OPTIMIZING AND MAINTAINING EFFICACY OF FUNGICIDES FOR *VENTURIA EFFUSA* AND MECHANISMS OF RESISTANCE TO THE DMI FUNGICIDES

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(Under the Direction of Timothy Brenneman)

ABSTRACT

Pecan scab, caused by the fungal pathogen *Venturia effusa*, is the most economically significant pathogen affecting commercial pecan (*Carya illinoinensis*) production in the southeastern United States. Control of the disease is reliant on multiple fungicide applications per season. The efforts described here are aimed at improving fungicide rotation plans and managing fungicide resistance in pecan orchards. The results indicate that *V. effusa* is extremely sensitive to pydiflumetofen, a novel succinate dehydrogenase inhibitor (SDHI) fungicide, with a baseline EC_{50} of 0.0011 µg/ml, and the combination product, Miravis Top, is exceptionally effective at controlling scab. Amistar Top, the previous industry standard, continues to provide excellent control in orchards without Quinone outside inhibitor (QoI) resistance, but efficacy in orchards with resistance to the QoIs is comparable to that of stand-alone difenoconazole. We found reduced sensitivity to tebuconazole to be widespread across 11 locations in southern GA. Some isolates grew uninhibited on 10 µg/ml of tebuconazole, whereas historic isolates from 25 years ago showed no growth on 1.0 µg/ml. Sequence analysis of the *CYP51* gene revealed multiple mutations, including the G444D, G357H, I77T, and I77L in resistant isolates, which also exhibited overexpression of the *CYP51* gene. We investigated the potential of micronized sulfur as a mixing partner with various modes of action to explore if any additive or synergistic response occurred. Micronized sulfur did not result in increased efficacy when added to

fungicides for control of *V. effusa*. Finally, it is not currently known if isolates of *V. effusa* from different pecan cultivars differ in their response to certain fungicides. We evaluated isolates from different cultivars within the same orchards to determine why the cultivar's susceptibility to *V. effusa* infection seemed to change over time, as reported by multiple commercial growers. *In vitro* assays were conducted to screen isolates of *V. effusa* against tebuconazole, fentin hydroxide, dodine, and thiophanate methyl; the isolates were screened for mutations that lead to QoI resistance. There were no consistent differences in the fungicide sensitivity of isolates from different cultivars at any location that would explain the differential disease development. The results of this research will help improve fungicide rotation programs, establish resistance monitoring to the SDHI fungicides, as well as aid in managing fungicide resistance in commercial pecan orchards.

INDEX WORDS: *Venturia effusa*, fungicides, resistance, DMIs, pecan, management

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

History, background, and production of *Carya illinoinensis*

Pecan [*Carya illinoinensis* (Wangenheim) K. Koch] belongs to the walnut family, Juglandaceae, and is a deciduous tree that is primarily grown for its seed, which are an edible nut. Indeed, pecan is a hickory species, and the genus name '*Carya*' is Greek for "nut" (Alden, 1995). Pecan is the only major tree nut crop that has a North American center of origin and is considered to be one of the most valuable nut species in the United States. Within North America, pecan is native to the alluvial soils of the lower Mississippi River basin and its tributaries (Bonner & Maisenhelder 1974; United States Department of Agriculture, 1979). In natural habitats, pecan is found in areas containing well-drained loam soils on riverfront high-grounds that are not subject to flooding (Peterson 1990). Pecan's botanical range extends from northern Indiana south to the Gulf coast of Louisiana and Texas, and from Tennessee and Alabama west to Kansas and central Texas (Betts, 1945; Clark, 1973; Bonner & Maisenhelder 1974). The word "pecan" is a Native American word of Algonquin origin, and refers to "nuts that require a stone to crack." It is speculated that the Native Americans were among the first to cultivate pecan as an agronomic crop (National Pecan Shellers Association, 2018). Production of pecan on a commercial scale began in the mid to late 1800s as vegetative propagation methods were developed that allowed clonal multiplication of improved cultivars. The majority of the early cultivars developed during the 1800s were failures; however, at the time pecan breeders developed some of the most successful and widely used cultivars that are still grown to this day, including Schley, Western Schley, Stuart, and Desirable (Sparks 1992). In the early to mid-

1900s, rigorous efforts were invested in planted seedling orchards in hopes of finding improved cultivars that would produce sizable nuts that were easy to shell (Wood et al. 1990). Several pecan orchards were established in southwest Georgia and east-central Texas, which shifted the major production region from Texas, Mississippi, and Louisiana to southwestern Georgia (Wood et al. 1990).

Pecan trees are long-lived and are capable of growing to heights of 30 to 45 m, with a trunk diameter of 1.8 to 2.1 m (Duncan & Duncan 1988). The tree has a shallow-furrowed and flat-ridged trunk that is gray in color. The branches are ascending, forming an irregular, rounded crown. Young twigs are hairy and grey brown, but become furrowed and rough with maturity. The trees have compound leaves, and the leaflets are typically dark green, pointed, narrow, and curved at the tips and contain toothed/serrated margins. The age at which the trees begin nut production varies drastically between cultivars, with most commercially grown trees producing nuts beginning in year 4 or 5. The optimum nut-bearing age is estimated to be 75 to 225 years (Knapp & Rice 1998; Peterson 1990). Pecan typically flowers from March to May shortly after bud break when the leaves are expanding. The nuts usually mature from September to October. The nuts typically reach maturity and dislodge from the fruit pericarp (which generally remains in the tree) starting in September and ending in December (Bonner & Maisenhelder 1974; Hodges & Switzer 1979).

Pecan is a monoecious tree crop with unisexual flowers of both sexes borne in clusters located in the same tree. Staminate flower catkins develop at the base of emerging shoots. The long and slender catkins are 7 to 12 cm in length and usually less than 1 cm in width. Each catkin may contain up to 115 staminate flowers. Each staminate flower contains a leafy bract with 3 to 5 anthers. Each anther contains up to 2,000 pollen grains. After the catkins have fully elongated,

the shoot continues to grow several more inches. Catkins are usually aborted directly following bud break, but are often present with young leaves as well. Each shoot usually produces 8 to 10 compound leaves containing 13 to 17 leaflets per leaf before pistillate flower production is initiated. If the tree contains an adequate level of carbohydrates, the pistillate flowers will form during the early growth stages in the spring. It takes approximately 4 to 6 weeks for the pistillate flowers to become visible. The pistillate flowers form a typical spike shape, with the point of growth remaining vegetative until several pistillate flowers are formed. The number of pistillate flowers is determined by the variety and vigor of the tree. The individual pistillate flowers typically measure from 5.5 to 8 mm in length, much of which is enclosed in a green, pubescent calyx. This calyx has four sections, which enclose the pistillate flower and form the tapering bract that is 3 to 4 mm in length. The bracts may be erect or expanded outward, depending on cultivar or perhaps genotype. The vegetative growth within the flower abscises, producing a scar at the inflorescence terminal. This abscission is most likely the result of depleted carbohydrates. The stigmas may undergo protandrous dichogamy, with the stigma becoming receptive following pollen shed, or protogynous dichogamy, with the stigma becoming receptive prior to pollen shed. Dichogamy prevents self-pollination, which may lead to poor kernel quality or nut abortion (Wells 2007). Different pecan cultivars may differ in their annual occurrence of stigma receptivity and pollen release; therefore, planting multiple cultivars with complementary flowering characteristics within the same orchard guarantees pollination (Wells 2007; Wood 2011). The stigma is coated in a viscous fluid, which aides in retaining pollen grains that are carried by wind to the stigma during the period of receptivity. Fertilization takes place approximately 5 to 7 weeks following pollination. Fertilized female flowers develop into the fruit, and nut encapsulated therein, in the pecan tree. The fruit typically form in clusters of 3 to

12, each of which consist of three parts: the kernel, shell, and shuck. The kernel is the edible portion of the nut that is found within the enclosed shell, which is housed within the shuck (Sparks 1992). During the first half of fruit development, nut size increases rapidly. During the second half of development, the ovary wall and the two cotyledons are formed to complete kernel development. The calyx, (which in pecan develops into the shuck) covers the developing fruit until fruit maturation is complete. The calyx is composed of four quarters or sections that separate at maturity, revealing the nut (Sparks 1992). The nut contains a middle septum separating the two cotyledons, which is parallel to the inflorescence axis. The stigma's plane is also parallel to the axis (Adriance 1931). Once the nut is ready to be dislodged from the tree, the calyx dehydrates and separates from the shell, allowing the nut to fall from the tree. In commercial settings, pecan trees are shaken using machines to facilitate the dislodging of the nuts. Once the nuts are on the ground, machines are used to harvest and separate them from plant debris.

The United States is the world's leading pecan producing country. Other countries that grow pecans at a commercial scale include: Brazil, Australia, South Africa, Israel, Mexico, Peru, Chile, Argentina, Uruguay, and China. Pecan production has gradually increased in the United States, expanding from 2.2 million pounds of in-shell nuts produced per annum in 1920 to approximately 280 million pounds produced in 2017 (Clevenger et al. 2017; National Pecan Shellers Association 2018; NASS 2018). In 2014, United States pecan exports were valued at $446 million. Hong Kong, China has historically been the primary destination for U.S. pecan exports, with sales of $118 million in 2014. Vietnam was the second most valuable pecan export destination, with pecan exports valued at $87.6 million. Mexico purchased $43.8 million of pecans from the U.S. in 2014 (FAS, 2015), but is rapidly increasing their own production and is

now exporting pecans. Within the U.S., AL, AZ, AR, CA, FL, GA, KS, LA, MS, MO, NM, NC, OK, SC, TX, KY, MD, TN, and VA are all states with commercial pecan production. In 2019, the top three pecan producing states were New Mexico, Georgia, and Texas, respectively (NASS 2019).

In 2014, Georgia was the leading pecan producing state (76 million pounds), followed by New Mexico (67 million pounds), Texas (61 million pounds), and Arizona (21 million pounds) (NASS 2015). In 2016, Georgia had 158,905 acres of pecan trees under production, with a farm gate value of $355,854,324. The top pecan producing counties for the state of Georgia are Dougherty county, claiming 12% of Georgia's pecan production with 16,500 acres, Mitchell county, claiming 10% of Georgia's pecan production with 16,137 acres, and Lee county, claiming 8% of Georgia's pecan production with 10,500 acres (Stubbs 2017). The acreage of pecan in Georgia has experienced significant expansion since the industry was established in the early 1900's. The first expansion occurred around 1900 as a result of low land prices influenced by the coupling of low cotton prices and exaggerated reports of income from pecan production. As a result, thousands of acres of pecan orchards were planted as a real estate enterprise that was sold to investors. The result was several poorly managed and unproductive orchards and a decrease in popularity of pecan as a lucrative income source, at least until production practices were improved (Wood et al. 1990). As a result of the extensive planting in the early 1900's, Georgia's production exceeded 15.5 million pounds between 1930 and 1940. Georgia was producing more pecans than any other state by 1950 and is the second largest pecan production state today, averaging 82 million pounds of pecans per annum from 2017 to 2019, with a value exceeding $145 million (USDA, 2020). For the first time since 2006, New Mexico pecan production surpassed Georgia's production in 2018. The reason for the fall in Georgia production

was of the impact of Hurricane Michael decimating many of the pecan orchards in the most important production areas of Georgia. Furthermore, during the pecan growing season in 2018, the weather conditions were conducive for disease, particularly pecan scab (caused by the plant pathogenic fungus *Venturia effusa* (G. Winter) Rossman & W.C. Allen), which negatively impacted the yield (NASS 2019).

In the 1960s, another era of expansion in pecan acreage occurred and continued into the early 1980s. New pecan orchards were planted in Georgia, Texas, Oklahoma, and in the more arid southwestern U.S. Thousands of acres were planted in western Texas, California, New Mexico, and Arizona, mostly the cultivar Western Schley. The planting boom was a result of increased demand for domestic pecan use and the availability of new, improved cultivars (Wood et al. 1990; Wells 2014). Since 1980, pecan exports from the U.S. have grown by almost 2000% (Lillywhite et al. 2014). In 2004, only 2 million pounds of the U.S. pecan crop were exported to China. In 2009, over 80 million pounds, or nearly 25% of the total U.S. pecan crop, were exported from the U.S. to Asian markets, primarily China. The increase was a product of the Chinese middle class's rapidly growing incomes. The result was record pecan prices, increasing from $1.43 per pound to $2.30 per pound of in-shell pecans between 2009 and 2010. The prices rose again in 2011, to $2.43 per pound of in-shell pecans (USDA 2012). As a result of higher pecan prices, more acreage was planted to pecan in all U.S. pecan production areas (USDA 2012). In 2017, the U. S. had approximately 377,500 acres of bearing pecan orchards, yielding an average of 778 lb/acre with a cumulative value of $ 684,348,000 for in shell pecans (NASS 2018).

Pecan trees have a natural tendency to bear fruit in 2-year cycles, producing a large crop one season followed by a negligible or nonexistent crop the next. This phenomenon is known as

alternate bearing. Almost all tree-fruit crops exhibit some level of alternate bearing; however, the issue is particularly severe in pecan (Monselise & Goldschmidt 1982). The severity of alternate bearing in pecan is linked to three traits that are inherent to the species: 1) the timing of fruit maturity; 2) the inherent characteristics of fruit development; and 3) the kernel's chemical composition (Sparks 1974). Compared to other fruit crops, pecan fruit mature later in the season, which leaves little time prior to leaf shed for carbohydrate storage to reach the level needed to support the following season's developing flower initials and fruit growth. Kernels of pecan contain approximately 70% lipids, which are metabolically expensive to produce. The accumulation of dry matter within the nut occurs predominantly at the end of the growing season. The coupling of these traits and heavy fruit-set leads to depletion of carbohydrate reserves within the tree, especially the roots, at the end of the season and results in a carbohydrate deficit the following season when flowering and nut development is expected to occur. The carbohydrate deficit ultimately leads to poor fruit-set the following season (Malstrom 1974; Smith & Waugh 1938; Wood 1989). Improved horticultural practices in pecan have contributed to a reduction in severity of alternate bearing. Ensuring the availability of adequate light, water, and nutrients is important for maintaining acceptable carbohydrate reserves. Chemical or mechanical thinning of fruits may help reduce the severity of alternate bearing in certain cultivars of pecan (Smith & Gallot 1990). One of the most important methods for suppressing alternate bearing is to prolong leaf retention to increase carbohydrate reserves for the following season (Wood 1999; Worley 1979a; Worley 1979b; Worley 1973). Pecan scab is a leading cause of premature defoliation in pecan; therefore, mitigation of the disease is crucial for the suppression of alternate bearing (Dodge 1966; Worley & Harmon 1969). Despite advances in

horticultural methods, alternate bearing remains one of the key problems in commercial pecan production (Conner & Worley 2000).

Venturia effusa and other pecan pathogens

Taxonomy and nomenclature. Pecan is susceptible to infection from many pathogens. The most economically important disease that affects pecan is pecan scab, caused by the fungal pathogen *V. effusa* (syn. *Fusicladium effusum*) (Figure 1.1). The pathogen belongs to the phylum Ascomycota, subphylum Pezizomycotina, class Dothideomycetes, order Pleosporales, and family Venturiaceae (Bock 2013; Index Fungorum 2019). *V. effusa* has been documented causing disease on *Carya tomentosa*, *C. aquatica*, *C. glabra*, *C. cordiformis*, *C. ovata*, and *C. illinoinensis*, which are all native to the southeastern U. S., however, *C. illinoinensis* is the only commercially cultivated species (Schubert et al. 2003). *V. effusa* was first discovered on the leaves of a mockernut hickory [*Carya tomentosa* (Lam.)] in 1882, in Illinois by F. S. Earle (Demaree 1928). The isolates were sent to Berlin, Germany, where the pathogen was named *Fusicladium effusum* by G. Winter in 1885 (Demaree 1928). In 1888, Ellis and Everhart (1888) described a fungus on *Carya illinoinensis*, which they named *Fusicladium caryigenum* because they believed it was a different species to the fungus described on *C. tomentosa* in 1885. In 1928, Demaree assigned the pecan fungus a new name, *Cladosporium effusum* (G. Winter) Demaree. Later, Gottwald (1982) determined that the fungus should be *Cladosporium caryigenum* (Ellis and Lang.) after observing the conidial morphologies and developmental characteristics. Based on additional information using molecular tools, Schnabel et al. (1999), Schubert et al. (2003) and Beck et al. (2005) concluded that the fungus was best classified as *F. effusum*. Crous et al. (2007) used modern molecular techniques to demonstrate that *F. effusum* was a member of the genus *Venturia*. Finally, in 2016, the name was changed to *Venturia effusa* by Rossman & Allen

(2016). Since there was extensive use of the generic term *Venturia, Venturia* was applied to the pecan scab pathogen in preference to the alternative generic synonym *Fusicladium* (Bock 2013; Rossman et al. 2016).

Reproductive biology. Under natural conditions *V. effusa* reproduction has been documented to be solely asexual. The asexual reproductive structures are conidia, which can be produced in scab lesions formed on the adaxial or abaxial sides of the leaves, the shucks, and the host's shoots. Conidia infect the host and cause disease. The conidiophores of this fungus are solitary, erect, occasionally branched, dark brown and ascending. The conidiophores are smooth, cylindrical, septate and have thick walls. Conidia are 22 to 130 µm long and 4 to 6 µm wide. The conidial cells are approximately 10 to 40 µm long, integrated and indeterminate with 1 or more denticle-like conidiogenous loci that are 1.5 to 3 µm wide (Demaree, 1928). The conidia undergo blastic formation into catenulate, branched chains that may contain more than 100 conidia that are produced from the base of the conidiophore. The conidia are pale olive-brown to brown in color. The shape of the conidia may be described as subcylindrical, pyriform, ellipsoid to fusiform, and typically have a smooth surface, although protuberant scars may be present. The conidia range from 10 to 24 µm in length and 5 to 10 µm in width (Gottwald 1982; Partridge & Morgan-Jones 2003; Schubert et al. 2003; Bock 2013). Although the sexual stage of *V. effusa* has never been observed in nature, a sexual structure has been produced *in vitro*. The sexual phase of *V. effusa* occurs as a pseudothecium producing ascospores. Although the sexual stage has never been reported in nature, the population genetic characteristics of the fungus, genetic diversity, and the mating type equilibrium being present at most scales in nature are compelling arguments for regular sexual recombination in the field (Young et al. 2018; Charlton et al. 2020).

Distribution and economic impact. *V. effusa* was first reported in the U. S. and is believed to have originated there. Due to dissemination with its host, *V. effusa* is now widespread in areas where pecans are grown and where weather for the pathogen is suitable, particularly those areas that are humid and wet during the growing season (Schubert & Braun 2002; CABI/EPPO 2021). *V. effusa* has been reported in the United States (Winter 1885), Mexico (Garza-Lopez et al. 1996), South Africa (Doidge et al, 1953; Crous et al. 2000), Argentina (Mantz et al. 2008), Brazil (CABI/EPPO 2021), Uruguay (CABI/EPPO 2021), Paraguay (CABI/EPPO 2021), China (CABI/EPPO 2021), and New Zealand (Pennycook 1989). Within the U.S., *V. effusa* is prevalent in the southeastern states where the warm, humid climate is conducive to the spread and survival of the fungus. Cole (1953) reported that *V. effusa* has been documented in all states where pecan is grown, including the more arid southwestern states; although *V. effusa* is rare in more arid regions. Infection with *V. effusa* results in reduction of nut size, quality, and yield on susceptible cultivars (Gottwald & Bertrand 1988; Sanderlin 1994; Stevenson & Bertrand 2001). Premature nut drop may occur, and complete yield loss is possible if the disease outbreak is early severe enough (Hunter 1983). Infection with *V. effusa* may result in reduction of net photosynthetic and dark respiration rates of foliage and fruit, reduction of nut number, reduction in nut weight, reduction in oil content, reduction in moisture content, and reduction in protein content (Gottwald & Bertrand 1983; Gottwald & Wood 1985). Infection of the leaves may result in premature defoliation and reduction in photosynthetic area. Severe outbreaks of scab may induce alternate bearing, which is the most important economic problem that that the North American pecan industry faces (Smith & Weckler 2011). The economic impact of *V. effusa* in pecan is most studied in North America, however, its presence in South Africa and South America indicates that it has the potential to cause epidemics in other pecan

production areas. Fungicides are the primary tool used to mitigate scab, but they are both economically and environmentally expensive (Gottwald & Bertrand 1988). The combined cost of the damage caused by, and control of this pathogen in the state of Georgia alone was estimated to be $78.7 million, $25.7 million, and $65 million and the estimated crop losses were 15%, 10%, and 12% in 2013, 2014, and 2015 respectively (Brock & Brenneman 2013, 2014, 2015). Thus, the combined economic impacts of *V. effusa* by increasing the cost of production and reducing crop yields threatens the sustainability of pecan production, and the heavy use of fungicides also impacts the environment.

Epidemiology. *V. effusa* overwinters as black stromata within lesions on twigs, shoots, leaves, and shucks from the previous season (Demaree 1924). Conidia are typically produced in early spring on stromata located on the previous season's lesions, which serves as the primary inoculum source for the disease and are primarily distributed by wind and rain (Demaree 1924; Gottwald 1982; Gottwald & Bertrand 1982; Latham 1982). The stromata produce the dark brown conidiophores which gives rise to chains of up to nine single-celled conidia that are light brown in color (Demaree 1928). A combination of rapid decreases in relative humidity, increases in temperature, and infrared radiation are factors that result in an increased abundance of air-borne conidia (Gottwald 1982; Gottwald & Bertrand 1982). In 1982, Gottwald and Bertrand observed that the aerial concentration of conidia peaked around mid-day, and typically displayed diurnal periodicity. Severe scab outbreaks on susceptible cultivars are especially common in locations with frequent rainfall, high humidity, and high temperature; however, pecans grown at higher altitudes tend to have less severe outbreaks of scab (Sparks et al. 2009). In dry areas where pecan production is only possible with irrigation, *V. effusa* is usually not a problem, even on susceptible cultivars. Infection anywhere within the tree canopy is possible; however, it is typically most

severe in the lower canopy probably due to rain-splash dispersing conidia from upper canopy to lower canopy, and conditions in the lower canopy being more conducive to disease development (Bock et al. 2013). The conidia are typically dispersed from overwintering shoot lesions and old shucks to the young leaves directly following bud break, and subsequently from lesions that form that season on developing leaves and fruit (Gottwald & Bertrand 1982). Temperatures ranging from 15 to 25°C with a period of leaf wetness lasting 10 to 48 hours is optimal for *V. effusa* infection. Because scab is polycyclic with a 7- to 18-day latent period, multiple generations of the fungus can occur within a season if the weather is compliant (Gottwald & Bertrand 1982; Turechek & Stevenson 1998). Conidial dispersal occurs from April to November, with the bulk of the dispersal occurring between June and October (Gottwald & Bertrand 1982).

Only the current season's host tissues are susceptible to infection, and conidia can germinate within 3 hours of inoculation under ideal infection conditions (Latham & Rushing 1988; Rushing & Latham 1991). Within 12 hours following inoculation, the hyphae that develop from the appressoria penetrate the host cuticle. Subcuticular hyphal growth occurs along the anticlinal walls of the epidermal cells and through the middle lamella of adjoining epidermal cells. Within 144 hours of germination, hyphal growth becomes intercellular, and bulbous, melanized cells emerge on the branches of the subcuticular hyphae. These cells erupt through the cuticle and provide a base from which conidiophores are produced. Approximately 168 hours after germination, the conidia develop. The foliage is most susceptible 7 to 24 days after emergence, however, fully expanded and mature leaves are mostly resistant to infection (Gottwald & Bertrand 1982; Turechek & Stevenson 1998). There is speculation that resistance in more mature leaves is due to a change in the structure of trichomes and plant derived phenolics (Wetzstein & Sparks 1983). There is little difference in conidial germination of *V. effusa* on

leaves from resistant, susceptible, or non-host cultivars; however, on resistant cultivars, no subcuticular development of hyphae has been observed, while on leaves of susceptible cultivars, subcuticular hyphal growth occurs (Yates et al. 1996). The fruit of pecan are susceptible to *V. effusa* infection throughout the season (Gottwald & Bertrand 1983). Gottwald and Wood (1984) found that fatty acids in the fruit negatively impacted the sporulation of this pathogen. Both the timing and severity of fruit infection dictate the degree of yield loss. Severe early nut infections can result in premature fruit abortion. There is a positive correlation between earlier fruit infection and higher yield loss (Gottwald & Bertrand 1983; Stevenson & Bertrand 2001).

V. effusa displays pathogenic diversity on its host (Demaree & Cole 1929; Converse 1960; Graves 1975; Conner & Stevenson 2004). Isolates of *V. effusa* are pathogenic to one or more cultivars of pecan, but not pathogenic to other cultivars of pecan (Converse 1960; Conner & Stevenson 2004). A comprehensive investigation of cultivar specificity has not been conducted. Furthermore, cultivars that were previously resistant to *V. effusa* are reported to have become susceptible due to adaptation of the pathogen to the resistance in that host cultivar (Goff et al. 1989; Goff et al. 1996).

Symptoms and signs. Pecan scab is easily detected and identified based on visual inspection for characteristic symptoms and signs of the disease that may develop on the current season's fruit, foliage, shoots, dormant buds, and catkins of the host. Typical symptoms include dark black lesions with a velvety appearance on the surface of the affected plant part (Nolen 1926; Demaree 1924, 1928; Littrell 1980; Goff et al. 1996). Symptoms are similar on all infected plant tissues. Premature defoliation and fruit abortion may occur if the infection is severe.

The mycelium of *V. effusa* is composed of septate, branched, olivaceous brown to brown-black hyphae that are, on average, 1 to 3 µm wide. When growing on plant tissue, the fungal

colony is hypophyllous and maculicolous. The stroma develop poorly on leaves, but become well developed on twigs and fruit shucks (Gottwald 1982; Partridge & Morgan-Jones 2003; Schubert et al. 2003). On the leaves, lesions are approximately 1 to 5 mm in diameter and are olive-brown to black. The small lesions may expand and coalesce to form larger, irregularly-shaped lesions. The lesions appear velvety or rough in appearance when conidial sporulation within the stromata occurs. Lesions are typically found on the abaxial surface of the leaf; however, they may be found on both sides of the leaf and in association with the veins or midrib (Demaree 1924; Bertrand 2002). As the lesions age, they harden and develop dark grey, silver, or brown spots that extend through the leaf in a translaminar fashion. The senescent lesions may separate from the leaf tissue and drop to the ground, resulting in visible holes in the older leaves. The lesions on the foliage serve as a source of inoculum for developing fruit and for that season's developing shoots (Nolen 1926; Demaree 1928; Schubert et al. 2003).

Pecan trees are usually used for nut production, therefore the greatest economic damage results from fruit infections, which occur between the first stages of fruit growth through shell hardening. Shuck infections begins as small, circular lesions that develop slowly and can reach diameters of up to 12 mm, however, most lesions stop expansion once they reach a diameter of 3 mm. These larger lesions are often attributed to early infection that expands with the fruit during the fruit growth phase. The color of the lesions is typically olive-brown to gray during the early stages. Once the lesions age and become larger, they develop an olive-brown color with a grey border which appears jagged and irregular shaped (Demaree 1924; Bertrand 2002). Under favorable conditions, the lesions can increase in diameter, coalesce, and develop a velvety appearance. Larger lesions may become brown, cracked, and slightly raised. If infection is severe, the pathogen may penetrate deeper and cause the shuck to adhere to the shell of the nut.

Particularly on fruit, black stromata may form on the lesions, producing a dark, velvety growth of conidiophores (Nolen 1926; Demaree 1928, Schubert et al. 2003). Severe infection may result in underdevelopment or premature abortion of the fruit. Infection occurring after shell hardening is considered to be a cosmetic issue rather than economically damaging (Demaree 1924; Gottwald & Bertrand 1983; Hunter 1983). *V. effusa* can infect pecan plants from the seedling stage to the end of a tree's life, which may exceed 100 years in a commercial setting (Nolen 1926; Demaree & Cole 1929).

On the current-season shoots, the disease appears as olive brown to black colored lesions that have an approximate diameter of 0.5 to 3 mm (Demaree 1924). The margins of the lesions may be moderately raised and contain a dark fungal growth in the center. Lesions on twigs may develop stroma that overwinter, developing conidia in the following spring, similar to the overwintering lesions on the shucks. The symptoms that develop on the dormant buds and bracts/pedicels of the catkins are described as modest and typified as smaller, black lesions (Nolen 1926; Demaree 1924; Demaree 1928). Young, actively growing foliar tissues are more susceptible to infection by *V. effusa,* but become more resistant as they mature and form a thick cuticular layer (Demaree 1924; Littrell & Bertrand 1981).

The identity of *V. effusa* can be confirmed by collecting conidia from lesions and observing them microscopically (Schubert et al. 2003). *V. effusa* can be isolated from the lesions and grown *in vitro* on potato dextrose agar (PDA), oatmeal agar, or other media (Barnes 1964). The fungal colonies are slow growing and take approximately 3 weeks to grow to approximately 2.5 cm in diameter on PDA. Modern molecular techniques including DNA sequencing may be used to confirm the identity of the fungus.

