ETIOLOGY OF *PSEUDOCERCOSPORA* LEAF SPOT DISEASES ON *LIQUIDAMBAR STYRACIFLUA* AND *LOROPETALUM CHINENSIS*

by

GULCIN EKEMEN

(Under the Direction of Jean L. Williams-Woodward)

ABSTRACT

Cercospora liquidambaris, *Pseudocercospora liquidambaricola* and *Pseudocercospora turberculans* have been described as causing a leaf spot disease on American sweetgum (*Liquidambar styraciflua*). A leaf spot disease on Chinese fringe flower (*Loropetalum chinense*) has also been attributed to *P. liquidambaricola* in China. A formal report of this pathogen on loropetalum in the USA has not been completed. Confusion in pathogen identity has implications for regulatory and management issues. Isolates collected from leaf spot-infected sweetgum and loropetalum were compared morphologically, phylogenetically, and through cross-inoculation. The ITS, ACT, EF-1, and LSU gene regions were sequenced. Isolates from sweetgum and loropetalum separated into two phylogenetic groupings. Morphologically, sweetgum isolates were similar to the description of *P. liquidambaricola*, while loropetalum isolates had characteristics distinctly different. Sweetgum and loropetalum isolates were not cross-pathogenic. An in-vitro fungicide sensitivity assay also revealed possible fungicide resistance developing in loropetalum leaf spot isolates to the benzimidazole fungicide, thiophanate methyl.

INDEX WORDS: *Pseudocercospora liquidambaricola., Cercospora liquidambaris, leaf spot disease, American sweetgum, Chinese fringe flower, fungicide resistance*

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DEDICATION

This thesis is dedicated to Nurten Ekemen, Hasan Nazim Ekemen and Elcin Ekemen. Thank you very much for supporting and loving me in every decision I make.

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CHAPTER 1

INTRODUCTION

A fungal leaf spot disease on the popular ornamental shrub, Chinese fringe flower (*Loropetalum chinense Oliv.*) has been seen within ornamental plant production nurseries since at least 2015 in the southeastern USA (UGA Plant Disease Clinic; J. Williams-Woodward, personal communication). The leaf spot disease mostly affects the lower leaves and can cause premature defoliation if infection is severe. The putative identity of this causal pathogen was originally described as *Pseudocercospora liquidambaris* from China (Liu and Guo, 1998). However, the taxonomy of the pathogen is still unclear. The taxonomy of this group of fungi is uncertain and in need of a comprehensive phylogenetic study. This pathogen also infects American Sweetgum (*Liquidambar styraciflua*) and Chinese Sweetgum (*Liquidambar formosana*). However, taxonomy of isolates originating from sweetgum have been moved to *Pseudocercospora liquidambaricola* (J.M. Yen) based upon morphological characteristics, with *P. liquidambaris* declared invalid and the original epithet of *Cercospora liquidambaris* being retained until phylogenic relationships are determined (Braun et al., 2015).

The taxonomic confusion with the sweetgum pathogens and only a minor mention of the pathogen on *Loropetalum* has led to confusion in correctly naming the leaf spot pathogen on *Loropetalum*. This could have regulatory implications. Several years ago, the California State Department of Agriculture wanted to reject a shipment of *Loropetalum* from a Georgia plant nursery because the plants had leaf spots. The pathogen had not been identified in California at the time. Since the pathogen was identified as existing in the USA on sweetgum, the shipment was not returned to Georgia (J. Williams-Woodward, personal communication). Currently, the leaf spot pathogen on *Loropetalum* has yet to be officially described in the USA; however, it appears to be widely distributed within the southeastern USA affecting numerous *Loropetalum* cultivars in nurseries and landscapes.

Management of this leaf spot pathogen has also not been evaluated. General recommendations from fungal leaf spot control such as avoiding prolonged leaf wetness, sanitation to remove fallen leaf litter, and fungicide recommendations for products listing Cercospora leaf spot control on their labeling.

The objectives of the research are to: 1) identify morphologically and phylogenetically the leaf spot pathogens recovered from American Sweetgum and *Loropetalum chinense*, 2) prove pathogenicity of the isolates using cross-inoculations, and 3) evaluate fungicide efficacy against the leaf spot pathogen on *Loropetalum chinense*.

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CHAPTER 2

LITERATURE REVIEW

Chinese Fringe Flower, *Loropetalum chinense*

Loropetalum or Chinese Fringe Flower (*Loropetalum chinense* Oliv.) is in the family Hamamelidaceae, and is native in China, Japan and South-eastern Asia. The Hamamelidaceae family also includes other ornamental plant taxa such as *Fothergilla* (witch-alder) and *Hamamelis* (witch-hazel). Loropetalum is well-adapted and widely used across the southern United States (USDA Zones 7a-10). Use within landscapes has grown considerably since 1989 when the first red-foliaged cultivars were introduced (Chappell et al., 2017). It is a fast-growing shrub with flashy, colorful (white, yellow, pink, red) blooms. There are 56 named cultivars (as of 2017) that have either green or reddish foliage that can grow to a height and width of 10-15 feet (Chappell et al., 2017). Loropetalum sales are not nationally ranked; however, almost all nurseries in the southeastern USA produces at least one cultivar (Chappell et al., 2017).

Loropetalum is a relatively pest-free woody ornamental plant. Insect pests are primarily Ambrosia beetle and aphids, which can affect nursery and landscape plantings (Chappell et al., 2017). Insect damage is relatively minor. Ambrosia beetle often attacks plants stressed by drought or poor nutrition. Aphids are more common and mostly affect the plant by accumulating large amounts of honeydew that leads to black sooty mold growth on the leaves.

Few plant diseases affect Loropetalum. Root rot due mostly to *Phytophthora cinnamomi* and *P. nicotianae* is common within plant production nurseries. Wet rooting media and heat stress associated with growing containerized plants favors root disease development.

Loropetalum was also identified as a host for the Sudden Oak Death pathogen, *P. ramorum* (Blomquist et al., 2012). Disease management includes avoiding excessive irrigation, following good nursery sanitation practices, and timely preventative fungicide applications. Bacterial stem gall, caused by *Pseudomonas amygdali* pv. *loropetali* pv. nov., induces callused knots, swollen stems, and sometimes plant death has become a major concern (Harmon et al. 2018). The disease is likely spread during pruning operations and from propagating infected plants. Disinfesting pruning tools and using copper-containing fungicides may help reduce this disease.

The other disease affecting Loropetalum is a fungal leaf spot disease, which is the subject this research. The leaf spot pathogen is thought to be *Pseudocercospora liquidambarcola* (J.M. Yen) U. Braun (formerly *Cercospora liquidambaris* and *C. liquidambaricola*) (Braun, 2000; Braun et al., 2015), which also causes a leaf spot disease on American and Chinese Sweet Gum (*Liquidambar styraciflua* and *L. formosana*, respectively). The pathogen on Loropetalum was originally classified as *Pseudocercospora liquidambaris* (Liu and Guo, 1998). There is taxonomic uncertainty with this pathogen that needs phylogenetic study. Symptoms of infection on Loropetalum include purplish-colored, angular to irregular leaf spots that are mostly seen on the lower plant canopy. Infected leaves often prematurely drop from the plant. Defoliation can be severe under wet, humid environmental conditions. Management includes avoiding overhead sprinkler irrigation and periods of prolonged leaf wetness, propagating only from uninfected plants, and preventative fungicide applications to reduce infection and disease spread.

American Sweetgum, *Liquidambar styraciflua*

American sweetgum (*Liquidambar styraciflua* L.) is a deciduous shade and forest tree that is one of several *Liquidambar* species worldwide. Sweetgum was formerly placed in the

Hamamelidaceae family, but are now placed into a separate Altingiaceae family (Ickert-Bond and Wen, 2013). The name Liquidambar consists of Latin "liquidus" that means liquid and Arabic "amber", which means beautifully scented substances (Arslan and Şahin, 2016). There are several main *Liquidambar* species including *L. orientalis* known as the Oriental or Turkish sweetgum and is native to the eastern Mediterranean area and *L. acalycine* (Chang's sweet gum) and *L. formosana* (Chinese sweetgum or Formosan gum) that are native for southern China.

