	MZ540588
CP2054	*Pythium graminicola*	2020	B	MZ540589
CP2055	*Pythium graminicola*	2020	B	MZ540590
CP2056	*Pythium torulosum*	2020	B	MZ540591
CP2057	*Pythium graminicola*	2020	B	MZ540592
CP2058	*Pythium graminicola*	2020	B	MZ540593
CP2059	*Pythium graminicola*	2020	B	MZ540594

CP2060	*Pythium graminicola*	2020	B	MZ540595
CP2061	*Pythium graminicola*	2020	B	MZ540596
CP2062	*Pythium graminicola*	2020	B	MZ540597
CP2063	*Pythium graminicola*	2020	B	MZ540598
CP2064	*Pythium graminicola*	2020	B	MZ540599
CP2065	*Pythium graminicola*	2020	B	MZ540600
CP2066	*Pythium graminicola*	2020	B	MZ540601
CP2067	*Pythium torulosum*	2020	B	MZ540602
CP2068	*Pythium catenulatum*	2020	B	MZ540603
CP2069	*Pythium graminicola*	2020	B	MZ540604
CP2070	*Pythium coloratum*	2020	B	MZ540605
CP2071	*Pythium graminicola*	2020	B	MZ540606
CP2072	*Pythium graminicola*	2020	B	MZ540607
CP2073	*Pythium graminicola*	2020	B	MZ540608
CP2074	*Pythium graminicola*	2020	B	MZ540609
CP2075	*Pythium graminicola*	2020	B	MZ540610
CP2076	*Pythium graminicola*	2020	B	MZ540611
CP2077	*Pythium periilum*	2020	B	MZ540612
CP2078	*Pythium tardicrescens*	2020	B	MZ540613
CP2079	*Pythium graminicola*	2020	B	MZ540614
CP2080	*Pythium graminicola*	2020	B	MZ540615
CP2081	*Pythium graminicola*	2020	B	MZ540616
CP2082	*Pythium graminicola*	2020	B	MZ540617
CP2083	*Pythium periplocum*	2020	D	MZ540618
CP2084	*Pythium graminicola*	2020	B	MZ540619
CP2085	*Pythium graminicola*	2020	B	MZ540620
CP2086	*Pythium catenulatum*	2020	B	MZ540621
CP2087	*Pythium graminicola*	2020	B	MZ540622
CP2088	*Pythium graminicola*	2020	B	MZ540623
CP2089	*Pythium graminicola*	2020	B	MZ540624

CP2090	*Pythium graminicola*	2020	B	MZ540625
CP2091	*Pythium graminicola*	2020	B	MZ540626
CP2092	*Pythium graminicola*	2020	B	MZ540627
CP2093	*Pythium graminicola*	2020	B	MZ540628
CP2094	*Pythium graminicola*	2020	B	MZ540629
CP2095	*Pythium graminicola*	2020	B	MZ540630
CP2096	*Pythium graminicola*	2020	B	MZ540631
CP2097	*Pythium graminicola*	2020	B	MZ540632
CP2098	*Pythium graminicola*	2020	B	MZ540633
CP2099	*Pythium graminicola*	2020	B	MZ540634
CP20100	*Pythium graminicola*	2020	B	MZ540635
CP20101	*Pythium graminicola*	2020	B	MZ540636
CP20102	*Pythium graminicola*	2020	B	MZ540637
CP20103	*Pythium graminicola*	2020	B	MZ540638
CP20104	*Pythium graminicola*	2020	B	MZ540639
CP20105	*Pythium graminicola*	2020	B	MZ540640
CP20106	*Pythium graminicola*	2020	B	MZ540641
CP20107	*Pythium graminicola*	2020	B	MZ540642
CP20108	*Pythium graminicola*	2020	B	MZ540643
CP20109	*Pythium graminicola*	2020	B	MZ540644
CP20110	*Pythium graminicola*	2020	B	MZ540645
CP20111	*Pythium graminicola*	2020	B	MZ540646
CP20112	*Pythium graminicola*	2020	B	MZ540647
CP20113	*Pythium graminicola*	2020	B	MZ540648
CP20114	*Pythium graminicola*	2020	B	MZ540649
CP20115	*Pythium graminicola*	2020	B	MZ540650
CP20116	*Pythium graminicola*	2020	B	MZ540651
CP20117	*Pythium graminicola*	2020	B	MZ540652
CP20118	*Pythium graminicola*	2020	B	MZ540653
CP20119	*Pythium graminicola*	2020	B	MZ540654

CP20120	*Pythium graminicola*	2020	B	MZ540655
CP20121	*Pythium graminicola*	2020	B	MZ540656
CP20122	*Pythium torulosum*	2020	B	MZ540657
CP20123	*Pythium torulosum*	2020	B	MZ540658
CP20124	*Pythium arrhenomanes*	2020	B	MZ540659
CP20125	*Pythium torulosum*	2020	B	MZ540660
CP20126	*Pythium inflatum*	2020	B	MZ540661
CP20128	*Pythium irregulare*	2020	B	MZ540662
CP20129	*Pythium sylvaticum*	2020	F	MZ540663
CP20130	*Pythium sylvaticum*	2020	F	MZ540664
CP20131	*Pythium graminicola*	2020	B	MZ540665
CP20132	*Pythium graminicola*	2020	B	MZ540666
CP20133	*Pythium graminicola*	2020	B	MZ540667
CP20134	*Pythium graminicola*	2020	B	MZ540668
CP20135	*Pythium graminicola*	2020	B	MZ540669
CP20136	*Pythium graminicola*	2020	B	MZ540670
CP20137	*Pythium graminicola*	2020	B	MZ540671
CP20138	*Pythium graminicola*	2020	B	MZ540672
CP20139	*Pythium inflatum*	2020	B	MZ540673
CP20140	*Pythium graminicola*	2020	B	MZ540674
CP20141	*Pythium graminicola*	2020	B	MZ540675
CP20142	*Pythium graminicola*	2020	B	MZ540676
CP20143	*Pythium sylvaticum*	2020	F	MZ540677
CP20144	*Pythium attrantheridium*	2020	F	MZ540678
CP20145	*Pythium attrantheridium*	2020	F	MZ540679
CP20146	*Pythium graminicola*	2020	B	MZ540680
CP20147	*Pythium tardicrescens*	2020	B	MZ540681
CP20148	*Pythium attrantheridium*	2020	F	MZ540682
CP20149	*Pythium irregulare*	2020	B	MZ540683
CP20150	*Pythium irregulare*	2020	B	MZ540684

CP20151	*Pythium graminicola*	2020	B	MZ540685
CP20152	*Pythium graminicola*	2020	B	MZ540686
CP20153	*Pythium graminicola*	2020	B	MZ540687

[z] Accessions based on amplification of the ITS region.

Table 2.2 Effective concentration of fungicide to limit 50% of colony growth (EC50) (µg/µL) of *P. graminicola, P. sylvaticum, P. attrantheridium, P. torulosum,* and *P. inflatum in vitro.*

	EC50[z] Values by Fungicide		
Pythium spp.	**Mefenoxam**	**Ethaboxam**	**Picarbutrazox**
P. attrantheridium	0.02	0.10	0.0009
P. graminicola	0.05	0.10	0.0003
P. inflatum	0.14	0.08	0.0005
P. sylvaticum	0.04	0.21	0.0022
P. torulosum	0.04	>10.00[y]	0.0010

[z] EC50 values calculated using linear regression of percent inhibition data points against logarithmic transformation of the concentrations (µg/µL) in the amended petri plates.
[y] Calculated EC50 value exceeded the measured range.

Table 2.3 *In vitro* fungicide sensitivity of twelve *Pythium* species to mefenoxam, ethaboxam, and picarbutrazox at three temperatures.

		Percent inhibition ± SE (range)[z]		
Pythium spp.	**ºC**	**Mefenoxam**	**Ethaboxam**	**Picarbutrazox**
P. arrhenomanes (n = 3)[y]	18	47.5 ± 7.1 (9.2-74.1) cde	91.8 ± 3.6 (71.1-100.0) a	88.3 ± 1.1 (80.8-94.3) bc
	21	40.3 ± 4.7 (14.8-63.0) def	77.3 ± 1.9 (68.1-85.8) bc	83.8 ± 1.1 (75.7-90.7) cd
	25	38.1 ± 3.3 (23.8-59.2) defg	85.6 ± 4.00 (61.6-100.0) ab	70.7 ± 3.9 (53.3-94.2) ef
P. attrantheridium (n = 3)	18	39.5 ± 5.3 (13.4-74.0) def	52.7 ± 2.6 (37.1-69.3) ef	85.81 ± 3.03 (67.0-100.0) bc
	21	30.5 ± 6.0 (2.9-69.2) efgh	45.5 ± 2.5 (34.6-61.6) fg	87.0 ± 4.5 (58.9-100.0) bc
	25	19.2 ± 6.6 (0.0-64.6) hijk	40.3 ± 2.0 (26.1-51.2) fg	64.6 ± 4.4 (35.4-82.6) fg
P. catenulatum (n = 1)	18	76.1 ± 1.7 (70.8-79.0) a	95.0 ± 0.4 (94.0-95.9) a	70.8 ± 0.8 (69.1-73.2) ghi
	21	63.1 ± 0.6 (61.5-64.7) abc	100.0 ± 0.0 (100.0-100.0) a	67.2 ± 0.03 (67.1-67.3) ij
	25	45.1 ± 1.1 (41.3-48.4) cde	100.0 ± 0.0 (100.0-100.0) a	53.1 ± 1.0 (49.4-56.0) k
P. coloratum (n = 3)	18	50.5 ± 3.4 (42.0-84.2) bcd	24.8 ± 5.4 (0.0-61.7) ijk	55.5 ± 6.7 (35.9-100.0) def
	21	40.3 ± 4.8 (28.0-88.0) def	18.9 ± 5.6 (0.0-55.2) jkl	45.7 ± 8.1 (22.8-100.0) efg
	25	9.9 ± 5.1 (0.0-61.2) jkl	13.5 ± 6.0 (0.0-57.6) lmn	24.3 ± 10.9 (0.0-97.4) ghij
P. graminicola (n = 3)	18	41.4 ± 6.8 (20.6-87.4) de	59.9 ± 1.7 (51.3-69.2) def	100.0 ± 0.0 (100.0-100.0) ab
	21	27.8 ± 5.1 (2.4-62.2) fghi	50.2 ± 1.5 (41.5-59.4) f	100.0 ± 0.0 (100.0-100.0) a
	25	8.3 ± 3.0 (0.0-33.4) kl	35.4 ± 1.7 (25.2-48.8) ghi	81.9 ± 1.7 (70.7-96.2) cd
P. inflatum (n = 3)	18	19.3 ± 2.4 (2.2-25.3) hijk	71.4 ± 2.4 (58.9-81.9) cd	95.4 ± 0.6 (91.1-97.9) ab
	21	19.6 ± 3.5 (0-32.5) hijk	64.0 ± 2.7 (55.0-76.5) cde	92.7 ± 0.4 (90.1-94.2) abc
	25	16.3 ± 2.4 (5.7-27.6) hijkl	51.0 ± 2.2 (43.6-61.1) f	89.0 ± 0.6 (85.2-90.5) bc
P. irregulare (n = 3)	18	22.7 ± 4.970 (0.0-46) hi	18.6 ± 5.2 (0.0-51.3) kl	75.6 ± 3.8 (60.2-100.0) def
	21	14.0 ± 3.0 (0.0-34.6) ijkl	16.41 ± 6.4 (0.0-68.6) klm	69.4 ± 4.7 (50.2-100.0) ef
	25	4.4 ± 2.0 (0.0-22.0) l	8.1 ± 3.7 (0.0-37.2) lmno	49.4 ± 6.4 (22.8-93.2) hij
P. periilum (n = 1)	18	50.1 ± 0.4 (49.3-50.8) bcde	60.3 ± 0.3 (59.7-60.9) cdef	100.0 ± 0.0 (100.0-100.0) ab
	21	30.1 ± 4.8 (21.8-38.5) efghi	55.0 ± 1.5 (52.4-57.5) def	100.0 ± 0.0 (100.0-100.0) ab

