DEVELOPMENT OF RAPID IN VITRO ASSAYS AND CURRENT STATUS OF FUNGICIDE SENSITIVITY IN THE PECAN SCAB PATHOGEN *FUSICLADIUM EFFUSUM*

by

MURAT SEYRAN

(Under the supervision of KATHERINE STEVENSON and TIMOTHY BRENNEMAN)

ABSTRACT

Isolates of *F. effusum* from three baseline orchards in Georgia were tested for sensitivity using a mycelial growth assay in microtiter plates. The 50% effective dose ($EC_{50}$) values were estimated for azoxystrobin, dodine, fentin hydroxide, and propiconazole. The role of alternative oxidase inhibitors salicylhydroxamic acid and propyl gallate in quinone outside inhibitor assays also was evaluated. Both chemicals were found to be toxic to *F. effusum* and therefore were not used in the assays. A more rapid assay was developed based on conidial germination and micro-colony growth. Isolates of *F. effusum* from 35 commercial orchards were profiled using the new assay and *in vitro* resistance to fentin hydroxide, dodine, thiophanate-methyl, propiconazole, and azoxystrobin was detected in 20, 4, 6, 21, and 19 of the 35 orchards respectively. Taxonomy of *F. effusum* in the family Venturiaceae was supported based on the sequenced cytochrome b gene.

INDEX WORDS: alternative oxidase inhibitors, benzimidazoles, cytochrome b gene, disease management, fungal taxonomy, fungicide sensitivity, fungicide resistance, fungicide resistance monitoring, guanidines, organotins, scab management, sterol inhibitors

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# DEDICATION

*Pro prosum plebes*

For good people

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And, I salute Mustafa Kemal and his soldiers.

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# CHAPTER 1

# INTRODUCTION AND LITERATURE REVIEW

## Pecan Production and Pathology

Pecan (*Carya illinoinensis* (Wangenheim) K. Koch.), is the only native nut tree crop cultivated commercially in the United States (3). In 2005, U.S. growers produced 280 million pounds and the production in Georgia was 70 million pounds (2). Mexico is the second largest pecan producer in the world following the United States. Relatively small amounts of pecans are produced by Australia, Brazil, Israel, Peru, South Africa and Thailand (3,111). The utilized pecan production in the U.S. generally ranges from 85-150 million pounds and is fairly stable (111). However, the grower price of shelled pecans in the U.S. has gradually increased from $1.68 in 1980 to $3.32 per lb. in 2005 (111).

The major economic constraints of pecan production in the southeastern U.S. are drought stress, insect pests and diseases. Pecan scab caused by the fungus *Fusicladium effusum* (Winter) is the most devastating disease of the pecan plant and is the major target for disease control (10,123). The humid and warm climate in the Southeastern states favors pecan scab development, especially during the rainy periods. Without appropriate control measures pecan scab can cause complete yield loss on susceptible cultivars (10,96). In the state of Georgia during the years 2004, 2005, 2006, and 2007, reduction in crop value due to pecan scab disease was estimated as 4, 3, 0.1, and 0.05 % respectively (14,15,16,17). And, in these years, the estimated cost of control was 13.8, 13.8, 13.5, and 17.4 million dollars, with the total loss values 16.7, 16.9, 13.64, and 18.04 million dollars, respectively. (14,15,16,17). Disease control programs target

primarily pecan scab, and other fungal diseases are usually suppressed as well. Other important pecan pathogens are: bacterial leaf scorch caused by *Xylella fastidiosa*, downy spot caused by *Mycosphaerella caryigena*, powdery mildew caused by *Microsphaera alni*, Phytophthora shuck and kernel rot caused by *Phytophthora cactorum*, zonate leaf spot caused by *Cristulariella pyramidalis*, and anthracnose caused by *Glomerella cingulata* (94,96,104,119).

## Disease cycle and Epidemiology of Pecan Scab Disease

Pecan scab epidemics occur as early, mid and late season infections on pecan foliage and nut shucks (55). The pecan scab pathogen overwinters as subcuticular stroma in plant tissues such as twigs, leaves and shucks (31,32). The conidiophores arise from the fungal stroma and produce elliptical conidia with a dark brown color and thick walls (32). These conidia serve as primary inoculum for pecan scab epidemics and infect young unfolding leaves, twigs, shoots, and catkins in the spring (31,32,56,93). Relative humidity, infrared radiation and vegetative wetness are the major factors that influence discharge of conidia (53,54). A decrease in relative humidity and increase in temperature accompanied by an increase in solar radiation, like the drying period after a rain shower, increase the concentration of airborne conidia (53,54). Once liberated, conidia are dispersed by wind, rain splash and wash-off (92). Moisture in the form of rain, fog or dew is essential for infection (36,55,56). Forty-eight hours of continuous leaf wetness is optimum for infection and less than 9 h results in a low infection rate (55). Optimum temperature for infection is between 15ºC and 25ºC (56). Secondary cycles of infection occur throughout leaf and nut development (32). Physical effects of pecan scab infection include reduction of healthy foliar and fruit surface area, stunting and deformation of leaves, and premature nut and leaf drop. Even at low infection rates, the amount of carbon gain, respiration,

oil content, tissue moisture, leaf chlorophyll content, photosynthesis and water consumption levels can be reduced (35,57,60). Pecan scab infection also affects the biochemical content of the leaf and leaf surface chemical composition (35,57,91,144). As result of these physiological events, reduction of the energy reserves of the plant can cause irregular bearing, poor nut quality, and low yield (35,57,118). Premature nut drop also occurs as a result of early season infection, especially on susceptible cultivars (35,57,69,118). The pecan is most vulnerable to scab damage from the onset of fruit development until the shell hardening period; primary effects of pecan scab disease are premature nut drop and reduction in nut size (length and weight) (69,198).

## Pecan Scab Management

Pecan scab management is based on selecting resistant cultivars and preventive fungicide applications (10128). Establishing an orchard with resistant cultivars is the ideal control method; however differences in nut quality between the susceptible and resistant cultivars are an important economic constraint for commercial production (128). Growers often prefer susceptible cultivars because of their high market demand. However, relative resistance varies depending upon geographic location and scab strains found there (23,96). Resistance mechanisms include faster cell wall maturation in resistant cultivars, probably limiting subcuticular development of the pecan scab fungus (23,36,147,148). Additionally, the incubation period was significantly shorter on the more susceptible cultivar Wichita and the fungus produced more spores per lesion and more lesions per leaf compared to the more resistant cultivar Elliot (139). Tissue age is another major factor that affects resistance to scab infection (139). Under favorable conditions for disease development, the young and immature leaves of the more resistant cultivars can be infected by the pecan scab fungus only shown at a very low

rate and fungus did not develop very much (148). Pecan scab infection is highly cultivar specific; fungal isolates from one cultivar are often unable to infect other cultivars (22,23). However, over time new infectious biotypes of *F. effusum* have been observed on previously resistant cultivars (10,23,96, 128). For example, the cultivars Curtis and Stuart had been introduced as very resistant to scab during the 1940s and 50s, respectively, but are considered susceptible today (10,23,96,128).

The commercial production of high quality pecans of susceptible cultivars depends on multiple fungicide applications per season (135). These fungicides include guanidines, organotins, benzimidazoles, sterol demethylation inhibitors (DMIs), and quinone outside inhibitors (QoIs). Fungicides with each of these modes of action and biochemical attributes have been deployed in spray programs against pecan scab (70,96,135). Fungicide applications can be scheduled with either a calendar based program, or a weather-driven system such as Au-Pecan model (11). In calendar based fungicide application regimes, fungicides are applied every 10-14 days during the pre-pollination and 10-21 days post pollination periods (135). The other method for timing fungicide applications is AU-Pecan, was adapted at the University of Georigia from the Au-Pnut system developed by Auburn University for peanut leaf spot. The system is based on occurrence of rain or heavy fog and the average probability of rain over the next 5 days (11). Field tests with Au-Pecan have provided the same amount of pecan scab control with an average of 2 or fewer applications per season (11,70).

## Mode of Action of Pecan Scab Fungicides

**Guanidines**. Dodine is a member of the guanidines chemical group, and was registered for pecan scab control in 1963, (96). Its mode of action is denoted as multi site-contact activity

(47). Today it is still used for pecan scab control and is marketed as Elast (70). Dodine is a surface protectant compound with a strong diffusing capability in the leaf cuticle or in surface lipids (64). Dodine n-dodecylguanidineacetate is a cationic surfactant, with an alkyl group, hydrophobic apolar dodecyl chain group, and positively charged hydrophilic polar guanidine head (19,20,64,71,95). The alkyl group near the hydrophobic region of the molecule unspecifically binds to anionic sites (64). The metabolic activity of dodine *in planta* has been shown (129,130). For example in barley leaves dodine accelerates protein breakdown and inhibits membrane-bound peroxidase enzyme activity in maize scutellum (129,130).

Based on several studies, the antifungal mechanism of dodine may be related to interaction with anionic sites of the fungal membrane, disruption of the membrane permeability, and competition with cationic enzymes for the anionic substrate sites in the fungal cell (64,108). Additionally, dodine targets the mitochondrial membrane anionic sites and enzymes (64). Dodine interacts with the fungal cellular membrane, and its neutralization capability has been detected on *Neurospora crassa* conidia (64,108). Additional effects of dodine on cell membranes were revealed in studies on yeast and bacterial cells. For example, dodine affected permeability of yeast cells in a dose-dependent manner (64). In *Pseudomonas syringae*, a low concentration of dodine caused leakage of K, UV-absorbing materials, and ribose-containing materials and resulted in cell death (19,20). Another study on *Phycomyces blakesleeanus* demonstrated that cytoplasmic lethality was related to activity of dodine (108). In fungal cells the dodine molecule binds to the anionic surface structures of the cationic enzymes like NAD+ (108). In yeast cells for example, dodine interacts with alkylammonium chlorides and inhibits yeast alcohol dehydrogenase enzyme activity (64,108).

**Organotins**. The organotin fungicide fentin hydroxide was registered in 1967 for pecan scab control (96). It is still in use as a surface protectant and marketed under the trade names Super-Tin or Agri-Tin (70). Organotin compounds were originally developed as thermal stabilizers during the 1930s, and are also used as catalytic agents, wood preservatives, pvc stabilizers, and marine antifouling paints (64.109). Additionally these broad-spectrum materials are applied to repel rodents and insects, control parasites in chickens (e.g. *Raillientina cesticillus*), and as an antibacterial in hospitals (e.g. *Stapyhylococcus aureus*), or as a larvacide, mollusicide, algaecide or fungicide (64,109). This broad-spectrum activity, which includes anti-feeding activity on Lepidoptera and Coleoptera larvae, is an advantage for integrated pest management, but generates ecological concerns due to potential effects on non-target organisms (64). For example, isolates of entomopathogenic fungus *Beauveria bassiana* collected from pecan orchards after the applications to control pecan nut weevil (*Curculio caryae*) have been found to be more resistant to fentin hydroxide, dodine and propiconazole compare to parent isolate (125). Cationic and anionic moieties, and the geometry around the tin atom of the organotin compounds have a role in antifungal activity (7,38,73,105). Triphenyltins inhibit oxidative phosphorylation by inducing an anion-hydroxide exchange in the bilayer membrane of the mitochondria, binding to the ion channels on the mitochondrial surface and interacting mitochondrial membrane enzymes i.e. ATP synthase and ATPase (7,38,47,59,64,138,142). However, they also react with other target site in the fungal cell i.e. thiol-groups of amino acids and peptides (37,64).

**Methyl Benzimidazole Carbamates**. Thiophanate-methyl, a member of methyl benzimidazole carbamates group (MBC), is marketed on pecans as Topsin-M (70). MBCs are antimitotic agents that inhibit cell division and other tubulin derived cellular processes. They

possess a site specific activity toward fungal beta-tubulin (47,64,65,79). MBCs have been used as effective antifungal compounds in the fields of human and veterinary medicine and plant protection since 1960 (65,79).

All eukaryotic cells possess microtubules, which are essential components of mitotic spindles, cytoskeletons and flagella (79). Microtubules are composed of two subunits, alpha and beta-tubulin (79), and benzimidazole fungicides target the beta-tubulin subunit specifically. Glu-198 and phe-200 amino acid locations are the particular binding sites detected in several organisms such as *Aspergillus nidulans*, and protozoan parasites *Encephalitozoon* (27,28,64,79). MBCs suppress the function of microtubules, thereby inhibiting mitosis during hyphal growth and spore germination (27,28,79).

**DeMethylation Inhibitors (DMIs)**. Sterol demethylation inhibitors (DMIs) have been used for pecan fungal disease control since 1988 (10). DMI fungicides registered for use on pecan include propiconazole and fenbuconazole, marketed under the trade names, Orbit or Propimax and Enable respectively (47,70). Additionally, fungicides sold under the trade names Quilt and Stratego are mixtures of azoxystrobin and trifloxystrobin, respectively, with propiconazole (70).

The sterol biosynthesis inhibitors used in agriculture are classified in to three different groups (i.e. DMIs, amines, and hydroxyanilides) based on their different inhibition sites of the ergosterol biosynthesis pathway (45,83). The DMI fungicides hinder the production of ergosterol by inhibiting C-14 demethylation of the precursor molecules lanosterol or 24-methylenedihydrolanosterol (81). They were first introduced as agricultural fungicides beginning with pyridines and pyrimidines in 1970, followed by imidazoles in 1972, and triazoles in 1973. New DMIs continue to be developed because of their desirable qualities of a single-site mode of

action and broad spectrum activity (81,115). There are now more than 35 DMIs, and they were the leading selling fungicide class as of 1999 (89).

**Quinone Outside Inhibitors (QoIs)**. The QoIs currently registered for pecan fungal disease control are azoxystrobin, kresoxim-methyl, pyraclostrobin, and trifloxystrobin, marketed under the trade names Abound or Quilt, Sovran, Headline, and Stratego, respectively (70). Additionally, azoxystrobin and trifloxystrobin are available as mixtures with propiconazole. QoI fungicides bind to the ubiquinone oxidizing pocket (Qo site) of the cytochrome bc1 (complex III) of the respiration chain which is located on the inner mitochondrial membrane of the target fungal cell (8,51,146). Binding of the QoI molecule to the enzyme complex inhibits electron transfer between cytochrome b and cytochrome bc1 complex and prevents production of ATP (8,51,146).

In the nature, organisms inhibit the respiration chain of their antagonists with secondary metabolites and quinone is the target site of strobilurins produced by *Storobilurus tenacellus* to inhibit its fungal antagonists by binding to Pro270 of cytochrome bc1 enzyme complex (51,113,132,146,153). However, despite its superior activity as a fungicidial compound, strobilurins were modified by companies to add photostability, systemicity and mobility in the plant (8,51). And with their traits such as low environmental risks, broad-spectrum activity, xylem systemicity, metabolic stability, translaminar movement, and phloem mobility, sales have reached over 10% of the fungicide market with $ 620 million in 1999 (8) However, QoI fungicides possess different structural and biochemical traits; for example azoxystrobin has an (E)-methyl methoxyiminoacetate group, but kresoxim-methyl contains an (E)-methyl Beta-methoxyacrylate (8,51). Azoxystrobin has low movement potential into the leaf, metabolic stability in the leaf, translaminar movement, and xylem systemic activity. Trifloxystrobin and

kresoxim-methyl also have very low movement potential into leaf, however they have vapor activity, and pyraclostrobin has very low movement into the leaf but it has translaminar movement and is metabolically stable in the leaf (8). Strobilurins effectively control spore germination and zoospore motility which are high energy consumption stages (8).

## Fungicide Resistance

Fungicide resistance is the selection of a resistant fungal strain from among a population and the manifestation of disease with the recommended dose of the fungicide (63,72,90,117). However, under field conditions, resistance does not occur simultaneously among all individuals of the population (117). Initially, the naturally resistant biotypes or mutants survive in the fungicide exposed population. Then, due to the selection pressure of consecutive applications of the fungicide, the resistant individuals increase their proportion among the population. Eventually, the fungicide cannot suppress the target fungal population and economic loss occurs (51,117).

The most important factor that determines the risk of resistance is the fungicide mode of action. Fungicides with a site-specific mode of action are more prone to lose their effectiveness as a consequence of resistance. Different site-specific fungicide groups possess different levels of resistance risk. For example benzimidazoles and QoIs are high risk while, guanidines and DMIs have moderate level of resistance risks (12). However, site-specific fungicides have several advantages compared to multi-site fungicides, specifically their effectiveness at low dosages, target specificity, and compatibility with integrated pest management programs. Additionally, they have environmental and toxicological advantages such as low toxicity towards non-target species, low mammalian toxicity, and low level of residues in food (63,72,115,146).

Consequently, single-site fungicides serve as essential tools against fungal pathogens of crops around the world. (117). Another important determinant for resistance is systemicity of the fungicide. Systemic compounds are more at risk for resistance compare to protectant fungicides i (12). The risk of resistance should be evaluated for each pathogen and fungicide combination, based on data at the molecular, genetic, biochemical, physiological and population levels (51).

One phenomenona associated with fungicide resistance is cross resistance where resistance to one fungicide also confers resistance to another fungicide in the same group (13). These fungicides usually carry the same specific moiety that interacts with the fungal enzyme or target site. Based on the configuration and the interaction of the target region and the fungicide, resistant mutants may be resistant to other members of the same group. Cross resistance has been demonstrated for a number of pathogens and fungicide groups, including MBCs, QoIs and DMIs (6,39,66,143). Occassionally resistance to one fungicide group can lead to increased sensitivity to other fungicides, which is called negative cross resistance (12,13). And, another phenomenona is multiple resistance, where fungi obtain resistance to more than one class of fungicides (1,78,86,126).

To detect development of fungicide resistance prior to a control failure, the first step is to construct a baseline sensitivity profile of the target fungal pathogen. The baseline sensitivity is the estimated response level of the fungal population that has not been exposed to the fungicide (90,116). Establishing baseline sensitivity profiles involves determining the distribution of $EC_{50}$ values (effective concentration inhibiting the growth or development by 50%) for each fungicide. Resistance monitoring is then conducted by testing suspected isolates and comparing results to the baseline data (13,116). Routine monitoring of a large number of isolates is more easily accomplished by selecting a single discriminatory concentration (the subsequent lethal

concentration of the fungicide after $EC_{50}$). However, fungicide resistance in DMIs occurs via gradual shifts, and to set precise concentrations for both baseline and treated populations requires high sample sizes (89). The traditional bio-assay techniques to detect fungicide resistance are labor intensive, costly, and usually give a fungicide resistance profile from less than 1% of the population. Other techniques utilizing microtiter plates, and conidia germination assays could be adapted in order to increase the sample size, efficiency and likelihood of detection (12,90,131).

Molecular techniques such as polymerase chain reaction (PCR) have also been used to confirm resistance in some fungicide groups like MBCs, DMIs, and QoIs (9,30,97). Resistance to these fungicides may occur as the result of single-site mutations on the nuclear or mitochondrial genome. The detection of these mutations is possible with the identification of the gene sequence for the fungicide target site by the application of PCR with site-specific primers (13,24,42). Availability of the sequence data bases enables the detection of single site point mutations of the gene in question. However it is possible that development of resistance mechanisms may progress through different mutation pathways. In several case studies, high field resistance has not been correlated with the expected point mutations (87).

**Resistance to Dodine**. Fungicide resistance action committee (FRAC) guidelines consider dodine to be an at-risk chemistry due to the documented cases of resistance in the field and laboratory (47). In 1969, five years after its first introduction to market, dodine resistance was first detected in *Venturia inaequalis* following intensive use in apple orchards of New York (50). Dodine resistance in *V. inaequalis* was found to be a non-durable trait with different sensitivity levels (149), but sensitivity did not return completely to baseline levels. A survey in 1997 revealed that the frequency of resistant isolates in sampled populations was as high as 10-14% (85).

Dodine resistance in *V. inaequalis* may have gone undetected for many years because of the gradual decline in sensitivity (126). This resistance was found to be dependent on polygenic mechanisms (75). Fungal crossing studies revealed that dodine resistance was based on at least two independent genes (110,149). The mechanism of dodine resistance is likely to be a generic system, thus in *V. inaequalis* 20% of isolates were resistant to both benomyl and dodine (126). Additionally, the resistance to DMI (fenarimol) developed in a higher frequency at the orchard where dodine resistant isolates of *V. inaequalis* were detected previously (86).

One theory about dodine resistance is based on the multi-drug resistance system (34), whereby some fungi exhibit resistance to fungicides and natural toxins, resulting in increased virulence (33,121,122,134). All organisms have toxin transport mechanisms, the two major types are the ATP-binding cassette and major facilitator super-family (29). In nature, fungal organisms are faced with a variety of natural toxins, plant defense compounds, synthetic antifungal compounds and even endogenous toxics substances that should be expelled from the fungal cell (34). Fungi have an ATP-binding cassette (ABC) drug transporter system that is an energy dependent efflux activity in order to exclude toxic substances such as fungicides, plant phytoalexins and antibiotic substances of antagonistic organisms outside the cell. This task is performed by plasma membrane drug transporter systems, like ABC transporters (34). The overproduction of ABC transporters is associated with multi-drug resistance (MDR) (33,134). Increased tolerance to azole fungicides in laboratory mutants has been demonstrated to be under the control of ABC transporters (134). For example, an ABC transporter BcatrB deficient mutants of *B. cinerea* were suppressed more by *Pseudomonas spp.* antibiotics (phenazins (2,4-diacetylphloroglucinol, phenazine-1-carboxylic acid and phenazine-1-carboxamide (PCN)), the phytoalexin resveratrol of grapevine, and fenpiclonil fungicide, compared to the wild type strain

of *B. cinerea* (121,122). Interestingly, the BcatrB ABC transporter system could be the mechanism in the case of multiple resistance of *B. cinerea* to benzimidazole and dicarboximide fungicides (13). Additionally, *Gibberella pulicaris* Gpabc1 ABC transporter genes were found to be related to virulence of the pathogen due to the increasing tolerance of ? Gpabc1 mutants towards phytoalexins of the host potato tubers (41). In another study of *Mycosphaerella graminicola*, MgAtr4-deficient mutants were less virulent on several wheat cultivars compared to the parent strain of the fungus (134). The predisposition to resistance is controlled by several genes, and the polygenic trait is related to resistance to dodine with increased multi-drug transporter activity (34).

**Resistance to Organotins**. The organotins are considered a medium to low risk fungicide group and *Cercospora beticola*, which causes leaf spot of sugar beet, is the only pathogen with detected field resistance (44,47). Resistance was first noted in *C. beticola* in Greece (49), and resistant isolates were found to be 32 times more resistant to fentin acetate and fentin hydroxide than was a baseline population (49). However, the *C. beticola* resistance in Greece was found to be a non-durable trait (78). In 2004, a study performed in the same area found the frequency of *C. beticola* strains resistant to fentin acetate to have declined from the 1978 level of 29% to 2-3% and 8-11% at two locations, respectively (78). Additionally, fentin hydroxide resistance was found to be non-durable in *C. beticola* isolates in Minnesota and North Dakota (124). Isolates resistant to 1-2 ppm fentin hydroxide were detected in 1994, however, the frequency of resistant isolates declined with reduced numbers of applications of fentin hydroxide in subsequent years (124). In 2006, none of the isolates were found to be resistant (124). The mechanism of resistance to organotin fungicides remains unknown at this time.

**Resistance to Methyl Benzimidazole carbamates**. Methyl benzimidazole carbamates (MBC) is the FRAC code number 1 fungicide group, and is denoted as a high-risk chemistry group (47). Based on the most recent list of FRAC, 114 plant pathogens have been detected with resistance to different MBCs in laboratory and field studies since their first introduction in the 1960s (44). Resistance to MBCs occurs around the taxol-binding site the beta-tubulin with different single-site amino acid mutations (e.g. E198A, E198G, E198K, F167, Q134, Glu198Gle and F200Y) in the gene locus known as the benzimidazole box (26,150,154). In MBC resistance, both positive cross-resistance and negative cross resistant to N-phenylcarbamates are prevalent (44). For example, *Rhynchosporium secalis* (barley leaf blotch) Glu198Gle mutants resistant to carbendazim became sensitive to diethofencarb (65). This mutation was associated with a modified configuration of the beta-tubulin target site and subsequent increase in affinity to different moieties of fungicides (65). However, resistance to MBCs does not affect the sensitivity of mutant isolates to other fungicides. For example, MBC-resistant *Phoma clematidina* isolates were sensitive to difenoconazole, azoxystrobin and kresoxim-methyl (140), and isolates of *Monilinia fructicola* resistant to thiophanate-methyl were sensitive to iprodione and tebuconazole (150).

As mentioned above, positive cross resistance is very common within the MBC group (26,97). For example, benomyl-resistant strains of *Botrytis cinerea*, *Penicillium brevicompactum*, and *P. corymbiferum* showed cross resistance to thiophanate and thiophanate-methyl (48). MBC fungicides have different chemical attributes, but all of them convert to the same toxophoric compound which is benzimidazole carbamic acid, methyl ester (BCM) or ethyl ester (BCE). The difference is the conversion rate, which is dependent on pH and temperature (48), and in benomyl this is higher than for thiophanate-methyl. In *Helminthosporium solani*, the $EC_{50}$ values

for thiophanate-methyl and benomyl were different, and thiophanate-methyl was less toxic than carbendazim (26).

In MBC resistance, the different point mutations may be associated with different levels of resistance. For example, in *M. fructicola,* the mutation at codon 6 shows low level resistance, however the mutation at codon 198 associates with a high level of field resistance (97). The thiophanate-methyl-resistant isolates of *H. solani* from potato also showed resistance to benomyl (97). Additionally, different levels of resistance ranging from low (2-30 µg/ml) to high (above 30 µg/ml) levels have been observed in MBC-resistant *M. fructicola* in stone fruits (97,150). Based on the *M. fructicola* study, different levels of resistance to MBCs are associated with different fitness attributes in some plant pathogenic fungi. In the case of *M. fructicola,* low resistant isolates show cold sensitivity and high resistant isolates show heat sensitivity (97). Similar results were observed in the entomopathogenic fungus *Beauveria bassiana* (154). MBC-resistant mutants with Q134 and F167 beta-tubulin based resistance showed less thermotolerance and fewer hydrophobin-like proteins in the conidial cell walls (154). Thiophanate-methyl-resistant isolates of *M. fructicola* were equally competitive with wild type isolates; however, only 2-3% of the resistant population showed a high level of resistance to thiophanate-methyl (150). Isolates of *Phoma clematidina* resistant to thiophanate-methyl, benomyl and other MBCs were less virulent compared to sensitive isolates (140).

The durability of resistance to MBCs is apparently a case specific situation, and the resistance can appear sporadically. The number of thiophanate-methyl resistant isolates in a population of *C. beticola* increased approximately 3 fold in one growing season (143), but MBC resistance has also been shown to decline (75% to 22%) over time (143). In sugarbeet areas during the 1970s, the frequency of benomyl-resistant *C. beticola* was 80-90%. Based on surveys

in 1995-1996 the frequency of resistance had decreased to 20-25%. However, frequency of the resistant strains of *C. beticola* increased rapidly after only one benomyl application (78).