Management. While orchard management practices such as tree spacing, hedging, and improved air flow can help control scab epidemics, chemical control is undoubtedly the most widely used method to manage scab on susceptible cultivars of pecan. The first instance of fungicide utilization to mitigate scab was Bordeaux mixture (Demaree 1925; Large 1965). Bordeaux mixture is still used today, but mostly for organic pecan production. Modern fungicides have been successfully used since the 1960s to manage infection by *V. effusa* (Large 1965). In the U. S., there are now several mode of action groups of efficacious fungicides available for mitigation of scab and include guanidines, phosphonates, organotin compounds, methyl benzimidazole carbamates (MBCs), dithiocarbamates, demethylation inhibitors (DMIs), quinone outside inhibitors (QoIs), and, most recently, the succinate dehydrogenase inhibitors (SDHIs) (Fungicide Resistance Action Committee [FRAC] Code U12, P7, 30, 1, M03, 3, 11, and 7 respectively) (Brock et al. 2007b; Bock et al. 2012; Bock et al. 2013; FRAC 2021) (Table 1.1). In commercial pecan production, fungicides are typically applied using an air-blast sprayer. Pecan trees grow to heights of more than 30 meters, which poses an issue due to the limitations of even large orchard air-blast sprayer abilities to propel fungicides to such lofty heights. Results show that even powerful air-blast sprayers are only able to provide spray coverage for adequate disease control up to heights of 10 to 14 meters (depending on cultivar, orchard and season), leaving up to 66% of the tree with inadequate fungicide coverage (Bock et al. 2013). Aerial applications of fungicide is possible and may be necessary in areas where *V. effusa* is severe to obtain complete coverage of taller trees. Aerial application of fungicides does reduce disease severity in the upper canopy of tall trees, and may be particularly valuable at times when the ground is too wet for a ground-based airblast sprayer to enter the orchard. In mature orchards, aerial application does not generally provide complete coverage of the lower canopy, so effective

ground-applied sprays are still needed. However, the resulting coverage in the upper portions of the canopy from aerial application does ensure reduced disease at heights greater than 10 to 14 m (Bertrand & Brenneman 2001; Sumner 2004; Reilly et al. 2007; Bock & Hotchkiss 2020).

The guanidine group was first introduced in 1957 (Morton & Staub 2008). Dodine (*n*-dodecylguanidine acetate) is the only chemical in the group registered to control scab. The guanidines are designated by FRAC in the group U12, because they have an unknown mode of action, although it is speculated activity is due to cell membrane disruption. Dodine has been used to control various fungal diseases on crops as diverse as pecan, strawberry, apple, cherry, and pear, and is used as a protectant for prevention of scab (Brock & Brenneman 2015c; Sisler & Ragsdale 1981). The guanidines have a low to medium risk of resistance, however resistance management is still recommended. Dodine is recommended as a tank-mixing partner with other pre-pollination preventative fungicides for scab management, but it is used primarily as a post-pollination treatment where it can be effective when applied alone or as a mixing partner with other efficacious fungicides (Brock & Brenneman 2015c). There is very little to no translocation of guanidines within the plant, making it difficult to control fungal pathogens that are not in direct contact with the fungicide (Curry 1962). Although high levels of resistance to dodine have not been observed with *V. effusa*, it has, however, been documented in the closely related apple scab pathogen, *V. inaequalis* (Koller et al. 2005).

Phosphite fungicides (FRAC Code P7), also known as the phosphonates, are host plant defense inducers (FRAC 2021). Phosphite fungicides are composed of salts and esters of phosphorous acid ($HPO(OH)_2$). Plants absorb phosphite fungicides, which are incorporated into cells as phosphite ions ($H_2PO_3^-$). Phosphite ions also show direct fungitoxicity against certain pathogens (Fenn & Coffey 1984; Wilkinson et al. 2001), including *V. effusa* (Bock et al. 2012).

There are a few cases of phosphite sensitivity variation in pathogens, suggesting that resistance to the phosphites is possible (Wilkinson et al. 2001; Fenn & Coffey 1984; Brown et al. 2004). Phosphite fungicides exhibit symplastic mobility in most plants. The translocation in the phloem makes phosphites useful for controlling many root diseases including those caused by *Pythium* and *Phytophthora* species (Landschoot 2016). Phosphite fungicides are especially effective against oomycetes, but also have activity on some fungal pathogens, such as *V. effusa* (Bock et al. 2012, 2013a; Fenn & Coffey 1984; Kessmann et al. 1994; Jackson et al. 2000; Gozzo 2003; Miller et al. 2006; Ouimette & Coffey 1989; Percival et al. 2009). There are many commercial fungicide products within the group that are labeled for management of pecan scab. Phosphites are especially efficacious in controlling leaf scab (Brock & Brenneman 2015c).

Organotin compounds (FRAC Code 30) are inhibitors of oxidative phosphorylation and ATP synthase across the mitochondrial inner membrane (Ayoko et al. 2003; Sisler & Ragsdale 1981; von Ballmoos et al. 2004; FRAC 2018a). The group includes the three active ingredients fentin acetate, fentin chloride, and fentin hydroxide, all in the triphenyl tin compound group. Organotin compounds, have high fungistatic/fungicidal effects against many different species of pathogenic fungi (Dylag et al. 2010). There has been some documentation of resistance to organotin compounds, but they are currently classified as low to medium risk of resistance (Giannopolitis 1978; Brent 1995; Ioannidis & Karaoglanidis 2000; FRAC 2018a; FRAC 2019). Organotin fungicides are typically applied as a protectant to control various Plant Dis.s (Sisler & Ragsdale 1981). In 1967, fentin hydroxide, also known as triphenyl tin hydroxide (TPTH), was the first organotin fungicide to be labeled for use on pecan (Brock & Brenneman 2015c; Littrell & Bertrand 1981). Fentin hydroxide can be applied both pre- and post-pollination when used for

controlling or preventing *V. effusa*. The management recommendations are similar to those of dodine (Brock & Brenneman 2015c).

MBCs (FRAC Code 1), often referred to as the benzimidazoles, are systemic fungicides (FRAC 2018a). MBCs impede nuclear division by binding to the β-tubulin subunit and inhibiting the production of microtubules. Suppression of microtubule formation leads to disruption of mitosis during spore germination and hyphal growth, resulting in a fungistatic effect (Davidse 1973; Davidse 1986; Davidse & Flach 1978; Ishii 1992; Hollomon et al. 1998; Sisler & Ragsdale 1981). MBCs are considered as having high risk of resistance development (FRAC 2018a). Resistance has developed in many fungal species (Bollen & Scholten 1971; Wicks 1974; Yuan & Zhou 2005; Chung et al. 2009). For control of *V. effusa* on pecan, it is recommended to use the MBC thiophanate-methyl as a tank mix partner with either dodine or TPTH, but not as a stand-alone product (Brock & Brenneman 2015c; Stevenson 1998).

DMIs (FRAC Code 3) function by targeting and inhibiting the function of the sterol 14a-demethylase *CYP51* enzyme, which is an important enzyme involved with ergosterol biosynthesis and cell viability (FRAC 2018a). Rendering the *CYP51* region catalytically inactive prevents the demethylation of lanosterol and eburicol, which, in turn, inhibits the production of ergosterol, which is a major component in fungal membranes (Köller 1992; Kwok & Leoffler 1993; Price et al. 2015). The process ultimately results in the disruption of membrane structure and hinders active membrane transport due to the coupling of two factors: the buildup of 14a demethylated sterols, and the curtailment of ergosterol within the cell, resulting in a fungistatic effect (Price et al. 2015). The DMIs are considered to be at medium risk of resistance development, and resistance has been observed in various fungal species (de Waard et al. 1982; Köller 1988; de Waard & Nistelrooy 1990; Kendall et al. 1993; Golembiewski et al. 1995; Köller

1996; Hollomon et al. 1997; Gisi et al. 2000; FRAC 2018a). There are two subgroups of the DMI fungicides: the triazoles and the triazolinthiones. All but one of the DMI fungicides used in agriculture belong to the triazole subgroup. Although the results are similar in various fungi, triazoles vary in their activity. Triazoles do not inhibit spore germination because spores typically contain adequate levels of sterol needed for the development of germ tubes. In some cases, spores have sufficient levels of sterol to produce infection structures; therefore, in these situations, triazoles may not be effective against infection of the host tissue. DMIs have acropetal penetrant phytomobility, meaning that they are xylem mobile and move along a water-dependent gradient. The DMI fungicides labeled for use on pecan for scab control in the U. S. include tebuconazole, difenoconazole, mefentrifluconazole, propiconazole, tetraconazole, fenbuconazole, and metconazole. Flutriafol is also labeled for use on pecan, but not for scab control. DMI fungicides may be applied pre-pollination as stand-alone products or as tank-mix partners with dodine, a QoI, or TPTH for management of scab. When using DMIs for post-pollination scab control, it is recommended to use them in mixtures with dodine, a QoI, or TPTH (Brock & Brenneman 2015c).

QoI fungicides (FRAC Code 11) function through the inhibition of fungal mitochondrial respiration. They do this by binding to the Q_o-center on cytochrome b (complex III) and obstructing the transfer of electrons between cytochrome c_1 and cytochrome b, thus disrupting the fungal energy cycle by inhibiting ATP production (Bartlett et al. 2001; Bartlett et al. 2002; Fernandez-Ortuno et al. 2008; Gisi et al. 2002). The QoIs are highly efficacious against a wide range of crop diseases. Azoxystrobin in particular has historically had outstanding commercial success due to the fact that it controls Ascomycetes, Basidiomycetes, Deuteromycetes, and Oomycetes (Bartlett et al. 2001). When using QoIs for the management of pecan scab, they can

be used as pre-mix combinations, usually with a DMI, or used as a stand-alone product. Stand-alone QoI fungicides labeled for use on pecan in the United States include kresoxim-methyl, azoxystrobin, and pyraclostrobin. Pre-mix combinations of QoIs and DMIs labeled for use on pecan include azoxystrobin and tebuconazole, azoxystrobin and difenoconazole, azoxystrobin and propiconazole, and trifloxystrobin and tebuconazole (Brock & Brenneman 2015c). Tank mixtures of QoIs and DMIs are recommended for post-pollination control of *V. effusa*, while stand-alone products or tank mixtures of QoIs and DMIs may be recommended for pre-pollination control (Brock & Brenneman 2015c).

SDHIs (FRAC group 7) were first used in 1966 (FRAC 2018a). There are currently 17 SDHI compounds belonging to different chemical groups with various modes of action labeled for managing Plant Dis.s (Sierotzki & Scalliet 2013; FRAC 2018a). SDHIs are generally broad–spectrum with respect to biological activity, comparable to the QoIs; however, SDHIs lack activity on oomycetes (Sierotzki & Scalliet 2013). The SDHIs function by targeting the succinate dehydrogenase reductase protein within complex 2 of the mitochondrial membrane. The protein has four subunits: two hydrophobic subunits (SDHA and SDHB), and two hydrophilic subunits, (SDHC and SDHD). Within the protein, the fungicide binds to the ubiquinone-binding site (Q_p) located between the SDHB and SDHD to block access to the substrate and prevent succinate oxidation from cycling, which ultimately inhibits fungal respiration (Sierotzki & Scalliet 2013). There is medium to high risk of resistance associated with fungicides in the SDHI group and resistance has been observed in several pathogens (Georgopoulos et al. 1972; Van Tuyl 1975; Georgopoulos & Ziogas 1977; White & Thorn 1980; Grouet et al. 1981; Keon et al. 1991; Broomfield & Hargreaves 1992; Newcombe & Thomas 2000; Menzites et al. 2005; Avenot & Michailides 2007; Avenot et al. 2008; McGarth 2008; Stevenson et al. 2008; Miyamoto et al.

2009; Miyamoto et al. 2010; Stammler et al. 2011; Sierotzki & Scalliet 2013; FRAC 2018a). SDHIs are not yet widely used to control pecan scab, but will likely be used more extensively in the future.

A standard program for scab prevention in Georgia consists of 7 to 10 fungicide treatments per season, applied every 10 to 14 days beginning at bud break and lasting until pollination. From pollination to shell hardening, fungicide applications are recommended on 14- to 21-day intervals. The schedule and frequency of these fungicide applications depends on environmental conditions throughout the growing season and may be modified to fit the specific needs of an orchard (Brock et al. 2007a). Applications early in the season are primarily used to control leaf scab, while applications made later in the season are used to control fruit scab. Early infection of the fruit has proven to severely influence yield compared to infections occurring closer to harvest (Gottwald & Bertrand 1983; Stevenson & Bertrand 2001). During periods of frequent rainfall, spray intervals must be adjusted to every 7 to 10 days due to the increased risk of fungal infection. Interval reduction due to frequent precipitation has the potential to significantly increase the number of fungicide applications per season (Latham 1995). Fungicide applications to manage scab may also be scheduled using predictive models that utilize weather data (Brenneman et al. 1998; Brock et al. 2007a; Payne & Smith 2012). The weather-based predictive models are designed to provide a more sustainable yet effective fungicide application schedule, leading to fewer applications of fungicides in drier years compared to a calendar-based program (Brenneman et al. 1998). However, the time required to actually spray a large orchard (often 1-2 weeks) often limits the utility of advisories on a crop like pecan.

Biological control of pests and pathogens is becoming increasingly important in the quest to reduce pesticide use and the risk of resistance to pesticides, and is an important component of

integrated pest management systems. Biological control options for pecan scab have been limited in the past, however, *Bacillus mycoides* has shown some level of activity as a biological control agent. *B. mycoides* is a microbial agent that significantly reduces the severity of pecan scab by inducing a systemic acquired resistance response (Brenneman 2009). Bordeaux mixture has also proven to have some fungicidal effect on pecan scab (Bock et al. 2018). Another study tested multiple organic products against pecan scab for several years, and found that extract of Giant Knotweed and Bordeaux mixture appear to have great potential as organic fungicides for use against pecan scab. This same study found that, in some, but not all years of the study, applications of compost tea, sodium bicarbonate, *B. subtilis*, sulfur, and cuprous oxide were able to reduce scab infections compared to the nontreated control (Bock et al. 2019).

Cultural control measures are not considered effective for control of scab, however there are practices that may help reduce the risk and incidence of infection. When establishing a new orchard in humid areas or in areas where scab regularly occurs, it is important to ensure adequate tree spacing to maximize sunlight penetration and air flow in the tree canopy to reduce wetness duration and the risk of scab infection (Cooper & Johnson 1986). Planting resistant cultivars of pecan is one of the best ways to manage scab; however, there have been instances of resistant cultivars becoming susceptible over time (Goff et al. 1989; Goff et al. 1996). In the southeastern U. S., cultivars that display high levels of resistance to scab include Kanza, Elliott, Gloria Grand, Curtis, Excel, and Barton. Sumner displays moderate resistance. Cultivars that are especially susceptible to scab include Wichita, Schley, and Desirable, and should be avoided in areas that are wet or low-lying (Bock 2013). Research on the genetics of resistant cultivars suggests that the additive activity of multiple genes plays a role in scab resistance (Thompson & Grauke 1994). Recent efforts using RAPDs, AFLPs and SSRs have identified markers that may be useful

for selecting for scab resistance (Grauke et al. 2003; Beednagari et al. 2005). Using more modern molecular techniques, candidate genes for pathogen and pest resistance in pecan have been identified, which will likely contribute to future scab resistance breeding (Lovell et al. 2021). Establishing orchards in areas where *V. effusa* is uncommon due to unfavorable environmental conditions is another method of minimizing the risk and severity of scab. Removing sources of primary inoculum including the previous season's shucks, leaves, and fallen branches after harvest can aide in mitigating the spread of scab, although the polycyclic nature of the disease suggests that it may be of limited value (Bock 2013). Hedge pruning may also be implemented to reduce the height of the tree and allow better fungicide coverage (Bock et al. 2017). The general health of the tree may influence susceptibility to *V. effusa*. A study performed by Wood et al. (2012) suggests that adequate nickel availability may help reduce the severity of scab on susceptible trees. Reducing the spread of scab by preventing transportation of the pathogen or infected host could be effective in preventing long range dispersal; however, there are currently no specific regulatory control measures in place regarding the transportation of *V. effusa* other than the standard regulations regarding the import and export of plant material (Bock 2013).

Fungicide resistance

Farmers have been applying fungicides to crops to protect from infection by pathogens for over 200 years. The application of an aqueous solution of sodium chloride to the seed of wheat to protect against fungal invasion of *Tilletia caries* in the mid-18th century was the first recorded incidence of the application of a solution with the intent to control the growth of a fungus. The use of copper sulfate later proved to be effective in mitigating the growth of fungi. Bordeaux mixture for control of powdery mildew on grape was the beginning of widespread

usage of fungicides to mitigate fungal diseases (Brent 2012). Today, fungicides have become an integral part of food production and are widely used across the world to help increase crop yields, improve crop quality, and to ensure the stability of crop production (Lucas et al. 2015). There are 129 fungicide chemical groups with 10 known modes of action, several with multiple modes of action, and several with unknown modes of action (FRAC 2018a). The intense use of fungicides has inevitably led to resistance developing in many fungal pathogens to several fungicide classes. Fungicide resistance can be defined as "a stable, heritable trait that leads to a reduction in sensitivity to a fungicide" (McGrath 2015). Although fungicides have been used for hundreds of years, the first documented cases of fungicide resistance date back only to the 1960s and included reduced sensitivity to aromatic hydrocarbons in *Penicillium* species that cause storage rots of citrus, and resistance of the apple scab fungus, *V. inequalis*, to dodine (Brent 2012). One of the first cases of resistance in the U. S. was the documentation of reduced sensitivity of cucurbit powdery mildew (*Podosphaera xanthii*) to benomyl (McGrath 2001). Resistance to fungicides remained relatively uncommon until the introduction and wide usage of a novel classes of fungicides with specific modes of action in the 1970s (Brent 2012). Since the 1970s, the resistance of different pathogens to fungicides has increased (Lucas et al. 2015).

In cases of practical resistance to fungicides, the recommended rate of fungicide fails to control disease because the pathogen population has shifted to one that is mostly resistant. The phenomenon may occur in either a qualitative or quantitative manner (Brent & Hollomon 2007; FRAC 2016b). Qualitative resistance, also called single-step resistance, occurs when a single mutation develops in the fungal gene that encodes the target site of the fungicide that consequently prevents the fungicide from binding to the target. Over time, continued exposure to the fungicide results in two distinct sub-populations that differ sensitivity to the particular

fungicide, resulting in an abrupt loss of sensitivity to the fungicide (Brent & Hollomon 2007; FRAC 2016b). Quantitative resistance, also referred to as multi-step resistance, is the product of multiple mutations in the genome encoding the target site of the fungicide, each of which has a relatively small impact on the binding affinity between the fungicide and the target, which ultimately results in a gradual shift from a sensitive population to an insensitive population over a period of time (Brent & Hollomon 2007; FRAC 2016b). The likelihood and magnitude of resistance to fungicides largely depend on chemical, biological, and agronomic factors (FRAC 2013; Kuck 2005). For fungicides, spray coverage, application rate, formulation, mode of action, and frequency of usage all play a role in increasing the risk of resistance to fungicides (Dekker 1982; Hollomon 2015a). Inadequate spray coverage leads to poor disease control, resulting in a larger fungal population. A more intensive spray program will reduce the overall size of the pathogen population, thereby exposing fewer individuals to selection by the fungicide (Damicone & Smith 2009). However, increased fungicide dosage has been shown to increase selection for fungicide resistance in multiple studies. This is a direct consequence of eliminating the susceptible individuals from the population (Dekker 1982; Metcalfe et al. 2000; O'Hara et al. 2000; Genet et al. 2006; Mavroeidi & Shaw 2006; Russell 2009; Shaw 2009; van den Bosch et al. 2011). Exclusive and repeated use of fungicides with the same mode of action increases the risk of resistance by further selecting for resistant individuals of the pathogen. Using fungicides from multiple classes will help ensure that those individuals resistant to one mode of action will be controlled by the application of a different mode of action (Russell 1995; Lucas et al. 2015).

Many modern selective fungicides interrupt cellular processes and bind to specific target proteins; these fungicides are considered single-site or site-specific. Some fungicide classes act on multiple cellular processes and are referred to as multisite inhibitors (Lucas et al. 2015).

Single-site fungicides are usually highly active and are often systemic, controlling diseases effectively at low concentrations. After the application of a single-site fungicide, most of the pathogen population is eliminated or rendered incapable of completing their lifecycle, resulting in a selection for individuals that are resistant to that particular fungicide. Insensitivity to single-site fungicides can be the product of a single mutation in the genome, resulting in alteration of the target protein. For multi-site fungicides, insensitivity is the product of multiple mutations that affect multiple target proteins. In this way, it is more likely that pathogens will develop insensitivity to single-site fungicides than to multi-site fungicides. The mutations are usually heritable, which, over several generations, can result in entire pathogen populations that are resistant to a particular fungicide (Kent 1995; Lucas et al. 2015). Other pathogen qualities that affect the prevalence and evolution of fungicide resistance include the spatial and temporal abundance of inoculum, propagule dispersal methods, asexual vs. sexual reproduction, and the pathogen's lifecycle (monocyclic vs. polycyclic). For *V. effusa*, it has also been suggested that substantial biological variability in sensitivity to different fungicides is present within orchard-scale populations and spatio-temporal attributes of the populaitons may vary in two-dementional space (Standish et al. 2021). All pathogens differ and do not have the same risk for development of resistance (Hollomon 2015a).

Environmental and cultural factors summarize the agronomic risk associated with pathogen resistance to fungicides. Weather conditions including rainfall, temperature, humidity, and windspeed all influence propagule development, quantity, and dispersal (Kuck 2005). Cultural factors including irrigation, fertilization, tillage practices, use of resistant cultivars, and other sanitary measures may influence quantities of primary inoculum and disease development. Crop rotation is often a critical component of resistance management, but of course cannot be

utilized with a perennial crop like pecan. If fungicide resistant individuals are present in an area, the probability of fungicide resistance issues is increased (Kuck 2005).

Resistance of *V. effusa* to certain fungicides has been documented. Littrell (1976) was the first to report fungicide resistance in the pathogen. He found that practical fungicide resistance of *V. effusa* to the MBC fungicide, benomyl, was present in Georgia and Alabama. Since that initial discovery, there have been several reports of reduced sensitivity of *V. effusa* to the DMIs, including to tebuconazole, fenbuconazole, and propiconazole, and the organotin fentin hydroxide (Reynolds et al. 1997; Seyran et al. 2010a; Stevenson et al. 2004, 2015). The risk of *V. effusa* developing resistance to the active ingredients of fungicides labeled for scab management pathogen is considered to be from "low to medium" to "high" (Bertrand & Brenneman 2001; Bock et al. 2015; FRAC 2018a). The risk of resistance may be further exacerbated by the inability to provide sufficient spray coverage to tall pecan trees (Bock et al. 2013b). Other characteristics of *V. effusa* that may increase the risk of resistance to fungicides developing include its ability to overwinter, the polycyclic nature of scab, the ability to produce large quantities of inoculum (conidia), and the probable occurrence of sexual recombination (although this has not yet been observed in nature) (Demaree 1924; FRAC 2013; Young et al. 2018; Bock et al. 2017; Charlton et al. 2020). Application of fungicides is the primary management strategy used to control scab on pecan; therefore, it is imperative to monitor the efficacy of the fungicides and identify and mitigate any issues of resistance in *V. effusa* as they arise, providing an opportunity to preserve efficacy (Littrell & Bertrand 1981; Stevenson 1998; Brenneman et al. 1998; Stevenson et al. 2004; Isakeit 2010; Seyran et al. 2010a). Exploration of alternative disease management strategies is not only due to the desire to preserve fungicide efficacy and reduce the risk of resistance, but may also be justified by the need to minimize the use of fungicides for

health and environmental reasons (Schnabel & Parisi 1997; Agostini et al. 2003; Gozzo 2003; Percival & Haynes 2008; Percival et al. 2009).

Fungicide groups and characteristics. Guanidines inhibit spore germination and prevent sporulation of fungi. Dodine is the only fungicide in this group, and is used extensively in management of *V. effusa* (Isakeit 2010). Guanidines operate by multi-site mode of action, and are considered to be at moderate risk of fungicide resistance. The observed resistance is quantitative, but the exact mechanism of resistance is unknown. Resistance has been documented in the apple scab pathogen, *V. inaequalis*, which is closely related to *V. effusa* (Szkolnik & Gilpatrick 1969; Jones & Walker 1976; Köller et al. 1999; Carisse & Jobin 2010; Isakeit 2010). Seyran et al. (2010a) found reduced sensitivity of *V. effusa* to dodine in 12% of the commercial orchards sampled in 2008.

Phosphites are considered to be at low risk of resistance development (FRAC 2018a), and are thought to elicit systemically acquired resistance (SAR) in certain plant species (Guest & Grant 1991; Kessmann et al. 1994; Sticher et al. 1997; Becot et al. 2000; Jackson et al. 2000; Gozzo 2003; Percival et al. 2009; Miller et al. 2006). Bock et al. (2012) evaluated efficacy of phosphites in four field experiments over a two-year period. Both phosphites and TPTH reduced scab severity on foliage equally well, and gave equally good control of disease early in fruit development. However, late in the season scab severity on phosphite-treated trees was most often greater than those receiving TPTH. Sensitivity variability to phosphorous acid has been observed in isolates of *Phytophthora cinnamomi* (Wilkinson et al. 2001). Similarly, Brown et al. (2004) found insensitivity to the phosphite active ingredient, fosetyl-aluminum, in several species of oomycetes in commercial lettuce fields in California. Resistance to the phosphite fungicides has not yet been documented for *V. effusa*.

Organotin fungicides are multi-site inhibitors and are considered to be at low risk of resistance (FRAC 2018a). Resistance to TPTH, commonly referred to as fentin hydroxide, has only been documented in *Cercospora beticola*, isolated from sugar beets (Bugbee 1995; Bugbee 1996; Campbell et al; 1998; Ioannidis & Karaoglanidis 2000; Karaoglandis et al. 2003). Resistant isolates were less competitive than sensitive isolates as a result of the fitness cost associated with increased resistance and do not persist in nature once the use of the fungicide is terminated. The TPTH-resistant strains have developed cross resistance to oligomycin, which disengages oxidative phosphorylation, suggesting that the TPTH-resistant strains may have resistance to any fungicide that has a similar mode of action (Isakeit 2010; Sisler 2014). Resistance of *V. effusa* to the organotin fungicides has not yet been reported; however, reduced sensitivity to TPTH has been reported (Seyran et al. 2010a; Standish et al. 2018). One factor that may prevent the buildup of a population resistant to TPTH is the fact that the most highly insensitive isolates have an apparent fitness cost and do not persist from one season to the next (Standish et al. 2018). In 2008, Seyran et al. (2010a) found that 60% of samples treated with 30 μg/ml TPTH displayed reduced sensitivity.

MBC fungicides are single-site inhibitors and are considered to be at high risk of resistance development (FRAC 2016a; FRAC 2018a). Practical resistance to the MBC fungicides is quite common, and has been documented in approximately 80 different plant pathogens (FRAC 2018b). The mechanism of resistance is a nucleotide point mutation located within the β-tubulin gene, resulting in substitutions of amino acids within the target protein. Three different mutations have been described as the most common mechanisms of resistance. One substitution occurs at position 198 (E198A/G/K), when alanine, glycine, or lysine is substituted for glutamic acid. Other substitutions occur at position 200 (F200Y), where tyrosine is substituted for

phenylalanine, and at position 240 (L240F), where phenylalanine is substituted for leucine. The three substitutions have been found to individually confer resistance at variable levels due to a decreased affinity for fungicide binding at the target site (Koenraadt et al. 1992; Yarden & Katan 1993; Albertini et al. 1999; Lehner et al. 2015). Resistant isolates of various fungi to this fungicide class were later discovered in the U.S. (Giannopolitis 1978; Bugbee 1995; Campbell et al. 1998; FRAC 2016a). Cross resistance between fungicide products within the MBC class has also been documented in *Helminthosporium solani* isolates due to similar modes of action. Indeed, the cross-resistant individuals were resistant to all active ingredients within the MBC fungicide class (Cunha & Rizzo 2003). Interestingly, and according to Brent and Hollomon (2007), negative resistance has been documented, where isolates resistant to the MBC fungicides were more sensitive to the FRAC Code 10 fungicides (N-phenyl carbamates).

Reduced sensitivity of *V. effusa* to the MBC fungicides has been documented on multiple occasions. The first instance was documented in 1975, when benomyl-resistant isolates were found in Georgia and Alabama pecan orchards after only three consecutive years of benomyl application (Littrell 1976). Management of scab in the orchards was based on repeated applications of benomyl only, and approximately 25% of the samples contained resistant isolates (Littrell 1976). Insensitivity to thiophanate-methyl was detected in 2008, even after a significant reduction in usage. Nearly 18% of isolates from orchards in Georgia displayed reduced sensitivity when compared to isolates that had never been exposed to the product (Seyran 2010a).