	25	16.1 ± 2.0 (12.2-20.0) hijkl	39.8 ± 1.7 (35.7-43.8) fghi	78.0 ± 2.1 (73.5-82.2) cdef
P. spinosum	18	38.2 ± 4.0 (11.7-72.8) defg	5.9 ± 2.1 (0.0-23.2) mno	57.5 ± 0.9 (50.6-63.4) hg
(n = 3)	21	27.2 ± 4.1 (0.0-51.0) ghi	3.3 ± 1.6 (0.0-20.4) no	51.2 ± 0.8 (45.2-56.4) hij
	25	6.0 ± 2.1 (0.0-23.2) l	0.0 ± 0.0 (0.0-0.0) o	25.4 ± 0.9 (18.5-30.2) k
P. sylvaticum	18	65.6 ± 6.2 (35.9-100.0) ab	37.8 ± 10.8 (0.0-100.0) ghi	44.3 ± 0.9 (38.0-50.4) j
(n = 3)	21	46.3 ± 4.8 (26.7-77.2) cde	26.5 ± 7.34 (0.0-86.0) ijk	40.2 ± 2.9 (27.0-73.0) j
	25	34.5 ± 5.3 (9.4-60.2) efgh	4.0 ± 1.9 (0.0-21.6) no	3.2 ± 1.5 (0.0-18.4) l
P. tardicrescens	18	31.1 ± 7.6 (0.0-88.7) efgh	49.9 ± 1.8 (40.2-59.5) f	100.0 ± 0.0 (100.0-100.0) cd
(n = 3)	21	20.4 ± 4.4 (2.9-50.8) hij	39.9 ± 1.5 (30.6-47.0) fgh	99.0 ± 0.4 (96.9-100.0) ab
	25	14.0 ± 3.0 (0.0-30.2) ijkl	29.6 ± 0.9 (23.7-36.6) hij	82.0 ± 1.9 (65.0-96.0) ab
P. torulosum	18	73.3 ± 1.5 (61.2-81.7) a	0.0 ± 0.0 (0.0-0.0) o	91.2 ± 0.4 (89.2-94.1) abc
(n = 3)	21	69.9 ± 0.8 (65.8-74.1) a	0.7 ± 0.3 (0.0-3.0) o	86.5 ± 0.5 (82.5-90.1) bc
	25	46.2 ± 1.3 (34.8-52.1) cde	2.7 ± 1.2 (0.0-19.2) o	79.5 ± 0.6 (75.0-81.9) cde

[z] Percent inhibition calculated as (1 – (treated colony (mm) / untreated control (mm)) * 100%. Values are least square means of three replicates per isolate and temperature over two experimental runs.
[y] Number of isolates per species evaluated for sensitivity to fungicides.

Table 2.4 Corn percent-germination rates by treatment in *Pythium* greenhouse panel.

Treatment	% Germination Rate (Mean ± SE)
Control[z]	100.0 ± 7.8 a[y]
P. graminicola	100.0 ± 11.1 ab
P. irregulare	100.0 ± 11.1 ab
P. sylvaticum	100 ± 11.1 ab
P. torulosum	100.0 ± 11.1 ab
P. arrhenomanes	87.5 ± 11.1 ab
P. attrantheridium	87.5 ± 11.1 ab
P. catenulatum	87.5 ± 11.1 ab
P. periilum	87.5 ± 11.1 ab
P. tardicrescens	87.5 ± 11.1 ab
P. spinosum	75.0 ± 11.1 ab
P. coloratum	62.5 ± 11.1 ab
P. inflatum	50 ± 11.1 b

[z] All treatments compared to an uninoculated control set
[y] Least square means separations based on Tukey's HSD

Table 2.5 Sweet corn percent-germination rates by treatment in *Pythium* greenhouse panel.

Treatment	% Germination Rate (Mean ± SE)
Control[z]	87.5 ± 16.0 a[y]
P. arrhenomanes	75.0 ± 22.6 a
P. attrantheridium	75.0 ± 22.6 a
P. catenulatum	50.0 ± 22.6 a
P. coloratum	50.0 ± 22.6 a
P. graminicola	75.0 ± 22.6 a
P. inflatum	50 ± 22.6 a
P. irregulare	75.0 ± 22.6 a
P. periilum	75.0 ± 22.6 a
P. spinosum	75.0 ± 22.6 a
P. sylvaticum	100.0 ± 32.0 a
P. tardicrescens	100.0 ± 22.6 a
P. torulosum	100.0 ± 22.6 a

[z] All treatments compared to an uninoculated control set
[y] Least square means separations based on Tukey's HSD

Figure 2.1 Corn plant roots showing *Pythium* infection manifesting as discoloration.

Figure 2.2 Number of *Pythium* isolates collected and identified from corn tissue by year. Clade designations based on Lévesque and de Cock (2010

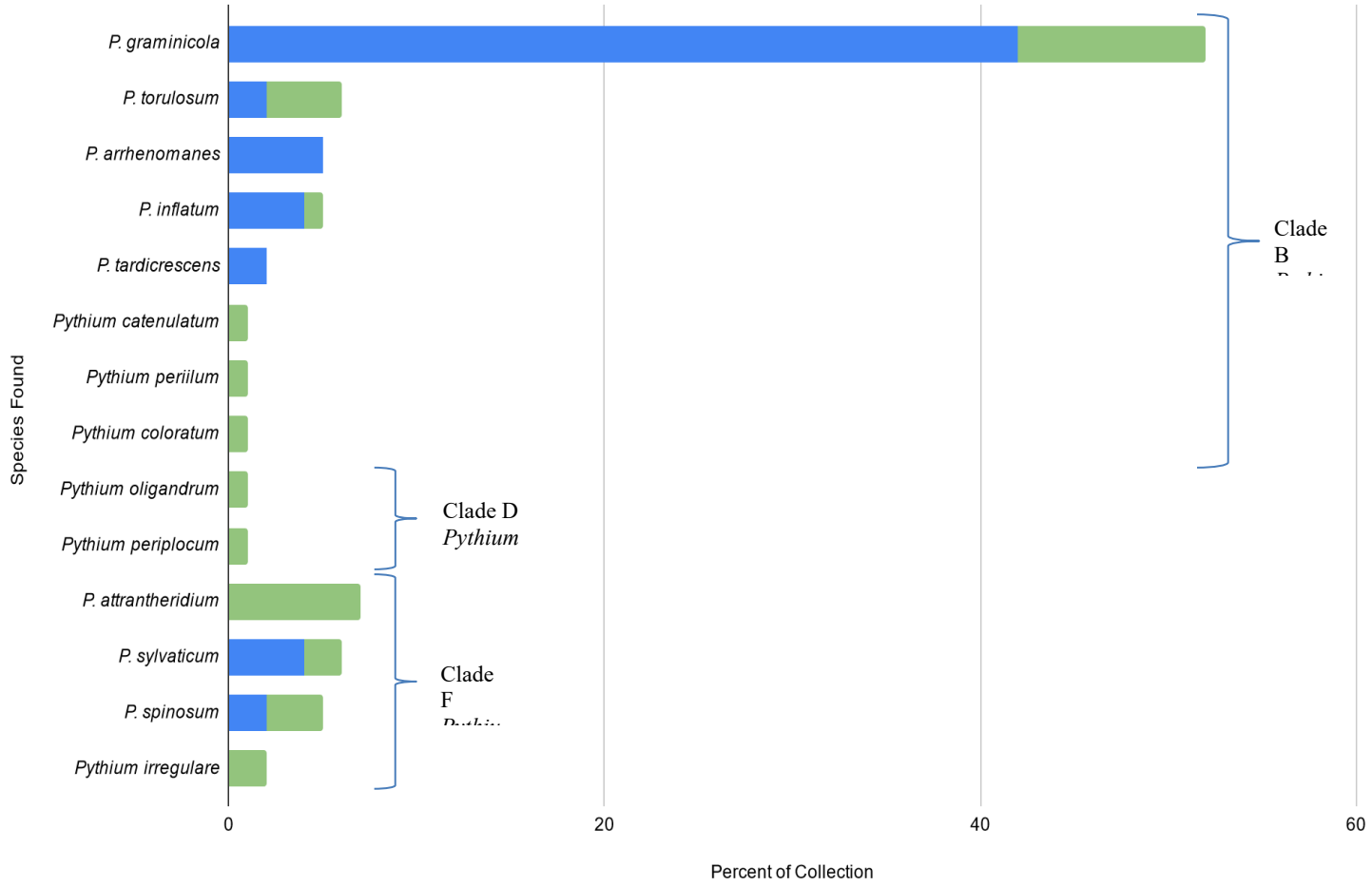


Figure 2.3 Measurement of *Pythium* colony on amended medium with digital calipers for evaluation of EC50.