Liquidambar styraciflua is native to the eastern and southeastern USA from Florida northward into Connecticutt and westward to Texas, as well as scattered in areas of Mexico and Central America (Lingbeck at al., 2015). Sweetgum is well-adapted to a wide range of soil types preferring deep, moist and fertile soils in USDA hardiness zones 5-9. The trees are large reaching heights over 60 feet. The leaves are glossy, 3-6 inch wide and palmate with 3-7 deep lobes. Leaves are stat-shaped and when crushed are aromatic. The fast-growing are often used in landscapes and parks as they have good red, orange, and yellow fall color. Sweetgum is also known for its round, hard, spiny fruit containing seed capsules.

Sweetgum trees have many uses. Antimicrobial essential oils ("storax") are derived from the leaves that are important to the cosmetics industry, in soaps and perfumes (Adams et al., 2014). Storax was also used by ancient civilizations for the treatment of skin infections, wounds and throat problems. Sweetgum is also used as a timber species, who's wood is used for furniture and pulp. Sweetgum trees may also have use as a source of bioenergy. Bio-oil, cellulosic ethanol and wood pellets are products of sweetgum trees (Wright and Cunningham, 2008).

The fungi causing leaf spots on Sweetgum

The leaf spot disease of *Liquidambar styraciflua* which is caused by the fungal pathogen of *Cercospora* genus was published in 1892 as a result of the study of Cooke and Ellis. They named the pathogen *Cercospora liquidambaris* (Cooke and Ellis, 1982). The fungus belonging to the genus *Cercospora* were usually based on the host that is isolated, when they were named (Chupp 1954, Ellis 1971). The name of the fungus causing this disease has changed many times since the time of this definition. This pathogen by scientists who continued their research within years was sometimes defined by different names in different genres and sometimes published in different genres, based on their different characteristics within the same genus. The name change of pathogen has not been clarified till today, while it may not be evident in the future.

In general, the species are morphologically limited by their anamorph and teleomorph characteristics (Chand et al., 1954). The genus *Cercospora* has been studied to classify in a variety of categories. These classification studies are based on both morphological characteristics and phylogenetic similarities. Conidia and conidiophore are studied as anamorphic characters. This genus is classified like as the length, width, base, shape, color and other visible futures of the conidian, and then conidiophore length, diameter, fascicles, geniculation and stromata by Charles Chupp in 1954. Chupp (1954) recognized 1,419 species in his study of the genus *Cercospora*. Chand et al. (1954) mentioned that in the studies based on the teleomorph feature, it is often not enough to use the analysis of ITS sequence and, in some cases, to give a general information in defining these species. The phylogenetic study can be carried out with the cooperation of CAL, ACT, EF, HIS or sequence of MAT which can be preferred for species and AFLP analysis and special development test specificity assays can be used.

Kobayashi and Nakashima (2002) mentioned that from 1888 until 1978, Four *Cercospora* species were identified by taking Liquidambar as a base. They were *Cercospora tuberculans* (Ellis and Everh.,1888), *C. liquidambaris* (Cooke and Ellis,1892), *C. liquidambaris* Sawada (1943-Latin name without legitimate and invalid name-), and C. liquidambaricola (J.M. Yen,1978), respectively. *Cercospora turberculans*, which was recorded in the USA, was found to be different in 1953 due to the morphological comparison of Chupp with Japanese liquidambar fungus. Years later, this fungus was recorded for *Liquidambar formasana* Hance host in Taiwan (Hsieh, 1983).

R. M. Leahy (1985) stated that the most common leaf spot disease on sweetgum was caused by *Cercospora Liquidambari*s Cooke and Ellis (1892). The description of leaf spot disease was that spots were angular to subcircular between 2-10 mm, dark brown colored with mostly surrounded by a narrow-raised border and pathogen sporulation exists on both surfaces on infected leaves. He mentioned in his study that recognition of this disease on foliage was easy with characteristic angular dark brown leaf spots. Also, the application of benomyl which was systemic fungicide should be applied at the first visible sign of disease and sanitation of infected trees were suggested as a control mechanisms of leaf spot disease on American sweetgum.

Many scientists agree that the correct identity of *Cercospora liuidambaris* can be revealed by lectotypication (Braun, Crous, and Nakashima, 2015). In 1891, Atkinson drew attention to the samples collected from the Alabama. The remaining remains are still unclear, while these samples from Atkinson's herbarium at CUP, is recognized as a lectotype which has conidiogenous loci and conidial hila are darkened, thickened and conidia look *Cercospora*-like but pigmented by U. Braun, P.W. Crous, C. Nakashima (2015). *Cercospora liquidambaris*, which had only been previously reported in America, and also *Cercopsora liquidambaris* *sawada*, without the definition of Latin, were recorded for the *Liquidambar formosana* host in Taiwan (Braun et al.,2015) Chupp (1954) published that *Cercospora liquidambaris sawada* (1943) was synonym of *Cercospora liquidambaris* Cooke and Ellis.

According to the design of lectotype samples from Atkinson's herbarium at CUP, Identity of *Cercospora liquidambaris* was shared detail by Braun et al., (2015) in their study. That Leaf spots are amphigenous, subcircular to angular-irregular, dingy greyish green, brown, 0.5-4 mm diameter, margin uncertain or darker, narrow. Mycelium are internal and external, and hyphae are branched, septate, subhyaline to pale olivaceous or brownish, 1.5-4 µm wide. Stromata is small or lacking, 10-60 µm diameter. Conidiophores in small to large fascicles arise from substomatal hyphae or stromata, or solitary, 25-90 x 3-5 µm. 1-8 septate and light to medium brown, paler at the tip. Conidia are solitary, narrowly obclavate-cylindrical, filiform-subacicular, straight to curved, 40-150 * (2.5-4(-4.5) µm diameter, thin walled subhyaline to pale olivaceous and 3-12 septate.

In 1990, Goh and W.H. Hsieh identified a new species in Taiwan, *Pseudocercopsora liquidambaris*, with the Latin definition as a result of the reexamination of *Cercospora liquidambaris sawada* (1943). Prior to this, there was a lot of discussion with the definition of *Pseudocercopsora liquidambaris* based on *Cercospora liquidambaris sawada* (1943), although it was considered unclear because the Latin definition of this fungus was not made. Guo and W.H. Hsieh (1995) and Liu and Guo (1998) added new information both *Pseudocercopsora liquidambaris* Goh et W.H. Hsieh was a synonym of *Cercospora liquidambaris* Cooke et Ellis and after the reexamination of Yen’s specimens, *Cercospora liquidambaricola* J.M.Yen (1978) was added a synonym of *Pseudocercopsora liquidambaris* Goh et W.H. Hsieh .

Braun et al., (2015) mentioned one more cercosporoid species identification which can cause the leaf spot disease on Liquidambar genus; *Pseudocercospora tuberculans* Ellis and Everh (1888), Braun (1999). The leaf spots are absent or sometimes with slight discolorations on the upper leaf surface and mycelium is internal. Stromata is dark brown to blackish, absent to well-developed, 10-80 µm diameter. Conidiophores are in small to large fascicles, loose to usually dense, subcylindrical to conical, pale olivaceous to olivaceous-brown, unbranched, 10-35 x 3-7 µm wide, 0-1 septate. Conidia are solitary, cylindrical to somewhat obclavate-cylindrical, straight to slightly, 20-80 x 4-6.5 µm wide, pale olivaceous to brownish and have 1-6 septate. Chupp (1954) did differentiate *Pseudocercospora tuberculans* Ellis and Everh (1888), Braun (1999) from *Pseudocercospora liquidambaricola* by reexamination of the samples collected by Geo. V. Nash in 1895.