The DMI fungicides are heavily used in commercial agricultural settings, including in pecan orchards for control of scab, due to their effectiveness against a wide variety of fungi and their relatively low cost (Price et al. 2015). They are considered to be at medium risk of

resistance development (FRAC 2018a). DMIs function by inhibiting sterol biosynthesis and are considered to be single site inhibitors. The triazoles are a subgroup of the DMIs, and contains the majority of the products used in scab management. Resistance is known in several fungal species, and many of the mechanisms of resistance are known. Cross resistance is present among triazoles that are active against the same pathogen (Hull et al. 2012). Although DMIs are sterol biosynthesis inhibitors, cross resistance to other sterol biosynthesis inhibitor classes, including the amines, the ketoreductase fungicides, and the sterol biosynthesis inhibiting (SBI) fungicides, has not been reported (FRAC 2018b).

Practical resistance to the DMI fungicides has been documented in 20 species of plant pathogenic fungi, and is often a consequence of repeated and exclusive applications of the fungicide class over time (Ma & Michailides 2005). The mechanism of resistance in these fungal species is typically attributed to overexpression of the *CYP51* enzyme, point mutations in the *CYP51* gene, or reduced accumulation of the fungicide within the fungal cells due to overexpression of genes encoding efflux transporters (Schnabel & Jones 2001; Ma et al. 2006; Price et al. 2015; Ziogas & Malandrakis 2015; FRAC 2016a).

Mutations in the *CYP51* gene are undoubtedly the most commonly documented mechanism of resistance in field isolates of DMI resistant pathogens. The binding affinity of the fungicide for the target enzyme is reduced as a result of the mutations, which leads to tolerance to the fungicide (Price et al. 2015). Some pathogens have developed multiple mutations in the gene, an example being *Zymoseptoria tritici*, which has developed over 30 different substitutions and deletions within the *CYP51* region (Cools & Fraalje 2013). Different combinations of mutations result in varying levels of sensitivity to the fungicide (Price et al. 2015). Prominent

target site mutations within the *CYP51* gene code for amino acid substitutions at V136A, I381V, Y137F, and A379G (FRAC 2018b).

Overexpression of the *CYP51* gene is not as commonly observed as point mutations, but it has been documented in several fungal pathogens, including *Monilinia fructicola* (Luo & Schnabel 2008a; Luo & Schnabel 2008b), *Pyrenophora tritici* (Stammler et al. 2009), *Phyllosticta brassicae* (Carter et al. 2014), *Z. tritici* (Cools et al. 2012), *Blumeriella jaapi* (Ma et al. 2006; Proffer et al. 2006), *V. inaequalis* (Schnabel & Jones 2001), and *Penicillium digitatum* (Hamamoto et al. 2000; Hamamoto et al 2001). Overexpression is a product of variations in the promoter region of the *CYP51* gene by the addition of tandem repeats or compatible elements (Price et al. 2015). It is postulated that amplified mRNA levels are associated with the resulting increase in cellular *CYP51* levels (target of the fungicide), resulting in decreased sensitivity to the fungicide (Price et al. 2015).

Overexpression of genes encoding the ATP-binding cassette (ABC) and major facilitator superfamily (MFS) transporters may also contribute to reduced sensitivity to fungicides by exporting them from within the cell to the extracellular space (Ziogas & Malandrakis 2015). The transporters, also called efflux pumps, are common in human pathogens, but there is only limited evidence of the mechanism for resistance in plant pathogens. Examples of plant pathogens using efflux pumps for resistance to the DMI fungicides include *Penicillium digitatum* (Nakaune et al. 1998; Nakaune et al. 2002) and *Botrytis cinerea* (Hayashi et al. 2001; Hayashi et al. 2003),

In 2003, monoconidial isolates of *V. effusa* were tested and were found to have reduced sensitivity to propiconazole (0.2 μg/ml) when compared to isolates collected 8 years earlier (Reynolds et al. 1997; Stevenson et al. 2004). Seyran et al. (2010a) found considerable reductions in sensitivity in 63% of their orchard samples treated with propiconazole (1.0 μg/ml)

compared to samples collected from orchards with no fungicide applications made within the last 30 years. In 2016 and 2017, Standish et al. (2018) used a similar *in vitro* bioassay to compare the efficacy of different fungicides and fungicide mixtures on the severity of *V. effusa* infection. Relative germination (RGe) of *V. effusa* and scab severity on both the leaves and nuts was significantly lower on trees treated with azoxystrobin, tebuconazole + azoxystrobin, or tebuconazole + TPTH when compared to the nontreated control and trees treated with only tebuconazole. The variable RGe was used because tebuconazole inhibits conidial germination, therefore it is an acceptable way to measure fungicidal activity for this particular fungicide/pathogen interaction. Reduced sensitivity *in vitro* was associated with increased scab severity in tebuconazole-treated orchards (Standish et al. 2018). Although the resistance of *V. effusa* to the DMI fungicides has been reported, the mechanism of resistance is unknown.

The QoIs inhibit fungal pathogens by impeding the ability of the fungus to make energy (Gisi et al. 2002; Gisi & Sierotzki 2008). QoIs are single site inhibitors and are considered to be at high risk of resistance development (FRAC 2018a). There are at least 50 known species of fungi that have developed resistance to QoI fungicides (FRAC 2018b). Prior to the use of QoI fungicides as an agricultural tool, resistance to QoIs due to several mutations in the *CYTB* gene occurred in a spectrum of organisms including mice, protozoa, yeast fungi, sea urchin, bacteria, and algae (Di Rago et al. 1989; Geier et al. 1992; Brasseur et al. 1996; Gisi & Sierotzki 2008). The first detection of resistance to the QoI fungicides in fungi was reported in isolates of *Blumeria graminis* f. sp. *tritici* (Bartlett et al. 2002). Subsequently, substitution of glycine by alanine at position 143 (G143A) was found in resistant isolates of *Pseudocercosporella fijiensis* (syn. *Mycosphaerella fijiensis*) and *B. graminis* f. sp. *tritici* (Sierotzki et al. 2000a, b). The substitution is an example of a single nucleotide polymorphism (SNP) in the *CYTB* gene. This

SNP is responsible for high levels of resistance, and sometimes complete resistance to the QoI fungicides (Gisi & Sierotzki 2008). The same mutation was later described in other important plant pathogens including *V. inaequalis*, *Pseudoperonospora cubensis*, *Plasmopara viticola*, and *Z. tritici* (Heaney et al. 2000; Steinfeld et al. 2002; Gisi et al. 2002). A second mutation was discovered, and is involves substitution of phenylalanine by leucine at position 129 (F129L) (Gisi & Sierotzki 2008). The F129L mutation was found in several plant pathogens, including *Pyrenophora teres* (Sierotzki et al. 2005) and *Pythium aphanidermatum* (Gisi et al. 2002). The mutation results in partial resistance, which reduces the effectiveness of the fungicide (Gisi & Sierotzki 2008). A third mutation that confers partial resistance to QoIs is a substitution of arginine for glycine at position 137 (G137R) (Gisi et al. 2007; Sierotzki et al. 2007; Fernández-Ortuño et al. 2008).

QoI fungicides are extensively used in the management of *V. effusa* in pecan orchards. There have been no reports of resistance of *V. effusa* to the QoI fungicides in the field; however, resistance may occur in the future if heavy usage of the QoIs within pecan orchards continues. Standish et al. (2016) assessed the risk of QoI resistance in *V. effusa* by characterizing a partial fragment of the *CYTB* gene. Sequence analysis of the fragment (1,919 bp) exposed the occurrence of a 1,407-bp intron positioned directly downstream of position 143. The intron was present in all 125 isolates included in the study from several different counties in the state of Georgia. There were no substitutions at position 129 or 143; however, glycine was exchanged for serine at position 137 in 7 isolates. The study provided strong evidence that the G143A mutation is unlikely to occur in *V. effusa* due to the presence of the intron; however, other mutations within the *CYTB* gene could lead to resistance in the future (Standish et al. 2016). The intron occurs in other pathogens, including, *M. fructicola, B. cinerea, M. laxa, Alternaria solani, P.*

teres, *Phyllosticta ampelicida*, and various species of rust fungi (Grasso et al. 2006a; Grasso et al. 2006b; Sierotzki et al. 2007; Banno et al. 2009; Luo et al. 2010; Miessner and Stammler 2010; Miessner et al. 2011).

The SDHI fungicides function by inhibiting cellular respiration. They are single-site inhibitors and are considered to be at medium to high risk of resistance development (FRAC 2018a). There have been 14 cases of fungal pathogens developing resistance to the SDHI fungicides due to different mutations in genes encoding the mitochondrial succinate dehydrogenase (*SDH*) enzyme. Reports of resistance to carboxins and associated potential mutations in *Ustilago maydis* and *Aspergillus nidulans* were found in the 1970s (Georgopoulos et al. 1972; Van Tuyl 1975; Georgopoulos & Ziogas 1977). The resistant isolates were selected following UV radiation on fungicide-amended agar. Results of this study indicated that mutations in the *SDH* genes acted as the mechanism of resistance (Georgopoulos et al. 1972; Van Tuyl 1975; Georgopoulos & Ziogas 1977). In more recent years, following increased utilization of SDHI fungicides, resistance has been observed in *A. alternata* (Avenot & Michailides 2007; Avenot et al. 2008), *B. cinerea* (McGrath 2008, Stammler et al. 2011, Walker et al. 2011), *Corynespora cassiicola* (Miyamoto et al. 2009), *Podosphaera xanthii* (Miyamoto et al. 2010), and *Stagonosporopsis spp.* (formerly *Didymella bryoniae*) (Stevenson et al. 2008). More than 30 mutations causing resistance to the SDHI fungicides have been identified in field populations of various pathogens (Sierotzki & Scalliet 2013). The only known mechanism of resistance for the SDHI resistant pathogens are mutations in the *SDH* genes. A Qo (exterior quinone oxidizing pocket) site SDHB histidine exchanged with a tyrosine is the most common mutation; however, alternative amino acid replacements were reported (Sierotzki & Scalliet 2013). Other enzyme subunit genes may contain mutations (*SDHC* and *SDHD*). Under field

conditions, multiple mutations appear to be selected in most pathogens; however, they seldom occur in the same isolate. Besides mutations in the *SDH* genes, no alternative mechanism of resistance has been reported for pathogens exhibiting resistance to the SDHI fungicides (Sierotzki & Scalliet 2013). Cross resistance among SDHIs has been detected in resistant isolates; however, patterns of cross resistance within this chemical class are complex due to the fact that many mutations confer complete cross resistance while others do not (Ishii et al. 2010; Scalliet et al. 2012). There is not yet any information on resistance of *V. effusa* to the SDHI fungicides due to the limited use of SDHIs in commercial pecan production.

Resistance to fungicides is becoming more prevalent and is a topic of major concern within the agricultural community. Fungicide resistance management and prevention is a major topic of study within the field of plant pathology, and in all pesticide-based research programs. In order to study resistance risk and prevalence, baseline sensitivity must be established. Identifying baseline fungicide sensitivity is particularly important to scientists working with fungicide resistance management; however, it is also a key task assumed by scientists working in the crop protection industry, and consumes a substantial portion of the registration process for all pesticides (Russell 2004). Baseline sensitivity can be defined as: “A profile of the sensitivity of the target fungus to the fungicide constructed by using biological or molecular biological techniques to assess the response of previously unexposed fungal individuals or populations to the fungicide” (Russell 2004). Practical utilization of baseline sensitivity creates a frame of reference for the accepted sensitivity of the pathogen to a fungicide. Pathogen populations or isolates with a sensitivity profile that is outside of the accepted baseline response are typically considered resistant or less sensitive to the fungicide under study (FRAC 1991; Russell 2004).

Justification and objectives.

Scab management is effectively accomplished by multiple preventative fungicide applications that are based on a calendar schedule (Brock et al. 2007a). The combination of repeated applications of the same fungicides or fungicides with the same mode of action combined with the high reproductive capacity of *V. effusa* makes the fungus a prime candidate for developing resistance to fungicides (FRAC 2013a). There have been a few instances of reduced sensitivity developing to modes of action that are key components of the fungicide application program for *V. effusa* (Littrell 1976; Stevenson et al. 2004; Seyran et al. 2010a; Standish et al. 2018). Maintaining the efficacy of existing fungicide classes that are used and managing resistance is a major concern for the pecan industry.

V. effusa is the most economically significant pest to commercial pecan growers in the southeastern U.S., and control of the pathogen is often the farmer's largest operating cost. The objectives of this research aim to better understand fungicidal activity on *V. effusa* and to improve the effectiveness of fungicide rotations and pre-existing resistance management plans that are used to combat this pathogen. The specific objectives are to (1) evaluate the efficacy of currently used and new fungicides on pecan scab and evaluate the efficacy of pre-mixed fungicides and their individual components, (2) determine the inherent activity and develop baseline sensitivity of *V. effusa* to pydiflumetofen, (3) identify the resistance mechanism(s) of *V. effusa* to the DMI fungicides, (4) Evaluate the response of *V. effusa* to applications of different fungicide classes with sulfur as a mixing partner, and (5) evaluate pecan cultivar sensitivities to *V. effusa* within orchards with similar histories of fungicide exposure.

To accomplish these objectives, field and lab experiments were conducted during 3 growing seasons (2019, 2020, and 2021). The methods included: (1) Conducting field trials in

several orchards across southern Georgia and incorporating older and newer combination fungicides as well as their individual components into the spray regime to investigate relative contribution and efficacy of these treatments. (2) Utilize isolates baseline isolates stored since 1996 that have never been exposed to SDHI fungicides for *in-vitro* sensitivity testing to various concentrations of pydiflumetofen (an SDHI) to determine discriminatory concentrations effective enough to inhibit growth of 50% of the population (EC_{50}). (3) Screening for amino acid changes within the *CYP51* gene and investigating the potential for overexpression of the *CYP51* gene which may lead to resistance to the DMI fungicides, as well as test various DMI fungicides in the field and tebuconazole in the lab using a rapid assay (Seyran et al. 2010a). (4) Conducting field trials in several orchards across southern Georgia and incorporating various fungicides belonging to different fungicide classes applied with and without sulfur to evaluate sulfur as a mixing partner for control of leaf and nut scab. (5) Take isolates from orchards containing multiple different cultivars with a history of exposure to the same fungicide programs and screen those isolates for the G137S mutation (Standish et al. 2016) which leads to QoI resistance, and also test them for sensitivity to different active ingredients such as tebuconazole, fentin hydroxide, thiophanate-methyl, and dodine.

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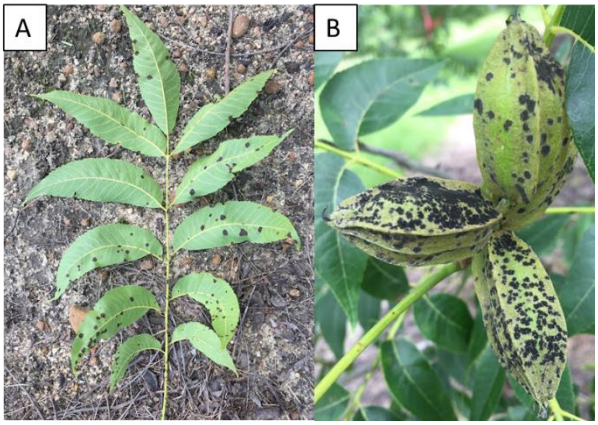

Figure 1.1. Symptoms of scab caused by *V. effusa* on pecan leaves (A), and pecan fruits (B).

Table 1.1. List of fungicide classes labeled for use on pecan for mitigation of *V. effusa*.

FRAC Group	*Fungicide Class*	*A.I.s Available for Pecan Growers*	*Resistance in V. effusa*
P07	Phosphites	phosphorous acid	None
1	Methyl Benzimidazole Carbamates (MBCs)	thiophanate-methyl	first reported in 1976 (Littrell 1976)
U12	Guanidines	dodine	First reported in 2010 (Seyran et al. 2010a)
30	Organotins	triphenyltin hydroxide	First reported in 2010 (Seyran et al. 2010a)
11	Quinone Outside Inhibitors (QoIs)	azoxystrobin, kresoxim-methyl, pyraclostrobin, propiconazole, trifloxystrobin	First reported in 2019 (Standish et al. 2019a)
3	Demethylation Inhibitors (DMIs)	tebuconazole, propiconazole, difenoconazole, mefentrifluconazole, fenbuconazole, tetraconazole, metconazole, flutriafol	First reported in 2010 (Seyran et al. 2010a)
7	Succinate Dehydrogenase Inhibitors (SDHIs)	pydiflumetofen, fluopyram	none
M03	Dithiocarbamates	ziram	none

CHAPTER 2

BASELINE SENSITIVITY OF *VENTURIA EFFUSA* TO PYDIFLUMETOFEN AND THE RELATIVE CONTRIBUTION OF FUNGICIDES APPLIED ALONE OR AS MIXTURES FOR MANAGEMENT OF PECAN SCAB

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Abstract

Pydiflumetofen, a new succinate-dehydrogenase inhibiting (SDHI) fungicide, was recently introduced to the market and was labeled for use on pecan in late 2019. The primary disease of pecan is scab caused by *Venturia effusa*. There are a variety of fungicides labeled for use on pecan to control scab, but this is the first SDHI to be widely used. With the known potential for pathogens to develop resistance to the SDHIs, there is a need to document the pre-exposure sensitivity of *V. effusa*. A set of 50 isolates collected from a baseline orchard in 1993 and stored in sterile water was selected for use in the study. Mycelial growth of the isolates on agar amended with 9 concentrations of pydiflumetofen ranging from 0.0002 to 6 µg/ml was measured. EC_{50} values ranged from <0.0002 µg/ml to 0.0307 µg/ml and the average EC_{50} value was 0.0011 µg/ml. Pydiflumetofen is used on pecans as Miravis Top, a premix with the demethylation inhibitor (DMI) fungicide, difenoconazole. To validate efficacy in the field of individual and combinations of fungicides, the formulated products Miravis (pydiflumetofen), Inspire (difenoconazole), Miravis Top (pydiflumetofen + difenoconazole), Abound (a Quinine outside inhibitor [QoI], azoxystrobin) and Amistar Top (difenoconazole + azoxystrobin) were applied to replicated pecan terminals on unsprayed trees in 11 orchards across South Georgia in 2019 and 2020. The mean efficacy (% control) with Miravis, Inspire, Miravis Top, Abound, and Amistar Top was 96.5%, 74.0%, 97.8%, 45.5%, and 78.9%, respectively. The excellent activity shown by pydiflumetofen applied alone or in mixes reflects the high level of *in vitro* sensitivity of *V. effusa*. The activity of Amistar Top, the previous industry standard, was less consistent, most likely due to the lower level of control contributed by azoxystrobin, a QoI. Resistance to the QoIs has been documented previously and appears to be widespread in some orchards with resulting loss of efficacy. Fortunately, the rate of difenoconazole in Miravis Top was very active

against scab at all locations, thus providing a strong companion to pydiflumetofen to help prevent resistance to this new and valuable class of fungicides.

Introduction

Pydiflumetofen (Syngenta Crop Protection, Greensboro, NC), a novel succinate-dehydrogenase inhibiting (SDHI) fungicide, has recently been labeled for use on pecan against scab, caused by the plant pathogenic fungus *Venturia effusa* (G. Winter) Rossman & W. C. Allen. *V. effusa* is the most economically significant disease affecting pecan (*Carya illinoinensis* (Wangenh.) K. Koch) production in the U. S. The fungus produces abundant conidia and has a high reproductive potential, so mitigating the spread and development of *V. effusa* early in the growing season is crucial for preventing yield loss (Gottwald & Bertrand 1982). Yield loss occurs through either reduction in photosynthetic capacity caused by leaf scab, or direct loss associated with scab on the fruit causing reduced size, premature fruit drop, or causing 'stick-tights' where the shucks cling to the fruit, precluding nut processing (Demaree 1928). *V. effusa* can be managed by the use of resistant cultivars, cultural methods, or fungicides. Although the use of resistant cultivars has been widely adopted, *V. effusa* is pathogenically and genetically diverse, which has resulted in some resistant cultivars becoming susceptible as the pathogen adapts to the resistance (Demaree and Cole 1929; Sparks 1992; Conner and Stevenson 2004; Bock et al. 2017). Chemical control is the most widely used and effective method for mitigating scab (Brock and Bertrand 2007a). Control of *V. effusa* in commercial pecan orchards typically consist of 7 to 10 fungicide applications per season, at 10 to 21-day intervals, depending on the weather and growth phase of the tree (Brock et al. 2007). In the United States, several classes of fungicides are labeled for use on scab, including phosphites, guanidines, organotin compounds, methyl benzimidazole carbamates (MBCs), quinone outside inhibitors (QoIs), demethylation

inhibitors (DMIs), and most recently, the succinate dehydrogenase inhibitors (SDHIs) (Fungicide Resistance Action Committee [FRAC] Code P7, U12, 30, 1, 11, 3, and 7 respectively) (Brock et al. 2007; FRAC 2021; Standish et al. 2021).

The SDHI fungicides are in FRAC group 7, and act by targeting the succinate dehydrogenase (*SDH* complex II) enzyme in the mitochondrial respiration chain, which is a critical component of the tricarboxylic cycle and is connected to electron transport within the mitochondria (Keon et al. 1991). *SDH* complex II is comprised of four subunits (A, B, C, and D), and the binding site of SDHI fungicides is the ubiquinone binding site, located within subunits B, C, and D (Stammler et al. 2007a). The SDHI fungicides are considered to be of high risk to resistance development, and resistance has been observed in many different pathogens (Keon et al. 1991; Avenot et al. 2008; Stevenson et al. 2008; Miyamoto et al. 2009; Bardas et al. 2010; Miyamoto et al. 2010; Ishii et al. 2011; Avenot et al. 2012; Gudmestad et al. 2013; Sierotzki & Scalliet 2013).

Fluopyram was the first SDHI fungicide to be labeled for use on pecan and has good activity against scab. However, fluopyram has not been widely used, primarily due to its relatively high price. Recently (since late 2019), the SDHI pydiflumetofen (Miravis Top) has been available to pecan growers, and is already well established as a premium product for post-pollination sprays due to its high level of activity and competitive price. Currently there is no known resistance in *V. effusa* to SDHIs. However, due to the high risk of resistance development of fungi to the SDHI fungicides and based on SDHI resistance that has developed in other pathogens (Avenot et al. 2008; Avenot et al. 2012; Keinath, 2012; Gudmestad et al. 2013; FRAC 2021), resistance development in *V. effusa* to the SDHI fungicides is a serious concern. Thus, it is critical to develop baseline sensitivity of *V. effusa* to pydiflumetofen to

monitor the status of any sensitivity shifts in the population in the future. Pydiflumetofen is currently among the most effective fungicides used to control *V. effusa* in commercial pecan orchards, and can legally be applied up to 4 times per growing season with a minimum application interval of 14 days. Current recommendations are to use other, more systemic products (such as phosphites) prepollination, and subsequently alternate Miravis Top with protectant fungicides including the organotin, triphenyl tin hydroxide (TPTH) and the guanidine, dodine to prevent over-exposure to any single mode of action.

Resistance has been documented to several different classes of fungicides currently labeled and used on pecans (Littrell 1976; Reynolds et al. 1997; Seyran et al. 2010; Stevenson et al. 2015: Standish et al. 2019). In the mid-1970s, the first documented case of *V. effusa* developing resistance to a fungicide was recorded in pecan orchards in Alabama and Georgia in relation to reduced sensitivity of *V. effusa* to benomyl, an MBC fungicide (Littrell 1976). Later, in 2008, and in 2015, reduced sensitivity of *V. effusa* to fentin hydroxide (TPTH) was documented (Seyran et al. 2010; Stevenson et al. 2015). In the early 2000s, resistance of *V. effusa* to the DMI fungicides was documented both *in-vitro* and *in-vivo* (Stevenson et al. 2004; Seyran et al. 2010; Stevenson et al. 2015). More recently, resistance of *V. effusa* to the QoI fungicides has been documented both *in-vitro* and *in-vivo*, and was found to be associated with a single nucleotide point mutation, a G137S substitution in the *cytochrome b* gene (Standish et al. 2019; Herrington, 2019).

Multiple risk factors contribute to the likelihood of fungicide resistance developing in *V. effusa*, one of the more significant being the large tree size (which can exceed 30 M) which makes adequate fungicide coverage almost impossible (Bock et al. 2015), resulting in a gradient in disease in fungicide treated trees (Bock et al. 2013, 2016, 2017). Other risk factors include

having a long spray application season each year (5 months), the fact that the pathogen can reproduce rapidly and is aerially dispersed, the perennial nature of the crop, and the recent finding that the pathogen is capable of sexual reproduction (Bock et al. 2017; Charlton et al. 2020; Young et al. 2018). The crop's perennial nature and longevity means that no crop rotation is possible, and the same pathogen population is resident on the same trees year after year and repeatedly subject to any fungicide selection applied. Considering these factors, and the track record of resistance developing to the SDHIs in other pathosystems (Avenot and Michailides 2007; Avenot et al. 2008a; Miyamoto et al. 2009; Amiri et al. 2010; Avenot and Michailides. 2010; Ishii et al. 2011; Wharton et al, 2012; Avenot et al. 2012), there is a real risk of SDHI resistance developing in *V. effusa*.

In an effort to prevent the development of fungicide resistance in *V. effusa*, or to manage populations with various levels of fungicide sensitivity, many of the sprays applied to mitigate pecan scab are combination products with more than one active ingredient, each representing a different mode of action. The sprays may be tank mixes of individual products, or commercial premixes. The underlying concept of using combination products is that if an isolate develops resistance to one of the modes of action (MOAs), it will be suppressed by the other MOA in the product. Mikaberidze et al. (2014) conducted an experiment to determine if high-risk fungicides could be used in mixtures without selecting for fungicide resistance. They found that fitness cost to the pathogen played a significant role. If fitness costs were absent, then the high-risk component would eventually become ineffective, but if significant fitness costs were associated with resistance development, the high-risk fungicide would maintain its efficacy within the mixture. There have been other studies that addressed the question of whether fungicide mixtures or alternating applications of single MOA are the most effective way to prevent fungicide

resistance. One study found that alternating fungicide MOAs was superior for mitigating resistance development compared to using combination products (Kable & Jeffery, 1980). However, other studies have concluded that combinations of fungicides with different MOAs are better at mitigating resistance development than alternating the MOAs (Skylakakis 1981; Hobbelen et al. 2013). Regardless of the method employed, it is widely agreed that spray programs need to include multiple MOAs as a foundation for fungicide resistance management. Since resistance has been observed to many of the active ingredients used to suppress *V. effusa*, it is critical to determine the relative contribution of each individual active ingredient within the combination products. Using active ingredients that the pathogen has developed resistance to provides little protection to a companion "at risk" fungicide.

One way to ensure that a single active ingredient is not used exclusively is to formulate it as a premix with another fungicide of a different MOA. One of the most effective scab fungicides used on pecans is Amistar Top, a premix of azoxystrobin and difenoconazole. However, resistance issues with both Group 11 and Group 3 fungicides have raised concern about the continued efficacy of Amistar Top. In late 2019, Miravis Top was labeled for use against *V. effusa* on pecan. Miravis Top also contains difenoconazole, but it is partnered with pydiflumetofen. Miravis Top is now being used extensively in commercial pecan production; therefore, baseline sensitivity data are essential to monitor any shifts in sensitivity in the population in the future. Also, an understanding of the scab control provided by Miravis Top and the companion fungicides in the field is needed. Thus, the objectives of this study are to develop baseline sensitivity data of *V. effusa* to pydiflumetofen, and establish efficacy of Miravis Top and Amistar Top and their component fungicides for controlling scab.

Materials and Methods

Sampling locations and fungal cultures. In 1993, samples of pecan leaves with actively sporulating lesions of *V. effusa* were collected from orchards in Troup and Jeff Davis counties in Georgia. The orchards were completely unmanaged (i.e. no pesticide applications), with few if any commercial pecan production sites within a 50-mile radius. The orchards served as an excellent source of isolates for baseline studies in 1993 due to the fact that the trees had not been exposed to fungicides, and there was minimal risk of *V. effusa* being blowing in from sprayed orchards. The cultivar sampled was Schley, which is highly susceptible to infection by *V. effusa*. Single lesions were removed from leaves using a cork borer, and were gently dabbed onto plates containing potato dextrose agar (PDA) with the sporulating side of the lesions facing down and were incubated in the dark at 25°C for 18 to 24 h. Individual germinated conidia were then transferred to plates of PDA containing 50 µg ml^{-1} each of streptomycin sulfate, chloramphenicol, and tetracycline to inhibit bacterial contamination. The cultures were incubated at 25°C for approximately 16 weeks. Sections of each culture were removed with a scalpel and placed in glass vials containing sterilized water, and were sealed with lids and securely wrapped with parafilm for long-term storage in 1996. These were the same isolates used for determining sensitivity of *V. effusa* to propiconazole and fenbuconazole *in vitro* by Reynolds et al. in 1997. The cultures remained in long-term storage in water for 24 years. In 2020, small fragments of the cultures were removed from storage, and were placed onto PDA amended with antibiotics (50 mg/L streptomycin sulfate, tetracycline, and chloramphenicol) in Petri dishes. These historic monoconidial isolates were chosen due to the fact that they have never been exposed to SDHI fungicides, or to any other fungicides. The isolates are the oldest and most fungicide-naive *V.*

effusa isolates known that are available for use, and are ideal for baseline sensitivity establishment even though SDHIs have been commercially used for over two years.