**Resistance to Demethylation Inhibitors**. Resistance to DMI fungicides is a quantitative type of resistance that develops slowly under field conditions. Resistance to DMIs is controlled by several genes, and is partially reversible in the absence of DMI exposure (45,89,107). The mechanisms of DMI resistance in plant pathogenic fungi are thought to be target site mutations in 14a-demethylase mutation cyp51 gene (e.g. V136A, Y137F, I381V), target site over-production associated with tandem repeats of the upstream promoter of CYP51, ATP-binding cassette (ABC) transporter systems, and destabilization of the toxic sterols (74,89,99). For example, in *Uncinula necator* isolates resistant to triadimenol, a mutation at codon 136 of the CYP51 gene resulted in an amino acid change from tyrosine to phenylalanine in the 14a-demethylase substrate recognition site (30). In this case, the switch to phenylalanine which does not contain a hydroxyl group resulted in an increased hydrophobicity at the active site without a configuration change in the enzyme system (30). Consequently, the increased hydrophobicity is hypothesized as the reason for resistance; however, the resistant strains do not always possess this mutation at their target site (30). Another example of 14a-demethylase (CYP51) gene alteration based resistance is the I381V substitution and deletion of codons 469 and 460 in resistant isolates of *Mycosphaerella graminicola.* The resistant isolates to tebuconazole (triazole) with this mutation showed negative cross resistance to prochloraz (imidiazole) (43). In this regard, the I381V mutation was suggested as an adaptive advantage rather than a direct resistance conferred by altered configuration of the target site (43). However, the CYP51 mutations were not detected in the resistant isolates of *M. graminicola* and *Venturia nashicola* resistant to DMIs (24,155). In the second type of resistance, the resistant *Blumeriella jaapii*

isolates expression of 14a-demethylase level was found to be 5 to 12 fold higher than in the sensitive isolates (99). Hypothetically, presence of the retrotransposons in some of the isolates resulted in hyper-expression of the gene product that is the target-site of the DMIs (99). Additionally, there were an extra four copies of the tandem repeat upstream promoter CYP51 detected in the imazalil-resistant isolates of *Penicillium italicum* (74). The third mechanism of DMI resistance relates to activity of the ATP-binding cassette (ABC) transporters. For example, in *M. fructicola*, the gene expression of ABC transporter gene MfABC1 was induced with the presence of DMIs myclobutanil and propicanazole in resistant and sensitive isolates (120) and a multidrug transporter AtrBp from *Aspergillus nidulans;* AtrBp deficient mutants showed increased sensitivity to azoles, other fungicides and natural compounds (1).

Another characteristics of DMI resistance is the reduced fitness of resistant strains without DMI exposure (67,76). In vitro growth rates and pathogenicity were found to be reduced by DMI resistance in *C. beticola* (101). This fitness-cost will cause a DMI-resistant population to gradually lose the resistance over time, due to the lack of DMI exposure. However, the population with DMI-resistance history may return more quickly to the level of resistance compare to DMI-sensitive populations (68). Several in vitro studies revealed the unstable nature of DMI resistance. For example, after cold storage DMI resistant *C. beticola* strains were found to be more sensitive to flutriafol (77). After 34 months in cold storage, the sensitivity of the propiconazole-resistant strains of *Monilinia fructicola* increased almost three fold (25). Interestingly, after eight consecutive transfers on PDA, some *M. fructicola* isolates showed reduced resistance to propiconazole (25). Similarly, cold storage of DMI-resistant strains of *V. inaequalis* for 2 to 10 months decreased resistance to flusilazole (82).

Generally, cross resistance to other DMIs is often observed in resistant fungal populations (39,43,52,84,99). However, cross resistance may not be the general rule for all DMIs. For example, propiconazole-resistant isolates of *Sclerotinia homoeocarpa* were cross resistance to other DMIs, but not to triadimefon (100). Additionally, cross resistance between DMIs, amines and hydroxyanilides has not been observed (44).

**Resistance to Quinone Outside Inhibitors (QoIs)**. The target site of the QoI fungicides is the mitochondrial cytochrome b, a region under the control of the mitochondrial genome (51). Mutations in the mitochondrial genome always occur during mitosis, usually during the mycelial growth stages of fungal pathogen development (51). Three different types of amino acid substitutions have been detected in 25 plant pathogenic fungi that confer resistance to QoIs (46). These mutations are 1) G143A, which is the conversion of the amino acid glycine to alanine, on the cytochrome b gene complex codon number 143; 2) F129L, which is the conversion of the amino acid phenylalanine to leucine, at the cytochrome b gene complex codon number 129; and 3) G137R, which is the conversion of the amino acid glycine to arginine, on the cytochrome b gene complex codon number 137. The resistance conferred by these mutations has different characteristics. G143A confers complete resistance with a resistance factor higher than 100 (46), and QoI fungicides cannot control G143A mutant isolates in the field. At least 22 plant pathogenic fungi have this mutation, including *Blumeria graminis f.sp. tritici, Sphaerotheca fuliginea, Plasmopara viticola, Pseudoperonospora cubensis, Mycosphaerella fijiensis* and *V. inaequalis* (51). In *Blumeria (Erysiphe) graminis f.sp. tritici*, the frequency of azoxystrobin-resistant strains with the g143A mutation in the population increased from 2% to 58% after only two azoxystrobin applications (42). By contrast, F129L and G137R mutations confer partial

resistance with a relatively low resistance factor of 5-15, and the QoIs provide sufficient control of these isolates (51).

Some fungi are naturally resistant to QoI fungicides. For example, in the strobilurin-A producing fungus, *Strobilurus tenacellus* comparative sequence analysis of the cytochrome b gene showed glutamine in position 254 and aspartate in position Asn261 compared to the non-strobilurin producing basidiomycete *Mycena viridmarginata*. More interestingly, analysis of the cytochrome b gene of *Mycena galopoda* showed a difference at the Gly 143 as alanine and Gly153 as serine (88). Additionally, apple bitter rot (*Colletotrichum spp.*) and black pox (*Helminthosporium papulosum*) pathogens were found to be immune to kresoxim-methyl during fungicide testing (151).

Cross resistance among QoI fungicides is common in plant pathogenic fungi, especially in isolates that carry the G143A mutation (80141). In *Pyricularia grisea*, for example, the mutation at the G143A location imparts a strong cross resistance to different QoIs. On the other hand, the F129L mutation in *P.grisea* conveyed a low level of resistance to azoxystrobin and little or no resistance to trifloxystrobin (80). Similarly, F129L mutants of *Alternaria solani* were less sensitive to azoxystrobin in vitro, but the sensitivity level did not change towards other QoIs famoxadone and fenamidone (106).

Another resistance mechanism of the QoI fungicides in some fungi is alternative oxidase (AOX), which is an alternative respiration system (145). AOX is controlled by the nuclear genome and utilizes the electrons for ATP production (145). In AOX, the electrons transfer directly from ubiquinone complex and bypass complex III (145,146,152). However, AOX is not effective enough to support the energy needs for fungal infection in plants. For example, metominostrobin activated the AOX of *Pyricularia oryzae* and *B. cinerea,* but, the fungi entered

a stage of fungistatis before infection took place (151). In another assay, production of ATP was reduced by 40% with AOX compared to cytochrome bc 1 based respiration system (146). Metominostrobin and rice flavonoids inhibited growth of *P. oryzae* in vitro assays, indicating that the plant phenolics captured the electrons before utilization by AOX (151). Therefore, in order to suppress the AOX, salicylhydroxamic acid (SHAM), cyanide and similar agents are often used in vitro with QoI resistance assays to mimic the effect of plant phenolics *in planta* (145,146).

There are several studies indicating the possible role of the AOX system in QoI resistance and pathogenicity. In *M. grisea*, AOX-deficient mutants showed less pathogenicity *in planta* and were suppressed effectively with azoxystrobin compared to wild type isolates (4). Expression of the alternative oxidase gene MfAOX1 increased with the presence of the azoxystrobin in some *M. fructicola* isolates (120). The fungicide SSF-126, which is a cytochrome bc1 inhibitor, induced activation of AOX in *M. grisea*, and hydrogen peroxide was found to be another inducer of AOX production in *M. grisea* (152). In vitro conidia germination assays performed with wild-type *M. grisea* and AOX deficient mutants indicated that AOX provides rescue capacity in the presence of azoxystrobin (5). AOX systems were found to be active in the absence of QoIs. In *V. inaequalis* for example, 38% of the baseline isolates exhibited active AOX during the conidial germination stage (102).

Not all QoI resistance has been associated with cyt bc1 mutations and AOX systems. For example, a highly resistant field strain of *V. inaequalis* resistant to trifloxystrobin in Germany did not have any mutation at the cytochrome b gene, and the AOX regulation was not the mechanism of resistance (133). Additionally, field studies have provided evidence of both quantitative and qualitative QoI resistance in *V. inaequalis* without any mutations in cytochrome

b gene (87,102). Similarly, in QoI-resistant isolates of *Podosphaera fusca,* neither point mutations nor AOX-related resistance was detected, but the resistant biotypes were also resistant to other QoIs (40). Recently, the drug transporter system MgMfs1 was found to be the mechanism of QoI resistance in some isolates of *Mycosphaerella graminicola* (114).

Thus far, resistance to QoIs has not been detected in some pathogens, including many rusts. The amino acid sequences of the cytochrome b gene from some plant pathogenic fungi revealed that many rust pathongens (e.g. *Puccinia recondita f.sp. tritici, P. graminis f.sp. tritici, P. striiformis f.sp. tritici, P. coronata f.sp. avenae, P. hordei, P. recondita f.sp. secalis, P. sorghi, P. horiana, Uromyces appendiculatus, Phakospore pachyrhizi, Hemileia vastatrix* and *A. solani*) have an intron region just after the G143 amino acid locus on the mitochondrial genome. However, other plant pathogenic fungi (e.g. *A. alternata, Blumeria graminis, Pyricularia grisea, Mycosphaerella graminicola, M. fijiensis, V. inaequalis* and *P. viticola*) do not have that intron region (61,62). Interestingly, unlike *A.solani, A. alternata* does not possess an intron after the G143 amino acid locus, and the G143A mutation for resistance to QoIs has been detected in *A. alternata* (98). A similar relationship was detected in *Pyrenophora teres* and *P. tritici-repentis*. The G143A mutation and a high level of resistance were detected in *P. tritici-repentis,* but not in *P. teres* (127). The cytochrome b gene sequence of *P. teres* indicates the presence of an intron after the G143 locus unlike the *P. tritici-repentis* gene sequence (127). As seen in *Alternaria spp*. and *Pyrenophora spp.*, the G143 based mutation is a species-specific trait (61,62). The authors associated the history of sensitivity to QoIs in rust pathongens with the lethal impact of the G143A mutation due to the existence of an intron just after G143 region on the amino acid sequence of fungi (61,62.127). Therefore, characterization of the pecan scab cyt bc1 gene

sequence profile was defined as an important factor to accurately assess the resistance risk level of QoI fungicides.

## Fungicide Resistance Management and the Current Status of Fungicide Resistance in *F. effusum*

To prevent development of field resistance, general strategies have been suggested by experts in the fungicide industry. However, as mentioned above, each specific fungicide host and pathogen interaction should be evaluated individually. Additionally, manufacturers provide specific resistance management outlines for each compound for growers to follow (18,90). The main principle for retarding fungicide resistance is to reduce selection pressure by limiting exposure to the fungicide. For example, a maximum of 4 sprays per season in DMIs, and rotate or mix them with fungicides with a different mode of action such as benzimidazoles, QoIs or dodine (18,135).

Another strategy to prevent resistance is to implement integrated pest management strategies, selecting resistant or tolerant plant varieties, and applying sanitation practices (12,72,89,90). The mycelial development stages of target fungi should not be exposed to fungicides with a high level of persistence (benzimidazoles) (12,13). In the case of quantitative resistance, eradicant and curative applications of the fungicide should be avoided, especially for DMIs (89). Additionally, the usefulness of the DMIs and organotins may be regained by halting the application of the fungicides for at least 2-3 years (89).

Specific fungicide management recommendations for pecan scab have been developed following the same principles mentioned above. Growers should obey the labeled rate of all products, and all fungicides should be applied as a preventive towards the pre-infection stage of

disease development (12,13,89). Additionally, post pollination application of QoIs should not follow the pre-pollination application of QoIs (kresoxim-methyl, pyraclostrobin, trifloxystrobin), and the same is true of DMIs (propiconazole and fenbuconazole) (18). Currently, formulated mixtures are available consisting of combinations of azoxystrobin or trifloxystrobin with propiconazole, and two other tank-mix combinations are recommended, fentin hydroxide–dodine and dodine-fenbuconazole (18).

In *F. effusum*, early records indicated development of resistance to benomyl just one year after its introduction in 1974 (96). Resistance to DMIs was detected in the pecan scab pathogen following grower reports in 2002. In vitro tests based on relative growth on 0.2 mg/L propiconazole revealed a significant decline in sensitivity of pecan scab monoconidial isolates from 6 of 7 orchards tested (112,136.137). Additionally, in vitro assays performed in 2004 with the isolates from suspected orchards indicated a similar shift in sensitivity in terms of relative growth. However, the nut scab severity in the suspected orchards treated with fenbuconazole was similar to those DMI-sensitive orchards, suggesting that DMIs were still performing adequately despite the shift in sensitivity (137). In another study, baseline distributions for the QoI kresoxim-methyl indicated mean $ED_{50}$ values 0.095 mg/L, and 0.035 mg/L for in vivo, and in vitro assays respectively (21). However, prior to this study no baseline sensitivity data was published for dodine, fentin hydroxide and thiophanate-methyl, in *F. effusum*.

## Research Objectives

Pecan production in southeastern U.S. relies on effective control of pecan scab, caused by *F. effusum*. The most effective tool to control this pathogen on susceptible cultivars is the intensive application of fungicides. All fungicides deployed in pecan disease management have

some level of resistance risks. Fungicide resistance monitoring is essential to evaluate the effectiveness of the pecan scab fungicides in individual orchards with different histories of fungicide use. To do this, baseline sensitivity data is needed for all five classes of fungicides, currently used for scab control, results compared to population from sprayed orchards.

The specific objectives of this research were as follows:

1. Determine baseline sensitivity distributions of isolates *F. effusum* to azoxystrobin, propiconazole, thiophanate-methyl, dodine and fentin hydroxide, using mycelial growth and spore germination assays.

2. Develop a rapid and reliable sensitivity assay technique (e.g. bulk spore germination or germ tube growth assay) for routine monitoring of sensitivity of *F. effusum* isolates to azoxystrobin, thiophanate-methyl, dodine, triphenyltin hydroxide, and propiconazole.

3. Determine current site-specific fungicide sensitivity profiles for representative commercial orchards in different pecan-growing regions of Georgia.

4. Investigation of the intron status of mitochondrial cytochrome bc1 gene sequence to evaluate the risk of resistance to QoI fungicides.

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# CHAPTER 2

# BASELINE SENSITIVITY PROFILE OF *FUSICLADIUM EFFUSUM* TO DODINE, FENTIN HYDROXIDE, THIOPHANATE-METHYL AND PROPICONAZOLE[1]

[1]Seyran, M., Stevenson, K. L., and Brenneman, T. B. 2008. To be submitted to Crop Protection

## Abstract

Seyran, M., Stevenson, K. L., and Brenneman, T. B. 2008. Baseline sensitivity profile of *Fusicladium effusum* to dodine, fentin hydroxide, thiophanate-methyl and propiconazole. Submitted to Plant Disease.

Commercial production of pecans in the southeastern U.S. relies on fungicide applications to control scab, caused by *Fusicladium effusum*. To monitor development of fungicide resistance in the pathogen, baseline sensitivity profiles to the major scab fungicides were established. Eighty-nine monoconidial isolates of *F. effusum* were obtained from three orchards in Georgia with no known fungicide history. Isolates were tested for sensitivity to dodine, fentin hydroxide, propiconazole and thiophanate-methyl using a mycelial growth assay in liquid medium in microtiter plates. The 50% effective dose ($EC_{50}$) values ranged from 0.008 to 0.595 µg/ml with a median of 0.0484 µg/ml for dodine (n=66), 0.115 to 4.540 µg/ml with a median of 0.759 µg/ml for fentin hydroxide (n=45), and 0.0114 to 0.793µg/ml with a median of 0.0614 µg/ml for propiconazole (n=65). All tested isolates were sensitive to thiophanate-methyl. All isolates grew well at 0.1 µg/ml (mean relative growth = 97.8%) and failed to grow at 1.0 µg/ml (mean relative growth = 3.3%). These sensitivity data will serve as baselines for future fungicide sensitivity monitoring in commercial pecan orchards.

## Introduction

Pecan scab, caused by the fungus *Fusicladium effusum* (Winter), is the most devastating disease of the pecan plant and is the major target for disease control (3,27). The humid and warm climate in the southeastern United States favors disease development, and disease can be especially severe during rainy years. Without appropriate control measures, pecan scab can cause complete yield loss on susceptible cultivars (3,20). Pecan scab management is based on selecting resistant cultivars and preventive fungicide applications (3,4,20,29). Establishing an orchard with resistant cultivars is the ideal control method (29). However, the nut quality difference between susceptible and resistant cultivars is an important consideration and "resistant" cultivars have a long history of becoming susceptible when they are widely grown (3,20,29). The commercial production of high quality pecans of susceptible cultivars depends on multiple fungicide applications per season (3,4).

Fungicides used for pecan scab disease control are dodine, a guanidine fungicide (FRAC code M7); fentin hydroxide, an organotin fungicide (FRAC code 30); thiophanate-methyl, a benzimidazole fungicide (FRAC code 1); fenbuconazole and propiconazole, demethylation inhibitor (DMI) fungicides (FRAC code 3); kresoxim-methyl, azoxystrobin, pyraclostrobin, and trifloxystrobin, quinone outside inhibitor (QoI) fungicides (FRAC code 11) (3,10,31). These fungicides are used either alone or as mixtures (17,31). Fungicide applications are scheduled with either a calendar-based program, or by the weather-driven Au-Pecan advisory program (2,31). All fungicide groups deployed in pecan disease management have some level of risk for development of resistance (10,16,17,31). Fungicide Resistance Action Committee (FRAC) guidelines consider dodine, fentin hydroxide, thiophanate-methyl, QoI group member fungicides and DMIs as moderate, medium, high, high and medium risk, respectively (10,16,17,31).

To detect shifts in fungicide sensitivity prior to control failures in the field, a baseline sensitivity profile of the target fungus is required (5,25,26). This profile is the estimated response level of fungal populations to each fungicide that have not been exposed to the fungicide (5,25,26). Some baseline data is available for *F. effusum* to DMIs and QoIs; however little sensitivity data is available for dodine, fentin hydroxide and thiophanate-methyl (6,24). However, early records indicated development of resistance to benomyl just one year after its introduction in 1974, and reduced sensitivity to DMIs was first detected in pecan scab in 2002 (19,32). The objectives of this study were to (i) test the feasibility of a microtiter plate technique to evaluate dose response of *F. effusum* to propiconazole, thiophanate-methyl, dodine, and fentin hydroxide, and (ii) determine or validate baseline sensitivity distributions of *F. effusum* to propiconazole, thiophanate-methyl, dodine and fentin hydroxide using a microtiter plate assay. Although it is preferable to establish baseline sensitivity profiles prior to introduction and use of a fungicide, sampling isolated, non-sprayed orchards is probably the best option for estimating baseline sensitivities for products already in use.

## Materials and Methods

**Collection of *F. effusum* isolates.** Pecan leaves with actively sporulating scab lesions were collected in 2007 from plantations located in Clarke, Jeff Davis, and Washington Counties in Georgia. These locations had no known history of fungicide use and were located at least 80 km from commercial orchards where fungicides have been used. Individual lesions were cut with a cork borer and gently rubbed on the surface of water agar (WA) amended with antibiotics (chloramphenicol, streptomycin sulfate, and tetracycline, each at 50 µg/ml). The conidia were incubated for 24 h at 25ºC in the dark. Single germinated conidia were transferred to potato

dextrose agar (PDA) with antibiotics (same concentrations as described above) and incubated at 25ºC dark for 6 weeks.

**Microtiter assay to determine fungicide sensitivity.** Technical grade fentin hydroxide, dodine, thiophanate-methyl and propiconazole were kindly provided by E.I. Du Pont de Nemours & Co. Inc., Wilmington DE; Agriphar S.A., Ougrée, Belgium; Cerexagri, King of Prussia, PA; and Novartis Crop Protection, Inc., Greensboro, NC. The propiconazole and fentin hydroxide were dissolved and serially diluted in acetone to obtain final concentrations of 0, 0.0001, 0.001, 0.01, 0.03, 0.1, 0.3, 1.0, or 3.0 µg/ml, and dodine was dissolved and serially diluted in methanol to obtain the same concentrations. Due to its mode of action, only three concentrations of thiophanate-methyl were tested in addition to a non-amended medium (control). The fungicide was dissolved and serially diluted in acetone to obtain final concentrations of 0, 0.1, 1.0, or 10.0 µg/ml. Fungicide stock solutions were added to potato dextrose broth (PDB) to obtain the final concentrations described above. The fungicide-amended broth was transferred to each well of 96-well microtiter plates (150 µl per well). A 3-mm diameter plug from each fungal colony growing on PDA was placed in a glass tube containing 50 ml sterile PDB and the agar disk homogenized with a tissuemizer (IKA Labortechnik T25 Basic, IKA Works, Inc. Willmington, NC) to obtain a uniform suspension of mycelial fragments. Fifty microliters of fungal suspension was transferred to each well of the microtiter plates with three replicate plates of each isolate and fungicide concentration. One of the replicate plates was used to measure initial light absorbance with an automated microtiter plate reader at a wavelength of 405 nm. The other two replicate plates were wrapped with Parafilm and incubated for 7 days in the dark at 25ºC on a rotary shaker set at 100 rpm.

Relative growth (RG) of the isolates in each well was calculated by the formula:

$$RG = (F_7 - F_0)/(C_7 - C_0),$$

where $F_0$ and $F_7$ = light absorbance values for wells with fungicide-amended medium on days 0 and 7, respectively; and $C_0$ and $C_7$ = light absorbance values for the control wells with non-amended medium (control) on days 0 and 7, respectively.

**Data analysis**. The 50% effective dose ($EC_{50}$) value for each isolate was calculated by regressing the probit-transformed RG values on the $log_{10}$-transformed fungicide concentrations using SAS PROC REG (SAS 9.1, SAS Institute Inc., Cary, NC). Median $EC_{50}$ values were calculated for each location and for combined locations. Student's t-tests were used to compare mean $log_{10}$-transformed $EC_{50}$ values between fungicides and locations. The log-normality of the calculated $EC_{50}$ values was tested by the Shapiro-Wilk test for each location and fungicide. A correlation analysis was performed on the $log_{10}$-transformed $EC_{50}$ values for azoxystrobin, dodine, fentin hydroxide and propiconazole using SAS PROC CORR (SAS ver. 9.1, SAS Institute, Cary NC).

## Results

**Sensitivity distributions.** For dodine, $EC_{50}$ values of the 34 Clarke County isolates ranged from 0.008 to 0.595 µg/ml with a median of 0.059 µg/ml (Table 2.1). $EC_{50}$ values of the 22 Jeff Davis County isolates ranged from 0.015 to 0.131 µg/ml with a median of 0.040 µg/ml (Table 2.1). $EC_{50}$ values of the 8 Washington County isolates ranged from 0.007 to 0.097 µg/ml with a median of 0.047 µg/ml (Table 2.1). Median $EC_{50}$ values for dodine were similar among the three locations and the values in each group were log-normally distributed (Table

2.1). For all 67 isolates combined, the median $EC_{50}$ value for dodine was 0.048 µg/ml (Table 2.1), and combined values were log-normally distributed (Prob < W = 0.695) (Fig. 2.1).

For fentin hydroxide, $EC_{50}$ values of the 30 Clarke County isolates ranged from 0.114 to 2.476 µg/ml with a median of 0.504 µg/ml (Table 2.1). $EC_{50}$ values of the 9 Jeff Davis County isolates ranged from 0.386 to 4.540 µg/ml with a median of 1.872 µg/ml (Table 2.1). $EC_{50}$ values of the 6 Washington County isolates range from 0.257 to 1.218 µg/ml with a median of 0.862 µg/ml (Table 2.1). The $EC_{50}$ values in each group were log-normally distributed (Table 2.1). For all 45 isolates combined, the median $EC_{50}$ value for fentin hydroxide was 0.760 and the combined values were log-normally distributed (Prob < W = 0.619) (Fig. 2.2).

For propiconazole, $EC_{50}$ values of the 36 Clarke County isolates ranged from 0.011 to 0.343 µg/ml with a median of 0.055 µg/ml (Table 2.1). $EC_{50}$ values of the 23 Jeff Davis County isolates ranged from 0.027 to 0.792 µg/ml with a median of 0.093 µg/ml (Table 2.1). $EC_{50}$ values of the 6 Washington County isolates ranged from 0.039 to 0.587 µg/ml with a median of 0.086 µg/ml (Table 2.1). Median $EC_{50}$ values for propiconazole were similar among the three locations and the values in each group were log-normally distributed (Table 2.1). For all 65 isolates combined, the median $EC_{50}$ value for propiconazole was 0.061 µg/ml (Table 2.1), and the combined values were log-normally distributed (Prob < W = 0.098) (Fig. 2.3).

For the 89 isolates of *F. effusum* from three different locations that were tested for sensitivity to thiophanate-methyl, the dose response was discontinuous, so $EC_{50}$ values could not be estimated. All isolates grew only slightly or not at all on medium containing 1.0 or 10.0 µg/ml thiophanate-methyl, with mean RG values of 0.033 and 0.018, respectively. However all isolates grew well on medium containing 0.1 µg/ml of thiophanate-methyl, with a mean RG value of 0.978.

Weak, but significant positive correlations were observed between $log_{10}$-transformed $EC_{50}$ values for fentin hydroxide and azoxystrobin (r =0.32629, p =0.035) (Fig. 2.4), and between propiconazole and azoxystrobin ( r =0.31298, p =0.0212 ) (Fig. 2.5). A weak, but significant negative correlation was observed between $log_{10}$-transformed $EC_{50}$ values for propiconazole and dodine (r =-0.27412, p =0.0492 ) (Fig. 2.6).

## Discussion

Pecan growers in the southeastern U.S. are encouraged to use all available cultural practices to reduce overall disease pressure (3). However, most orchards still require 7 to 10 or more fungicide applications per year (4). Considering the high reproductive capacity of the pathogen and the perennial nature of the crop, the potential for development of fungicide resistance is significant (31). Specific management guidelines have been developed to prevent the occurrence of fungicide resistance (4,31). All pecan scab fungicides should be applied preventively to target the pre-infection stage of disease development (4,31). Additionally, post pollination application of azoxystrobin should not follow the pre-pollination application of other QoIs (kresoxim-methyl, pyraclostrobin, trifloxystrobin), and post pollination application of DMIs (propiconazole and fenbuconazole) should not follow the pre-pollination application of DMIs (31). Several formulated mixtures containing two active ingredients are also available to ensure that single products are not used too frequently (31). In addition to these practices, routine fungicide sensitivity monitoring of *F. effusum* populations can be valuable for assessing the effective of resistnce management practices and can provide an early warning to growers (31).

Fungicide resistance can be monitored by testing the sensitivity of suspected isolates and comparing results to previously determined baseline data (5,25,26). However, fungicide

resistance to DMIs and some other fungicides occurs via gradual shifts, and accurate monitoring and detection of shifts requires high sample sizes (5,16,17,25,26). In this study, eighty-nine monoconidial isolates of *F. effusum* were cultured from three different baseline populations in Georgia.

The microtiter plate technique was used to efficiently handle the large number of fungicides and concentrations included in this study, as has been demonstrated previously with other pathogens and fungicides (1,7,8,9,18,22,23,28,30). In a previous study, Cancro determined sensitivity of conidia and mycelium of the pecan scab fungus to kresoxim-methyl by using microtiter plate assay (6). We have shown this method to be effective for determining sensitivity of *F. effusum* to fungicides in four different classes. The four fungicides evaluated in this study vary in their inherent resistance risk from moderate to high and cases of resistance have been documented to each of them. For some fungicides (e.g. the benzimidazoles), resistance developed very suddenly but for others (e.g. dodine and DMIs), resistance developed more gradually. Regardless of the mechanism, it is essential to have an accurate baseline sensitivity profile for each compound to serve as a reference for future studies. Since a true baseline was never established prior to the introduction of these fungicides, the current sensitivity profile of isolates from isolated, non-treated trees is the best available alternative.

