THE GENETIC IDENTIFICATION, EPIDEMIOLOGY, AND CHEMICAL CONTROL OF THE CAUSAL AGENT OF PECAN ANTHRACNOSE, *COLLETOTRICHUM*

by

THOMAS INGRAM

(Under the Direction of TIMOTHY BRENNEMAN)

ABSTRACT

New genetic data shows pecan anthracnose to be caused by species within the *Colletotrichum gloeosporioides* species complex. Conidial germination, appressoria formation, and latent infections are all higher at 20 °C and 25 °C, compared to 30 °C and 35 °C. Latent infections were found in South Georgia orchards in late July 2011, and early June in 2012. Symptoms did not always develop, even when numerous latent infections were present. Field applications of strobilurins, triazoles, phosphites and organotin combinations were effective in significantly reducing the number of foliar latent infections in 2011 and 2012. In the greenhouse the fungicides Super-Tin, Quadris Top, and ProPhyt significantly reduced the number of foliar latent infections in pre- and post-inoculation treatments. A range of sensitivities to the triazoles, strobilurins or organotins was found in 39 isolates tested, but only 3 were completely resistant to thiophanate-methyl.

INDEX WORDS: *Colletotrichum*, pecan anthracnose, organotin, triazole, strobilurin, phosphite, latent infection, fungicide sensitivity, GADPH

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B.S., Ohio University, 2010

A Thesis Submitted to the Graduate Faculty of the University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2013

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December 2013

ACKNOWLEDGEMENTS

First and foremost I would like to thank my advisor Dr. Tim Brenneman for his guidance, and wisdom throughout my project. I would also like to thank my committee members Dr. Brannen, Dr. Gold, Dr. Stevenson, and Dr. Wells for their advice and critical analysis on my project. For technical assistance I would like to acknowledge Bonnie Evans, Kippy Lewis, and last but not least Pat Hilton. Andrew Moore contributed a great deal with the phylogenetics portion of this paper. A great supporter of this project for many years has been Dr. Randy Hudson. Financial support for this project came from the Georgia Agricultural Commodity Commission for Pecans, and the Georgia Pecan Growers Association. Luxembourg-Pamol, Inc. also provided financial support for portions of the project. For providing insight and samples for my project I would also like to acknowledge Mike Hotchkiss.

I also want to thank my mother and father, for all their love and support. I would also like to thank Dr. Arthur Trese and Dr. Sarah Wyatt. My undergraduate advisor Dr. Trese not only inspired me to pursue a career in plant pathology, but he also aided and advised me to that goal. It was Dr. Sarah Wyatt's class "Plant Genetics" that sparked my interest in biology.

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INTRODUCTION AND LITERATURE REVIEW

Introduction

Pecan trees (*Carya illinoinensis* (Wang) K. Koch) are deciduous hickory trees in the *Juglandaceae* family; they are native to many parts of the southern regions of the United States, but are not native to Georgia. In the United States pecan trees are grown commercially for their nuts, with 40% of that production taking place in the state of Georgia. The Georgia Farm Gate report placed the value of pecan production in Georgia at $170 million in 2009, $233 million in 2010, and $319 million 2011.

Pecan anthracnose, caused by the ascomycete *Glomerella cingulata* (Stoneman) Spauld. & H.. Pecan anthracnose was first reported in Georgia in 1914 (43), with a documented resurgence in 1989 (7). Pecan anthracnose has been found as far away as Argentina (33). *Glomerella cingulata* has two anamorphs that cause disease on pecan trees, *Colletotrichum gloeosporioides* and *Colletotrichum acutatum* (31). An increase in pecan anthracnose incidence is highly correlated with heavy rainfall, especially in early spring. Severity increases as the season progresses, the disease will often cause leaf drop in the late fall; fall defoliation is linked to lower yield and nut quality (6, 51), shucks are also susceptible to infection.

The financial loss due to pecan anthracnose in 2009 was estimated at $3.4 million (8). However, the damage from foliar disease is difficult to quantify due to the nature of carbohydrate storage in perennials. Damage to the shucks is frequently mistaken for damage from other

causes. The damage to pecan production is also likely to result in a lower yield in the year directly following a year with severe foliar disease and early defoliation (51).

Symptoms of pecan anthracnose include a necrotic leaf scorch, and late summer shuck infection. The infection of pecan shucks usually results in a large slightly sunken brown lesion (1-3 cm in diameter). Most of the shuck lesions will begin at the basal end of the shuck, or around the middle area. When infected shucks in clusters come in contact with each other, infections will often develop at those contact points. Shuck infections can cause significant kernel reduction and aborted kernel development (6, 31, 43). Pecan anthracnose is reported to have an unusually long latent period; it can take weeks to months from the time of initial inoculation to symptom development. Both ascospores and conidia can be found in the field and in culture, and both of these spore types can cause infection. Conidia are the predominant spore type found in the field and in culture (31, 37, 43). In apple, different *Colletotrichum* isolates can cause different types of disease, although all can cause bitter rot symptoms on the fruit, not every isolate was able to infect apple leaves (18). Pink conidial masses can be observed emerging from acervuli with setae on heavily infected leaves and shuck (42).

Disease Development & Biology

Symptoms of pecan anthracnose on the foliage can produce symptoms similar to those caused by other diseases including bacterial leaf scorch. In the literature, these have been collectively referred to as Fungal Leaf Scorch (FLS). Anthracnose produces large brown lesions which usually develop on the margins and progresses towards the middle of the leaves. Lesions usually have a dark margin adjacent to healthy tissue. High humidity and prolonged leaf wetness are thought to be the driving factors behind leaf lesion progression. Although many necrotrophic organisms can be found in these lesions, the primary causal agents of FLS are *Colletotrichum*

gloeosporioides and *Colletotrichum acutatum* (31, 43). The taxonomy of both *C. gloeosporioides* and *C. acutatum* are in a state of flux, and they are currently described as species complexes, each containing over 20 species. At the moment there is no genetic data on which *Colletotrichum* species within these two complexes exist on pecan. Pecan anthracnose isolates have previously been identified by sequencing ITS regions, or by colony and conidial morphology, which only identify what species complex they belong to.

The conidia of *Colletotrichum* species are mainly distributed by rain/wind driven rain. The mucilaginous layer covering an individual conidium makes wind distribution difficult (36, 38). As with most *Colletotrichum* diseases, the initial infection begins with the penetration of the plants epidermal cells via appressoria; once the fungus has bypassed this layer of defenses it colonizes the epidermal cells biotrophically. This biotrophic phase is difficult to observe because the infected tissue is not brown/necrotized (20, 37). If an incompatible interaction between the fungus and the plant occurs, the plant will kill the tissue surrounding the fungal infection site, in order to wall off the infection, thus preventing disease (34). Because *Colletotrichum* species are hemibiotrophs they feed off of both living and dead tissue (25). The asexual stage (*Colletotrichum*) grows on the living and dead tissue, while the sexual stage (*Glomerella*) will only be found on dead tissue (23). Both ascospores from perithecia and conidia can cause pecan anthracnose (43).

Latent infections of *Colletotrichum* can be revealed by freezing leaves or treating them with an herbicide such as paraquat (5, 11). The process of revealing latent infections of *C. acutatum* has been demonstrated in sweet cherry. Sweet cherry leaves were frozen or dipped in paraquat, then exposed to 25° C and 100% relative humidity for six days. This treatment results in conidial masses oozing from lesions where the latent infections were laying idle (5).

According to preliminary experiments; at 25 °C conidia germinate on a wet pecan leaf surface between 2-4 hours after deposition. Appressorial formation follows conidial germination around 4-6 hours after landing on a wet leaf surface. The biotrophic stage/latent infection stage begins around hour 10. During the biotrophic stage, the primary infection hyphae colonize the plant cells surrounding the initial infection site, but remain symptomless (34). After *Colletotrichum* has formed latent infections on pecan leaves, the fungus can remain dormant for weeks to months (43). Relatively little is known about how the fungus switches from its latent phase to its more destructive necrotrophic stage in pecans tissue. In some post-harvest disease systems, *Colletotrichum* switches from biotrophic to necrotrophic once the host reaches a certain stage in its growth (41)

Disease Management

Almost every managed pecan orchard in Georgia is sprayed with fungicides to fend off pecan scab (*Fusicladium effusum*), while pecan anthracnose is mostly a secondary target. For this reason all the fungicides we studied for efficacy against *Colletotrichum gloeosporioides* are already in use against *F. effusum*. Fungicides are applied with air blast sprayers, usually spraying several chemicals in one mix. Contact/protectant fungicides are important for preventing infection. Popular fungicide chemistries used in pecans are organotins, triazoles, guanidines, benzimidazoles, and strobilurins (32, 45). Although the most important fungal disease of pecans is pecan scab, other fungal diseases plague pecan growers as well, including downy spot (*Mycosphaerella caryigena*), zonate leaf spot (*Cristulariella moricola),* and powdery mildew *(Microsphaera ulni*) (15, 30, 40). Pre-pollination, April to May, fungicides are sprayed in 10-14 day intervals. Post-pollination fungicides are sprayed at 10-21 day intervals, with the majority of spray programs being completed by shell hardening (32).

Organotins are broad spectrum multisite fungicides, located in the C6 FRAC (Fungicide Resistance Action Committee) group. Triphenyltin hydroxide (TPTH or Super-tin) is an organotin in use in pecans against pecan scab and other fungal diseases, including *Colletotrichum* (28). The mode of action of organotins on fungi is not well described, but they are thought to inhibit the mitochondria of fungi (1). TPTH is applied to pecan foliar and fruit tissue throughout the growing season as a protectant. Organotins inhibit spore germination and growth, thus preventing infection (44). Because organotins like TPTH have multiple sites of action, it is uncommon for fungi to evolve resistance to them, however, it does exists in some plant pathogens, like *Cercospera beticola* on sugar beet (Table 1.1) (27). Fungicide resistance to the organotin fentin hydroxide has been reported in pecan scab isolates from multiple counties in Georgia (46).

Guanidines are also broad spectrum multisite fungicides in the U12 FRAC group. The mode of action for guanidines is unknown, but the mode of action is thought to involve disruption of cell membranes. Guanidines like dodine can target the mitochondrial membrane of fungal cells, as well as certain enzymes involved in oxidative phosphorylation (10, 35). Dodine, labeled as Elast, has been available for use against pecan scab since 1963. In pecans dodine is used as a protectant with minimal systemic activity (32). Fungicide resistance to dodine has been recorded in the apple scab pathogen, *Venturia inaequalis*, as well as *Nectria haematococca* (*Fusarium solani*) (9, 24, 26). Guanidines have little known activity on *Colletotrichum* species, but are applied in many pecan orchards for pecan scab (3, 9, 30).

Triazoles are single-site, systemic fungicides with translaminar movement in plant tissue. They are in FRAC group G1, and because of their single site mode of action are considered a medium risk for resistance build-up in fungal pathogen populations (16). Triazoles are part of the

demethylation inhibitors (DMI) fungicides which are part of the sterol-biosynthesis-inhibiting fungicides. Triazoles inhibit the production of sterol, an important component of fungal cell walls (29, 39). Fungal resistance to triazoles is common, and mutations in the cyp51 (erg11) gene prevent the fungicide from inhibiting the biosynthesis of sterol. Because triazoles inhibit sterol synthesis instead of directly killing the fungus, spore germination assays may be inadequate in differentiating sensitive and resistant isolates because spores usually contain a reserve of ergosterol that allows them to germinate despite the presence of the fungicide (29). Resistance to DMI fungicides is known in the *Colletotrichum* genus (50). Cross-resistance is also known to occur with this fungicide class, once a pathogen is resistant one fungicide, it typically becomes resistant to most fungicides in the same class of DMIs (22). Quadris-top is a mix of two fungicide active ingredients: difenoconazole, a triazole; and azoxystrobin, a strobilurin. Tebuconazole (Folicur), propiconazole (Orbit/Propimax), and fenbuconazole (Enable) are other DMI fungicides registered for use on pecans.

Quinone outside inhibitors (QoI) are single-site, systemic fungicides with xylem movement. QoIs are commonly referred to as strobilurins, and are located in the C4 FRAC group. The target of all QoI fungicides is the cytochrome b complex in the respiratory chain complex III, which is located in the mitochondria. When QoI fungicides bind to the cytochrome b complex they inhibit the electron transfer between cytochrome b and cytochrome bc1, this prevents the mitochondria from producing ATP (39). The risk of fungicide resistance is high in this class. Resistance to QoIs in *Colletotrichum* occurs when there is a mutation in the cytochrome b gene that prevents the fungicide from binding to the complex. Mutations in this gene often lead to cross resistance to other QoIs (52). Azoxystrobin (Abound), kresoxim-methyl (Sovran), pyraclostrobin (Headline), are labeled for use on pecan scab.

Thiophanates are broad spectrum systemic fungicides that target β-tubulin assembly. This fungicide group, FRAC group B1 the methyl benzimidazole carbamates (MBC), is at high risk for fungicide resistance, due to its very specific mode of action (16). By suppressing the activity of β-tubulin, thiophanates can shut down mitosis, which inhibits spore germination and hyphal growth (42). In *Colletotrichum cereale* resistance against this fungicide has been brought about by a mutation in the β-tubulin 2 gene (52). Thiophanate-methyl is a common MBC used on pecans (Topsin-M). Fungicide resistance to benomyl and thiophanate-methyl have been reported in pecan scab (32, 45).

Phosphite fungicides are used in a variety of crops around the world, usually against Oomycete pathogens such as *Pythium* and *Phytophthora*. This fungicide can act directly by inhibiting oxidative phosphorylation, or indirectly by tagging pathogen cell walls so plants can detect their presence (12, 19). The main way phosphites are thought to increase resistance to pathogens is by stimulating the plant defense system of whatever plant they are sprayed on. By priming the host for infection they heighten the hosts ability to ward off infection (19). Phosphites have also been shown to have activity on *Colletotrichum* in apples. Phosphite has been shown to significantly reduce the incidence and severity of *Colletotrichum gloeosporioides* in apple leaves (2). Phosphites have also been used to target several other diseases in apples, with mixed success. Phosphites such as ProPhyt have been used to control pecan scab. In 2009 and 2010 phosphite did as well as TPTH in controlling scab symptoms on pecan leaves, but sometimes was less effective on fruit scab (4).

The most successful disease management strategies occur before the disease ever becomes established in an orchard, and utilize integrated approaches to minimize disease pressure. Pruning and removal of dead plant tissue are two important cultural practices that can

greatly reduce the amount of primary inoculum within a field by removing plant material where *Colletotrichum* can overwinter (14). Other strategies employed include the selection of resistant varieties. Typically pecans are bred for nut size/quality, and disease resistance is often a secondary quality. Several cultivars are known to be susceptible to anthracnose, including Desirable (48). Resistance to *Colletotrichum* can take many forms, from qualitative to quantitative resistance. Qualitative resistance to pathogens usually involves the presence of resistance genes. Resistance genes detect the presence of the pathogen and initiate cell death to wall off the fungus from the healthy plant tissue (21, 47). Quantitative resistance involves multiple genes that all contribute to inherent or induced resistance to pathogens. Even though quantitative resistance is typically less powerful than qualitative resistance at preventing symptom development, quantitative resistance usually lasts longer (17). Resistance against pecan scab has been documented in various pecan cultivars, however, resistance against anthracnose is less well understood in pecans (13, 48, 49) .

OBJECTIVES

1. Identify the pathogen(s) causing pecan anthracnose using conventional morphological traits and molecular analysis
2. Validate the freezing method of identifying latent infections on pecan foliage
3. Determine the effects of temperature on the different stages of the infection process of pecan anthracnose
4. Monitor development of *C. gloeosporioides* in inoculated and naturally infested pecan foliage
5. Determine the efficacy of fungicides in reducing latent infections in the field
6. Measure pre- and post-inoculation effects of fungicides on foliar latent infections
7. Asses the *in vitro* sensitivity of 39 *Colletotrichum* isolates to four fungicide classes

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CHAPTER 2

GENETIC IDENTIFICATION AND INFECTION PROCESS OF *COLLETOTRICHUM* SPECIES INFECTING PECANS IN GEORGIA

[1] Ingram, T.W., Moore, A., and Brenneman, T.B. To be submitted to *Plant Disease*.

Abstract

Both *C. gloeosporioides* and *C. acutatum* species complexes are reported to cause pecan anthracnose. The most prevalent species complex in Georgia is *C. gloeosporioides*, including *C. gloeosporioides, C. siamense*, and *C. fructicola*. The conidial germination, formation of appressoria, and latent infection development were higher at 20°C and 25°C, relative to 30°C and 35°C. Conidia germinate and appressoria form 2-4 hours after inoculation. However, functional latent infections that are able to survive surface disinfestation do not form until 8-10 hours after inoculation. Assays of pecan foliage in the field in 2011 showed that foliar latent infections were not detected until late July, and then increased dramatically in August. In 2012, foliar latent infections were detected over a month sooner, in early June. Pecan foliage inoculated with *C. gloeosporioides* conidia in the field developed latent infections and in 2012 had more necrosis than was present in non-inoculated control trees.

Introduction

Colletotrichum is a large genus of fungi that infect a great many plant species, and cause significant yield losses throughout the world (8, 13). In pecan trees *Colletotrichum gloeosporioides* and *Colletotrichum acutatum* can cause the disease known as pecan anthracnose. In 2009 pecan anthracnose caused 3.4 million dollars of damage in Georgia alone (5, 7). In the past 3 years pecan anthracnose has also been reported in Australia and Argentina (18, 31). Very few intensive studies have been conducted on pecan anthracnose since it was first described in 1914 by Fredrick V. Rand (3, 17, 27, 33). Pecan anthracnose is known to have a long latent period, and is associated with cool wet weather; however, the epidemiology and infection process of the causal agent is not well understood (6, 28).

Causal agent. Within the genus *Colletotrichum* are species complexes that contain many closely related species. Although both *C. gloeosporioides* and *C. acutatum* are species complexes that have both been found to cause pecan anthracnose, the *C. gloeosporioides* species complex is clearly dominant in Georgia (3, 17, 27). There are 22 species of fungi that were previously referred to as *C. gloeosporioides*; over the past few decades genetic data combined with morphological factors have aided in the separation of these species (8, 12). Members of the *C. gloeosporioides* species complex may be identified by its grey/white mycelia, oblong shaped conidia, and by sequencing segments of the ITS region (31). Species within the *C. gloeosporioides* complex can be distinguished based on sequences of regions of housekeeping genes such as calmodulin (CAL), glyceraldehyde-3-phosphate dehydrogenase (GADPH), or glutamine synthetase (GS) (8, 31). The genetic diversity of *Colletotrichum* species causing pecan anthracnose is not well understood. The vast majority of *Colletotrichum*

isolates from pecan in previous studies were identified using colony morphology and/or conidia shape (3, 17, 27).

Infection process and latent infections. Conidia are often the primary inoculum for diseases caused by *C. gloeosporioides*, and ascospores are typically rare, depending on the host species (8, 31). Conidia germinate on plant tissue at temperatures between 15 and 30°C and high relative humidity (90-100%). The optimum temperature for pecan anthracnose infections has been reported to be 20°C, and *C. gloeosporioides* does not grow above 32°C or greater (15, 21, 31). Between 2 and 4 h after inoculation, appressoria begin to form, melanize, and form penetration pegs that penetrate the plant epidermis. The fungus then forms an endophytic biotrophic relationship with the host plant, which can remain dormant in the plants for days, weeks, or even months before causing visible necrosis (2, 14, 15, 19, 27, 28). During the latent stage, the infection hyphae does not penetrate the plasma membrane of the host cell, which enables the fungus to retain undetected by the host (2, 16, 22, 23, 25). According to published reports of pecan anthracnose, the latent stage is rarely shorter than 2 weeks, can last months, and sometimes symptoms may never appear (6, 17, 27, 28). During this latent stage the fungus is difficult to detect. Several methods for revealing latent infections have been described, and usually involve destructive sampling (4, 22, 28, 30). A recent study on sweet cherry showed that treating infected but symptomless leaves with herbicides or by freezing allowed the fungus to grow freely on the dead tissue, and thus revealed the location and number of latent infections (4). Similar techniques have been used to reveal latent infections in grape, raspberry, strawberry, and tomato (9, 30).

Epidemiology of pecan anthracnose. Overwintering sites for pecan anthracnose include nut shucks or peduncles produced during the previous year, and stem cankers (28).

Primary infections can occur anytime between bud break in April, through July (27, 28). The symptoms of pecan anthracnose include shuck rot and leaf scorch, and both significantly reduce yield (7, 14, 27). The shuck rot usually presents as a large sunken lesion, typically starting at the base of the shuck (17, 27, 33). The foliar symptoms consist of brown to tan necrotic lesions, which can cause leaf drop if the symptoms cover over 50% of the leaf surface (17, 27, 33). Because of its long latent period, pecan anthracnose is difficult to observe during years when the disease pressure is low (6, 17, 27). The latent infection technique has also been used to show that asymptomatic pecan fruits were latently infected by *Colletotrichum* months before showing symptoms in mid-August (28). Pecan trees defoliate in late fall. Late season fungicide applications can increase the amount of time that leaves remain on the trees (33, 34). Whether this is because the fungicides are inhibiting *Colletotrichum* is not known. Most of the damage caused by pecan anthracnose is from the destruction of the host tissue during nut development (17, 27-29). However, *Colletotrichum* is a the primary causal agent of fungal leaf scorch, but more information is need regarding pathogen identification and disease development (17, 29, 33).