Yen and Braun (2000) described a new species *Pseudocercospora liquidambaricola* as a synonym of *Cercospora liquidambaris sawada*, *Pseudocercopsora liquidambaris* Goh and W.H. Hsieh and as a basionym of *Cercospora liquidambaricola* J.M. Yen (1978). Braun et al. (2015) reported the key to tree cercosporoid species on Altinginacea. At the first-time report, Guo and Hsieh in 1995; Guo et al. in 1998 mentioned a new host *Loropetalum chinense* in China for this fungus. *Pseudocercospora liquidambaricola* is one of them which can be distinguished in this key by a large leaf spot. The leaf spots are subcircular to angular-irregular, 1-10 mm diameter or larger, angular spots often vein-limited, pale olivaceous, brown to dark brown with a dark border with a diffuse yellowish halo. Mycelium are internal and external, and hyphae are branched, 1.5–4 µm wide, subhyaline to pale olivaceous-brown thin walled, smooth. Conidiophores in small to large fascicles, loose to dense arise from stromata, lateral erect, straight, unbranched, solitary 5-35 x 2-5 µm diam, 0-2 septate and subhyaline to olivaceous-brown. Conidia are single, cylindrical to obclavate-cylindrical, straight to curved, 20-100 * 2-4 µm wide, subhyaline to pale olivaceous or olivaceous-brown, and have 2-10 septate, thin walled, smooth, tip obtuse to subacute, 1-2 µm with unthicken and not dark hila.

To summarize, if the definition of these fungus that are described by the name of the leaf spot disease on Liquidambar genus will be reviewed; *Cercosopra tuberculans* Ellis and Everh (1888), *Cercospora liquidambaris* Cooke and Ellis ex G.F. Atk. (1892), *Cercospora sawada* (1943 - without Latin description), *Cercospora liquidambaricola* J.M.Yen (1978), *Pseudocercopsora liquidambaris* Goh and W.H. Hsieh (1990), *Pseudocercospora tuberculans* Ellis and Everh (1888), Braun (1999), *Pseudocercospora liquidambaricola* Yen and Braun (2000), finally *Pseudocercospora neoliqudambaris* Nakashima and Kobayashi (2002). These pathogens cause the different type of spots in infected leaves and have different characteristic feature.

Pseudocercospora neoliquidambaris Nakashima and Kobayashi (2002) mentioned on *Liquidambar formosana* Hance (Hamamelidaceae) that as a basionym of *Cercospora liquidambaris* Cooke and Ellis ex G.F.Atk. (1892), *Pseudocercopsora liquidambaris* Goh et W.H. *Cercospora liquidambaricola* J.M.Yen (1978)Hsieh (1990) and synonym of), *Cercospora* sawada (1943 - without Latin description), *Cercospora* liquidambaricola J.M.Yen (1978). However, this fungus caused the sooty leaf spot disease instead of the leaf spot disease. The description was that leaf spots abgular to irregular in shape, brown to grayish-brown, 5-10 mm diameter, conidiophores fascicular, geniculate, simple brown to pale brown at the bottom part, hyaline to upper part with the unclear conidial scars 10-35 x 2-2.5 µm wide. Conidia are obclavate, subhyaline to plae brown, straight or curved within truncate and thin bottom end, conic at the upper part, 32-78 x 2-3 µm diam with 3-9 septate and smooth.

DNA sequences and Phylogenetic Analyses

Fungal pathogens that cause disease in plants described in plant science until 1990 were generally identified by considering their morphological features and host range. With the study conducted by White et al. (1990), phylogenetic field research has created a different perspective for scientists. In this study, phylogenetic analysis of isolated pathogens was carried out with guidance on the studies of *Pseudocercospora* and *Cercospora* genera in consequence of the lack of sufficient phylogenetic studies of isolated pathogens.

The first study in connection with the genus *Cercospora* and *Pseudocercospora* was conducted by Stewart et al. (1999) in seven different genera of Mycosphaerelle anamorphs. Two of them are *Cercospora* and *Pseudocercospora*, while the rest were *Passalora*, *Paracercospora, Ramulispora, Pseudocercosporella* and Mycocentrospora. PCR amplification and sequence analysis were performed using a combined ITS, F63 and R635 primer set containing the 5n8S rRNA gene and the 5 'end of the 25S gene. Cercosporoid taxa length were examined between 502 bp in *Paracercospora* species and 595 bp in *Pseudocercospora* species. The four clade *Cercospora, Pseudocercospora / Paracercospora*, *Passalora* and *Ramulispora* were completely supported by phylogenetic analysis, while the results showed that *Paracercospora* was synonymous with *Pseudocercospora sp.*

In order to confirm the hypotheses in the historical development of the genus *Cercospora*, in 2001 Goodwin et al. Conducted a comprehensive phylogenetic analysis of Mycospherella and its associated anamorph genera. They sequenced 15 isolates from five *Cercospora* and *Mycospharella* strains based on the ITS nDNA loci. The ITS region was amplified by using ITS1 and ITS5 primers. In the DNA analysis performed in three steps, firstly all sequences were aligned multiple times to identify closely related taxa groups, followed by a separate alignment for each group, and finally all profiles were aligned together based on the original dendrogram. In the light of phylogenetic analyzes, most of the *Cercospora* species formed a single monophyletic group with a high bootstrap support of 97%. Only *Cercospora kalmiae* and *Cercospora arachidicola* were clustered with *Pseudocercospora* and *Passalora* species respectively. The *Asteromella brassiceae*, which was the sister of the *Cercospora* cluster and was anamorphic to mycospheralla brassicola, separated many species of *Cercospora* from the clusters of *Aracercospora*/ *Pseudocercospora / Cercospora kalmiae* cluster.

Cercospora agavicola was identified by Ayala-Escobar et al. In 2005 on the agave tequilana plant, which is of great economic importance in Mexico. This new disease-causing pathogen, which was first encountered in 2003, was sent to U. Braun for identification. Phylogenetic analysis study based on multiple loci sequence data derived from the ITS nrDNA region, and part of the elongation factor 1-a, calmodulin, actin and histone H3 genes. As a result of the phylogeny study, approximately 500,315,230 and 410 bases were obtained from ITS, EF, ACT, CAL, and His, respectively. According to these results, the sequence was demonstrated by a section homogeneity test using sequence data in which all loci could be combined in a single assay. The isolated pathogen in this study, apart from the other *Cercospora* species in the tree, formed a separate branch in the *Cercospora* genus.

Crous et al. (2013) conducted a very important study on the phylogenetic lineages of *Pseudocercospora*. In this study, the partial 28S nuclear ribosomal RNA gene of the selected set of isolates was sequenced to provide a broad outline of *Pseudocercospora* and aligned species. The pathogens collected from various plant families by direct isolation method were cultivated on 2% malt agar and subjected to DNA isolation. After this procedure, four different gene regions were studied for phylogenetic analysis. These four regions were selected as ITS, ACT, LSU and EF-1α. On the other hand, phylogenetic research pathogens were taxonomically identified based on herbarium materials. The phylogenetic analysis was initially performed by only analyzing the resolution of the LSU gene region and the rest by ITS, ACT and EF-1a multi locus. In this study, as in many studies, ITS gene loci resolution was not sufficient to reveal the difference between species. As a result, *Pseudocercospora* complex species were grouped in clades from 1 to 14 according to the LSU resolution. These 14 clades and the genera to which they were solved were described in detail and presented in the study as figured. 146 of the isolates were presented as a species belonging to *Pseudocercospora* by both morphological and phylogenetic analyzes and many new species were identified based on the same features. These groups and the species they represent are described in detail in the study. Another significant consequence of this study was that species belonging to this genus identified in America or Europe cannot be used in the same name even if they cause the same disease on the same host in Africa, South America or Asia.