*F. effusum* was found to be slightly more sensitive to dodine than the genetically related fungus *Venturia inaequalis*. $EC_{50}$ values ranged from 0.007 to 0.595 µg/ml with a median $EC_{50}$ of 0.051 µg/ml compared to a range of 0.024 to 1.2 µg/ml and a mean $ED_{50}$ of 0.17 µg/ml for *V. inaequalis* with another method (14). In our assay, the median $EC_{50}$ value for fentin hydroxide was estimated as 0.758 µg/ml, which is consistent with a mean $EC_{50}$ value of 0.941 µg/ml based on a similar microtiter plate assay conducted in 1997 (unpublished data). For the 9 monoconidial

isolates of *F.effusum* cultured in 2007 from the Jeff Davis County location, $EC_{50}$ values for fentin hydroxide ranged from 0.386 to 4.540 µg/ml with a median of 1.872 µg/ml. $EC_{50}$ values of monoconidial isolates cultured from the same location in 1997 ranged from 0.027 to 26.065 µg/ml with a mean of 1.238 µg/ml (unpublished data). In our assay, growth of all 89 isolates of *F. effusum* was strongly inhibited by 1.0 µg/ml thiophanate-methyl but all isolates were only slightly inhibited at 0.1 µg/ml. For *V. inaequalis*, a discriminatory concentration of 0.5 µg/ml was selected to distinguish between sensitive and resistant isolates, which is biologically consistent with the response observed in *F. effusum*. In our study, for propiconazole the median $EC_{50}$ value of the combined data from all locations was 0.061 µg/ml, which is quite similar with the mean $EC_{50}$ 0.143 µg/ml determined in 1994 with another method (24). For propiconazole, $EC_{50}$ values for isolates obtained from the Jeff Davis County location ranged from 0.027 to 0.792 µg/ml with a median of 0.093 µg/ml and with isolates obtained in 1994 from the same location and tested for sensitivity to propiconazole using a conventional mycelial growth assay on fungicide-amended PDA, $EC_{50}$ values ranged from 0.013 to 1.102 µg/ml with a mean of 0.121 µg/ml (24). The results were quite similar, despite the difference in methodology. In contrast to the observed correlation between sensitivity of fentin hydroxide and azoxystrobin in *F. effusum* in this study, no correlation was detected between the sensitivity of fentin acetate (organotin) and flutriafol (DMI) in *Cercospora beticola* (12). A similar positive correlation between the sensitivity of *F. effusum* to azoxystrobin and propiconazole detected in our study was reported, in *Uncinula necator* between the sensitivity to azoxystrobin and myclobutanil (34). However, this type of positive correlation was not observed between the sensitivity profiles of azoxystrobin and fluquinconazole in *Mycosphaerella graminicola*, flusilazole, metconazole (DMIs) and azoxystrobin in *Septoria tritici*, trifloxystrobin, pyraclostrobin (QoIs) and difenoconazole in *C.*

*beticola* and difenoconazole and azoxystrobin in *Phoma ligulicola* (11,13,21,33). Additionally, in our study, a negative correlation between the sensitivity of *F. effusum* to dodine and the DMI fungicide propiconazole was detected. However, *V. inaequalis* isolates resistant to dodine developed resistance to the DMI fungicide fenarimol more rapidly compared to dodine sensitive isolates, which was evaluated as an indication of multiple resistance (15).

This work documents the baseline sensitivity profiles of the pecan scab fungus, *F. effusum*, to fungicides representing four chemical classes. This is the first report of a baseline sensitivity of this pathogen to dodine, fentin hydroxide and thiophanate-methyl and confirms earlier data on sensitivity to propiconazole. As previously indicated, these fungicides have been widely used for scab control over the past 10 to 50 years. Therefore, it is difficult to ensure that the populations that we sampled had not ever been exposed to these fungicides. Therefore, the sampling sites used in this study were chosen specifically because of their considerable distance (approximately 80 km) from commercial orchards where fungicides were more recently used. Long-range aerial dispersal of *F. effusum* conidia from commercial orchards to the baseline locations is theoretically possible. However, the consistency among $EC_{50}$ values of baseline isolates cultured and tested over time, and the similarity in sensitivities of the genetically related apple scab fungus suggests a low frequency of migration and limited impact of fungicide drift. These sensitivity data will serve as baselines for future fungicide sensitivity monitoring in commercial pecan orchards.

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**Table 2.1.** Sensitivity of *Fusicladium effusum* isolates cultured from three different locations in Georgia to dodine, fentin hydroxide and propiconazole

| Fungicide | Location | $EC_{50}$ value (µg/ml) | | Number of isolates | Normality test (Prob < W)[z] |
|---|---|---|---|---|---|
| | | Range | Median | | |
| Dodine | Clarke Co. | 0.008 – 0.595 | 0.059 | 34 | 0.7215 |
| | Jeff Davis Co. | 0.015 – 0.131 | 0.040 | 22 | 0.5005 |
| | Washington Co. | 0.007 – 0.097 | 0.047 | 8 | 0.4742 |
| | Combined | 0.007 – 0.595 | 0.051 | 67 | 0.6195 |
| Fentin hydroxide | Clarke Co. | 0.114 – 2.476 | 0.504 | 30 | 0.0838 |
| | Jeff Davis Co. | 0.386 – 4.540 | 1.872 | 9 | 0.9124 |
| | Washington Co. | 0.257 – 1.218 | 0.862 | 6 | 0.1370 |
| | Combined | 0.114 – 4.540 | 0.758 | 45 | 0.2042 |
| Propiconazole | Clarke Co. | 0.011 – 0.343 | 0.055 | 36 | 0.9838 |
| | Jeff Davis Co. | 0.027 – 0.792 | 0.093 | 23 | 0.0857 |
| | Washington Co. | 0.039 – 0.587 | 0.086 | 6 | 0.3545 |
| | Combined | 0.011 – 0.792 | 0.061 | 65 | 0.0984 |

[z] W = Shapiro-Wilks statistic; Prob < 0.05 indicates the $\log_{10}$-transformed $EC_{50}$ values are not normally distributed.

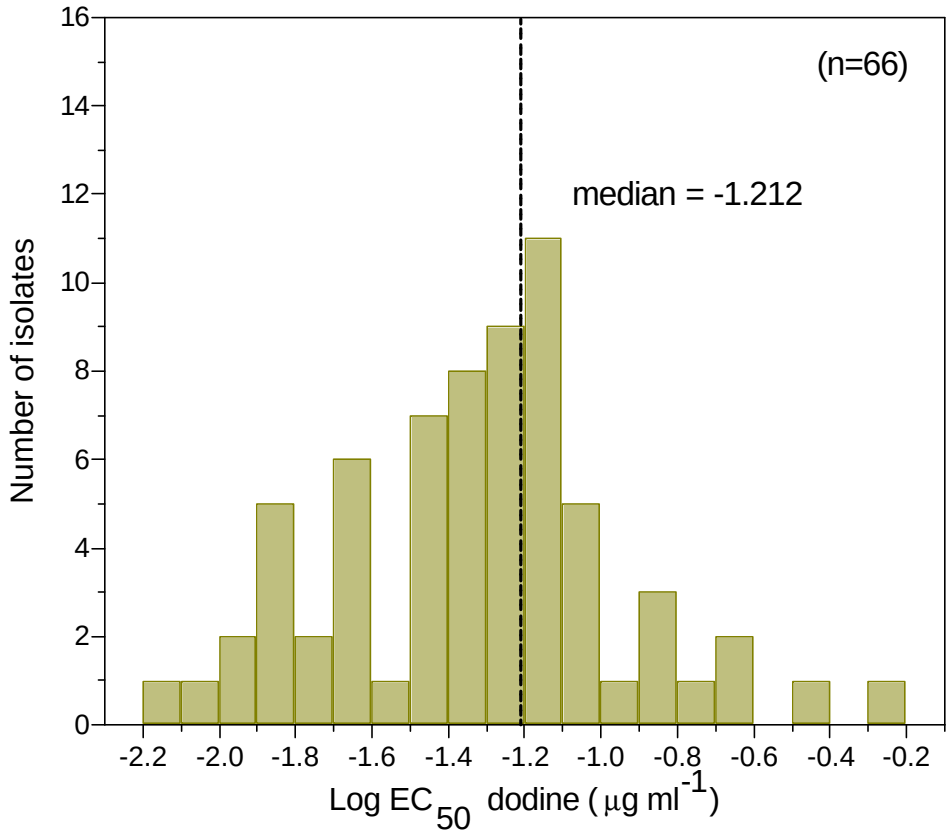


**Fig. 2.1.** Frequency distribution of $EC_{50}$ values for isolates of *Fusicladium effusum* to dodine.

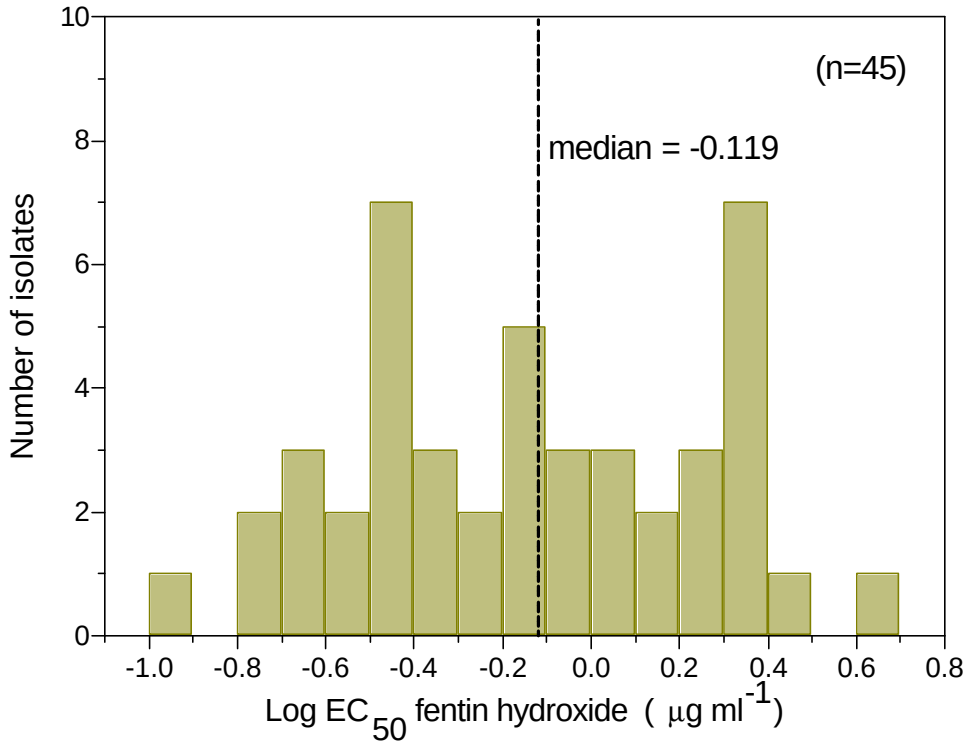


**Fig. 2.2.** Frequency distribution of $EC_{50}$ values for isolates of *Fusicladium effusum* to fentin hydroxide.

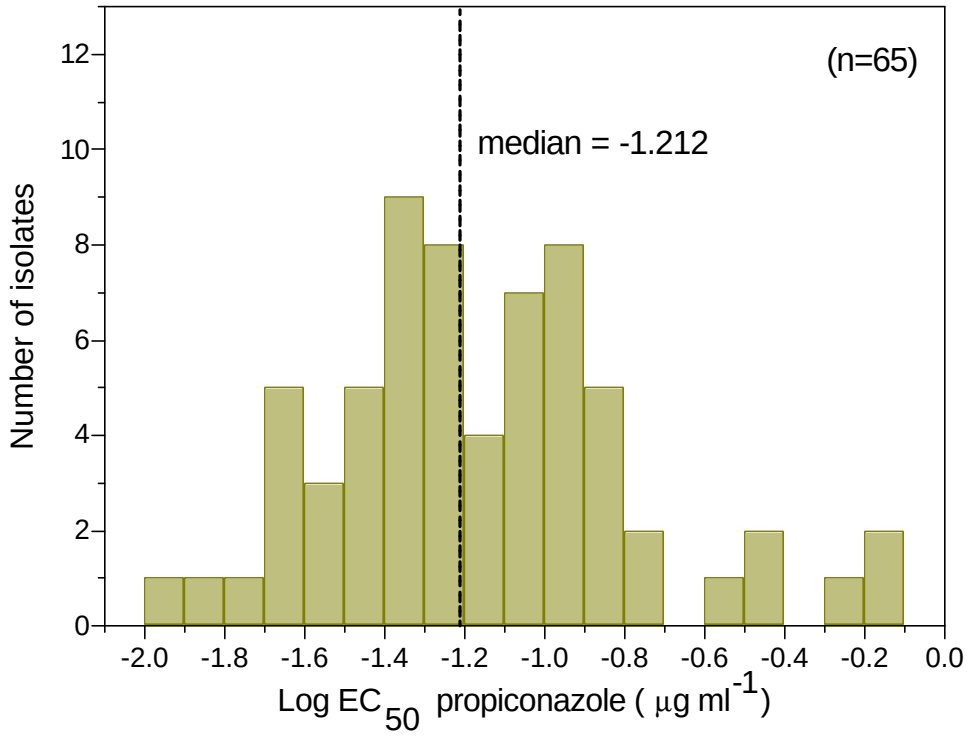


**Fig. 2.3.** Frequency distribution of $EC_{50}$ values for isolates of *Fusicladium effusum* to propiconazole.

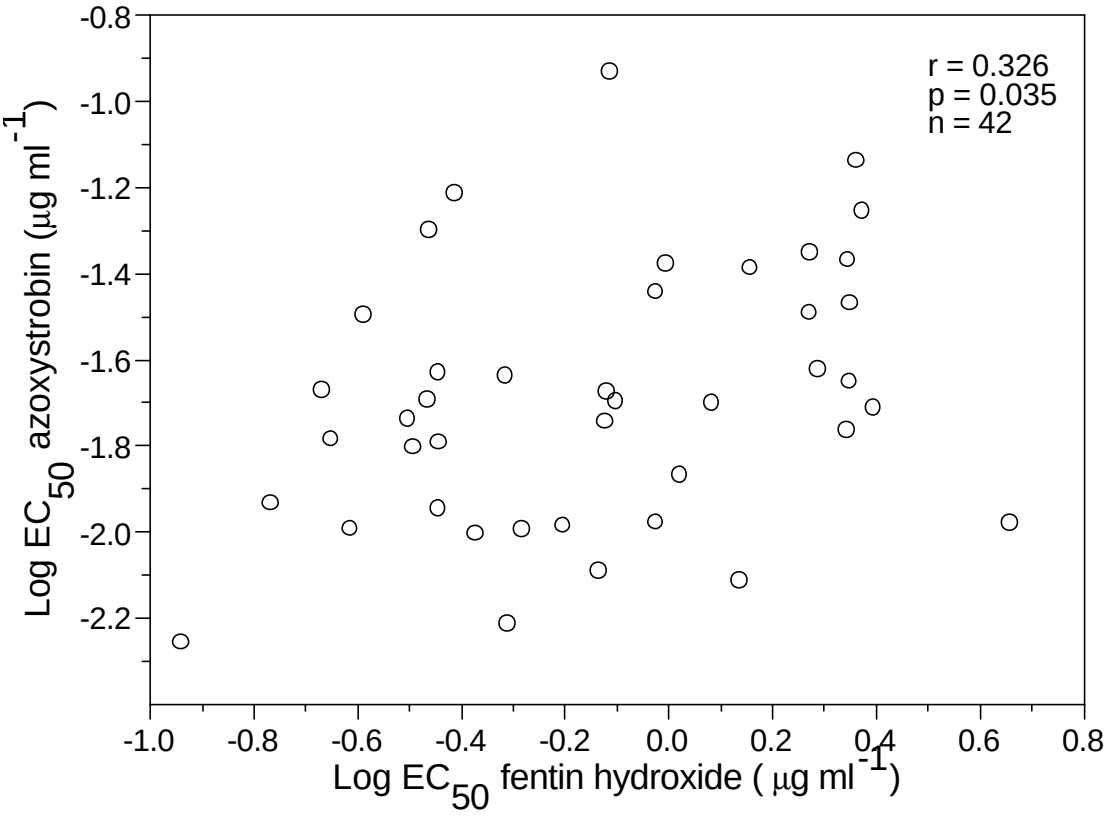


**Fig. 2.4.** Correlation between sensitivity of *Fusicladium effusum* to fentin hydroxide and azoxystrobin.

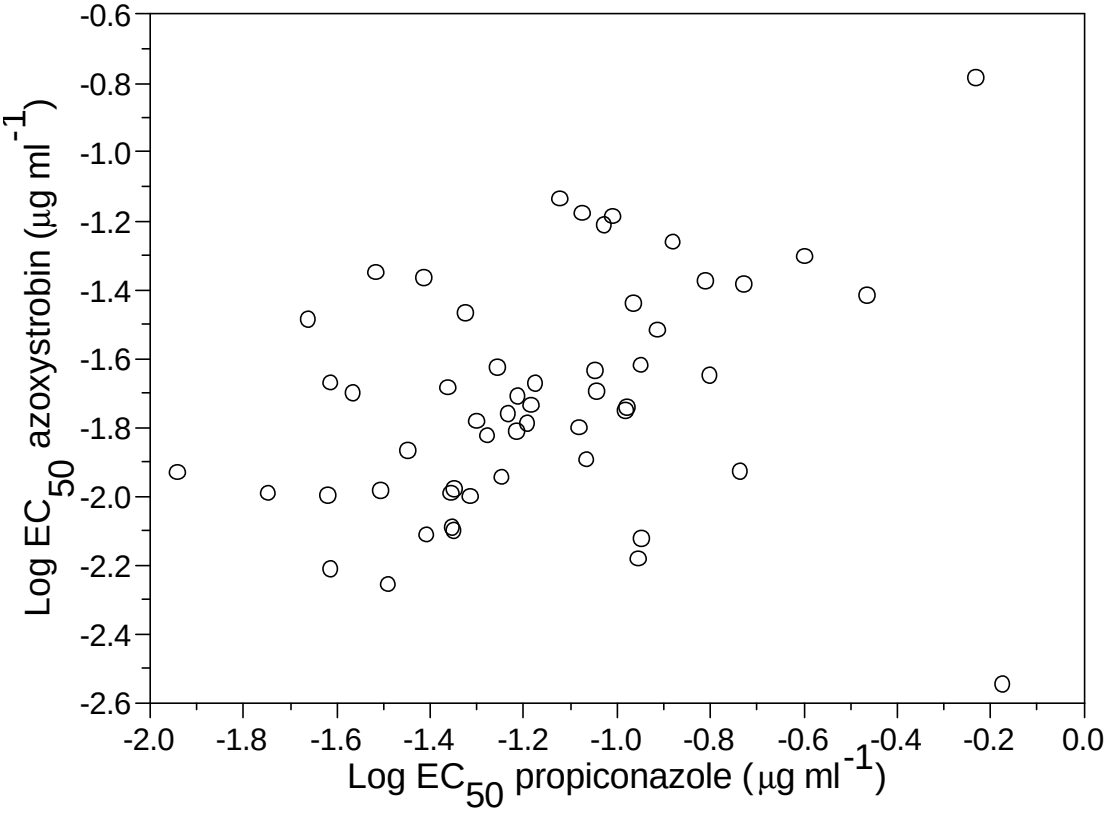


**Fig. 2.5.** Correlation between sensitivity of *Fusicladium effusum* to propiconazole and azoxystrobin.

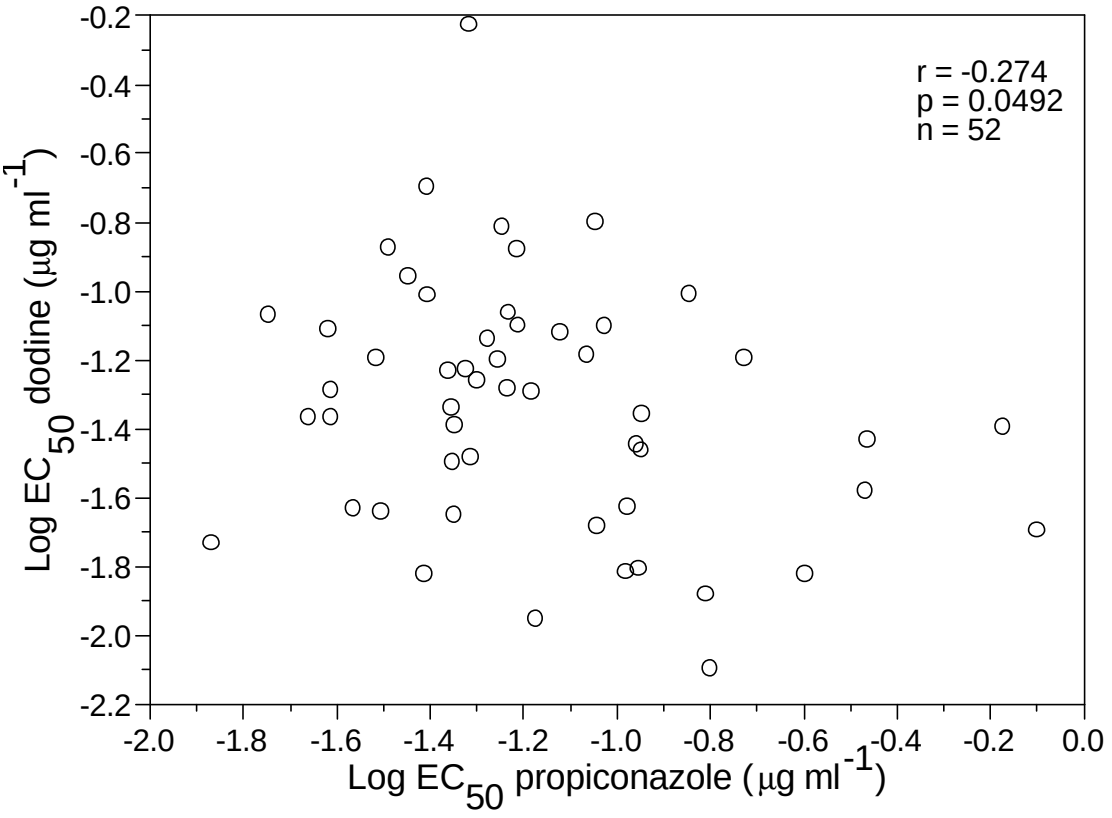


**Fig. 2.6.** Correlation between sensitivity of *Fusicladium effusum* to propiconazole and dodine.

# CHAPTER 3

# IN VITRO TOXICITY OF ALTERNATIVE OXIDASE INHIBITORS TO *FUSICLADIUM EFFUSUM* AND BASELINE SENSITIVITY TO AZOXYSTROBIN[1]

[1]Seyran, M., Stevenson, K. L., and Brenneman, T. B. 2008. To be submitted to Pest Management Science.

## Abstract

Seyran, M., Stevenson, K. L., and Brenneman, T. B. 2008. In vitro toxicity of alternative oxidase inhibitors to *Fusicladium effusum* and baseline sensitivity to azoxystrobin. To be submitted to Pest Management Science.

Pecan production in the southeastern U.S. relies on effective fungicides to control pecan scab, caused by *F. effusum*. Several quinone outside inhibitors (QoIs) are registered for pecan scab control and sensitivity monitoring is essential due to the high level of resistance risk. Baseline sensitivity to azoxystrobin was determined for 89 monoconidial isolates of *F. effusum* obtained from three orchards in Georgia with no known fungicide history. Salicylhydroxamic acid (SHAM) was added to the fungicide-amended medium to suppress alternative oxidase (AOX). However, the $EC_{50}$ values of azoxystrobin with SHAM were not distributed log-normally and were different from the values of azoxystrobin without SHAM. The effects of AOX inhibitors SHAM and propyl gallate on *F. effusum* without fungicide were investigated in vitro in the absence of a QoI fungicide. Even at the concentrations tested, both SHAM and propyl gallate (0.01 and 0.25 mM, respectively) inhibited hyphal growth of *F. effusum* in liquid medium and micro-colony growth on solid medium. Due to the toxic effects of SHAM to *F. effusum*, the sensitivity data obtained for azoxystrobin without SHAM will serve as baselines for future fungicide sensitivity monitoring in commercial pecan orchards.

## Introduction

Pecan scab, caused by the fungus *Fusicladium effusum* (Winter), is the most devastating disease of the pecan plant and is the major target for disease control (8,47). The humid and warm climate in the southeastern U.S. favors disease development, and disease can be especially severe during rainy years. Without appropriate control measures, pecan scab can cause complete yield loss on susceptible cultivars (8). Pecan scab management is based on selecting resistant cultivars and 7-14 preventive fungicide applications per season (8,11). Fungicide classes currently used for scab control are guanidines, organotins, benzimidazoles, demethylation inhibitors and quinone outside inhibitor (QoI) fungicides (11,51). Relative to the other fungicide groups, QoIs have some technical advantages, such as low environmental risk, broad-spectrum activity, xylem systemicity, metabolic stability, translaminar movement, reduced mammalian toxicity and phloem mobility (6,46,57,58). QoI fungicides are site-specific and bind to the ubiquinone oxidizing pocket (Qo site) of the cytochrome bc1 (complex III) of the respiration chain, which is located at the inner mitochondrial membrane of the target fungal cell (6,46,57,58). The presence of the fungicide molecule inhibits the electron transfer between cytochrome b and cytochrome bc1 complex and interrupts ATP production (6,46,57,58). Due to the inhibition of electron flow through complex III by QoI fungicides, an alternative respiration system located on the mitochondrial membrane can be activated. The electrons transfer directly from the ubiquinone complex, bypassing complex III, and are utilized by an alternative oxidase (AOX) system thus avoiding the fatal effects of the QoI fungicides (56,57,58). Therefore many in vitro assays with QoI fungicides include AOX inhibitors such as salicylhydroxamic acid (SHAM) in the medium to suppress alternative respiration (2,3,4,13,16,17,24,27,31,33,34,36,37,41,42, 44,45,49, 55, 59, 60,) (Table 3.1).

To detect shifts in fungicide sensitivity prior to a control failure, a baseline sensitivity profile of the target fungus is needed for each class of fungicide used. This profile is the estimated response level to each fungicide of fungal populations that have not been exposed to the fungicide (12). During preliminary experiments to determine baseline sensitivity of *F. effusum* to the QoI fungicide azoxystrobin, inhibitory effects of 100 µg/ml (0.653 mM) SHAM on fungal growth of *F. effusum* were observed. Although SHAM has been frequently used in similar studies with other fungi, inhibitory effects during in vitro experiments have not been reported previously (2,3,4,13,16,17,24, 27,31,33,34,36,37,41,42,44,45,49,55,59,60).

Fungicide sensitivity assays are critical dose response studies, and evaluation of the results relies on the dose response differences at low concentrations. Thus, any additional inhibitory effect of secondary chemicals may lead to erroneous results. The objectives of this study were to (i) determine baseline sensitivity distributions of *F. effusum* to azoxystrobin with or without AOX inhibitors using a microtiter plate assay, and (ii) investigate the fungitoxicity of SHAM and propyl gallate on *F. effusum* using a micro-colony growth assay on solid medium and a hyphal growth assay in liquid medium.

## Materials and Methods

**Collection of *F. effusum* isolates.** Pecan leaves with actively sporulating scab lesions were collected in 2007 from orchards located in Clarke, Jeff Davis and Washington Counties. These locations had no known history of fungicide use and were isolated from commercial orchards where fungicides have been used. Individual leaf lesions were cut with a 6-mm diameter cork borer and gently rubbed on the surface of water agar (WA) amended with antibiotics (chloramphenicol, streptomycin sulfate, and tetracycline, each at 50 µg/ml). The

conidia were incubated for 24 h at 25ºC in the dark. Single germinated conidia were transferred to potato dextrose agar (PDA) with antibiotics (same concentrations as described above) and incubated at 25ºC dark for 6 weeks.