OBJECTIVES

1. Identify the fungal species causing pecan anthracnose using molecular analysis.
2. Validate the freezing method of identifying latent infections on pecan foliage.
3. Determine the effects of temperature on the pecan anthracnose infection process.
4. Measure development of pecan anthracnose symptoms and infections in inoculated and naturally infested pecan foliage.

Materials and Methods

Identification of *Colletotrichum* spp. causing pecan anthracnose. In 2009 and 2010, 57 isolates of *Colletotrichum* spp. were collected from diseased pecan leaves or shucks. Three historical isolates collected in 1989 and 1990 from Ducker Plantation in Dougherty County, Georgia were also available from the USDA culture collection in Byron, GA. These isolates were stored in slant tubes on potato dextrose agar (PDA). All 60 isolates were grown on PDA. The DNA of all 60 isolates was extracted with a MO-BIO UltraClean Microbial DNA Isolation Kit. The DNA samples were stored at -80 °C. Primers for the polymerase chain reaction were designed for amplification in the GADPH coding region conserved in fungi (31). The forward and reverse primers were GDF1 (5'-GCCGTCAACGACCCCTTCATTGA-3'), and GDR1 (5'-GGGTGGAGTCGTACTTGAGC ATGT-3'), respectively (24). The cycling parameters were a denaturation cycle of 94 °C for 4 minutes, 34 cycles of 94 °C for 45 seconds, 60 °C for 45 seconds, and 72 °C for 45 seconds, followed by a final cycle of 72 °C for 10 minutes (24). PCR amplification was confirmed using gel electrophoresis. PCR product of the 60 isolates was sent to Eurofins MWG Operon for sequencing. The forward and reverse sequences were aligned using Geneious (Version 6.1, Auckland, New Zealand). The aligned sequences were identified using BLAST against the NCBI nucleotide database (1).

Latent infection procedure. In order to reveal asymptomatic latent infections, pecan leaflets were removed from the plants and placed in a -20 °C freezer for 5 hours. Directly after the freezing process, leaves were disinfested by placing them in 70% ethanol for 1 minute, rinsed in tap water, then placed in a 10% bleach/distilled water solution (0.5% NaOCl) for 1 minute, then rinsed with distilled water. After disinfestation the leaves were placed in sealed plastic bags, along with a moist paper towel to keep the moisture high. The leaves in the bags

were exposed to 12 hours of light, and kept at room temperature (20-25 °C). After three to seven days of incubation, the latent infections in the leaves produced small dark lesions, with concentric circles of salmon pink-orange conidial masses that emerge from acervuli. The latent infections were counted, and the average number of latent infections is measured per leaf, or per leaflet.

The effects of temperature on the infection process of *Colletotrichum gloeosporioides*. To determine when pecan foliage develops latent infections of *C. gloeosporioides* at different temperatures, three month old pecan seedlings were inoculated with a conidial suspension strain G1069. The conidia were suspended in distilled deionized water at a concentration of 10^6 conidia/ml, and applied to the pecan seedlings leaflets with a spray bottle until runoff. Immediately after inoculation, the seedlings were covered with plastic bags to maintain high humidity, and placed in incubators at 20, 25, 30 or 35 °C. Every two hours for 18 hours two leaflets were removed from each incubator, and stained with Remel lactophenol alanine blue dye for two minutes. After staining the leaflets were placed in distilled deionized water for one minute to remove excess dye. Fifty conidia were observed on the leaflets with the aid of a microscope (200X), and rated as non- germinated, germinated, or appressoria formed. At the same sampling times, three leaflets were removed from the plants, and exposed to the latent infection procedure. Throughout the experiment the plants were lightly sprayed with distilled deionized water to maintain humidity and leaf wetness. The plants were only removed from incubation chambers during sampling or re-watering.

Detection of foliar latent infections in pecan orchards. In 2011 and 2012, two orchards were sampled for the presence of latent infections. The first orchard was at the UGA Ponder Farm in Tift County GA. Most of the trees in the Ponder Farm orchard were sprayed

with fungicides; however, the trees sampled were not. The second orchard sampled was a managed, commercial orchard, owned by the Hudson Pecan Company, just north of Fitzgerald GA in Ben Hill County. The Hudson orchard was sprayed with fungicides both years it was sampled. Samples were collected only from unsprayed trees from May through October in 2011, and May through November in 2012. The leaves were collected from Desirable cultivar pecan trees. Every 2 weeks 40 pecan leaves were removed arbitrarily from five different pecan trees in each orchard. Of the 40 leaves sampled, 20 were collected from the base of their shoot, and 20 were terminal collected from the end of the shoot. The number of leaflets per leaf usually varied from 9 to 13. Less than 1 hour after being removed from the trees, the leaves were frozen to reveal latent infections, using the latent infection procedure, as described previously. The number of latent infections per leaf and per leaflet was recorded. The mean number of latent infections per leaf was determined for each sampling date.

Field inoculations. To better understand pecan anthracnose symptom development, trees were inoculated every two weeks from June through October in 2011 and 2012, and rated every 2 weeks for symptom development. The plants were inoculated with a 10^6 conidia/ml suspension from the *C. gloeosporioides* isolate G1069. The conidia were obtained from a one week old colony, grown on Difco PDA. The inoculation consisted of spraying the control terminal per tree with distilled water, and the inoculation terminal per tree with the conidial suspension until runoff. The terminals were inoculated and covered with a plastic bag between 5 and 7 PM EST, and the bags were removed between 9 and 10 AM the following day. The trees were unsprayed Desirable cultivar pecan trees at the UGA Ponder Farm with a history of some pecan anthracnose. Ten trees were chosen for this study, and every 2 weeks a new asymptomatic

terminal on each tree was inoculated. One leaf per day was removed arbitrarily from each terminal the day after inoculation, and frozen to reveal the latent infection procedure. The control terminals were similarly sampled for latent infections. Every 2 weeks, the third leaf from the base of all 10 terminals was rated for visible symptoms of anthracnose on a 0-10 scale, with 0 being 0% necrosis, and 10 being 100% necrosis. The mean disease severity (% necrosis) was determined for all the inoculated terminals, and compared to the average mean disease severity on control terminals. Significant differences in severity between inoculated and non-inoculated leaves were determined using a t-test with at α=0.05.

Results

***Colletotrichum* species infecting pecans in south Georgia.** Of the 60 *Colletotrichum* isolates evaluated, 59 were identified as being in the *C. gloeosporioides* complex (Table 2.1). The other isolate was identified as belonging to the *C. acutatum* complex. This was one of the three historical isolates from Dougherty County in 1990. *C. siamense* was the most frequent isolate, with a total of 40, and was present in every orchard tested. Ten and nine of the isolates were identified were identified as *C. gloeosporioides* and *C. fructicola*, respectively. The one *C. acutatum* species complex isolate was identified as *C. nymphaeae*.

The infection process of pecan anthracnose. There was no spore germination at 2 h after inoculation, but it increased rapidly from 4-6 h at all temperatures (Fig. 2.1 & 2.2). Appressorium formation began between 2 and 4 h after inoculation, but developed more slowly. At 30°C appressorium formation was greatly inhibited (Fig. 2.3 & 2.4), and at 35°C no appressoria were observed. A small number of latent infections formed between 6 and 8 h, with the most latent infections forming 8 h or more after inoculation, and continuing to rise

for another 10 h. The frequency of latent infections was lower at 30°C, relative to 20°C and 25°C, and no latent infections were observed at 35°C (Fig. 2.5 & 2.6).

Latent infections in non-inoculated pecan orchards. In 2011 foliar latent infections of pecan anthracnose were first detected on 18 June, followed by a significant increase in latent infections in early August (Fig. 2.7). The number of latent infections remained high in the Ponder Farm orchard, but in the Hudson orchard, the number of latent infections increased in early August, then decreased in late August, then increased again in October. In 2012 latent infections were first detected as early as 6 June in both orchards (Fig. 2.8). In both years, the number of latent infections fluctuated greatly after their initial development.

Field inoculations. In both years the number of latent infections per leaf was significantly higher in the inoculated terminals, compared to the controls, but latent infections also were observed in the non-inoculated pecan leaves. In 2011 there was no significant difference between the inoculated and non-inoculated terminals (Fig. 2.9). In 2012, symptoms were first seen in mid-September in both treatments, but developed faster in the inoculated terminals, resulting in significantly higher disease severity compared to the controls (Fig. 2.10). Interestingly, no symptoms developed until approximately 3 months after the initiation of latent infections. Also, no symptoms of anthracnose were observed on nut shucks on the control or inoculated terminals

Discussion

Genetic diversity of *Colletotrichum* species infecting pecans. The predominant species complex infecting pecans in south Georgia appears to be *C. gloeosporioides*. The three species

within the *C. gloeosporioides* species complex infecting pecans are most likely *C. gloeosporioides, C. siamense* and *C. fructicola*. More research is needed to determine if there are any practical differences between these species that might impact disease management. About 2/3's of the *C. gloeosporioides* species complex isolates were *C. siamense*, making it the dominant species isolated in south Georgia. Both *C. siamense*, and *C. fructicola* are in the 'Musae clade' within the *C. gloeosporioides* species complex (26, 31, 32). The 'Musae clade' and the 'Kahawae clade' are the two main groupings of species within the *C. gloeosporioides* species complex (31). *C. gloeosporioides* falls just outside the 'Musae clade', and is basal relative to all the Musae clade species. One future line of work would be to distinguish whether any of the species are more pathogenic on fruits vs. foliar tissue, which is a characteristic observed in bitter rot of apple, caused by *C. gloeosporioides* and *C. acutatum*. None of the three historical isolates from Dougherty County were identified as *C. siamense*, while 8 out of 10 of the 2009 isolates from Dougherty County were *C. siamense* (Table 2.1). This population shift may or may not have led to the increase in pecan anthracnose observed in Georgia in the past decade.

The infection process of pecan anthracnose. Results confirmed that the optimum temperature for infection of pecan leaflets by *C. gloeosporioides* is between 20°C and 25°C. At 30°C conidia germinated nearly as well as at lower temperatures, but fewer appressoria, and latent infections were formed. While the fungus developed well at 35°C, fewer appressoria and latent infections were formed at this temperature, compared to lower temperatures. These results are consistent with previous research on pecan anthracnose, and other *Colletotrichum* diseases (15, 20, 27, 28). Severe epidemics of pecan anthracnose are highly correlated with cool wet

growing seasons that may be associated with severe el Niño events, which can cause the pacific jet stream to pass across the southern United States (10, 11).

Latent infections in south Georgia orchards. Latent infections of pecan anthracnose were first detected in south Georgia orchards at different times in 2011 versus 2012. The observed difference in disease development between these years may be due to differences in environmental conditions in the 2 years, with significantly more rainfall occurring in 2012. In 2012, infections were found about 1 month before those in 2011. Because this disease usually requires a long latent period before forming symptoms, the longer the pathogen is in the host, the more likely symptoms will form. The number of latent infections fluctuated greatly in both orchards in both years. In both orchards in both years the trees were mostly symptomless. Symptom development is usually associated with stress factors, and 2011 and 2012 may have not had the conditions conducive for symptom development. The reason for the high number of latent infections in the commercial orchard, which was sprayed regularly with fungicides is not known. This site has a history of the disease however, and may have had a high level of overwintering inocula. Overall there was more rain in the commercial orchard, especially in 2012. In June 2012 there was also some phytotoxicity on the commercial orchard trees that were sampled. Despite this burn, there were more latent infections in those trees than in the non-burned trees in the same area. It is likely that any injury to the foliage could lead to more severe anthracnose, given the hemibiotrophic nature of the pathogen. Overall this data set shows when the infections begin, and demonstrates that the latent infection procedure can be used for understanding epidemics during their asymptomatic stage.

Symptom development in the field. Pecan trees of the cultivar Desirable that were inoculated with *Colletotrichum gloeosporioides* conidia showed few symptoms in 2011, and more in 2012, but only very late in the year. In 2011 there was no statistically significant difference between the amounts of necrosis on the inoculated leaves, compared to the control leaves. The lack of symptom development may have been the result of different weather conditions in 2011. Cumulative rainfall recorded at the UGA Ponder Farm from 1st May through 31st October was 58.6 cm with 44 rainy days in 2011, compared to 86.8 cm and 73 rainy days in 2012. In 2009, when there was a pecan anthracnose epidemic documented in Georgia, the total annual rainfall was 151.2 cm. There was also a great deal of black aphid damage in the Ponder Farm orchard in 2012, which may or may not have increased the severity of the inoculated terminals. Black aphid damage is characterized by the presence of chlorotic yellowing around the necrotic lesions the aphids create (14). Because *Colletotrichum* is a hemibiotroph, plant stressors such as insect damage, fruiting stress, mechanical wounding and nutritional imbalances may encourage pathogen and disease development (17, 29, 33). This study has helped describe the conditions required for initial infection of pecan anthracnose, but the stress factors associated with symptom expression are not fully understood. It is apparent that large numbers of latent infections can be present in an orchard without ever producing visible symptoms, even in 'Desirable' pecan trees known to be susceptible to pecan anthracnose (33).

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Table 2.1. BLAST results of aligned GADPH sequences. Species frequency for each county. *C. siamense*, *C. gloeosporioides* and *C. fructicola* are all in the *C. gloeosporioides* species complex, while *C. nymphaeae* is in the *C. acutatum* species complex.

Sampling Location	*C. siamense*	*C. gloeosporioides*	*C. fructicola*	*C. nymphaeae*
Ben Hill Co.	9	0	2	0
Irwin Co.	6	0	2	0
Tift Co.	2	1	2	0
Lowndes Co.	7	6	1	0
Dougherty Co.	8	3	1	1
Brooks Co.	1	0	1	0
Peach Co.	7	0	0	0
Total	**40**	**10**	**9**	**1**

Figures 2.1-2.6 Seedlings from Desirable variety pecan trees, inoculated with conidia from *C. gloeosporioides* strain G1069 at hour 0. Percent conidial germination, appressorium formation and average number of latent infections sampled at 4 temperatures. Samplings were conducted every two hours for 18 hours.

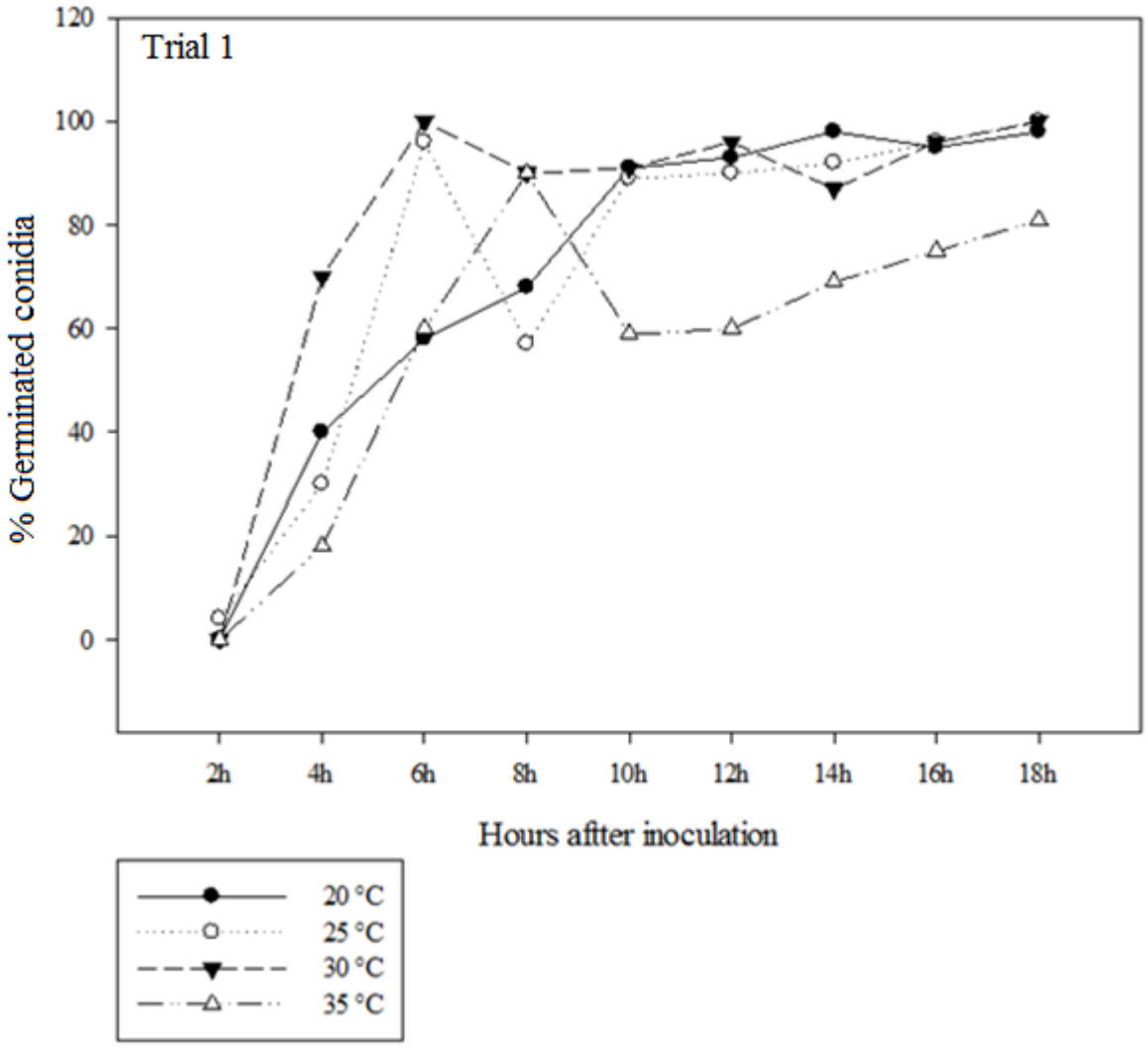

Figure 2.1. Percent conidial germination at various temperatures, 2-18 hours after inoculation, trial 1 conducted in 2011.

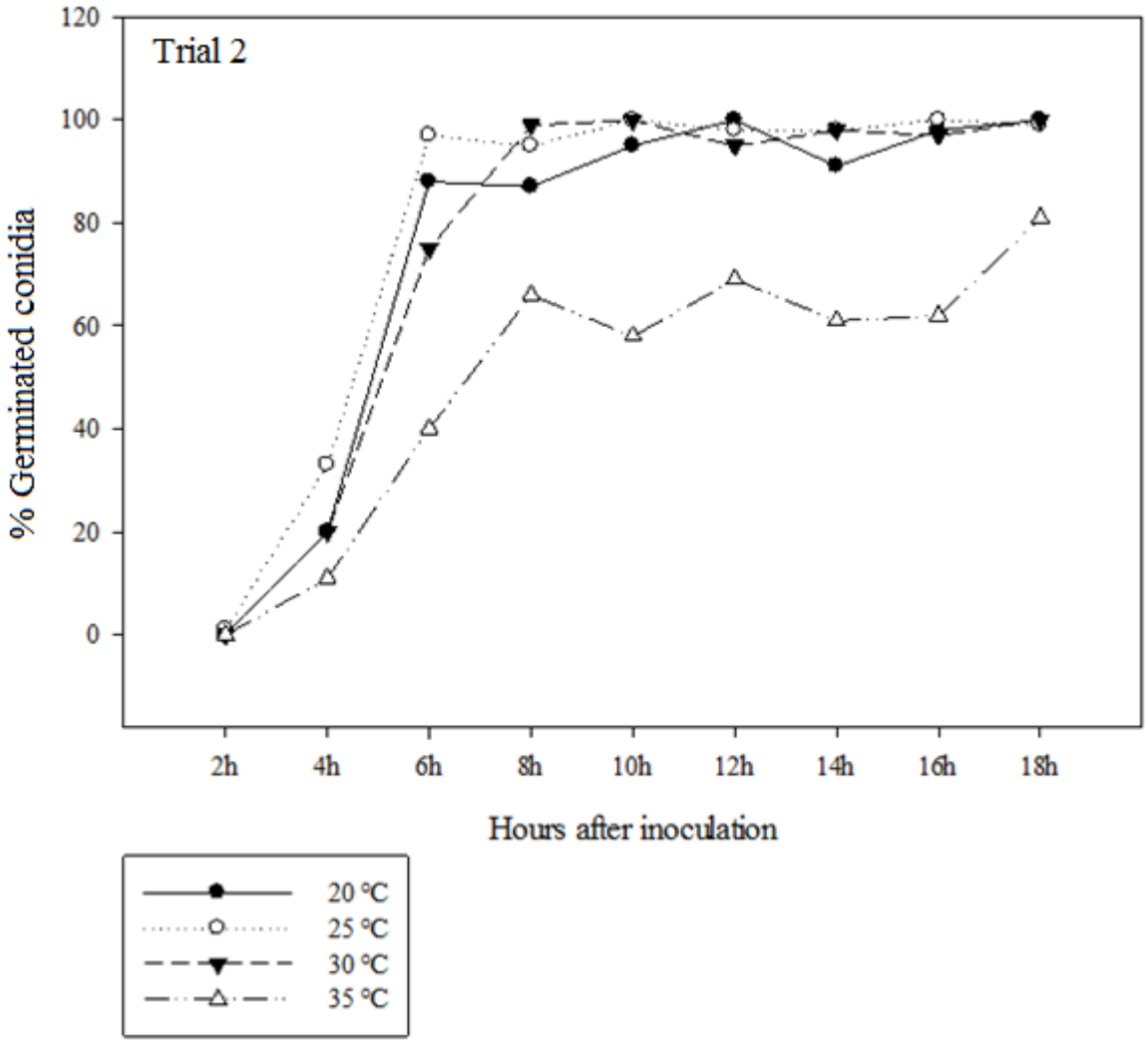

Figure 2.2. Percent conidial germination at various temperatures, 2-18 hours after inoculation, trial 1 conducted in 2011.