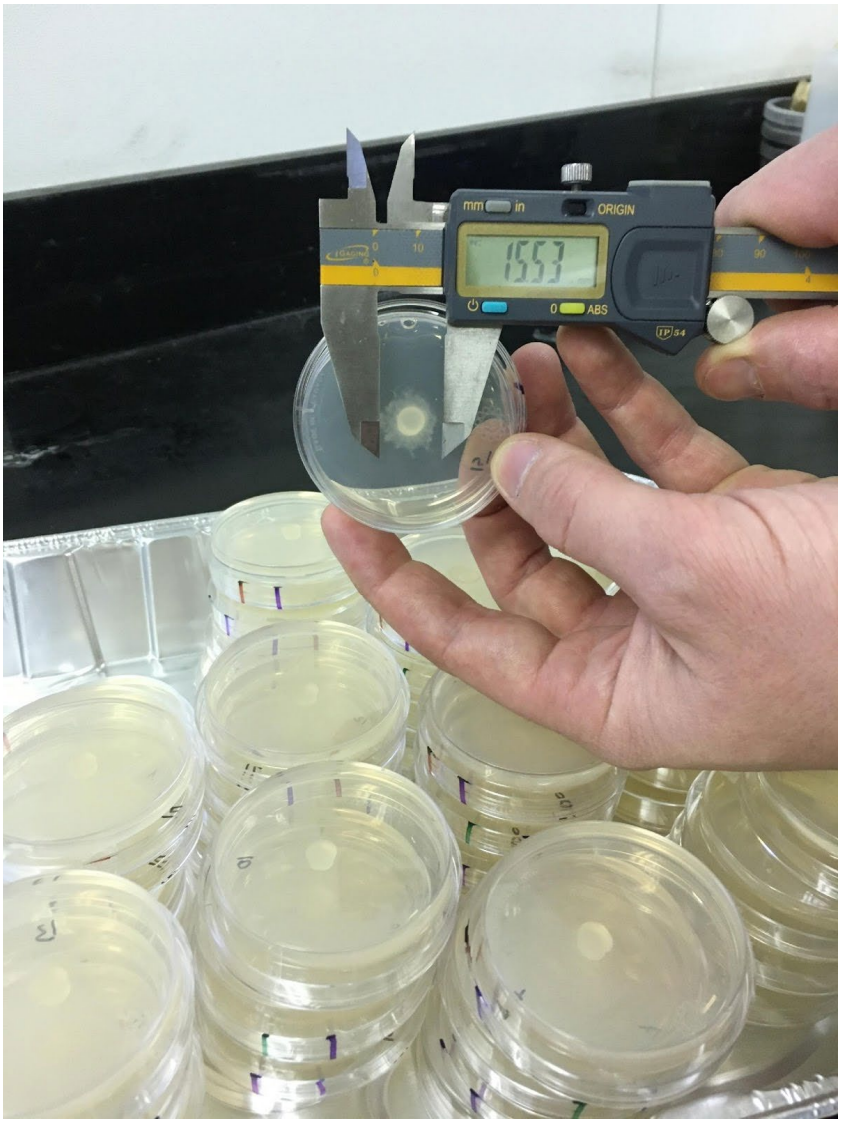
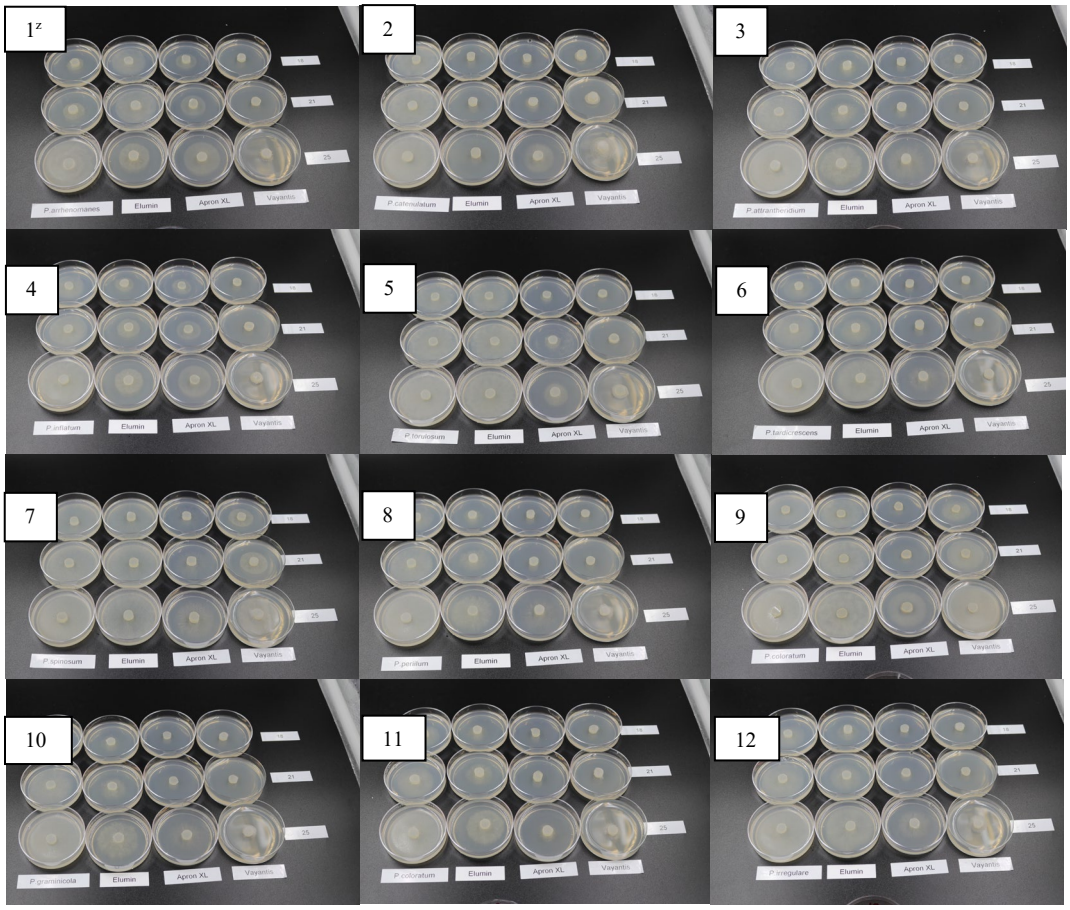
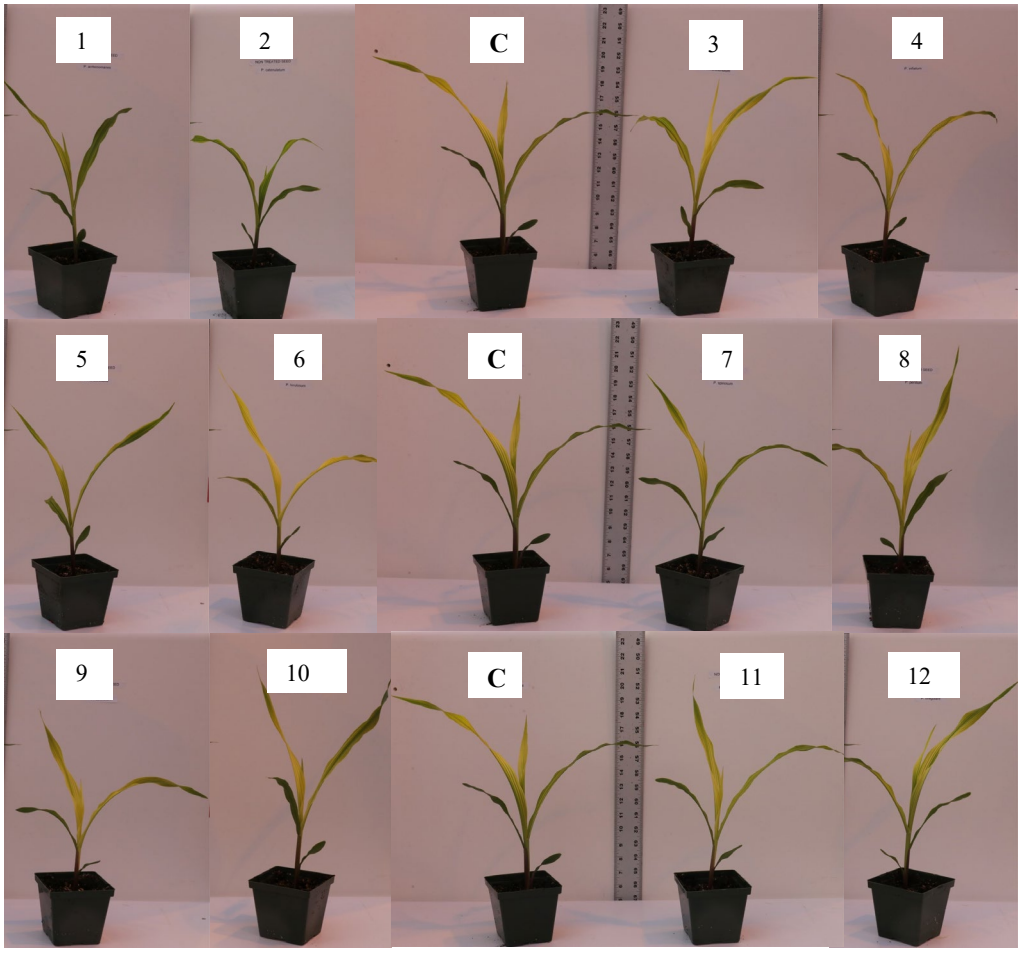


Figure 2.4 Temperature *in vitro* trials of *Pythium* species on media containing mefenoxam (ApronXL), ethaboxam (Elumin), and picarbutrazox (Vayantis) at 18ºC, 21ºC, and 25ºC.



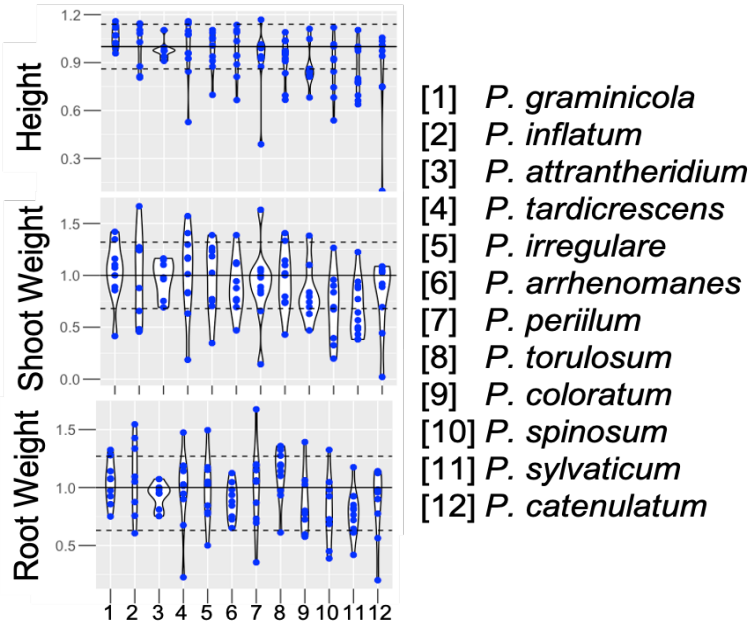
[z] All species tested fungicides compared to non-inoculated control (left column) at 3 temperatures (rows). *P. arrhenomanes* (1), *P. catenulatum* (2), *P. attrantheridium* (3), *P. inflatum* (4), *P. tardicrescens* (5), *P. torulosum* (6), *P. spinosum* (7), *P. periilum* (8), *P. coloratum* (9), *P. graminicola* (10), *P. sylvaticum* (11), and *P. irregulare* (12).

Figure 2.5 Greenhouse *Pythium* panel comparisons to control. Ruler added for scale.



[z] All species compared to non-inoculated (C) control, *P. arrhenomanes* (1), *P. catenulatum* (2), *P. attrantheridium* (3), *P. inflatum* (4), *P. tardicrescens* (5), *P. torulosum* (6), *P. spinosum* (7), *P. periilum* (8), *P. coloratum* (9), *P. graminicola* (10), *P. sylvaticum* (11), and *P. irregulare* (12).

Figure 2.6 Greenhouse assessment of the impact of individual *Pythium* spp. on corn growth. At 18 d post-inoculation, plants were assessed for changes in plant height, shoot weight and root weight. Trials were repeated three independent times. Only surviving plants were included in this analysis. Values from within each trial were normalized for the average control values (horizontal line at 1). Dashed lines indicate 1 standard deviation of the control data



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Chapter 3

SEASON-LONG EFFECTS OF PYTHIUM IN CORN

Abstract

Root rot and damping-off of corn (*Zea mays* L.) caused by species of the oomycete *Pythium* can lead to severe yield losses in Delaware and the Mid-Atlantic region United States, especially in wet years. Early season symptoms of *Pythium* infection in corn include pre- and post-emergence damping-off, stunting, and lesions on root and hypocotyl tissue that can lead to plant death. While many plants may succumb, not all plants that have the early symptoms of *Pythium* infection will die. In many cases, stunted plants will survive the season, but limited data exists on the development of surviving infected plants compared to symptomless healthy plants. To test season-long effects of *Pythium* infection, sixty on-farm plots in DE cornfields were tracked throughout the season for symptom development, mid-season and late-season flexural rigidity, and end of season stalk width, root weights, and kernel counts. A subset of 16 paired sets were designated comparing *Pythium* symptomatic plants against an adjacent asymptomatic control plant. *Pythium* infected plants showed significantly less stalk flexural rigidity ($p < 0.0001$) mid-season and late season. Post-harvest analyses of shoot diameter at the first node, root weight, and kernel count were all significantly lower ($p < 0.0001$) in *Pythium* infected plants. Extrapolated yield estimates identified reductions of $8.09 - $16.19 per hectare due to stand loss and losses of $10.93 per hectare for every 5% increase in *Pythium* infected ears.

Introduction

Corn (*Zea mays* L.) is the most significant grain crop by hectares grown in Delaware, accounting for over 120 million dollars of income annually (NASS 2020). Uniform germination and development of healthy corn stands able to produce robust ears are the basis for profitable production (Rojas et al. 2019). Seedling diseases that reduce stand and season long plant quality, such as Pythium root rot caused by *Pythium* spp., have been prominent in the Mid-Atlantic region (MAR) in recent years. Wet springs, earlier planting dates, reduced tillage, and other environmental fluctuations may all play a role in the level of disease observed. In the past ten years, the planting date of corn in Delaware has shifted to be one week earlier from April 19 in 2012 to April 11 in 2015 (NASS and DDA 2013; 2015). Corn planted into cooler soil temperatures takes longer to germinate, providing a longer window for seedling pathogen interactions (Broders et al. 2007; Matthiesen et al. 2016; Martin and Loper 1999). Yield loss estimates from 2012 valuate losses due to *Pythium* at approximately 93.4 million bushels of corn, translating to over 373.6 million dollars lost in a single year in United States and Ontario (Mueller et al. 2016).