**Baseline sensitivity of *F. effusum* to azoxystrobin.** Technical grade azoxystrobin provided by Syngenta Crop Protection, Inc., Greensboro, NC was dissolved and serially diluted in acetone to obtain final concentrations of 0, 0.0001, 0.001, 0.01, 0.03, 0.1, 0.3, 1.0, or 3.0 µg/ml in potato dextrose broth (PDB). A suspension of SHAM was prepared in acetone (SHAM forms a turbid suspension in acetone; DMSO was used as solvent in subsequent experiments) and added to a duplicate set of azoxystrobin-amended PDB to provide a final SHAM concentration of 100 µg/ml (0.653 mM). The broth amended with fungicide and/or SHAM was transferred to each well of 96-well microtiter plates (150 µl per well). A plug of each fungal colony was cut with a sterile 3-mm diameter cork borer and placed in a glass tube containing 50 ml sterile PDB. Fungal colonies were homogenized with a tissuemizer (IKA Labortechnik T25 Basic, IKA Works, Inc. Wilmington, NC) to obtain a uniform suspension of mycelial fragments. Fifty microliters of fungal suspension was transferred to each well of the microtiter plates. Three replicate plates of each isolate and fungicide concentration were prepared. One of the replicate plates was used to measure initial light absorbance with an automated microtiter plate reader at a wavelength of 405 nm. The other two replicate plates were wrapped with Parafilm and incubated in the dark at 25 ºC on a rotary shaker at 100 rpm for 7 days. After incubation, the plates were measured as described above. Relative growth (RG) of the isolates in each well was calculated by the formula:

$$RG = (F_7 - F_0)/(C_7 - C_0),$$

where $F_0$ and $F_7$ = light absorbance values for wells with fungicide-amended medium on days 0 and 7, respectively; and $C_0$ and $C_7$ = light absorbance values for the control wells with non-amended medium on days 0 and 7, respectively.

**Effects of AOX inhibitors on hyphal growth in liquid medium.**. Propyl gallate and SHAM were dissolved and serially diluted in dimethyl sulfoxide (DMSO) and added to PDB to obtain final concentrations of 0.005, 0.025, 0.05, 0.25, 0.5 mM and 0.01, 0.05, 0.1, 0.5, 1 mM, respectively. In preliminary experiments, SHAM was found to be insoluble in acetone, methanol, and acetone-methanol (50/50 v/v). Therefore, experiments were repeated by dissolving both of the AOX inhibitors in DMSO and including a DMSO-amended control. The AOX inhibitor-amended broth was transferred to corresponding wells of 96-well microtiter plates (150 µl per well). Eight monoconidal isolates with two replicates were tested as described above.

**Effects of AOX inhibitors on micro-colony development on solid medium.** The fungitoxic effects of SHAM and propyl gallate on hyphal growth were evaluated by dissolving and serially diluting them in DMSO and adding them to autoclaved and cooled potato dextrose agar (PDA) to obtain final concentrations of 0.05, 0.1, 0.5, 1, 5 mM and 0.005, 0.05, 0.1, 0.5, 1 mM, respectively. All media were dispensed into disposable plastic petri plates (100×15 mm). Control plates were prepared with DMSO. Pecan leaves with actively sporulating scab lesions were collected in 2008 from the nonsprayed orchards mentioned previously in Jeff Davis and Washington counties. A solution of sterile distilled water with antibiotics (chloramphenicol, streptomycin sulfate, and tetracycline each at 50 µg/ml) and one drop of Tween 20 per 100 ml was used to dislodge conidia from three individual lesions from each location. The conidia from the each lesion were dislodged by using a pipette for each lesion, 9 µl was pipetted onto the surface of each lesion and then retracted repeatedly 3-5 times before returning it to a micro-

centrifuge tube with 750 µl total volume of suspension. This step was repeated five times to dislodge enough conidia. The presence of dislodged conidia in the suspension was confirmed by dropping 9 µl on a microscope slide and observation with a microscope (100X). Nine microliters of conidial suspension dropped on each AOX inhibitor amended and non-amended medium (control) plates with two replicates was spread over the surface of PDA plates with a sterile glass rod. The conidia were incubated for 72 h at room temperature (23-25°C ) to determine the fungal growth in terms of micro-colony diameter using a microscope integrated camera system (MoticCam 2300, 3.0 MPixel USB 2.0, Motic Inc., Richmond, British Columbia, Canada). Colonies were viewed with a dissecting microscope at a magnification of 32X and the images were stored on a computer. Single colonies growing apart from other colonies and contaminants were randomly selected for measurement. Micro-colony diameter for each single conidial colony was defined as the longest axis of hyphal growth measured by using the Motic Images Plus 2.0 software (provided by the camera manufacturer).

**Data analysis**. The 50% effective dose ($EC_{50}$) values were calculated by regressing the probit-transformed relative growth against the $\log_{10}$-transformed fungicide concentrations by SAS 9.1 (SAS Institute Inc., Cary, NC, USA). Median $EC_{50}$ values were calculated for each location and combined locations. A paired t-test was used to compare mean $\log_{10}$-transformed $EC_{50}$ values of azoxystrobin with or without SHAM. The log-normality of the calculated values was tested by Shapiro-Wilk test for each locations of the fungicide with or without SHAM. A one-tailed Dunnett's test ($a = 0.05$) was used to determine whether colony diameter/light absorbance values for different concentrations of SHAM or propyl gallate were significantly less than the control (DMSO only).

## Results

**Baseline sensitivity of *F. effusum* to azoxystrobin.** For azoxystrobin without SHAM, $EC_{50}$ values for 67 isolates ranged from 0.002 to 0.182 µg/ml with a median of 0.02 µg/ml and was log-normally distributed (Prob < W =0.477) (Table 3.2) (Fig. 3.1). For azoxystrobin with SHAM (100 µg/ml), $EC_{50}$ values for 21 isolates ranged from 0.028 to 0.765 µg/ml with a median of 0.049 µg/ml and was not log-normally distributed (Prob < W = 0.003) (Table 3.2) (Fig. 3.1). Of the 89 isolates tested, $EC_{50}$ values were estimated with and without SHAM for 18 common isolates and the median $EC_{50}$ values of these isolates were more than two-fold higher in azoxystrobin with SHAM (0.049 µg/ml) compared to without SHAM (0.02 µg/ml) (Fig. 3.3). Based on results of a paired t-test of the $log_{10}$-transformed $EC_{50}$ values, there was a statistically significant difference between sensitivity of these 18 isolates to azoxystrobin with and without SHAM ($p < 0.0001$).The median light absorbance in control wells with SHAM was calculated as 0.311 which is 55 % less than control wells without SHAM (Fig. 3.2). The other 68 isolates did not show any dose response to azoxystrobin on SHAM-amended media and could not be used in the analysis.

**Effects of AOX inhibitors on hyphal growth in liquid and solid media.** Growth of *F. effusum*, as measured by light absorbance, was significantly lower in liquid medium amended with 0.5 mM or 1.0 mM SHAM compared to the non-amended control (DMSO only) (Fig. 3.4). Growth of *F. effusum* on solid medium amended with 0.01, 0.05 or 0.1 mM SHAM was not significantly less than the non-amended control (Fig. 3.5,). Growth of *F. effusum*, as measured by light absorbance, was significantly lower in liquid medium amended with 0.5 mM or 0.025 mM propyl gallate compared to the non-amended control (DMSO only) (Fi.g, 3.6). Growth of *F.*

*effusum* on solid medium amended with 0.25, 0.5 or 1 mM propyl gallate was not significantly less than the non-amended control (Fig. 3.7).

## Discussion

Fungicide resistance can be monitored by testing the sensitivity of suspected isolates and comparing results to previously determined baseline data (12). In 2007, eighty-nine monoconidial isolates of *F. effusum* were cultured from three different orchards in the state of Georgia which represent the baseline population. While it is preferable to establish baseline sensitivity profiles prior to introduction and use of a fungicide, sampling isolated, non-sprayed orchards is probably the best option for products already in use. In this study, sensitivity of *F. effusum* to azoxystrobin, a QoI fungicide, was profiled using microtiter plates. In 2000, the same method was used to obtain baseline sensitivity data for *F. effusum* to the QoI fungicide kresoxim-methyl (13,14). Despite the fact that azoxystrobin and kresoxim-methyl are very different QoIs, the estimated mean and median $EC_{50}$ values, respectively, for these fungicides was estimated as 0.02 µg/ml (13,14). Similarly, Cancro was not able to estimate $EC_{50}$ values for a majority of *F. effusum* isolates due to the lack of dose response to kresoxim-methyl when SHAM was added to the medium (13). She also noted that the estimated $EC_{50}$ values from in vitro assays with SHAM were significantly higher than the values without SHAM (14).

SHAM has been used in many in vitro studies to inhibit AOX with the concentration range of 0.2 – 2.0 mM, and except for *Colletotrichum graminicola*, inhibition of fungal growth was not noted. (2,3,4,16,17,24, 27,31,33,34,36,37,41,42,44,45,49,55,59,60) (Table 3.1). For example, the fungicide sensitivity of *Venturia inaequalis* was tested by two different research groups using different concentrations of SHAM (30.62 and 100 µg/ml) (31,41,42,50) (Table 3.1).

In contrast, FRAC guidelines do not recommend the use of SHAM for in vitro studies with *V. inaequalis*, *Phakopsora pachyrhizi*, or *Botrytis cinerea* but do suggest using SHAM for *Alternaria solani*, *Rhynchosporium secalis* and *Pyrenophora teres* (19) (Table 3.1). The FRAC guidelines do suggest using 0.2 mM SHAM to inhibit AOX of *Mycosphaerella graminicola*, however one study demonstrated that a 10-fold higher concentration of SHAM was required to inhibit AOX of this organism (Table 3.1) (19,59). In another study, the authors suggested using different concentrations of SHAM for different stages of *Magnaporthe grisea*, specifically 100 µg/ml SHAM for conidia and 150 µg/ml SHAM for the mycelial stage (Table 3.1) (2,3,19). The most common concentration is 100 µg/ml (0.653 mM) of SHAM, which has been used in 14 previous studies (2,3,4,16,17,24, 27,31,33,34,41,42,44,45,55). This concentration inhibited the fungal growth of *F. effusum* by approximately 50 %. Additionally, there has not been a common solvent to dissolve the SHAM. Solvents reported include DMSO, methanol, acetone, ethanol, and a acetone/methanol mixture (2,3,4,13,20,21,23,29,30,31,35,36,40,41, 43,44,48, 49,51,52, 59, 61,66). One protocol suggested warming the methanol to 37?C in a water bath to dissolve 100 µg/ml of SHAM (44). However, even after exposure to room temperature, this amount of dissolved SHAM forms solid crystals in acetone, methanol and acetone-methanol mixtures immediately after dissolving (unpublished data).

There are different theories about the role of AOX in QoI sensitivity assays. One theory is that AOX in fungi is not active during infections *in planta*, but only serves to maintain viability of the fungus in vitro (2,3,4,56,58). Therefore AOX should be inhibited during in vitro studies (2,3,4,56,58). Other evidence suggests that the fungal AOX system is active during infections *in planta*, but can be inhibited by plant secondary metabolites (2,3,4,56,58). Thus during in vitro experiments, fungal AOX should be inhibited by SHAM to mimic the plant

secondary metabolites (2,3,4,56,58). Clearly fungitoxic effects of SHAM on *F. effusum* were observed in this study. Other hydroxamic acid derivatives naturally existing in plant species have been found to be effective inhibitors of fungal organisms such as *Fusarium subglutinans*, *Fusarium culmorum*, *Gaeumannomyces graminis* var. *tritici Cephalosporium gramineum*, and *Candida albicans* (9,10,38,54). In 1971, SHAM was used to inhibit the cyanide-resistant respiration (AOX) of *Neurospora crassa* mutant (32). However, structural differences between plant and fungal AOX systems were defined (48,53) There are efforts to synthesize better AOX inhibitors based on the SHAM moiety to control AOX of *Trypsonoma brucei,* since AOX is the only respiration mechanism this organism utilizes (43). Metabolic pathways other than AOX, such as fungal peroxidase, melanin biosynthesis, esterases, and laccase, have also been shown to be inhibited by SHAM (5,15,21,52). Fungal peroxidase as an enzyme, and melanin as a fungal antioxidant, play an important role in preventing fatal effects of oxygen radicals which are released during respiration in the presence of QoI molecules (22,23,35). Therefore, the inhibition of these critical antioxidant systems by SHAM could generate fatal effects prior to the activation of the AOX system. Additionally, in some fungi exposed to kresoxim-methyl, plant secondary metabolites inhibited the superoxide dismutase and other antioxidative stress responses (28,29,30). Juglone, which is both a pecan and a fungal secondary metabolite, did not suppress the AOX of *Fusarium decemcullulare* (39). In fact, low concentrations of juglone actually increased the AOX activity (39).

Even at low concentrations, SHAM caused some suppression of growth of *F. effusum* (Fig. 3.8). We also do not know the concentration of SHAM or propyl gallate required to inhibit AOX in *F. effusum*. Additionally, FRAC does not recommend the use of SHAM for in vitro assays of *Venturia inaequalis*, which is genetically related to *F. effusum* (7,19). Consequently,

the sensitivity data obtained for azoxystrobin without SHAM will serve as baselines for future fungicide sensitivity monitoring in commercial pecan orchards.

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**Table 3.1.** Concentrations of SHAM and solvents used with QoI fungicides in previous studies with plant pathogenic fungi

| Organism | Concentration of SHAM (mM) | Solvent | References |
|---|---|---|---|
| *Alternaria spp.* | 0.65 | methanol | 19,33,34 |
| *Alternaria solani* | 0.65 | methanol | 19,44,45 |
| *Botrytis cinerea* | 0.5 | methanol | 37 |
| *Botrytis cinerea* | 0[b] | b | 19 |
| *Cercospora beticola* | 0.5 | methanol | 36 |
| *Colletotrichum graminicola* | 0.65 | acetone & methanol | 4 |
| *Gaeumanomyces graminis* var. *tritici* | 0.2, 0.3 | DMSO | 25 |
| *Gaeumanomyces graminis* var. *tritici* | 0.3 | NA | 26 |
| *Magnaporthe grisea* | 098 | acetone & methanol | 2[c] |
| *Magnaporthe grisea* | 0.65, 0.98 | NI | 3 |
| *Septoria tritici* (*M. graminicola*) | 2.0 | methanol | 59 |
| *Mycosphaerella graminicola* | 0[b] | NI | 18 |
| *Mycosphaerella graminicola* | 0.2 | NI | 19 |
| *Phakopsora pachyrhizi* | 0[b] | b | 19 |
| *Pyrenophora spp.* | 0.3 | NI | 49 |
| *Pyrenophora teres* | 0.2 | NI | 19 |
| *Pyricularia grisea* | 0.65 | methanol | 55 |
| *Pyricularia oryzae* | 1.0 | DMSO | 27 |
| *Phoma ligulicola* | 0.65 | acetone | 24 |
| *Podosphaera fusca* | NI | NI | 16[c] |
| *Podosphaera fusca* | 0.65, 1.63, 3.27 | ethanol | 17[c] |
| *Rhynchosporium secalis* | 0.2 | NI | 19 |
| *Ustilago maydis* | 0.5 | methanol | 60 |
| *Venturia inaequalis* | 0.65 | acetone & methanol | 31[c] |
| *Venturia inaequalis* | 0.65 | methanol | 41[c],42 |
| *Venturia inaequalis* | 0.2 | DMSO | 50 |
| *Venturia inaequalis* | 0[b] | b | 19 |

a The authors used 0.65 mM (100 µg/ml) of SHAM for conidia germination and 0.98 mM (150 µg/ml) of SHAM for mycelial growth assays.
b The use of SHAM or other AOX inhibitors not suggested.
c The use of SHAM on a leaf assay.
NI , not indicated

**Table 3.2**. Sensitivity of *Fusicladium. effusum* isolates from three pecan orchards in Georgia with no history of fungicide use to azoxystrobin with and without SHAM (100µg/ml)

| | | $EC_{50}$ value (µg/ml) | | | |
|---|---|---|---|---|---|
| **Fungicide** | **Location** | **Range** | **Median** | **Number of Isolates** | **Shapiro-Wilk test** (Prob < W ) |
| Azoxystrobin | Clarke Co. | 0.005 to 0.118 | 0.021 | 35 | 0.837 |
| | Jeff Davis Co. | 0.003 to 0.065 | 0.017 | 24 | 0.485 |
| | Washington Co. | 0.010 to 0.182 | 0.02 | 7 | 0.155 |
| | Combined results | 0.002 to 0.182 | 0.02 | 67 | 0.477 |
| Azoxystrobin + SHAM | Clarke Co. | 0.028 to 0.765 | 0.047 | 11 | 0.0001 |
| | Jeff Davis Co. | 0.032 to 0.193 | 0.094 | 7 | 0.721 |
| | Washington Co. | 0.046 to 0.279 | 0.082 | 3 | 0.616 |
| | Combined results | 0.028 to 0.765 | 0.049 | 21 | 0.003 |

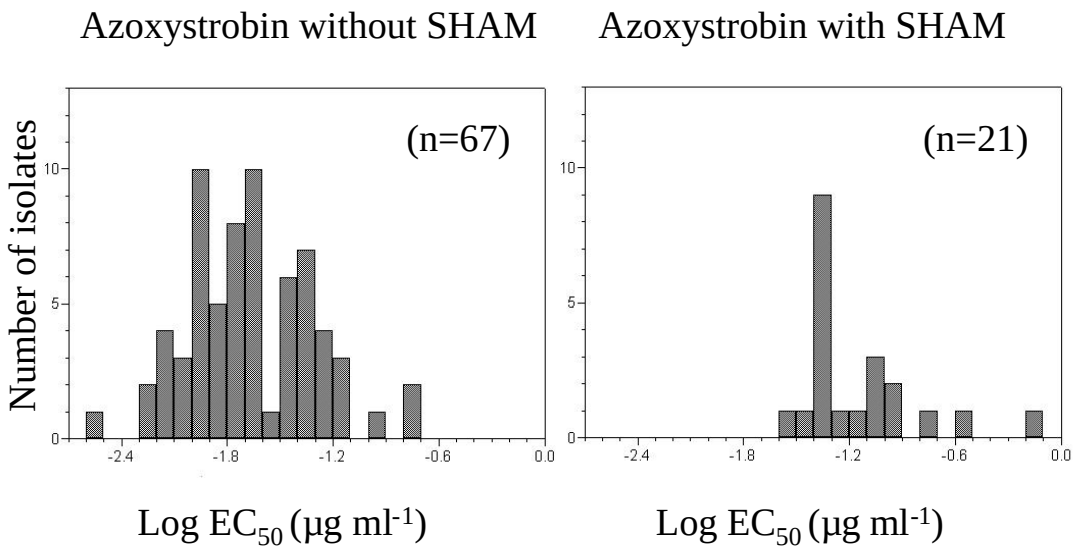


**Fig. 3.1.** Frequency distribution of $EC_{50}$ values for isolates of *Fusicladium effusum* from baseline orchards to azoxystrobin with and without SHAM (100 µg/ml).

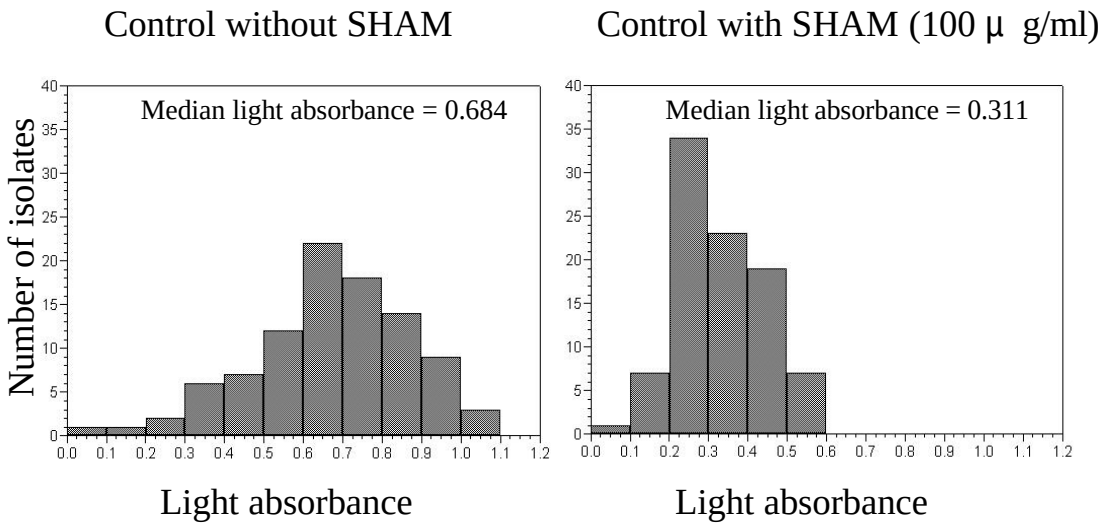


**Fig. 3.2.** Frequency distribution of light absorbance in control wells of PDB amended with acetone without and with SHAM (100 µg/ml).

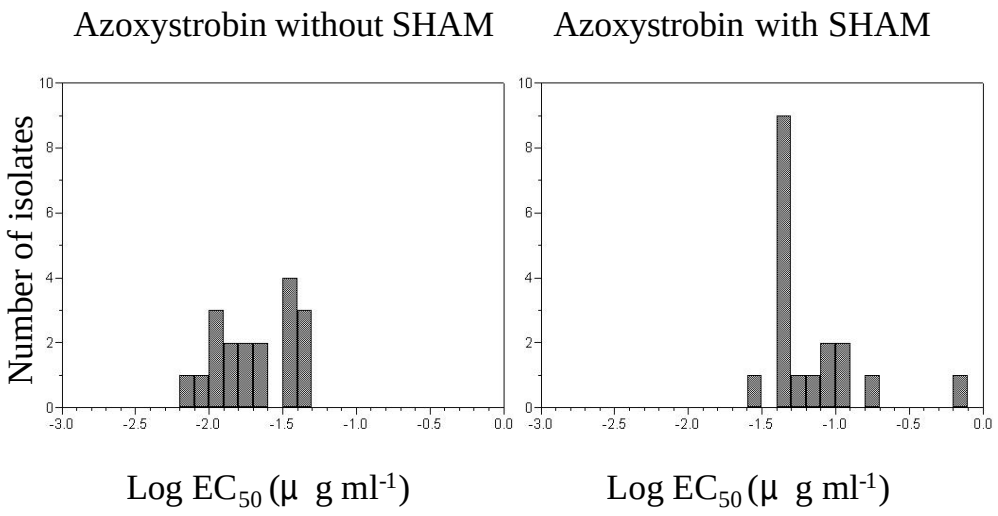


**Fig. 3.3.** Comparison of $EC_{50}$ values for 18 isolates of *Fusicladium effusum* to azoxystrobin with and without SHAM (100 µg/ml).

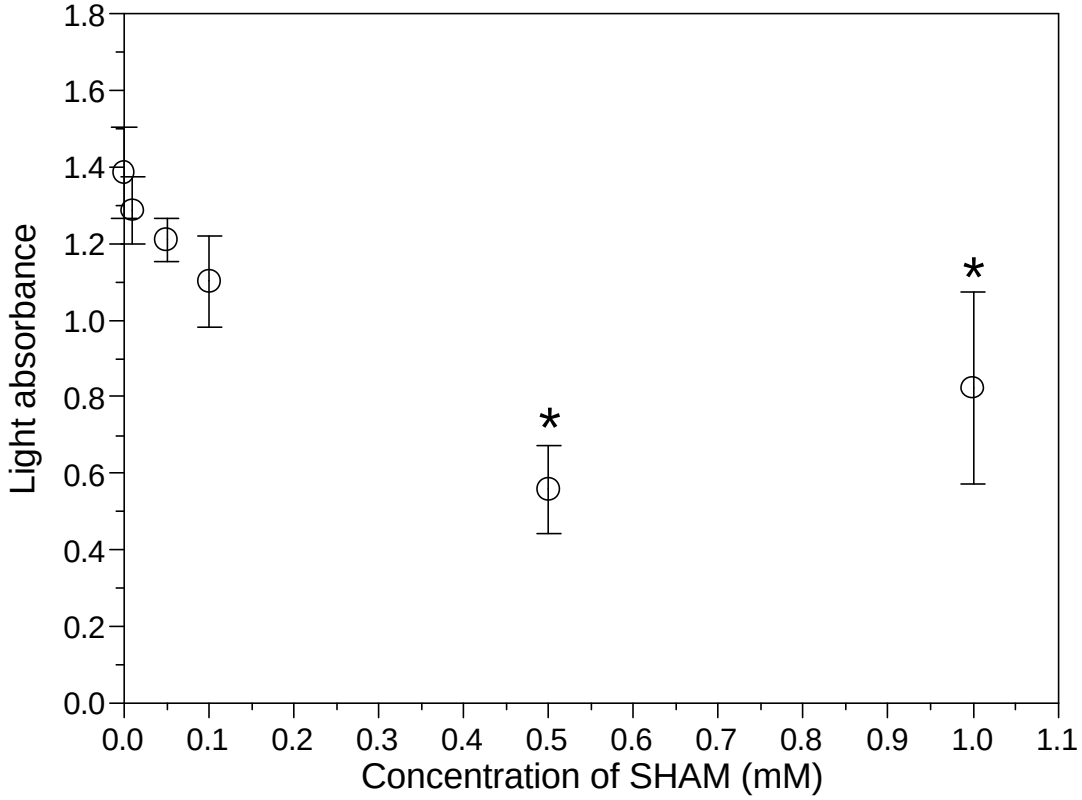


**Fig. 3.4.** Dose response of *Fusicladium effusum* to SHAM in liquid medium in microtiter plates as measured by light absorbance at a wavelength of 405 nm. Values are means of 8 isolates, vertical bars indicate standard errors of the mean, and asterisks indicate light absorbance values that are significantly lower than the non-amended control (DMSO only).

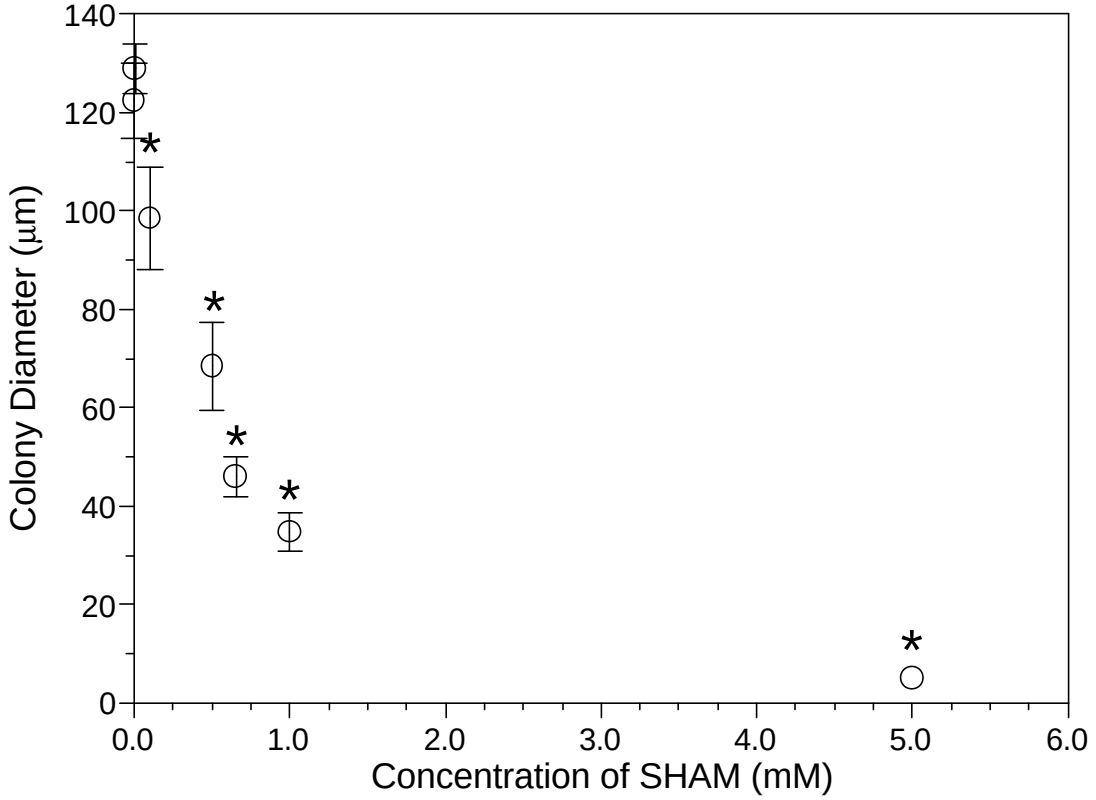


**Fig. 3.5.** Dose response of *Fusicladium effusum* to SHAM on solid medium. Values are means of 6 isolates, vertical bars indicate standard errors of the mean, and asterisksthe growth values that are significantly lower than the non-amended control (DMSO only).