Fungicide sensitivity assay. In total, 50 monoconidial isolates of *V. effusa* from the 1993 sample were randomly selected from a larger set in long-term storage and were used to determine baseline sensitivity to pydiflumetofen. Technical grade pydiflumetofen (99.5% a.i.; Chem Service, Inc., West Chester, PA) was dissolved in acetone to obtain a stock solution of 3 mg/ml. Pydiflumetofen concentrations of 6.0, 2.0, 0.6, 0.2, 0.06, 0.02, 0.006, 0.002, and 0.0002 µg/ml within the media were obtained though serial dilution and were added to autoclaved PDA once the temperature of the PDA cooled to at least 45°C. The final concentration of acetone within the fungicide-amended and non-amended (acetone only) medium was 0.1% by volume.

The isolates of *V. effusa* were grown on PDA and incubated at 25°C for 6 weeks in the dark. After 6 weeks, the colonies were cut from the agar and homogenized in 1 ml of autoclaved water to provide a uniform suspension of mycelial fragments. Using a 4-mm diameter cork borer, wells were made in the fungicide-amended and non-amended media, and 20 µl of the mycelial suspension was added to each well in the fungicide amended and non-amended plates. The plates were incubated in the dark at 25°C for 4 weeks. Following the incubation period, the diameter of each fungal colony was measured, and the diameter of the well was subtracted so that the mean adjusted colony diameter could be determined for each isolate and fungicide concentration. Relative growth (RGr) values for each isolate and fungicide concentration were calculated as the ratio between the adjusted colony diameter on fungicide-amended medium and the adjusted colony diameter on non-amended medium. There were 2 reps of each isolate per fungicide concentration, and the experiment was repeated to verify the results.

Field efficacy experiment. In both 2019 and 2020, a total of 11 pecan orchards were selected to test efficacy of various fungicides. The pecan orchards were located in Berrien, Crisp, Dougherty, Lanier, Sumter, Tift, and Wilcox counties in southern Georgia. The orchards varied in age, tree spacing, disease pressure, and cultivar. Scab susceptible cultivars were selected, including Cunard, Desirable, Pawnee, and Wichita. The experimental design in each orchard was a randomized complete block design. Each location contained 8 consecutive trees that were left untreated from fungicides, but not from their regular insecticide routine, from nut set to harvest. Each tree at a location represented a block and contained one replicate of each of 6 different treatments. Thus, the treatments were applied directly to individual fruiting terminals on the pecan trees that were flagged with different colored ribbons to indicate which treatment the terminal received. Each terminal contained one or more clusters of pecans. The treatments were applied by spraying to initial runoff using a handheld sprayer (Project Source model #5318). The six treatments were: Inspire (difenoconazole; Syngenta Crop Protection, Greensboro, NC) applied at 490 milliliters per hectare (ml/ha), Abound (azoxystrobin; Syngenta Crop Protection, Greensboro, NC) applied at 797 ml/ha, Amistar Top (difenoconazole + azoxystrobin; Syngenta Crop Protection, Greensboro, NC) applied at 1001 ml/ha, Miravis (pydiflumetofen; Syngenta Crop Protection, Greensboro, NC) applied at 380 ml/ha, Miravis Top (pydiflumetofen + difenoconazole; Syngenta Crop Protection, Greensboro, NC) applied at 1001 ml/ha, and a non-treated control. Amistar Top was selected because it was the previous industry standard for controlling scab in commercial pecan production and is widely used. Miravis Top was selected because it is the labeled product that contains pydiflumetofen, and Miravis was used because it is a stand-alone pydiflumetofen product. Inspire was selected because difenoconazole is one of the components of both Amistar and Miravis Top, and Abound (azoxystrobin) is one of the

components of Amistar Top. Amistar Top and Miravis Top were applied at the maximum labeled rates. Miravis, Abound, and Inspire were applied to deliver the same amount of active ingredient as were in the Miravis Top and Amistar Top (i.e. applying 490 ml/ha of Inspire achieves the same volume of difenoconazole as applying 1001 ml/ha of Amistar Top). The fungicides were diluted to an equivalent spray volume of 946.3 L/ha which is typical of a commercial air-blast sprayer. Treatment applications were not initiated until after pollination to ensure that terminals selected for treatment were fruiting. The treatments were applied on a bi-weekly basis until harvest, resulting in a total of 7 and 8 applications in 2019 and 2020, respectively. Disease severity was rated visually by estimating the percentage (0-100%) of the surface of each fruit on the terminal that was covered with scab lesions several weeks after the final application. All disease ratings were conducted by one person to avoid statistical error.

Data Analysis. The statistical software SAS V9.4 (SAS Institute, Cary, NC) was used for all analyses. To calculate EC_{50} values in the baseline study, the Univariate procedure was used to first examine the distribution of the data. Second, EC_{50} values for each isolate were calculated by regressing the percentage of fungal growth inhibition against the logarithmic value of pydiflumetofen concentration by using PROC REG. There was not a normal distribution, therefore the data were log-transformed and a Shapiro-Wilk test was conducted to test normality. The distribution of EC_{50} values were lognormal, therefore the antilog of the log-transformed EC_{50} values were calculated to provide the geometric mean EC_{50}. Coefficient of variation was reported in the PROC TTEST statement and represents standard deviation.

The data from the field experiments were first analyzed using the Univariate procedure to check for normality. Data were combined across year and location for most treatments. Data were analyzed separately by location for the Abound treatment due to a significant interaction of

treatment*location caused by the variability of efficacy across locations. Locations containing little (<10% scab severity) to no disease pressure on nontreated terminals were excluded from the dataset. The data were analyzed using a generalized linear mixed model with the GLIMMIX procedure to check for effects of treatments, locations and interactions. The treatment mean 95% confidence interval were calculated. Random effects were block, location, and cultivar. A Tukey-Kramer test was conducted as the mean separation procedure. To test for associations between the single active ingredient products and combination products, a correlation analysis was conducted across locations using PROC CORR.

Results

Baseline sensitivity assay. Many of the historic scab isolates were viable even after storage in sterile water for 24 years. The calculated EC_{50} values for the 50 isolates ranged from <0.0002 µg/ml to 0.0307 µg/ml, with a mean EC_{50} value of 0.0011 µg/ml (Table 2.2), showing that baseline isolates of *V. effusa* are highly sensitive to pydiflumetofen. Frequency distributions were not normally distributed (Figure 2.1). We found that the baseline sensitivity of *V. effusa* to pydiflumetofen displayed a fairly broad range of EC_{50} values, but even the highest values were very low compared to sensitivity to many other fungicides. The coefficient of variation of *V. effusa* EC_{50} values to pydiflumetofen was 130%, indicating moderate to high levels of variability in the sensitivity of *V. effusa* to pydiflumetofen.

Field experiment. Symptoms of nut scab were first observed in the orchards in early to mid-June in both 2019 and 2020. In 2019, environmental conditions were reasonably conducive for *V. effusa* growth and development, with plentiful and frequent rainfall, and high temperatures/humidity in southern Georgia. Disease pressure varied significantly from location to location, with nut scab severity on the untreated terminals ranging from 0-100% at the

different locations (P > 0.0001) (Figure 2.2; Table 2.1). Cultivars were different at some locations and likely contributed to some of the observed differences in addition to those due to environmental factors, with more northern orchards in the region having inherently lower scab pressure.

Miravis and Miravis Top were the most efficacious treatments to reduce scab, while Abound was usually the least efficacious (Table 2.3). Applications of Inspire resulted in a mean of 71% and 79% control of *V. effusa* across the locations in 2019 and 2020, respectively; Abound resulted in a mean of 51% and 48% control in 2019 and 2020, respectively; and Amistar Top resulted in 72% and 85% control in 2019 and 2020, respectively, suggesting that difenoconazole is the active ingredient that is the most efficacious in the Amistar Top combination product. This observation is likely due to some of the orchards having populations of *V. effusa* displaying resistance to azoxystrobin, thus rendering Amistar Top less effective. Control with Inspire was highly correlated with Amistar Top ($r = 0.9555$; $p = 0.0008$) compared to Abound ($r = 0.7702$; $p = 0.0428$). Efficacy of azoxystrobin was highly variable among locations, ranging from a mean of 2% to 90% control for individual orchards (Figure 2.3). There was a significant interaction of treatment × location due to the variability associated with azoxystrobin ($P < 0.0001$) (Table 2.1). Control using Miravis was 93% and 99% in 2019 and 2020, respectively; control with Inspire was 71% and 79% in 2019 and 2020, respectively, and with Miravis Top was 97% and 98% in 2019 and 2020, respectively, indicating that pydiflumetofen is the more effective active ingredient in Miravis Top (Figure 2.4). Control using Miravis Top was highly correlated with the control achieved using both Inspire ($r = 0.8473$; $p = 0.0161$) and Miravis ($r = 0.7423$; $p = 0.0056$).

Discussion

Miravis Top, labeled for use on pecan in late 2019, showed 98% control of scab in this study, and was uniformly efficacious across all locations. Amistar Top has been the pecan industry standard for controlling scab since it was labeled for use on pecan in 2012, under the name of Quadris Top. Since its first use, there have been reports of decreased efficacy, presumably due to the presence of the G137S mutation that leads to decreased sensitivity to the QoI fungicides (Standish et al. 2019). In this regard, the efficacy of azoxystrobin varies considerably among orchards. Presence or absence of the G137S mutation, as well as variation in disease pressure, may explain the range in efficacy observed among locations for Abound and Amistar Top. With the azoxystrobin component of Amistar Top being compromised in many orchards (Herrington, 2019), the difenoconazole component of the premixture is likely providing the bulk of the scab control, resulting in increased selection that may lead to eventual resistance development to the DMI, difenoconazole. Although there has not yet been decreased sensitivity of *V. effusa* found to difenoconazole, there has been resistance of this pathogen reported to other DMIs, such as tebuconazole and propiconazole (Reynolds et al. 1997; Seyran et al. 2010; Stevenson et al. 2015). Difenoconazole is a newer DMI fungicide, but resistance has been reported in the closely related pathogen *Venturia inaequalis*, and was found to be a result of overexpression of the *CYP51A1* gene (Villani et al. 2016). There is no baseline sensitivity data of *V. effusa* to difenoconazole, therefore minor reductions in sensitivity may go unnoticed. Resistance development of *V. effusa* to difenoconazole would be unfortunate due to it's use as a component in popular premixes such as Amistar Top and Miravis Top. In locations where resistance to azoxystrobin was not observed, including locations 10 and 11 in this study, Amistar

Top remains an effective fungicide, and can be used successfully in fungicide rotation programs (Figure 2.3; Table 2.2).

Commercial pecan production is expanding at a rapid pace, with more orchards being established in various regions of the world each year. In those areas where pecan is being grown and weather is conducive for scab, *V. effusa* is the greatest threat to yield potential. In the southeastern U.S., where pecan production is prolific, and susceptible cultivars are widely grown, adequate scab control measures are required. Aside from cultivar selection, the most effective way to control *V. effusa* is by the frequent application of fungicides during the growing season. Our bi-weekly application of the fungicides included in the study is a realistic reflection of their field efficacy, and the results can be extended to application of the products using air-blast sprayers. Despite using a hand-held sprayer, we applied the products at recommended rates in appropriate volumes to runoff to reflect commercial practice. Furthermore, all products were applied in an identical manner, ensuring comparisons, and minimizing issues of incomplete coverage that are a problem with commercial applications using an airblast sprayer (Bock et al. 2013). Even more importantly, our results provide a comprehensive evaluation of fungicide efficacy in "real world" orchards with a wide range of previous fungicide exposure histories and levels of resistance exhibited in the *V. effusa* populations. Growers would not be willing to sacrifice large replicated blocks of unsprayed trees to scab that would be required with traditional applications using an air-blast sprayer, but they are more than willing to provide eight trees that remain unsprayed to inform which chemistries provide most control of scab in their orchard. Thus, we contend that application using hand held sprayers provides an excellent method to compare products for differences in efficacy. The range of scab severity among orchards was surprising (0 – 100%), although the sites with lower scab pressure sites were consistently those

to the north which historically have had less disease pressure. However, we acknowledge that these were on farm locations, and while clearly marked not to be sprayed, there is always a possibility that they may have received one or more fungicide applications by the grower. Air flow and tree spacing also play a significant role in development of *V. effusa*. Some of the locations in this study were positioned where wind is able to move freely through the orchard (locations 1, 4, 5, and 8), and others were located in areas where the air flow is limited by other vegetation (locations 2, 3, 6, 7, 9, 10, and 11), which could aide in explaining the variability in scab pressure from location to location.

With widespread adoption of Miravis Top, and the potential for resistance development to both pydiflumetofen and difenoconazole, it is crucial that baseline sensitivity be established for pydiflumetofen. The coupling of high-level performance in the field and pricing being competitive with other fungicide alternatives suggest that Miravis Top will likely become the new industry standard. Although labeled for a maximum of four applications per year on pecans, widespread use makes it even more important that sensitivity to Miravis Top be periodically tested from multiple orchards, and any observation of decreased sensitivity in the field be reported and action taken to reverse the trend. Since resistance in *V. effusa* to pydiflumetofen has not yet been observed or defined, the development of a true discriminatory concentration to use for detecting shifts in sensitivity in isolates is limited. However, by utilizing the concentration response curve we have prepared, we suggest a preliminary discriminatory concentration of 0.6 µg/ml, which is, on average, a point of complete inhibition for our baseline isolates. Resistance of different fungi to the SDHI fungicides has been shown to be quantitative for some pathogens (Gudmestad et al. 2013; Fernandez-Ortuño et al. 2017), as well as qualitative for others (Wharton et al. 2012; Fernandez-Ortuño et al. 2012). Mechanisms of resistance to the SDHIs has

been investigated in multiple pathosystems, and was found to be attributed to one or more mutation in the *SDH* genes (Honda et al. 2000; Stammler et al. 2007b; Avenot et al. 2008b; Shima et al. 2009; Avenot et al. 2012). Our choice to conduct a mycelial inhibition assay was based on the inability to induce sporulation using the 24-year-old isolates. The EC_{50} values derived from conidial germination inhibition assays could be lower, as was found by Wang et al. (2020) for determining baseline sensitivity of *Fusarium virguliforme* to the SDHI fungicide, fluopyram. The EC_{50} values could be higher too, as was found by Vega and Dewdney (2015) while screening *Alternaria alternata* isolates against the SDHI fungicide boscalid. Differing EC_{50} values derived from mycelial inhibition assays compared to conidial germination assays could be due to a difference in the starting materials. The mycelial growth assays utilize actively growing mycelia, whereas conidial inhibition assays utilize conidia that are in a state of dormancy (Gougouli and Koutsoumanis 2013).

All isolates that were tested in this study were highly sensitive to pydiflumetofen. Based on the results of the study, there were no significant differences in EC_{50} values between the two baseline locations. Extremely high sensitivity of fungi to pydiflumetofen is not uncommon. There have been several other baseline studies of plant pathogenic fungi to pydiflumetofen, many of which found very low EC_{50} values (Neves and Bradley 2019; Breunig and Chilvers 2021), and some of which found slightly higher EC_{50} values (Miller et al. 2020; Neves and Bradley 2021). Due to the nature of SDHI fungicides and their medium to high risk of resistance development, fungicide rotation with different fungicide classes combined with resistance monitoring programs are highly desirable and encouraged. If shifts in sensitivity to pydiflumetofen are suspected, the procedures outlined here can be implemented to compare isolates with those used in the current study. Any shift toward higher EC_{50} values in the future

will indicate the potential for reduced efficacy of pydiflumetofen. Miravis Top is a valuable tool that is available to commercial pecan growers to combat scab, and maintaining its efficacy as long as possible will be a benefit to the commercial pecan industry as well as the broader plant protection industry.

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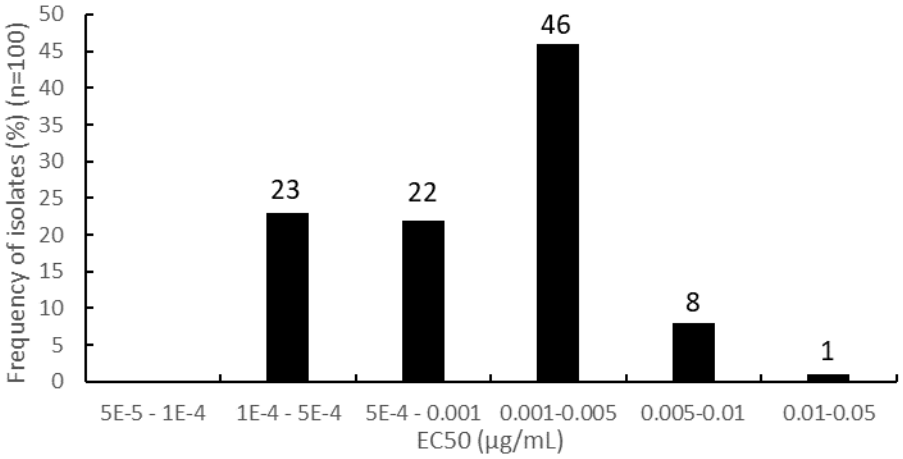

Figure 2.1. Baseline sensitivity frequency distribution of *Venturia effusa* to pydiflumetofen. Numbers above bars on graph represent the percentage of isolates that fall into that category.

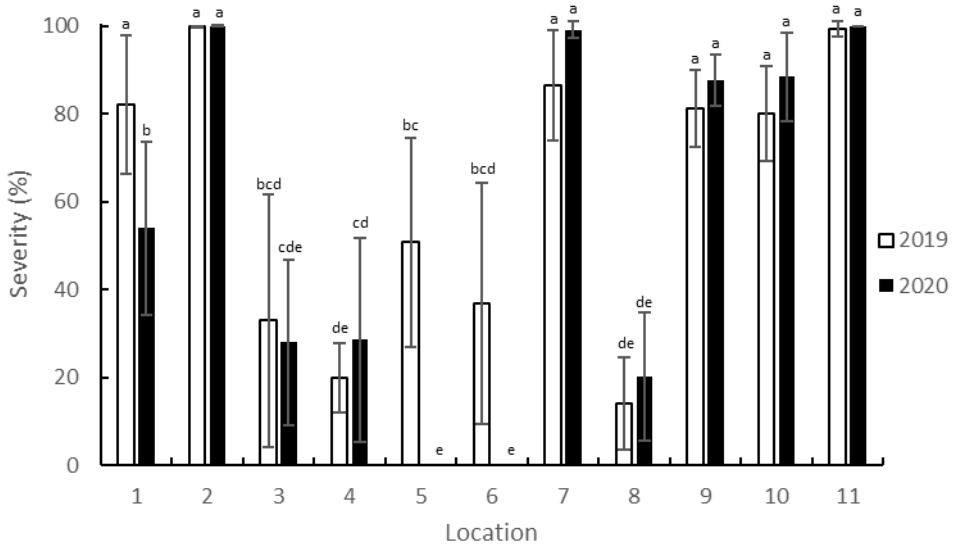

Figure 2.2. Severity of symptoms of scab (caused by *Venturia effusa*) on nontreated pecan fruit at 11 different locations across southern Georgia in 2019 and 2020. Different letters indicate significant differences among locations within a year ($P \leq 0.05$) based on Tukey Kramer mean

separation procedure. The * indicates orchards in which no data were collected due to the absence of scab in the orchard that year. Bars represent standard deviation from the mean at each location.

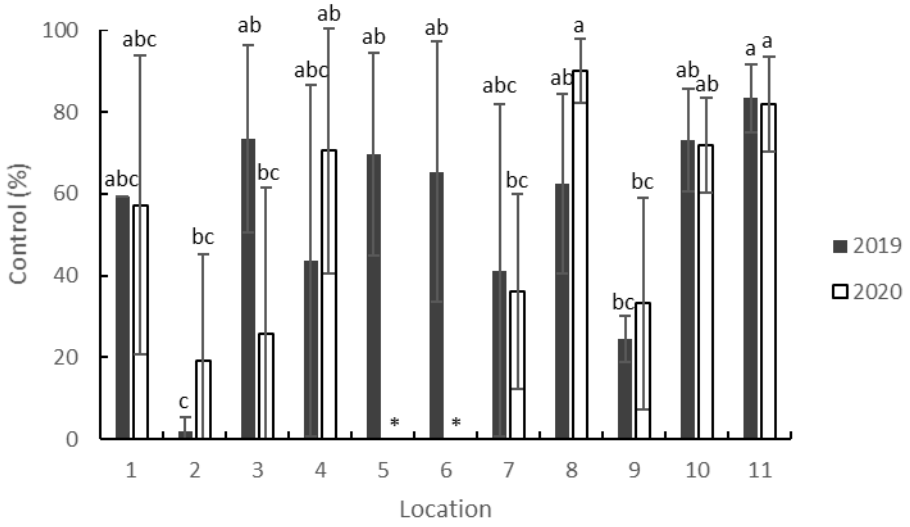

Figure 2.3. Average efficacy (percent reduction in severity of symptoms of scab (caused by *Venturia effusa*) compared to the control) of Abound (azoxystrobin) at each of the 11 pecan orchard locations in 2019 and 2020. Different letters indicate statistical differences among locations within a year ($P \leq 0.05$) based on Tukey Kramer mean separation procedure. The * indicates orchards in which no data were collected due to the absence of scab in the orchard that year. Bars represent standard deviation at each location.

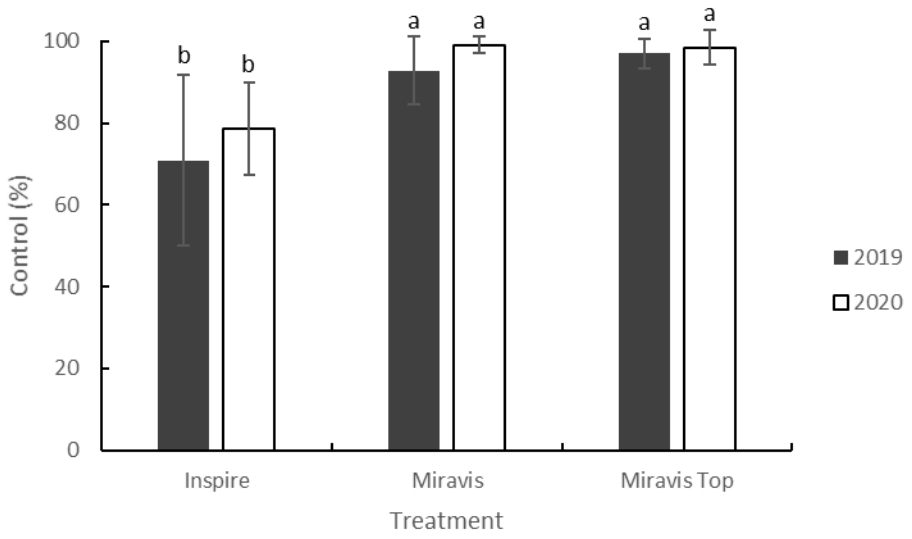

Figure 2.4. Average efficacy (percent reduction in severity of symptoms of scab (caused by *Venturia effusa*) compared to the control) of Inspire (difenoconazole), Miravis (pydiflumetofen), and Miravis Top (difenoconazole + pydiflumetofen) from all 11 pecan orchard locations in 2019 and 2020. Different letters indicate statistically significant differences ($P \leq 0.05$) among treatments within each year based on the Tukey Kramer mean separation procedure.

Table 2.1. Type III test of fixed effects table generated from statistical analysis in SAS 9.4. "Num DF" indicates the number of degrees of freedom in the model. "Den DF" represents the number of degrees of freedom associates with the model errors. "Pr > F" represents the p-value associated with the F statistic.

Effect	*Num DF*	*Den DF*	*F Value*	*Pr > F*
Location	6	37	25.20	<.0001
Treatment	4	352	143.22	<.0001
*Location*Treatment*	24	352	8.03	<.0001

Table 2.2. The range in EC_{50} values of isolates of *Venturia effusa* to the SDHI pydiflumetofen. Isolates were collected from pecan orchards in Jeff Davis and Troup counties Georgia in 1996. Orchards were isolated and had not received any fungicide treatments for at least 25 years.

NUMBER OF ISOLATES	COUNTY	EC_{50} RANGE
31	Troup	<0.0002 – 0.0066
19	Jeff Davis	<0.0002 – 0.0307

Table 2.3. Efficacy of different fungicide treatments for controlling scab on pecan fruit (caused by *Venturia effusa*) compared to the control) in 11 different orchards in southern Georgia in 2019 and 2020. Different letters indicate significant differences within that location and year ($P \leq 0.05$) based on the Tukey Kramer mean separation procedure.

Location	Year	County	Cultivar	Treatment	Rate (ml/ha)	Control (%)
1	2019	Dougherty	Desirable	Amistar Top	1001	39.0 B
				Inspire	490	50.8 AB
				Abound	797	59.2 AB
				Miravis	380	93.0 A
				Miravis Top	1001	94.2 A
1	2020			Amistar Top	1001	90.3 A
				Inspire	490	81.6 AB
				Abound	797	57.2 B
				Miravis	380	99.3 A
				Miravis Top	1001	96.5 A
2	2019	Dougherty	Cunard	Amistar Top	1001	44.5 B
				Inspire	490	58.2 B
				Abound	797	2.0 C
				Miravis	380	88.4 A
				Miravis Top	1001	95.7 A
2	2020			Amistar Top	1001	75.0 B
				Inspire	490	63.0 B
				Abound	797	19.3 C
				Miravis	380	99.4 A
				Miravis Top	1001	99.1 A
3	2019	Dougherty	Desirable	Amistar Top	1001	60.7 A
				Inspire	490	66.4 A
				Abound	797	60.2 A
				Miravis	380	96.0 A
				Miravis Top	1001	100 A
3	2020			Amistar Top	1001	42.5 BC
				Inspire	490	82.8 AB
				Abound	797	25.8 C

				Miravis	380	96.0 A
				Miravis Top	1001	92.8 A
4	2019	Sumter	Cunard	Amistar Top	1001	83.4 A
				Inspire	490	76.5 AB
				Abound	797	50.9 B
				Miravis	380	96.2 A
				Miravis Top	1001	94.9 A
4	2020			Amistar Top	1001	90.0 AB
				Inspire	490	88.2 AB
				Abound	797	70.4 B
				Miravis	380	99.5 A
				Miravis Top	1001	100 A
5	2019	Crisp	Pawnee	Amistar Top	1001	86.8 A
				Inspire	490	80.3 A
				Abound	797	69.8 A
				Miravis	380	90.4 A
				Miravis Top	1001	99.4 A
5	2020			Amistar Top	1001	-
				Inspire	490	-
				Abound	797	-
				Miravis	380	-
				Miravis Top	1001	-
6	2019	Wilcox	Desirable	Amistar Top	1001	95.7 A
				Inspire	490	97.2 A
				Abound	797	65.5 B
				Miravis	380	100 A
				Miravis Top	1001	100 A
6	2020			Amistar Top	1001	-
				Inspire	490	-
				Abound	797	-
				Miravis	380	-
				Miravis Top	1001	-
7	2019	Berrien	Pawnee	Amistar Top	1001	78.3 AB
				Inspire	490	87.0 A
				Abound	797	41.3 B
				Miravis	380	98.5 A
				Miravis Top	1001	98.3 A
7	2020			Amistar Top	1001	85.3 AB
				Inspire	490	75.0 B
				Abound	797	36.1 C
				Miravis	380	99.7 A
				Miravis Top	1001	99.1 A
8	2019	Lanier	Pawnee	Amistar Top	1001	84.6 A
				Inspire	490	81.7 A
				Abound	797	62.4 B
				Miravis	380	92.4 A
				Miravis Top	1001	90.4 A
8	2020			Amistar Top	1001	95.3 A
				Inspire	490	92.7 A
				Abound	797	89.8 A
				Miravis	380	99.4 A
				Miravis Top	1001	99.0 A
9	2019	Berrien	Cunard	Amistar Top	1001	62.6 B
				Inspire	490	71.1 B
				Abound	797	24.6 C

				Miravis	380	89.2 A
				Miravis Top	1001	93.5 A
9	2020			Amistar Top	1001	87.9 AB
				Inspire	490	81.0 B
				Abound	797	33.2 C
				Miravis	380	98.1 A
				Miravis Top	1001	99.0 A
10	2019	Tift	Wichita	Amistar Top	1001	95.4 A
				Inspire	490	82.0 B
				Abound	797	73.1 B
				Miravis	380	95.9 A
				Miravis Top	1001	98.4 A
10	2020			Amistar Top	1001	97.8 A
				Inspire	490	84.4 B
				Abound	797	71.9 C
				Miravis	380	100 A
				Miravis Top	1001	99.9 A
11	2019	Tift	Desirable	Amistar Top	1001	92.4 AB
				Inspire	490	87.2 AB
				Abound	797	83.4 B
				Miravis	380	92.4 AB
				Miravis Top	1001	99.0 A
11	2020			Amistar Top	1001	94.3 AB
				Inspire	490	85.5 BC
				Abound	797	82.0 C
				Miravis	380	99.9 A
				Miravis Top	1001	99.3 A

CHAPTER 3

MULTIPLE MUTATIONS AND OVER EXPRESSION IN THE *CYP51A&B* GENES LEADS TO DECREASED SENSITIVITY OF *VENTURIA EFFUSA* TO TEBUCONAZOLE

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Abstract

Venturia effusa, the causal agent of scab, is the most important disease limiting pecan production in the southeastern U.S. Several demethylation inhibiting (DMI) fungicides are labeled for use on pecan and the DMI fungicides are used heavily in commercial pecan orchards to control scab. To compare the efficacy of different DMI fungicides commonly used to control scab, field studies were undertaken at multiple locations applying fungicide to individual, fruiting pecan terminals. *In vitro* sensitivity assays were conducted to test the sensitivity of isolates of *V. effusa* from multiple locations to a range of concentrations of tebuconazole. Both field and *in vitro* studies confirm high levels of resistance to tebuconazole at multiple locations. To investigate the mechanism of resistance, the two copies of the *CYP51* gene, *CYP51A* and *CYP51B* of resistant and sensitive isolates were sequenced and the coding and promoter region of the genes were scanned for mutations. In the *CYP51A* gene, mutation at codon 444 (G444D) and in the *CYP51B* gene, mutations at codon 357 (G357H), and 177 (I77T and I77L) were found in resistant isolates but not in sensitive isolates. Expression analysis of both *CYP51A* and *CYP51B* genes revealed enhanced gene expression in the resistant isolates compared to the sensitive isolates. There was a 3.0 and 1.9-fold increase in gene expression in the resistant isolates compared to the sensitive isolates for the *CYP51A* and *CYP51B* genes, respectively. Therefore, two potential mechanisms: multiple point mutations and gene over expression in the *CYP51* gene of *V. effusa* isolates were revealed as likely reasons for the observed resistance in isolates of *V. effusa* to the DMI fungicide tebuconazole. The results facilitate the detection of DMI resistance, and will aid in management of fungicide resistance in pecan and other phytopathogens to extend the lifespan of the DMI fungicides.