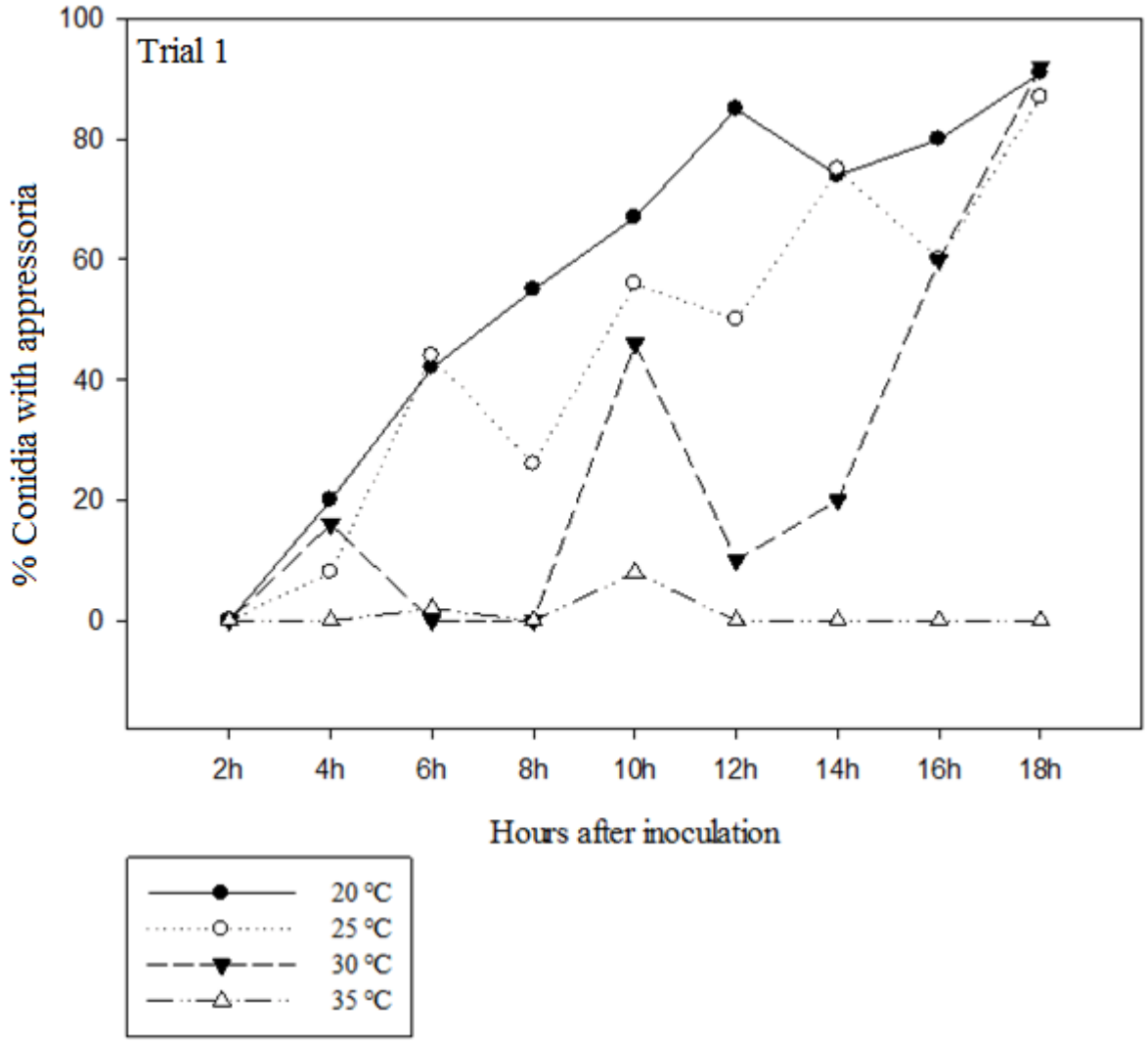

Figure 2.3. Percent of conidia with appressoria at different sampling points, 2-18 hours after inoculation.

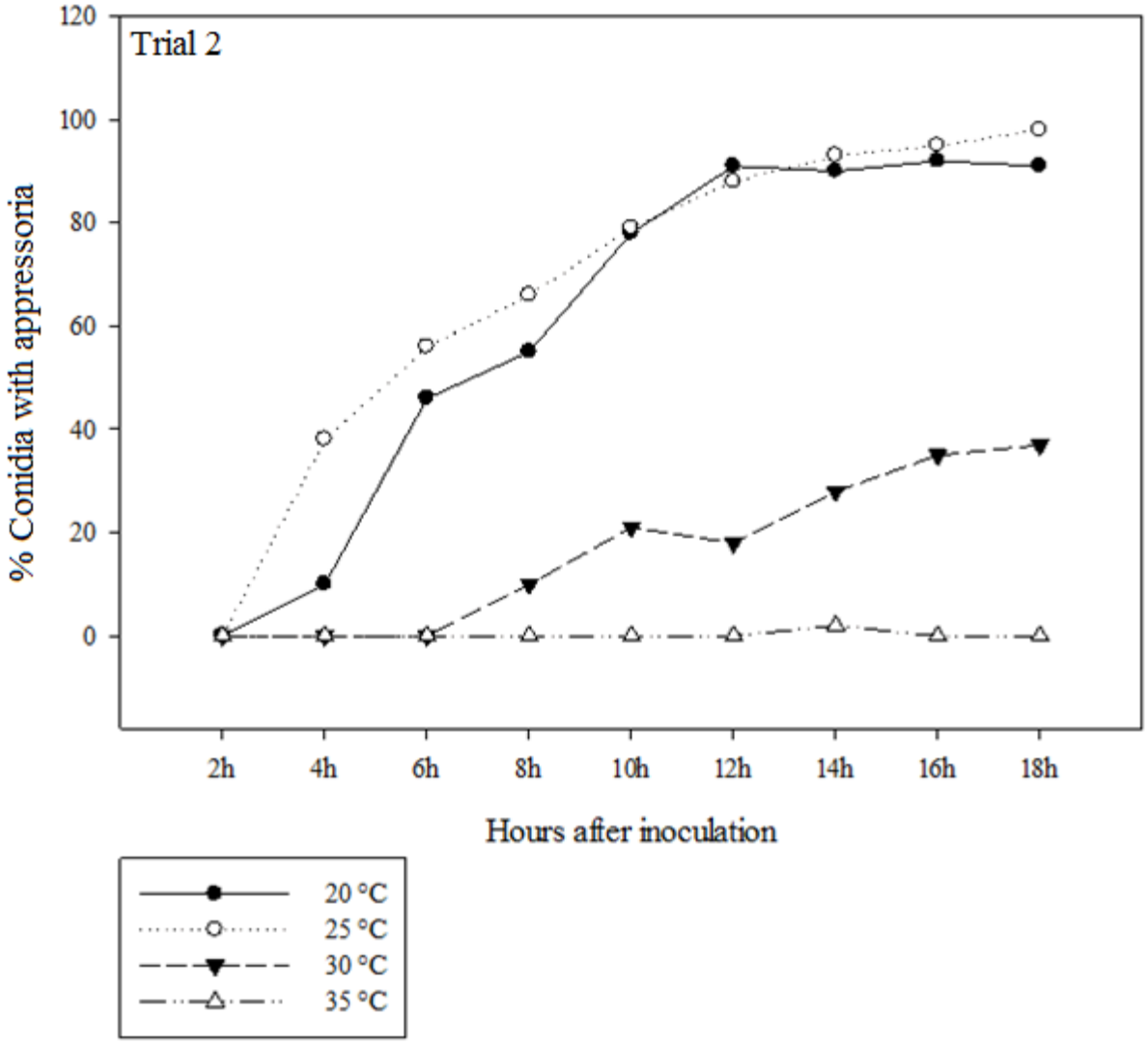

Figure 2.4. Percent conidia with appressoria at 2 hour intervals, 2-18 hours after inoculation. 100 conidia were rated per sample time, per temperature.

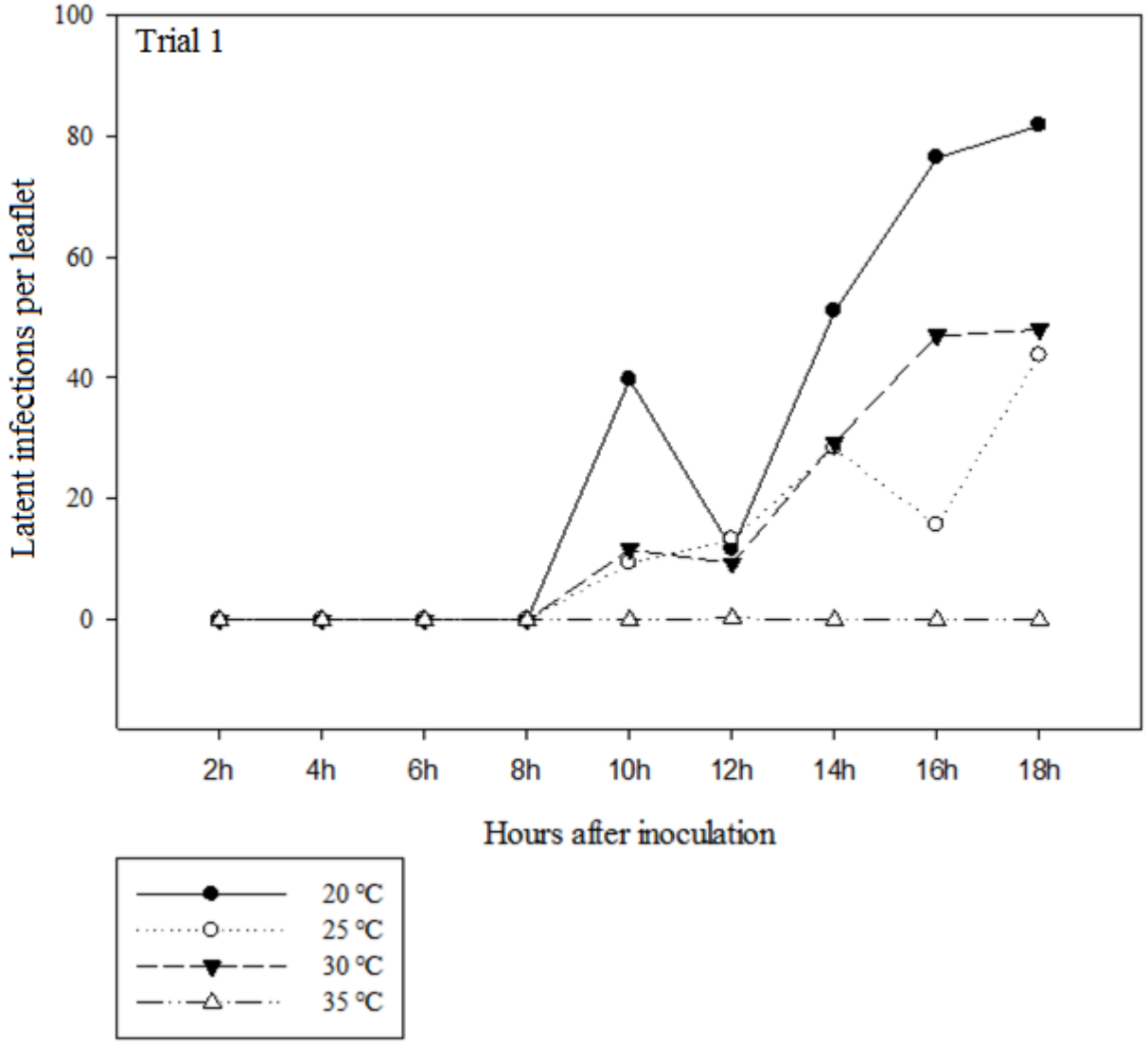

Figure 2.5. The average number of latent infections at different samplings points, 2-18 hours after inoculation.

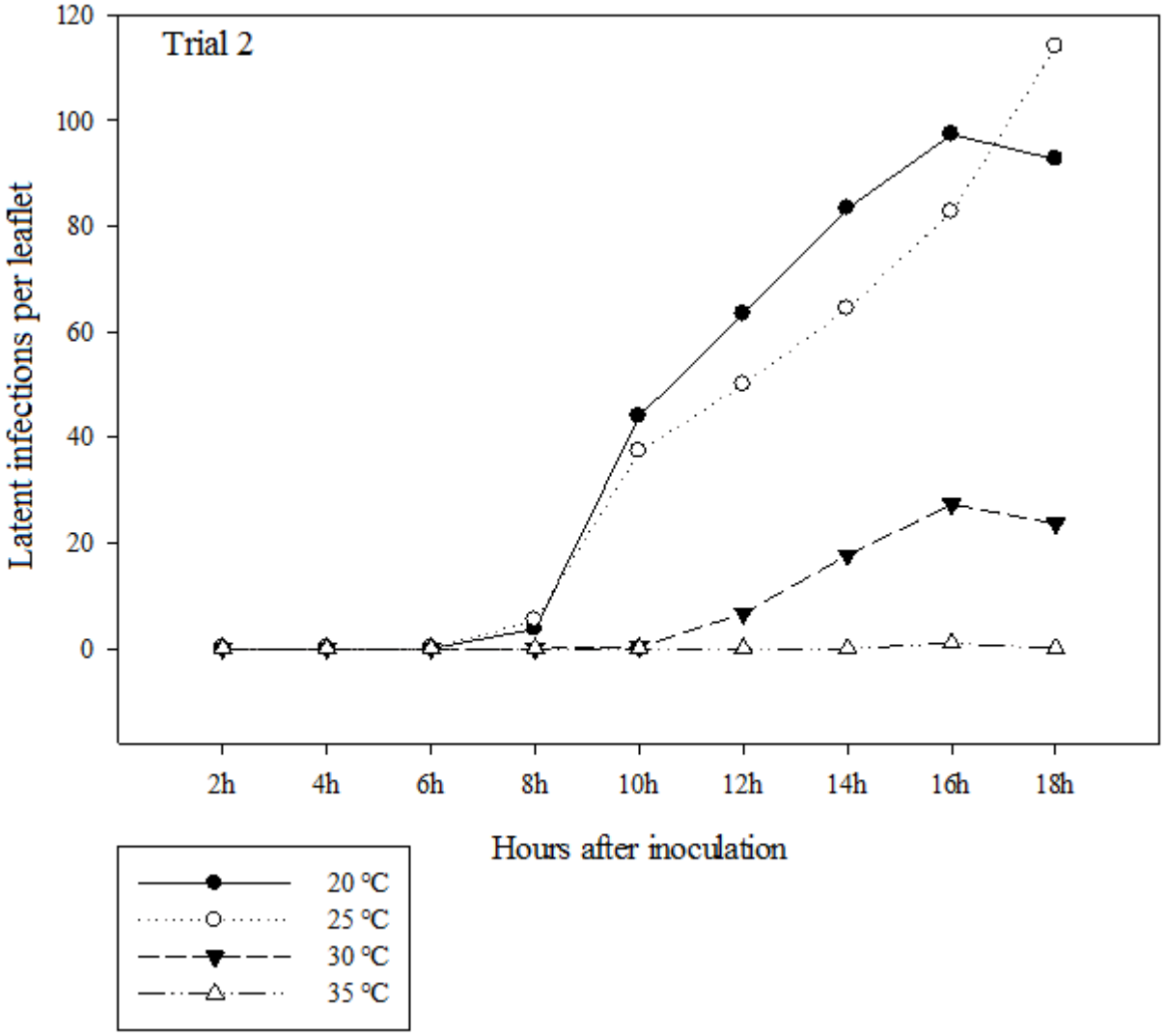

Figure 2.6. The average number of latent infections per leaflet at different samplings points 2-18 hours after inoculation.

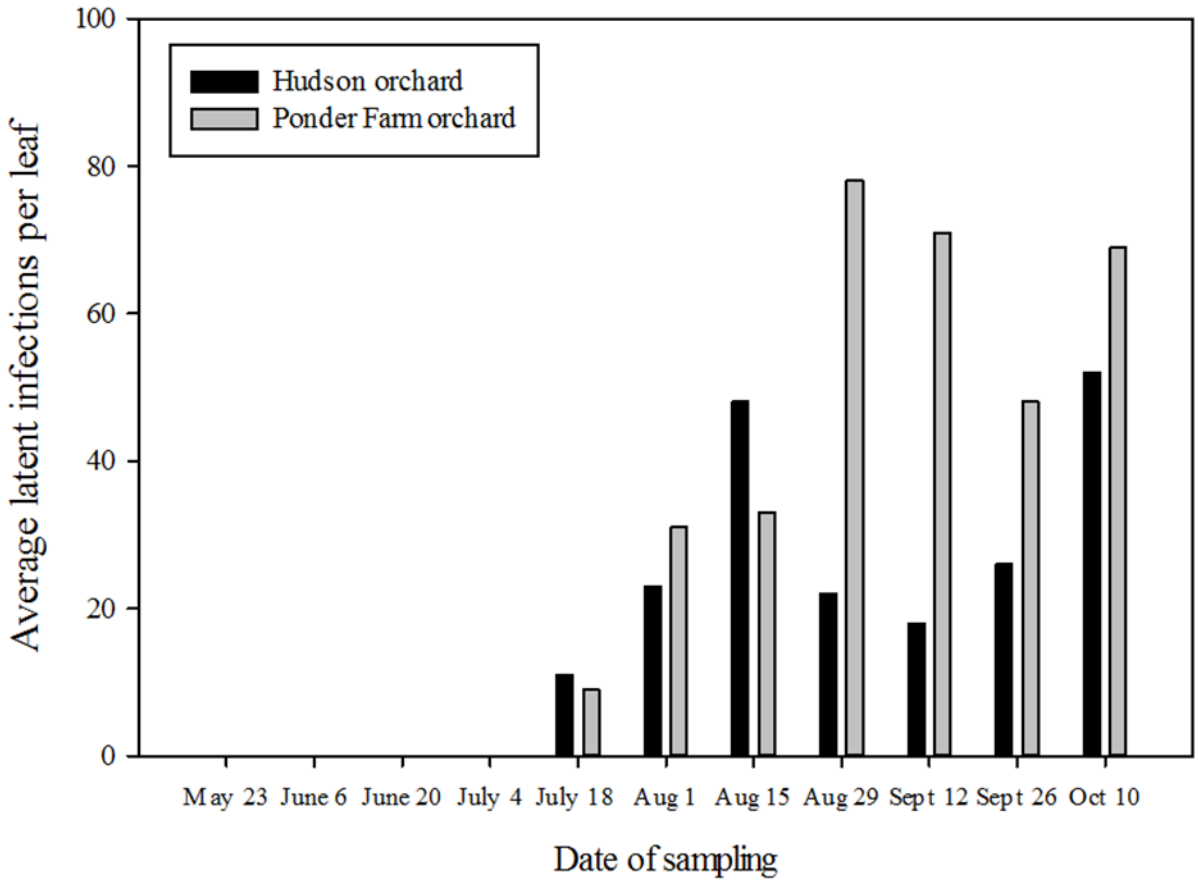

Figure 2.7. The average number of foliar latent infections, per leaf, in two south Georgia orchards in 2011 (n=40). Samples were removed from orchards every two weeks and exposed to the latent infection procedure. The average number of latent infections is displayed per orchard per sample.