In 2013, Groenewald et al. examined 360 *Cercospora* isolates, collected from 161 hosts from 39 countries in the field of molecular phylogeny in the study of Species concepts in *Cercospora*: spotting the weeds among the roses. In this study, 49 host families and 39 countries were conducted to get result. The phylogenetic analysis based on the internal transcribed spacer regions and intervening 5.85nrRNA, calmodulin, actin, histone H3 and translation elongation factor 1-alpha genes. As a supplementary, additional primers were added in this study. However, none of single locus was found for DNA barcode. The result of unified alignment included 361 taxa (including the outgroup taxon) and 471, 263, 199, 240 and 347 characters (including alignment gaps) were used in the ITS, TEF, ACT, CAL and HIS partitions, respectively. Phylogenetic analyses were supported with Bayesian tree in where obtained from the combined five major primers alignment. The result of DNA phylogeny, the ITS region offered limited and general solutions for almost full of *Cercospora* species. The TEF (translation elongation factor 1-alpha) was able to be allocated 33 of the 73 species clades and however, ACT (actin) regions distinguished 43 of the 73 species clades. Also, most of the variation observed for *Cercospora* sp. explained from this ACT region. The CAL region was able to distinguish 34 of the 73 species clades. The HIS region clarified for most of the variation observed for *C. armoraciae* and was responsible for the divide of *C. beticola* into two clades. An important another outcome of this study was that there were several species originally called *C. apii s.lat*. based on morphology (Crous and Braun 2003), they were separated into different phylogenetic species. This also showed that morphology alone did not provide a sufficient basis for species discrimination of the genus *Cercospora*. In this study, Groenewald et al. were stored new sequences at NCBI's GenBank nucleotide database (www. Ncbi.nlm.nih.gov) and the alignment and phylogenetic tree in TreeBASE (www.treebase.org).

The study of Multi-gene analysis of *Pseudocercospora spp.* from Iran was investigated by Bakhshi et al. (2014). In this study, phylogenetic research of nineteen isolates from seven different plant families and eight hosts was conducted with the combination of LSU, ITS, TEF1-α, and ACT loci. Amplification of the LSU, ITS, ACT and TEF1-α loci obtained 900, 700, 200 and 300 bases, respectively. According to the results of LSU sequence data, all isolates were found in *Pseudocercospora* s. str. clade. The multi-gene phylogeny data from the combined sequence analyses noted eight clades. Three of these showed the known species - *P. atromarginalis, P. norchiensis* and *P. vitis*. -, while the five of them were new based on the phylogenetic analyses, described as novel two species.

In 2014, Shivas et al. identified the new species of *Cercospora* and *Pseudocercospora* by analyzing both morphological characteristics and partial nucleotide sequencers of isolates for three loci genes ITS, the large subunit region of rDNA (LSU) and EF-1a in the study of “Novel species of *Cercospora* and *Pseudocercospora* (*Capnodiales, Mycosphaerellaceae*) from Australia”. In this study, the identification of new taxon was made based on the phylogeny of LSU region by using the study of Crous et al. (2013). The LSU sequences analyzed with maximum likelihood (ML) and Bayesian inference. The pathogens isolated from Ischaemum (*Cercospora ischaemi*, BRIP 56010) belonging to Poaceae family showed similarity to the genus of *Cercospora*, on the other hand, the other three isolates (BRIP 58545, BRIP 58549 and BRIP 58550) belonged in the *Pseudocercospora spp.* These four new species have been described in terms of morphological and phylogenetic aspects.

A study by Bakhsi and et all. In 2015 aimed that describe freshly collected *Cercospora* spp. samples from the north and northwest of Iran to derived cultures and DNA sequence data. They sequenced the internal transcribed spacer regions (ITS locus, including ITS1, 5.8S nrRNA gene and ITS2), together with other primers of translation elongation factor 1-alpha (TEF1-α), actin (ACT), calmodulin (CAL) and histone H3 (HIS) and supported 20 lineages with in the Bayesian tree. Single conidial colonies obtained from 161 isolates of 74 host species were subjected to DNA extraction after being kept in darkness for 10 days established on 2% MEA media. Approximately 700, 300, 200, 450 and 400 bp were obtained from ITS, TEF1-α, ACT, CAL and HIS loci, respectively. The resolution of the ITS region had limited almost all *Cercospora* species same as Groenewald (2013). Just only it was able to distinguish *C. chenopodii, C. solani* and *C. sorghicola* from the other species examined. Based on the ACT region, they were allocated nine of the 20 species, 45% clade recovery. Whereas seven of the 20 species including were distinguished in the TEF1-α phylogeny,35% of lineages. Eight out of the 20 lineages were able to differentiate based on the CAL region, just only 40% of clade recovery. Based on the HIS region, they distinguished 10 of the 20 lineages. One of the other important results of this study was the identification of six new species; *C. convolvulicola, C. conyzae-canadensis, C. cylindracea, C. iranica, C. pseudochenopodii and C. sorghicola.* In addition, the existing *Cercospora* species which were including *C. apii, C. armoraciae, C. beticola, C. cf. richardiicola, C. rumicis, Cercospora sp. G* and *C. zebrina* were defined in new hosts.

Bakhsi et al. (2015) collected samples infected with *Cercospora* spp. from northern and northwestern Iran to obtain cultures and DNA sequence data. They sequenced the internal transcribed spacer regions (ITS locus, including ITS1, 5.8S nrRNA gene and ITS2), translation elongation factor 1-alpha (TEF1-α), actin (ACT), calmodulin (CAL) and histone H3 (HIS) regions and supported 20 lineages with in the Bayesian tree. DNA was extracted from single conidial cultures obtained from 161 isolates from 74 host species were grown on 2% malt extract agar (MEA) medium in the dark for 10 days. Approximately 700, 300, 200, 450 and 400 bp were obtained from ITS, TEF1-α, ACT, CAL and HIS loci, respectively. The resolution of the ITS region had limited almost all *Cercospora* species same as Groenewald (2013). It was only able to distinguish *C. chenopodii, C. solani* and *C. sorghicola* from the other species examined. Based on the ACT region, they were allocated nine of the 20 species, 45% clade recovery. Whereas seven of the 20 species were distinguished in the TEF1-α phylogeny (35% of lineages). Eight out of the 20 lineages were differentiated based on the CAL region, just only 40% of clade recovery. Based on the HIS region, they distinguished 10 of the 20 lineages. One of the other important results of this study was the identification of six new species; *C. convolvulicola*, *C. conyzae*-canadensis, *C. cylindracea, C. iranica, C. pseudochenopodii* and *C. sorghicola*. In addition, the existing *Cercospora* species (*C. apii, C. armoraciae, C. beticola, C. cf. richardiicola, C. rumicis, Cercospora sp. G* and *C. zebrine*) were defined in new hosts.

Raja et al. (2017) conducted an important study in order to use phylogenetic analyses as a standard procedure to classify fungi in addition to morphological characteristics. They mainly focused on the problems encountered in the identification of new species of phenotypic characters used as the only option until 1990 (White et al.). Some examples of these problems were the difficulties in distinguishing certain fungal species according to asexual or sexual spore production structures with very small nuances and the difficulties and limitations of in vitro growth and storage of these species. In addition, different names for sexual and asexual stages of the same fungus would cause nomenclature confusion. They chose three different gene regions for their phylogenetic analysis, the large subunit (nrLSU-26S or 28S), the small subunit (nrSSU-18S), and the entire internal transcribed spacer region, and evaluated these gene regions in two stages. On the one hand, they discussed ITS resolution alone to learn their limitations and advantages, while on the other hand they resolved phylogenetic relationships with multi locus gene analysis. Another problem they encountered was the limitations of the DNA barcoding search using NCBI-BLAST system, and they found directions to get it better for users. Finally, while investigating various protein-coding genes to support ITS resolution, they examined the methods required to construct a phylogenetic tree after DNA analysis.

As a result, it is emphasized that in identification of fungi, morphological features should be added in addition to their cultural and molecular characteristics and single or multi locus gene analyzes should be performed. There is also a description section in the process features for PCR which can be used as a guide for the primers that can be used in many gene regions. In the research conducted on the ITS regions limits, it was emphasized that these gene regions might be used in many GenBank research points, but especially in the searches within the BLAST system, more attention should be paid. In addition, if some information could not be obtained from ITS gene regions analysis for some fungal genus, multiple gene locus analyzes or the use of accessory protein encoding genes were recommended.

In this study, as another consequence, in order to compare the analyses data in the databases, it was accomplished that if the necessary information cannot be obtained by using the customized gene regions in the ribosomal DNA repeat units, the protein-coding genes might be used for comparing or definition of fungi. And it should also be stored in the GenBank database with reference to the original source of sequences obtained at the end of the researches. This barcoding system may be explanatory if there was not enough identification information for a fungus. One of the most important results was that there were many fungal species that can be sequenced in the ITS region and these were available in the International Sequence Data Base system (INSD).