Symptoms of *Pythium* in corn can begin with pre-emergence damping-off, where the seed or young seedling will become infected underground and die before emerging from the soil; or as post-emergence damping-off, in which the plants germinate and emerge from the soil before exhibiting symptoms of "wetness," wilting, and chlorosis before dying completely (Johann 1928;

Matthiesen et al. 2016). These forms of infection result in reduced stand and growers must decide if the yield penalty of later panting date for re-plant outweighs the potential yield loss of marginal stands. Due to the sandy soils found in Delaware, just under half of the corn planted is irrigated (NASS 2020). In intensively managed irrigated plantings, corn is typically planted at populations of 11,331 to 15,378 plants per hectare, while dryland populations tend to range from 8,094 to 11,736 plants per hectare. The increased plant density, frequency of moisture, and higher residue of irrigated fields tend to favor issues from *Pythium.* The colonization of young corn roots and hypocotyls by pathogenic *Pythium* species will result in the appearance of brownish-yellow lesions that progress to darker colors, often resulting in tissue necrosis, tissue sloughing, and girdling, all of which can contribute to the decline of the plant (Rao 1978; Rojas et al. 2019). Often young corn plants can survive *Pythium* infection only to show stunting compared to uninfected plants (Broders et al. 2007). Lower internode stalk rots and lodging in older plants has also been attributed to *Pythium* infection (Elliot 1942). Lodging susceptibility (flexural rigidity) can be mechanically estimated through the analysis of the force-rotation and resistance data collected while the corn plant is bent from an upright position, within its linear-elastic range (before breakage) (Erndheim et al. 2020).

In other regions, early infection by *Pythium* has been associated with poor performance throughout the season, but no studies investigating the season-long impacts of *Pythium* have been conducted in Delaware. Improved

understanding of plant potential would aid grower when making replant decisions and considering yield potential and profit margins following early season *Pythium* infections.

The objectives of this study were to track plants that survived early season *Pythium* infection in comparison to symptomless neighboring plants to ascertain the specific effects that early season stunting has on corn plant performance and grain yield.

Materials & Methods

On- Farm Stand Count Tracking. Linear plots were established across three cornfields with field histories of *Pythium* damping-off in Sussex County, Delaware. The first field site was planted in Dekalb 61-41 on May 14, 202, the second in Axis 65D28 on May 16, 2020 and the third in Dekalb 61-41 on May 14, 2020. All plots were planted at 14,973 sd/hectare. Linear plot locations within established fields were determined based on the presence of at least one symptomatic seedling showing stunting and/or wilting at vegetative growth stages two to three (V2-V3). Each linear plot was 5.3 meters long to simulate $1/1000^{th}$ of an acre (4 square meters). All symptomatic plants were flagged for tracking and GPS coordinates were noted. Stand counts within the linear sections were recorded weekly to note plants succumbing to post-emergence damping-off as well as growth stage of plants within linear segments (Table 3.1). A subset of paired sets was established where a symptomatic plant was

paired with a symptomless neighbor for more detailed observation throughout the season. Nearby plants showing symptoms of *Pythium* such as wilting, stunting, and post-emergence damping-off were destructively sampled and brought to the lab for isolate recovery and pathogen identification for verification of the presence of *Pythium.*

Isolate Collection and Molecular Identification. Following seedling flagging at V2-V3, corn seedlings were collected in areas close to the marked linear sections in each of the three on-farm partnership fields. Symptomatic seedlings were transported to the lab and washed with tap water to remove excess soil. Plants with roots and hypocotyls showing symptoms such as brown or yellow lesions and sloughing roots were taken from the plants and 5 mm pieces of infected tissue were surface disinfested in 0.825% sodium hypochlorite solution for 30 s and then rinsed in deionized (DI) water for 30 s. The clean root and hypocotyl segments were plated onto PARP selective medium (V8 agar (20 g/l Bacto agar (BD Difco), 200 ml/l clarified V8 juice) amended with pimaricin, ampicillin, rifampicin, and PCNB [Terraclor 75% WP, Southern Ag Insecticides, Inc.]) (Jeffers and Martin 1986). Isolates were left to incubate for five to seven days. Any samples showing growth of glassy, hyaline hyphae were sub-cultured onto plates containing PARP medium in repetition to get the isolates into pure culture.

To extract DNA, three 6.75 mm plugs from each pure culture isolate were added to 100 x 15 mm petri dishes containing 20 ml of potato-dextrose broth

(PDB) (Difco). Plates were left to fully colonize at 23°C. Isolates found to be free of contamination were vacuum-aspirated through autoclave-sterilized filter paper in a Buchner funnel. The tissue, once dried, was transferred to 1.5 ml microcentrifuge tubes and moved to -2°C storage before DNA extraction.

DNA from the vacuum-aspirated, frozen tissue underwent maceration with 400 µl of cell lysis solution (Qiagen, Hilden, Germany) with sterilized plastic pestles. Tubes were vortexed for 20 s and incubated at 55°C after 3 µl Puregene proteinase K (Qiagen) had been added to each sample. The samples were incubated for 30 min at 37°C after treatment with 3 µl of RNase (Qiagen) and then amended with 200 µl of protein precipitation solution (Qiagen) and incubated on ice for 1 h. Following cold incubation, the samples were centrifuged for 5 min and the supernatant was pipetted into new microcentrifuge tubes that contained premeasured aliquots of 400 µl isopropanol to allow the DNA to precipitate. Tubes were centrifuged for 3 min, the supernatant was poured off and the remaining DNA pellet was washed with 200 µl of 70% ethanol and centrifuged. Tubes were allowed to dry on a heating block before 50 µl of sterile, nuclease-free water was added to rehydrate. Concentration and purity of the DNA was measured using spectrophotometry, and samples were diluted to a standardized concentration of 20 ng ml^{-1} before storage at -2 °C.

Each isolate was amplified at three loci with polymerase chain reaction (PCR): partial beta tubulin (*BT*), cytochrome c oxidase subunit II (COII), and nuc rDNA internal transcribed spacer (ITS1-5.8S-ITS2 =ITS) (Villa et al. 2006; Martin

2000). PCR reactions were 50 µl and consisted of 10 X DreamTaq Master Mix, 10 µM of each forward and reverse primer: ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3'); BT5 (5′-GTATCATGTGCACGTACTCGG-3′) and BT6 (5′-CAAGAAAGCCTTACGACGGA-3′); and FM66 (5'-TAGGATTTCAAGATCCTGC-3') and FM58 (5'-CCACAAATTTCACTACATTGA-3'), and 20 ng of genomic DNA. Thermal cycling protocol was as follows: 94°C for 5 min followed by 35 cycles of DNA denaturation for 45 s at 94°C; primer annealing for 30 s at 53°C; and primer extension for 90 s at 72°C.held at A final extension step of 72°C for 10 minutes completed the cycle.

Gel electrophoresis at 90V for 40 m verified the amplification of the target gene regions. ExoSAP-IT (USB products Affymetrix, Inc. ThermoFisher Scientific, Walthan, Massachusetts) was used to clean the amplicons before being sent to Eurofins Genomics (Louisville, KY) for sequencing. Sequences were analyzed in CLC Main Workbench 20.0.4 (CLC Bio: a Qiagen Company, Germany) and sent to the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) for comparison to previously identified specimen sequences. Sequences from the ITS region were deposited into GenBank (Table 3.2).

Rigidity Sampling. Within the subset of paired plants, mid-season, flexural rigidity was measured (non-destructively) as a mechanically-quantified push-test using the Device for Assessing Resistance to Lodging IN Grains (DARLING) when plants were near vegetative tassel (VT) (Cook et al. 2019). To operate the DARLING, the operator stands the device next to the stalk and lines up the force sensor against the stalk. The operator then steps on the ground platform, initiates the test protocol program, and pushes the DARLING's vertical arm forward, bending the stalk (Figure 3.1). The DARLING measures both force and rotation from the sensors and the data collected are used to calculate the moment of maximum bending applied during push and the flexural rigidity of the corn stalk. Measurements of the symptomatic plants were compared with the adjacent, non-symptomatic control plant. When plants reached dent (reproductive stage 5, R5), DARLING push tests were performed again, this time pushing each plant to stalk failure. All measured paired sets were then excavated and brought to the lab for further analysis.

Stalk, Root, and Ear Measurement. Using the destructively samples paired sets, stalk width, root weights, and kernel counts were conducted. At the lab, stalks were cut below the first node to separate the roots. Ears were shucked from the main stalk and stalk circumference was measured at the first node. Roots were washed in tap water, dried on paper towels, and weights were measured. After weighing, root tissue bearing darkened lesions, or other *Pythium*-indicative symptoms were excised from the root ball. Root pieces were cut into 5mm

sections and symptomatic tissue pieces that showed yellow and brown lesions and necrosis were surface disinfested in 0.825% sodium hypochlorite solution for 30 s and rinsed in deionized (DI) water for 30 s. The disinfested root and hypocotyl root segments were plated onto a PARP selective medium (V8 agar (20 g/l Bacto agar (Fisher Scientific), 200 ml/l clarified V8 juice) amended with pimaricin, ampicillin, rifampicin, and PCNB [Terraclor 75% WP, Southern Ag Insecticides, Inc.] Isolates were identified to species using the steps described above.

Data Analysis. Data was analyzed for normality, the effects of the location of observation within the three fields, as well as the effects of the timing of measurement with mixed-model analysis of variance (ANOVA) using PROC GLIMMIX in SAS 9.4 (SAS Institute, Cary, North Carolina). The stand count values from each linear plot were grouped by field. The location of the linear plots and the time points at which the stand counts were taken were treated as random effects, the stand count was treated as a fixed effect. Fixed effects were tested for significance at $\alpha=0.05$, and significant differences among stand count were separated using paired *t* tests. Principal Component Analyses (PCA) were performed with *ggfortify* (version 0.4.11) package in R (R Core Team 2020; Tang et al. 2016; Hoikoshi and Tang 2018).

Results

A total of 60 linear plots were established across three fields. Twenty-four linear plots were established in fields 1 and 2 and twelve in field 3. From initial stand count data, all fields experienced plant death due to *Pythium* damping off.

Symptom development was most severe in Field 3, which contained greater numbers of symptomatic plants and more areas of low, wet, waterlogged soils (Table 3.1). Based on calculations from the difference between each field, stand counts were compared across the season and flagged symptomatic plants were monitored for death or continued growth through the season. From stand count extrapolations comparing the plants that emerged at the beginning of the season to those that were still present at the end, Field 1 experienced estimated losses close to 4.5% with 83.3% of early season symptomatic plants surviving to harvest (Table 3.1). Field 2 showed estimated losses nearing 3.5% with 74% of early season symptomatic plants surviving to harvest. Field 3 suffered the heaviest losses at around 4.9% within the observation time with only 54.6% of symptomatic plants surviving to harvest.

Early season sampling, isolation, and sequencing found that the corn plants in all three fields were predominantly infested with *P. graminicola* followed closely by *P. torulosum* (Figure 3.2). Other species present were *P. attrantheridium, P. oligandrum, P. spinosum,* and *P. irregulare.*

Within the linear plots, sixteen paired sets of the surviving flagged plants with one of their immediate adjacent symptomless neighbor plants were identified for detailed observation. Mid-season push tests with the DARLING as compared to their adjacent partner control plant showed that the flagged symptomatic plants had significantly lower stalk flexural rigidity ($p < 0.0001$) (Figure 3.3). Late season DARLING measurements of flexural rigidity found that flagged symptomatic plants continued to have significantly less flexural rigidity ($p < 0.0001$) than their adjacent neighbor close to harvest (Figure 3.4).