Introduction

Pecan is an important crop in the southeastern United States, and is increasing in importance in other countries (Blayney et al. 2017). The U.S. produced 302 million pounds of in-shell pecans in 2020, and the state of Georgia is the second leading producer of pecans in the U.S., producing 142 million pounds in 2020 (USDA NASS 2021). Pecan scab is caused by the plant pathogenic fungus *Venturia effusa*, which is a pathogen that thrives in high temperatures, high humidity, and frequent rainfall conditions, which are common to the southeastern United States (Latham 1982; Latham 1995). Scab infections can result in significant yield losses to pecan if left unmanaged (Gottwald & Bertrand 1983; Hunter 1983). Although the most effective method for controlling *V. effusa* is to plant resistant cultivars, selection has resulted in adaptation of the pathogen to be pathogenic on historically resistant cultivars (Demaree and Cole 1929; Sparks 1992; Conner and Wells 2007; Conner and Stevenson 2004; Bock et al. 2014, 2017). Thus, frequent use of fungicides is the most widely adopted management approach for controlling scab in commercial pecan orchards in the Southeast. However, fungicide control of scab is one of the largest costs to pecan growers in the region.

Demethylation inhibiting (DMI) fungicides were first introduced for use against Plant Dis.s in the 1970s and are now the most widely adopted and important group of fungistatic agents used worldwide both in medicine and in agriculture (Szkolnik 1981). Since their discovery, over 30 DMI fungicides have been synthesized for use in agriculture (Brent, 2012). They function by impeding sterol C-14 a-demethylation of 24-methylenedihydrolanosterol, which is a critical component in the formation of ergosterol in fungi. Ergosterol is vital to fungal cell membranes and regulates membrane permeability and fluidity, which is why the enzymes that synthesize ergosterol are important targets for anti-fungal agents (Price et al. 2015). Since

the DMIs have been so widely used and repeatedly applied to large areas of crops year after year, there have been many reports of resistance in fungi, beginning in the 1980s, only a decade after their release (Fletcher and Wolfe, 1981; Heaney et al., 1984; Stanis and Jones, 1985). DMI fungicides are single-site, broad-spectrum fungicides that are used for pre- and post-infection control of various pathogens (FRAC, 2021). Development of resistance to the DMI fungicides has led to decreased sensitivity of various pathogens to a range of DMI fungicides, including the apple scab pathogen *Venturia inaequalis*, a close relative to the pecan scab pathogen, *V. effusa* (Villani et al. 2015). DMI fungicides were first labeled for use against *V. effusa* in 1988 and are heavily used to combat scab each year (Bertrand and Hadden 1992). The DMIs labeled for use in pecan include difenoconazole, propiconazole, fenbuconazole, tetraconazole, metconazole, flutriafol, mefentrifluconazole, and tebuconazole. Isolates of *V. effusa* collected in 2003 were found to be less sensitive to propiconazole when compared to the baseline isolates reported in 1997 (Reynolds et al. 1997; Stevenson et al. 2015). Standish et al. (2018) reported field resistance of *V. effusa* to tebuconazole, and confirmed the reduced sensitivity using an *in vitro* assay. In a separate study, Standish et al. (2019) demonstrated insensitivity to tebuconazole to be phenotypically stable.

To date, resistance to DMI fungicides has been reported in 37 fungal species. Cross resistance has been documented among DMI fungicides used against the same pathogen (Hsiang et al, 1997; Karaoglanidis and Thanassoulopoulos 2003; Thomas et al. 2012; Ishii, et al. 2021). Cross resistance has been observed in *V. effusa* among tebuconazole, fenbuconazole, and propiconazole, but has not yet been found for difenoconazole, which is widely applied to control scab (Reynolds et al. 1997; Stevenson et al. 2015). Resistance in DMIs is typically caused by amino acid changes in the *CYP51* gene, overexpression of the *CYP51* gene, or by efflux pumps

reducing intracellular fungicide accumulation within the pathogen (FRAC 2020; Price et al. 2015; Ziogas and Malandrakis 2015). Tucker et al. (2019) investigated the mechanism of resistance to DMI fungicides in the pathogen *Blumeria graminis* and found 5 separate amino acid substitutions in the *CYP51B* target gene, four of which were novel, showing that multiple mutations may confer resistance to DMI fungicides. *V. inaequalis*, a close relative of *V. effusa*, also displays resistance to the DMI fungicides. Villani et al. (2016) investigated the mechanisms of DMI resistance in *V. inaequalis* and found overexpression of the *CYP51A1* gene in resistant isolates compared to the sensitive isolates. Hayashi et al. (2002) demonstrated that ABC transporters can lead to decreased sensitivity of *Botrytis cinerea* to DMI fungicides. The results from these studies show that several potential factors may be contributing to resistance of *V. effusa* to the DMI fungicides.

The fungicide resistance studies describing resistance in *V. effusa* to the DMIs to date have reported only phenotypic data, and the specific mechanism of resistance remains unknown (Seyran et al. 2010; Stevenson et al. 2015; Standish et al. 2018). The goal of the current study was to determine the mechanism of resistance of *V. effusa* to the DMI tebuconazole to provide further insights regarding DMI insensitivity prevalence in commercial pecan orchards in the southeastern U.S.

Materials and Methods

Evaluation of field sensitivity of *V. effusa* to DMI fungicides. In 2019 and 2020, 11 pecan orchards were selected in Tift, Berrien, Wilcox, Lanier, Crisp, Dougherty, and Sumter counties in Georgia. The orchards were chosen as they were planted in scab susceptible cultivars including Cunard, Desirable, Pawnee, and Wichita. The orchards varied in tree spacing, age, disease pressure, and environmental conditions. At each location, 8 consecutive trees within a

row received no commercial fungicide applications from nut set to harvest. The experimental design was a randomized complete block design at each location, with each tree being a block with one replicate of each of the 4 treatments. Thus, the treatments were applied directly to individual fruiting terminals on the pecan trees that were flagged with different colored ribbons to indicate which treatment the terminal received. Each terminal contained one or more clusters of pecans. The treatments were applied by spraying to initial runoff using a handheld sprayer (Project Source model #5318). The treatments were as follows: Orius 3.6F (tebuconazole; Makhteshim Agan of North America, Inc. Raleigh, NC) at 584.6 milliliters per hectare (ml/ha), Inspire (difenoconazole; Syngenta Crop Protection, Greensboro, NC) applied at 489.6 ml/ha, Cevya (mefentrifluconazole; BASF Corporation, Research Triangle Park, NC) applied at 365.4 ml/ha, and an untreated control (Table 3.1). The terminals received the same application on a 14-day schedule from bud break to shell hardening for a total of 7 applications in 2019 and 8 applications in 2020. The severity of scab symptoms was estimated after shell hardening on each of the fruit on each terminal by visual observation using a 0-100% rating scale. Relative control (%) for each treatment was calculated based on the severity on the nontreated control.

Determination of in-vitro sensitivity of *V. effusa* to tebuconazole. At each location in both 2019 and 2020, leaf and nut scab samples were taken in July from multiple untreated trees and were placed in Ziplock bags containing a wet paper towel to help induce sporulation. After 24 hours, the samples were used to conduct an *in vitro* sensitivity assay to tebuconazole, following the procedure described by Seyran et al. (2010). There were two plates (repetitions), with three groups of conidia from different scab lesions per plate. The concentrations of tebuconazole in the media were 0, 1, 3, and 10 µg/mL, prepared by serial dilution from technical grade tebuconazole (Chem Service Inc, West Chester, PA; 98.1% purity). Conidia that

germinated and grew on media amended with 10 µg/mL tebuconazole were considered to be highly resistant, and were removed from the fungicide amended media under a dissecting microscope using a sterile needle and were plated as monoconidial isolates on non-amended potato dextrose agar (PDA) in Petri-plates to be used for later molecular analysis to determine the mechanism of tebuconazole resistance. The monoconidial isolates of *V. effusa* that were considered to be sensitive to tebuconazole were isolated from two baseline orchards in 1993 and 1994, stored in sterile water in 1996, and were revived in 2020 by culture on PDA. The isolates were collected for a baseline sensitivity study in *V. effusa* to the DMIs propiconazole and fenbuconazole in Troup and Jeff Davis counties in Georgia, and have never been exposed to modern fungicides (Reynolds et al., 1997). Additional mycelial growth assays were performed to confirm the sensitivity of these isolates to tebuconazole. The resistant isolates were from orchards in Berrien and Dougherty counties in Georgia, collected in 2020. All monoconidial isolates cultured on PDA were incubated in the dark at 25°C for 4 weeks to reach acceptable colony size (15 – 25 mm in diameter). A plug of PDA with mycelium of *V. effusa* was taken from each colony using a 4 mm cork borer, and was homogenized in 1 ml sterile water in a microfuge tube using a bead beater for 20 seconds. Tebuconazole amended media was prepared in Petri-plates containing 0, 1, 3, and 10 µg/mL tebuconazole exactly as described before with the previous assay. Using a 4 mm cork borer, two 4 mm wells were prepared near the center of each plate of the fungicide amended media, approximately 30 mm apart, and 20 ul of the mycelial slurries were pipetted into the wells. The Petri-plates were incubated in the dark at 25°C for 4 weeks, and the diameters of the colonies were measured using a ruler.

Investigating mechanisms of resistance

DNA extraction. All monoconidial isolates were grown on PDA amended with antibiotics (50 mg/L each of streptomycin, rifampin and chloramphenicol) prior to DNA extraction. The isolates were incubated in the dark for 4 weeks at 25°C prior to extraction. After 4 weeks, the colonies had reached sufficient diameter (15 – 25 mm), and 50 to 100 mg of mycelium was collected from the surface of the agar using a scalpel. 500 µL lysis buffer (Norgen DNA Isolation Kit; Norgen Biotek Corp., Thorold, ON, Canada) and fifteen to twenty small glass beads were added to a 1.5 ml safe-lock tube (Eppendorf Canada Ltd, Mississauga, ON, Canada), and by using a FastPrep FP 120 cell distributor (Qbiogene, Carlsbad, CA) were homogenized twice at speed 4.0 for 30 seconds each. After lysis, the DNA was extracted using an UltraClean Microbial DNA Isolation Kit (Qiagen, Germantown, MD) following the manufacturer's protocol. The purity and quantity of DNA were measured with a NanoDrop spectrophotometer (Nanaodrop Lite, Thermo Scientific, Waltham, MA). A polymerase chain reaction (PCR) was used to amplify the DNA of both the *CYP51A* and *CYP51B* genes using the SYBR Green PCR Master Mix (Thermo Fisher Scientific Inc, Waltham, MA) and the primers listed (Table 3.2). PCR conditions varied for each primer set that was used to amplify different fragments of the *CYP51* genes. For sequencing of the *CYP51* genes, DNA was extracted from 3 sensitive isolates and 8 resistant isolates using the protocol described above.

Primer Design. Six sets of primers were designed to sequence the *CYP51A* gene including the coding region based on the sequence obtained from GenBank (Table 3.2). The *V. effusa* albino strain chromosome 1 sequence with the accession number CP042185.1 was used as a reference sequence (Winter et al. 2020). The 1686 bp sequence of the *CYP51A* gene on chromosome 1 was at 371163 to 372848 bp. The primer pairs

CYP51A_I1I2_F1/CYP51A_I1I2_R1, CYP51A_I3_F1/CYP51A_I3_R1 and CYP51A_End_F1/CYP51A_End_R1 were designed to amplify a 1578 bp fragment of the coding region. The primer pair CYP51A_P1 - 80 F/CYP51A_P1 - 988 R were developed to amplify part of the *CYP51A* coding region and the upstream 368 bp of promoter regions at the 5' end based on the GenBank accession number CP042185.1 (370698bp to 373113bp). A 610 bp fragment including 198 bp downstream of the 3′ end of the *CYP51A* gene was amplified using the primer set CYP51A_P2-1,724 F/CYP51A_P2-2,333 R. The primer set CYP51A_ORF_F1/CYP51A_ORF_R1 was developed to amplify the whole coding region at once using conventional PCR for subsequent sequencing of the gene (Table 3.2, Figure 3.2). Similarly, for the sequencing of the *CYP51B* gene, 8 sets of primer pairs were designed based on the sequence of *V. effusa* albino strain chromosome 5 obtained from NCBI GenBank (Winter et al. 2020). The GenBank accession number used as the reference for the sequence is CP042189.1, with a position from 1774471 to 1778656 bp that covers the whole *CYP51B* gene. The primer pair CYP51B_F1 - 29 F/ CYP51B_F1 - 1,029 R set was designed to amplify an amplicon of 1001bp including a part of the upstream promoter region (~50bp) and a part of the coding region (847bp) with one intron region. The primer set CYP51B_F2 - 566 F/CYP51B_F2 - 1,734 R was designed to sequence the 1173 bp of the coding region. The CYP51B_F3-1541F/ CYP51B_F3-2437R, and CYP51B_F4-36F/ CYP51B_F4-807R primer sets were designed to amplify fragments of the coding region including the second intron site. The remaining three sets of primers including CYP51B_F5_I1_F/CYP51B_F5_I1_R, CYP51B_F6_Mid_F/CYP51B_F6_Mid_R and CYP51B_F7_I2_F/CYP51B_F7_I2_R were developed to amplify the coding region including the third and fourth intron sites to amplify the remaining portion of the *CYP51B* coding region, totaling 4123 bp. The primer set CYP51B_F8 -

1,611 F/CYP51B_F8 - 2,354 R amplified a 744 bp fragment including 284 bp downstream of the 3′ end of the *CYP51B* gene for the sequencing of the terminator region of the gene (Table 3.2, Figure 3.3)

Sequencing and analysis of the *CYP51A* and *B* sequences. PCR products were separated by gel electrophoresis on a 1% agarose gel (BioRad, Hercules CA) stained with GelRed (Biotium, Fremont, CA) and run in 1X Tris/Borate/EDTA buffer at 90 V for 40 minutes. Images of the gel were captured on a UV Geldoc gel imager (Analyik Jena, Upland, CA). DNA was purified using the Quantum Prep PCR Kleen Spin purification kit (BioRad, Hercules, CA) using the protocol provided by the manufacturer. Purified DNA was sent to Retrogen Inc. (San Diego, CA) for Sanger sequencing with both forward and reverse internal primers for each primer set. Once sequencing results were obtained, introns were removed, the DNA was aligned, translated to amino acid sequence, and screened for possible mutations using Geneious Prime software V 2019.2.3 (https://genious.com).

***CYP51A* and *B* gene expression**. All isolates were grown on PDA amended with antibiotics (50 mg/L each of streptomycin, rifampin and chloramphenicol) prior to RNA extraction. RNA was extracted from each isolate using the RNeasy kit (Qiagen, Germantown, MD) following the manufacturer's protocol. Synthesis of cDNA from the extracted RNA was achieved using the iScript™ cDNA synthesis kit (BioRad, Hercules, CA) following the manufacturer's protocol. Expression analysis was conducted through a real-time quantitative PCR (qPCR) assay using a BioRad CFX connect real-time system (BioRad, Hercules, CA) to quantify the expression of *CYP51A* and *B* genes from all the isolates using the primers listed in (Table 3.1). The SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Inc., Hercules, CA, United States) was used for qPCR analysis. The total reaction volume was 10 µl containing

5 µl of SYBR® Green Supermix, 0.4 µl of 1000 nM of each forward and reverse primers (Table 3.2), and 2 µl (10 pg) of cDNA; the balance of volume was made up with molecular grade water. The thermal cycling protocol for the expression study had an annealing/extension temperature of 60°C, and a melt curve analysis was included. The CFX Maestro™ Software (Bio-Rad Inc., Hercules, CA, United States) was used to analyze all qPCR data. Relative gene expression was calculated as the ratio between *CYP51A* and *B* and the reference control gene, β-actin, following the $2^{-\Delta\Delta Ct}$ equation (Livak and Schmittgen, 2001).

Data Analysis

For the field experiment data comparing DMI fungicide treatments, the Univariate procedure was used to confirm that data were normally distributed. Data were then analyzed using a generalized linear mixed model (PROC GLIMMIX) in SAS V9.4 (SAS Institute, Cary, NC), with block, cultivar, and year as random effects, and location and treatment as fixed effects. Location was treated as a fixed effect due to a significant interaction of treatment*location (Table 3.3). Locations containing little (<10% scab severity) to no disease pressure on nontreated terminals were excluded from the dataset. The 95% confidence intervals of the treatment means were calculated. A Tukey-Kramer test was conducted as the mean separation procedure.

The qPCR *CYP51A* and *B* expression data are presented as mean ± the standard error of the mean (SEM). Graph preparations and analysis of the data were performed using GraphPad Prism 8 software (GraphPad Software, Inc. San Diego, CA). Data were subjected to a mean separation procedure using a two-tailed Student *t*-test at $P < 0.05$.

Results

Evaluation of field sensitivity of *V. effusa* to DMI fungicides. The field experiment showed variability in scab pressure among locations, with the nontreated terminals at different locations having scab severity ranging from 0 to 100% in 2019 and 2020 ($P < 0.0001$). Repeated applications of Inspire resulted in 70.9 and 78.7% control, repeated applications of Cevya led to 67.3 and 67.0% control, and repeated applications of Orius 3.6F led to 22.4 and 25.2% control in 2019 and 2020, respectively. At most locations, the scab control efficacy of Inspire and Cevya were statistically similar, while the efficacy of Orius 3.6F was significantly less than that achieved by application of either Inspire or Cevya (Figure 3.1; Table 3.4).

Determination of in vitro sensitivity of *V. effusa* to tebuconazole. Results from the *in vitro* assay of isolates from the different locations revealed variability in sensitivity to tebuconazole. At 1 µg/mL tebuconazole, the relative growth values (RGr) ranged from 48 to 173%, at 3 µg/mL RGr ranged from 23 to 133%, and at 10 µg/mL the RGr ranged from 3 to 86%. While variability was evident, all locations presented high levels of resistance to tebuconazole (Table 3.5). The highly resistant isolates that were grown on tebuconazole amended media all grew well on amended media containing 10 µg/mL tebuconazole, with RGr values ranging from 58% to 110% at 10 µg/mL tebuconazole, and a mean RGr value of 71% (Table 3.6). Since resistant isolates of fungal pathogens may be defined as having 50% or higher RGr in the presence of the discriminatory concentration (1 µg/mL tebuconazole), the resistant isolates with over 50% growth on 10 µg/mL tebuconazole confirm that the isolates are highly resistant to tebuconazole (Russell, 2004). All sensitive isolates (from 1993) were completely suppressed (0% RGr) by 1 µg/mL tebuconazole (Table 3.6).

Sequence analysis of *CYP51A* and *B*. Several nucleotide anomalies were observed in both the *CYP51A* and *B* genes; however, most of the single nucleotide polymorphisms were silent mutations that did not lead to a change in the translated amino acid sequence. In the *CYP51A* gene, there was one mutation (the G444D mutation) that did impact the translated amino acid sequence. The G444D mutation was present in 4 of the 8 resistant isolates but not in any of the sensitive isolates. The change in sequence resulted in an amino acid switch from Glycine to Aspartate at location 444 (Figure 3.2). In the *CYP51B* gene, amino acid mutations were present at 2 locations. The I77T mutation was present in 5 of the resistant isolates and none of the sensitive isolates and occurred as a result of the single nucleotide base substitutions of thymine to cytosine at nucleotide location 230, resulting in the amino acid change of Isoleucine to Threonine at location 77. The I77L mutation occurred in 1 resistant isolate and occurred as a result of a single nucleotide base substitution of thymine to adenine at nucleotide location 230, resulting in an amino acid change of Isoleucine to Leucine at location 77. The G357H mutation was present in 6 of the 8 resistant isolates and none of the sensitive isolates and occurred as a result of a single nucleotide base substitution of adenine to thymine at nucleotide location 1071, resulting in an amino acid change of Glycine to Histidine at location 357 (Figure 3.3).

Gene expression. Relative expression (RE) analysis revealed that resistant isolates expressed both *CYP51A* and *CYP51B* genes more than the sensitive isolates. This analysis revealed that the resistant isolates with the G444D mutation expressed the *CYP51A* gene more compared to the resistant isolates that did not contain the mutation. The sensitive isolates mean RE was 1.48, whereas the resistant isolates with the G444D mutation had a mean RE value of 5.96, and the resistant isolates without the G444D mutation had a mean RE value of 2.80 (Figure 3.4). With the *CYP51B* gene, sensitive isolates had a lower RE compared to the resistant isolates.

Sensitive isolates had a mean RE value of 0.53, while the resistant isolates had a mean RE value of 1.02 (Figure 3.5).

Discussion

Our results indicate that difenoconazole and mefentrifluconazole are both highly active on *V. effusa*, while tebuconazole is not, due to the presence of resistant isolates. Confirmation of reduced sensitivity led to the collection of resistant isolates and investigation of the exact mechanism of resistance. Although some possible mechanisms of resistance in *V. effusa* to the DMI fungicides are presented, definitive conclusions regarding the mechanism of resistance should not be made. We suggest two mechanisms based on the results of this study, including mutations in both the *CYP51A* and *B* genes (G444D, G357H, I77T, and I77L) (Figure 3.2, 3.3; Table 3.7), as well as 3.0- and 1.9-fold increases in expression of the *CYP51A* and *B* genes, respectively (Figure 3.4, 3.5). Mutations in the *CYP51* gene are a common cause of resistance to DMIs and have been found in several other pathogens (Pereira et al. 2016; Tucker et al. 2019; Muellender et al 2020). Overexpression of the *CYP51* genes is also commonly found in resistant isolates of various pathogens (Ma et al. 2006; Rallos & Baudoin 2016; Zhang et al. 2020). We anticipated either mutations or overexpression of the *CYP51* gene to be the cause of the resistance, and we found mutations and overexpression to be present in DMI resistant isolates of *V. effusa*. The resulting amino acid changes and overexpression in isolates of a single pathogen species exhibiting resistance to the DMI fungicides is also not uncommon (Stammler et al. 2009; Wei et al. 2020). While this study did not address ABC transporters and other efflux transporters, it is possible that they may also play a role in resistance to the DMI fungicides in in *V. effusa*, and future studies should be aimed at investigating ABC transporters and other efflux transporters as potential sources of DMI resistance.

The DMI fungicides are used heavily in commercial pecan orchards to control *V. effusa*. Understanding fungicide sensitivity in *V. effusa* can aide in the further development and strengthening of fungicide resistance management rotation programs. The results help outline the necessity for further fungicide research and development required to control *V. effusa* efficiently and sustainably. Difenoconazole is the most widely used DMI fungicide in commercial pecan orchards, and is labeled for use on pecan only as a mixture combined with other active ingredients. The popular combination products are Amistar Top (difenoconazole + azoxystrobin) and Miravis Top (difenoconazole + pydiflumetofen). Combination products with more than one active ingredient in different fungicide classes can increase disease control, and contribute to delaying fungicide resistance in certain pathogens. However, if one of the active ingredients in the premixture begins to lose its efficacy due to resistance development in the pathogen, the other active ingredient in the combination is more at risk of resistance development. So far, no resistance of *V. effusa* to difenoconazole has been reported, and combination products containing difenoconazole remain popular among commercial pecan growers. However, there is currently no baseline sensitivity data pertaining to *V. effusa* sensitivity to difenoconazole, therefore slight shifts in sensitivity may go unnoticed and cannot be confirmed in the lab. Although resistance to difenoconazole has not been reported for *V. effusa*, it has been reported for several other pathogens, including *V. inaequalis, Lasiodiplodia theobromae, Penicillium spp, Botrytis cinereal, Alternaria spp, Aspergillus fumigatus,* etc. (Villani et al. 2015; Ali & Amiri 2018; Li et al. 2020; Lichtner et al. 2020; Zhang et al. 2019; Wang et al. 2020; Zhang et al. 2020).

Mefentrifluconazole was recently labeled for use on pecan (Cevya), and although resistance has not yet been reported in *V. effusa*, resistance has already been detected in other pathosystems (Ishii et al. 2021). Mefentrifluconazole is not yet widely used in commercial pecan

orchards, but may play a role in future fungicide rotation programs. However, since Cevya is a stand-alone DMI fungicide that runs a high risk of resistance development, it's use should be limited, and it should only be applied in strict rotation and never applied alone in consecutive applications. Tebuconazole products are no longer widely used among commercial pecan growers due to very low activity as a result of fungicide resistance developing in *V. effusa*, as confirmed by our field and *in vitro* studies. Our results indicate that resistance of *V. effusa* to tebuconazole is widespread, and that the efficacy of tebuconazole across southern Georgia is low (Figure 3.1; Table 3.4, 3.5). Mefentrifluconazole and difenoconazole are both newer DMI fungicides, and are active on scab even when other DMI fungicides are not. Newer DMI fungicides maintaining efficacy while older DMI fungicides fall victim to resistance development is not a novel observation (Jørgensen et al., 2020; Ishii et al. 2021). It has been proposed that the high structural flexibility of the mefentrifluconazole molecule is to blame for the limited cross resistance being observed (Strobel et al. 2020). Because of flexible isopropanol linkers, mefentrifluconazole molecules are able to settle into the binding pocket of the *CYP51* enzyme, resulting in strong inhibition of enzymatic activity, even when target site alterations due to amino acid substitutions may be present (Strobel et al. 2020; Ishii et al. 2021)

The goal of determining the mechanism of resistance is to both add to our knowledge regarding resistance development in a pathogen, as well as providing a basis for developing detection methods that can be used rapidly to identify and track the specific resistance trait in orchard populations of *V. effusa* in the future. These rapid detection methods are not uncommon, and have been proven effective in various other pathogens in regard to resistance to the DMIs, as well as other fungicide groups (Chen et al. 2019; Shrestha et al. 2020). Since *V. effusa* is a very slow growing pathogen, taking approximately 30 days to for a colony to grow to diameter of 25

mm, a rapid method for detecting resistance to DMI (and other) fungicides would be a valuable tool to provide to growers and other stakeholders to better characterize the pathogen population present in their orchard, which ultimately will help optimize management of the populations of *V. effusa* to minimize risk of further development and spread of fungicide resistance, and improve the efficacy and sustainably of scab control.