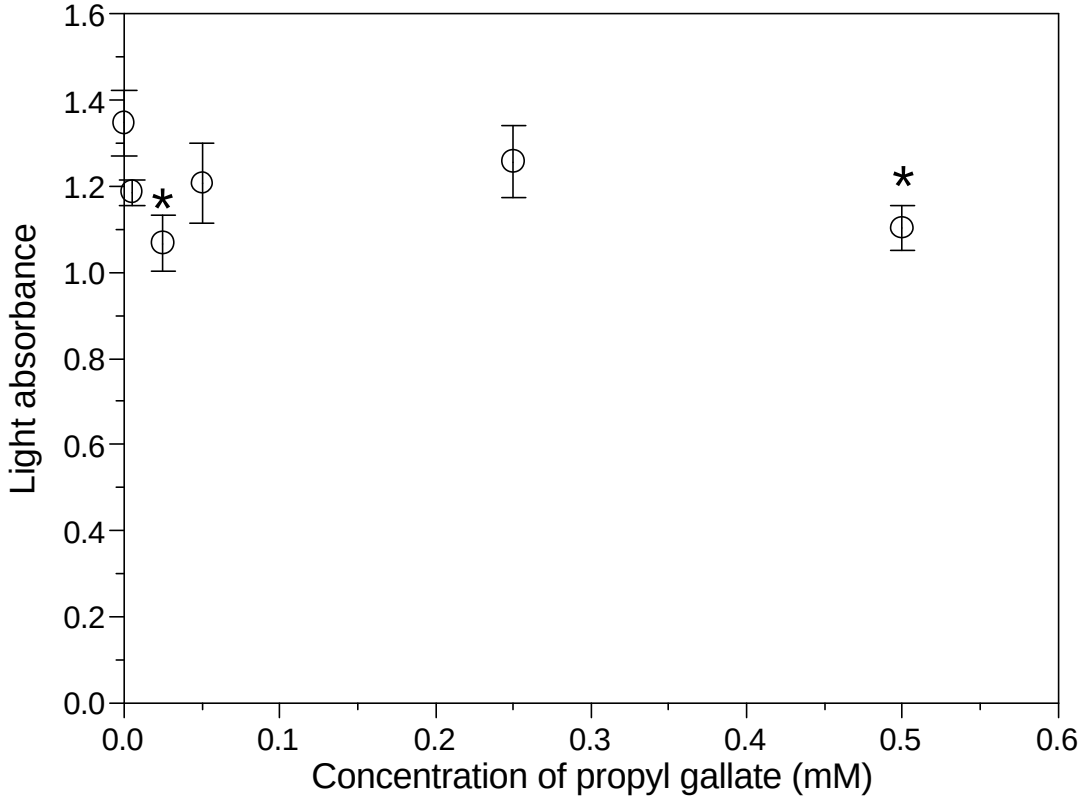


**Fig. 3.6.** Dose response of *Fusicladium effusum* to propyl gallate in liquid medium in microtiter plates as measured by light absorbance at a wavelength of 405 nm. Values are means of 8 isolates, vertical bars indicate standard errors of the mean, and asterisks indicate light absorbance values that are significantly lower than the non-amended control (DMSO only).

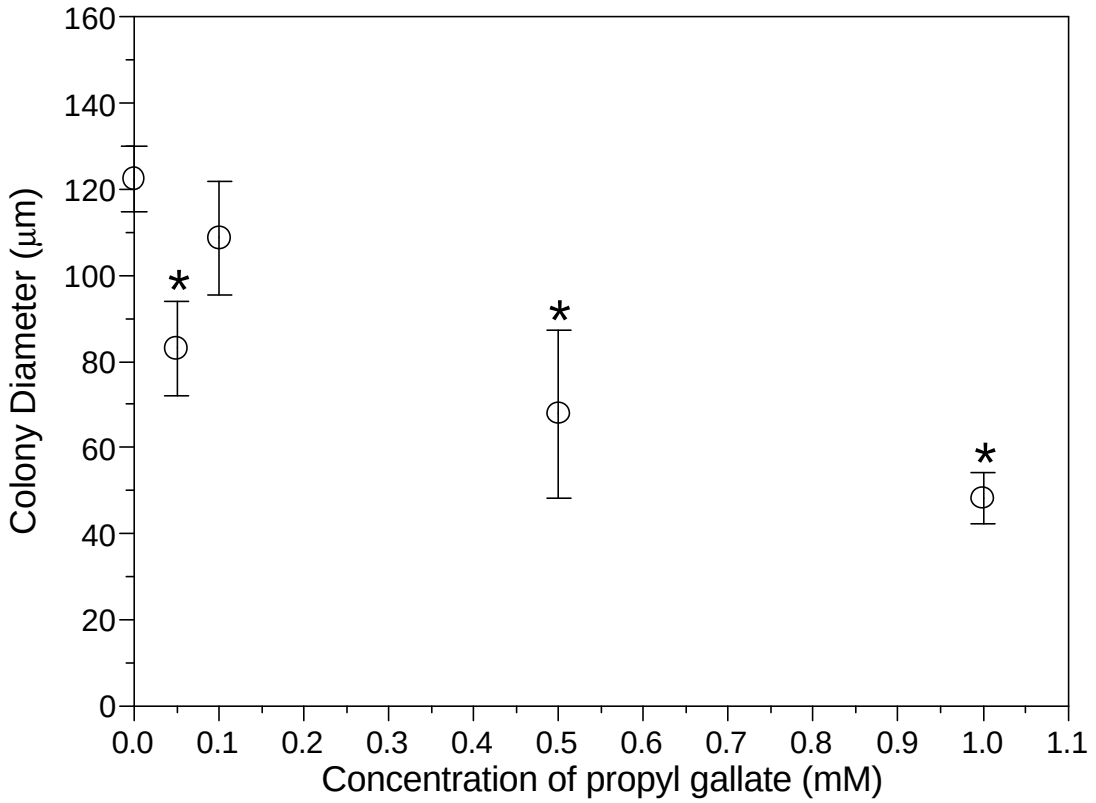


**Fig. 3.7.** Dose response of *Fusicladium effusum* to propyl gallate on solid medium. Values are means of 6 isolates, vertical bars indicate standard errors of the mean, and asterisksthe growth values that are significantly lower than the non-amended control (DMSO only).

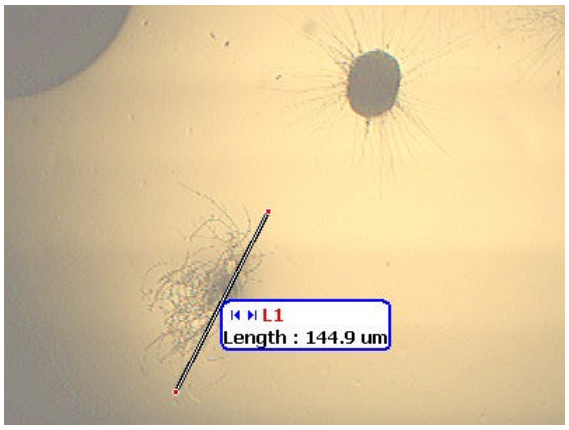


Image 1. Control

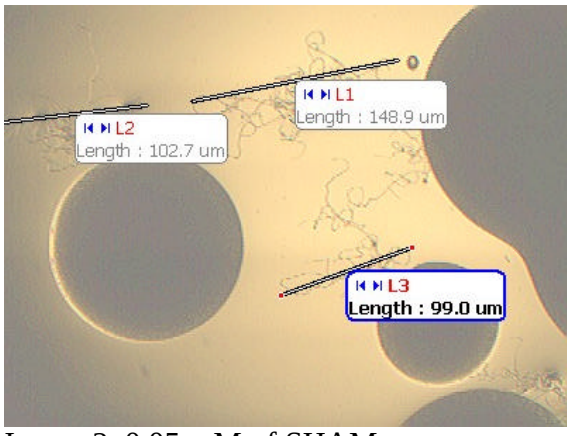


Image 2. 0.05 mM of SHAM

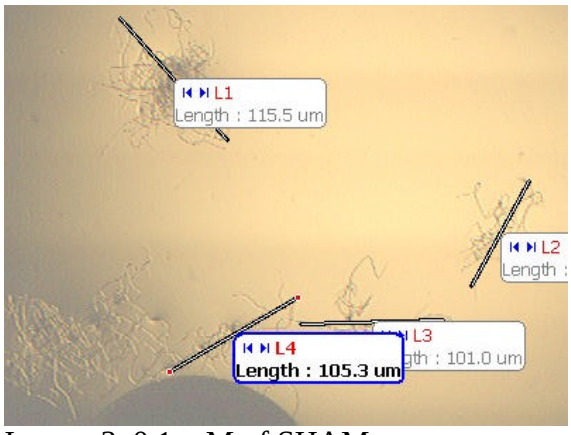


Image 3. 0.1 mM of SHAM

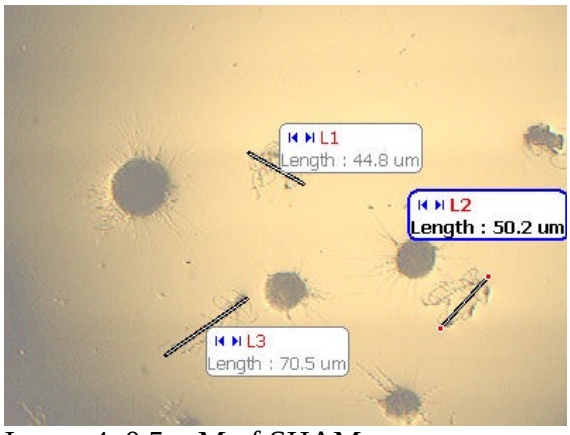


Image 4. 0.5 mM of SHAM

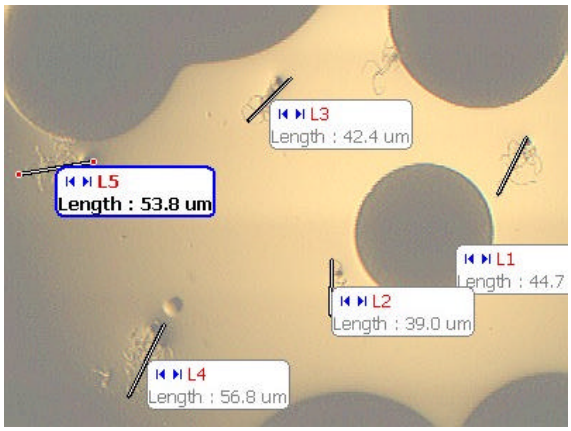


Image 5. 0.653 mM of SHAM

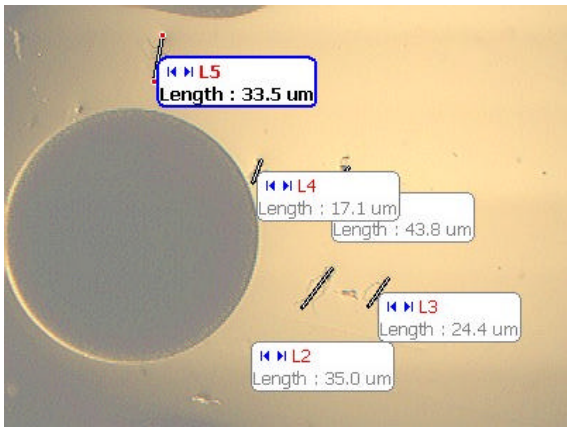


Image 6. 1 mM of SHAM

**Fig 3.8.** Three-day-old micro-colonies of *Fusicladium effusum* on SHAM-amended PDA. Measured colonies are *F. effusum* and other growing organisms are contaminants. The 5.0 mM concentration of SHAM was fatal to *F. effusum* and is not shown.

# CHAPTER 4

# A RAPID METHOD TO DETECT FUNGICIDE RESISTANCE IN *FUSICLADIUM EFFUSUM*[1]

[1]Seyran, M., Stevenson, K. L., and Brenneman, T. B. 2008. To be submitted to Crop Protection.

## Abstract

Seyran, M., Stevenson, K. L., and Brenneman, T. B. 2008. A Rapid Method to Detect Fungicide Resistance in *Fusicladium effusum*. To be submitted to Crop Protection.

Commercial production of pecans in the southeastern U.S. relies on fungicide applications to control scab, caused by *Fusicladium effusum*. These fungicides include guanidines, organotins, benzimidazoles, sterol demethylation inhibitors (DMIs), and quinone outside inhibitors (QoIs). During periods of intense disease pressure up to 10 or more times applications may be made per season and the potential development of fungicide resistance is a major concern. A rapid method was developed to determine baseline sensitivity profiles of protectant chemicals based on conidia germination, and for post-appresorial stage inhibitors based on micro-colony growth. This method uses conidia transferred directly from lesions to fungicide-amended media. Sensitivity profiles were determined for isolates from three non-sprayed baseline locations and from 35 commercial orchards with a history of fungicide use. In vitro fungicide resistance was detected in 20 orchards to fentin hydroxide, in 4 orchards to dodine, in 6 orchards to thiophanate-methyl, in 21 orchards to propiconazole, and in 19 orchards to azoxystrobin. The novel methodology enabled detection of fungicide resistance in 48 h by screening early developmental stages of the target pathogen *F. effusum*.

## Introduction

Pecan scab, caused by the fungus *Fusicladium effusum* (Winter), is the most devastating disease of the pecan plant and is the major target for disease control (5,41,53). The humid and warm climate in the southeastern United States favors disease development, and disease can be especially severe during rainy years. Without appropriate control measures, pecan scab can cause complete yield loss on susceptible cultivars (5,41,60). Integrated disease practices are utilized, but control of pecan scab relies heavily on preventive fungicide applications (3,62). Fungicides currently used for pecan scab disease control include dodine, a guanidine fungicide (FRAC code M7); fentin hydroxide, an organotin fungicide (FRAC code 30); thiophanate-methyl, a benzimidazole fungicide (FRAC code 1); fenbuconazole and propiconazole, demethylation inhibitor (DMI) fungicides (FRAC code 3); and kresoxim-methyl, azoxystrobin, pyraclostrobin, and trifloxystrobin, all quinone outside inhibitor (QoI) fungicides (FRAC code 11) (3,62). These fungicides are used either alone or as mixtures (3,39,62). Fungicide applications are scheduled with either a calendar-based program, or by the weather-driven AU-Pecan advisory program (6). All fungicide groups deployed in pecan disease management have some level of risk for development of resistance (25,62). Fungicide Resistance Action Committee (FRAC) guidelines consider dodine, fentin hydroxide, thiophanate-methyl, QoI fungicides and DMIs as moderate, medium, high, high and medium risk, respectively (25).

Resistance to fungicides has been reported previously in the pecan scab pathogen. Resistance of *F. effusum* to benomyl was noted just one year after its introduction in 1974, and reduced sensitivity to DMIs was detected in 2002 (40,41,63). Interestingly, resistance to fentin hydroxide, dodine and propiconazole was also detected in isolates of the entomopathogenic fungus *Beauveria bassiana* applied to control the pecan nut weevil (*Curculio caryae*) (58). To

detect shifts in fungicide sensitivity prior to control failures in the field, baseline sensitivity profiles were obtained for *F. effusum* to representative fungicides in each of the five groups using fungicide-amended potato dextrose agar and microtiter plate techniques (6,49,50). However, with a slow growing organism like *F. effusum*, these methods require approximately 8 weeks. Therefore, a fast and accurate method to test the sensitivity profile of a large number of *F. effusum* isolates was investigated.

The primary growth stage targeted by protectant fungicides is conidial germination, which involves a series of chemical and physiological reactions that occur between spore attachment and appressorium development (2,64). A conidia germination assay has proven effective to evaluate fungicide sensitivity of several plant pathogenic fungi to protectant fungicides (6). This is one of the methods suggested by FRAC to test the sensitivity of *Venturia inaequalis*, which is genetically related to *F. effusum* (22,53,55). However conidial germination assays may not be useful for some fungicide classes such as DMI fungicides (2,34,35,64). The DMI fungicides hinder the production of ergosterol by inhibiting the C-14 demethylation of the precursor molecules lanosterol or 24-methylenedihydrolanosterol (35). However, since fungal conidia generally contain enough ergosterol to support formation of a germ tube, the effects of inhibition of ergosterol production are usually not observed until after appressorium formation (34,35). Therefore post-appressorial stage screening is necessary to determine sensitivity to DMIs (34,35).

The objectives of this study were to (i) determine baseline sensitivity distributions of *F. effusum* to azoxystrobin, thiophanate-methyl, dodine, and fentin hydroxide based on conidia germination, and to propiconazole based on post-appressorial development, (ii) define discriminatory concentrations of these fungicides for fungicide resistance monitoring in

commercial pecan production sites, and (iii) determine the frequency of fungicide resistance in *F. effusum* populations in commercial pecan production sites where fungicides have been applied intensively via use of the rapid assay method.

## Materials and Methods

### Baseline sensitivity assays.

**Preparation of fungicide-amended medium.** Technical grade fungicides were provided by E.I. Du Pont de Nemours & Co. Inc., Wilmington DE (fentin hydroxide); Agriphar S.A., Ougrée, Belgium (dodine); Cerexagri, King of Prussia, PA (thiophanate-methyl); and Syngenta Crop Protection, Inc., Greensboro, NC (azoxystrobin and propiconazole). Fentin hydroxide was dissolved and serially diluted in acetone to obtain final concentrations of 0, 0.1, 1.0, 3.0, 10 and 30 µg/ml in water agar (WA) (40 g/L). Dodine was dissolved and serially diluted in methanol to obtain final concentrations of 0, 0.1, 0.3, 1.0, 3.0, and 10 µg/ml in WA. Thiophanate-methyl was dissolved and serially diluted in acetone to obtain final concentrations of 0, 0.1, 1.0, and 10 µg/ml in WA. Azoxystrobin was dissolved and serially diluted in acetone to obtain final concentrations of 0, 0.01, 0.03, 0.1, 0.3, and 1.0 µg/ml in WA. Due to the detected toxic effects on *F. effusum*, alternative oxidase inhibitor salicylhydroxamic acid (SHAM) was not added to the azoxystrobin-amended medium (10,51,52). Propiconazole was dissolved and serially diluted in acetone to obtain final concentrations of 0, 0.003, 0.01, 0.03, 0.1, 0.3, and 1.0 µg/ml in potato dextrose agar (PDA) (40 g/L) medium. All media were dispensed into disposable plastic petri plates (100×15 mm).

**Collection of *F. effusum* isolates and preparation of the conidial suspensions.** Pecan leaves with actively sporulating scab lesions were collected in 2008 from abandoned orchards located in Jeff Davis,Washington and Clarke counties. These locations had no history of fungicide use and were isolated from commercial orchards where fungicides have been used. Diseased leaves were refrigerated in sealed plastic bags until assayed. A stock solution of sterile distilled water with antibiotics (chloramphenicol, streptomycin sulfate, and tetracycline, each at 50 µg/ml) and Tween 20 (one drop per 100 ml) was prepared. Seven hundred and fifty microliters of the solution was transferred to 1.5-ml microcentrifuge tubes. Conidial suspensions from individual lesions were prepared by transferring 9 µl of this solution to the surface of a sporulating lesion, using a series of strokes (3-5) to dislodge conidia. Then the solution with conidia was transferred back to the tube. This step was repeated five times for each lesion. The presence of conidia in the tube was confirmed by placing 9 µl of conidial suspension on a microscope slide and examining under the microscope.

**Conidia germination and micro-colony assays.** For the baseline sensitivity assays for fentin hydroxide, dodine, thiophanate-methyl and azoxystrobin, a 9-µl drop of conidial suspension was placed on two replicate plates of WA amended with the fungicides and control plates amended only with methanol (dodine control) or acetone (fentin hydroxide, thiophanate-methyl and azoxystrobin control). Plates were incubated at room temperature (23-25 °C) for 48 h, and germination was assessed for 50 conidia per replicate. A conidium was considered germinated if the length of the germ tube was at least four times the diameter of the conidium (Fig. 4.1).

Relative germination (RG) of the isolates was calculated by the formula:

$$RG = [(F_G)/(F_G + F_{NG})] / [(C_G)/(C_G + C_{NG})],$$

where $F_G$ and $F_{NG}$ = number of germinated and non-germinated conidia on fungicide-amended medium, respectively; and $C_G$ and $C_{NG}$ = number of germinated and non-germinated conidia on acetone or methanol amended medium, respectively.

For baseline sensitivity assays for propiconazole, 9 µl of conidial suspension was dropped on propiconazole-amended medium and control plates with two replicates. The conidia were spread over the surface of the medium with a sterile glass rod, and the plates were incubated for 72 h at room temperature (23- 25 °C). Colony diameter was measured using an integrated microscope camera system (MoticCam 2300, 3.0 MPixel USB 2.0, Motic Inc. Richmond, British Columbia, Canada) (Fig. 4.2). Plates were viewed under a compound microscope at a magnification of 32X, and the images were transferred to a computer. For each concentration, 10 single, well separated colonies were arbitrarily selected and the maximum colony diameter of each was measured using the Motic Images Plus 2.0 software (Provided by the camera manufacturer). Relative fungal growth was calculated by the formula:

$$RG = (DF_{10})/ (DC_{10}),$$

where, $DF_{10}$ = mean colony diameter of ten distinct colonies on fungicide amended medium and $DC_{10}$ = mean colony diameter of ten distinct colonies on non-amended medium (control).

**Data analysis**. The 50% effective dose ($EC_{50}$) value for each isolate was calculated by regressing the probit-transformed RG against the $log_{10}$-transformed fungicide concentrations using SAS PROC REG (SAS 9.1, SAS Institute Inc., Cary, NC). Median $EC_{50}$ values were calculated for each location and for combined locations. Student's t-tests were used to compare mean $log_{10}$-transformed $EC_{50}$ values between fungicides and locations. The log–transformed

$EC_{50}$values for each location and fungicide were tested for normality using the Shapiro-Wilk test. A correlation analysis was performed on the $log_{10}$-transformed $EC_{50}$ values for azoxystrobin, dodine, fentin hydroxide, thiophanate-methyl and propiconazole using SAS PROC CORR (SAS ver. 9.1, SAS Institute, Cary NC).

**Resistance monitoring in commercial orchards.**

**Preparation of fungicide-amended medium.** To evaluate samples from commercial orchards, single discriminatory concentrations of each fungicide were selected based on the sensitivity of baseline populations. Concentrations chosen were 30 µg/ml for fentin hydroxide, 3 µg/ml for dodine, 1 µg/ml for thiophanate–methyl, 10 µg/ml for azoxystrobin, and 1 µg/ml for propiconazole. Fungicide-amended media were prepared as described above.

**Collection of *F. effusum* isolates and preparation of the conidial suspension.** To obtain isolates from commercial orchards for comparison to baselines, pecan leaves and nuts with symptoms of scab were collected in 2008 from 34 commercial orchards in 16 counties in Georgia and one orchard in Lee County, Alabama (Fig. 4.3). Diseased tissues were kept in sealed plastic bags and refrigerated until needed. Infected leaves or nuts from each location were arbitrarily divided into three groups and 15 individual lesions were selected from each group for a total of 45 lesions per orchard. The procedure described previously was used to dislodge conidia. However, to monitor variability among the population, the conidia dislodged from 15 lesions were combined in one single tube and three different tubes were prepared for each orchard.

**Conidia germination and micro-colony assays.** To detect isolates of *F. effusum* with reduced sensitivity to fentin hydroxide, dodine, thiophanate-methyl, or azoxystrobin, conidial suspension was plated on fungicide-amended WA. Due to the presence of secondary organisms

and to reduce their frequency, more suspension (19 µl) was used in testing samples from commercial orchards. Three samples per each of three orchards were evaluated per plate by placing the 19 µl of suspension in designated circles on the plate to keep them separate. Additionally, to test the quality of the fungicide-amended medium, 9 µl of conidia suspension from 15 lesions from a baseline location was also placed in a separate area on each test plate.

The relative germination (RG) of the isolates was calculated by the formula:

$$RG = (F_G)/(F_G + F_{NG}) / (C_G)/(C_G + C_{NG}),$$

where $F_G$ and $F_{NG}$ = number of germinated and non-germinated conidia on fungicide-amended medium, respectively, $C_G$ and $C_{NG}$ = number of germinated and non-germinated conidia on acetone- or methanol-amended medium, respectively, and the mean RG of three samples per orchard was calculated.

To detect propiconazole-resistant isolates of *F. effusum,* 19 µl of conidial suspension from each of the three groups per location was placed on two replicates of propiconazole-amended PDA and PDA amended only with acetone (control). The conidia were spread, incubated, and evaluated as described for the baseline assays. Relative fungal growth was calculated by the formula:

$$RG = (DF_{10}) / (DC_{10}),$$

Where $DF_{10}$ = mean colony diameter of ten distinct colonies on fungicide amended medium, $DC_{10}$ = mean colony diameter of ten distinct colonies on control plate, and the mean RG of three samples per orchard was calculated. The orchards were divided into 3 groups, based on the level of resistance. If the relative germination or relative growth on the discriminatory concentration was 0-39% the level of resistance was defined as low, 40-69% as medium and 70-100% as high.

## Results

**Baseline sensitivity distributions.** For dodine, $EC_{50}$ values of the 37 Jeff Davis County isolates ranged from 0.08 to 1.27 µg/ml with a median of 0.33 µg/ml (Table 4.1). $EC_{50}$ values of the 28 Washington County isolates ranged from 0.13 to 1.22 µg/ml with a median of 0.26 µg/ml (Table 4.1). $EC_{50}$ values of the 7 Clarke County isolates ranged from 0.59 to 1.29 µg/ml with a median of 0.68 µg/ml (Table 4.1). Estimated $EC_{50}$ values for dodine for isolates from Jeff Davis and Clarke Counties were log-normally distributed, but those from Washington Co. was not (Table 4.1).The median $EC_{50}$ value for dodine for the 72 isolates from three different locations combined was 0.32 µg/ml and the values were log-normally distributed (Prob < W = 0.1617). For all baseline isolates combined, mean relative germination on 0.1, 0.3, 1.0, 3.0 µg/ml dodine was 90%, 61%, 20% and 0%, respectively. Therefore 3.0 µg/ml was selected as an appropriate discriminatory concentration for dodine resistance monitoring (Fig. 4.4). Based on the frequency distribution of $\log_{10}$-transformed $EC_{50}$ values, the probability of an isolate from the sampled baseline population having an $EC_{50}$ value greater than 3.0 µg/ml is $1.87\times10^{-7}$.