More recently in 2018, Vaghefi et al. investigated the pathogenicity and evolutionary limitations of *Cercospora beticola*, which causes *Cercospora* leaf spot disease in Beta vulgaris. The Mixed Yule Coalescent (GMYC), Poisson Tree Processes (PTP), and Bayesian factor delimitation (BFD) were performed on sequences of six loci in 102 isolates. The ITS, ACT, CAL, HIS, and EF were amplified in all isolates as described in a total alignment length of 1873 bp, including 95 polymorphic sites, for a total of 21 unique multi-locus haplotypes and the codes for cercosporin protein in *Cercospora* species of the cfp gene was also partially sequenced in a total alignment length of 2727 bp as sixth loci. Multi-species coalescent analysis based on GMYC and PTP could not identify *C. apii* and *C. beticola* separately in the identification of phylogenetic species, while BFD-based study bolstered these two pathogens as distinguished species. A significant recombination was observed between the CAL, ACT and HIS loci, while there was no sign of recombination in the ITS and TEF 1-a paired with the remaining three loci.

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CHAPTER 3

COMPARISON OF *PSEUDOCERCOSPORA* SPP. RECOVERED FROM *LIQUIDAMBAR STYRACIFLUA* AND *LOROPETALUM CHINENSE*

INTRODUCTION

The production and landscape use of Chinese fringe flower (*Loropetalum chinense* Oliv.) has grown dramatically over the past decade in the southeastern USA, particularly with the introduction of dwarf, purple-leaf cultivars. Plant disease issues on loropetalum are few, and include mostly Phytophthora root rot, bacterial stem gall, and a fungal leaf spot (Chappell et al., 2017). The leaf spot disease increases in occurrence and severity under warm, humid, wet conditions. Infection causes purplish, angular to irregularly-shaped leaf spots that are mostly seen within the lower plant canopy. Infected leaves often turn reddish or yellow and drop prematurely from the plant. On new growth, the leaf spots often look like dull, discolored patches that may go unnoticed.

The leaf spot pathogen on *L. chinense* was originally described as *Pseudocercospora liquidambaris* from China (Liu and Guo, 1998). However, the identity of the pathogen on loropetalum is unclear. This pathogen is also described as infecting American Sweetgum (*Liquidambar styraciflua*) and Chinese Sweetgum (*Liquidambar formosana*). The taxonomy of the sweetgum pathogen is very confused and requires phylogenetic study to properly deduce genetic relatedness.

The sweetgum leaf spot pathogen was originally described as *Cercospora liquidambaris* Cooke & Ellis ex G.F. Atk. on American sweetgum in Alabama (Atkinson, G.F., 1892). However, Atkinson's original description more closely resembles the description of *C. tuberculans* Ellis & Everh. (Chupp, 1954), which also causes a leaf spot disease on American sweetgum, and has been subsequently renamed, *Pseudocercospora tuberculans* (Ellis & Everh) U. Braun (Braun, 2000). Chupp (1954) re-described *C. liquidambaris* based upon a sweetgum specimen from Florida collected in 1895. This pathogen was then moved to the genus, *Pseudocercospora,* as *P. liquidambaris* Goh & W.H. Hsieh (Hsieh and Goh, 1990) based upon conidiophore and conidial morphology. Evaluation of voucher specimens in Taiwan and China from *L. formonsana* showed that *P. liquidambaris* was synonymous with the described fungus, *Cercospora liquidambaricola* (J.-M. Yen) (1978), and the pathogen was renamed *Pseudocercospora liquidambaricola* (J.-M. Yen) U. Braun (Braun, 2000). The name *P. liquidambaris* has thus been declared invalid. Furthermore, the original name of *Cercospora liquidambaris* has been tentatively retained as a separate species since some morphological characteristics of North American sweetgum herbaria specimens are more similar to *Cercospora* or *Passalora* complex than to *Pseudocercospora*, and phylogenic relationships have not been determined (Braun et al., 2015).

Taxonomic confusion of the leaf spot pathogen on American sweetgum, and that of Chinese fringe flower (*L. chinense*) needs to be resolved. If the leaf spot pathogens from both American sweetgum and Loropetalum are the same, then the isolates should be able to cross-infect the other host. Cross-inoculation will confirm pathogencity and can help identify the biological relatedness of the isolates from the two disparate hosts. Accurate identification of a pathogen is key to understanding its pathogenicity and biology, which is fundamental to developing successful disease management strategies (Crous et al., 2015). Therefore, the objectives of this study are 1) collect isolates causing leaf spot disease on American sweetgum and Loropetalum, 2) characterize the isolates morphologically and phylogenetically, and 3) prove pathogenicity by cross-inoculating American sweetgum and Loropetalum with isolates derived from the other host.

MATERIALS AND METHODS

Isolate Collection. *Pseudocercospora* spp. isolates were recovered from American sweetgum (*L. styraciflua*) and *L. chinense* foliage samples collected from June 2017 through October 2018. A total of 40 American sweetgum foliage samples were collected from roadways and forested areas from multiple trees at eight distinct locations in northern GA and one location in TN (Table 3.1). A total of 25 loropetalum samples were collected from multiple plants within cultivar blocks at three ornamental plant nurseries in GA and AL, and from individual plants in four landscapes in GA (Table 3.2). Symptomatic leaves from a sample collection site were placed into plastic zip-top bags and stored at 4°C until processed.

Sporulation was induced by incubating samples in a moist chamber for 2-3 days at 22-23°C. Individual leaf spots were examined under a stereoscope to locate conidiophores and conidia. Conidia were removed from the leaf surface using a heat-sterilized needle and lightly streaked across the surface of potato dextrose agar (PDA: 39 g/L; BD Difco) amended with 250 µg ampicillin trihydrate (Sigma Aldrich, St. Louis, MO) in 100 x 15 mm disposable plastic petri plates (Fisher Scientific, Suwanee, GA). Plates were wrapped with strips of parafilm and incubated at 24-25°C under white fluorescent light on a 12-hr light/dark cycle until fungal growth was evident (usually within 3-5 days). Colonies were transferred to clarified V8-juice agar medium (200 ml clarified V8® juice [Campbell Soup Co., Camden, NJ], 17 g Bacto Agar, 800 ml deionized water) to maintain isolates and to induce sporulation.

A total of 169 single-spore isolates corresponding to individual leaf spots were obtained from the 40 American sweetgum samples (Table 3.1). A total of 71 isolates corresponding to individual leaf spots were obtained from the 25 loropetalum samples (Table 3.2).

Morphological comparison of leaf spot symptoms and fungal structures. Leaf spot symptoms from *L. styraciflua* and *L. chinense* samples were photographed, described, and measured using a digital caliper (Mitutoyo America Corporation, Aurora, IL) in the transverse and longitudinal directions.

Conidia for each fungal isolate were obtained by removing a 4-mm diameter agar disc containing superficial hyphae and conidia from a 14-18 day old culture grown on V8 juice agar medium (200 ml clarified V8® juice [Campbell Soup Co., Camden, NJ], 17 g Bacto Agar, 800 ml deionized water) at 24°C under white fluorescent lights with a 12 hr light/dark diurnal light cycle. Agar discs were placed into a sterile 1.5 ml microcentrifuge tubes containing 1 ml of sterile deionized water, crushed and vortexed to release conidia. A 100 µl conidial suspension was pipetted and spread using a sterile, bent glass rod across the surface of fresh V8 juice agar plates and incubated for 14-21 days at 24°C under white fluorescent lights with a 12 hr light/dark diurnal light cycle. Agar plates were flooded with a 0.01% Tween 20 solution and agitated with a small, sterile, natural-hair paint brush to dislodge conidia. The conidial suspension was examined microscopically and length and median width of a minimum of 50 conidia per sample location were measured and photographed using a Dino-Lite USB microscope camera and DinoCapture 2.0 microscope imaging software (Dino-Lite/Dunwell Tech, Inc., Torrance, CA). Conidial measurements for the sweetgum and loropetalum isolates were analyzed using ANOVA General

Linear Model (Minitab Statistical Software, Minitab LLC, State College, PA) to determine differences among host groups and same-host isolates from different locations with mean separation using Tukey's HSD post hoc analysis.