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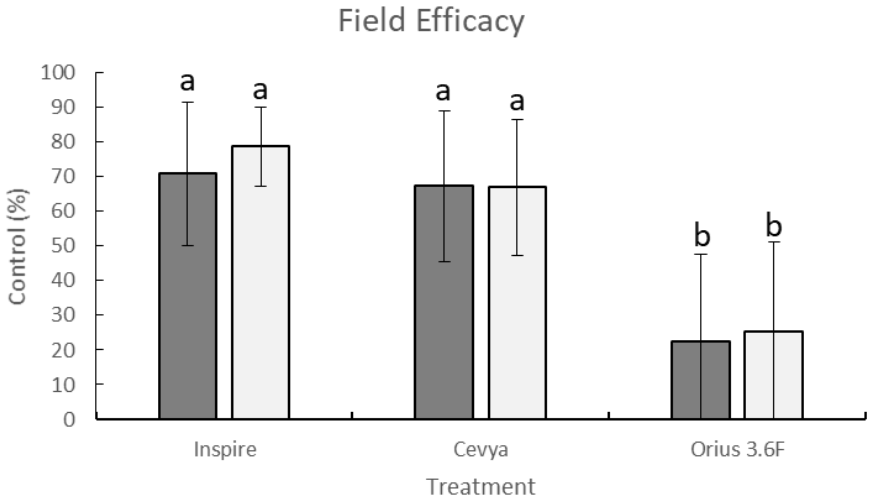
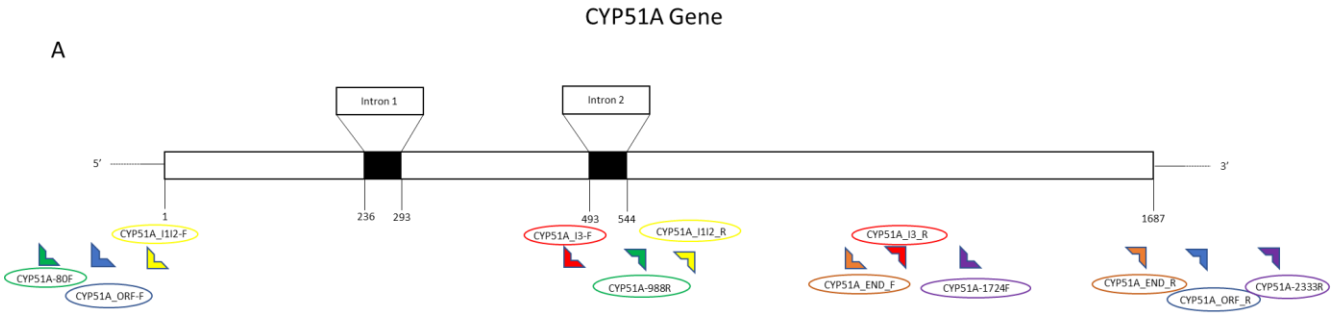


Figure 3.1. Field efficacy (percent reduction in severity of symptoms of scab (caused by *Venturia effusa*) compared to the control) of Inspire (difenoconazole), Cevya (mefentrifluconazole), and Orius 3.6F (tebuconazole). Different letters indicate statistical differences based on the Tukey-Kramer mean separation procedure. Bars on graph represent standard deviation from the mean.

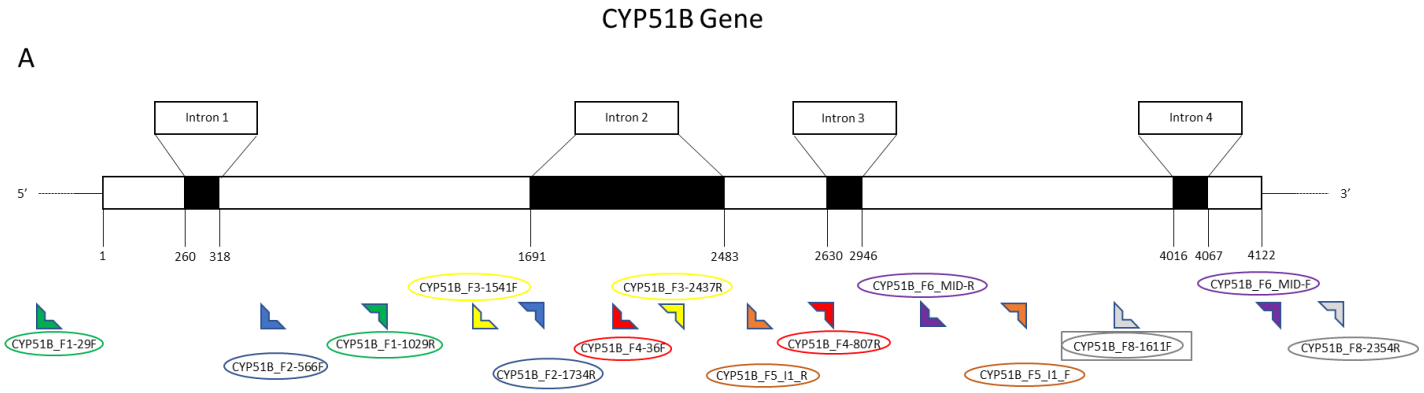


B

Isolate	Nucleotide (148)	Amino Acid (444)
Sensitive	144-TACGGTTAC	440-WKKAIENPL
Resistant	144-TACGATTAC	440-WKKATENPL

Figure 3.2. Schematic of the *CYP51A* gene and the detected mutation in *Venturia effusa*. (A) Exon and Intron organization of *CYP51A*. Primers are indicated by 90° symbols at the bottom of the schematic, and primer names are listed above or below the primer symbol. Each primer set contains the same color symbol and circle surrounding the name of the primer. (B) Characteristics of sensitive and resistant isolates comparing nucleotide and amino acid mutations indicated with a red nucleotide or amino acid identifier. The mutation described here is the G444D amino acid substitution.

A



B

Isolate	Nucleotide (230)	Amino Acid (77)	Nucleotide (1071)	Amino Acid (357)
Sensitive	226-GCTATAGAGA	73-WKKAIENPL	1067-TTCAAAAAG	253-KTAFGKAVD
Resistant	226-GCTACAGAG	73-WKKATENPL	1067-TTCATAAAG	253-KTAFHKAVD

Figure 3.3. Schematic of the *CYP51B* gene and the detected mutations in *Venturia effusa*. (A) Exon and Intron organization of *CYP51B*. Primers are indicated by 90° symbols at the bottom of the schematic, and primer names are listed above or below the primer symbol. Each primer set contains the same color symbol and circle surrounding the name of the primer. (B) Characteristics of sensitive and resistant isolates comparing nucleotide and amino acid mutations indicated with a red nucleotide or amino acid identifier. The mutations described here are the I77T/L and G357H amino acid substitutions.

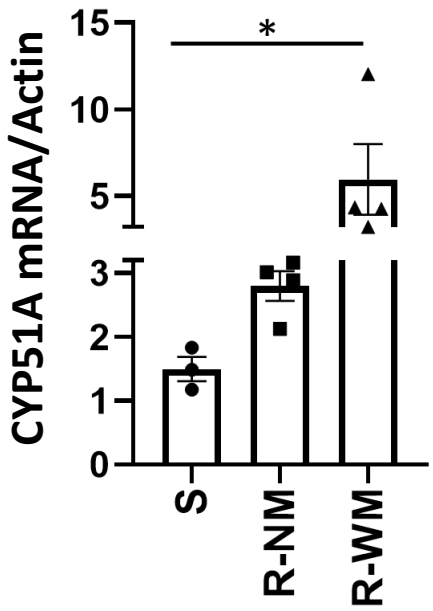


Figure 3.4. Relative expression of the *CYP51A* gene in *Venturia effusa*. Asterix (*) indicates a statistical difference between resistant and sensitive groups. The "S" on the x-axis indicates the sensitive isolates, while the "R-NM" represents the resistant isolates without the G444D mutation and the "R-WM" represents the resistant isolates with the G444D mutation. The circles represent individual sensitive isolates, while the triangles and squares represent the resistant isolates with and without the G444D mutation, respectively.

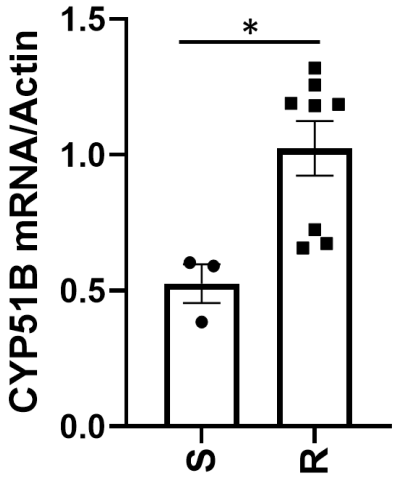


Figure 3.5. Relative expression of the *CYP51B* gene in *Venturia effusa*. Asterix (*) indicates statistical differences between resistant and sensitive groups. The "S" on the x-axis indicates the sensitive isolates, while the "R" represents the resistant isolates. The circles indicate individual sensitive isolates, while the squares indicate individual resistant isolates.

Table 3.1. List of demethylation inhibitor fungicide products and active ingredients compared for efficacy for controlling scab (caused by *Venturia effusa*) on pecan in field trials conducted in 2019 and 2020 in southern Georgia.

Fungicide product	***Active ingredient***	***Rate/ha***
Orius 3.6F	Tebuconazole	584.6 ml
Inspire	Difenoconazole	489.6 ml
Cevya	Mefentrifluconazole	365.4 ml
Nontreated	N/A	N/A

Table 3.2. The primers used for amplifying the *CYP51A* and *CYP51B* genes of *Venturia effusa*, and the primers used for RT-qPCR *CYP51A* and *CYP51B* gene expression assay.

Purpose	***Primer name***	***Primer sequence (5'-3')***	***Gene***	***Size (bp)***
Sequencing	CYP51A_ORF_F1	AATGGAAGGGTCCTCGCATG	*CYP51A*	2009
	CYP51A_ORF_R1	AGTTCGAAGCCGCCTAGAAC	*CYP51A*	
	CYP51A_I1I2_F1	CAGGCTACAATTCTGCCGC	*CYP51A*	674
	CYP51A_I1I2_R1	TGGATGAGGGTGACATAGGA	*CYP51A*	
	CYP51A_I3_F1	CGGTTCCGACGTCGTCTATG	*CYP51A*	687
	CYP51A_I3_R1	AGACACGAAGCTGTTCCTGG	*CYP51A*	
	CYP51A_End_F1	TACCTCGTCCTGGATCCTCC	*CYP51A*	605
	CYP51A_End_R1	GAGAGGTCCGGAGAAGAGGG	*CYP51A*	
	CYP51A_P1 - 80 F	TTGACTTGGATGTTGAGGCG	*CYP51A*	909
	CYP51A_P1 - 988 R	TGGAAGTCAGCGTATGTTCC	*CYP51A*	
	CYP51A_P2-1,724 F	TATTCCCACTTCGCACATCC	*CYP51A*	610
	CYP51A_P2-2,333 R	AGTCTTCCTTGGTCTACTTCG	*CYP51A*	
	CYP51B_F1 - 29 F	AGCCAACTGGTGAGATACGAC	*CYP51B*	1001
	CYP51B_F1 - 1,029 R	TCGAAGAGGAGACAACGGG	*CYP51B*	
	CYP51B_F2 - 566 F	AGTCCGGAGCTACAATTGCC	*CYP51B*	1169
	CYP51B_F2 - 1,734 R	AACAGGCACCTTTCCCTCAC	*CYP51B*	
	CYP51B_F3 - 1,541 F	AATACGACGTACTCATCCTCCC	*CYP51B*	897
	CYP51B_F3 - 2,437 R	TTGGTTCCTGAGCGTGTCAC	*CYP51B*	
	CYP51B_F4 - 36 F	ATTGCGCAAATTCGATCGG	*CYP51B*	772
	CYP51B_F4 - 807 R	AATCGTAAACCACTCCCTCG	*CYP51B*	
	CYP51B_F5_I1_F	TTTCCATGACCTTTTGCGCG	*CYP51B*	640
	CYP51B_F5_I1_R	GGCAGAATGCGAATCCGAAC	*CYP51B*	
	CYP51B_F6_Mid_F	TCGCATGATGGAGTGGATGG	*CYP51B*	539
	CYP51B_F6_Mid_R	CTTCGTCCTCTCTCCAGGGA	*CYP51B*	
	CYP51B_F7_I2_F	ATCGTCGCTGGATTCATCGG	*CYP51B*	596
	CYP51B_F7_I2_R	CTCTCAGTTCTCGGATCGCC	*CYP51B*	
	CYP51B_F8 - 1,611 F	AGAAACCGAATGGACAATCCC	*CYP51B*	744
	CYP51B_F8 - 2,354 R	AAGCTCGCTAGTGGTTTATCG	*CYP51B*	
RT-qPCR	Ve-CYP51A-qPCR-S2 - 1,509 F	GCCAGCACTCATCTTCCAG	*CYP51A*	100
	Ve-CYP51A-qPCR-S2 - 1,608 R	CAGACACGAAGCTGTTCCTG	*CYP51A*	
	Ve-CYP51B-qPCR - 542 F	TTCACGGATGGACAACAGGG	*CYP51B*	107
	Ve-CYP51B-qPCR - 648 R	ATCTTCAATACTCGGGAGGCC	*CYP51B*	
	Ve-Actin-qPCR-S1 - 92 F	TGCATACGATCCGAGATACCTG	Actin	84
	Ve-Actin-qPCR-S1 - 175 R	ATTGTTTGGGTGAGCTTGGC	Actin	

Table 3.3. Type III test of fixed effects table generated from statistical analysis in SAS 9.4. "Num DF" indicates the number of degrees of freedom in the model. "Den DF" represents the number of degrees of freedom associates with the model errors. "Pr > F" represents the p-value associated with the F statistic.

Effect	*Num DF*	*Den DF*	*F Value*	*Pr > F*
Location	6	37	6.41	0.0001
Treatment	2	186	155.83	<.0001
*Location*Treatment*	12	186	2.58	0.0035

Table 3.4. Percent scab control comparing different DMI products and active ingredients in field trials at each of 11 locations in 2019 and 2020. Means with different letters are significantly different at each location for each year based on the Tukey-Kramer mean separation procedure. The "-" symbol indicates missing data due to lack of scab pressure at that location in that year.

Location	*County*	*Treatment*	*2019 % control*	*2020 % control*
N	Dougherty	Orius 3.6F	2.7 B	49.7 B
		Inspire	50.8 A	81.6 A
		Cevya	56.5 A	80.8 A
B3	Dougherty	Orius 3.6F	30.8 A	46.5 A
		Inspire	66.4 A	82.8 A
		Cevya	63.7 A	44.3 A
JB	Sumter	Orius 3.6F	51.3 A	45.2 B
		Inspire	76.5 A	88.2 A
		Cevya	75.3 A	81.4 A
PKD	Dougherty	Orius 3.6F	3.8 B	6.9 B
		Inspire	58.2 A	63.0 A
		Cevya	48.5 A	64.8 A
JB2	Crisp	Orius 3.6F	52.5 B	-
		Inspire	80.3 AB	-
		Cevya	89.7 A	-
SH	Wilcox	Orius 3.6F	57.1 B	-
		Inspire	97.2 A	-
		Cevya	81.4 AB	-
BP	Berrien	Orius 3.6F	14.6 B	39.1 B
		Inspire	71.1 A	81.0 A
		Cevya	57.7 A	65.5 A
JD	Lanier	Orius 3.6F	48.3 B	77.6 A
		Inspire	81.7 A	92.7 A
		Cevya	80.5 A	88.0 A
CR	Berrien	Orius 3.6F	55.7 A	9.5 B
		Inspire	89.5 A	75.0 A
		Cevya	75.0 A	62.7 A
PW	Tift	Orius 3.6F	9.8 B	12.9 B
		Inspire	82.0 A	84.4 A
		Cevya	81.2 A	76.4 A
PD	Tift	Orius 3.6F	49.8 B	29.8 B
		Inspire	87.2 A	85.5 A
		Cevya	82.2 A	73.5 A

Table 3.5. Results from the rapid assays to determine *V. effusa* isolate resistance to tebuconazole in 2019 and 2020 from the 11 locations where the field experiments were conducted in southern Georgia. Relative growth (RGr) values are presented in the far-right columns for both years.

Location	***County***	***Concentration***	***2019 RGr***	***2020 RGr***
N	Dougherty	1 µg/ml	100%	100%
		3 µg/ml	100%	61%
		10 µg/ml	86%	16%
B3	Dougherty	1 µg/ml	100%	56%
		3 µg/ml	100%	54%
		10 µg/ml	44%	5%
JB	Sumter	1 µg/ml	100%	97%
		3 µg/ml	85%	46%
		10 µg/ml	25%	9%
PKD	Dougherty	1 µg/ml	49%	48%
		3 µg/ml	46%	57%
		10 µg/ml	19%	21%
JB2	Crisp	1 µg/ml	48%	27%
		3 µg/ml	23%	9%
		10 µg/ml	3%	0%
SH	Wilcox	1 µg/ml	100%	N/A
		3 µg/ml	87%	N/A
		10 µg/ml	36%	N/A
BP	Berrien	1 µg/ml	1%	72%
		3 µg/ml	58%	32%
		10 µg/ml	25%	10%
JD	Lanier	1 µg/ml	25%	3%
		3 µg/ml	11%	0%
		10 µg/ml	1%	0%
CR	Berrien	1 µg/ml	N/A	74%
		3 µg/ml	N/A	58%
		10 µg/ml	N/A	8%
PW	Tift	1 µg/ml	67%	82%
		3 µg/ml	43%	51%
		10 µg/ml	3%	12%
PD	Tift	1 µg/ml	76%	66%
		3 µg/ml	63%	23%
		10 µg/ml	15%	4%

Table 3.6. Sensitivity of the isolates of *Venturia effusa* used to determine the mechanism of resistance to tebuconazole at 1, 3, and 10 µg/ml. The assay confirmed that the sensitive isolates were highly sensitive to tebuconazole while the resistant isolates were highly resistant to tebuconazole.

Isolate name	***Georgia county***	***Sensitivity status***	***RGr at 1 µg/ml***	***RGr at 3 µg/ml***	***RGr at 10 µg/ml***
T11	Troup	Sensitive	0%	0%	0%
T15	Troup	Sensitive	0%	0%	0%
T37	Troup	Sensitive	0%	0%	0%
108	Berrien	Resistant	100%	98%	100%
241	Berrien	Resistant	100%	73%	58%
253	Berrien	Resistant	100%	73%	58%
254	Berrien	Resistant	92%	100%	67%
407	Dougherty	Resistant	100%	76%	67%
410	Dougherty	Resistant	100%	85%	62%
482	Dougherty	Resistant	97%	75%	66%
803	Dougherty	Resistant	100%	77%	77%

Table 3.7. The isolates of *Venturia effusa* used to determine the mechanism of resistance to tebuconazole, and the mutations that were observed in the resistant isolates. Sensitive isolates are presented had no mutations in the sequences defined. The G444D mutation represents a glycine to aspartic acid amino acid substitution. The I77T mutation represents an isoleucine to threonine amino acid substitution. The I77L mutation represents an isoleucine to leucine amino acid substitution. The G357H mutation represents a glycine to histidine amino acid substitution.

Sensitivity	***Isolate Name***	***CYP51A Gene***	***CYP51B Gene***
Sensitive	T11	None	None
	T15	None	None
	T37	None	None
Resistant	407	G444D	I77T & G357H
	410	G444D	I77T & G357H
	482	G444D	I77T & G357H
	241	None	I77L & G357H
	253	None	I77T & G357H
	254	None	I77T & G357H
	803	G444D	None
	108	None	G357H

CHAPTER 4

COMBINATIONS OF MICRONIZED SULFUR WITH DIFFERENT FUNGICIDES FOR MANAGEMENT OF PECAN SCAB (CAUSED BY *VENTURIA EFFUSA*)

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Abstract

Management of pecan scab, caused by *Venturia effusa*, relies heavily on the use of fungicides. Scab control can be challenging due to the polycyclic nature of the disease, resistance development to multiple fungicide classes in the pathogen, and incomplete coverage of larger trees. In other pathosystems the addition of micronized sulfur has enhanced the control of disease achieved with certain fungicides. The aim of this study was to explore whether control of scab of pecan could be enhanced by combining micronized sulfur with labeled fungicides from different groups. Field experiments were conducted in 2019, 2020, and 2021 in commercial orchards in several counties in southern Georgia. Efficacy of control on leaf and nut scab were determined throughout the season by visual assessment, and phytotoxic effects were monitored. Application of micronized sulfur alone provided little to no control of scab, and the addition of micronized sulfur to each fungicide that we tested did not result in any increase in efficacy, and in some cases resulted in slight antagonism. Phytotoxic effects were found to be associated only with the phosphite fungicide group, but the addition of micronized sulfur significantly decreased the phytotoxic effects observed. The application of micronized sulfur may have other benefits to the pecan trees, but enhanced control of scab is not one of them, at least when applied at labeled rates in the high-volume applications normally used by pecan growers.

Introduction

Scab, a disease of pecan (*Carya illinoinensis* (Wangenh.) K. Koch) caused by the plant pathogenic fungus *Venturia effusa* (G. Winter) Rossman & W. C. Allen, can result in devastating yield loss. Control of scab is one of the largest input costs to pecan growers in the southeastern U.S. (Demaree 1924; Wells 2014). Although the use of resistant cultivars is the most effective method of managing scab, the lack of durable resistance (in some cases resistance has broken down due to adaptability of *V. effusa*) and low nut quality or yield in resistant cultivars, has resulted in scab susceptible cultivars being widely cultivated in the region (Conner and Wells 2007). The frequent summer rains in the southeastern U.S. are very conducive for scab development (Gottwald 1985; Sparks et al. 2009), thus requiring frequent application of multiple fungicide classes using air-blast sprayers as the primary method of control for commercial pecan growers (Brock and Bertrand 2007a). There are several fungicide classes that contain products labeled for use on pecan, including the demethylation inhibitors (DMIs), quinone outside inhibitors (QoIs), succinate dehydrogenase inhibitors (SDHIs), methyl-benzimidazole carbamates (MBCs), organotin compounds, phosphites, guanidines, and dithiocarbamates (Fungicide Resistance Action Committee (FRAC) codes 3, 11, 7, 1, 30, 33, U12, and M3, respectively) (Bock et al. 2017). During the growing season, fungicides are typically applied on a 10 to 14-day schedule from bud break to pollination and a 14 to 21-day schedule from pollination to shell hardening when the foliage has become less susceptible to infection (Brock and Bertrand 2007a; Gottwald and Bertrand 1983). Fungicide applications made prior to pollination are used to prevent initial infection, thus slowing progression of scab on susceptible shoot and leaf tissue. Applications made after pollination are used to prevent infection of developing fruit clusters. Infections of fruit earlier in the season have been found to have a

greater impact on yield and nut quality compared to infections that occur during the later stages of fruit development later in the season, particularly if infection of the fruit occurs after shell hardening (Gottwald and Bertrand 1983). Weather dependent fungicide application models have been proposed to conserve fungicide use and help delay resistance development (Brenneman et al. 1998; Brock and Bertrand 2007b; Payne and Smith 2012). Similar to many other pathosystems, fungicide resistance has developed in *V. effusa* to many of the FRAC groups used for control of scab. Reduced sensitivity or complete resistance of *V. effusa* has been associated with the MBCs, DMIs, QoIs, guanidines, and the organotins (Littrell 1976; Reynolds et al. 1997; Seyran et al. 2010a; Standish et al. 2019a; Standish et al. 2019b; Stevenson et al. 2015).

Until the 1940s, chemical disease control relied primarily upon applications of inorganic substances, one of which was elemental sulfur. Sulfur is one of the oldest fungicides known, and it is still in use today (Russell 2005). As early as 1824, sulfur dust was used to control powdery mildew on peaches, as well as to control other pathogens (Robertson 1824). In 1833 the preparation of boiling lime and sulfur together to form “lime sulfur” was recommended for controlling powdery mildew of grape (Kendrick 1833). In 1848 elemental sulfur was recommended for control of powdery mildew on several crops (Russell 2005). Throughout fungicide history, there has been a pattern of using sulfur as a means to control Plant Dis.s, whether used stand-alone, or in a mixture. New ways to use sulfur to combat plant pathogens are being developed.

In the early 2000s, it was reported that combining elemental sulfur (FRAC Code M2) with myclobutanil (FRAC Code 3) provided superior control of powdery mildew of nectarine, caused by *Sphaerotheca pannosa* when compared to either the sulfur or myclobutanil alone (Reuveni 2001). In 2008, the addition of sulfur to propiconazole was shown to increase control

of brown rot caused by *Monolinia fructicola* on peach compared to propiconazole alone (Holb and Schnabel 2008). Culbreath et al. (2019) evaluated different combinations of micronized sulfur as a mixing partner with DMI fungicides for control of late leaf spot (*Nothopassalora personata*) of peanut. They found that applications of stand-alone sulfur provided minimal control of late leaf spot; however, when mixed with DMI fungicides, the mixtures resulted in control that was superior to the DMI treatments alone. It is possible that the synergistic effect between certain fungicide groups and sulfur could be used to improve control of diseases in other plant pathosystems, or with other fungicide classes.

As noted, there are several classes of fungicides labeled to control scab on pecan (Bock et al. 2017; Wells 2021). In 2019, mefentrifluconazole, a DMI fungicide, was labeled for use on pecan. The possible benefit of combing mefentrifluconazole, or other classes of fungicide to improve control of scab has not been explored. The objectives of this research were to 1) evaluate the efficacy of mefentrifluconazole versus two previously labeled DMI fungicides against scab on pecan when applied with and without micronized sulfur, and 2) evaluate the effect of sulfur applied alone or as a mixing partner with fungicides from other classes to control scab.

Materials and Methods

In 2019 and 2020, field experiments were conducted in 11 pecan orchards across southern Georgia. The orchards were located in Tift, Sumter, Crisp, Wilcox, Berrien, Lanier, and Dougherty counties and contained one of four cultivars: Pawnee, Desirable, Cunard, or Wichita (Table 4.1). The cultivars were selected as they are susceptibility to infection by *V. effusa*. The orchards represent a range of environmental and physical conditions in southern Georgia, but all were selected for having a known history of scab epidemic development. At each location the

experiments were randomized complete block designs. Each experiment consisted of 8 pecan trees that were not treated with fungicides by the orchard owner. Each tree represented a block at that location. In the canopy of each tree, six different treatments were applied to individually selected, fruiting terminals; the treatments were micronized sulfur (United Phosphorous, Inc., King of Prussia, PA), Cevya, (mefentrifluconazole; BASF, Ludwigshafen, Germany) micronized sulfur mixed with Cevya, Inspire (difenoconazole; Syngenta Crop Protection, Inc., Greenville, NC), Orius 3.6F (tebuconazole; Adama, Raleigh, NC), and an untreated control (Table 4.2). The treatments were applied at the maximum labeled rate to the same terminals on a biweekly basis from nut set to shell hardening by using a handheld sprayer (Project Source model #5318), for a total of 7 applications in 2019 and 8 applications in 2020. Two liters of solution were mixed for each treatment at a dilution equivalent to a spray volume of 935 L/ha (an application volume commonly used to apply fungicide in commercial pecan orchards). The treatment solutions were manually agitated prior to application and were applied to runoff. The incidence and severity of scab was visually estimated on the fruit in July and August using the percentage scale (0-100%). Relative control (%) was calculated based on the severity ratings from the treatments and the nontreated control as follows: (1 - (severity of treated terminal/severity of nontreated terminal)).

In 2021 a third experiment was conducted to evaluate the potential for synergism between sulfur and other fungicide classes, including phosphites, DMIs, QoIs, organotins, guanidines, and MBCs). The specific treatments evaluated were Kphite (salts of phosphorus acid; Plant Food Systems, Inc, Zellwood, FL), Orius 3.6F (tebuconazole; Adama, Raleigh, NC), Abound (azoxystrobin; Syngenta Crop Protection, Inc., Greensboro, NC), Super Tin (fentin hydroxide; United Phosphorous, Inc., King of Prussia, PA), Elast (dodine; Arysta Lifescience Benelux, Ougree, Belgium), and Topsin (thiophanate-methyl; Cerexagri-Nisso LLC, King of

Prussia, PA), each applied alone and mixed with micronized sulfur. Micronized sulfur alone and a nontreated control were included (Table 4.3). Each fungicide was applied at the maximum labeled rate, and the test was repeated at 4 orchard sites in southern Georgia. The orchards were located in Tift and Berrien counties and contained the cultivars Desirable, Wichita, or Pawnee (Table 4.4). The experiment was a randomized complete block design, with 8 pecan trees (the trees were not treated with fungicides by the orchard owner). Each tree constituted a block, and the fungicides were applied as described for the 2019 and 2020 experiments using a hand-held sprayer applying the different treatments to single terminals. The applications were initiated soon after budbreak in April and repeated at 14-day intervals for a total of 9 applications for the season. Phytotoxicity ratings on the foliage were conducted in late May and mid-June by visually estimating the amount of bronzing or necrosis on all the leaves on the treated terminals (using a 0-100% scale). Leaf scab incidence (percentage of leaflets per compound leaf that had symptoms of scab) and leaf scab severity (using a 0-100% scale) ratings were assessed on July 12, 2021 by visual observation. Nut scab incidence (percentage of nuts per cluster with scab) and nut scab severity (using a 0-100% scale) ratings were evaluated on July 12, 2021 and on August 18, 2021. From the incidence or severity ratings, relative control (%) was calculated as described earlier.

For quantifying the interactions between each fungicide and sulfur, the expected efficacy of the mixture (M_{Exp}) was calculated as described by Culbreath et al. (2019): $M_{Exp} = (E_S + E_A) – (E_S \text{ X } E_A)$, where M_{Exp} represents the expected efficacy of the combination, while E_S represents the control with sulfur alone, and E_A represents the control with the fungicide alone. For each location, the mean observed control and expected control were calculated for each mixture, and a 95% confidence interval was calculated for the means. The mixture was considered synergistic if

the mean observed control was significantly higher than the expected control. The mixture was considered antagonistic if the mean observed control of the mixture was significantly lower than the mean control of the stand-alone product. The calculations were performed for both leaf and nut scab data.