In the lab, following washing, root balls were weighed and *Pythium*-symptomatic plants were found to have significantly lower ($p<0.0001$) root weights (90 g) than the symptomless partners (237 g) (Figure 3.5, 3.6). Stalk circumference of *Pythium*-infected plants vs controls were also shown to be significantly lower ($p < 0.0001$) with controls averaging close to 82.6 mm and *Pythium*-infected plants averaging 56.9 mm (Figures 3.7, 3.8). Kernel estimates were taken from the shucked ears based on number of kernels longitudinally along the ear multiplied by the number of kernels around the widest part of the ear (Figure 3.9). *Pythium*-infected plants averaged significantly ($p < 0.0001$) fewer kernels on plants, averaging 231 whereas control estimates averaged 587 (Figure 3.10).

Discussion

All three fields sampled and tracked were planted at 14,973 sd/H. Field 1 and 2 had starting counts that were comparable and similar stand count losses throughout the season (Table 3.1). Field 3 had a lower topology and had a history of waterlogging and *Pythium*-related losses. Based on the planting rate of 14,973 seeds per hectare and the observed emergence at the beginning of the season, Field 1 had an average emergence of 97.3%, whereas Field 2 had an average emergence of 96.8%. Field 3 had an average emergence of 79.9%, coinciding the history of soil wetness and presence of soil microorganisms like *Pythium* that contributed to pre-emergence damping-off and seed rot preventing the germination of the planted seed.

The observed losses were consistent throughout the season between the three fields in which stand count losses were sustained between 6/12/20 and 6/26/20 and appeared to level off by the middle of the season (Figure 3.11). Field 3 appeared to suffer more losses in the early time frame. Close to the end of the season, in the second week of August, a relatively steep reduction in stand count coincided with a high-wind rain event that caused stem breakage and lodging resulting in the non-*Pythium* related death of several plants in the tracking plots.

Comparing initial stand counts, post-emergence (6/12/20) (Table 3.1) and final stand counts at the end of the season before harvest (8/26/20) stand reductions of 4.5% in Field 1, 3.5% in Field 2 and 4.9% in Field 3 were observed due loss of plants from *Pythium*. Based on the estimations by NASS (2020), yield loss due to missing stand would have averaged 2.91 bushels/ha or approximately $11.65/ha lost at $4/ bushel corn prices in Field 1. In Field 2 stand loss accounted for losses of 2.27 bushels/ha, totaling $9.06/ha lost. Field 3 with heavy symptomology at just above 95% survival is estimated to have losses of 3.17 bushels/ha, totaling $12.72/ha lost.

In symptomatic plants, the species *P. graminicola* represented over 30% of the collection. This species is well documented in the literature for being pathogenic to corn plants (Broders et al. 2007; Hampton and Buchholtz 1959; Rao 1976 and 1978; Van der Plaats-Niterink 1981). Other species identified included *P. torulosum, P. attrantheridium, P. oligandrum,* and *P. irregulare,* all

of which have been reported on corn in the United States previously (Broders et al. 2007; Dorrance et al. 2004; Matthiesen et al. 2016; Radmer et al. 2017; Rojas et al. 2019; Van der Plaats-Niterink 1981). The sampling from the beginning of the season yielded about 60% *P. graminicola*, followed by about 23% *P. torulosum*, then small percentages of *P. spinosum, P. attrantheridium,* and *P. oligandrum.* The late season sampling and isolation from tracked *Pythium* and control plant roots yielded multiple species, however, *P. graminicola* accounted for 98% of isolates recovered. Interestingly, 10 samples of *P. graminicola* were isolated from asymptomatic control plant roots.

The DARLING push tests to measure stalk flexural rigidity were conclusive, showing that the plants that sustained early *Pythium* infection demonstrated significantly ($p < 0.0001$) less resistance to bending than their controls (Figure 3.3, 3.4). Corn planted at 14,973 sd/ha germinate and grow in very close proximity. It is likely that the early-season infection, when survived, causes stunting in individual plants, as the corn grows taller, the canopy closes, and the neighboring roots spread out, the stunted corn has less access to light, water, and soil nutrients perpetuating and worsening the development of the individual plant. Sampling and comparison of the root stocks, stalk circumference, and kernel estimates (Figure 3.6, 3.8, 3.10) from the cobs at post-harvest, point to similar trends in the perpetuation of a reduction of quality in all metrics as compared to neighboring controls.

The kernel estimates from the harvested, paired plants proved to be significantly less in plants tracked with early season-*Pythium* infection, consistent with the other metrics tested. The tracked *Pythium* plants performed poorly for the entire season and produced small, malformed ears with very few kernels (Figure 3.10). The effects on yield in 100% disease scenario were estimated to approach losses of $218.59/ha. Delaware farmers, more likely to encounter 1, 2, or 5% disease incidence in a field can expect losses ranging from $2.19/ha, $4.54/ha, or up to $10.93/ha, respectively. This estimate can provide a baseline connection for farmers of incidence of *Pythium*-symptomatic corn plants surviving in the field to the direct monetary effects on yield. This metric can be used to assist farmers in weighing options for the cost and yield effects of replanting as compared to leaving the field as is with the *Pythium*-symptomatic plants. The estimate should be applied on a case-by-case basis based on the individual costs of replanting for each farm.

Depending on the severity of *Pythium* infestation causing early-season damping-off in fields, farmers will consider replanting sections of fields or entire fields to attempt to counter yield loss. The early season effects of *Pythium* infection and rates of plant death are well documented, however, it is also valuable for a farmer to consider the how post-disease survival with developmental limitations in the size and performance of the plants can result in smaller ears that produce less kernels and therefore continue to impact yield. This study begins to elucidate those long-term effects and how the effects of

Pythium impact yield season-long. Though *P. grmainicola* was the most common species found, alternative directions could include controlled inoculation trials of single plants within asymptomatic fields with *P. torulosum, P. spinosum, P. attrantheridium* and others to discover how different species may affect long term development of corn plants.

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Table 3.1 Average stand count losses between 6/12/20 and 8/26/20 at on-farm partnership field sites.

Average Stand Count Losses by Field						
Field	**Initial Count[z]**	**Flagged[y]**	**Final Count**	**Flagged**	**Losses (%)**	**Symptomatic Survivors[x]**
1 (n = 24)[w]	14,569/ha	36	13,913/ha	30	4.5%	83.3%
2 (n = 24)	14,500/ha	35	13,986/ha	26	3.5%	74.0%
3 (n = 12)	11,958/ha	22	11,372/ha	12	4.9 %	54.6%

[z] Stand counts estimated by replicated observation points within a field measured to be 1/1000th of an acre (4.05 m^2). Averages were calculated within fields and extrapolated to reflect hectare estimates.

[y] Symptomatic plants that were flagged at the beginning of the season were tracked throughout.

[x] Based on the number of plants flagged as symptomatic at the beginning of the season compared to those flagged plants that were still present at harvest.

[w] Number of observation points within each field used to achieve averages.

Table 3.2. Genbank accessions and species identification of *Pythium* species isolated at on-farm partnership in 2020.

Isolate No.	Species	Collection Year	Clade	#Accession
CP2001	*Pythium graminicola*	2020	B	MZ540536[z]
CP2002	*Pythium graminicola*	2020	B	MZ540537
CP2003	*Pythium graminicola*	2020	B	MZ540538
CP2004	*Pythium graminicola*	2020	B	MZ540539
CP2005	*Pythium graminicola*	2020	B	MZ540540
CP2006	*Pythium graminicola*	2020	B	MZ540541
CP2007	*Pythium graminicola*	2020	B	MZ540542
CP2008	*Pythium graminicola*	2020	B	MZ540543
CP2009	*Pythium torulosum*	2020	B	MZ540544
CP2010	*Pythium attrantheridium*	2020	F	MZ540545
CP2011	*Pythium attrantheridium*	2020	F	MZ540546
CP2012	*Pythium oligandrum*	2020	D	MZ540547
CP2013	*Pythium graminicola*	2020	B	MZ540548
CP2014	*Pythium graminicola*	2020	B	MZ540549
CP2015	*Pythium graminicola*	2020	B	MZ540550
CP2016	*Pythium spinosum*	2020	F	MZ540551
CP2017	*Pythium coloratum*	2020	B	MZ540552
CP2018	*Pythium spinosum*	2020	F	MZ540553
CP2019	*Pythium spinosum*	2020	F	MZ540554
CP2020	*Pythium graminicola*	2020	B	MZ540555
CP2021	*Pythium graminicola*	2020	B	MZ540556
CP2022	*Pythium graminicola*	2020	B	MZ540557
CP2023	*Pythium graminicola*	2020	B	MZ540558
CP2024	*Pythium graminicola*	2020	B	MZ540559
CP2025	*Pythium graminicola*	2020	B	MZ540560
CP2026	*Pythium graminicola*	2020	B	MZ540561
CP2027	*Pythium torulosum*	2020	B	MZ540562
CP2028	*Pythium graminicola*	2020	B	MZ540563
CP2029	*Pythium torulosum*	2020	B	MZ540564

CP2030	*Pythium torulosum*	2020	B	MZ540565
CP2031	*Pythium graminicola*	2020	B	MZ540566
CP2032	*Pythium torulosum*	2020	B	MZ540567
CP2033	*Pythium graminicola*	2020	B	MZ540568
CP2034	*Pythium torulosum*	2020	B	MZ540569
CP2035	*Pythium torulosum*	2020	B	MZ540570
CP2036	*Pythium graminicola*	2020	B	MZ540571
CP2037	*Pythium torulosum*	2020	B	MZ540572
CP2038	*Pythium graminicola*	2020	B	MZ540573
CP2039	*Pythium torulosum*	2020	B	MZ540574
CP2040	*Pythium graminicola*	2020	B	MZ540575
CP2041	*Pythium graminicola*	2020	B	MZ540576
CP2042	*Pythium graminicola*	2020	B	MZ540577
CP2043	*Pythium tardicrescens*	2020	B	MZ540578
CP2044	*Pythium irregulare*	2020	B	MZ540579
CP2045	*Pythium graminicola*	2020	B	MZ540580
CP2046	*Pythium graminicola*	2020	B	MZ540581
CP2047	*Pythium graminicola*	2020	B	MZ540582
CP2048	*Pythium graminicola*	2020	B	MZ540583
CP2049	*Pythium attratheridium*	2020	F	MZ540584
CP2050	*Pythium graminicola*	2020	B	MZ540585
CP2051	*Pythium graminicola*	2020	B	MZ540586
CP2052	*Pythium coloratum*	2020	B	MZ540587
CP2053	*Pythium graminicola*	2020	B	MZ540588
CP2054	*Pythium graminicola*	2020	B	MZ540589
CP2055	*Pythium graminicola*	2020	B	MZ540590
CP2056	*Pythium torulosum*	2020	B	MZ540591
CP2057	*Pythium graminicola*	2020	B	MZ540592
CP2058	*Pythium graminicola*	2020	B	MZ540593
CP2059	*Pythium graminicola*	2020	B	MZ540594
CP2060	*Pythium graminicola*	2020	B	MZ540595