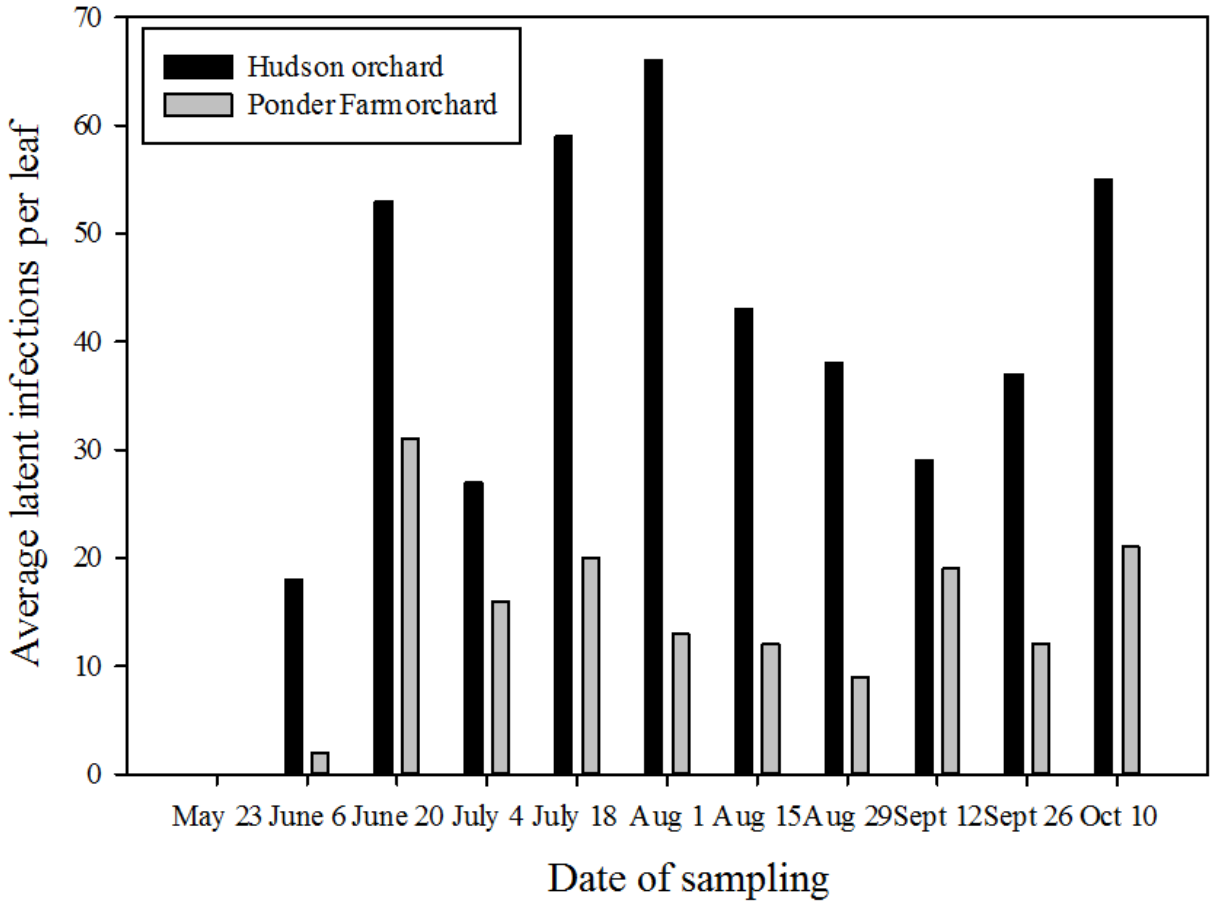

Fig. 2.8. The average number of foliar latent infections, per leaf, in two south Georgia orchards in 2012 (n=40).

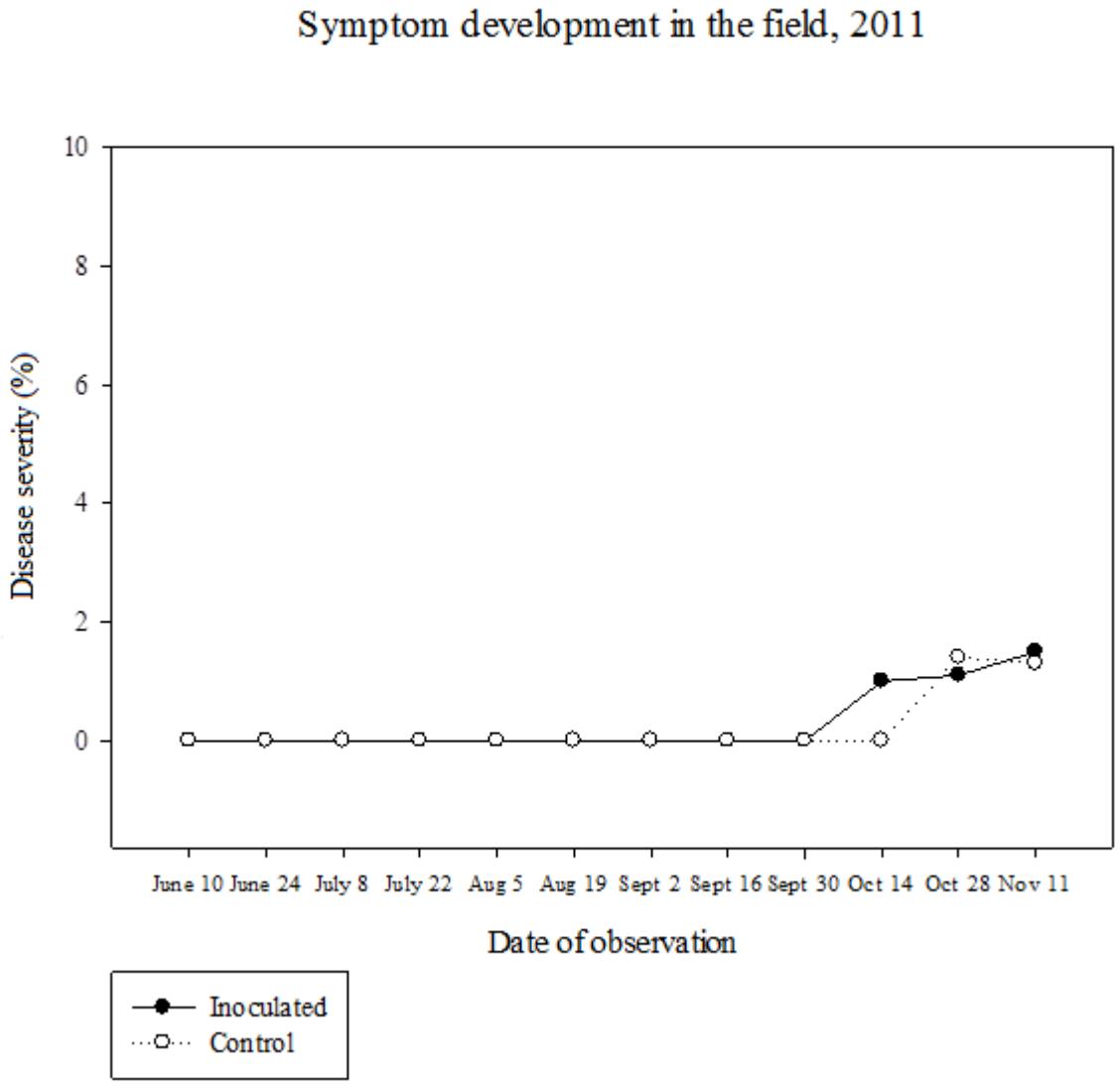

Fig. 2.9. Symptom development in inoculated trees in an unsprayed orchard in 2011. In this year there was no significant difference between % necrosis on the inoculated terminals and the control terminals.

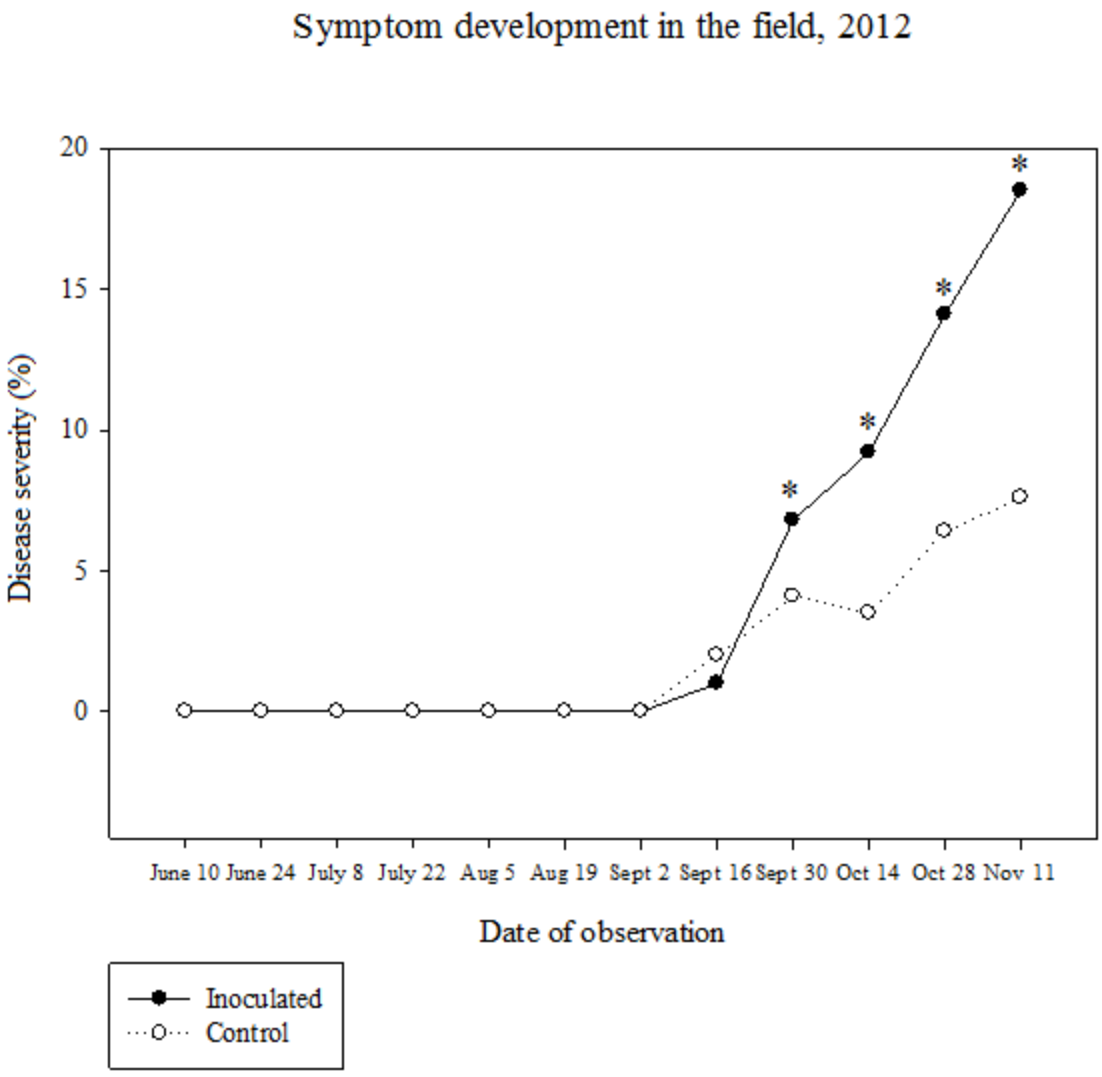

Figure 2.10. Symptom development in inoculated trees in an unsprayed orchard in 2012. Sample points represent the average % necrosis at that date. Inoculated terminals had significantly higher levels of necrosis than control terminals. A significant difference between inoculated and non-inoculated is indicated by an asterisk (*)

CHAPTER 3

THE PRE- AND POST-INFECTION ACTIVITYOF FUNGICIDES CONTROLLING PECAN ANTHRACNOSE

[1] Ingram, T.W., Stevenson, K.L., and Brenneman T.B. To be submitted to *Plant Disease*.

Abstract

Foliar applications of thiophanates, triazoles, strobilurins, phosphites, and organotins are frequently applied to control fungal diseases, including pecan anthracnose. In 2011 and 2012, the field efficacy of five fungicide combinations were rated using a latent infection sampling technique, and compared to non-treated controls. The fungicide applications were either a strobilurin-triazole mix, a phosphite, or an organotin guanidine mix. All five fungicide combinations significantly reduced the number of latent infections, relative to the non-treated controls. In a greenhouse experiment, Quadris Top and Super Tin prevent latent infections when applied before inoculation. Quadris Top, Super Tin, and ProPhyt can all reduced the number of latent infections, in post inoculation treatments. In *in vitro* fungicide sensitivity assays, no complete resistance to tebuconazole, azoxystrobin, and triphenyltin-hydroxide was found in seven orchards in South Georgia. The ranges of EC_{50} values for tebuconazole, azoxystrobin, triphenyltin-hydroxide and thiophanate-methyl were 0.117-1.296, 0.00061-0.19959, 0.044-2.219 and 0.694-3.276 µg/ml respectively.

Introduction

Pecan trees (*Carya illinoinensis* (Wangenh.) K. Koch) are deciduous hickory trees in the *Juglandaceae* family. Pecans are native to many parts of the southern regions of the United States, but are not native to Georgia, where 40% of the U.S. pecan crop is produced. The Georgia Farm Gate report placed the value of pecan production in Georgia at $170-320 million in recent years. Fungal diseases cause significant yield losses in pecan production, and the primary means of controlling these diseases are fungicides (41). Pecan anthracnose, caused by *Colletotrichum* spp., is typically a late-season disease that can reduce yield by causing leaf scorch or shuck rot (11, 12, 51, 52, 67). Cumulative effects of premature defoliation are difficult to quantify, but in the past 10 years there has been an increase in the reports of pecan anthracnose, and in 2009, the yield loss in Georgia due to pecan anthracnose was $3.4 million (12).

Pecan anthracnose is caused by two distinct species of *Colletotrichum*, *C. gloeosporioides* and *C. acutatum* (40, 51, 58, 67). Recent studies have shown that *C. gloeosporioides* and *C. acutatum* are species complexes that contain multiple species (18, 49, 65). Species complexes are groups of fungi that were previously identified as belonging to one species because of their morphological characteristics, but were later found to be multiple species, as more genetic and morphological data was gathered (18, 29, 65). In Georgia the dominant species complex causing pecan anthracnose is *C. gloeosporioides*, based on the grey-white mycelia and oblong shaped conidia (40). Pecan anthracnose has also been reported in Australia and Argentina, and in both cases the species were in the *C. gloeosporioides* complex. The primary infections are caused by conidia from overwintering acervuli, and less often by ascospores from perithecia. These conidia and ascospores germinate on leaves and shucks to

form appressoria that penetrate the plant epidermis to form a biotrophic relationship with the plant cells after only 10 to 12 hours of cool, wet conditions (18, 34, 45, 48, 65). After several weeks, or even months, the infected pecans may begin to show symptoms on the leaves and shucks. Foliar symptoms of pecan anthracnose are a necrotic lesions that continue to expand until leaf drop, and can form as early as July, but are usually not apparent until much later in the growing season, from August to October (11, 51, 58, 67). The shuck rot associated with pecan anthracnose is a dark, sunken, necrotic lesion, usually starting at the stem-end of the nut. Under high-moisture conditions, concentric rings of acervuli are formed within lesions, which often produce salmon pink to orange conidial masses (12, 51, 52).

Because the fungus often lays dormant for a long period of time, there is a window of opportunity after the plant becomes infected to control the disease with fungicides (10, 18, 32, 34, 45, 65). This latent/asymptomatic stage of the disease can be revealed in some hosts by freezing the tissues, followed by surface disinfestation with 10% bleach and 70% ethanol solutions for 1 minute each (10). The infections remain intact and unaffected by the freezing and surface disinfestation processes, providing them the opportunity to grow freely on the necrotic tissue of the host. Within 7 days of incubation in a moist chamber at 22-26 °C, the fungus will produce a small circular or irregularly shaped dark lesion with small-salmon pink to orange colored conidial masses. Each lesion represents an infection site where a conidium or ascospore landed on the plant tissue and formed a successful biotrophic relationship (10, 34, 43, 45, 46).

Various management strategies used to combat fungal diseases include the planting of disease-resistant cultivars, and pruning (63). Pruning can help control diseases in several ways, firstly by removing overwintering structures that grow in twig lesions and the previous year's peduncles, and secondly by increasing air flow that reduces humidity and leaf wetness, which are

conducive to disease development (25, 39) However, fungicides are currently widely used as a primary means of disease management. Fungicides are applied in orchards using air blast sprayers, or less frequently with airplanes, sometimes with adjuvants that may increase uniformity of deposition, plant uptake, etc. (7). Fungicide spray programs usually start in early April, and fungicides are applied on 14 to 21-day intervals until August or September (25, 39). Fungicides used for anthracnose include the thiophanates (MBCs), organotins, QoIs, phosphonates, and the triazoles/sterol inhibitors (DMIs), all of which are applied to manage scab (*Fusicladium effusum*), anthracnose, and other fungal diseases of pecan (25, 39, 41).

Thiophanates, in the methyl benzimidazole carbamates (MBC) group, are a chemical group of fungicides that inhibit assembly of β-tubuline during mitosis in fungi (28). Thiophanates are protectant and eradicant fungicides that can move systemically through plants, and have activity on a number of fungal and oomycete plant pathogens (15, 26, 28). The thiophanate used in pecan production is thiophanate-methyl, sold under the trade name Topsin-M. Resistance to this fungicide is qualitative, and in *Colletotrichum* spp. is usually caused by a mutation in the β-tubulin (*TUB2*) gene. This mutation results in the substitution of a codon for glutamic acid (GAG) in the susceptible fungi, to alanine (GCG) in the resistant types (38). Resistance to thiophanates often results in complete field resistance. Cross-resistance among MBC fungicides, such as the benzimidazoles; benomyl, carbendazim, fuberidazole, and thiabendazole is common. Resistance to thiophanate-methyl has been reported in other pecan pathogens such as the pecan scab pathogen (*Fusicladium effusum*) (55).

Organotin fungicides have been used in pecan orchards since 1967 (41). Organotins, such as fentin hydroxide, are broad-spectrum protectants that adhere to the plant cuticle and prevent fungal pathogens from forming infections (36, 53). Fentin hydroxide is registered for use in

pecan orchards under the trade names Agro-Tin or Super-Tin. It is a broad spectrum pesticide that inhibits oxidative phosphorylation in the mitochondria of fungi and a variety of other organisms (61). Resistance to organotins is typically quantitative, involves multiple gene mutations, and builds up over years or even decades in managed orchards from repeated organotin applications (36, 55, 56).

Several triazoles, with the larger group of demethylation inhibitors (DMI) fungicides, have been registered for use in pecan orchards since the late 80s. DMI fungicides inhibit the synthesis of ergosterol, an important cell and mitochondrial membrane component in fungi. DMI's prevent or eradicate fungal infections by inhibiting hyphal growth inside a plant (47), but are not as effective against spore germination. When ergosterol synthesis is inhibited by DMI fungicides, fungi build up toxic amounts of other sterol compounds such as lanosterol and 12-mehyl-3,6-diol (37). DMI fungicides are xylem systemic within plants, and some of them exhibit significant acropetal movement, after being taken up through the leaves and stems (26). Resistance to DMI fungicides is quantitative, and is usually the result of multiple genes developing mutations. A mutation in the gene that codes for cytochrome p450 monooxygenase has been correlated with reduced sensitivity (13, 31, 66). Other mutations block the buildup of the toxic sterols that accumulate after ergosterol is inhibited (64). As these mutations accumulate in different strains of the fungus, the field efficacy of triazoles diminishes (59). Reduced sensitivity to DMI fungicides has been observed in several other *Colletotrichum* species (44, 66).

Quinone outside inhibitor (QoIs) fungicides were introduced to pecans in the 90s, and are used as protectants. QoIs inhibit ATP production in fungi by binding to the outer oxidizing pocket of cytochrome b (47). QoI fungicides prevent spore germination and inhibit mycelial growth on and in plant tissue. As a group they have less systemic movement than DMI's [40],

but some individual fungicides exhibit significant acropetal movement (8). QoIs provide a high level of protection against a large number of fungal diseases (8, 47). In pecan orchards, QoIs are often used in combination with DMIs to provide multiple levels of protection, and to decrease the risk of fungicide resistance (13, 14, 60). While the DMI fungicides are highly systemic and eradicate fungal infections inside the host tissue, QoIs typically inhibit spore germination and prevent infections from forming (8, 47). Resistance to QoI fungicides can be quantitative, but field resistance is typically qualitative. In *Colletotrichum* spp. and many other plant pathogens it is usually caused by a mutation in the cytochrome *b* gene (*cytb*) at position 143, with the substitution of glycine (GGT) to alanine (GCT) (4, 6, 27). Cross resistance is common within the QoIs, just as it is within the DMI fungicides; if a fungus is qualitatively resistant to one, it is resistant to all of them (14, 27, 33).

Phosphorous acid salts, known as phosphites, were introduced at first as fertilizers in combination with phosphate, and later by themselves as fungicides once it was found that they have antifungal activity (2, 16, 17, 20, 21, 24). They were originally used to control diseases incited by various oomycetes. Although there is some debate regarding their mode of action, foliar applications of phosphites have been shown to reduce the severity of apple leaf scorch (*Colletotrichum gloeosporioides*) in Brazil by 62%, and can significantly reduce the incidence and severity of *Colletotrichum graminicola* on turf grass (2, 16). In addition to anthracnose diseases, phosphites have also been effective in controlling pecan scab (9, 20). Phosphite (PO_3^{3-}) fungicides are typically a phosphite ion (PO_3^{3-}) attached to potassium, or aluminum (62). Because phosphites are highly systemic, and do not fertilize the plant as phosphate does, they can cause the plant to reduce its phosphorous uptake and cause phosphorous deficiency that can

reduce plant growth, or cause foliar necrosis (30, 42). The phytotoxic effects of phosphites are also relatively more prevalent in younger plants (20, 42).