Data Analysis

All field experiment data were analyzed using SAS V9.4 (SAS Institute, Cary, NC). To check for normality, the Univariate procedure was performed. Data were confirmed to be normally distributed. The data for the 2019 and 2020 field experiments were analyzed combining years and all 11 locations. The data from the 2021 field experiments were analyzed combining the data from the 4 locations. Treatment data were also analyzed separately for each cultivar to investigate differences of treatments on different cultivars. The data were analyzed using a generalized linear mixed model (GLIMMIX procedure) as a 7x2 factorial, with location, block, location x fungicide, location x sulfur (yes or no), and location x fungicide x sulfur as random effects, and fungicide treatments considered fixed effects. A Tukey-Kramer test was conducted as the mean separation procedure between each treatment as well as for the treatments with and without sulfur.

Results

In the 2019 and 2020 field experiments, symptoms of nut scab were first observed in early to mid-June. Scab severity was highly variable on nontreated terminals among the different locations in both 2019 and 2020, with some orchards having 0% scab severity on fruit clusters at the end of the growing season, while other orchards had fruit clusters with 100% scab severity ($P<0.0001$) (Figure 4.1). Stand-alone applications of Cevya resulted in 67.3 and 67.0% control in

2019 and in 2020, respectively. Stand-alone applications of micronized sulfur resulted in 8.7 and 10.9% control in 2019 and in 2020, respectively. When Cevya and micronized sulfur were combined, the combination resulted in 45.0 and 62.6% control in 2019 and in 2020, respectively. The efficacy of Cevya compared to Cevya mixed with micronized sulfur was not significantly different, therefore no synergism was observed. The application of Inspire resulted in 70.9 and 78.7% control in 2019 and in 2020, respectively. The efficacy of Cevya compared to Inspire was not significantly different, but the efficacy of Cevya and Inspire compared to Orius 3.6F was significantly different. Orius 3.6F resulted in only 22.4 and 25.7% control in 2019 and 2020, respectively. Both Cevya and Inspire were more efficacious than Orius 3.6F for controlling scab (Figure 4.2).

In the 2021 field experiments, first symptoms of leaf scab were observed in mid to late April, and first symptoms of fruit scab in early June. During the pecan growing season in Tifton, Georgia in 2021, there were 54 rainfall events, resulting in 59.7 cm of total rainfall from May 1 to September 1, which exceeds the average 42.5 rainfall events resulting in 50.3 cm of total rainfall during the specified time period (georgiaweather.net). Frequent, heavy rains post-pollination led to severe fruit scab, with a mean severity of 24.4, 14.4, and 4.5% for leaf scab, a mean incidence of 88.2, 82.5, and 44.9 for leaf scab, and a mean severity of 100, 99, and 84% for fruit scab by the end of the season on untreated Wichita, Desirable, and Pawnee terminals, respectively. Addition of sulfur did not improve efficacy for any of the products tested, therefore no synergism was observed for any treatment (Table 4.5). For the Elast treatment, slight antagonism was observed for leaf scab control when micronized sulfur was added to the mixture ($P=0.0003$). This is likely due to a mixing incompatibility of the Elast and micronized sulfur. Despite vigorous agitation, the Elast and micronized sulfur would not homogenize, and formed

clumps that settled to the bottom of the sprayer. Incompatibility was not observed for any other treatment tested. There was an antagonistic response with fruit scab control when sulfur was added to Super Tin (P = 0.0095). Kphite was the most efficacious treatment for controlling leaf scab. Interestingly, phytotoxicity was less severe on terminals that were treated with the Kphite + micronized sulfur compared to the stand-alone Kphite application for all three cultivars. Mean phytotoxicity ratings of 13.0, 11.5, and 16.6% were observed on terminals where Kphite was applied, and 4.7, 3.9, and 9.1% phytotoxicity ratings were observed on foliage where Kphite + micronized sulfur was applied to Wichita, Desirable, and Pawnee, respectively. Phytotoxicity was not observed for any other treatment. For fruit scab management, Super Tin and Elast were the most effective treatments (Table 4.5). Abound was a superior treatment on Wichita, but was mediocre on Desirable, and was the worst treatment for controlling fruit scab on Pawnee. Stand-alone applications of micronized sulfur provided little control of leaf or fruit scab, resulting in a mean control of 22.7 and 5.2%, for leaf and fruit scab, respectively.

Discussion

Micronized sulfur was found to be an inadequate mixing partner for various fungicides for control of *V. effusa*, although it did help suppress foliar phytotoxicity caused by the phosphite fungicide applications. Sulfur is a multi-site fungicide, used primarily as a protectant. The mechanism of fungistatic effects due to sulfur are largely unknown, but are speculated to be associated with the interference of mitochondrial respiration, thus inhibiting conidial germination (Cooper and Williams, 2004; Gogoi et al., 2013). Most fungicides used to control scab have single-site modes of action. Theoretically, the addition of a multi-site fungicide, especially one that improves efficacy of the mixture, would help delay development of fungicide resistance in the partner fungicides in question. The findings of Culbreath et al. (2019) provided a rationale

for the addition of micronized sulfur to pecan fungicide rotation programs to improve efficacy and thus delay development of fungicide resistance in at-risk groups. This study aimed to determine if the addition of micronized sulfur increased efficacy of the fungicides commonly used to control scab. The SDHI fungicides were not included because pydiflumetofen, the primary SDHI fungicide used for scab control, results in nearly 100% control when applied alone. Pydiflumetofen is only labeled for use on pecan as Miravis Top (Syngenta Crop Protection, Inc., Greenville, NC), which is a highly effective premix of pydiflumetofen and difenoconazole (Moore et al. unpublished).

Similar to Culbreath et al. (2019), we found that the application of stand-alone micronized sulfur did not result in suitable control of our target pathogen. For each pecan cultivar, the application of stand-alone micronized sulfur every 14 days was statistically similar to applying no fungicides whatsoever to the terminals for the duration of the season. Sulfur has been found to be phytotoxic to some crops (Johnson and Mayberry, 1980; Cantonwine et al., 2008), but no phytotoxicity was found to be associated with sulfur in this trial on pecan. Phytotoxicity has been observed on peanut when wettable powder formulations of sulfur have been applied (Cantonwine et al. 2008), but micronized sulfur is speculated to be less toxic to foliage than wettable powder formulations, and no phytotoxic effects were observed on peanut in the Culbreath et al. (2019) study, where micronized sulfur was used.

Although no synergistic effects of micronized sulfur were observed in the control of scab on pecan, there may be other benefits to applying sulfur to the canopy of the tree, including control of other pathogens or insects, decreasing phytotoxicity caused by phosphite fungicides, and reducing the potential for sulfur deficiency within the orchard. The addition of sulfur to the DMI fungicides has been shown to result in synergism in some pathosystems (Culbreath et al.

2019); however, when sulfur was combined with either Cevya and Orius 3.6F, no additional efficacy on *V. effusa* was observed. The lack of synergy might be partly explained by the dilution effect in application of fungicides to pecan. Pecans are sprayed with air-blast sprayers, which require a large volume of spray to be applied to the canopy of the tree. Typical fungicide applications for pecan use is 935 liters per hectare (L/ha), where the application volumes for most other crops are in the140 to 187 L/ha range. We applied the maximum labeled rate of 6.7 kg of sulfur per hectare; however, when diluted in 935 liters, the mix is 5- to 10-fold more dilute than if applied at 94 to 187 L/ha, which is the volume of solution commonly used to deliver fungicide to peanut. It should be noted that recent studies have shown the potential to significantly reduce spray volumes on pecans and still maintain adequate disease control (Bock et al., 2021). Such reductions in spray volume may increase the likelihood of micronized sulfur enhancing disease control in pecan with various fungicides, assuming treatment rates remain the same.

Phytotoxic effects of the phosphite, Kphite on foliage were observed, but were reduced when mixed with micronized sulfur, while maintaining excellent control of leaf scab. Phytotoxic effects of phosphites on pecan have been reported previously, as described by Bock et al. 2012. The apparent reduction in phytotoxicity could in part be due to the physical effects of the sulfur, lessening the direct and immediate exposure of the Kphite to the leaf's surface. The physical effect of sulfur could also explain the slightly decreased efficacy of the Kphite + micronized sulfur combination compared to the Kphite alone. Although the micronized sulfur decreased the efficacy of the combination, it was still one of the most effective treatments to reduce leaf scab. For this reason, using phosphite fungicides pre-pollination is recommended for control of leaf scab (Wells 2021). After pollination, most leaves have been produced on the terminal and

"harden off", developing a thick cuticular layer, making them less likely to develop further scab (Demaree 1924; Latham 1979; Gottwald 1985; Turechek and Stevenson 1998; Brock and Brenneman, 2020). Super Tin and Elast treatments were highly effective against fruit scab, which was not unexpected. For this reason, both Super Tin and Elast are recommended as a major component of fungicide rotation plans to manage fruit scab (Brock and Brenneman, 2020).

Another interesting finding was the variable levels of scab control that Abound provided at the different orchard locations on the various pecan cultivars. Such variability has been observed previously and was explained by the presence or absence of the G137S mutation that confers resistance of *V. effusa* to the QoI fungicides (Standish et al., 2016; Herrington 2019). Scab control was poor on cultivars Desirable and Pawnee, but on Wichita, the most scab-susceptible cultivar in the study, Abound was one of the most efficacious fungicides.

One goal of this study was to determine the efficacy of Cevya applied alone to control scab. The results show that mefentrifluconazole has comparable efficacy to that of difenoconazole for controlling scab, and both were superior to the control provided by the DMI tebuconazole. Cross resistance among the DMI fungicides has been found in *V. effusa* with fenbuconazole, propiconazole, and tebuconazole, but has not yet been observed for mefentrifluconazole or difenoconazole (Reynolds et al. 1997; Stevenson et al. 2015). Tebuconazole has been widely used for many years, and resistance of *V. effusa* to tebuconazole is commonly observed, which was reflected by the poor efficacy of tebuconazole observed at all locations in these experiments (Seyran et al. 2010a; Standish et al. 2019a). DMI fungicides are considered to be at high risk of resistance development, therefore Cevya should not be applied multiple times, nor consecutively in an orchard. According to the label, Cevya can only be applied 3 times per year at 365 ml/ha or 5 times per year at 219 ml/ha. The benefit to having

Cevya labeled for use on pecan is that it adds another active ingredient to the arsenal that commercial pecan growers have available to combat scab. With more active ingredients, the potential for fungicide rotations increases, which helps delay fungicide resistance development.

Although the findings indicate that sulfur is an inadequate mixing partner with the fungicides tested for control of scab, there are some valuable implications that this test highlights. The use of the phosphite fungicides pre-pollination, the use of Super Tin, Elast, and Cevya post-pollination, as well as the effect of sulfur reducing phytotoxicity from the phosphites are valuable takeaways. Commercial pecan growers are eager to add new chemistries to their toolbelt for mitigation of *V. effusa*, as well as to preserve the chemistries that are currently available. With effective fungicide rotations in place, and by using some of the findings from the current study, growers should have the ability to provide reasonable control of this disease, as well as delay fungicide resistance development.

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Table 4.1. List of pecan orchards used in 2019 and 2020 field experiments.

Location name	*GPS coordinates*	*Georgia County*	*Cultivar*
N	31.499450, -84.278361	Dougherty	Desirable
B3	31.522600, -84.079592	Dougherty	Desirable
JB	32.014708, -84.201233	Sumter	Cunard
PKD	31.528711, -84.364462	Dougherty	Cunard
JB2	32.000343, -83.937048	Crisp	Pawnee
SH	31.854409, -83.356105	Wilcox	Desirable
BP	31.028016, -83.287860	Berrien	Cunard
JD	31.058426, -83.157004	Lanier	Pawnee
CR	31.108749, -83.214079	Berrien	Pawnee
PW	31.512288, -83.640974	Tift	Wichita
PD	31.509773, -83.641328	Tift	Desirable

Table 4.2. The fungicide treatments applied to compare scab (caused by *Venturia effusa*) control efficacy on fruit in field experiments in pecan orchards southern Georgia in 2019 and 2020. In the first column, FRAC stands for Fungicide Resistance Action Committee.

FRAC class	*Product*	*Rate/ha*	*Rate a.i./ha*	*Active ingredient*
3	Cevya	365.4 mL	146.3 g	Mefentrifluconazole
3	Cevya +	365.4 mL	146.3 g	Mefentrifluconazole
M2	Microthiol S	6.7 kg	5.4 kg	Sulfur
M2	Microthiol S	6.7 kg	5.4 kg	Sulfur
3	Inspire	489.6 mL	122.1 g	Difenoconazole
3	Orius 3.6F	584.6 mL	252.3 g	Tebuconazole
N/A	nontreated	N/A	N/A	N/A

Table 4.3. The fungicide treatments applied without and with micronized sulfur to compare scab (caused by *Venturia effusa*) control efficacy on leaves and fruit in field experiments in pecan orchards southern Georgia in in 2021. In the first column, FRAC stands for Fungicide Resistance Action Committee.

FRAC class	***Product***	***Rate/ha***	***Rate a.i./ha***	***Active ingredient***
P07	Kphite	4.7 L	2.5 kg	Salts of Phosphorous Acid
P07	Kphite +	4.7 L	2.5 kg	Salts of Phosphorous Acid
M2	Microthiol S	6.7 kg	5.4 kg	Sulfur
3	Orius 3.6F	584.6 mL	252.3 g	Tebuconazole
3	Orius 3.6F +	584.6 mL	252.3 g	Tebuconazole
M2	Microthiol S	6.7 kg	5.4 kg	Sulfur
11	Abound	796.5 mL	198.4 g	Azoxystrobin
11	Abound +	796.5 mL	198.4 g	Azoxystrobin
M2	Microthiol S	6.7 kg	5.4 kg	Sulfur
30	Super Tin	876.9 mL	420.3 g	Triphenyltin Hydroxide
30	Super Tin +	876.9 mL	420.3 g	Triphenyltin Hydroxide
M2	Microthiol S	6.7 kg	5.4 kg	Sulfur
U12	Elast	2.6 L	1.1 kg	Dodine
U12	Elast +	2.6 L	1.1 kg	Dodine
M2	Microthiol S	6.7 kg	5.4 kg	Sulfur
1	Topsin	1.5 L	784.6 g	Thiophanate-methyl
1	Topsin +	1.5 L	784.6 g	Thiophanate-methyl
M2	Microthiol S	6.7 kg	5.4 kg	Sulfur
M2	Microthiol S	6.7 kg	5.4 kg	Sulfur
N/A	nontreated	N/A	N/A	N/A

Table 4.4. List of pecan orchards used in 2021 field experiments.

Location name	***GPS coordinates***	***Georgia County***	***Cultivar***
PN	31.506893, -83.641830	Tift	Desirable
CR	31.108749, -83.214079	Berrien	Pawnee
PW	31.512288, -83.640974	Tift	Wichita
PD	31.509773, -83.641328	Tift	Desirable

Table 4.5. Mean efficacy of fungicide treatments applied with or without micronized sulfur to compare scab (caused by *Venturia effusa*) control efficacy on leaves and fruit in field experiments in pecan orchards acrosds southern Georgia in 2021. Means with different letters in the same column are significantly different based on Tukey-Kramer mean separation procedure ($P < 0.05$). Means with different letters in the same row for leaf scab control and nut scab control are significantly different based on Tukey-Kramer mean separation procedure ($P < 0.05$).

	Leaf scab (%)		**Fruit scab (%)**	
Fungicide	***Stand-alone***	***With Sulfur***	***Stand-alone***	***With Sulfur***
Nontreated	0.0 D	22.7 D	0.0 D	5.2 D
Abound	58.5 B	56.0 B	32.4 BC	37.1 AB
Elast	59.3 B	34.0 CD	52.0 AB	53.7 A
Kphite	86.0 A	83.5 A	27.1 C	26.6 BCD
Teb	40.5 C	37.2 C	20.4 CD	13.7 CD
Tin	52.6 BC	43.9 BC	56.7 A	40.0 AB
Topsin	55.4 B	45.0 BC	27.1 C	34.5 ABC

Figure 4.1. Fruit scab severity on untreated terminals in pecan trees in field experiments across southern Georgia in 2019 and 2020. Means with different letters are significantly different based on Tukey-Kramer mean separation procedure ($P < 0.05$). Bars indicate standard deviation from the mean.

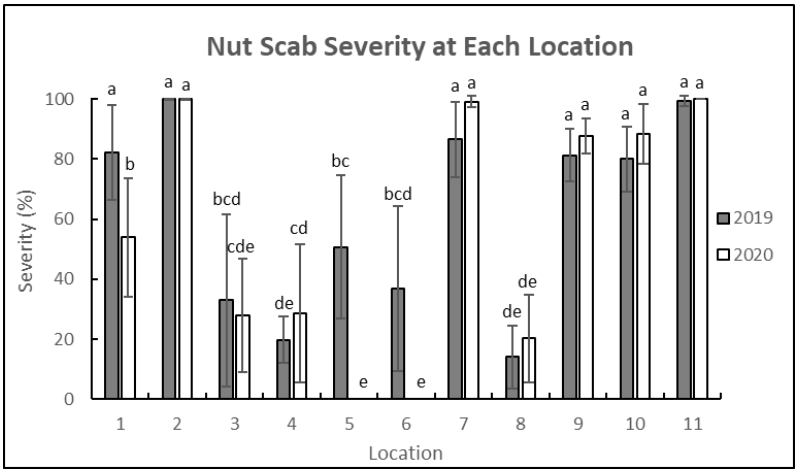
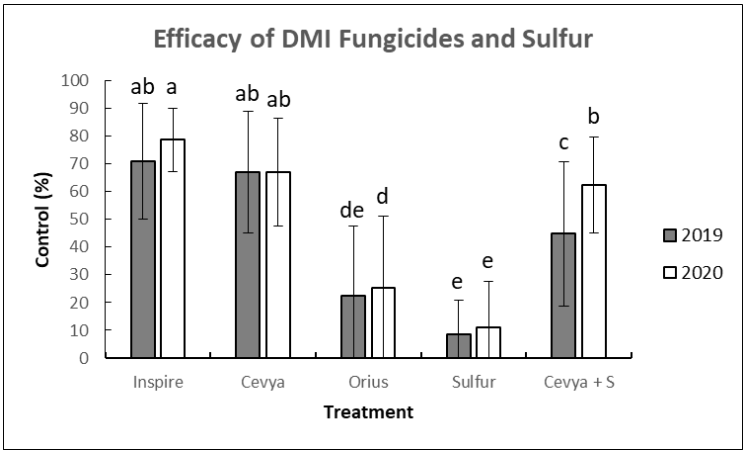


Figure 4.2. Comparison of efficacy of Inspire (difenoconazole), Cevya (mefentrifluconazole), Orius 3.6F (tebuconazole), micronized sulfur, and Cevya combined with micronized sulfur for controlling nut scab in field experiments in South Georgia in 2019 and 2020. Means with different letters are significantly different based on Tukey-Kramer mean separation procedure ($P < 0.05$). Bars indicate standard deviation from the mean.



CHAPTER 5

FUNGICIDE SENSITIVITY OF PECAN SCAB FROM DIFFERENT CULTIVARS OF PECAN FROM THE SAME ORCHARDS WITH SIMILAR HISTORIES OF FUNGICIDE EXPOSURE

Moore, L.C., Bock, C.H., and Brenneman, T.B. To be submitted to PhytoFrontiers, December 17, 2021.

Abstract

Venturia effusa, the causal agent of pecan scab, is the most economically damaging disease affecting commercial pecan production in the southeastern U.S. In 2018 and 2019, multiple commercial pecan growers reported an abnormal pattern of scab development in pecan orchards where they reported that the disease was unusually different on pairs of pecan cultivars – despite application of the same control measures, the cultivar with historically less disease had severe scab, yet the other, with historically more disease, had little to no scab severity. To address the potential cause of this phenomenon, we collected isolates from the different cultivars in multiple pecan orchards and performed rapid assays to test for sensitivity to various fungicides including fentin hydroxide, tebuconazole, thiophanate methyl, and dodine. We sequenced the cytochrome b gene of multiple isolates of each cultivar and screened for the G137S mutation that causes resistance of *V. effusa* to the QoI fungicides. The results indicate that fungicide sensitivity is not contributing to the phenomenon being observed, and additional research on this topic should be addressed in the future.

Introduction

Pecan (*Carya illinoinensis* (Wangenh.) K. Koch) production has been increasing in several regions of the world, and as a specialty crop, pecan is becoming increasingly important. The U.S. is one of the global leaders in pecan production, and the state of Georgia is the biggest producer of pecans in the U.S. (USDA NASS 2021). Scab, caused by the plant pathogenic fungus *Venturia effusa* (G. Winter) Rossman & W. C. Allen, is the most damaging disease of pecan in the southeastern U.S. (Demaree 1924; Wells 2014). *V. effusa* thrives in areas with high temperature, high humidity, and high rainfall, which is representative of the climate of the southeastern states (Gottwald 1985; Sparks et al. 2009). Conidia of *V. effusa* are dispersed in rain splash and wind, and are capable of infecting developing fruit, mature fruit, twigs, shoots, buds, and young, expanding foliage that has not yet developed a thick cuticular layer (Latham 1979; Gottwald and Bertrand 1982; Gottwald 1985; Turechek and Stevenson 1998; Stevenson and Bertrand 2001; Brock and Brenneman, 2020). The primary method for controlling *V. effusa* in commercial pecan orchards is through the use of fungicides, which are typically applied 7 to 10 times per growing season from just after budbreak to shell hardening. The fungicides are applied on a 10- to 14-day schedule pre-pollination and a 14- to 21-day schedule post-pollination (Brock and Bertrand 2007). Current modes of action labeled for use on pecan include the guanidine, phosphite, demethylation inhibitors (DMI), strobilurin (QoI), succinate dehydrogenase inhibitor (SDHI), methyl-benzimidazole carbamate (MBC), organotin, and dithiocarbamate classes of fungicide (Bock et al. 2012; Brock and Brenneman 2020).

As with many other pathosystems, fungicide resistance is an issue in commercial pecan orchards. The polycyclic nature of scab, the ability *V. effusa* to survive as stromata on the host between growing seasons, the perennial nature of the crop, and the fact that sexual recombination

in nature likely occurs, all contribute to a higher risk of fungicide resistance developing in the pathogen (Demaree 1924; FRAC 2019; Young et al. 2018; Charlton et al. 2020). The risk is compounded further by the very large size of pecan trees which can exceed 30 m in height, thus making uniform, efficacious fungicide coverage challenging (Bock et al. 2013; Bock et al. 2015; Bertrand and Brenneman 2007). Decreased sensitivity of *V. effusa* to the QoIs, DMIs, organotins, guanidines, and the MBCs has been reported in commercial pecan orchards in the southeastern U.S. (Littrell 1976; Reynolds et al. 1997; Seyran et al. 2010; Stevenson et al. 2015; Standish et al. 2019). Partial resistance to the QoI fungicides has been reported and attributed to the novel G137S mutation described by Standish et al. (2019b). Mechanisms of resistance in *V. effusa* to the other fungicides used to control scab are currently unknown. Understanding the mechanism of resistance to different fungicides can aide in efficient fungicide resistance monitoring programs using molecular techniques (Standish et al. 2020).

An alternative method for mitigating infection of pecan by *V. effusa* is to select resistant cultivars at orchard establishment. The most commonly grown cultivars with moderate to high resistance to *V. effusa* include Stuart, Kanza, Elliott, Gloria Grand, Curtis, Excel, Barton, and Sumner, among others (Thompson and Grauke 1994; Wells and Conner 2015). Widely grown cultivars that are more susceptible to infection by *V. effusa* include Wichita, Schley, Pawnee, Cunard, and Desirable, among others (Thompson and Grauke 1994; Wells and Conner 2015). The more highly resistant cultivars including Excel are nearly immune to scab, although in recent very wet years scab has been observed on some resistant cultivars too. Indeed, the history of resistant pecan cultivars shows a pattern where the cultivar becomes increasingly susceptible over a period of years as it becomes more widely grown and the pathogen population adapts to that cultivar's resistance (Sparks 1992; Goff et al. 1996). As with many other crops, there is a

tradeoff in planting disease resistant cultivars. Scab-resistant cultivars of pecan are typically lower yielding than the susceptible cultivars, and the nut size and quality is typically inferior compared to susceptible cultivars, which, combined with the lack of long-term data for newer cultivars, explains the large acreages of susceptible cultivars that are planted across the southeast (Wells 2012; Thompson and Conner 2012). The most widely-grown cultivars in the southeastern U.S. are Desirable and Pawnee, which are both highly susceptible to infection by *V. effusa* (Wells, 2014).

Different races of *V. effusa* tend to be highly cultivar specific (Hunter et al., 1986). The first report of differences in virulence among isolates of *V. effusa* was by Demaree and Cole (1929), who conducted cross inoculations of the pathogen on different cultivars to demonstrate that at least four different races of *V. effusa* existed that varied in their capacity to infect the pecan cultivars. Conner (2002) conducted a detached leaf assay to study race-specific resistance to *V. effusa*. Monoconidial isolates from the cultivars Desirable, Wichita, Cape Fear, and Elliot showed that the population of *V. effusa* is composed of different races that are highly specific to the host cultivar that can be infected (Conner 2002; Conner and Stevenson 2004). According to grower reports, it is apparent that the host cultivar specializations may not be completely uniform across the region, and that localized differences occur, presumably due to adaptation of the scab pathogen to specific cultivars. Often, pecan orchard managers have a good knowledge of the relative scab susceptibility of the cultivars in their orchards, and use that information to optimize fungicide programs.

In 2018 and 2019, several pecan growers in southern Georgia observed an unusual pattern of scab development relative to cultivar susceptibility in their orchards. The concerns were based on observations made early in the growing season, and were in regard to orchards

that contained either Stuart and Desirable, or Pawnee and Desirable growing in the same orchard. Growers reported that cultivars Stuart and Pawnee had more severe scab compared to cultivar Desirable. The unusual phenomenon had not been observed previously, despite the orchard managers having years of experience controlling scab in these same trees. Although the cultivar Stuart might develop severe scab, it is considered a moderately scab resistant cultivar, particularly when compared with either Desirable or Pawnee which are both known to be highly scab-susceptible.

Further investigation was conducted by plant pathologists, including location visits, and the unusual pattern of scab development between cultivars was confirmed. Also confirmed was that within the orchards, the same fungicide programs had been used on both cultivars. The anomaly resulted in the hypothesis that different pecan cultivars may have different "races" of *V. effusa*, and specifically that race on the cultivar Stuart or Pawnee had developed resistance to one or more commonly used fungicide class, while the Desirable "race" had not. Due to these observations and the resulting hypothesis, the aim of this study was to determine the cause of the abrupt, apparent change in scab cultivar susceptibilities in the mixed pecan orchards.

Materials and Methods

Cultivar race-related differential fungicide sensitivity of *V. effusa* could occur with multiple classes of fungicides, but QoIs (FRAC group 11) were considered the most likely candidate since they were used prepollination, and resistance to QoIs is well-documented in some scab populations (Standish 2016; Herrington 2019; Standish 2019b). However, since the current fungicide sensitivity of a population can reflect all previous fungicide exposure, sensitivity of *V. effusa* isolates was tested to multiple classes of fungicide with reported resistance in the pathogen.

Isolate collection and preparation. Multiple foliar samples of *V. effusa* were collected from five different orchards that reported the occurrence of unusual cultivar susceptibility to scab in Taylor, Sumter, Peach, and Ware counties in South Georgia. Pecan leaves with symptoms of *V. effusa* were collected from both cultivars at random from multiple locations within each orchard. The relative spacing of trees in orchards varied, but in all cases the cultivars were in close proximity and had been sprayed with the same pesticide programs each year.

A stock solution of Tween 20 (one drop per 100 ml) and sterile distilled water with antibiotics (tetracycline, streptomycin sulfate, and chloramphenicol, each at 50 µg /ml) was prepared. 750 ml aliquots of the solution were pipetted into 24 × 1.5 ml microcentrifuge tubes (3 per cultivar from each orchard). Three groups of 15 leaflets collected from each cultivar at each location were selected for conidial collection. Conidia were collected by carefully pumping 9 µl of the solution on a sporulating lesion of scab on each leaflet using a pipette. Thus the conidia collected from a lesion on each leaflet were bulked in the microcentrifuge tube to make a composite suspension of conidia from that cultivar and location (Seyran et al. 2010).

Sensitivity to DMIs, Guanidines, Organotins, and MBCs. Potato dextrose agar (PDA) in Petri plates amended with the aforementioned antibiotics was further amended with dodine (3 µg/mL), tebuconazole (1, 3, and 10 µg/mL), thiophanate-methyl (TPM) (1 µg/mL), or fentin hydroxide (TPTH) (3, 10, and 30 µg/mL) following the method described by Seyran et al. (2010). Each plate was divided into 3 sections to represent the three groups of 15 leaves described earlier. There were 2 replicates and 3 groups per cultivar from each location. Each of the 3 sections on each Petri plate was inoculated with 23 ul of the conidial suspension prepared as described earlier from the corresponding microcentrifuge tube. The Petri plates containing TPTH, dodine, and TPM were incubated in the dark for 48 hours at 25°C, and relative

germination (RGe) data were recorded using the methods described by Seyran et al. (2010). The tebuconazole amended Petri plates were incubated for 72 hours, and the relative growth (RGr) data were collected using the methods described by Seyran et al. (2010).