CP2061	*Pythium graminicola*	2020	B	MZ540596
CP2062	*Pythium graminicola*	2020	B	MZ540597
CP2063	*Pythium graminicola*	2020	B	MZ540598
CP2064	*Pythium graminicola*	2020	B	MZ540599
CP2065	*Pythium graminicola*	2020	B	MZ540600
CP2066	*Pythium graminicola*	2020	B	MZ540601
CP2067	*Pythium torulosum*	2020	B	MZ540602
CP2068	*Pythium catenulatum*	2020	B	MZ540603
CP2069	*Pythium graminicola*	2020	B	MZ540604
CP2070	*Pythium coloratum*	2020	B	MZ540605
CP2071	*Pythium graminicola*	2020	B	MZ540606
CP2072	*Pythium graminicola*	2020	B	MZ540607
CP2073	*Pythium graminicola*	2020	B	MZ540608
CP2074	*Pythium graminicola*	2020	B	MZ540609
CP2075	*Pythium graminicola*	2020	B	MZ540610
CP2076	*Pythium graminicola*	2020	B	MZ540611
CP2077	*Pythium periilum*	2020	B	MZ540612
CP2078	*Pythium tardicrescens*	2020	B	MZ540613
CP2079	*Pythium graminicola*	2020	B	MZ540614
CP2080	*Pythium graminicola*	2020	B	MZ540615
CP2081	*Pythium graminicola*	2020	B	MZ540616
CP2082	*Pythium graminicola*	2020	B	MZ540617
CP2083	*Pythium periplocum*	2020	D	MZ540618
CP2084	*Pythium graminicola*	2020	B	MZ540619
CP2085	*Pythium graminicola*	2020	B	MZ540620
CP2086	*Pythium catenulatum*	2020	B	MZ540621
CP2087	*Pythium graminicola*	2020	B	MZ540622
CP2088	*Pythium graminicola*	2020	B	MZ540623
CP2089	*Pythium graminicola*	2020	B	MZ540624
CP2090	*Pythium graminicola*	2020	B	MZ540625
CP2091	*Pythium graminicola*	2020	B	MZ540626

CP2092	*Pythium graminicola*	2020	B	MZ540627
CP2093	*Pythium graminicola*	2020	B	MZ540628
CP2094	*Pythium graminicola*	2020	B	MZ540629
CP2095	*Pythium graminicola*	2020	B	MZ540630
CP2096	*Pythium graminicola*	2020	B	MZ540631
CP2097	*Pythium graminicola*	2020	B	MZ540632
CP2098	*Pythium graminicola*	2020	B	MZ540633
CP2099	*Pythium graminicola*	2020	B	MZ540634
CP20100	*Pythium graminicola*	2020	B	MZ540635
CP20101	*Pythium graminicola*	2020	B	MZ540636
CP20102	*Pythium graminicola*	2020	B	MZ540637
CP20103	*Pythium graminicola*	2020	B	MZ540638
CP20104	*Pythium graminicola*	2020	B	MZ540639
CP20105	*Pythium graminicola*	2020	B	MZ540640
CP20106	*Pythium graminicola*	2020	B	MZ540641
CP20107	*Pythium graminicola*	2020	B	MZ540642
CP20108	*Pythium graminicola*	2020	B	MZ540643
CP20109	*Pythium graminicola*	2020	B	MZ540644
CP20110	*Pythium graminicola*	2020	B	MZ540645
CP20111	*Pythium graminicola*	2020	B	MZ540646
CP20112	*Pythium graminicola*	2020	B	MZ540647
CP20113	*Pythium graminicola*	2020	B	MZ540648
CP20114	*Pythium graminicola*	2020	B	MZ540649
CP20115	*Pythium graminicola*	2020	B	MZ540650
CP20116	*Pythium graminicola*	2020	B	MZ540651
CP20117	*Pythium graminicola*	2020	B	MZ540652
CP20118	*Pythium graminicola*	2020	B	MZ540653
CP20119	*Pythium graminicola*	2020	B	MZ540654
CP20120	*Pythium graminicola*	2020	B	MZ540655
CP20121	*Pythium graminicola*	2020	B	MZ540656
CP20122	*Pythium torulosum*	2020	B	MZ540657

[z] Accessions based on amplification of the ITS region.

Figure 3.1 Operational instructions for the DARLING from Erndwein et al. 2020.

Figure 3.2 Distribution of *Pythium* species isolated from on-farm partnership fields in 2020.

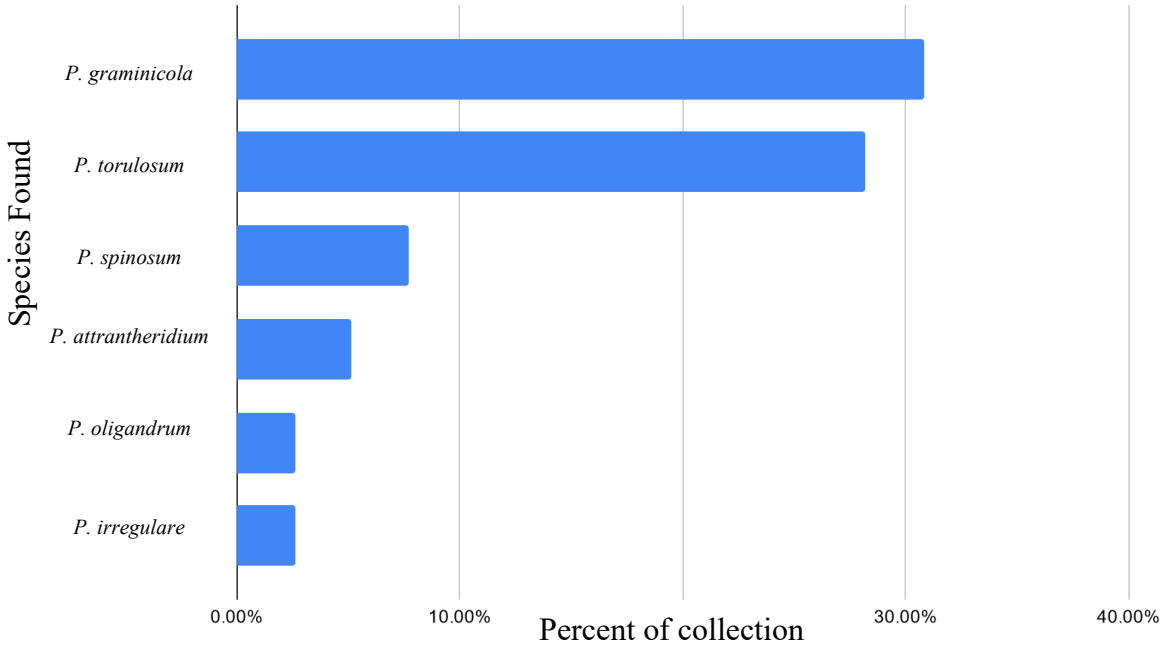
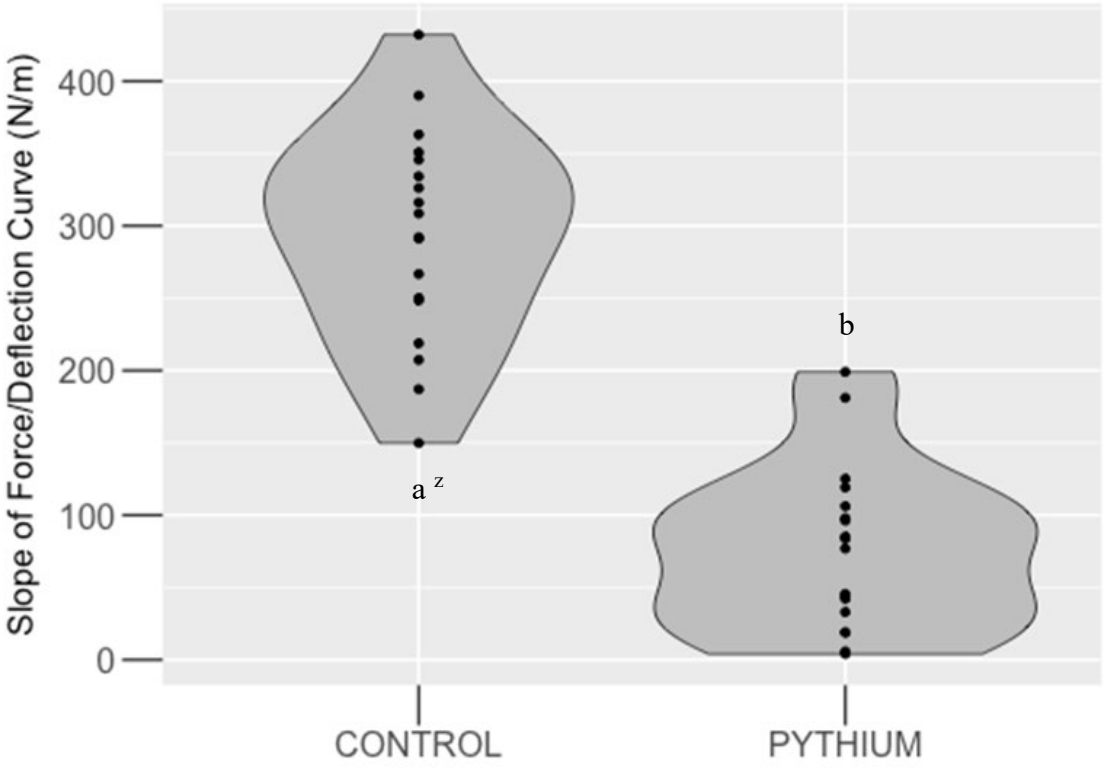
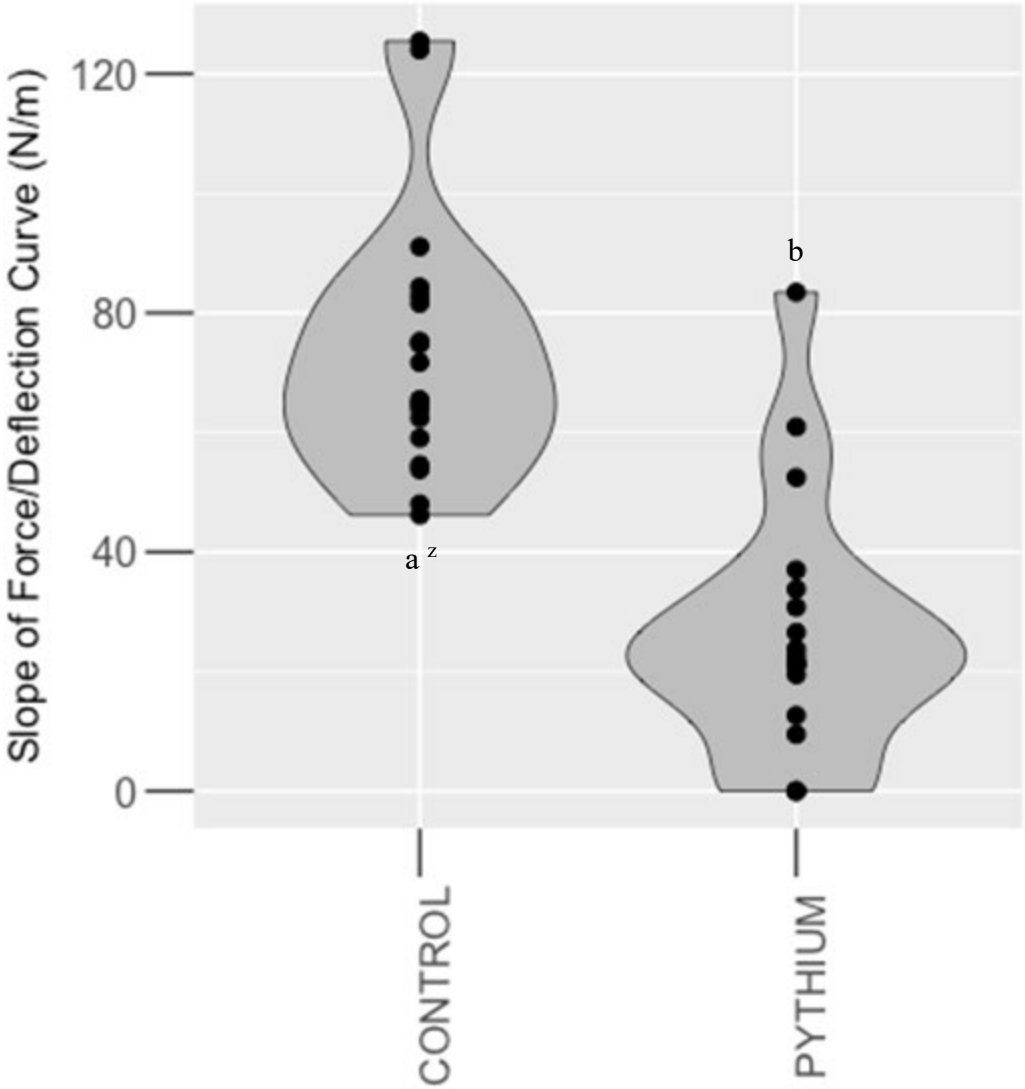


Figure 3.3 Mid-season DARLING measurements of flexural rigidity (slope of force/ deflection curve N/m) of *Pythium*-infected plants vs. adjacent controls. Figure courtesy of Erin Sparks, PhD.