For fentin hydroxide, $EC_{50}$ values of the 43 Jeff Davis County isolates ranged from 0.39 to 14.10 µg/ml with a median of 2.98 µg/ml (Table 4.1). $EC_{50}$ values of the 28 Washington County isolates ranged from 0.14 to 15.84 µg/ml with a median of 3.07 µg/ml (Table 4.1). $EC_{50}$ values of the 6 Clarke County isolates ranged from 5.37 to 17.32 µg/ml with a median of 11.97 µg/ml (Table 4.1). Estimated $EC_{50}$ values for fentin hydroxide for isolates from Jeff Davis and Clarke counties were log-normally distributed, but those from Washington County were not (Table 4.1). The combined $EC_{50}$ values for fentin hydroxide were not log-normally distributed (Prob < W = 0.0189). For all baseline isolates combined, mean relative germination on 0.1, 3.0, 10, and 30 µg/ml was 100%, 78%, 64%, 27% and 0%, respectively . Therefore, 30 µg/ml was

selected as an appropriate discriminatory concentration for fentin hydroxide resistance monitoring (Fig. 4.5). Based on the frequency distribution of $log_{10}$-transformed $EC_{50}$ values, the probability of an isolate from the sampled baseline population having an $EC_{50}$ value greater than 30 µg/ml is 0.00862,

For thiophanate-methyl, $EC_{50}$ values of the 36 Jeff Davis County isolates ranged from 0.05 to 0.46 µg/ml with a median of 0.35 µg/ml (Table 4.1). $EC_{50}$ values of the 24 Washington County isolates ranged from 0.14 to 0.46 µg/ml with a median of 0.35 µg/ml (Table 4.1). $EC_{50}$ values of the five Clarke County isolates ranged from 0.13 to 0.32 µg/ml with a median of 0.22 µg/ml (Table 4.1). Estimated $EC_{50}$ values for thiophanate-methyl for isolates from Clarke County were log-normally distributed, but those from Washington and Jeff Davis counties were not (Table 4.1).The median $EC_{50}$ value for thiophanate-methyl for the 65 isolates from three different locations combined was 0.32 µg/ml (Table 4.1), and the values were not log-normally distributed (Prob < W = 0.0001) (Fig. 4.6). For all baseline isolates combined, mean relative germination on 0.1, 1.0, and 10 µg/ml thiophanate-methyl was 99%, 0% and 0%, respectively. Therefore 1.0 µg/ml was selected as an appropriate discriminatory concentration for thiophanate-methyl resistance monitoring (Fig. 4.6). Based on the frequency distribution of $log_{10}$-transformed $EC_{50}$ values, the probability of an isolate from the sampled baseline population having an $EC_{50}$ value greater than 1.0 µg/ml is 0.00542.

For propiconazole, $EC_{50}$ values of the 47 Jeff Davis County isolates ranged from 0.002 to 0.07 µg/ml with a median of 0.02 µg/ml (Table 4.1). The $EC_{50}$ values of the 39 Washington County isolates ranged from 0.006 to 0.19 µg/ml with a median of 0.03 µg/ml (Table 4.1). $EC_{50}$ values of the 7 Clarke County isolates ranged from 0.006 to 0.04 µg/ml with a median of 0.01 µg/ml (Table 4.1). Estimated $EC_{50}$ values for propiconazole for isolates from the three locations

were log-normally distributed (Table 4.1).The median $EC_{50}$ value for dodine for the 93 isolates from three different locations combined was 0.02 µg/ml (Table 4.1), and was log-normally distributed (Prob < W = 0.4364) (Fig. 4.7). For all baseline isolates combined, mean relative growth on 0.003, 0.01, 0.03, 0.1, 0.3 and 1.0 µg/ml was 81%, 68%, 42%, 28%, 6% and 0%, respectively. Therefore, 1.0 µg/ml was selected as an appropriate discriminatory concentration for propiconazole resistance monitoring (Fig. 4.7). Based on the frequency distribution of $log_{10}$-transformed $EC_{50}$ values, the probability of an isolate from the sampled baseline population having an $EC_{50}$ value greater than 1.0 µg/ml is $1.49\times10^{-7}$.

For azoxystrobin, $EC_{50}$ values of the 42 Jeff Davis County isolates ranged from 0.01 to 1.67 µg/ml with a median of 0.34 µg/ml (Table 4.1). $EC_{50}$ values of the 23 Washington County isolates ranged from 0.03 to 1.96 µg/ml with a median of 0.20 µg/ml (Table 4.1). Median $EC_{50}$ values for azoxystrobin estimated from the three locations were log-normally distributed (Table 4.1).The median $EC_{50}$ value for dodine for the 65 isolates from two different locations combined was 0.25 µg/ml (Table 4.1), and was log-normally distributed (Prob < W = 0.2800) (Fig. 4.8). For all baseline isolates combined, mean relative germination on 0.01, 0.03, 0.1, 0.3, and 1.0 µg/ml was 100 %, 85 %, 75 %, 42 %, and 22 %. Therefore, 10 µg/ml, which is close to the solubility limit of the compound in the medium was selected as an appropriate discriminatory concentration for azoxystrobin resistance monitoring (Fig. 4.8). Based on the frequency distribution of $log_{10}$-transformed $EC_{50}$ values, the probability of an isolate from the sampled baseline population having an $EC_{50}$ value greater than 10 µg/ml is 0.000843.

Significant positive correlations were observed between $log_{10}$-tranformed $EC_{50}$ values for fentin hydroxide and azoxystrobin ($r = 0.444$, $p = 0.0004$) (Fig. 4.9), and between fentin hydroxide and dodine ( $r = 0.499$, $p < 0.0001$ ) (Fig. 4.10).

**Resistance monitoring in commercial orchards.** Samples from 35 commercial orchards were profiled and in vitro fungicide resistance was detected in 4 orchards to dodine, 20 orchards to fentin hydroxide, 6 orchards to thiophanate-methyl, 21 orchards to propiconazole, and 33 orchards to azoxystrobin.The level of dodine resistance was low in all four orchards where resistance was detected (Table 4.2). The level of fentin hydroxide resistance was low in 15 orchards, medium in 4 orchards, and high in 1 orchard, with 15 orchards having populations with sensitivity similar to baseline locations (Table 4.3). The level of thiophanate-methyl resistance was low in 5 orchards and medium in 1 orchard, and in 29 orchards the populations were sensitive to the fungicide (Table 4.4). The level of propiconazole resistance was low in 4 orchards, medium in 14 orchards and high in 2 orchards, with 15 orchards having populations that were sensitive to the fungicide (Table 4.5). The level of azoxystrobin resistance was low in 12 orchards, medium in 13 orchards and high in 6 orchards, with only four orchards having populations that were sensitive to the fungicide (Table 4.6). A significant positive correlation was determined between the relative germination values of dodine and thiophanate-methyl ($n=33$, $r=0.51$, $p=0.0023$) when evaluated across all locations.

## Discussion

Pecan growers are encouraged to use all available cultural practices to reduce overall disease pressure (5). However, most orchards still require 7-10 fungicide applications per year (3,41). Considering the high reproductive capacity of the pathogen and the perennial nature of the crop, the potential for development of fungicide resistance is significant (40,63).

Fungicide resistance can be monitored by testing the sensitivity of suspected isolates and comparing results to previously determined baseline data (6,49,50). Such baseline data is

available for *F. effusum* with other methods, such as microtiter plate assay (11,47,55,56,57). In this study, isolates from three different baseline locations in Georgia were used to profile fungicide sensitivity using conidia germination assays. In our assay, the median $EC_{50}$ value for dodine was estimated as 0.32 µg/ml with a range of 0.08-1.29 µg/ml. In 2007, with a microtiter plate assay the median $EC_{50}$ value for dodine was estimated as 0.051 µg/ml, with a range of 0.007-0.59 (55,57). In our assay, the median $EC_{50}$ value for fentin hydroxide was estimated as 3.14 µg/ml with the range of 0.14-17.32 µg/ml. In 2007, with a microtiter plate assay the median $EC_{50}$ value for fentin hydroxide was estimated as 0.76 µg/ml, with a range of 0.11-4.54 µg/ml, and in a previous study, the mean $EC_{50}$ value estimated as 0.94 µg/ml with a range of 0.008-26.065 (unpublished data). In our assay, the median $EC_{50}$ value for propiconazole was estimated as 0.02 µg/ml with a range of 0.006-0.19 µg/ml. In 2007, with a microtiter plate assay the median $EC_{50}$ value for propiconazole was estimated as 0.06 µg/ml, with a range of 0.011-0.79, and in 1994 using a conventional mycelial growth assay on fungicide-amended PDA the mean $EC_{50}$ was estimated to be 0.143 µg/ml with a range of 0.19 to 0.30 µg/ml (47,55,57). In our assay, the median $EC_{50}$ value for azoxystrobin was estimated as 0.25 µg/ml with a range of 0.01-1.96 µg/ml. In 2007, with a microtiter plate assay the median $EC_{50}$ value for azoxystrobin was estimated as 0.02 µg/ml, with a range of 0.002 to 0.182 µg/ml and in 2000 using a microtiter plate assay the mean $EC_{50}$ was estimated as 0.02 µg/ml with another QoI fungicide, kresoxim-methyl (11,55,57). The baseline isolates of *F. effusum* were strongly inhibited by 1.0 µg/ml of thiophanate-methyl both in our assays with conidia germination as well as in mycelial growth assays with microtiter plates in 2007 (55,57). Overall, in conidia germination assays the estimated $EC_{50}$ values for dodine, fentin hydroxide and azoxystrobin was significantly higher than in the microtiter plate assays, but were similar for thiophanate-methyl (55,57). The $EC_{50}$

values obtained using the micro-colony method for propiconazole were lower than the estimated $EC_{50}$ values using microtiter and fungicide-amended PDA methods (47,55,57). In our microtiter assays, there were significant positive correlations between baseline $EC_{50}$ values for propiconzole and azoxystrobin, and between $EC_{50}$ values for fentin hydroxide and azoxystrobin (55). In conidia germination assays, there were significant positive correlations between baseline $EC_{50}$ values for fentin hydroxide and azoxystrobin, and between $EC_{50}$ values for dodine and fentin hydroxide. Correlations of sensitivity between these groups of fungicides have not been reported previously in isolates from baseline populations.These positive correlations of sensitivity were not observed in isolates from commercial orchards. Among isolates from commercial orchards, only sensitivities to thiophanate-methyl and dodine were significantly and positively correlated. Similarly, in *V. inaequalis*,dodine-resistant isolates were found to be simultaneously resistant to benomyl (59).

Although not completely analogous to the previously determined sensitivity profiles, the rapid assay results should serve as a valid baseline for comparison of populations using the same method. Condial germination assays have several advantages compared either to microtiter plates or fungicide-amended PDA methods (Table 4.7). Conidial germination assays do not require the 6-8 weeks to grow pure cultures of monoconidial isolates of *F. effusum* (Table 4.7). The main difference is that the scale of observation is at the micrometer level which enables much earlier observations. Additionally, quality of the fungicide-amended medium can be easily checked by including a baseline isolate on each plate of medium.

Based on the rapid conidia germination and micro-colony growth methods, sensitivity profiles were determined for isolates from 35 commercial orchards in Georgia or Alabama (Fig. 4.2). In vitro fungicide resistance was detected to fungicides in all 5 chemical groups evaluated,

and in some cases, multiple resistances were detected (Table 4.8). Resistance to dodine was detected in populations of *F. effusum* from 4 of the 35 sampled locations (Table 4.2). Dodine was labeled in 1963 and is a member of the guanidines chemical group which are contact (protectant) fungicides with a multi-site mode of action (25,41). Based on several studies, the antifungal mechanism of dodine may be related to interaction with anionic sites of the fungal membrane, disruption of membrane permeability, and competition with cationic enzymes for the anionic substrate sites in the fungal cell (29). Additionally, dodine may target anionic sites and enzymes on the mitochondrial membrane (29). According to FRAC, dodine is considered an at-risk chemistry due to the cases of resistance that have been detected in the field and laboratory (25). In 1969, five years after its first introduction to the market, dodine resistance was first detected in *V. inaequalis* following intensive use in apple orchards of New York (27). Dodine resistance in *V. inaequalis* was found to be a non-durable trait with different tolerance levels, but populations did not return completely to baseline levels (68). Dodine resistance in *V. inaequalis* may have gone undetected because of the gradual decline in sensitivity (59). This resistance was found to be dependent on polygenic mechanisms and fungal crossing studies revealed that dodine resistance was based on at least two independent genes (32,68).

Resistance to fentin hydroxide was detected in our commercial orchard populations of *F. effusum* from 20 of the 35 sampled locations (Table 4.3). The organotin fungicide fentin hydroxide was registered in 1967 for pecan scab control (41). Organotins are applied as protectant fungicides, and the known mode of action is inhibition of oxidative phosphorylation (25,29). However, they may have other target sites in fungal cells due to potential reaction with thiol groups of amino acids and peptides (18,29). In fungi, organotin compounds inhibit oxidative phosphorylation by inhibiting ATP synthesis and inducing an anion-hydroxide

exchange in the bilayer membrane of mitochondria, presumably by blocking anion channels (12,29,65). The organotins are considered to have medium to low resistance risk; however, field resistance has been reported in *Cercospora beticola*, which causes leaf spot of sugar beet (25,26,54). In Greece, isolates resistant to fungicides fentin acetate and fentin hydroxide were found to be 32 times more tolerant to these fungicides in vitro (27). However, resistance of *C. beticola* to fentin hydroxide in Greece was found to be non-persistent, which suggests a fitness cost. Fentin hydroxide resistance also was found to be non-persistent in *C. beticola* isolates in Minnesota and North Dakota (54). Isolates resistant to 1.0- 2.0 ppm fentin hydroxide were detected in 1994, but the frequency of resistant isolates declined over time as the number of applications of fentin hydroxide decreased (54). In 2006, in vitro resistance could not be detected in isolates cultured from the location where resistance originally occurred (54). The mechanism of resistance to organotin fungicides remains unknown at this time.

Resistance to thiophanate-methyl was detected in our commercial orchard populations of *F. effusum* from 6 of the 29 sampled locations (Table 4.4). Thiophanate-methyl, a member of the methyl benzimidazole carbamate (MBC) group, was labeled for pecan scab control in 1968 (41). Resistance of *F. effusum* to another benzimidazole, benomyl, was reported in 60 locations in Georgia and one location in south Alabama in 1975, just one year after its introduction and widespread use (21,23,40,41). These fungicides are site-specific antimitotic agents that inhibit cell division and other tubulin-derived cellular processes (21,29,30,33). MBC fungicides (FRAC code 1) are thus considered as high risk for resistance (25). Based on the most recent list of plant pathogenic organisms resistant to disease control agents published by FRAC, resistance to MBCs has been detected in 114 plant pathogens in laboratory and field studies since their first introduction in the 1960s (21,23). Resistance to MBCs is conferred by several different single-

site point mutations in the gene locus known as the benzimidazole box resulting in amino acid substitutions E198A, E198G, E198K, F167, Q134, Glu198Gle and F200Y (13,69,71). In our *F. effusum,* populations, only low or moderate levels of thiophanate-methyl resistance were detected. This could be related to the limited use of thiophanate-methyl, or migration of resistant population from another location. However, in MBC resistance, the different point mutations may be associated with different levels of resistance. For example, in *Monilinia fructicola,* the mutation at codon 6 that results in a conversion of histidine to tyrosine confers a low level of resistance, but the mutation at codon 198 that results in a conversion of glutamic acid to alanine is associated with a high level of field resistance (42).

Resistance to the DMI fungicide propiconazole was detected in our commercial orchard populations of *F. effusum* from 21 of the 35 sampled locations (Table 4.5). DMI fungicides have been used for fungal disease control in pecans since 1988, and reduced sensitivity in the populations of *F. effusum* to DMIs was detected in 2004 (63). The DMI fungicides inhibit the production of ergosterol by interfering with C-14 demethylation of the lanosterol or 24-methylenedihydrolanosterol precursors (34,35). Resistance to DMI fungicides is a quantitative type of resistance that develops slowly under field conditions (6). The resistance is controlled by several genes, and is partially reversible in the absence of DMI exposure (38,49). Several different mechanisms of resistance to DMIs have been identified, including target site mutations ( e.g. V136A, Y137F, I381V) in the 14a-demethylase gene (cyp51), target site over-production associated with tandem repeats of the upstream promoter of cyp51, ATP-binding cassette (ABC) transporter systems, and destabilization of toxic sterols (6,15,17,20,31,43,46,52).

Resistance to the QoI fungicide azoxystrobin was detected in our commercial orchard populations of *F. effusum* from 19 of the 35 sampled locations (Table 4.6). QoI fungicide binds

to the ubiquinone oxidizing pocket (Qo site) of the cytochrome bc1 (complex III) of the respiration chain, which is located at the inner mitochondrial membrane of the target fungal cell (1,28,67). The presence of the strobilurin molecule inhibits the electron transfer between cytochrome b and cytochrome bc1 complex, which interrupts production of ATP (1,28,67). Three different types of amino acid substitutions that confer resistance to QoIs have been detected in 25 plant pathogenic fungi (20). These mutations are; 1) G143A, conversion of the amino acid glycine to alanine at codon 143, on the cytochrome b gene; 2) F129L, conversion of the amino acid phenylalanine to leucine at codon 129 on the cytochrome b gene; and 3) G137R, conversion of the amino acid glycine to arginine at codon 137 on the cytochrome b gene (24). Characteristics of the resistance vary, depending on specific mutations. The most common mutation associated with QoI resistance is G143A, which confers complete resistance with a resistance factor higher than 100. QoI fungicides cannot control G143A mutant isolates in the field (24). By contrast, F129L and G137R confer partial resistance with a relatively low resistance factor of 5 to 15, and are only occasionally found in QoI-resistant pathogens (20). QoIs can provide sufficient control of these isolates (24).

Another mechanism of resistance to QoI fungicides in some fungi is induction of alternative oxidase (AOX), an alternative respiration system (66,70). When conducting in vitro assays with QoI fungicides, AOX inhibitors such as salicylhydroxamic acid (SHAM) are often added to the medium to suppress AOX in plant pathogenic fungi (66,70). However, toxic effects of SHAM on *F. effusum* were detected in preliminary studies and the toxicity of alternative oxidase inhibitors SHAM and propyl gallate to *F. effusum* was confirmed in subsequent studies (11,55,56). Even at low concentrations, AOX inhibitors reduced growth of *F. effusum* to some degree (55,56). Additionally, FRAC does not recommend the use of SHAM for in vitro assays of

*V. inaequalis*, organism that is closely related to *F. effusum* (22,55). Therefore, the alternative oxidase inhibitors were not added to medium amended with azoxystrobin.

Not all QoI resistance has been associated with cyt bc1 mutations or AOX systems. For example, a highly trifloxystrobin-resistant field isolate of *V. inaequalis* in Germany did not show any mutation at the cyt b gene or AOX-related resistance (37). Field studies also indicate occurrence of both quantitative and qualitative QoI resistance in *V. inaequalis* without any cyt b mutations (37,44). Similarly, in QoI-resistant isolates of *Podosphaera fusca*, neither point mutations nor AOX-related resistance was detected, but the resistant biotypes were also resistant to other QoIs (19). Recently, the drug transporter system MgMfs1 was found to be the mechanism of QoI resistance in some isolates of *Mycosphaerella graminicola* (48).

In our study, fungicide resistance in *F. effusum* isolates was detected to the 5 fungicides evaluated, and multiple-resistance was detected in 29 of 33 resistant populations (Table 4.8). There was also a correlation between dodine and thiophanate-methyl resistant population. Similarly, in the genetically related organism *V. inaequalis*, the mechanism of dodine resistance was non-specific, thus 20% of isolates were resistant to both benomyl and dodine (59). Additionally, resistance to the DMI fenarimol developed to a higher frequency in an orchard where dodine resistant isolates of *V. inaequalis* were detected previously (36). A multi-drug resistance mechanism may also be associated with dodine resistance, whereby some fungi exhibit resistance to fungicides and natural toxins (16,17,51,52,61). All organisms have toxin transport mechanisms (14). The two major types are the ATP-binding cassette and major facilitator super-family (14). Fungi have an ATP-binding cassette (ABC) drug transporter system that is an energy dependent efflux activity in order to exclude toxic substances such as fungicides, plant phytoalexins and antibiotic substances of antagonistic organisms outside the

cell (17). This task is performed by plasma membrane drug transporter systems, like ABC transporters (17). The overproduction of ABC transporters is associated with multi-drug resistance (MDR) (16,61). Increased tolerance to azole fungicides in laboratory mutants has been demonstrated to be under the control of ABC transporters (16). For example, ABC transporter BcatrB deficient mutants of *B. cinerea* was suppressed more by antibiotics and fenpiclonil fungicide, compared to the wild type strain of *B. cinerea* (51,52). Interestingly, the BcatrB ABC transporter system could be responsible for multiple resistance of *B. cinerea* to benzimidazole and dicarboximide fungicides (6). And as mentioned above, the drug transporter system MgMfs1 was found to be the mechanism of QoI resistance in some isolates of *M. graminicola* (48). However, most of the field resistance to the fungicides was associated with point mutations and specific mechanisms mentioned above. In *F. effusum,* the actual mechanism of resistance in the field could be related to generic multidrug systems or selection of mutants with specific point mutations generating resistance to each group fungicide individually.

According to Palumbi (45), the annual cost of mutations to the United States economy is approximately 50 billion dollars. Mutations resulting in pesticide resistance cost approximately 10% of control cost, or an estimated 1.2 billion dollars per year, of which fungicide resistance is a subgroup (45). In the state of Georgia between the years of 2004 and 2008, the average cost of pecan scab control was estimated as 12 million dollars with the average total loss of 16.32 million dollars,and an average 1.8 % reduction in crop value (7,8,9,10). An additional 13.8 million dollars were spent for control, resulting in a total 16.7 million dollars loss to Georgia pecan industry. Based on the evalution ratio of Palumbi, the estimated cost of fungicide resistance in pecan scab management could be as much as 1.5-2.0 million dollars per year (7,8,9,10,45). Pecan growers rely on effective fungicides to manage diseases. These results

indicate that *F. effusum* is developing in vitro resistance to all five of our major classes of fungicides labeled on pecans. The rapid screening method described should enable further sampling and characterization of this, but much additional work is needed to determine the significance and relationship to disease control in the field.

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**Table 4.1.** Sensitivity of *Fusicladium effusum* isolates cultured from three different non-sprayed pecan orchards in Georgia to dodine, fentin hydroxide, thiophanate-methyl, propiconazole and azoxystrobin based on a rapid assessment of conidial germination and micro-colony growth

| Fungicide | Location | $EC_{50}$ value (µg/ml) | | Number of isolates | Shapiro-Wilk test (Prob<W) |
|---|---|---|---|---|---|
| | | Range | Median | | |
| Dodine | Clarke Co. | 0.08-1.27 | 0.33 | 37 | 0.9927 |
| | Jeff Davis Co. | 0.13-1.22 | 0.26 | 28 | 0.01 |
| | Washington Co. | 0.59-1.29 | 0.02 | 7 | 0.06 |
| | Combined results | 0.08-1.29 | 0.02 | 72 | 0.1617 |
| Fentin hydroxide | Clarke Co. | 0.39-14.10 | 2.98 | 43 | 0.1904 |
| | Jeff Davis Co. | 0.14-15.84 | 3.07 | 28 | 0.0548 |
| | Washington Co. | 5.37-17.32 | 11.97 | 6 | 0.4655 |
| | Combined results | 0.14-17.32 | 3.14 | 77 | 0.0189 |
| Thiophanate-methyl | Clarke Co. | 0.05-0.46 | 0.32 | 36 | 0.0003 |
| | Jeff Davis Co. | 0.14-0.46 | 0.35 | 24 | 0.0014 |
| | Washington Co. | 0.13-0.32 | 0.22 | 5 | 0.5754 |
| | Combined results | 0.05-0.46 | 0.32 | 65 | 0.0001 |
| Propiconazole | Clarke Co. | 0.002-0.07 | 0.02 | 47 | 0.3837 |
| | Jeff Davis Co. | 0.006-0.19 | 0.03 | 39 | 0.257 |
| | Washington Co. | 0.006-0.04 | 0.01 | 7 | 0.7182 |
| | Combined results | 0.006-0.19 | 0.02 | 93 | 0.4364 |
| Azoxystrobin | Clarke Co. | 0.01-1.63 | 0.34 | 42 | 0.0586 |
| | Jeff Davis Co. | 0.03-1.96 | 0.2 | 23 | 0.7699 |
| | Washington Co. | - | - | - | - |
| | Combined results | 0.01-1.96 | 0.25 | 65 | 0.28 |

**Table 4.2.** Sensitivity profiles of *Fusicladium effusum* to dodine in non-sprayed baseline locations (Jeff Davis, Washington, and Clarke Counties) and commercial orchards with reduced sensitivity in Georgia

| Location | Mean relative germination [z] (%) | Fungicide exposure history |
|---|---|---|
| Baseline Locations | | |
| Jeff Davis Co. | 0 | no |
| Washington Co. | 0 | no |
| Clarke Co. | 0 | no |
| | | |
| Commercial Orchards | | |
| Berrien Co. | 10 | yes |
| Lanier Co | 14 | yes |
| Pierce Co. Location 1 | 23 | yes |
| Pierce Co. Location 2 | 27 | yes |

[z] Determined as relative germination on 3 µg/ml

**Table 4.3.** Sensitivity profiles of *Fusicladium effusum* to fentin hydroxide in non-sprayed baseline locations ( Jeff Davis, Washington, and Clarke Counties) and commercial orchard samples (listed beneath the Washington Co. results) with reduced sensitivity in Georgia

| Location | Mean relative germination [z] (%) | Fungicide exposure history |
|---|---|---|
| Baseline Locations | | |
| Jeff Davis Co. | 0 | no |
| Washington Co. | 0 | no |
| Clarke Co. | 0 | no |
| | | |
| Commercial Orchards | | |
| Appling Co | 51 | yes |
| Calhoun Co. | 16 | yes |
| Colquitt Co. | 44 | yes |
| Dougherty Co. Sample 1 | 25 | yes |
| Dougherty Co. Sample 2 | 13 | yes |
| Dougherty Co. Sample 3 | 25 | yes |
| Dougherty Co. Sample 4 | 13 | yes |
| Dougherty Co. Sample 6 | 13 | yes |
| Dougherty Co. Sample 7 | 5 | yes |
| Dougherty Co. Sample 8 | 23 | yes |
| Lee Co. Alabama | 12 | yes |
| Lee Co. Georgia | 12 | yes |
| Peach Co Sample 1 | 77 | yes |
| Peach Co Sample 2 | 4 | yes |
| Schley Co. | 55 | yes |
| Screven Co. | 18 | yes |
| Sumter Co. | 64 | yes |
| Terrell Co. Sample 1 | 22 | yes |
| Terrell Co. Sample 3 | 23 | yes |
| Tift Co. | 3 | yes |

[z] Determined as relative germination on discriminatory concentration 30 µg/ml

**Table 4.4.** Sensitivity profiles of *Fusicladium effusum* to thiophanate-methyl in non-sprayed baseline locations ( Jeff Davis, Washington, and Clarke Counties) and commercial orchard samples (listed beneath the Washington Co. results) with reduced sensitivity in Georgia

| Location | Mean relative germination [z] (%) | Fungicide exposure history |
|---|---|---|
| Baseline Locations | | |
| Jeff Davis Co. | 0 | no |
| Washington Co. | 0 | no |
| Clarke Co. | 0 | no |
| | | |
| Commercial Orchards | | |
| Colquitt Co. | 23 | yes |
| Lanier Co. | 25 | yes |
| Pierce Co. Sample 2 | 45 | yes |
| Pierce Co. Sample 3 | 19 | yes |
| Schley Co. | 10 | yes |
| Tift Co. | 26 | yes |

[z] Determined as relative germination on discriminatory concentration 1 µg/ml

**Table 4.5.** Sensitivity profiles of *Fusicladium effusum* to propiconazole in non-sprayed baseline locations ( Jeff Davis, Washington, and Clarke Counties) and commercial orchard samples (listed beneath the Washington Co. results) with reduced sensitivity in Georgia

| Location | Mean relative germination [z] (%) | Fungicide exposure history |
|---|---|---|
| Baseline Locations | | |
| Jeff Davis Co. | 0 | no |
| Washington Co. | 0 | no |
| Clarke Co. | 0 | no |
| | | |
| Commercial Orchards | | |
| Appling Co | 54 | yes |
| Berrien Co. | 56 | yes |
| Calhoun Co. | 36 | yes |
| Dougherty Co. Sample 1 | 72 | yes |
| Dougherty Co. Sample 2 | 40 | yes |
| Dougherty Co. Sample 3 | 53 | yes |
| Dougherty Co. Sample 4 | 65 | yes |
| Dougherty Co. Sample 5 | 54 | yes |
| Dougherty Co. Sample 6 | 28 | yes |
| Dougherty Co. Sample 8 | 50 | yes |
| Lee Co. Alabama | 22 | yes |
| Lee Co. Georgia | 46 | yes |
| Lowdnes Co. Sample 1 | 65 | yes |
| Lowdnes Co. Sample 3 | 82 | yes |
| Lowdnes Co. Sample 5 | 79 | yes |
| Peach Co Sample 2 | 58 | yes |
| Peach Co Sample 3 | 42 | yes |
| Sumter Co. | 53 | yes |
| Terrell Co. Sample 1 | 45 | yes |
| Terrell Co. Sample 3 | 53 | yes |
| Tift Co. | 45 | yes |