During the infection process, *Colletotrichum* spp. infecting pecan are vulnerable at different stages to different fungicides (43, 45, 47). During and shortly after spore germination, DMIs, organotins and QoIs can prevent the fungus from infecting the host by inhibiting germination of conidia and ascospores (8, 45, 47). Once *Colletotrichum* spp. have formed appressoria and penetrated a leaf surface to form biotrophic relationships with their hosts, they are vulnerable to systemic fungicides such as DMIs, phosphites, thiophanates, and QoIs, to varying degrees (8, 16, 26, 47). Depending on the crop system, *Colletotrichum* will remain in an asymptomatic quiescent biotrophic stage until transitioning to its more destructive necrotrophic stage, in which secondary hyphae ramify throughout host tissue faster than the plant can defend itself (18, 22, 45). Systemic fungicides may reduce or completely inhibit the growth of *Colletotrichum* during its necrotrophic stage. QoIs may reduce hyphal growth to a small degree, but are relatively less systemic than other fungicides. In general QoIs are not as efficient at reducing hyphal growth as they are at reducing spore germination (8, 47). Because the phosphite chemistries may not directly inhibit fungal growth, they may reduce fungal growth within plants by stimulating plant defenses, or by binding to fungal cell walls and increasing the likelihood that the plant will detect the pathogen during its biotrophic stage (2, 20, 42, 47, 62).

The epidemiology of pecan anthracnose is not well understood, particularly as it relates to the latent infections. A better understanding of how different fungicides affect the pathogen during this period is also needed. One reason for the increase in disease incidence in recent years may be decreased sensitivity of pecan anthracnose to one or several fungicides used to control fungal diseases in pecan orchards. Therefore one objective of this work was to determine the

sensitivity of *Colletotrichum* isolates from Georgia pecan orchards to the fungicides representing the major classes used in pecans. An additional objective was to validate the use of a freezing technique to enumerate latent anthracnose lesions on pecan tissues. Once validated, the technique was then be used to evaluate the efficacy of some currently used fungicides for anthracnose control in the field. The freezing technique was also used on plants in the greenhouse to evaluate the pre- and post-infection activity of pecan fungicides applied at different times during the infection process. This will provide a better understanding of how to use different fungicides to optimize disease control in the field.

Materials and Methods

Latent infection procedure. To determine the presence of asymptomatic latent infections of *Colletotrichum* species in pecan foliage, leaves were removed from pecan plants and placed in -18 °C freezers for 5 hours. After the freezing process, the leaves are disinfested in diluted bleach (0.5% NaOCl) for 1 minute, washed with tap water, disinfested with a 70% ethanol solution, and then rinsed in distilled water. The leaves were then placed in sealed plastic bags with moist paper towels to maintain 100% relative humidity. The bagged leaves were kept at 20-25 °C, and exposed to 12 hours of fluorescent light per day. After 5-7 days of incubation, small dark brown lesions (3-10 mm in diameter) appear on the dead leaf tissue. *Colletotrichum* latent infections are distinguished from other fungal latent infections by the presence of sand grain sized conidial masses protruding from acervuli, usually in concentric rings on the lesions. Each lesion is counted as 1 latent infection, representing the site where a conidium landed, formed an appressorium, penetrated the leaf epidermis, and formed a biotrophic relationship with the host plant (10). Latent infections are counted in two ways in this study: average latent infections per leaf; average latent infections per leaflet. The different ways of counting latent

infections may or may not result in significant variations in analyzing the results. Depending on the cultivar, pecan leaves usually have anywhere from 9 leaflets per leaf, to 13 leaflets per leaf. Research on sweet cherry did not reveal significant variations in results when comparing average latent infections per leaf, and average latent infections per leaflet (10).

Effect of fungicides on latent infections in the field. In 2011 and 2012, pecan leaves were sampled from an orchard at the UGA Ponder Farm in Tift County for the presence of natural latent infections. The objective of this study was to determine the relative effects of the different fungicides on formation of latent infections. The experiment was designed as a randomized complete block design with 4 replications. The fungicide treatments were applied to 10 to 15-year-old pecan trees (cv. Desirable) planted in a 12.2 by 12.2 M pattern using a commercial air blast sprayer (888 L/ha) (Table 3.1). Every other tree was left unsprayed and served as a buffer between treated trees. Fungicides were applied on a 2-week schedule beginning in early April, for a total of 10 applications per year. To avoid over-exposure of at-risk chemistries, every other application in all treatments, except the nontreated control, was a combination Super Tin 4L (fentin hydroxide), applied at 0.44 L/Ha (liters/hectare), and Elast 400F (dodine) at 1.83 L/Ha. On the 7th of September in 2011 and 2012, approximately 125 leaflets per rep, with four reps, were collected from each treatment. Less than an hour after collection, the leaflets were frozen for 5 hours, disinfested, placed in moist chambers, and the number of latent infections per leaflet was counted 9 days later. The differences between each treatment were analyzed using Statistical Analysis Software 9.3 (SAS), with PROC MIXED.

Pre- and post-infection activity of 3 different fungicide treatments. In 2011 pecan seedlings, grown from seed collected from the cultivar Desirable pecan trees from the previous year, were grown in a greenhouse for 3 months. These 3 month old pecan seedlings were sprayed

with Super-Tin, Quadris-Top, or ProPhyt, to evaluate these fungicides ability to prevent latent infections from forming. The fungicides were mixed in water to a concentration equivalent to 935 L/Ha and sprayed to runoff on pecan leaflets using Garden Plus 0.40-Gallon Plastic Tank Sprayers. Either one or 10 days after being sprayed with fungicide, the plants were inoculated by misting with a suspension of *C. gloeosporioides* conidia, isolate G1069, (1×10^5 conidia/mL) in distilled, deionized water. Plastic bags were placed over the sprayed plants for 20 h, and control plants were sprayed with distilled water instead of fungicide. Each treatment group contained four plants, and at days 11, 21, and 36, two leaflets were removed from each plant, and exposed to the latent infection procedure (Table 3.2). The number of latent infections per leaflet was measured 7-9 days after they were collected. In 2012 this experiment was repeated, but with 6 plants per treatment, instead of 4 (Table 3.3). The average number of latent infections per leaflet for each treatment at each sampling day was compared using Statistical Analysis Software 9.3 (SAS), with PROC MIXED.

Post inoculation activity of fungicides on pecan foliage. In 2011 and 2012 three month old pecan seedlings, grown from seeds collected from Desirable cultivar pecan trees the previous year, were grown in a greenhouse in Tift County GA. These pecan seedlings were sprayed with Super Tin, Quadris-Top, or ProPhyt, 1 or 10 days after they were inoculated with conidia from *C. gloeosporioides*. The fungicides were applied to the pecan seedling foliage with Garden Plus 0.40-Gallon Plastic Tank Sprayers, using concentrations similar to those used in the field (Tables 3.2 & 3.3). In 2011, four plants per treatment were used, and in 2012, six plants per treatment were used. Control plants were sprayed with distilled deionized water. At days 11, 21, and 36 post inoculations, 2 leaflets from each plant were removed from the plant, and frozen to reveal latent infections, as previously described. The number of latent infections was determined for

each sampling day for each treatment (Table 3.1). The number of latent infections recorded for each of the different fungicide treatments and sampling times was compared, using Statistical Analysis Software 9.3 (SAS) PROC MIXED.

Colletotrichum spp. from seven counties in southern Georgia were isolated from infected pecan leaves and shucks by surface-disinfesting the tissue in 0.5% NaOCl and plating it on potato dextrose agar (PDA). Single germinated conidia were transferred from 1.7% Difco water agar (WA), to PDA. A total of 39 isolates was chosen for fungicide sensitivity assays, 36 isolated in either 2009 or 2010, and three historical isolates obtained from Cheyenne cultivar pecan trees in the Ducker Plantation, (Dougherty County) in 1989 or 1990. All 36 of the recent isolates were identified as being in the *Colletotrichum gloeosporioides* species complex and one of the historical isolates was classified as belonging to the *Colletotrichum acutatum* species complex (unpublished data).

In vitro fungicide sensitivity – mycelial growth assay

All isolates were grown on PDA plates for 7 days, then transferred to ¼ strength PDA (QPDA) plates, that were amended with the different concentrations of fungicide. Concentrations of each fungicide were selected based on preliminary experiments. All fungicides were technical grade, diluted in the appropriate solvent, and added to the media at 50-55 °C. Nonamended control plates containing the appropriate solvents were included with all assays. Thiophanate-methyl and tebuconazole were dissolved in acetone, and added to QPDA to make concentrations of 0, 0.1, 1.0, 3.0, and 10 µg ml^{-1}. There were three repetitions of each isolate and treatment, and the plates were incubated at 25 °C for seven days. Radial mycelial growth was measured, and the percent inhibition calculated as a percent of the growth on the nonamended

control plates. The EC_{50} and log EC_{50} value for each isolate was determined using Statistical Analysis Software 9.3 (SAS). Resistance or sensitivity was determined based on greater than 50% growth at the highest concentration of thiophanate-methyl, compared to acetone only control plates.

In vitro fungicide sensitivity – spore germination assay

Technical grade azoxystrobin or TPTH was dissolved in acetone to obtain stock concentrations. Concentrations evaluated were 3.0, 1.0, 0.1, and 0.01 µg ml^{-1} for azoxystrobin, and 10.0, 1.0, 0.1 and 0.01 µg ml^{-1} for TPTH. Control water agar plates contained 1 ml of acetone per liter of water agar, and all plates had 100 mg of salicylhydroxamic acid (SHAM) dissolved in 1mL of dimethylsulphoxide (DMSO) added to inhibit alternative respiration. Spore suspensions (1 x 10^6 conidia ml^{-1}) were made by adding conidia from 10-day-old cultures of each of 39 *Colletotrichum* spp. isolates to sterile tubes with distilled deionized water. 100 µl of spore suspension was added to each of three replicate plates per fungicide concentration. The plates were incubated at 25°C for 14 h, and then examined microscopically for spore germination. An arbitrarily selected sample of 50 conidia per plate were examined at 200 magnification, and each conidium was rated as germinated (hypha protruding from a conidium) or not germinated.

Results

Fungicide efficacy in the field. When tested for foliar latent infections, all the trees treated with fungicides had significantly fewer latent infections compared to the nontreated trees in 2011 (Fig 3.1). The trees with the fewest latent infections, with a mean of 0.26 latent infections per leaflet, were sprayed with ProPhyt, which was significantly lower than all other treatments. The treatments with the second lowest number of latent infections were Absolute and

Quadris-Top, with means of 0.75 and 0.74 latent infections per leaflet respectively. Sovran + Orius and Super Tin + Elast had the highest average number of latent infections, 1.1 and 1.31 respectively, but were still significantly lower than the nontreated control which had 2.2. In 2012, all the trees treated with fungicides again had significantly fewer latent infections than the non-treated control (Fig. 3.2). Quadris Top had the fewest latent infections, and all other fungicides had statistically similar numbers of latent infections. All treatments had fewer latent infections than the non-treated control which had 2.6 per leaflet (Fig 3.2).

Pre- and post-infection activity of fungicides on latent infections. Results of 2011 and 2012 were very similar, and in both years pre-inoculation sprays of Super Tin and Quadris-Top reduced latent infection formation when applied either 1 or 10 days prior to inoculation (Fig. 3.3, 3.4, 3.5, 3.6). ProPhyt applications did not prevent latent infections from forming at day 1 after inoculation, but at the 10 and 25 day post-inoculation (DPI) sampling, the number of viable latent infections declined and were not significantly different from the other two treatments. The length of time the fungicide was on the leaf (one versus 10 days) did not impact the level of activity for any of the three fungicides. In both years there was also a steady increase in the number of latent infections on the non-treated leaves during the 25 days of the experiment.

None of the treatments applied one day after inoculation reduced the number of latent infections compared to non-treated leaves when they were sampled at 10 DPI in 2011 and 2012 (Fig. 3.7, 3.8, 3.9, 3.10). However, in both years the number of latent infections was significantly lower in leaflets that received the post-inoculation applications of Super Tin, Quadris-Top, or ProPhyt than in the non-treated leaflets when sampled at 20 and 35 DPI. There was no significant difference in the number of latent infections among fungicides at all sampling times, except at 20 DPI in 2012 when fewer latent infections were observed in leaflets treated with Quadris Top

had fewer lesions than the other compared to leaflets treated with the other two fungicides. Differences among treatments in the later evaluation timings were the result of the number of latent infections in the non-treated pecan seedlings gradually increasing over time, and the number in the fungicide treatments gradually decreasing. When applied at 10 DPI, ProPhyt did not significantly reduce latent infections until 35 DPI, or 25 days after application.

In vitro fungicide sensitivity. Of the 39 isolates grown on thiophanate-methyl amended QPDA, three were tolerant to thiophanate-methyl. The three resistant isolates were not inhibited by any of the four concentrations of thiophanate-methyl evaluated, including the highest rate of 10 ppm. Two of these resistant isolates were historical isolates from Dougherty County that have been in culture since 1990; the other isolate was from the UGA Ponder Farm. Varying degrees of sensitivity to thiophanate-methyl were observed in the 36 isolates (Fig. 3.11) (Table 3.4). The effective concentration to inhibit 50% of mycelial growth (EC_{50}) for the sensitive isolates ranged from 0.69 to 3.28 µg ml^{-1} (Table 3.4). The median EC_{50} value for all isolates was 1.42 µg ml^{-1}. The median log EC_{50} value of all isolates grown on thiophanate-methyl was 0.15 µg ml^{-1}. The median EC_{50} values for each region ranged from 0.92-1.82 µg ml^{-1} (Table 3.4). Tift County isolates had the lowest level of sensitivity, while Peach County isolates had the highest sensitivity. The frequency distribution of log EC_{50} values was not significantly different from a normal distribution, according to Shapiro-Wilk $p<0.05$ (Fig. 3.11).

A wide range of EC_{50} values was detected (Fig 3.12). The median EC_{50} value for all isolates was 0.545 µg ml^{-1}. The median log EC_{50} value of all isolates grown on tebuconazole was 0.016µg ml^{-1}. The EC_{50} values of the isolates tested ranged from 0.12-1.3 µg ml^{-1}. The region that had the isolates with the lowest median EC_{50} value was Dougherty County, the historical

isolates, at 0.203 µg ml^{-1}. The region with the highest median EC_{50} value was Tift County at 0.674 µg ml^{-1} (Table 3.4).

In vitro fungicide sensitivity – spore germination assay. No complete resistance to azoxystrobin was observed, however, the EC_{50} value based on spore germination of the 39 isolates ranged from 0.0001 µg ml^{-1} to 0.1996 µg ml^{-1}. The log EC_{50} values for the isolates ranged from -3.21 to -0.70 µg ml^{-1} (Fig. 3.13). The median EC_{50} value for all isolates was 0.0158 µg ml^{-1}. The median log EC_{50} value of all isolates grown on azoxystrobin was -1.8 µg ml^{-1}. The lowest median EC_{50} value, 0.0042 µg ml^{-1}, was Dougherty County, and the highest median EC_{50} values were observed in samples from Peach County and Brooks County of 0.0655 and 0.0652 µg ml^{-1} respectively (Table 3.4).

The EC_{50} values for all the isolates grown on TPTH ranged from 0.044 to 2.219 µg ml^{-1} (Fig 3.9) (Table 3.4). The median EC_{50} value for all isolates was 0.783 µg ml^{-1}. The log EC_{50} values of all isolates grown on TPTH ranged from -1.36 to 0.35 µg ml^{-1}. The median log EC_{50} value of all isolates grown on TPTH was -0.11. Complete resistance to TPTH was not detected in any of the 39 isolates using this spore germination assay.

Discussion

The freezing procedure used to reveal latent infections was very effective and was successfully used to enumerate the severity of anthracnose infections. Because pecan anthracnose does not produce visible symptoms losses every year, sampling for asymptomatic latent infections provides an opportunity to measure disease incidence during years when symptoms are not apparent. Determining the frequency of latent infections of diseases with long latent periods, such as those caused by *Colletotrichum* spp., may prove to be an effective way of

measuring disease without requiring the environmental conditions necessary for symptom development. It also was instrumental in demonstrating the effects of the fungicides in this study on different components of anthracnose infections.

Although all products evaluated reduced the incidence of latent infections, the relative level of control was not the same in both years of the study. There are several possible explanation for this. For example, there was less rain in 2011 which may have increased the efficacy of protectant fungicides, because less fungicide would be lost due to wash-off (26, 37). Another explanation may be the variation associated with sampling only once a year (10). Multiple samples taken throughout the season may help detect treatment differences from the noise of the data. Another confounding factor is that every other fungicide application was a half rate of Super Tin, and a half rate of Elast. Although Elast has little to no effect on *Colletotrichum* spp., Super Tin is effective in preventing many fungal infections from forming (36). Depending on when conditions favorable for infection occurred, some of the disease control obtained could have been from the Super Tin/Elast applications.

When applied prior to inoculation, Super Tin and Quadris Top reduced latent infection formation. These results were expected because both fungicides have been reported to have protectant activity (5, 8). ProPhyt reduced latent infections over time, but had no immediate protectant effect, which is somewhat consistent with the literature on this fungicide (30, 57, 62). The consistent increase in the number of latent infections over time in the non-treated controls was not expected. This may have been due to conidia germinating on the leaf surface and forming new infections after the initial 20 hour incubation period. However, the plants were kept in the greenhouse and would have been exposed to minimal periods of leaf wetness needed for infection. Another possibility is that as infections developed over time, they became able to

withstand the fairly harsh disinfestation procedure utilized. Whatever the cause, clearly the fungicides were effective in reducing the incidence of this delayed pathogen development.

One of the more interesting findings of this study was the apparent post-infection activity of fentin hydroxide, a broad-spectrum, protectant fungicide commonly used on pecans. The anti-fungal activity of fentin hydroxide is well documented, and it is known to also inhibit bacterial growth and insect reproduction (1, 5, 35, 53), but it has no reported systemic movement in plants or post-infection activity on diseases. However, in this study the number of latent infections in plants sprayed with Super Tin was lower than the controls when those applications were made either 1 or 10 days after inoculation. The fact that these differences were not evident until 20 to 35 DPI when the infections in the nontreated control had increased would support the theory that TPTH was simply preventing further infections that occurred after the initial incubation period, as discussed above. Alternatively it is possible that latent infections of *Colletotrichum* are affected by conditions on the leaf surface, and the TPTH is acting on those latent infections over time. Preliminary studies have shown that latent infections are true endophytic infections, and not simply conidia or hyphae that are on the leaf surface. It is possible that Super Tin is eradicating latent infections systemically; however, it is more likely that the fungicide is either preventing new infections from forming or acting on the appressoria through the plant surface. Quadris Top reduced latent infections by both preventing and eradicating foliar latent infections. Quadris Top is a combination of two active ingredients: difenoconazole (DMI), and azoxystrobin (QoI). Many triazoles have some post-infection, systemic activity, and difenoconazole is probably responsible for eradicating the latent infections that become established in the plants in the post-inoculation test (23, 26, 37, 66). When applied prior to inoculation, azoxystrobin was effective at preventing latent infection formation. Azoxystrobin

has been shown to greatly reduce spore germination, and the buildup of toxic sterols usually kills spores that come into contact with this chemical (8, 27). Azoxystrobin has also been shown to have significant acropetal movement in peanut (*Arachis hypogaea*), but its systemic properties in pecan are not known (3). Although in this study, it was not possible to completely distinguish the relative effects of each active ingredient, we can say that their combination leads to a significant reduction in latent infections in both post- and pre-inoculation situations. Foliar applications of ProPhyt did not reduce latent infections from forming when applied either one or 10 days before inoculation, but the number of viable latent infections was consistently lower in subsequent samplings. The reasons for the delayed response would be similar to those discussed previously for the other fungicides, but apparently ProPhyt has no measurable effect on this pathogen during the initial infection events of spore germination, penetration, and establishment of an appressorium. The eradicant effect of ProPhyt was not measurable until 10 or 20 days after spraying this fungicide. One reason for this delayed effect may be the complex mode of action that ProPhyt has on fungal organisms. Phosphite chemistries like ProPhyt were traditionally used for Oomycete diseases, and because they are highly systemic they move easily from the upper parts of the plants to the roots (42, 62). Although ProPhyt did not have any measurable preventative activity, the pre-inoculation sprays of ProPhyt eradicated latent infections sooner than most post-inoculation sprays. One reason may be that it takes longer term exposure to the active ingredient, phosphite, is required to reduce latent infections. It is also possible that phosphite may enhance or prime the plants defense mechanisms of the plant host to fend off latent infections (57).

Three of the 39 isolates tested showed complete *in vitro* resistance to thiophanate methyl, and two of these were historical isolates from Dougherty County collected over 20 years ago.