[z] Letter separations based on Least Square Means ($p < 0.0001$).

Figure 3.4 Late-season DARLING measurements of flexural rigidity (slope of force/ deflection curve N/m) of *Pythium*-infected plants vs. adjacent controls. Figure courtesy of Erin Sparks, PhD.



[z] Letter separations based on Least Square Means ($p < 0.0001$).

Figure 3.5 Size comparison of *Pythium*-infected root stalk and adjacent control, ruler included for scale.

Figure 3.6 Root weight comparisons in grams of *Pythium*-infected plants (P) and adjacent controls (C). Significance of mean separation based on T-test ($p < 0.0001$).

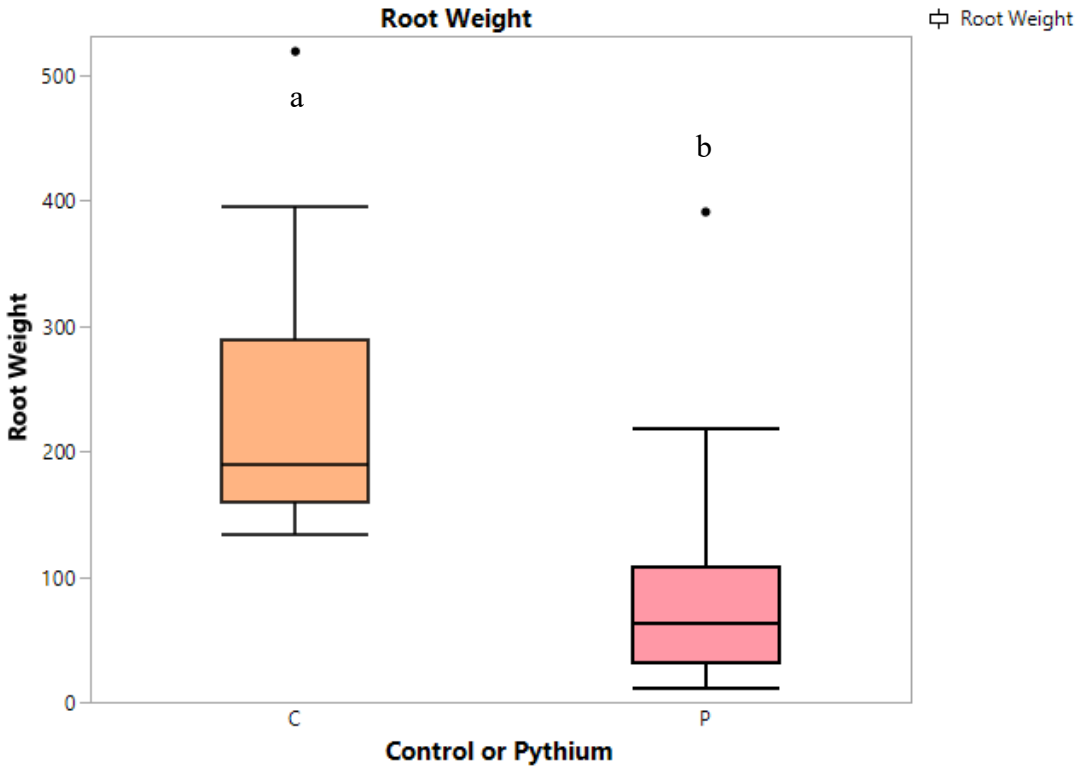


Figure 3.7 Split stalks of *Pythium*-infected corn plants and adjacent control. Marker included for scale.

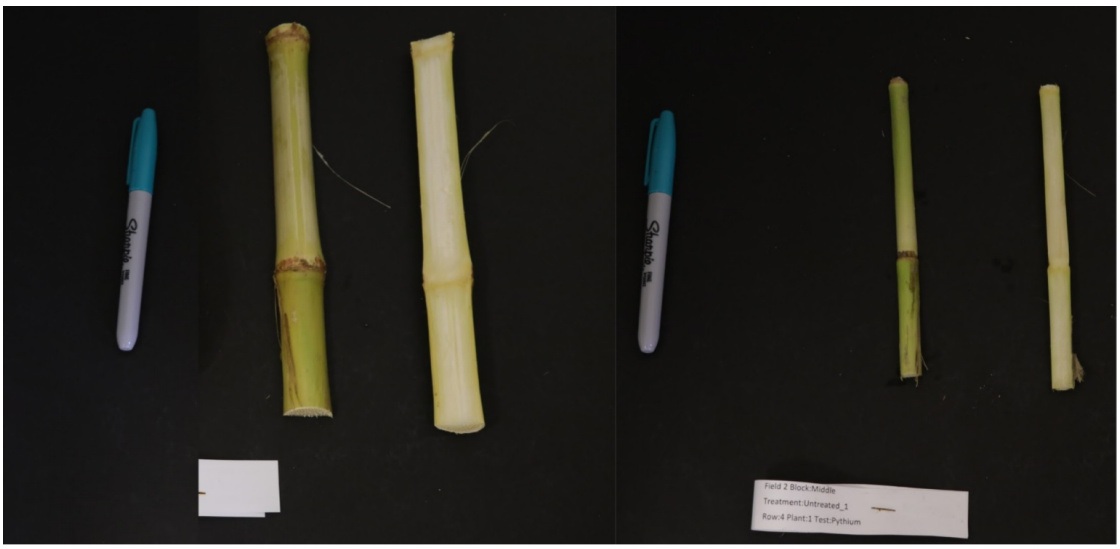


Figure 3.8 Shoot circumference comparisons (in) of *Pythium*-infected plants (P) and adjacent controls (C). Significance of mean separation based on T-test ($p < 0.0001$).

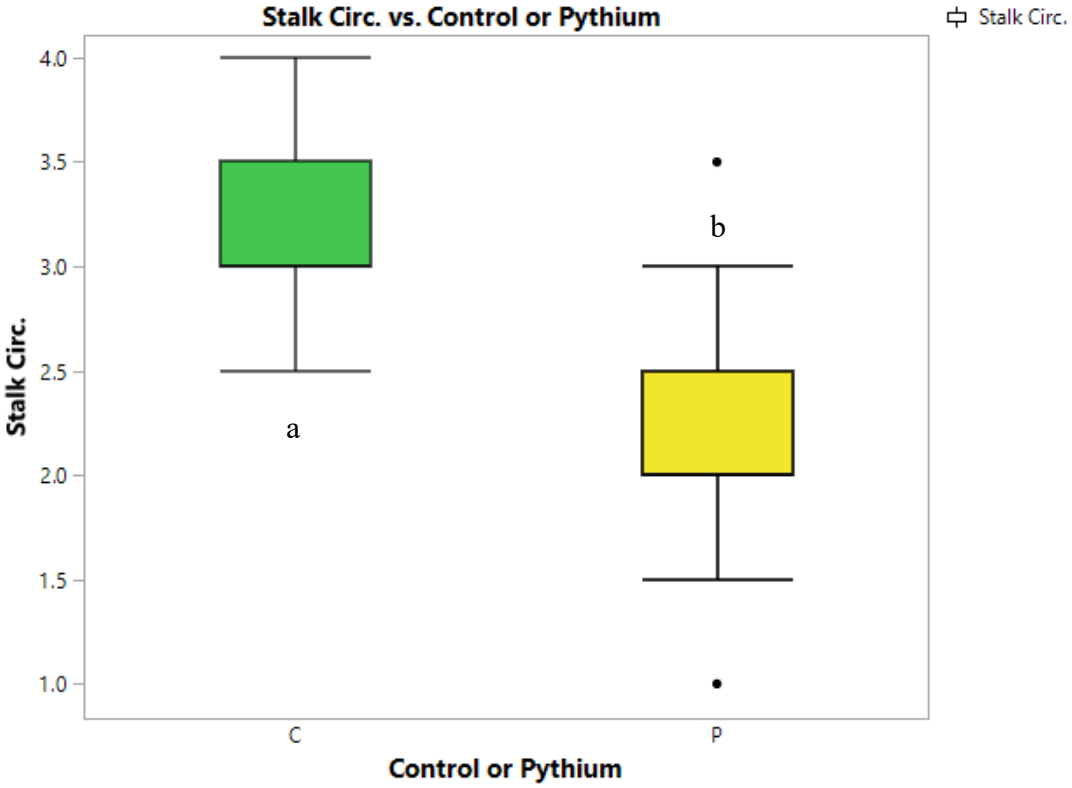


Figure 3.9 Example of shucked ears of *Pythium*-infected plants as compared to adjacent controls. Marker included for scale.

Figure 3.10 Averaged kernel estimate comparisons of *Pythium*-infected plants (P) compared to control (C) based on kernel count along the longitudinal axis of the ear multiplied by the kernel count along the widest latitudinal axis. Significance of mean separation based on T-test ($p < 0.0001$).