Resistance to QoIs. The conidial suspension previously described was used as a source of *V. effusa*. An aliquot of 23 ul was transferred to a Petri plate with PDA containing the aformentione antibiotics, and the volume was spread evenly using a sterile glass rod. The Petri plates with PDA were incubated in the dark at 25°C for 24 hours. Between 18 and 43 monoconidial isolates for each cultivar at each location were isolated by removing single, germinated conidia and placing them individually on fresh PDA plates amended with the aforementioned antibiotics. The monoconidial isolates were incubated in the dark at 25°C for 4 weeks for the colonies to reach 15–30 mm in diameter.

Two additional locations, one each in Dougherty and Ware counties, reported the same phenomenon in 2017. In both cases, trees of cultivar Stuart planted in the same orchard as cultivar Desirable trees and receiving the same fungicide applications exhibited more severe scab than the cultivar Desirable trees. Isolates from both cultivars (43 Desirable and 36 Stuart isolates from Dougherty County; 42 Desirable and 24 Stuart isolates from Ware County) were collected and had been placed in storage on PDA plates containing the aforementioned antibiotics in 2017. Since the isolates were mycelial, the rapid assay was not conducted for the two locations; however, the isolates were screened for sensitivity to the QoI fungicides. The isolates were transferred to fresh PDA in Petri plates, and incubated in the dark at 25°C for 4 weeks. After 4 weeks, all cultures had sufficient mycelial growth to extract genomic DNA using the UltraCLean Microbial DNA Islation Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. The cytochrome *b* gene was amplified using a polymerase chain reaction (PCR) protocol with

the FeCytb_F1 and FeCytb_R1 primers (Standish et al. 2016). PCR conditions were a 3-minute denaturation period at 94°C, 35 cycles of repeated amplification (94 °C for 30 seconds, 55°C for 45 seconds, and 72°C for 50 seconds), and the final extension was at 72°C for 10 minutes. Amplified DNA were removed immediately following PCR, and successful amplification was confirmed by gel electrophoresis on 1.5% agarose gels (Standish et al. 2016). The amplicons were purified following the protocol from the QiaQuick PCR Purification Kit (Qiagen, Valencia, CA). The purified DNA was sent to Eurofins MWG Operon LLC (Louisville, KY) for sanger sequencing. The resulting sequence and the forward and reverse primer sequences were analyzed using MEGA6 software (Tamura et al. 2013), and the sequences were aligned. Individual isolate sequences were screened for the G137S mutation that results in reduced sensitivity of *V. effusa* to the QoI fungicides (Standish et al. 2019).

Data Analysis

Data were analyzed using SAS V9.4 (SAS Institute, Cary, NC). Data were analyzed to investigate differences in fungicide sensitivity of *V. effusa* between cultivars at each location using a generalized linear mixed model (PROC GLIMMIX) with location and cultivar as fixed effects, and group/rep as random effects. Distribution of relative growth (RG) and relative germination (RGe) for each cultivar at each location were explored (PROC UNIVARIATE). The Tukey-Kramer test was used as the mean separation procedure between the different cultivars at each location.

Results

Sensitivity to DMIs, Guanidines, Organotins, and MBCs. There were very few significant differences in the sensitivity of *V. effusa* to TPTH, TPM, dodine, or tebuconazole

between the cultivars at each location. For isolates from the Sumter County orchard, the relative germination (RGe) of the conidia at the discriminatory concentration of 10 ppm for TPTH was 57% for cultivar Stuart and 70% for cultivar Desirable ($p = 0.3275$). For the same location, the RGe for TPM for cultivar Stuart was 8%, and for cultivar Desirable was 20% ($p = 0.3760$). For dodine, the RGe was 0% for both cultivars. For tebuconazole, the discriminatory concentration used for resistance monitoring was 1 ppm, and the relative growth (RGr) of the conidia for cultivar Stuart was 85%, and for cultivar Desirable was 81% ($p = 0.4282$). For isolates from the Peach County orchard #1, the RGe values for the discriminatory concentration for TPTH was 58% for both cultivars Pawnee and Desirable ($p = 0.7104$). The RGe for TPM was 14 and 15% for cultivars Pawnee and Desirable, respectively ($p = 0.9496$). For dodine, the RGe was 0% for both cultivars. For tebuconazole, the RGr values were 100 and 62% for the cultivars Pawnee and Desirable, respectively ($p = 0.1304$). For isolates from the Peach County orchard #2, the RGe values for the discriminatory concentration for TPTH was 63 and 42% for cultivar Pawnee and Desirable, respectively ($p = 0.1393$). The RGe for TPM was 43 and 31% for cultivars Pawnee and Desirable, respectively ($p = 0.3440$). For dodine, the RGe was 3 and 15% for cultivars Pawnee and Desirable, respectively ($p = 0.0089$). For tebuconazole, the RGr values were 100% both cultivars. For isolates from the Taylor County orchard, the RGe values for the discriminatory concentration of TPTH was 5 and 72% for cultivars Pawnee and Desirable, respectively ($p = 0.0017$). The RGe for the discriminatory dose of TPM was 54 and 6% for cultivars Pawnee and Desirable, respectively ($p = 0.0047$). For dodine, the RGe was 5 and 12% for cultivars Pawnee and Desirable, respectively ($p = 0.5990$). For tebuconazole, the RGr values were 100% for both cultivars (Table 5.1).

Resistance to QoIs. DNA was successfully extracted from all isolates, and the sequences were obtained for mutation screening (Table 5.2). As has been previously found, there were large differences among locations regarding incidence of the mutation, ranging from 0 to 67%. For the Sumter County orchard, 26% of the isolates from cultivar Stuart had the G137S mutation, while 14% of the isolates from cultivar Desirable had the G137S mutation. In the Peach County orchard #1, 28% of the cultivar Pawnee isolates had the mutation, while 67% of the cultivar Desirable isolates had the mutation. In the Peach County orchard #2, 53% of the cultivar Pawnee isolates had the mutation, while 31% of the cultivar Desirable isolates had the mutation. In Dougherty County, 24% of the cultivar Stuart isolates had the mutation, while 19% of the cultivar Desirable isolates had the mutation. In Ware County, none of the isolates from either cultivar had the mutation (Table 5.2). Isolates from the Taylor County orchard were not available for mutation screening, so the G137S data for that site is unknown. The data show no clear pattern associated with sensitivity of *V. effusa* to the QoI fungicides when considering the severity of scab between the different cultivars at the different locations. It does not appear that isolates of *V. effusa* from one cultivar consistently had a higher frequency of the mutation compared to isolates from the other cultivar.

Discussion

Pecan growers were concerned that *V. effusa* might be adapting to fungicides and becoming less predictable in response to control measures. The sudden and unexpected onset of scab on a cultivar that has historically been less susceptible is perplexing. The original hypothesis was that there are different races of *V. effusa* that infect each cultivar, and that isolates from one cultivar are becoming resistant to a commonly used fungicide, thus allowing the disease to develop only on that cultivar. Our results from the rapid assay suggest that there are

few if any differences in the sensitivity of the isolates from the two cultivars to discriminatory doses of TPTH, TPM, dodine, or tebuconazole. Nor were there differences in the sensitivity to the QoI fungicides, despite the fact that each grower used QoI fungicides, primarily kresoxim-methyl and azoxystrobin, early in the season. The results did not show any clear pattern that the isolates from the different cultivars differ in their sensitivity to the QoI fungicides based on the G137S mutation frequency, which is the only known mutation for QoI resistance in *V. effusa* (Standish et al. 2016).

Fungicide resistance is an issue for scab management on pecan, and although there are multiple chemistries labeled for use, many of them are becoming less effective due to resistance development. In order to preserve new and existing chemistries that are used to control scab, rotations of fungicides with different modes of action are recommended (Brent and Hollomon 1995; Brent and Hollomon 1998; Wells 2021). Relying on repeated applications of a single mode of action to control a pathogen will likely lead to resistance development in populations of *V. effusa* in that orchard, and likely surrounding orchards considering the ability of the pathogen to disperse (Gottwald and Bertrand 1982).

There is evidence that different races of *V. effusa* infect different pecan cultivars (Demaree and Cole, 1929; Conner, 2002). It is probable that races of *V. effusa* that infect cultivar Desirable are not equally capable of infecting cultivars Pawnee and Stuart (and vice versa), if indeed they could infect them at all. In the closely related apple scab (*Venturia inaequalis*) pathosystem, there is evidence of genetically distinct races of *V. inaequalis* infecting different cultivars of apple (Xu et al. 2013; Passey et al. 2016), similar to what may be occurring in the pecan scab pahtosystem. However, the nature of isolate-cultivar specificity for these pecan cultivars has yet to be demonstrated. One explanation for the results observed in this study is that

the isolates evaluated could freely move from one cultivar to another, thus any selection for reduced sensitivity would be expressed equally on both cultivars. A second possible explanation might simply involve differences between cultivars regarding the phenology of fruit development. Some cultivars develop fruit earlier in the growing season compared to other cultivars (Sparks 1991). As the shuck tissue starts to expand as the fruit grows, it is much more vulnerable to scab infection as protection from fungicides (particularly protectant types) is reduced, and the timing of rainfall events relative to this period of increased scab susceptibility could certainly influence incidence of infection events on those parts of the fruit less protected due to the expansion. Yet a third factor that can be considered is that fungicide resistance in *V. effusa* is not necessarily a stable trait (Standish et al. 2019a). Isolates of *V. effusa* with higher levels of TPTH resistance develop within a season, but were demonstrated to not persist through the winter to the following season (Standish et al. 2019a). Instability of fungicide resistance is well documented in various other pathosystems (Köller et al. 1991; Yourman et al. 2001; Karaoglandis and Thanassoulopoulos 2002; Cox et al. 2007; Ishii et al. 2015), and provides a potenital alternative explanation. It is likely that other interactions between the pathogen, the cultivar, the environment, and persistence of fungicide resistance exist based on fitness costs, but have yet to be explored.

Although the results do not resolve why one cultivar was unexpectedly and severely affected by scab while the other was not, but the results have ruled out some important hypotheses. The severe scab developing on one cultivar in a mixed planting, but not the other when treated with the same fungicides does not appear to be due to race related fungicide resistance. New hypotheses harnessing molecular techniques, and including race differentiation can be developed to explore reasons for the subject of research in this study and other aspects of

scab control using fungicides. The entire genome of *V. effusa* has now been sequenced, serving as a valuable resource for continued molecular, genetic, and genomic advances (Bock et al. 2016; Winter et al., 2020). Tools can be developed from the genomic resources and are needed to address scab management issues and provide practical tools for monitoring fungicide resistance in *V. effusa*.

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Table 5.1. Results of the rapid assay to screen isolates of *Venturia effusa* for sensitivity to fentin hydroxide (TPTH), thiophanate methyl (TPM), dodine, and tebuconazole. Results are presented as relative germination (%RGe) values for TPTH, TPM, and Dodine, and as relative growth (%RGr) values for tebuconazole. Higher RGe or RGr values indicate higher germination or growth, while lower RGe and RGr values indicate lower of germination and growth. Different letters indicate statistical differences between cultivars at individual locations according to Tukey-Kramer mean separation**.**

Treatment	***Concentration***	***Cultivar***	***Orchard (% RGe or RGr)***			
			Sumter	**Peach 1**	**Peach 2**	**Taylor**
TPTH	3 ppm	Stuart/Pawnee	73 A	58 A	63 A	34 A
		Desirable	73 A	64 A	95 A	85 A
	10 ppm	Stuart/Pawnee	57 A	58 A	63 A	5 A
		Desirable	70 A	58 A	42 A	72 B
	30 ppm	Stuart/Pawnee	0 A	2 A	1 A	0 A
		Desirable	2 A	1 A	1 A	0 A
TPM	1 ppm	Stuart/Pawnee	8 A	14 A	41 A	6 A
		Desirable	20 A	15 A	31 A	54 B
Dodine	3 ppm	Stuart/Pawnee	0 A	0 A	3 A	5 A
		Desirable	0 A	0 A	15 B	12 A
Tebuconazole	1 ppm	Stuart/Pawnee	85 A	100 A	100 A	100 A
		Desirable	81 A	62 A	100 A	100 A
	3 ppm	Stuart/Pawnee	60 A	44 A	72 A	92 A
		Desirable	55 A	55 A	51 A	100 A
	10 ppm	Stuart/Pawnee	27 A	13 A	13 A	63 A
		Desirable	23 A	10 A	7 A	76 A

Table 5.2. The percentage of the isolates of *Venturia effusa* that contains the G137S mutation from samples collected from two different cultivars interplanted at each location. The G137S mutation leads to partial resistance to the QoI fungicides. The "*" symbol in the cultivar column indicates which cultivar displays severe infection of scab.

Location	***Cultivar***	***Number of Isolates***	***G137S mutation (% of isolates)***
Sumter County	Stuart *	23	26
	Desirable	21	14
Peach County #1	Pawnee *	19	28
	Desirable	19	67
Peach County #2	Pawnee *	18	53
	Desirable	26	31
Dougherty County	Stuart *	36	24
	Desirable	43	19
Ware County	Stuart *	24	0
	Desirable	42	0

CHAPTER 6
SUMMARY AND CONCLUSIONS

Pecan is growing in importance and in area under cultivation in several regions of the world. Pecans are native to the U. S., and the nation is a major producer, with the majority of the crop being grown in the southeast, where temperatures, humidity, and rainfall are high. The environmental conditions in the southeastern U.S. are ideal for the development scab, caused by the plant pathogenic fungus, *Venturia effusa* (Gottwald 1985; Sparks et al. 2009). Indeed, scab is the most damaging and yield limiting disease affecting pecan production in the Southeast. Scab can be devastating if left unmanaged. In 2021, a particularly wet year, terminals in this study that were not sprayed with an efficacious fungicide would suffer nearly 100% yield loss. Several newer cultivars of pecan are more resistant to scab, but most of the cultivars currently grown in Georgia are at least moderately susceptible, and some of the more popular cultivars, such as Desirable and Pawnee are highly susceptible. Cultivar selection is one of the most important considerations for controlling scab in commercial pecan orchards, however, most scab 'resistant' cultivars lack the horticultural traits desired by commercial pecan growers and the nut traits preferred by consumers. Also, resistant cultivars often become increasingly susceptible to scab over a period of years (Sparks 1992; Goff et al. 1996). The scab fungus adapts to the resistance, and a population shift occurs resulting in previously resistant cultivars becoming susceptible. With potential changes in cultivar scab susceptibility, and the large acreages of susceptible orchards currently planted in the Southeast, fungicide applications must be the primary method

for controlling scab. Robust and effective fungicide rotation programs are critical to successfully and consistently producing pecans on a commercial scale in the southeastern U.S.

Fortunately, there are several fungicides belonging to different fungicide classes that are labeled for use on pecan, including fungicides belonging to the strobilurin (QoI), demethylation inhibitor (DMI), succinate dehydrogenase inhibitor (SDHI), methyl-benzimidazole carbamate (MBC), dithiocarbamate, guanidine, organotin, and phosphite groups. Unfortunately, resistance in *V. effusa* has evolved over time to several of these fungicide groups, including the QoIs, DMIs, MBCs, organotins, and the guanidines. With fungicide resistance being prevalent in *V. effusa* populations, it is imperative that research is continued to improve fungicide resistance management and develop fungicide rotation programs that minimize risk of resistance developing and extend the lifespan of the available chemistries.

In 2019 and 2020, there were 11 commercial pecan orchards in southern Georgia used to conduct several studies relative to fungicide-based control of pecan scab. While commercial orchards use integrated programs with multiple classes of fungicide, these studies used multiple applications of the same fungicide applied to single terminals to investigate the relative activity of individual products with multiple modes of action (i.e., some premixes), as well as their individual components when applied separately. The fungicide sensitivity of the *V. effusa* population in each orchard was assessed. A rapid resistance assay conducted as described by Seyran et al. (2010) to test isolates from each orchard to tebuconazole, fentin hydroxide, dodine, and thiphanate-methyl suggested that resistance to tebuconazole is widespread, but efficacy of dodine, thiophanate methyl, and fentin hydroxide still remains quite strong (appendix A). The results showed that a new fungicide, Miravis Top, is extremely effective against scab at all orchard locations, and will be an integral component of future fungicide rotation programs. For

effective fungicide resistance management, it is important that both components of a fungicide mixture provide efficacious disease control. Miravis Top contains both difenoconazole and pydiflumetofen, and each was highly effective at controlling scab at all locations when applied alone at the rate recommended in the commercial pre-mix. Amistar Top, another combination fungicide, also contains difenoconazole, but in combination with azoxystrobin. Amistar Top has been the industry standard for several years, but resistance to the azoxystrobin component has led to decreased efficacy at some locations (Herrington 2019). The efficacy of stand-alone azoxystrobin varied from location to location in the current study, presumably due to the presence or absence of the G137S mutation that results in resistance to the QoI fungicides. As stated previously, difenoconazole was highly effective at all locations, even though scab from some of the orchards was shown to have resistance to tebuconazole (an early generation DMI). No resistance was detected in *V. effusa* to the DMI difenoconazole, but applications of Amistar Top in locations where resistance to azoxystrobin is present would put increased selection on the difenoconazole component, increasing the risk for resistance developing to this important chemistry. From this study, it may be concluded that Miravis Top is particularly efficacious against scab, and that the SDHI component, pydiflumetofen, is an excellent addition to scab fungicide rotation programs. A further conclusion is that excessive applications of Amistar Top would put increased pressure on the difenoconazole component, which should be avoided. Development of resistance to difenoconazole by *V. effusa* would be disastrous since it is an integral component of multiple combination fungicides and is widely used in commercial pecan orchards.

In the combination fungicide study, both stand-alone pydiflumetofen as well as the combination of pydiflumetofen and difenoconazole were extremely effective at controlling scab,

resulting in almost 100% protection. With this magnitude of control from a single product, *V. effusa* is clearly highly sensitive to pydiflumetofen. Considering the control provided and the affordability of Miravis Top for commercial pecan growers, the product is likely to be widely used, and potentially abused on a large scale in the southeastern U.S. Since pydiflumetofen is a new product and is the first SDHI fungicide to be widely used on pecans, baseline sensitivity data was needed for *V. effusa* in order to monitor any future shifts in sensitivity that may evolve over time. Furthermore, SDHI fungicides are at high risk of resistance development, which means that it is likely that *V. effusa* will develop resistance to this product at some point in the future. In order to establish baseline sensitivity, a set of isolates from 1993 were used that had been in storage and had never been exposed to SDHI fungicides, or any other fungicide for that matter. An assay on SDHI amended media with 8 pydiflumetofen concentrations ranging from 0.0002 µg/ml to 6.0 µg/ml showed that the mean EC_{50} for *V. effusa* isolates never before exposed to pydiflumetofen was 0.0011µg/ml. The EC_{50} value is very low, and reveals just how sensitive *V. effusa* is to pydiflumetofen. The study will help in monitoring for resistance development to pydiflumetofen in the future. Screening of isolates should be conducted regularly to preserve the efficacy of pydiflumetofen which is a valuable tool for scab management.

Culbreath et al. (2019) found that stand-alone applications of various DMI fungicides did not control late leaf spot of peanut at high levels; however, when combined with micronized sulfur, the mixture resulted in synergistic effects that provided greater control than expected based on the simple additive activity of the combination. Subsequent studies showed similar increases in efficacy with both QoI and SDHI fungicides (Culbreath et al. unpublished). To test whether synergistic effects of micronized sulfur combined with fungicides from other classes could be used to enhance control of pecan scab, experiments were conducted at 4 locations,

applying the different treatments to individual terminals for the duration of the growing season. Full rates of stand-alone fungicides from every fungicide class labeled for use on pecan were included (excluding the SDHIs due to their incredibly high activity), as well as each fungicide combined with 6.7 kg/ha of micronized sulfur. The results indicated that the addition of micronized sulfur did not increase efficacy against scab for any of the fungicide classes that were tested. There may be other benefits of foliar applications of micronized sulfur to pecan trees. If increased efficacy had been observed, it may have allowed expanded use of some chemistries, particularly tebuconazole, where use has been seriously reduced due to resistance-induced loss of activity.

DMI fungicides are heavily used in commercial pecan production, yet the relative efficacy of some of the commonly used products belonging to this fungicide class are poorly characterized. The potential of a new DMI fungicide, mefentrifluconazole, as a control agent for scab, has not been established either. The same 11 aformentioned commercial pecan orchards were used to test the efficacy of difenoconazole, tebuconazole, and mefentrifluconazole using the same single terminal application method. Difenoconazole and mefentrifluconazole both had good activity against scab, and effectively controlled the disease at all locations. Tebuconazole, on the other hand, was not as efficacious as the other DMI fungicides, and resulted in very poor control, often statistically similar to the untreated control. An *in vitro* assay using 1, 3, and 10 ppm tebuconazole amended media with samples from each location to test for tebuconazole sensitivity in the lab showed that resistance of *V. effusa* to tebuconazole is widespread and was present at every location. The most resistant isolates from the assay were used to investigate the mechanism of resistance of *V. effusa* to tebuconazole; the isolates from 1993 were used as the sensitive checks, as confirmed by an additional assay to test sensitivity of individual isolates to

tebuconazole. DNA was extracted from all isolates, and the *CYP51A* and *CYP51B* genes were sequenced and screened for potential mutations. While several single nucleotide polymorphisms were found, most were silent and only a few led to amino acid changes. In the *CYP51A* gene, the G444D mutation was observed, and in the *CYP51B* gene, the G357H, I77T, and I77L mutations were observed. An expression analysis was conducted on both genes using qPCR. Both genes in the resistant isolates were more highly expressed compared to the genes in the sensitive isolates. Therefore, we conclude that the combination of the mutations and overexpression of the *CYP51A* and *CYP51B* genes may result in resistance of *V. effusa* to tebuconazole.

An additional study was initiated based on reports by multiple commercial pecan growers in 2018 and 2019 who observed an atypical pattern of scab severity relative to known cultivar susceptibility in orchards of mixed cultivars. The unexpectedly severe scab was noted early in the growing season in orchards of either cultivars Stuart and Desirable, or cultivars Pawnee and Desirable. The observations were that cultivars Stuart and Pawnee had severe *V. effusa* infections, while cultivar Desirable was relatively free of scab. The phenomenon is unusual, and has not previously been reported, and led to the hypothesis that the cultivars supported different races of *V. effusa*, and that one of the races had developed resistance to a commonly used fungicide group, while the scab race on the other cultivar had not. To test the hypothesis, multiple leaf samples containing active *V. effusa* lesions were sampled from each cultivar at each location, and an *in vitro* sensitivity assay was conducted to test the isolates sensitivity to dodine, tebuconazole, thiophanate-methyl, and fentin hydroxide. The results indicated that no meaningful difference in sensitivity to the fungicides was present between the *V. effusa* populations from the different cultivars. QoI fungicides had been heavily used in the orchards, and had been used in each orchard in at least one pre-pollination spray in the year the

phenomenon was noted. Sensitivity tests of *V. effusa* to alternate biosynthesis inhibitors like salicylhydroxamic acid (SHAM) prevented use of a simple bioassay to detect resistance to QoIs (Seyran et al. 2010). Thus, DNA was extracted from multiple monoconidial *V. effusa* isolates from each cultivar from each location, the cytochrome b region was sequenced, and was screened for the G137S mutation that results in reduced sensitivity of *V. effusa* to the QoI fungicides (Standish et al. 2019). The G137S mutation was present at most locations, with mutation frequencies ranging from 0to 67%, but there was no pattern to the differences in the frequency of the mutation between cultivars that developed more severe scab. The reason for the unexpected difference in cultivar susceptibility to scab in these orchards remains unknown, but the study ruled out differences in fungicide sensitivity as being the cause.

The objectives of this research aim to better understand fungicidal activity on *V. effusa* and to improve the effectiveness of fungicide rotations and pre-existing resistance management plans that are used to combat this pathogen. The specific objectives were to (1) evaluate the efficacy of currently used and new fungicides on pecan scab and evaluate the efficacy of pre-mixed fungicides and their individual components, (2) determine the inherent activity and develop baseline sensitivity of *V. effusa* to pydiflumetofen, (3) identify the resistance mechanism(s) of *V. effusa* to the DMI fungicides, (4) Evaluate the response of *V. effusa* to applications of different fungicide classes with sulfur as a mixing partner, and (5) evaluate pecan cultivar sensitivities to *V. effusa* within orchards with similar histories of fungicide exposure. We addressed each of the objectives with the research experiments that were designed, and have provided results that fill the knowledge gaps, and at the same time fulfill needs of fungicide management programs and sensitivity monitoring for the future. With the increased importance

of pecan, continued research on scab, the most damaging disease of the crop, is urgently needed and must be continued to address existing and future challenges.

APPENDIX A

RAPID ASSAY RESULTS FROM 11 LOCATIONS IN SOUTH GEORGIA

Location	*Concentration*	*RG % (2019)*	*RG % (2020)*
Nilo	3 µg/ml	91	33
	10 µg/ml	74	40
	30 µg/ml	0	0
Blue 3	3 µg/ml	100	94
	10 µg/ml	85	23
	30 µg/ml	0	0
Buchanan	3 µg/ml	94	98
	10 µg/ml	74	65
	30 µg/ml	0	0
Ducker	3 µg/ml	81	93
	10 µg/ml	47	24
	30 µg/ml	1	0
Hudson	3 µg/ml	89	-
	10 µg/ml	70	-
	30 µg/ml	0	-
Baker	3 µg/ml	69	30
	10 µg/ml	37	30
	30 µg/ml	1	0
Paulk	3 µg/ml	89	83
	10 µg/ml	70	68
	30 µg/ml	0	0
Dorsey	3 µg/ml	85	76
	10 µg/ml	25	1
	30 µg/ml	0	0
Ponder W	3 µg/ml	99	82
	10 µg/ml	81	29
	30 µg/ml	0	0
Ponder D	3 µg/ml	75	88
	10 µg/ml	65	29
	30 µg/ml	0	0
Ray	3 µg/ml	-	100
	10 µg/ml	-	7
	30 µg/ml	-	0

Table A.1. Rapid assay (method of Seyran et al. 2010) results for relative germination (RG%) of conidia of *Venturia effusa* from lesions on leaves collected from 11 orchard locations in South Georgia. The agar was amended with 3, 10, or 30 µg/ml triphenyltin hydroxide (TPTH), RG was calculated at each concentration, and the range of sensitivity in *V. effusa* to TPTH at the different locations is indicated. "-" represents missing data from that location for that particular year.

Location	*Concentration*	*RGr % (2019)*	*RGr % (2020)*
Nilo	1 µg/ml	100	100
	3 µg/ml	100	61
	10 µg/ml	86	16
Blue 3	1 µg/ml	100	56
	3 µg/ml	100	54
	10 µg/ml	44	5
Buchanan	1 µg/ml	100	97
	3 µg/ml	85	46
	10 µg/ml	25	9
Ducker	1 µg/ml	49	48
	3 µg/ml	46	57
	10 µg/ml	19	21
Baker	1 µg/ml	48	27
	3 µg/ml	23	9
	10 µg/ml	3	0
Hudson	1 µg/ml	100	-
	3 µg/ml	87	-
	10 µg/ml	36	-
Paulk	1 µg/ml	100	72
	3 µg/ml	58	32
	10 µg/ml	25	10
Dorsey	1 µg/ml	25	3
	3 µg/ml	11	0
	10 µg/ml	1	0
Ponder W	1 µg/ml	67	82
	3 µg/ml	43	51
	10 µg/ml	3	12
Ponder D	1 µg/ml	76	66
	3 µg/ml	63	23
	10 µg/ml	15	4
Ray	1 µg/ml	-	74
	3 µg/ml	-	58
	10 µg/ml	-	8

Table A.2. Rapid assay results for relative growth (RGr%) of colonies of *Venturia effusa* grown from samples of lesions on leaves collected from 11 orchard locations in South Georgia. The agar was amended with 3, 10, or 30 µg/ml tebuconazole. RGr was calculated at each concentration, and the range of sensitivity in *V. effusa* to tebuconazole at the different locations is indicated. "-" represents missing data from that location for that particular year.

Location	*RG % (2019)*	*RG % (2020)*
Nilo	6	6
Blue 3	26	2
Buchanan	85	20
Ducker	54	47
Hudson	12	-
Baker	8	10
Paulk	12	26
Dorsey	1	0
Ponder W	67	35
Ponder D	0	0
Ray	-	3

Table A.3. Rapid assay (method of Seyran et al. 2010) results for relative germination (RG%) of conidia of *Venturia effusa* from lesions on leaves collected from 11 orchard locations in South Georgia. The agar was amended with 1 µg/ml thiophanate-methyl (TPM). RG was calculated and the range of sensitivity in *V. effusa* to TPM at the different locations is indicated. "-" represents missing data from that location for that particular year.

Location	*RG % (2019)*	*RG % (2020)*
Nilo	7	28
Blue 3	0	0
Buchanan	18	2
Ducker	31	4
Hudson	6	-
Baker	16	1
Paulk	2	3
Dorsey	6	0
Ponder W	0	0
Ponder D	0	0
Ray	-	1

Table A.4. Rapid assay (method of Seyran et al. 2010) results for relative germination (RG%) of conidia of *Venturia effusa* from lesions on leaves collected from 11 orchard locations in South Georgia. The agar was amended with 3 µg/ml dodine. RG was calculated and the range of sensitivity in *V. effusa* to dodine at the different locations is indicated. "-" represents missing data from that location for that particular year.