[z] Determined as relative germination on discriminatory concentration 1 µg/ml

**Table 4.6.** Sensitivity profiles of *Fusicladium effusum* to azoxystrobin in non-sprayed baseline locations ( Jeff Davis, Washington, and Clarke Counties) and commercial orchard samples (listed beneath the Washington Co. results) with reduced sensitivity in Georgia only isolates with a medium or high level of resistance are shown

| Location | Mean relative germination [z] (%) | Fungicide exposure history |
|---|---|---|
| Baseline Locations | | |
| Jeff Davis Co. | 0 | no |
| Washington Co. | 0 | no |
| Clarke Co. | 0 | no |
| | | |
| Commercial Orchards | | |
| Appling Co | 42 | yes |
| Colquitt Co. | 71 | yes |
| Dougherty Co. Sample 1 | 55 | yes |
| Dougherty Co. Sample 3 | 84 | yes |
| Dougherty Co. Sample 4 | 44 | yes |
| Jefferson Co. | 55 | yes |
| Lee Co. Alabama | 82 | yes |
| Lee Co. Georgia | 65 | yes |
| Lowdnes Co. Sample 1 | 60 | yes |
| Lowdnes Co. Sample 2 | 41 | yes |
| Lowdnes Co. Sample 5 | 100 | yes |
| Peach Co Sample 1 | 52 | yes |
| Pierce Co. Sample 1 | 47 | yes |
| Pierce Co. Sample 2 | 42 | yes |
| Pierce Co. Sample 3 | 50 | yes |
| Schley Co. | 73 | yes |
| Sumter Co. | 63 | yes |
| Sumter Co. | 55 | yes |
| Terrell Co. Sample 1 | 74 | yes |

[z] Determined as relative germination on discriminatory concentration 10 µg/ml. Baseline isolates were tested on 1 µg/ml

**Table 4.7.** Comparison of different in vitro fungicide resistance screening methods for *Fusicladium effusum*

| | Fungicide Screening methods | | |
|---|---|---|---|
| | PDA Amended | Microtiter Plate | Conidial Assays |
| Duration | 8 weeks | 6 weeks | 2 days |
| Pure culture | Required | Required | Not required |
| Screening scale | Individual | Individual | Population |
| Observation method (unit) | Macroscopic (mm) | Light reflection (absorbance) | Microscopic (µm) |
| Quality control (medium) | Impractical | Impractical | Practical |
| Testing propagule | Mycelium | Mycelium | Conidia/Mycelium |

**Table 4.8.** List of the commercial pecan production sites with fungicide resistance, and in some locations multiple resistance were detected

| Sampling site | Fentin hydroxide | Dodine | Thiophanate-methyl | Propiconazole | Azoxystrobin |
|---|---|---|---|---|---|
| Appling Co. | M | | | M | M |
| Berrien Co. | | L | | M | L |
| Calhoun Co. | L | | | L | L |
| Colquitt Co. | M | | L | | H |
| Dougherty Co. 1 | L | | | H | M |
| Dougherty Co. 2 | L | | | L | L |
| Dougherty Co. 3 | L | | | M | H |
| Dougherty Co. 4 | L | | | M | M |
| Dougherty Co. 5 | | | | M | L |
| Dougherty Co. 6 | L | | | L | |
| Dougherty Co. 7 | L | | | | |
| Dougherty Co. 8 | L | | | M | L |
| Jefferson Co. | | | | | M |
| Lanier Co. | | L | L | | L |
| Lee Co. Alabama | L | | | L | H |
| Lee Co. Georgia | L | | | M | M |
| Lowndes Co. 1 | | | | M | M |
| Lowndes Co. 2 | | | | | M |
| Lowndes Co. 3 | | | | H | L |
| Lowndes Co. 4 | | | | | L |
| Lowndes Co. 5 | | | | H | H |
| Peach Co. 1 | H | | | | M |
| Peach Co. 2 | L | | | M | L |
| Pierce Co. 1 | | | | | M |
| Pierce Co. 2 | | L | L | | M |
| Pierce Co. 3 | | L | M | M | M |
| Schley Co. | M | | L | | H |
| Screven Co. | L | | | | M |
| Sumter Co. | M | | | M | M |
| Terrell Co. 1 | L | | | M | H |
| Terrell Co. 2 | L | | | M | L |
| Terrell Co. 3 | | | | | L |
| Tift Co. | L | | L | M | L |

Blank = sensitive; Relative growth or germination (RG)=0% on discriminatory concentration
L = low level of resistance; 1%=RG=39% on discriminatory concentration
M = medium level of resistance; 40%=RG=69% on discriminatory concentration
H = high level of resistance; 70%=RG on discriminatory concentration

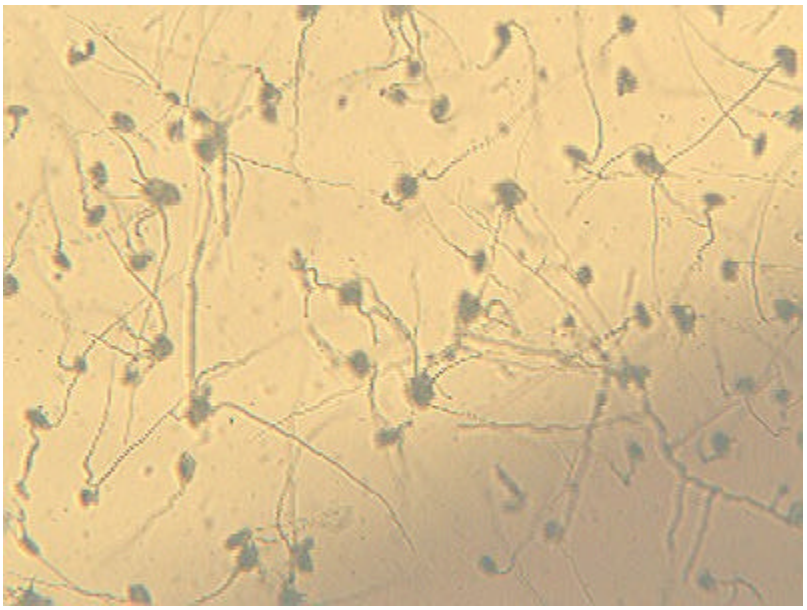

**Fig. 4.1.** Conidia of *Fusicladium effusum* on 30 µg/ml of fentin hydroxide in water agar. Abundant germ tubes are an indication of resistance to this fungicide.

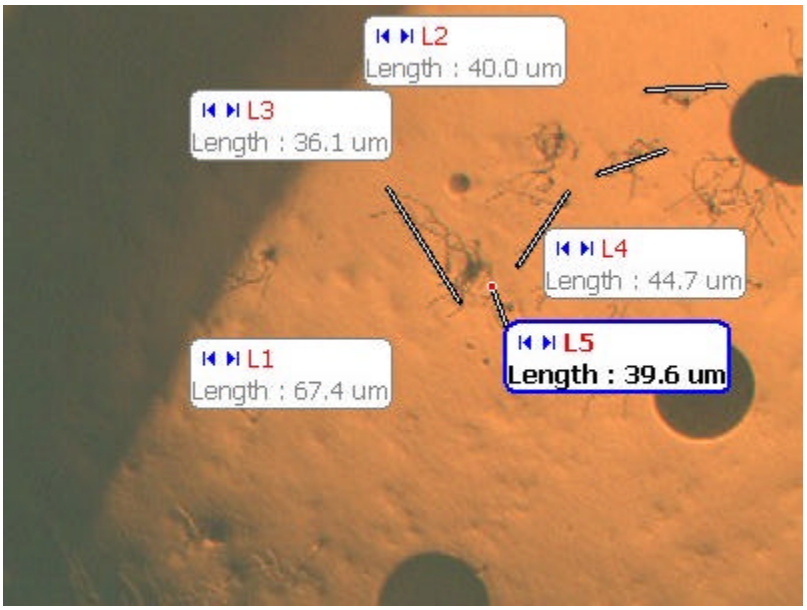


**Fig. 4.2.** Micro-colonies of *Fusicladium effusum* after 48 hours incubation on 1 µg/ml of propiconazole in PDA. Sensitivity was quantified by measuring micro-colony diameter.

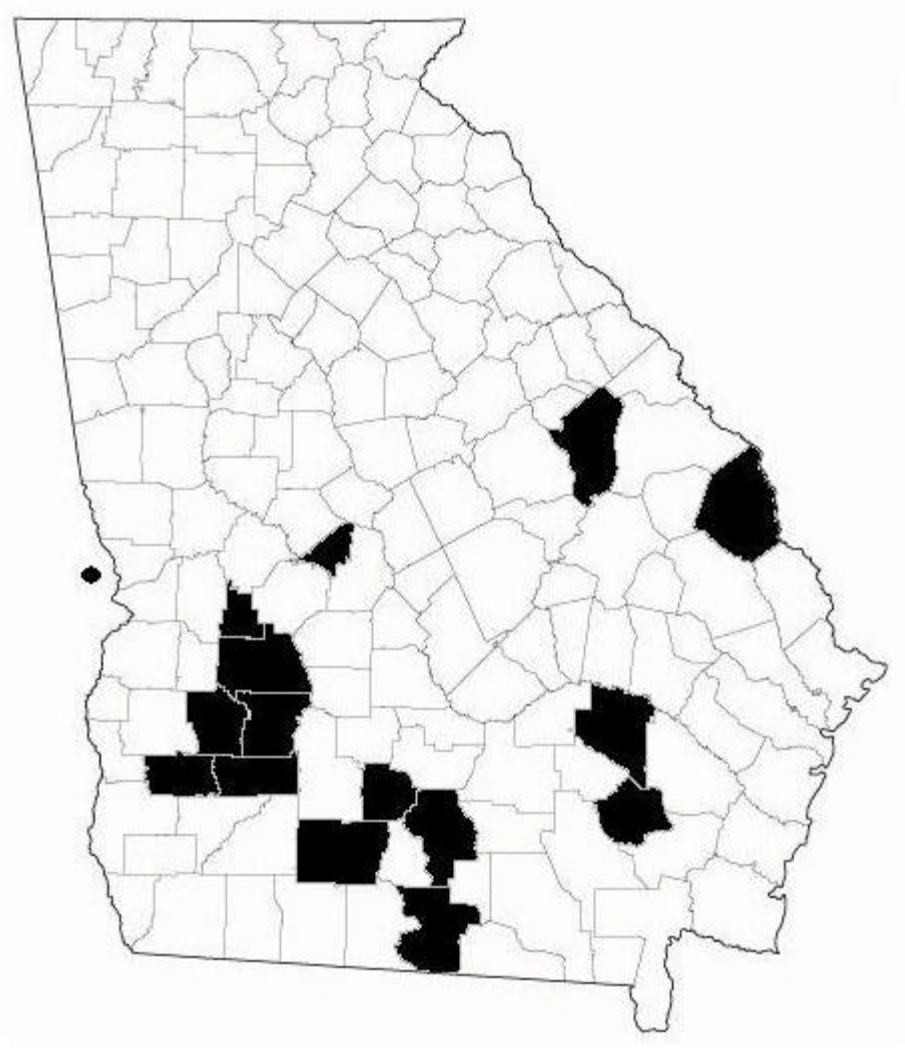

**Fig. 4.3.** Highlighted counties were the 34 commercial pecan production sites in Georgia and one location in Alabama where *Fusicladium effusum* populations were evaluated for fungicide sensitivity.

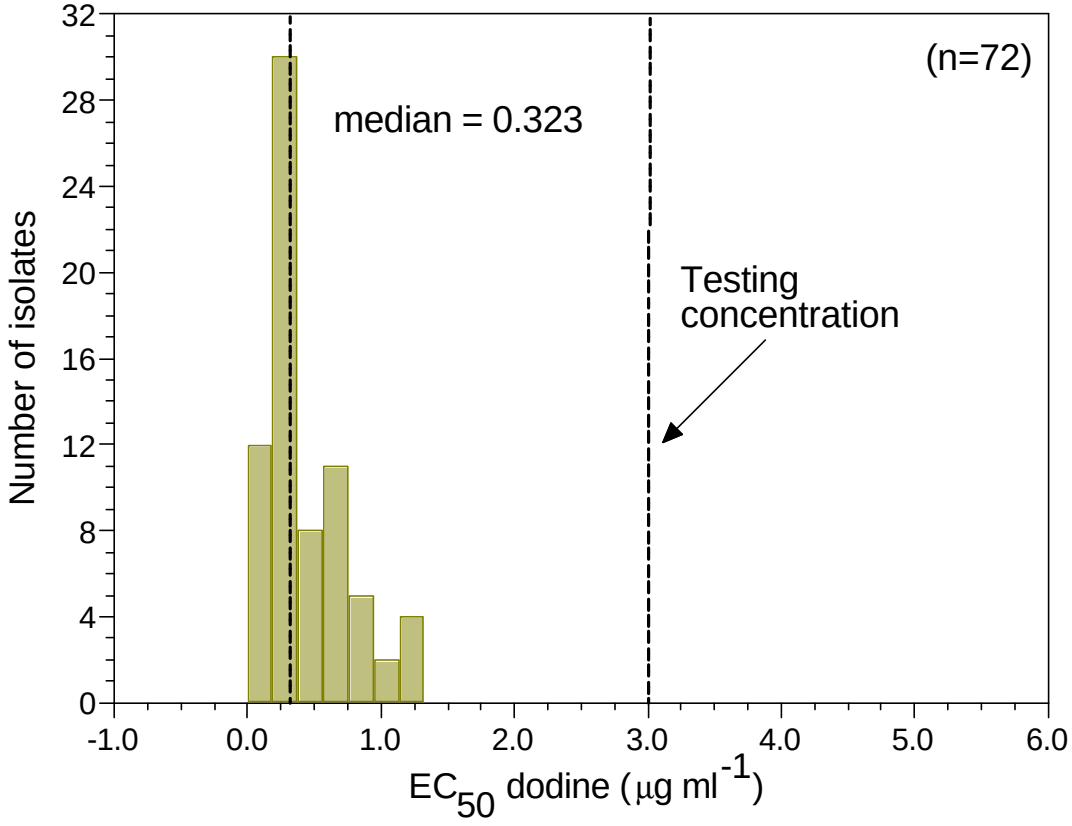


**Fig. 4.4.** Frequency distribution of $EC_{50}$ values of non-sprayed baseline locations (Jeff Davis, Washington, and Clarke Counties) and the discriminatory concentration of 3 µg/ml for isolates of *Fusicladium effusum* to dodine as determined via the rapid conidial germination assay.

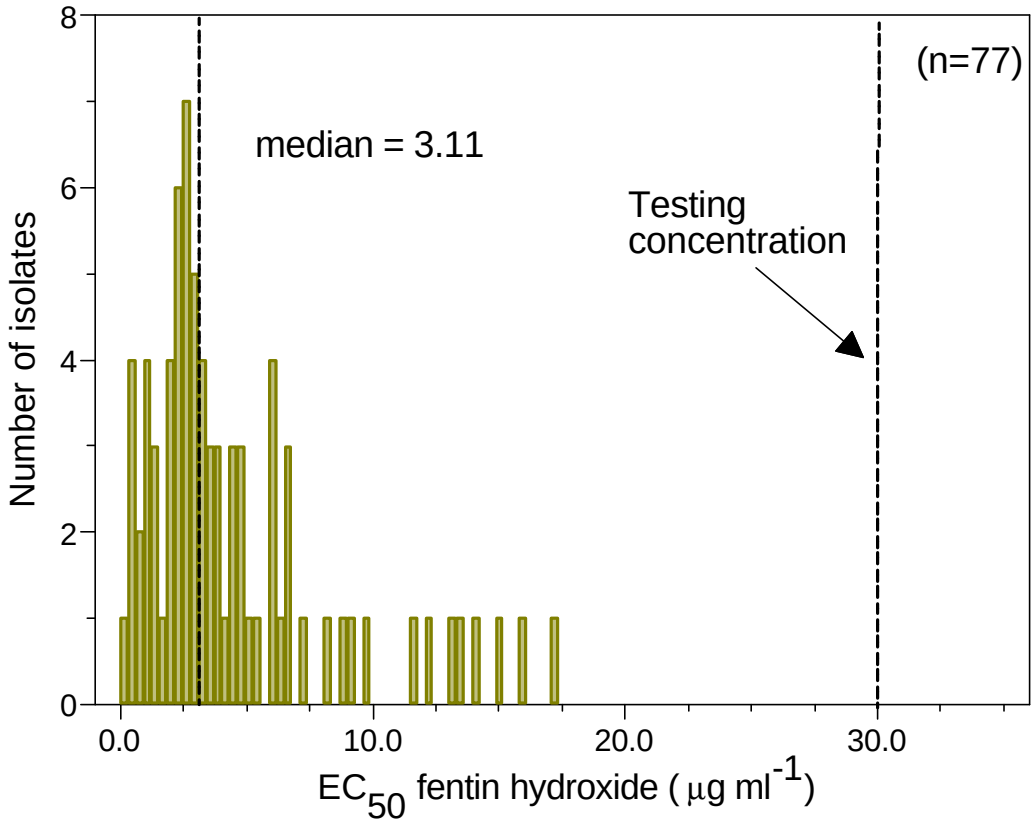


**Fig. 4.5.** Frequency distribution of $EC_{50}$ values of non-sprayed baseline locations (Jeff Davis, Washington, and Clarke Counties) and the discriminatory concentration of 30 µg/ml for isolates of *Fusicladium effusum* to fentin hydroxide as determined via the rapid conidial germination assay.

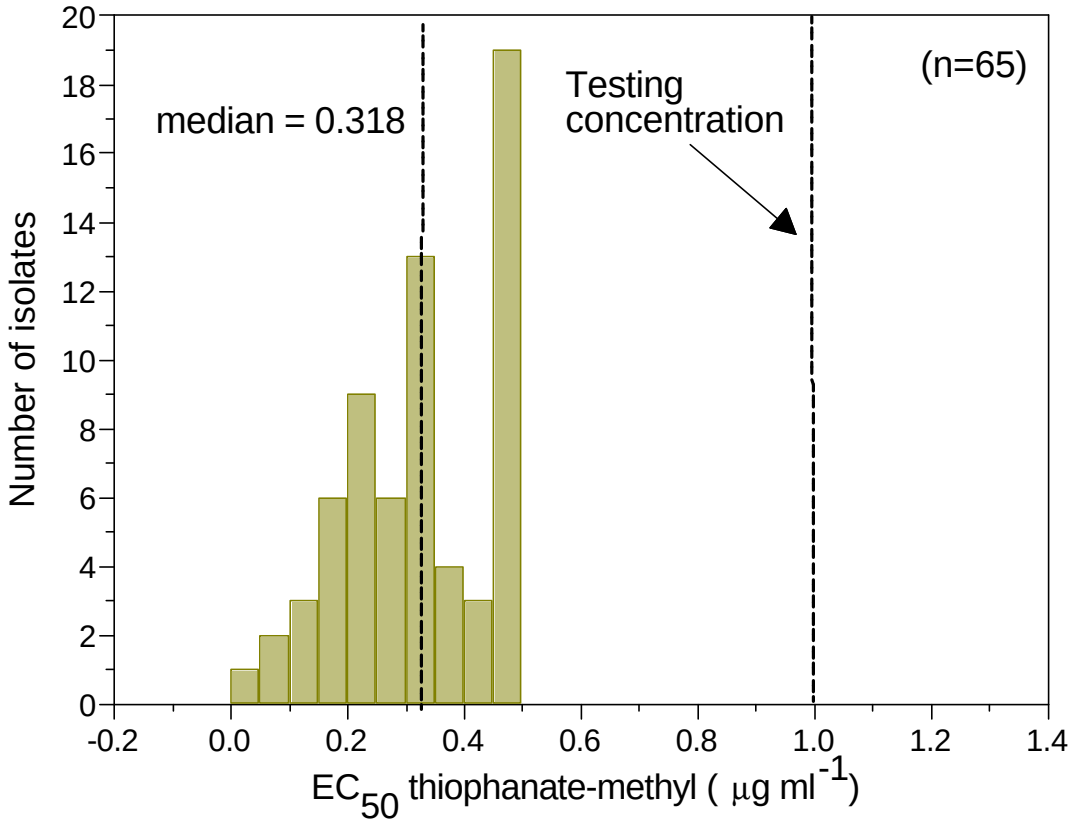


**Fig. 4.6.** Frequency distribution of $EC_{50}$ values of non-sprayed baseline locations (Jeff Davis, Washington, and Clarke Counties) and the discriminatory concentration of 1 µg/ml for isolates of *Fusicladium effusum* to thiophanate-methyl as determined via the rapid conidial germination assay.

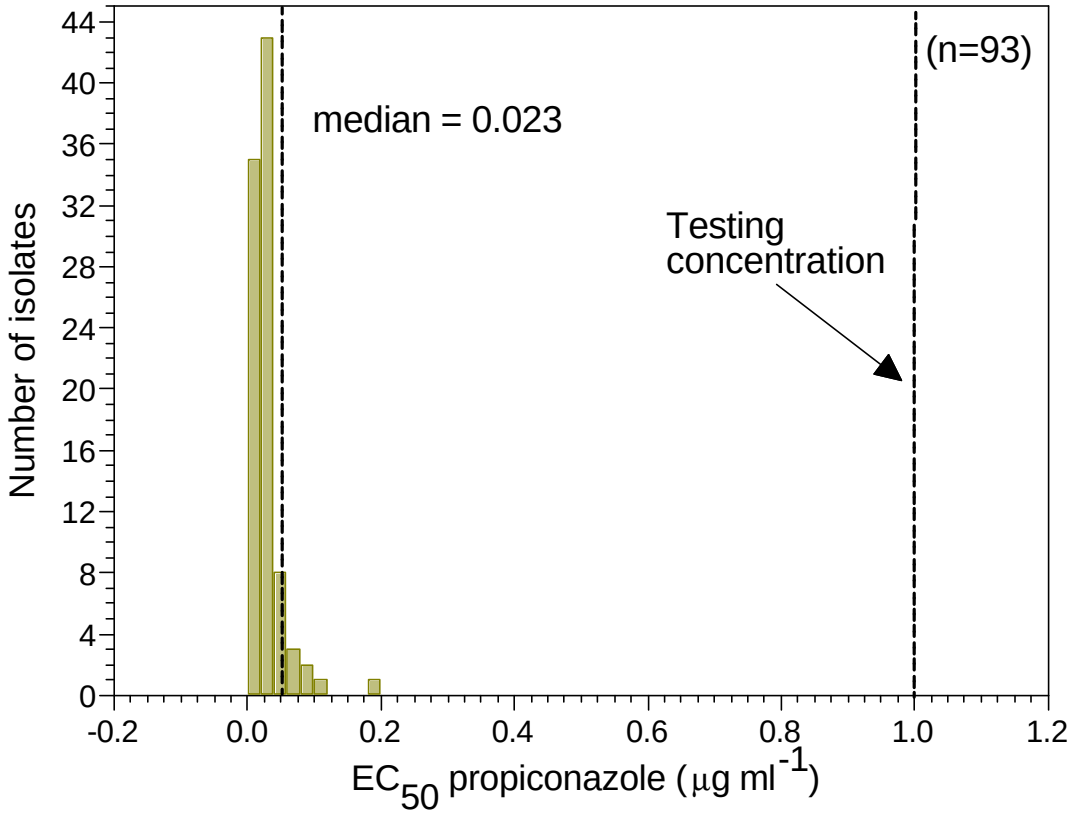


**Fig. 4.7.** Frequency distribution of $EC_{50}$ values of non-sprayed baseline locations (Jeff Davis, Washington, and Clarke Counties) and the discriminatory concentration of 1 µg/ml for isolates of *Fusicladium effusum* to propiconazole as determined via the micro-colony growth assay.

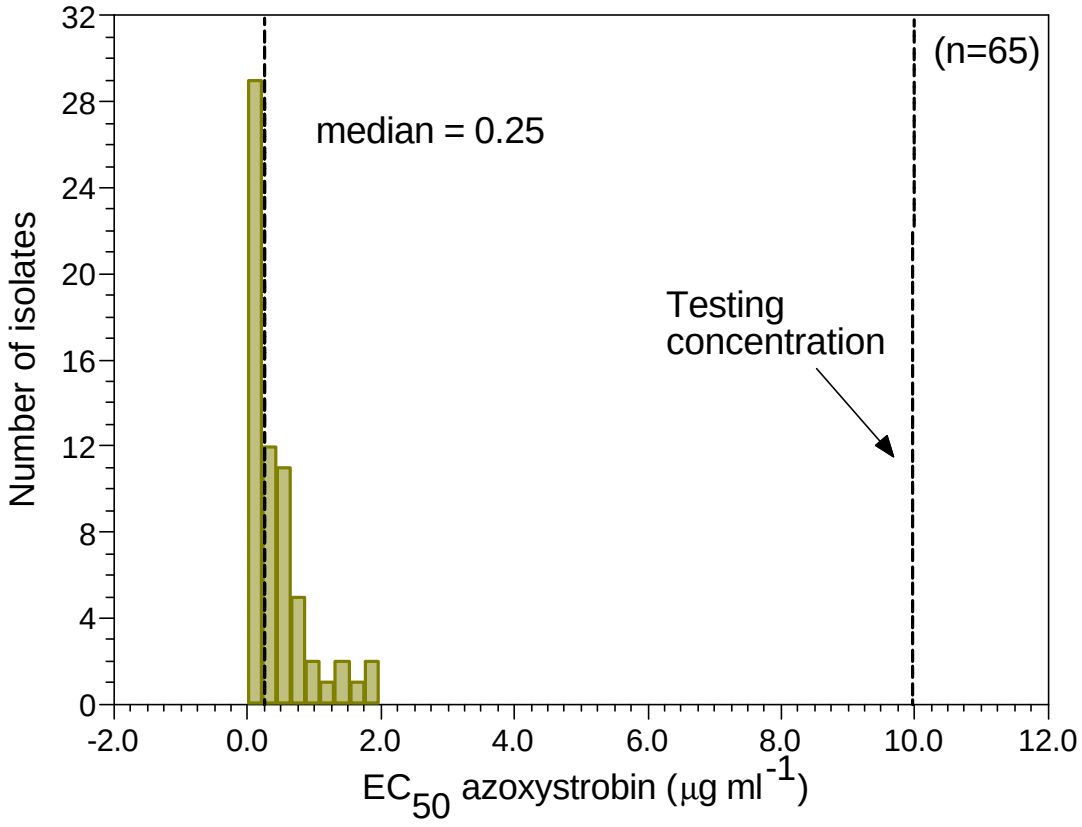


**Fig. 4.8.** Frequency distribution of $EC_{50}$ values of non-sprayed baseline locations (Jeff Davis, Washington, and Clarke Counties) and the discriminatory concentration of 10 µg/ml for isolates o *Fusicladium effusum* to azoxystrobin as determined via the rapid conidial germination assay.