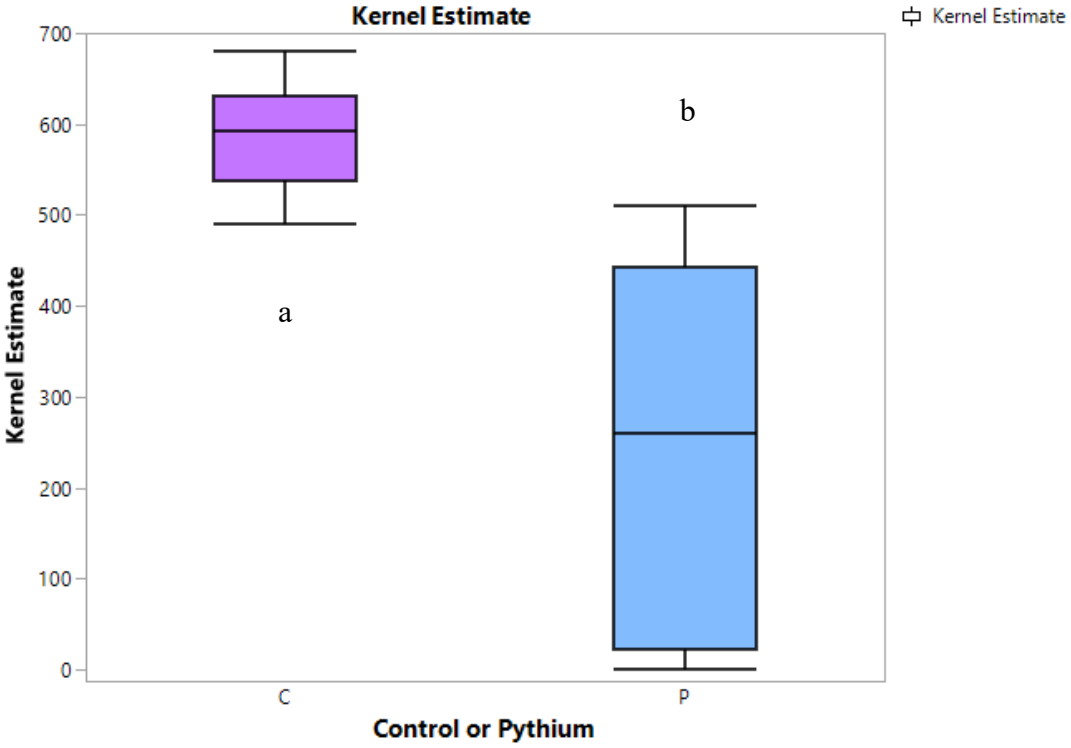
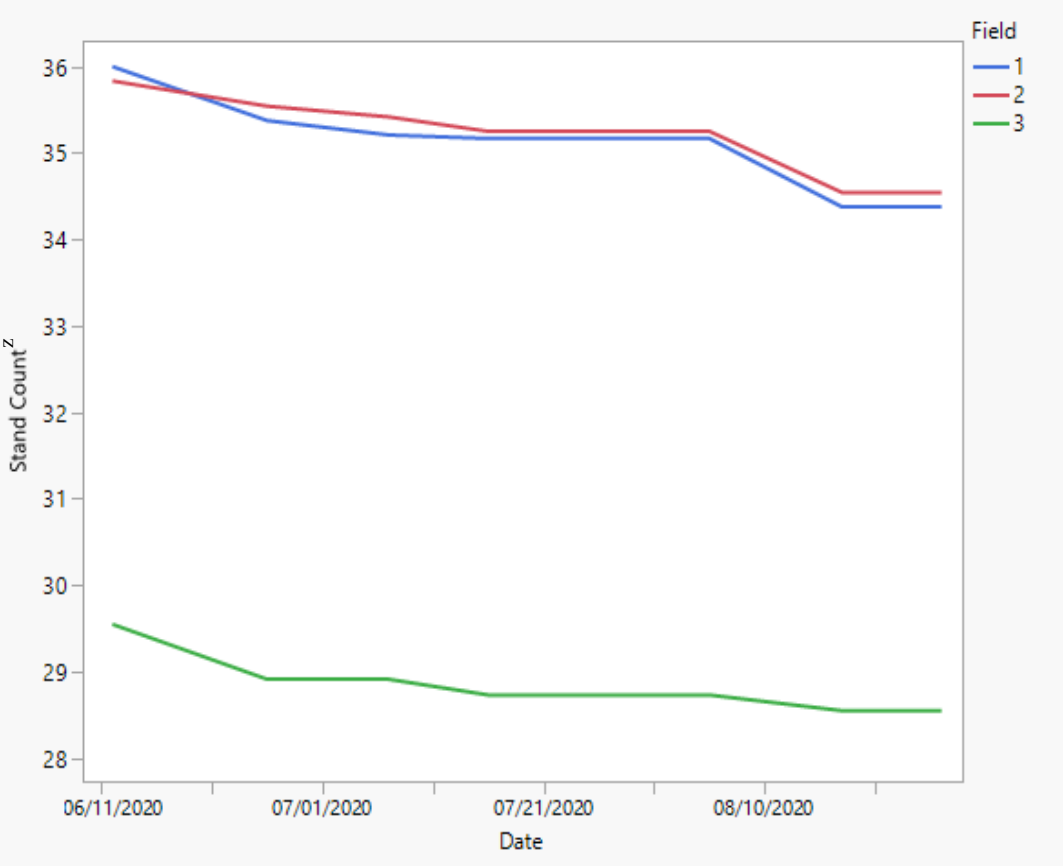


Figure 3.11. Corn stand count changes by field throughout the season (6/12/20 – 8/26/20).



[z] Stand counts extrapolated to x 1000 plants per 1 acre (0.404686 ha).

Appendix A

EVALUATION OF IRRIGATED FOLIAR FUNGICIDES FOR MANAGEMENT OF GRAY LEAF SPOT OF CORN IN DELAWARE, 2020

Published in *Plant Disease Management Network*

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CORN (*Zea mays* L. 'Dyna-Gro D48SS38RIB')
Gray Leaf Spot; *Cercospora zeae-maydis*

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University of Delaware, Georgetown, DE 19947

Evaluation of foliar fungicides for management of gray leaf spot of corn in Delaware, 2019

A trial was established at the Carvel Research and Education Center in Georgetown, DE in a pepperbox loamy sand that had been previously planted in soybeans. Corn was planted on 24 June at 31,000 sd/A. The trial was set up as a randomized complete block design with five replications. Plots consisted of four 30-in spaced rows, 25 ft long with 5 ft alleys between plots. Corn production practices were followed as described by the University of Delaware Cooperative Extension Service. Treatments included one non-treated control and 10 fungicide products. Fungicides were applied using a CO_2-pressurized backpack sprayer equipped with four 8002XR flat fan nozzles calibrated to deliver 20 GPA at 40 psi. Fungicides were applied at the R1 growth stage (3 Aug). Natural sources of inoculum present in the plots were relied upon for disease. Disease severity was measured as percent of ear leaf covered in grey leaf spot lesions on 5 leaves per plot on 10 and 17 Sep;

28 and 35 days after fungicide application. Yield was determined by harvesting the center two rows of each plot on 11 Oct and correcting to 15.5% moisture. Data were analyzed using ANOVA and means were separated using Fisher's Least Significant Difference (LSD; α=0.05).

Delayed planting date resulted in environmental conditions that favored development of grey leaf spot (GLS) prior to R1 growth stage and through the remainder of the season. Fungicides were applied at R1 and no phytotoxicity was observed in any of the treatments. All fungicides significantly reduced GLS compared to the non-treated control at 28 days after application (DAA). At 35 DAA, Revytek, Lucento, Miravis Neo, and Veltyma maintained the lowest disease severity values and all treatments had lower disease than the non-treated control. No significant differences in yield were observed.

Treatment and rate/A (growth stage at application)	NIS[z]	GLS Severity 28 DAA[y, x]	GLS Severity 35 DAA[y]	Yield[w]

Veltyma (R1) 7 oz/A	0.25% v/v Induce 90SL	28.2 abc	42.3 ab	134.8 a
Revytek (R1) 8 oz/A	0.25% v/v Induce 90SL	29.0 abc	32.6 a	153.7 a
Priaxor (R1) 4 oz/A	0.25% v/v Induce 90SL	26.8 abc	45.6 bc	138.3 a
Headline AMP (R1) 10 oz/A	0.25% v/v Induce 90SL	21.4 ab	47.5 bc	136.6 a
Topguard EQ (R1) 5 oz/A	0.25% v/v Penetrator Plus	27.7 abc	56.8 c	131.9 a
Lucento (R1) 5 oz/A	0.25% v/v Penetrator Plus	26.1 abc	36.7 ab	140.4 a
Delaro (R1) 8 oz/A	0.125% v/v Induce 90SL	31.7 bc	45.1 bc	144.1 a
Trivapro (R1) 13.7 oz/A	0.125% v/v Induce 90SL	28.2 abc	44.2 b	138.6 a
Miravis Neo (R1) 13.7 oz/A	0.125% v/v Induce 90SL	20.6 a	42.3 ab	147.4 a
Non-treated Control	--	57.4 d	71.0 d	152.9 a
$P > F$[x]		0.0001	0.0001	ns[v]

[z] Non-ionic surfactant added to each treatment.
[y] Grey leaf spot disease (GLS) severity was visually assessed as the average % ear leaf covered in lesions per plot; means of 5 leaves per plot were used for analysis.
[x] Means followed by the same letter are not significantly different based on Fisher's Least Significant Difference (LSD; α=0.05).
[w] Yield was calculated from the center two rows of each plot harvested 11 Oct and adjusted to 15.5% moisture.
[v] ns= not significant (α=0.05).

Appendix B

EVALUATION OF IRRIGATED FOLIAR FUNGICIDES FOR MANAGEMENT OF GRAY LEAF SPOT OF CORN IN DELAWARE, 2019

Published in *Plant Disease Management Network*

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CORN (*Zea mays* L.; 'Dyna-Gro D48SS38RIB')
Gray Leaf Spot; *Cercospora zeae-maydis*

J.T. Bickel and A.M. Koehler
University of Delaware, Georgetown, DE 19947

Evaluation of of foliar fungicides for management of gray leaf spot of corn in Delaware, 2019

A trial was established at the Carvel Research Station in Georgetown, DE. in a pepperbox loamy sand that had been previously planted in soybeans. Corn was planted on 24 June at 31,000 sd/A. The trial was set up as a randomized complete block design with five replications. Plots consisted of four 30-in spaced rows, 25 ft long and 10 ft wide with 5 ft alleys between plots. Fertility and weed control corn production practices were followed as described by the University of Delaware Cooperative Extension Service. Treatments included one non-treated control and 10 fungicide treatments. Fungicides were applied using a Co_2 pressurized backpack sprayer equipped with 8002XR flat fan nozzles calibrated to deliver 20 GPA at 40 psi. Fungicides were applied at the R1 growth stage (3 Aug). Natural sources of inoculum present in the plots were relied upon for disease. Disease severity was measured as percent of ear leaf covered in grey leaf spot lesions on 5 leaves per plot on 10 and 17 Sep, 28 and 35 days after fungicide application. Yield was determined by harvesting the center two rows of each plot on 11 Oct and correcting to 15.5% moisture. Data were analyzed using ANOVA and means were separated using Fisher's Least Significant Difference (LSD; α=0.05).

At 35 DAA, all fungicide plot disease severity averages were significantly different from the control (p=0.0001). Post-harvest analyses showed no significant difference yield from control plot (p=0.1161). In comparing statistically dissimilar data points from the Fisher's LSD of disease severity at 35 DAA, Revytek treated plots showed the least amount of ear leaf lesions as compared to plots treated with Priaxor, Headline, Delaro, Topguard EQ, and Triviapro. Similar analysis shows that Topguard EQ treated plots averaged the highest disease incidence as compared to plots treated with Veltyma, Lucento, Miravis Neo, Revytek, and Triviapro.

Treatment and rate/A (growth stage at application)	**NIS**	**GLS Ear Leaf Severity 28 DAA[z]**	**GLS Ear Leaf Severity 35 DAA**	**Yield[w]**
Veltyma (R1) 7 oz/a	0.25% v/v Induce	28.2 abc	42.3 ab	134.8 a

Revytek (R1) 8 oz/a	0.25% v/v Induce	29. 0 abc	32.6 a	153.7 a
Priaxor (R1) 4 oz/a	0.25% v/v Induce	26.8 abc	45.6 bc	138.3 a
Headline AMP (R1) 10 oz/a	0.25% v/v Induce	21.4 ab	47.5 bc	136.6 a
Topguard EQ (R1) 5 oz/a	0.25% v/v Penetrator Plus	27.7 abc	56.8 c	131.9 a
Lucento (R1) 5 oz/a	0.25% v/v Penetrator Plus	26.1 abc	36.7 ab	140.4 a
Delaro (R1) 8 oz/a	0.125% v/v Induce	31.7 bc	45.1 bc	144.1 a
Trivapro (R1) 13.7 oz/a	0.125% v/v Induce	28.2 abc	44.2 b	138.6 a
Miravis Neo (R1) 13.7 oz/a	0.125% v/v Induce	20.6 a	42.3 ab	147.4 a
Non- treated Control	--	57.4 d	71.0 d	152.9 a
Pr >F[x]		0.0001	0.0001	0.1161

[z] Disease severity rated as percentage of leaf covered in grey leaf spot lesions caused by *Cercospora zeae-maydis.*

[y]Means followed by the same letter are not significantly different based on Fisher's Least Significant Difference (LSD; α=0.05)

[x] Yield was calculated from the center two rows of each plot and adjusted to 15.5% moisture