Pathogenicity test with cross-inoculations. Cross-inoculation tests were conducted on containerized plant material, as well as in detached leaf assays. American sweetgum seedlings (32-46 cm in height) (Native Forest Nursery, Chatsworth, GA) were transplanted into a 9:1 composted pine bark:sand rooting medium mix in 3.15 L Treepots (13.5 x 30.5 cm; Stuewe & Sons, Inc., Tangent, OR) and grown for 2-5 months under greenhouse conditions in Athens, GA. Liners of cultivars of *L. chinense* (Ever Red®, Zhuzhou Fuschia, Ruby) were obtained from a local nursery (Griffith Propagation Nursery, Watkinsville, GA) growing in 8.9 x 10.2 cm square liner cups. Plants were maintained in the greenhouse for 3-5 months and observed for any existing symptoms of infection and to allow for any potential fungicide residual to wear off. All plants were irrigated daily and liquid-fertilized weekly with Peters 20-20-20. No additional fungicides or insecticides were used on the plants.

Three plants each of sweetgum and loropetalum were spray-inoculated separately with conidial suspensions composed of a mixture of three isolates originating from sweetgum and a conidial suspension from three isolates originating from loropetalum. Two plants of each host were used as uninoculated controls.

Inoculum was prepared from isolates grown on V8 juice agar for 14-18 days at 24°C under a 12/hr light/dark diurnal cycle as previously described. A conidial suspension was obtained by flooding agar plates and dislodging conidia with a small brush as previously described. Agar plates were washed with additional sterile deionized water to remove additional conidia. Conidia concentration was counted using a hemocytometer and adjusted to 10,000 conidia per milliliter. The conidial suspension was sprayed onto plants using hand-held spray bottle until foliage was thoroughly wet. Inoculated plants were moved into a mist chamber where a humidifier was used to maintain humidity at about 90%. Plants were evaluated weekly for 5 weeks.

Another set of plants were inoculated in the same manner as previously described, but with a suspension of 8×10^4 conidia per ml and the foliage was allowed to dry for 2 hrs at 22-23°C in the laboratory before being enclosed in individual clear plastic bags that had been misted lightly with deionized water to maintain humid conditions. This group of plants was placed into an incubator at 22-24°C with 12 hr diurnal light and humidity of 60-70% during the day and 90% at night. The plastic bags were then opened, and remained off the plants for 12 hrs. Plants were then lightly misted with deionized water and re-bagged for 12 hrs. This cycle of removing and re-bagging the plants was repeated for three days after which the plants were maintained in the incubator, irrigated daily as needed, and monitored for leaf spot development for 45 days.

Detached leaf assays were also conducted by surface disinfesting loropetalum and sweetgum leaves with 5% bleach solution for 1-3 minutes followed by rinsing in sterile deionized water for 1-3 minutes. Leaves were placed into replicate plastic, square pint containers lined with a wet paper towel or onto the surface of water agar (Bacto Agar; 15 g/L) in 100 x 15 mm disposable petri dishes. The abaxial leaf surface of the detached leaves of each plant species were injured with a sterile needle and inoculated separately with a 10 µl droplet of loropetalum and sweetgum mixed isolate conidial suspensions (8×10^4 conidia per ml) per leaf. A 10 µl droplet of sterile deionized water was used as a control. In addition, surface disinfested sweetgum and loropetalum leaves within pint container moist chambers or on water agar were injured using a sterile needle and inoculated separately with a 3-mm agar plug from an 18-day-old sweetgum and loropetalum isolate that was inverted and placed directly over the leaf wound. Inoculation with 3-mm plugs from fresh water agar plates served as a control. Pint containers were sealed with a plastic lid and water agar plates were placed within a lidded plastic container to ensure humid conditions and prevent leaf drying. Detached leaves were monitored every other day for leaf spot development beneath the conidial suspension droplet or agar plug for 3 weeks.

DNA extraction. Isolates collected from sweetgum and loropetalum leaf spots were grown on dilute V8 juice agar (50 ml clarified V8 juice, 950 ml deionized water) overlain with sterile cellophane for 14-18 days at 24°C under white florescent lights with a 12 hr light/dark diurnal cycle. A conidial suspension was used to inoculate plates as previously described and was preferred over transfers of agar plugs from the margins of actively growing cultures because it yielded more mycelium in a shorter period of time. Mycelium (approximately 10-30 mg) was collected by gently scraping the cellophane with a sterile scalpel blade and placed into 2-ml sterilized polypropylene microvials (Bio Spec Products Inc., Bartlesville, OK) containing two 0.5 mm sterilized glass beads. Mycelia was ground by quick-freezing the sample in liquid nitrogen and bead-beating twice (Mini-BeadBeater-1; Bio Spec Products Inc.). Genomic DNA for each isolate extracted using a DNeasy Plant Mini kit (QIAGEN, Valencia, CA) according to manufacturer’s protocols.

PCR Amplification, and sequencing. Five gene loci regions (ITS, ACT, EF-1, CAL, and LSU) were targeted for PCR amplification and subsequent sequencing for each of isolate. The universal primers ITS1 and ITS4 (White et al. 1990) were used to amplify the internal transcribed spacer region (~600 bp). Other regions amplified were the actin gene (ACT; ~370 bp) using ACT-512F (5'- ATG-TGC-AAG-GCC-GGT-TTC-GC -3') and ACT-783R (5'- TAC-GAG-TCC-TTC-TGG-CCC-AT – 3') (Carbone and Kohn, 1999); calmodulin gene (CAL; ~580 bp) using CAL-228F (5'- GAG-TTC-AAG-GAG-GCC-TTC-TCC-C -3') and CAL-737R (5'-CAT-CTT-TCT-GGC-CAT-CAT-GG - 3') (Carbone and Kohn, 1999); elongation factor 1-α gene (EF-1α; ~350 bp) using EF1-728F (5' – CATCGAGAAGTTCGAGAAGG – 3') and EF1-986R (5' - TAC-TTG-AAG-GAA-CCC-TTA-CC -3') (Carbone and Kohn, 1999); and the nuclear large subunit (LSU; ~1000 bp) using LROR (5' – ACC-CGC-TGA-ACT-TAA-GC – 3') and LR6 (5'- CGC-CAG-TTC-TGC-TTA-CC –3') (Vilgalys and Hester, 1990; Rehner and Samuels, 1995).

All PCR reactions were conducted using an Eppendorf 5331 MasterCycler thermal cycler as 25 µl reactions using Illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare, Pittsburgh, PA) to which 1 µl of each forward and revers primer, 1 µl of DNA template, and 22 µl of sterile molecular grade water was added. PCR conditions for ITS and LSU were the same and consisted of an initial denaturation of 5 min at 94°C followed by 34 cycles of 94°C for 1 min, 52°C for 1 min and 72°C for 1 min, then 5 min at 72°C, followed by a hold at 4°C (White, 1990). PCR products were visualized on a 1% agarose gel containing 8 µl ethidium bromide.

PCR conditions for ACT and CAL consisted of an initial denaturation step of 8 min. at 95°C followed by 35 cycles of 15 sec at 94°C, 20 sec at 55°C, 1 min at 72°C, followed by a final extension step of 5 min 72°C and hold at 4°C (Carbone and Kohn, 1999). The PCR protocol of EF-1α consisted of an initial denaturation step of 5 min at 96°C followed by 30 cycles of 30 sec at 96°C for, 30 sec at 52°C, and 1 min at 72°C followed by an extension step of 72°C for 5 min and hold at 4°C (Crous et al., 2004).

PCR products were purified using ExoSAP-IT (Thermo Fisher Scientific, Waltham, MA) where 5 µl of PCR product was mixed with 2 µl of ExoSAP-IT and incubated at 37°C for 15 min, followed by heating at 80°C for 15 min. DNA concentration was estimated using a

Nanodrop spectrophotometer ND-1000 (Nanodrop Technologies, Wilmington, DE) and product concentration was adjusted as needed for sequencing using both forward and reverse primers by EuroFins (Louisville, KY).