Although the number of historical isolates was small (these were the only isolates still available), the results correlate with what was known about fungicide resistance in pecan orchards in the 1970s and 80s. Because of high levels of resistance to MBC fungicides, their use in pecan orchards was greatly reduced in the 1980s and 1990s (41, 54). This change reduced the selection pressure for the mutation that causes qualitative resistance to thiophanates. Without the selection pressure present, the mutation becomes neutral, and after several generations, beneficial mutations in other regions of the chromosome may have caused a shift away from the resistance mutation. Isolates sensitive to thiophanate-methyl generally had significantly higher EC_{50} values than the other three fungicides tested (Table 4). With the exception of the historical isolates, none of the isolates tested have had significant exposure to thiophanate-methyl (Topsin-M). With little selection pressure present, the variation in sensitivity to thiophanate-methyl among these isolates may just be the random distribution found in natural populations. There is also a possibility that thiophanate-methyl resistance is widespread, and the sample size from each orchard was too small to detect it. Overall these results should be encouraging, but should not be taken out of context. Resistance to MBC class fungicides can occur quickly, and if MBC fungicides are included in spray programs, it is recommended to include at least one other fungicide to manage resistance (13, 14, 60). According to this study, azoxystrobin, TPTH, and tebuconazole exhibit higher intrinsic activity against *C. gloeosporioides* than thiophanate-methyl.

Reduced sensitivity to tebuconazole is typically multigenic, and builds up over time as various mutations accumulate in different strains (66). When grown on tebuconazole amended QPDA, isolates from certain regions were less sensitive, however, this reduced sensitivity has not been correlated with field resistance (14, 50). In isolates from each of the regions tested, the median EC_{50} values were less than 0.75 μg ml^{-1}. The three historical isolates from Dougherty

County were in culture since 1989 or 1990, meaning they had very little exposure to triazoles (three years at the most). Both the range (0.12-0.38 µg ml^{-1}) and the median (0.20 µg ml^{-1}) EC_{50} values of the historical isolates were lower than the *Colletotrichum* strains isolated in 2009 and 2010. This shift may indicate a reduction in sensitivity over time, as has been documented for DMIs in other pathogens, or may simply be a coincidence due to the small number of historical isolates. This difference in EC_{50} values over time may seem troubling, but is still relatively low. In many cases DMIs are being combined with QoIs to target fungi at different stages of the disease process, and prevent or at least delay development of resistance (13, 14, 47).

Tolerance to azoxystrobin was not observed in any of the isolates evaluated in the spore germination assay. The EC_{50} values ranged from 0.0006 to 0.1995 µg ml^{-1}, with a median of 0.015 µg ml^{-1}. The median EC_{50} value of the historical isolates was 0.0042 µg ml^{-1}, and although several present day isolates had high EC_{50} values, for each region the median EC_{50} values were less than 0.07 µg ml^{-1}. Resistance to QoIs is often complete resistance and qualitatively determined by a mutation in the gene that codes for the cytochrome-b complex (4). Based on these results, if qualitative resistance to azoxystrobin exists in the pecan anthracnose pathogen population, it is probably rare. Even the isolates with the highest EC_{50} values probably do not represent a risk of field resistance (4, 33).

The range of EC_{50} values for spores germinating on TPTH water agar plates was 0.04 to 2.22 µg ml^{-1}. The log EC_{50} values of the isolates appear to form two groups, one group of five isolates ranged from -1.36 to -0.76 µg ml^{-1}, the other group of isolates log EC_{50} values ranged from -0.35 to 0.34. The EC_{50} values of all the isolates as a group were not normally distributed, however, separating the five highly sensitive isolates and the 33 less sensitive resulted in two normally distributed groups. Some isolates with high EC_{50} values may be resistant to TPTH in

the field, however more research would be needed to determine this. Quantitative resistance in fungi exposed to TPTH exists in several pathogen systems (19). For example, the range of EC_{50} values in populations of pecan scab isolates (*Fusicladium effusum*) was 0.14-17.32 μg ml^{-1} (55). Organotin fungicides were introduced in 1967, and pecan anthracnose was first described in 1914, which means there has been over 40 years of exposure to this fungicide.

The latent infection procedure is an innovative and effective way of measuring disease incidence in the field and in greenhouses. Sampling for latent infections in pecan foliage sprayed with fungicide can help elucidate how and when they affect the different stages of infection. In every case the fungicides were more effective when the applied before the plants were inoculated. All fungicides and fungicide combinations applied in the field were effective in reducing pecan anthracnose latent infections. With the exception of a remnant of resistance to thiophanate-methyl, high levels of resistance to fungicides do not appear to be widespread in among isolates of *Colletotrichum* spp. from pecans in South Georgia, but there is evidence of some reduced sensitivity to tebuconazole and azoxystrobin *in vitro* when compared to historic isolates. This provides options to growers to utilize multiple modes of action and thus prevent or delay further shifts in fungicide sensitivity.

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Table 3.1. Fungicide applications in the Ponder Farm North Orchard. Rate/acre refers to the amount of active ingredient of fungicide applied to each acre. All treatments are alternated with Super Tin and Elast for resistance management.

Treatment	Fungicide class(es)	Rate/Acre	Applications
1. Super Tin 4L + Elast 400F	Organotin + Guanidine	0.87 L/ha.+3.65 L/ha	1 -10
2. Absolute 500SC + Induce	Strobilurin & Triazole	0.36 L/ha	2, 4, 6, 8, 10
Super Tin 4L + Elast 400F	Organotin + Guanidine	0.44 L/ha.+1.83 L/ha	1, 3, 5, 7, 9
3. Quadris-Top	Strobilurin & Triazole		2, 4, 6, 8, 10
Super Tin 4L + Elast 400F	Organotin + Guanidine	0.44 L/ha.+ 1.83 L/ha	1, 3, 5, 7, 9
4. Sovran + Orius	Strobilurin + Triazole	0.23 L/ha.+0.29 L/ha.	2, 4, 6, 8, 10
Super Tin 4L + Elast 400F	Organotin + Guanidine	0.44 L/ha.+1.83 L/ha	1, 3, 5, 7, 9
5. ProPhyt	Phosphite	2.92 L/ha.	2, 4, 6, 8, 10
Super Tin 4L + Elast 400F	Organotin + Guanidine	0.44 L/ha.+1.83 L/ha	1, 3, 5, 7, 9
6. Non-treated			

Table 3.2. 2011 and 2012 Pre-inoculation greenhouse experiment fungicide sprays. The seedlings were all sprayed with their respective treatments on day 0. Seedlings were inoculated 1 or 10 days after treatment application. Leaflets were sampled 1, 10, and 25 days after inoculation. Four plants per treatment were used in 2011 and 2012, respectively.

Treatment	Sprayed	Inoculated	Sampling days	Leaflets per sampling
1. Super Tin 4L	Day 0	Day 1	2, 12, 27	2 Per plant
2. Super Tin 4L	Day 0	Day 10	11, 21, 36	2 Per plant
3. Quadris-Top	Day 0	Day 1	2, 12, 27	2 Per plant
4. Quadris-Top	Day 0	Day 10	11, 21, 36	2 Per plant
5. ProPhyt	Day 0	Day 1	2, 12, 27	2 Per plant
6. ProPhyt	Day 0	Day 10	11, 21, 36	2 Per plant
7. Untreated (Distilled water)	Day 0	Day 1	2, 12, 27	2 Per plant
8. Untreated (Distilled water)	Day 0	Day 10	11, 21, 36	2 Per plant

Table 3.3. 2011 and 2012 post-inoculation fungicide sprays greenhouse experiment. Plants were inoculated on day 0, and treated with their respective treatments 1 or 10 days after inoculation. Leaflet sampling took place 10 20 and 35 days after inoculation. In 2011 there were 4 plants per treatment and 6 plants per treatment in 2012.

Treatment	Inoculated	Sprayed	Sampling days	Leaflets per sampling
1. Super Tin 4L	Day 0	Day 1	11, 21, 36	2 Per plant
2. Super Tin 4L	Day 0	Day 10	11, 21, 36	2 Per plant
3. Quadris-Top	Day 0	Day 1	11, 21, 36	2 Per plant
4. Quadris-Top	Day 0	Day 10	11, 21, 36	2 Per plant
5. ProPhyt	Day 0	Day 1	11, 21, 36	2 Per plant
6. ProPhyt	Day 0	Day 10	11, 21, 36	2 Per plant
7. Untreated (Distilled water)	Day 0	Day 1	11, 21, 36	2 Per plant
8. Untreated (Distilled water)	Day 0	Day 10	11, 21, 36	2 Per plant

Table 3.4. Sensitivity of *Colletotrichum* spp. isolated from pecan to thiophanate-methyl, tebuconazole, azoxystrobin, and fentin hydroxide. Only isolates sensitive to thiophanate-methyl were included in those sensitivity results.

		EC_{50} value (μg/ml)		
Fungicide	Location	Range	Median	Number of isolates
Thiophanate-methyl				
	Bibbin, Brooks Co.	.76-1.55	1.1563	6
	Lowndes Co.	1.03-3.28	1.49781	6
	Fitzgerald, Ben Hill Co.	0.69-2	1.708925	6
	Hudson Ben Hill Co.	1.21-1.66	1.29298	6
	Peach Co.	0.75-1.92	0.92581	6
	Tift Co.	0.70-2.39	1.82115	5
	Dougherty Co.	1.43	1.43	1
Tebuconazole				
	Bibbin, Brooks Co.	0.37-0.84	0.443525	6
	Lowndes Co.	0.23-0.79	0.63477	6
	Fitzgerald, Ben Hill Co.	0.30-0.90	0.55986	6
	Hudson, Ben Hill Co.	0.31-0.80	0.55824	6
	Peach Co.	0.35-0.77	0.52217	6
	Tift Co.	0.64-1.3	0.674125	6
	Dougherty Co.	0.12-0.38	0.2031	3

Azoxystrobin				
	Bibbin, Brooks Co.	0.001-0.122	0.065235	4
	Lowndes Co.	0.0007-0.014	0.00574	5
	Fitzgerald, Ben Hill Co.	0.0001-0.044	0.01576	5
	Hudson, Ben Hill Co.	0.0008-0.1132	0.00798	5
	Peach Co.	0.0285-0.0973	0.06559	5
	Tift Co.	0.0071-.1996	0.053475	6
	Dougherty Co.	0.0011-0.0138	0.00422	3
Fentin hydroxide				
	Bibbin, Brooks Co.	0.044-2.159	1.28569	6
	Lowndes Co.	0.589-1.609	0.810625	6
	Fitzgerald, Ben Hill Co.	0.503-1.446	0.58394	6
	Hudson, Ben Hill Co.	0.446-2.219	1.13705	5
	Peach Co.	0.049-2.210	0.81632	6
	Tift Co.	0.061-0.797	0.544495	6
	Dougherty Co.	0.536-1.842	1.07204	3

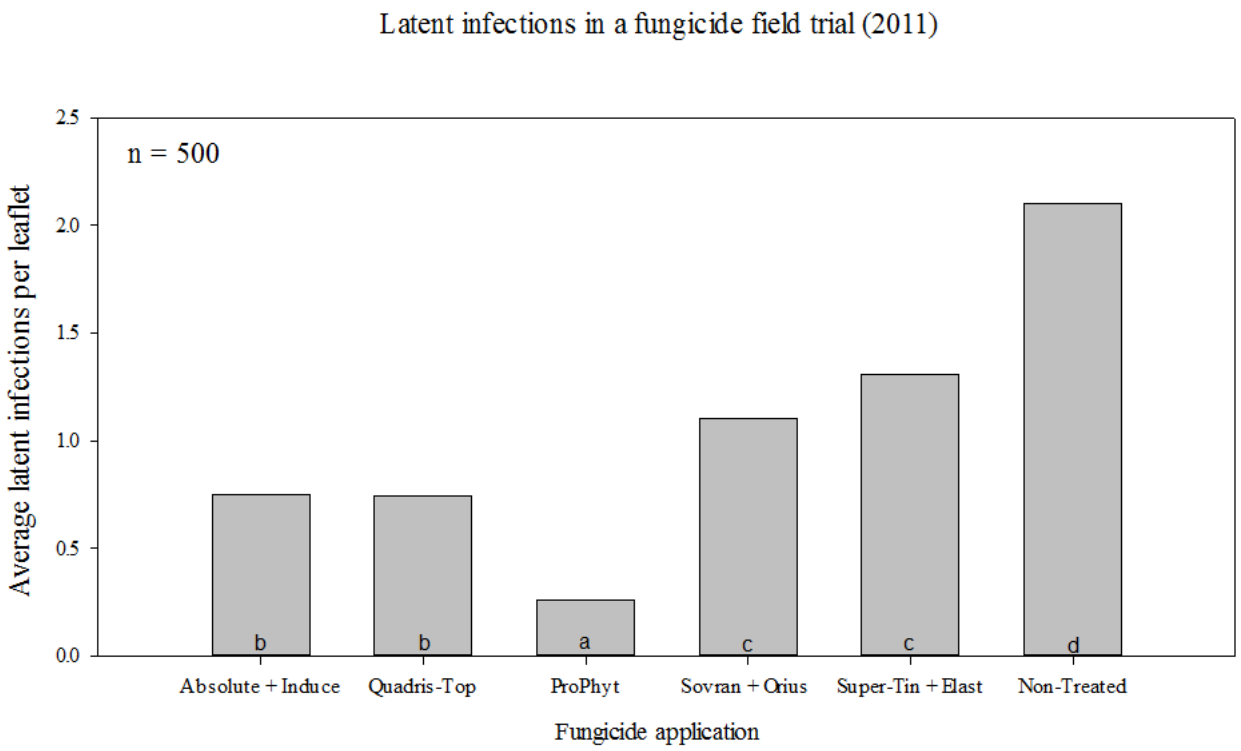

Figure 3.1. The average number of latent infections per leaflet, in 2011, from asymptomatic trees treated with different fungicide combinations. Statistical differences are designated with letters ($p>0.05$).

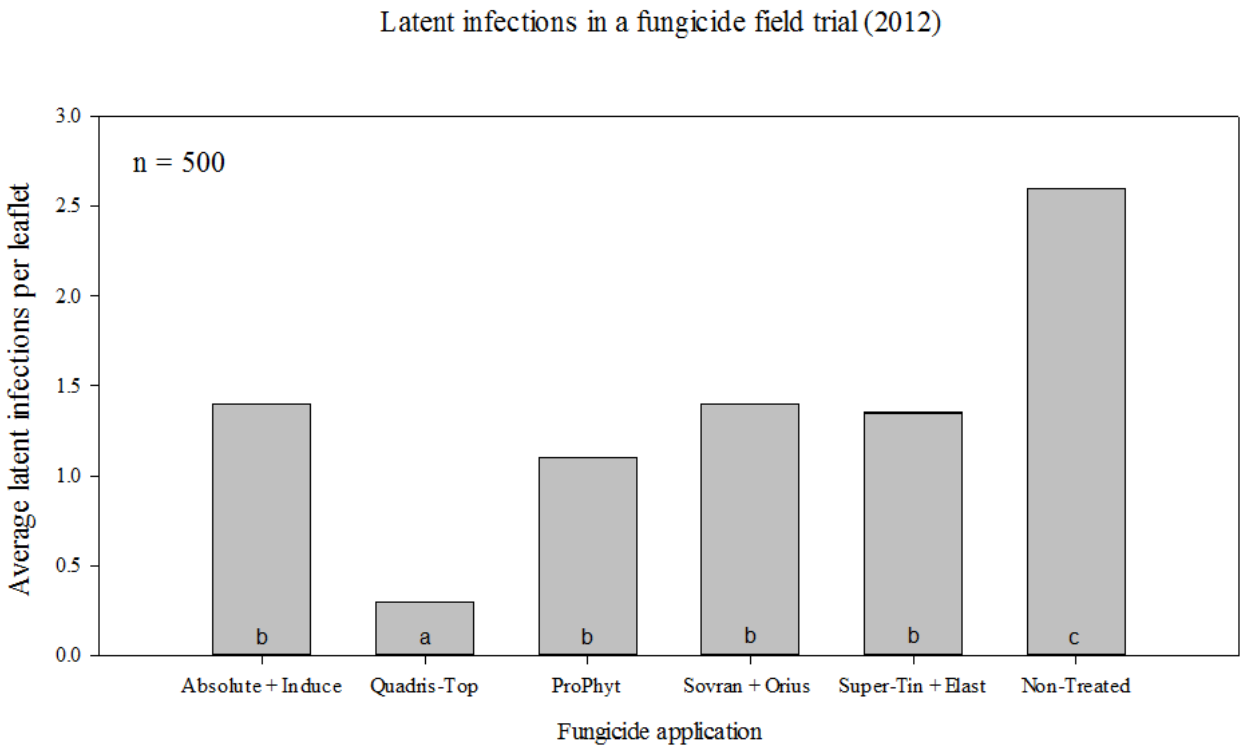

Figure 3.2. The average number of latent infections per leaflet, in 2012, from asymptomatic pecan trees treated with different fungicide combinations. Statistically significant differences are designated with letters ($p>0.05$).

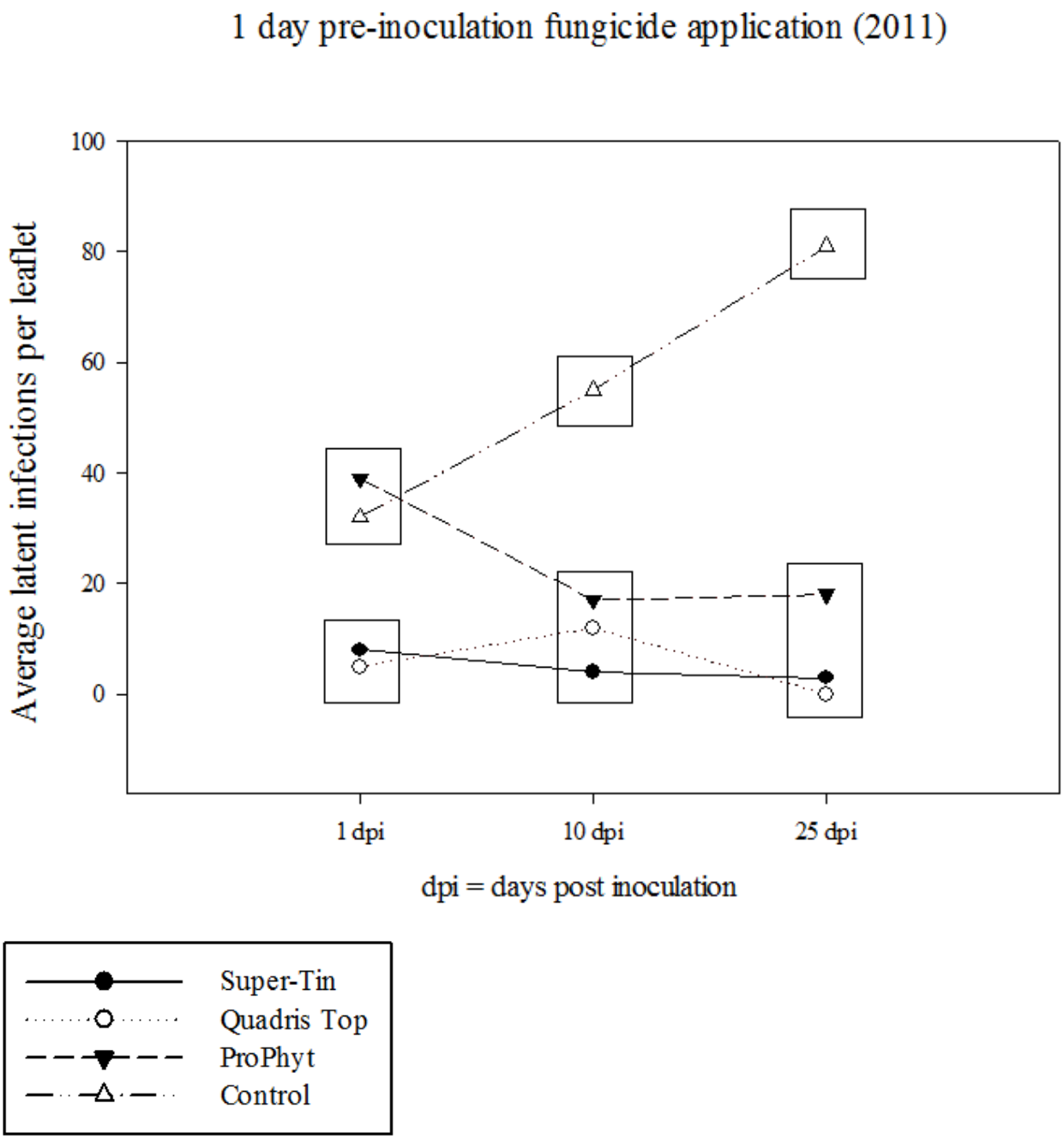

Figure 3.3. Average latent infections per leaflet in pecan seedlings sprayed with fungicides 1 day before they were inoculated in 2011 (pre-inoculation). Boxes indicate statistically similar averages of latent infections in leaflets sampled on the same dates ($p<0.05$).