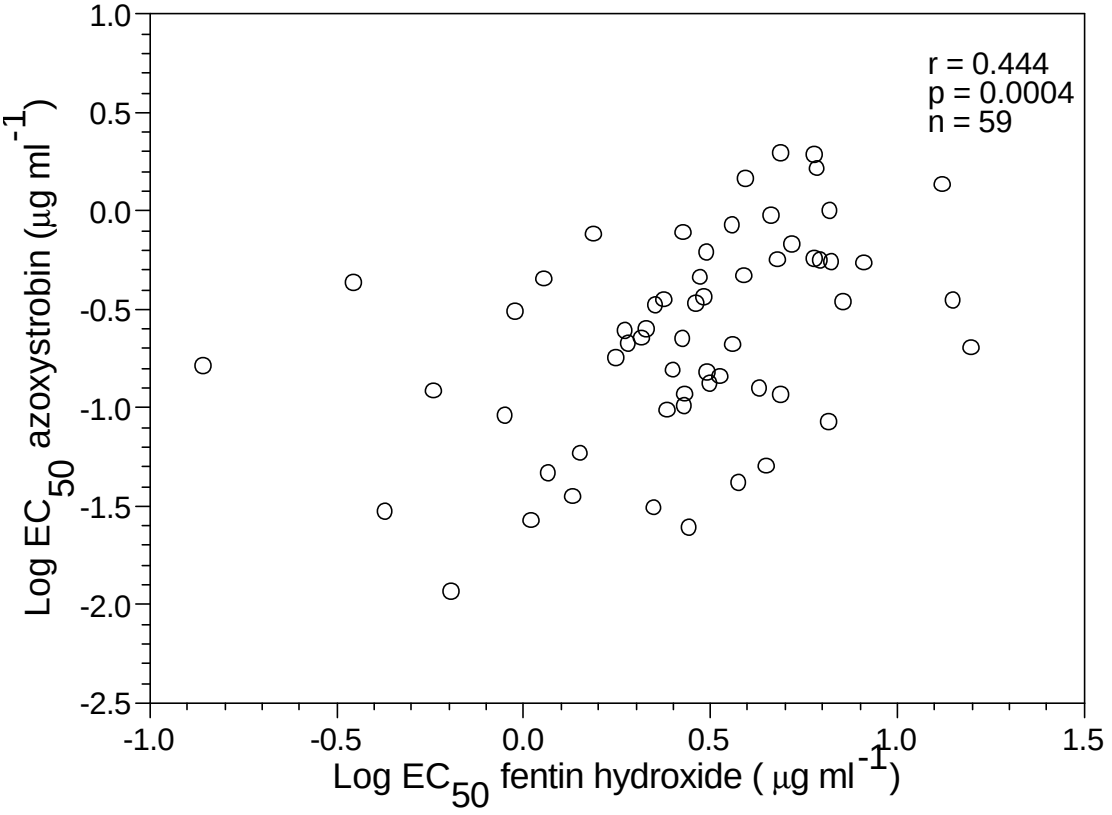


**Fig. 4.9.** Correlation between sensitivity of *Fusicladium effusum* to fentin hydroxide and azoxystrobin.

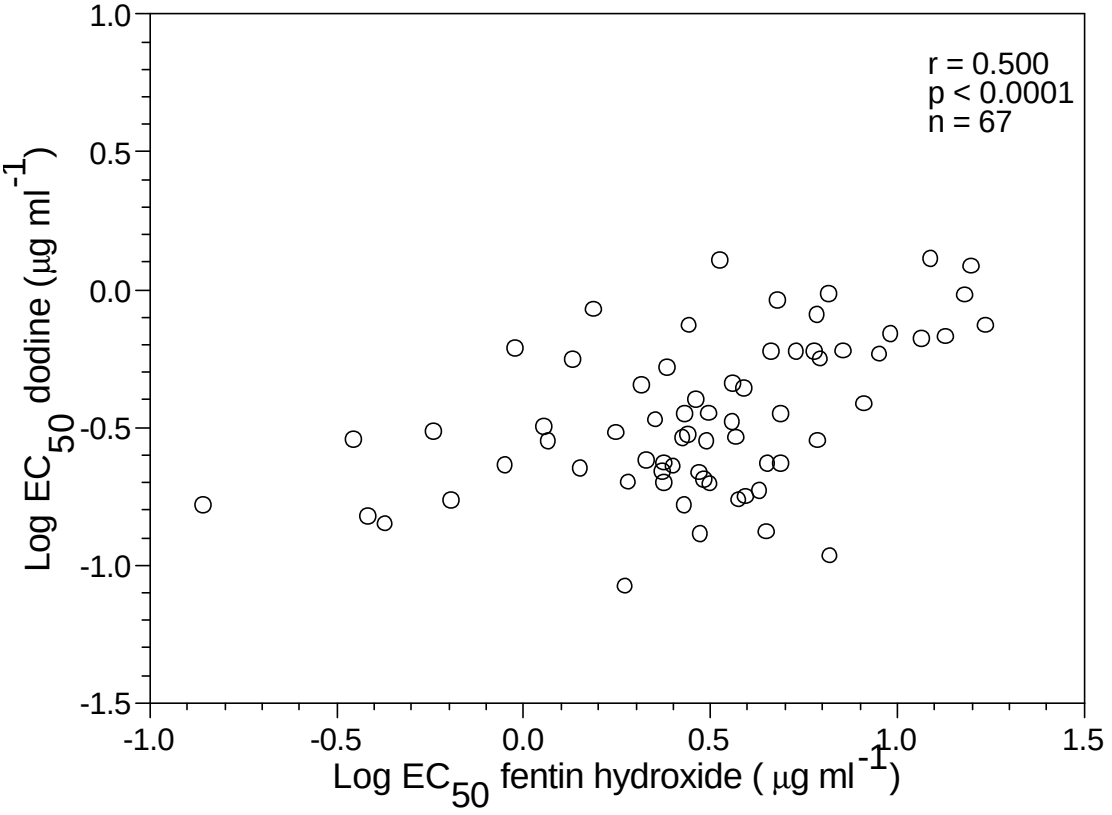


**Fig. 4.10.** Correlation between sensitivity of *Fusicladium effusum* to fentin hydroxide and dodine.

# CHAPTER 5

## PHYLOGENY OF THE PECAN SCAB FUNGUS *FUSICLADIUM EFFUSUM* (WINTER) BASED ON CYTOCHROME B GENE SEQUENCE[1]

[1] Seyran, M, Nischwitz, C., Lewis, K. J., Gitaitis, R. D., Steveson, K. L., and Brenneman, T. B. 2008. To be submitted to Mycological Progress.

## Abstract

Seyran, M, Nischwitz, C., Lewis, K. J., Gitaitis, R. D., Steveson, K. L., and Brenneman, T. B. 2008. Phylogeny of the pecan scab fungus *Fusicladium effusum* (Winter) based on cytochrome b gene sequence. Mycological Progress.

Pecan scab, caused by the fungus *Fusicladium effusum* (Winter), is the most devastating disease of pecan trees (*Carya illinoinensis* (Wangenh.) K. Koch) and is to blame for the majority of disease management efforts applied to that crop. The taxonomy of the fungus changed several times in the last decade and most recently using ITS nrDNA data and conventional taxonomic methods, the organism was renamed *Fusicladium effusum*. In our study, a conserved region of the mitochondrial cytochrome b gene was sequenced from three isolates of *F. effusum*. The obtained sequences showed 95% nucleic acid and 100% amino acid homology (201-266 amino acids on exon 5 of the cytochrome b gene) with *Venturia inaequalis* (NCBI GenBank accession number AF047029). And in the maximum parsimony tree based on nucleotide sequences *F. effusum* and *V. inaequalis* were clustered, with 92% bootstrap value. The taxonomic classification of pecan scab fungus was supported based on cytochrome b region.

## Introduction

The casual agent of pecan scab, *Fusicladium effusum*, is a hemibiothrophic, *forma specialis* fungus with an unknown telemorph stage Pecan scab is the most economically important disease of pecan *Carya illinoinensis* (Wang) K. Koch) in the southeastern U.S. (2,10). An accurate understanding of the taxonomy of this organism is essential if control practices and molecular methods developed with genetically related organisms, such as the apple scab fungus *Venturia inaequalis*, can be applied to *F. effusum*. The pecan scab fungus has been reclassified nine times since its first description by George Winter in 1882 (1,4,6,8,10). Most recently the organism was renamed *Cladosporium caryigenum*, *Fusicladium effusum, Fusicladosporium effusum*, and back to *Fusicladium effusum* in 1982, 2002, 2003, and 2005, respectively. The most recent taxonomic change was based on analyzing ITS nuclear DNA which placed the organism within the Venturiaceae without any subgroups or further classifications (1,4,6,6,10). Additionally, in a previous study pecan scab fungus was used as outgroup to investigate the taxonomy of the several genera in Venturiaceae (9). However, the *F. effusum* is a true Venturiaceae and this taxonomic position was also confirmed with our results. In our study, we investigated the phylogeny of the pecan scab fungus with mitochondrial DNA, specifically using highly conserved sequences from the cytochrome b gene.

## Materials and Methods

Pecan leaves with actively sporulating scab lesions were collected in 2007 from plantations located in Clarke, and Jefferson Davis Counties in Georgia. Individual lesions were cut with a cork borer and gently rubbed on the surface of water agar (WA) amended with antibiotics (chloramphenicol, streptomycin sulfate, and tetracycline, each at 50 µg/ml). The

conidia were incubated for 24 h at 25ºC in the dark. A single germinated conidium was transferred to potato dextrose agar (PDA) with antibiotics (same antibiotics and concentrations described above) and incubated at 25ºC in the dark for 6 weeks. A 3-mm diameter plug was sampled from the fungal colony growing on PDA and transferred to a glass tube containing 50 ml of sterile potato dextrose broth (PDB). The agar disk was homogenized with a tissuemizer (IKA Labortechnik T25 Basic, IKA Works, Inc. Willmington, NC) to obtain a uniform suspension of mycelial fragments. The fungal suspension was transferred to a glass test tube (25 x 150 mm) containing 100 ml of sterile PDB. Tubes were sealed with Parafilm and incubated at room temperature for 3 weeks on a rotary shaker at 75 rpm. After the formation of a mycelia mass, cultures were observed for possible contamination and if found to be pure were drained and transferred to a Petri plate. DNA was extracted using a ZR Fungal/Bacterial DNA Kit extraction kit, according to the manufacturer's protocol (Zymo Research Corp.625 W. Katella Ave., #30 Orange, CA 92867 USA). PCR was conducted using the primers Cytbf 5`- CCTTTGTATTAGCDGCWTTAG and Cytbr 3`- GGAGTTTGCATAGGRTTWGC with the concentraion of 25 pm/ µl which were designed by alinging the sequences on Clustal W. For PCR reactions, 12.5 µl of PCR mix (Promega # PRM 7502), and 2.0 µl of each of the forward and reverse primers was added to a PCR reaction tube (Promega BioSciences, San Luis Obispo, CA USA) to a final volume of 25 µl. Finally, 4.0 µl of extracted DNA template and 4.5 µl of water were added to each PCR reaction tube. The amplification was carried out in a PTC-100 programmable DNA thermal cycler (MJ Research, Watertown, MA, USA). The PCR reaction conditions were: initial warm-up period at 95 ºC for 1 minute, denaturation period at 95 ºC for 5 minutes, annealing phase at 52 ºC for 30 seconds and the elongation phase at 72 ºC for 30s. PCR amplifications were conducted for 39 cycles. The PCR reaction was completed by incubating the

tubes at 95 ºC for 30 seconds, 52 ºC for 30 seconds, and 72 ºC for 5 minutes. Then, the PCR product was stored at 4 ºC for subsequent use. PCR amplification products were detected by gel electrophoresis (3 V/cm) using a 1.0% agarose gel in a 0.04 M Tris-acetate 0.001 M EDTA (TAE) buffer (53). The DNA fragment was excised from the agarose gel and gel extraction was performed using a QIAquick gel extraction kit (Qiagen Sciences, Maryland, USA). The product was purified using a QIAGEN purification kit (QIAGEN Sciences, Maryland USA), and the PCR product was sent to the Davis Sequencing DNA Sequencing and Synthesis Facility (Davis Sequencing 1490 Drew Ave Suite 170 Davis, CA 95618). Obtained sequences were analyzed using a BLAST search (BlastX algorithm )of the NCBI data base and multiple sequence alignment by MAFFT v6.240 (http://align.genome.jp/mafft/). The phylogenetic tree was prepared with bootstrap analysis (2000 replicates) using PAUP 4.0 (Sinauer Assoc., Inc. Publishers, Sunderland, MA) (7).

## Results and Discussion

The cytochrome b gene of three different isolates of the pecan scab fungus (one of from Clarke County, and two from Jefferson Davis County) were sequenced. The size of each sequence obtained approximated 200 bp. The sequences were 100% homologous with each other and 95% homologous with NCBI GenBank accession number AF047029 of *V. inaequalis*. Furthermore, the resulting amino acids coded by the three pecan scab strains and *V. inaequalis* were 100% homologous and corresponded to the amino acids 201-266 on exon 5 of *V. inaequalis* (Table 5. 1)(12).

The pecan scab fungus sequences also were compared with several other Ascomycete fungi and BLAST search results were used to prepare a phylogenetic tree based on cytochrome b gene

sequences (Table 5.1) (Figure 5.1). 15 different taxa (three isolates of *F. effusum*) were examined with bootstrap analysis and parsimony tree, using PAUP 4.0 (Table 5.1) (7). Parsimony tree branch supporting clades were grouped, in clade A, three *F. effusum* isolates clustered with *V. inaequalis* 92% of the bootstrap value in Venturiaceae family which is a supporting data of the taxonomic position of pecan scab fungus (Fig. 5.1). In clade B, *Glomerella graminicola, Fusarium subglutinans, Verticillium dahliae*, and *Podosphaera fusca* were group with 88% bootsrap values and differ from Venturiaceae family by class (Fig. 5.1). In this clade *G. graminicola, F, subglutinans* and *V. dahliae* are member of Sordariomycetes class however, *P. fusca* is a member of different fungal class Leotiomycetes (Fig. 5.1). In clade C, *Phaesphaeria nodorum* and *Alternaria alternata* both are member of Pleosporales family were grouped with the bootstrap value of 100% and differ from Venturiaceae by family (Fig. 5.1). In clade D, *Aspergillus niger, Penicillim expansum* and *Neosartoya fisheri* all the members of Trichocomaceae family were grouped with the bootsrap value of 85% and differ from Venturiaceae family by class (Fig. 5.1). And in Clade E, *Cercospora kikuchii* and *Cercospora beticola* were grouped with 100% bootstrap value and differ from Venturiaceae family by subclass (Fig. 5.1). The wood decaying Leotiomycetes *Cadophora fastigiata* was selected as outgroup in parsimony tree analysis due to its distinct biological niche (Fig. 5.1) (3).

The cytochrome b gene has been used to classify different fungal species of *Verticillium* and subspecies of *Aspergillus* section *Nigri* (5,11). In our study, the phylogeny of the pecan scab fungus based on the conserved cytochrome b region was consistent with the phylogeny based on the ITS region that supports the most recent taxonomic classification of the pecan scab fungus as *Fusicladium effusum* (Winter) (1,4,10).

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**Table 5.1.** List of fungi and their GenBank accession used to obtain cytochrome b sequences for phylogenetic analysis and amino acid sequence homology with *Fusicladium effusum* isolates

| **Organism** | **Accession No.** | **% Homology** |
|---|---|---|
| *Fusicladium effusum* Clarke Co. | FJ407295 | - |
| *Fusicladium effusum* Jeff Davis 1 | FJ407296 | - |
| *Fusicladium effusum* Jeff Davis 2 | FJ407297 | - |
| *Venturia inaequalis* | AF047029 | 100 |
| *Glomerella graminicola* | AY285743 | 73 |
| *Verticillium dahliae* | ABM65032 | 70 |
| *Podosphaera fusca* | ABL75951 | 73 |
| *Fusarium subglutinans* | BAG12970 | 70 |
| *Phaeosphaeria nodorum* | YP_001427390 | 80 |
| *Alternaria alternata* | ABB54712 | 82 |
| *Aspergillus niger* | YP_337876 | 78 |
| *Penicillium expansum* | BAF98953 | 78 |
| *Neosartorya fischeri* | AAX39422 | 73 |
| *Cercospora kikuchii* | BAE53454 | 90 |
| *Cercospora beticola* | ABM53468 | 90 |
| *Cadophora fastigiata* | BAC78356 | 83 |

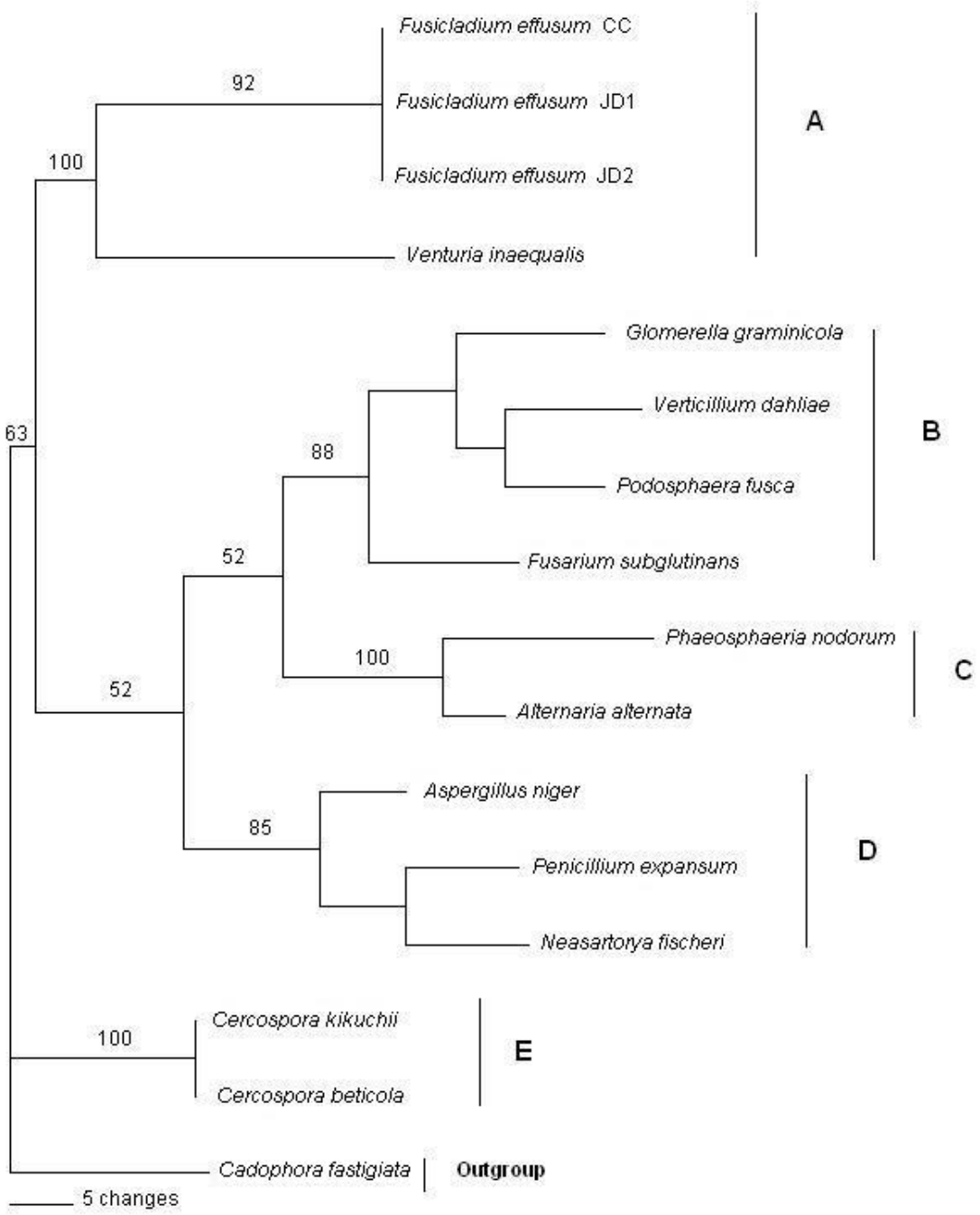


**Figure 5.1.** Maximum parsimony tree based on nucleotide sequences of the cytochrome b gene on exon 5 of *Fusicladium effusum* and other Ascomycetes fungi obtained from heuristic parsimony search and bootstrap analysis. Bootstrap values greater than 50% are shown at the nodes based on 2000 replications. Gaps were treated as missing data.

# CHAPTER 6

## SUMMARY

Pecan scab, caused by the fungus *Fusicladium effusum* (Winter), is the most devastating disease of pecan, and is the major target for disease control (3,5). Management of scab requires 7 to 10 or more fungicide applications per season. Fungicides in at least five different chemical classes are labeled for scab control. All of these fungicides have some level of risk for development of resistance (1,3,6), but with the exception of the demethylation inhibitors (DMIs), the sensitivity of *F. effusum* populations in Georgia is largely unknown (2,4). Fungicide resistance monitoring is essential to evaluate the effectiveness of the pecan scab fungicides. To detect development of fungicide resistance, prior to a control failure, the first step is to construct a baseline sensitivity profile of the target fungus. Baseline sensitivity profiles to the major scab fungicides were established with microtiter plates and spore germination assays. Eighty-nine monoconidial isolates of *F. effusum* were obtained from three orchards in Georgia with no known fungicide history. Isolates were tested for sensitivity to propiconazole, dodine, fentin hydroxide, azoxystrobin, azoxystrobin with the alternative oxidase inhibitor salicylhydroxamic acid (SHAM) and thiophanate-methyl using a mycelial growth assay in liquid medium in microtiter plates and spore germination assays. The 50% effective dose ($EC_{50}$) values ranged from 0.008 to 0.595 µg/ml with a median of 0.0484 µg/ml for dodine (n=66), 0.115 to 4.540 µg/ml with a median of 0.759 µg/ml for fentin hydroxide (n=45), and 0.0114 to 0.793µg/ml with a median of 0.0614 µg/ml for propiconazole (n=65) using micotiter plate assay. All tested

isolates were sensitive to thiophanate-methyl and failed to grow at 1.0 and 10.0 µg/ml. However, the $EC_{50}$ values of azoxystrobin with SHAM were not log-normally distributed and were different from the values of azoxystrobin without SHAM (2). Experiments in liquid and solid medium were performed to determine the effects of different concentrations of SHAM and another AOX inhibitor, propyl gallate, on growth. Both AOX inhibitors inhibited the growth of *F. effusum*. Therefore, azoxystrobin without SHAM was used for fungicide sensitivity monitoring in commercial pecan orchards A more rapid assay using conidia taken directly from lesions on leaves or nuts was developed for azoxystrobin, dodine, fentin hydroxide and thiophanate-methyl based on conidial germination, and for propiconzole based on micro-colony growth on medium amended with a discriminatory concentration of each fungicide. Samples from 35 commercial orchards were profiled using the new assay and in vitro fungicide resistance was detected to fentin hydroxide, dodine, thiophanate-methyl, propiconazole, and azoxystrobin in 20, 4, 6, 21, and 19 orchards, respectively. The partial sequence of the cytochrome b gene (amino acids 201-266 on exon 5 of *V. inaequalis*) of *F. effusum* was obtained with PCR; which is supporting the first and most recent fungal taxon of pecan scab fungus as *Fusicladium effusum* (5,7). In summary, based on my study the pecan scab fungicide resistance monitoring and the detection the resistance would be accomplished with developed techniques. This developed, fast, accurate, economical method to produce sensitivity profiles on individual orchards will enable growers to avoid losses from the unexpected occurrence of fungicide resistance.

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# APPENDIX

## DISCUSSION BASED ON THE INTRON STATUS OF MITOCHONDRIAL CYTOHROME B GENE SEQUENCE

Thus far, resistance to QoIs has not been detected in some pathogens, including many rusts. The amino acid sequences of the cytochrome b gene from some plant pathogenic fungi revealed that many rust pathongens (e.g. *Puccinia recondita f.sp. tritici, P. graminis f.sp. tritici, P. striiformis f.sp. tritici, P. coronata f.sp. avenae, P. hordei, P. recondita f.sp. secalis, P. sorghi, P. horiana, Uromyces appendiculatus, Phakospore pachyrhizi, Hemileia vastatrix* and *A. solani*) have an intron region just after the G143 amino acid locus on the mitochondrial genome. However, other plant pathogenic fungi (e.g. *A. alternata, Blumeria graminis, Pyricularia grisea, Mycosphaerella graminicola, M.. fijiensis, V. inaequalis* and *P. viticola*) do not have that intron region (1,2). Interestingly, unlike *A.solani, A. alternata* does not possess an intron after the G143 amino acid locus, and the G143A mutation for resistance to QoIs has been detected in *A. alternata* (3). A similar relationship was detected in *Pyrenophora teres* and *P. tritici-repentis*. The G143A mutation and a high level of resistance were detected in *P. tritici-repentis,* but not in *P. teres* (4). The cytochrome b gene sequence of *P. teres* indicates the presence of an intron after the G143 locus unlike the *P. tritici-repentis* gene sequence (4). As seen in *Alternaria spp*. and *Pyrenophora spp.*, the G143 based mutation is a species-specific trait (1,2). The authors associated the history of sensitivity to QoIs in rust pathongens with the lethal impact of the G143A mutation due to the existence of an intron just after G143 region on the amino acid sequence of fungi (1,2,4).

Therefore, the identification of the intron status of *F. effusum* cytochrome b gene was defined as a research objective to accurately assess the risk level of QoI fungicides. 25 monoconidial pecan scab isolates were used with the methods described previously in chapter 6 to sequence the region of interest the amino acid sequence of G143 and subsequent codon (5). The degenerate primers used to amplify the region of interest are shown in Table 6.1. The PCR

protocol was the same as that described in Chapter 6 and PCR protocols to sequence the cytochrome b sequence of *F. effusum*, did not yield a successful sequence of the region of interest (Table of appendix).The target site was successfully amplified with primers RU2 and FC4, but the resulting seqeucences were with noisy background and did not contain th region of interest (G143 and subsequent codon). However, in vitro resistance to QoIs in *F. effusum* was detected in 33 different locations with different level of frequency and at least in 19 locations with medium to high levels (5). Therefore an intron region after G143A is not likely to be present. But that is only true if the G143A mutation is responsible for the QoI resistance detected in the commercial orchard samples. The presence of this mutation has not been confirmed, so it could be some other mechanism.

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**Table of appendix.** List of the degenerate primers used to sequence the region of interest (G143) of *Fusicladium effusum*

| Code | Sequence |
|---|---|
| FX1 | GTAAGTGCGGTGCCTTGAATTGGT |
| FX2 | GTGCGGTGCCTTGAATTGGTACTG |
| FX3 | CCTTGAATTGGTACTGATATAGTC |
| RU1 | CATTCACGCCACGGAACTTAACCA |
| RU2 | CACGCCACGGAACTTAACCATGAC |
| RU3 | GGAACTTAACCATGACTATATCAG |
| RX1 | GAGAGAWACAAAAGGAGATWGGG |
| RX2 | GGGAAATCCACACAGTCCWTTG |
| FC1 | GCNTTCTTGGGTTATGTDTTACC |
| FC2 | GCNTTCCTGGGTTATGTDTTACC |
| FC3 | GCNTTCTTAGGTTATGTDTTACC |
| FC4 | GCNTTCCTAGGTTATGTDTTACC |
| FC5 | GGNTTCTTGGGTTATGTDTTACC |
| FC6 | GGNTTCCTGGGTTATGTDTTACC |
| FC7 | GGNTTCTTAGGTTATGTDTTACC |
| FC8 | GGNTTCCTAGGTTATGTDTTACC |
| FD1 | GAACDTTAGTTTGADCTTTAGG |
| FD2 | GAACDTTAGTTTGADCATTAGG |
| FD3 | GAACDTTAGTATGADCAATAGG |
| FD4 | GAACDTTAGTATGADCTATAGG |
| FD5 | GAACDTTAGTTTGADCTATAGG |
| FD6 | GAACDTTAGTTTGADCAATAGG |
| FD7 | GAACDTTAGTATGADCATTAGG |
| FD8 | GAACDTTAGTATGADCTTTAGG |
| FE1 | CGDTATHTACACDGTAACACTGC |
| FE2 | CGDTATHTACACDGTAATACTGC |
| FE3 | CGDTATHTACACDCTAATACTGC |
| FE4 | CGDTATHTACACDCTAACACTGC |
| FE5 | CGDTACHTACACDCTAACACTGC |
| FE6 | CGDTACHTACACDCTAATACTGC |
| FE7 | CGDTACHTACACDGTAATACTGC |
| FE8 | CGDTACHTACACDGTAACACTGC |
| FF1 | CHTTAGCAATGCACTACAAHCCT |
| FF2 | CHTTAGCAATGCACTATAAHCCT |
| FF3 | CHTTAGCAATGCATTACAAHCCT |
| FF4 | CHTTAGCAATGCATTATAAHCCT |