Phylogenetic Analysis. Sequences were trimmed and aligned using Geneious 10.1.3 software. Phylogenetic analyses were performed for Bayesian inference (BI) analyses of LR and SG isolates on LSU and concatenated, ITS, EF1-α, ACT loci were performed with Geneious 10.1.3 version program. Phylogenetic analyses were performed using MEGA X 10.1.5 (Kumar et al., 2018). The best evolutionary model for nucleotide substitutions for each region was inferred using MEGA 10.1.5.

Loropetalum (LR) and Sweetgum (SG) isolate phylogenics were inferred for LSU using the Kimura 2-parameter model (Kimura, 1980). The tree with the highest log likelihood (-1971.65) was chosen for further analysis. The percentage of trees in which the associated taxa clustered together was shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites [5 categories (+G, parameter = 0.0500)]. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 35 nucleotide sequences. There were a total of 1175 positions in the final dataset.

The Jukes-Cantor model was fit for ITS multiple alignment sequence data (Jukes and Cantor, 1969). The tree with the highest log likelihood (-974.40) was chosen. The percentage of trees in which the associated taxa clustered together was shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the MCL approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 37 nucleotide sequences. There was a total of 535 positions in the final dataset.

EF gene loci were inferred by using Kimura 2-parameter model (Kimura, 1980). The tree with the highest log likelihood (-1816.53) was shown. The percentage of trees in which the associated taxa clustered together was shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the MCL approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites [5 categories (+G, parameter = 0.7476)]. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 31 nucleotide sequences. There was a total of 359 positions in the final dataset.

The ACT gene region tree by using Kimura 2-parameter model with the highest log likelihood (-1027.93) was shown (Kimura, 1980). The percentage of trees in which the associated taxa clustered together was shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the MCL approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites [5 categories (+G, parameter = 0.3943)]. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 42 nucleotide sequences. There was a total of 225 positions in the final dataset.

RESULTS

*Leaf spot symptoms and morphological comparison and isolates***.** Leaf spots on American sweetgum leaves were usually subcircular, angular or irregular in shape and often occurred near leaf veins. Spots measurements ranged from 1.5 to 7.8 mm in diameter (Figure 3.1). The center of the spots ranged in color from brown to dark brown and turned gray or grayish white with age. The spot border was a dark brown. Often the spots were surrounded by halos of yellow to reddish-brown. Dense fascicles of conidiophores were seen within the leaf spots mostly on the abaxial leaf surface and rarely on the adaxial side. Conidiophores were small to mostly moderately large fascicles, loose to dense, arising from through stomata or erumpent (Figure 3.2). Conidia was observed cylindrical to obclavate-cylindrical, straight to curved, occasionally sigmoid, also around between 35-156 x 2-6 µm (Table 3.3), 2 to up 12-septate, subhyaline to pale olivaceous or more dark than olivaceous, thin-walled, smooth, with a sub-obtuse to obtuse apex and a short truncates base. The hila are mostly unthicken and not darkened (Figure 3.2).

Leaf spots from *Loropetalum chinense* were angular to irregular, and occasionally circular or subcircular (Figure 3.1). Most were vein-limited and ranged in size from 1.5 to 5 mm. Halos of diffuse yellow to reddish-brown may be seen on some cultivars. Leaf spots are generally dull grayish green, to purple-brown and may have a slightly darker border. The color of leaf spots is slightly different on the abaxial surface than the adaxial surface, and may appear darker brown to olivaceous-brown. On new growth, leaf spots may be inconspicuous to dull, diffuse spots. Dense to open fascicles are seen on both the adaxial and abaxial leaf surfaces. Conidiophores are mostly moderately large fascicles, seldom loose to mostly dense (Figure 3.3). Conidia is narrowly elongated cylindrical to obclavate-cylindrical, filiform, straight to curved, occasionally sigmoid, range in size from around 28-219 x 2-5 µm (Table 3.4), are subhyaline to pale olivaceous with 4 to 14-septations (usually around 10), thin-walled, smooth, with an obtuse apex and a short truncated base. The hila unthicken to slightly thickened, and is often darkened or refractive (Figure 3.3).

Comparing a total of 400 conidia from sweetgum isolates and 350 conidia from loropetalum isolates; length and width of the conidia were different ($P<0.05$) between the hosts. The length of the conidia originating from loropetalum samples were longer than that of sweetgum with a mean length of 103.6 µm for loropetalum isolates and a mean length of 96.5 µm for sweetgum isolates. The mean width of sweetgum isolates was wider than loropetalum isolates, 3.6 µm and 3.11 µm, respectively.

Pathogenicity of isolates using cross-inoculation. Inoculation of plants within the mist chamber was unsuccessful for both loropetalum and sweetgum plants. Inoculation results were improved using the bagged plant method within the incubator. After 14 days, sweetgum leaves inoculated with a sweetgum isolate conidial suspension developed dull gray green leafs potting. By 30-45 days, the leaf spots had become tan to brown with a slight grayish center similar to leaf spots seen on naturally-collected sweetgum leaves. No leaf spots symptoms developed on sweetgum leaves inoculated with a conidial suspension of isolates from loropetalum. Loropetalum plants did not develop leaf spots symptoms when inoculated with either loropetalum or sweetgum isolate conidial suspensions. Inoculations trials were repeated several times and loropetalum plants failed to develop symptoms. Infection using either a conidial suspension or agar discs in the detached leaf assays was variable. Darkened areas beneath the suspension droplet were seen when either host was inoculated with a conidial suspension from the same host; however, definitive leaf spots did not develop after 21 days. Fascicles of the pathogen were seen after 21 days on loropetalum detached leaf samples, but not on detached sweet gum leaves. Darkened spots did not develop when either host was inoculated with a conidial suspension from the other host. Darkened spots did not develop on leaves inoculated with a water control. From the inoculation studies, it does not appear that the isolates from sweetgum and loropetalum are cross-pathogenic.

DNA analyses and phylogenetic trees. A total of 71 and 169 isolates from loropetalum and sweetgum, respectively, were collected from 2017-2018. A subset of the isolates (25 and 40, respectively) were sequenced at five genetic regions.

The robustness of the trees were evaluated by 500 bootstrap replicates and between group mean distances were analyzed by using Kimura 2-parameter model (Kimura, 1980) in MEGA X 10.1.5 program (Kumar et al., 2018). The EF-1α gene region phylogeny was resolved in two groups (Figure 3.4). The distance of these LR and SG groups was 32.3%. In these groups, LR was divided into 3 clades with 100% bootstrap value. SG group, on the other hand, could not provide a significant resolution due to its low bootstrap percentage values.

According to sequence data of ACT gene region, this tree was resolved in two groups (Figure 3.5). The distance of these LR and SG groups was computed between group mean distance as 22%. With a bootstrap value of 99%, the LR group was separated into two main clades. SG group could not provide a significant result for clades separation cause of low bootstrap percentage.

The LSU gene region phylogeny was first resolved in two groups. SG group, which was one of these groups, was resolved into two main clades with a bootstrap value of 85% (Figure 3.6). The other group formed in this phylogeny tree was branched into two sub-groups. Unlike all other gene regions, only the DNA analysis of SG16 isolate constitutes the first of these subgroups, while the other sub-group consisted of sequences LR isolates with 100% bootstrap value. The LR sub-group showed in-house distinctions with fewer bootstrap percentages. In this phylogenetic tree, the separation distance to the two most dominant groups was 1.13%.

ITS phylogeny was first shown in two groups. LR group, which was one of these groups, was resolved into two main clades with a bootstrap value of 100% (Figure 3.7). The other group formed in this phylogenetic tree was branched into two sub-groups. Like the LSU gene region, DNA analysis of LR15 isolate constitutes the first of these subgroups, while the other sub-group consisted of sequences SG isolates with 79% bootstrap value was separated five clades. In this phylogenetic tree, the separation distance to the two most dominant groups was 2.33%.