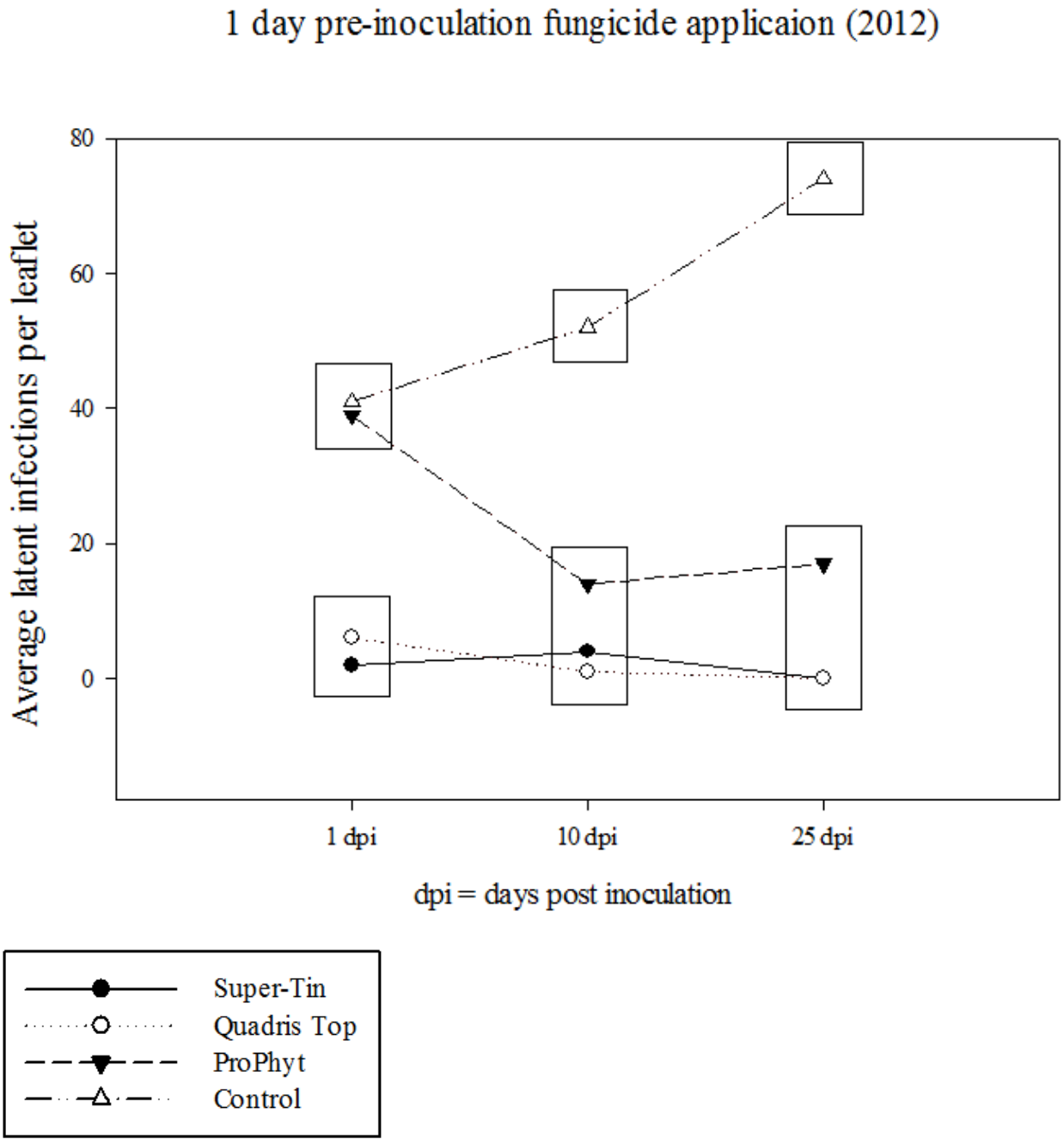

Figure 3.4. Average latent infections per leaflet in pecan seedlings sprayed with fungicides 1 day before they were inoculated in 2012 (pre-inoculation). Boxes indicate statistically similar averages of latent infections in leaflets sampled on the same dates ($p<0.05$).

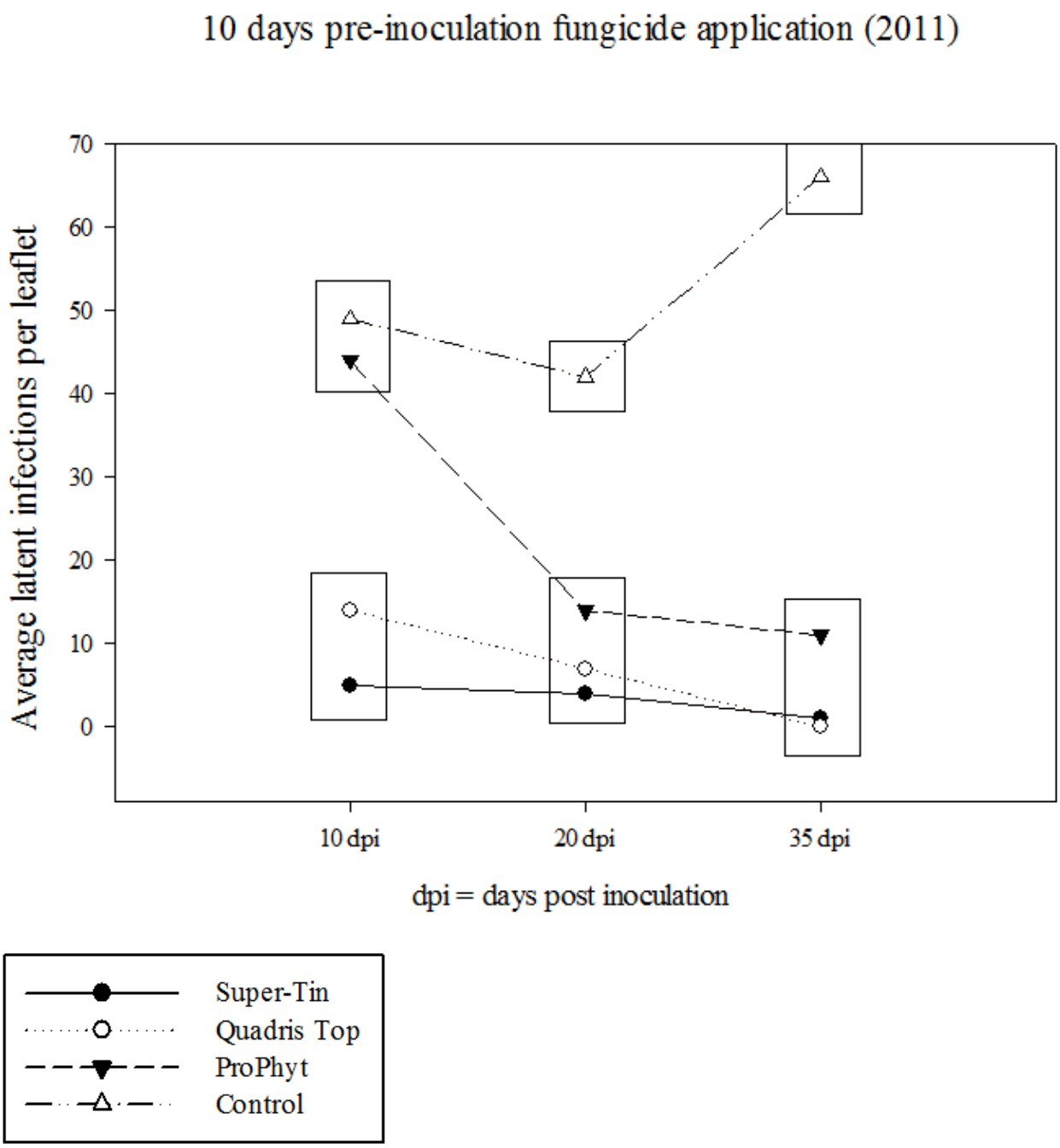

Figure 3.5. Average latent infections per leaflet in pecan seedlings sprayed with fungicides 10 days before they were inoculated in 2011 (pre-inoculation). Boxes indicate statistically similar averages of latent infections in leaflets sampled from different treatments on the same dates ($p<0.05$).

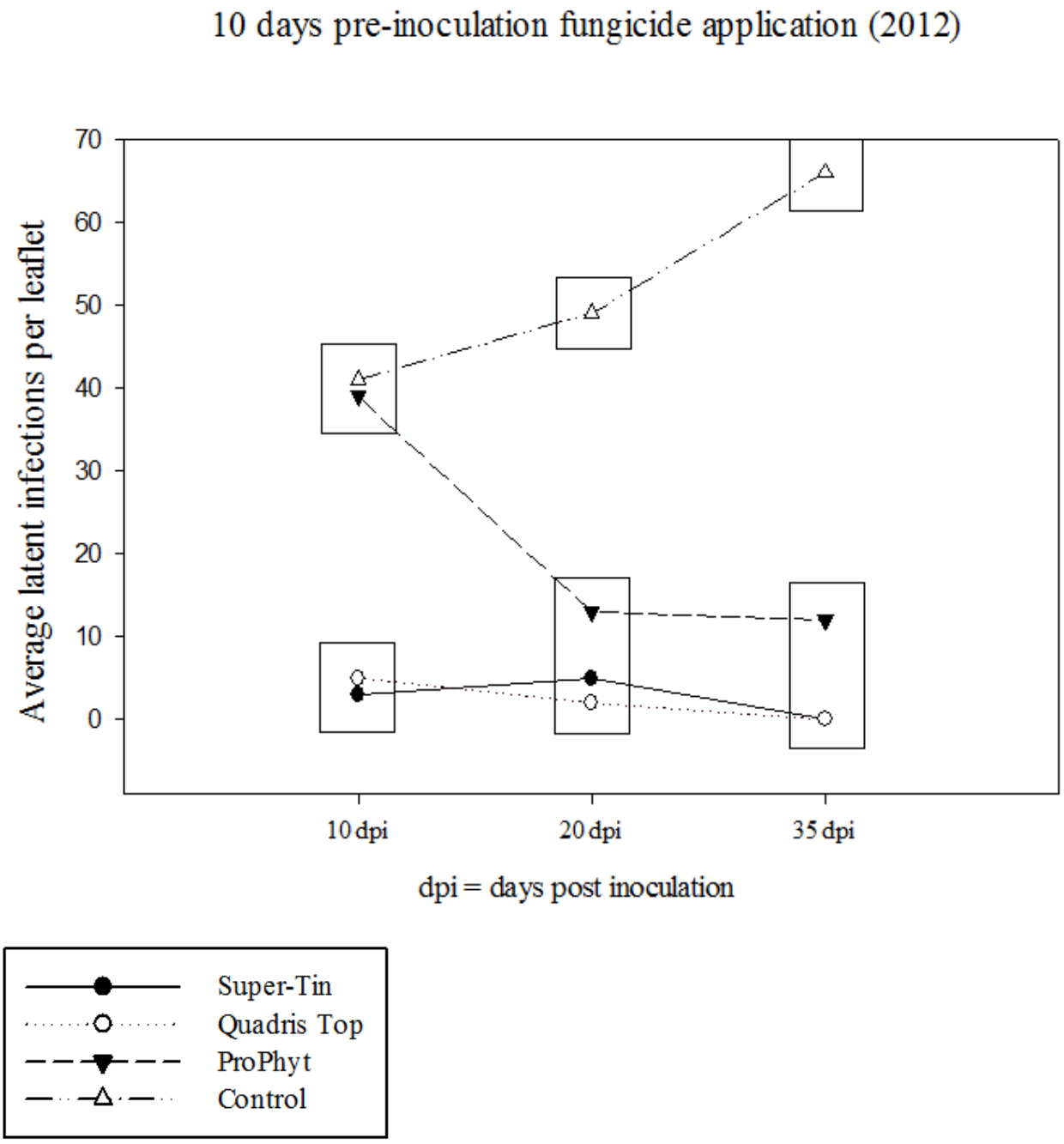

Figure 3.6. Average latent infections per leaflet in pecan seedlings sprayed with fungicides 10 days before they were inoculated in 2012 (pre-inoculation).

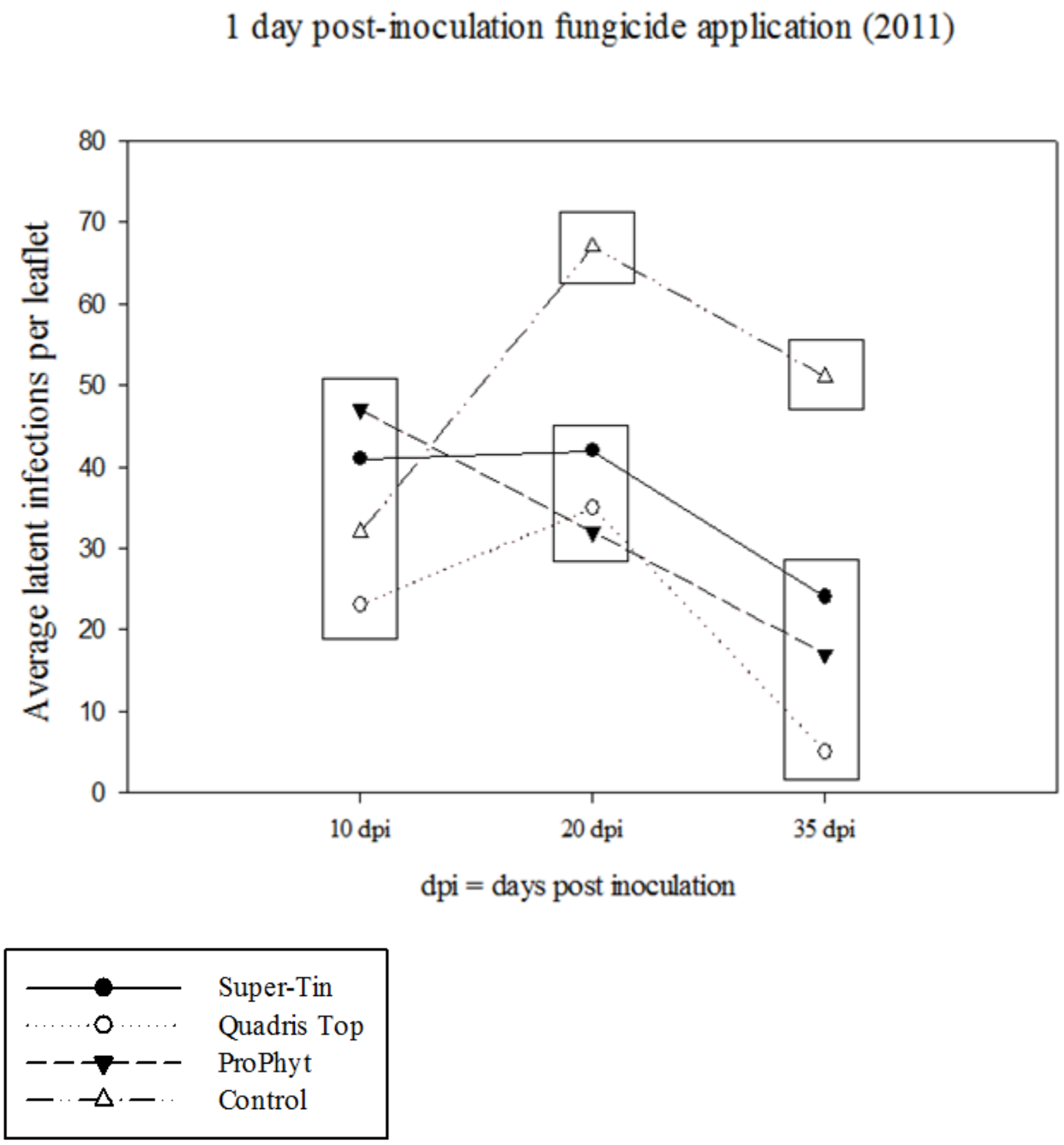

Figure 3.7. The average number of latent infections of leaflets sampled at 10, 20, or 35 days post inoculation in 2011. These pecan seedlings were sprayed with their respective fungicide 1 day after they were inoculated.

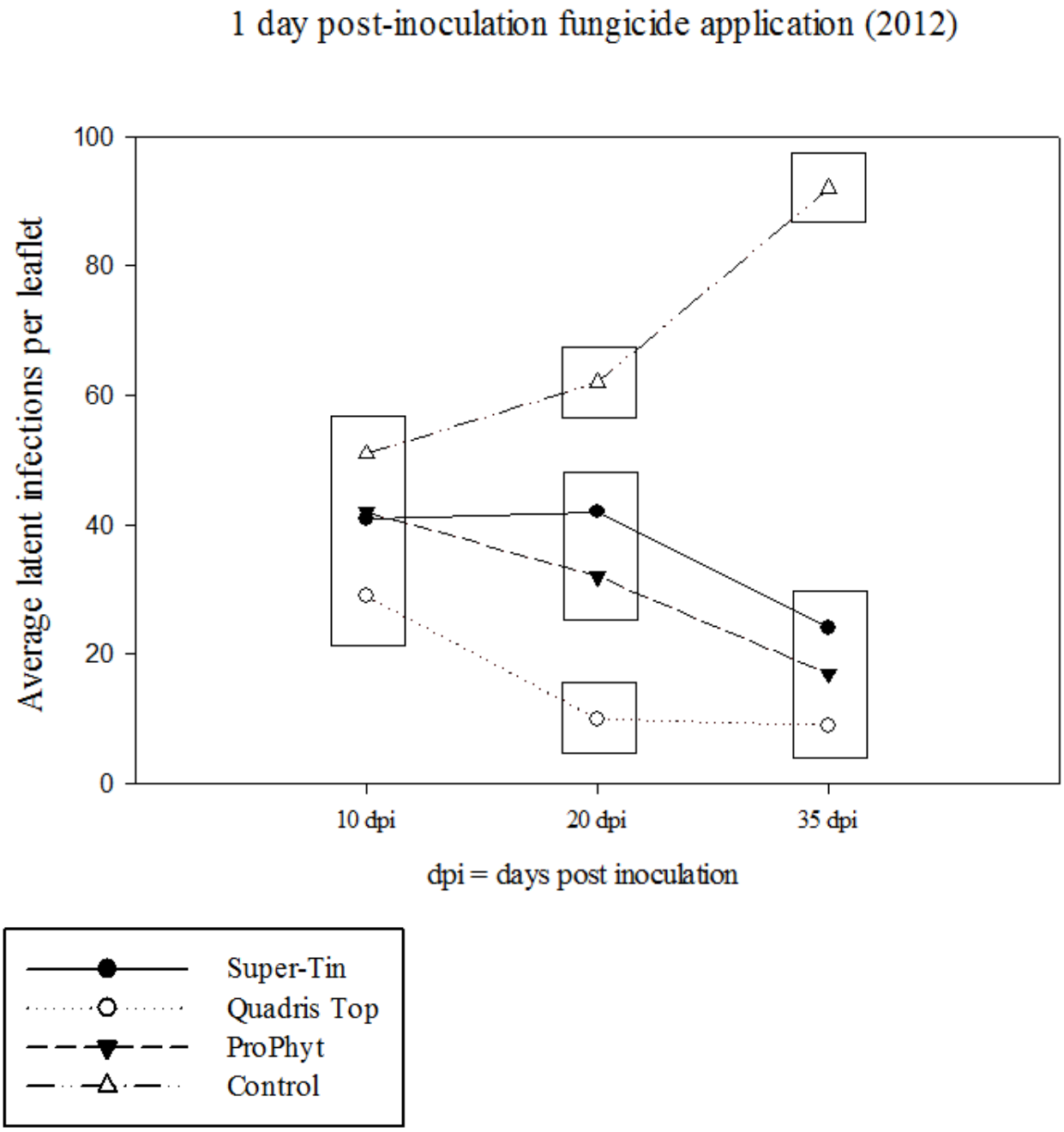

Figure 3.8. The average number of latent infections of leaflets sampled at 10, 20, or 35 days post inoculation in 2012. These pecan seedlings were sprayed with their respective fungicide 1 day after they were inoculated.

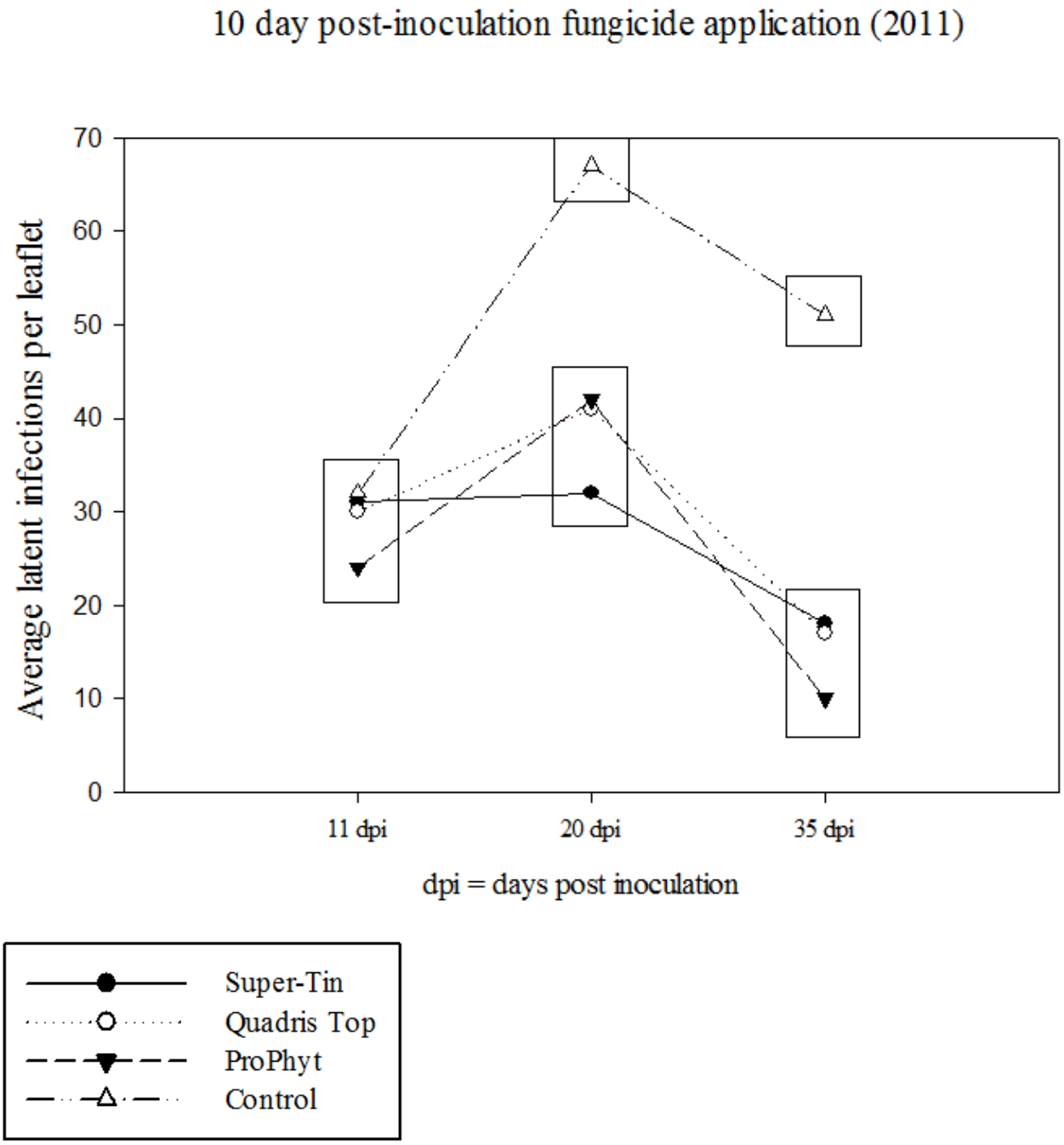

Figure 3.9. The average number of latent infections of leaflets sampled at 11, 20, or 35 days post inoculation in 2011. These pecan seedlings were sprayed with their respective fungicide 10 days after they were inoculated.

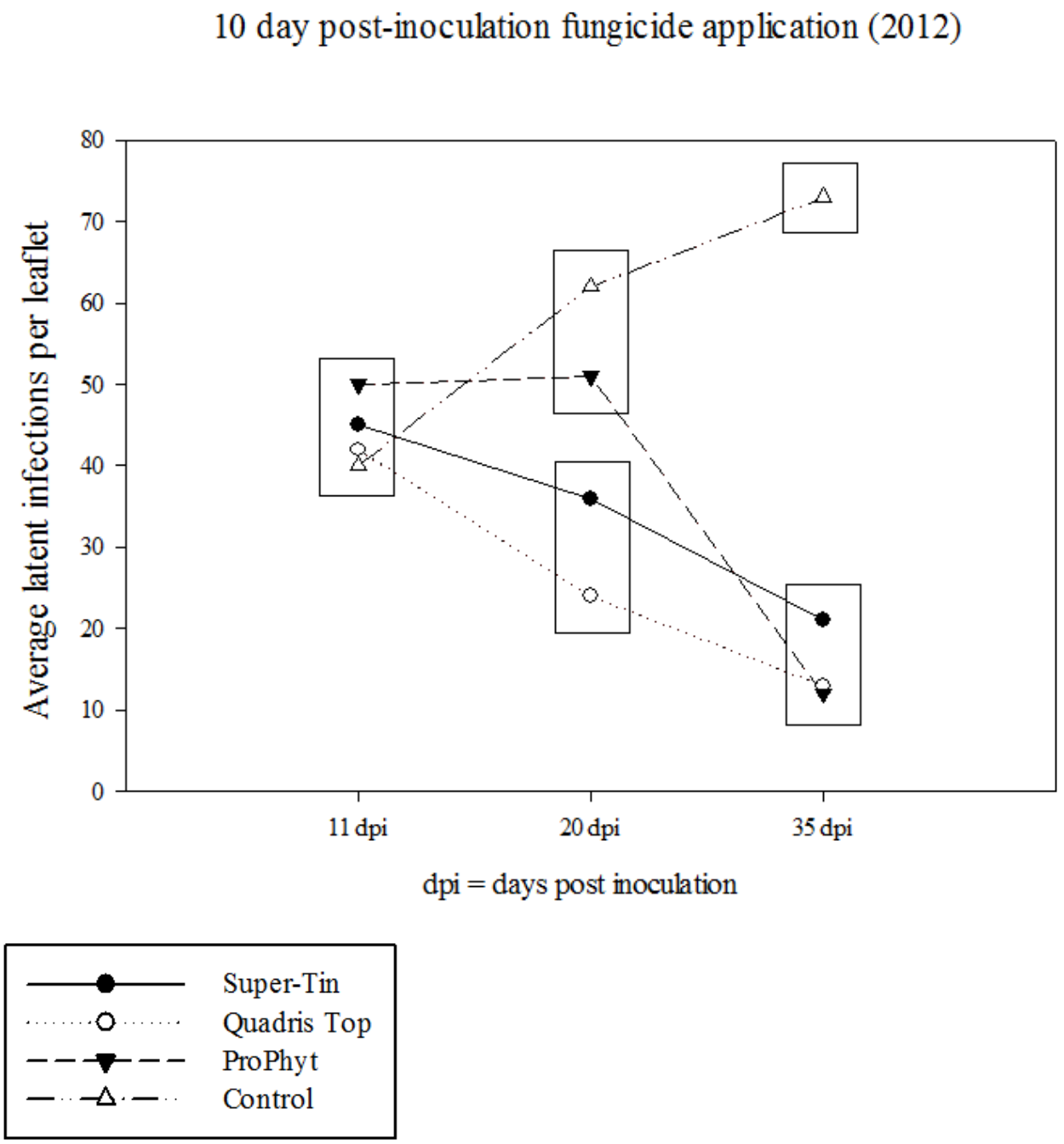

Fig. 3.10. The average number of latent infections of leaflets sampled at 11, 20, or 35 days post inoculation in 2012. These pecan seedlings were sprayed with their respective fungicide 10 days after they were inoculated.

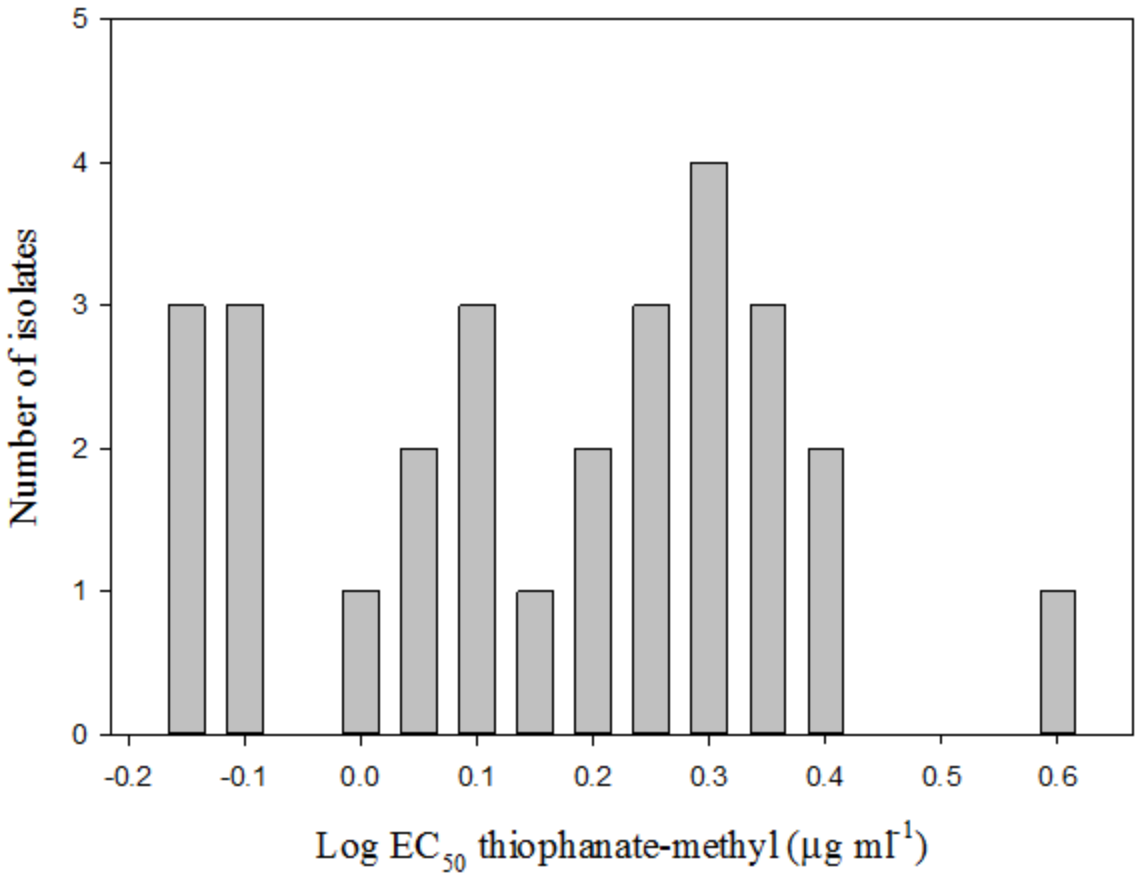

Fig. 3.11. Sensitivity distribution of 39 isolates of *Colletotrichum* spp. from pecan to thiophanate-methyl based on an *in vitro* mycelial growth assay.

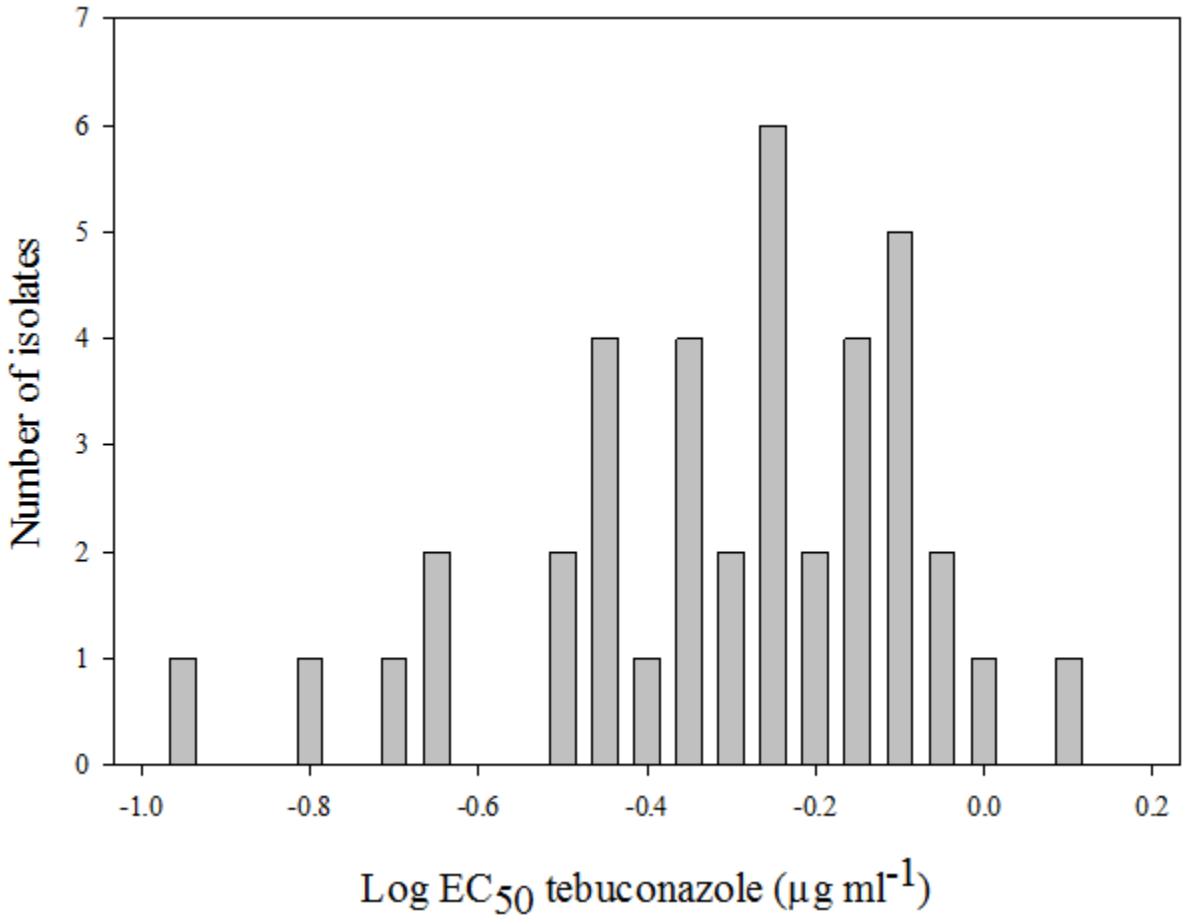

Figure 3.12. Sensitivity distribution of 39 isolates of *Colletotrichum* spp. from pecan to tebuconazole based on an in vitro mycelial growth assay.

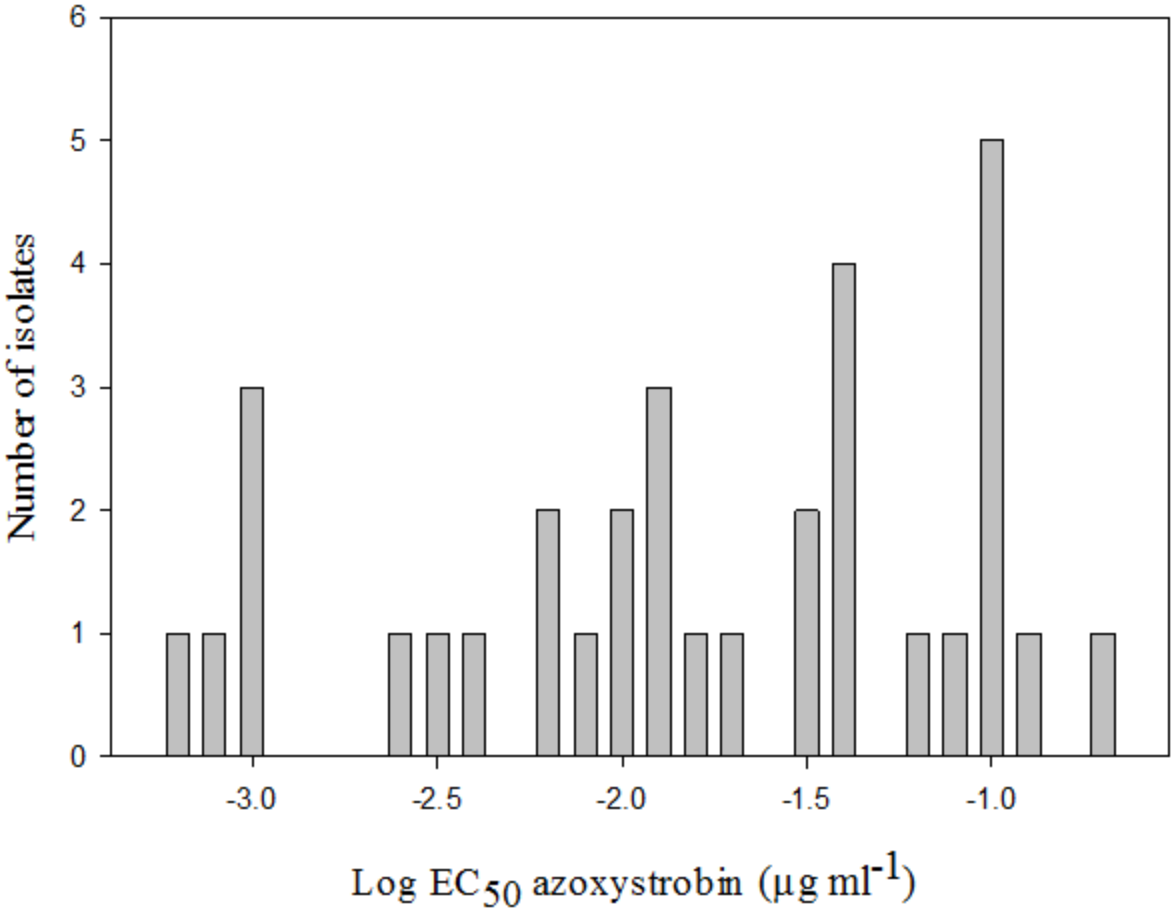

Figure 3.13. Sensitivity distribution of 38 isolates of *Colletotrichum* spp. from pecan to azoxystrobin in an *in vitro* conidial germination assay.

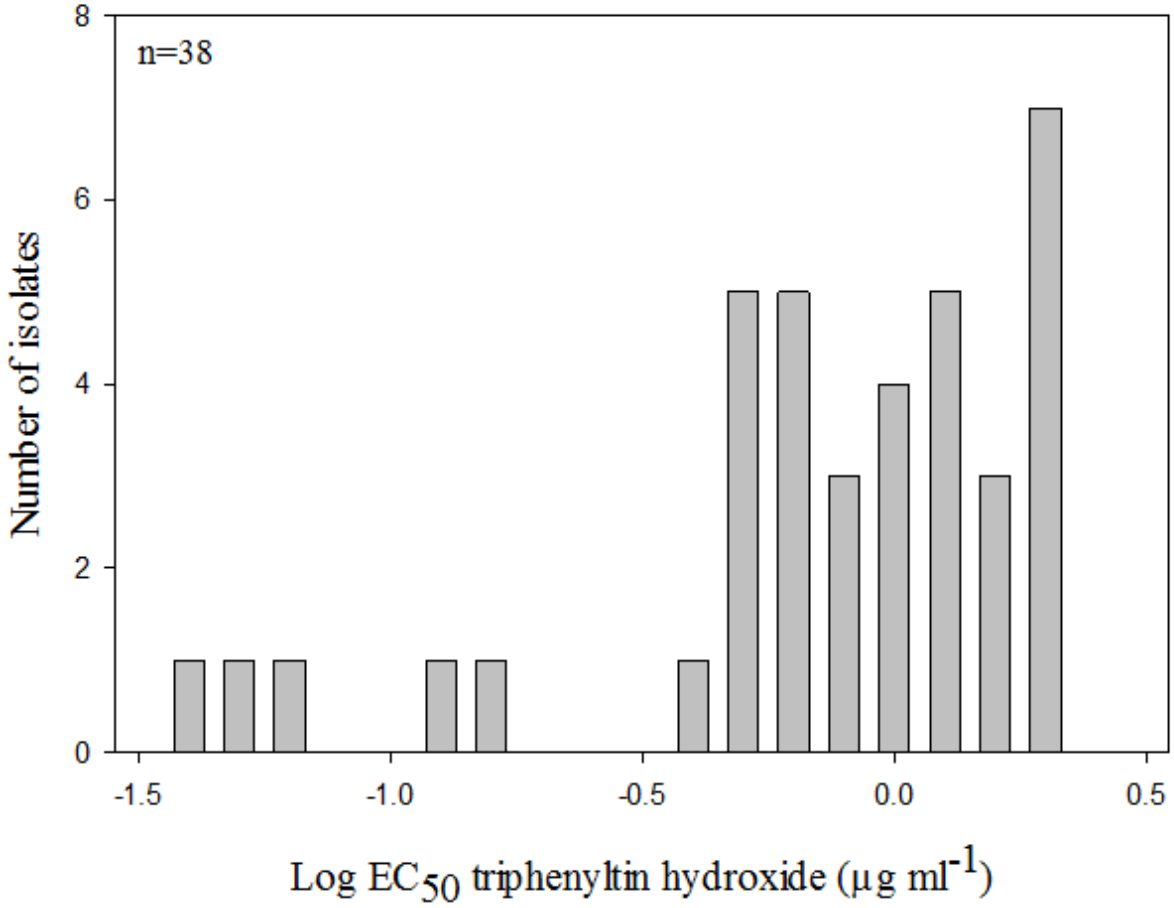

Figure 3.14. Sensitivity distribution of 38 isolates of *Colletotrichum* spp. from pecan to fentin hydroxide in an *in vitro* conidial germination assay.