DISCUSSION

Accurate identification and naming of fungal pathogens is important in communication, for understanding the biology and pathogenicity of the organism, and it can have disease management implications (Crous et al., 2015). The identity of the pathogen associated with leaf spot diseases on American sweetgum (*L. sytraciflua*) and Chinese fringe flower (*L. chinense*) has been confused through the reassigning of pathogens to different genera over time. This study attempted to elucidate the relatedness or its lack of relatedness between a pathogen recovered from sweetgum and loropetalum in southeastern USA, and purported to be the same species.

Based upon conidial and conidiophore morphological characteristics of the fungal isolates obtained from *L. styraciflua* in this study, as well as the leaf spot disease symptoms, compared to three fungal descriptions of leaf spot pathogens affecting American and Formosan sweetgum in Braun et al. (2015), the isolates more closely resemble that of *Pseudocercospora liquidambaricola* (J.M. Yen) U. Braun. In general, the American sweetgum isolates do not comply with the description of *P. tuberculans* Ellis & Everh., U. Braun, which is believed to possibly be the true identity of the first description of a leaf spot pathogen on American sweetgum (possibly mistakenly identified as *Cercospora liguidambari*s) in Alabama in 1892 (Atkinson, 1892; Braun, 2000). In the re-describing of *C. liquidambaris* by Chupp (1954) and the reclassification of this species to *Pseudocercospora liquidambaris* (Hsieh and Goh, 1990) and subsequently *P. liquidambaricola* (Braun, 2000). This may have confused the taxonomy. Braun et. al. (2015) states that the confused taxonomy may only be resolved through lectotypification and phylogentic study. Unfortunately, this confusion may have also encompassed the leaf spot pathogen affecting *L. chinense* in North America.

Loropetalum chinense was previously mentioned as a host in China for *Pseudocercospora liquidambaris* (Liu and Guo, 1998), which was reclassified as *P. liquidambaricola*. (Braun, 2000). Although leaf spot on loropetalum has been reported in disease clinics across the southeastern USA, a first report of this pathogen within the US has not been published. It was assumed that the identity of the leaf spot pathogen was *P. liquidambaricola* as reported in China. However, based upon conidial and conidiophore morphological characteristics, the pathogen recovered from *L. chinense* in the southeastern USA does not fit the description of *P. liquidambaricola*. The morphological characteristics more closely resemble *Cercospora liquidambaris* in having longer, thinner, paler conidia that often develop lateral outgrowths and a more distinct hilum (image not shown) and longer conidiophores that have an narrower and paler apex. Leaf spot disease symptoms also more closely align with the description of symptoms attributed to *C. liquidambaris*.

Furthermore, cross-inoculation trials of conidial suspension from sweetgum and loropetlaum isolates failed to cause disease symptoms of the alternate host. It does not appear that the sweetgum pathogen can infect loropetalum. The loropetalum pathogen did not infect sweetgum; however, since infection of loropetalum was also not detected using spray-inoculation methods, it cannot be definitively said that the loropetalum pathogen is unable to infect sweetgum. Until this study, no other investigation has attempted cross-inoculation of these pathogens on either host.

Although, the sweetgum pathogen has been described and reclassified multiple times, genetic sequence information has not (to date) been deposited within repositories. To our knowledge, this is the first study that has sequenced any gene region for the American sweetgum or loropetalum leaf spot pathogens. Neither leaf spot disease (as of yet) causes significant damage and plant loss within forested areas, nurseries, and landscapes. The leaf spot on sweetgum occurs regularly within the southeast in late summer and early fall. Since sweetgum is not considered a tree of high economic importance, the leaf spot is more of an aesthetic problem causing premature defoliation. Within ornamental plant nurseries, leaf spot on loropetalum can cause premature leaf drop, but is not considered a major disease problem and is managed as any other leaf spot caused by Cercosporoid pathogen (J. Williams-Woodward, personal communication).

Since genetic data is unavailable for these leaf spot pathogens, the data generated from this study may aid others in continuing phylogenetic analyses of this group of pathogens worldwide. Based upon the sequences from five gene regions in this study, with few exceptions, the sweetgum isolates separate into a different clade than the loropetalum isolates. When the results of EF and ACT phylogeny studies were examined, it was calculated that there was more than 20% distance between the two groups. In the ITS and LSU regions, one isolate from each group emerged in phylogenetic trees as sub-groups to the others. For this reason, the distance comparisons of the two groups were found to be between 1% and 3% at much higher percentages compared to the other two gene regions. Two isolates, SG-16 and LR-15 from sweetgum and loropetalum, respectively, were sub-grouped to the other group with very low bootstrap percentage values. There may be a number of reasons why this occurred. First, such a result might have been formed due to insufficient sequence data of both samples. However, when the consensus data in multiple alignments were examined this does not appear to be the cause. Another possibility is that these two isolates may be hybrids or did not have enough time to accumulate genetic differences because they are separated from the same ancestor in the recent past. Additional studies on these two isolates may be needed.

Based upon morphological and phylogentic comparisons between the fungal pathogen isolates recovered from American sweetgum (*L. styraciflua*) and *Loropetaulm chinense* leaf spot diseases, as well as the inability to show cross-pathogenicity, it is doubtful that the pathogen infecting sweetgum is the same as that infecting loropetalum. Therefore, the two pathogens should not share the same name, *P. liquidambaricola*. The morphological characteristics of the isolates recovered from American sweetgum does agree with the published description of *P. liquidambaricola* (Braun et al., 2015), and it should retain this name. However, additional phylogenetic studies should be conducted to determine whether the pathogen on loropetalum belongs as a subspecies, a new distinct species, or if it should be renamed *Cercospora liquidambaris* since morphologically it agrees with its published description. However, as Braun et al. (2015) indicates, this species name has been tentatively retained in *Cercospora s. lat.* until phylogenetic data is available. It is possible that information from this study may be used to aid in this process.

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Figure 3.1. Leaf spot disease on *Liquidambar styraciflua* (top row) and *Loropetalum chinense* (bottom row). Top row: Circular to irregular or vein-limited angular, brown to dark brown spots that turn grayish white with a dark border, 2-10 mm or confluent in size, may have diffused yellow halo. Bottom row: Small (1-4 mm), purplish to grayish green, angular to subcircular leaf spots with or without a slight pale halo on cultivars, Ruby (bottom left) and Emerald Snow® (bottom right).

Table 3.1. Isolates of *Pseudocercospora* sp. recovered from *Liquidambar styraciflua* in 2017-2018

Isolate ID	Sample Collection Location	Year Collected
SG-1, SG-2	Greensboro, GA (Oconee National Forest)	2017
SG-3, SG-4	Athens, GA (Whitehall Forest)	2017
SG5	Greensboro, GA (Oconee National Forest)	2017
SG-6, SG-7, SG-8	Athens, GA (Whitehall Forest)	2017
SG-9, SG-10	Nashville, TN	2017
SG-11, SG-12, SG-14, SG-15	Ellijay, GA	2017
SG-16	Greensboro, GA (Oconee National Forest)	2017
SG-17	Ellijay, GA	2018
SG-18	Greensboro, GA (Oconee National Forest)	2018
SG-19, SG-20, SG-21, SG-22	Watkinsville, GA (Durham Hort. Farm)	2018
SG-23, SG-24, SG-25	Athens, GA (Sandy Creek Park)	2018
SG-26, SG-27, SG-28	Athens, GA (Sandy Creek Park)	2018
SG-29, SG-30	Atlanta, GA	2018
SG-31, SG-32, SG-33, SG-34	Athens, GA (Sandy Creek Park)	2018
SG-35, SG-36, SG-37, SG-38	Athens, GA (Sandy Creek Park)	2018
SG-39	Watkinsville, GA (Durham Hort. Farm)	2018
SG-40	Athens, GA (UGA University Village)	2018

Table 3.2. Isolates of *Pseudocercospora* sp. recovered from *Loropetalum chinense* in 2017-2018.

Isolate	City of Sample Origin	Cultivar	Location	Year
LR-1	Dearing, GA (Clinic #45484)	Sizzling Pink	nursery	2017
LR-2	Watkinsville, GA (Post Office)	Ruby	landscape	2017
LR-3	Watkinsville, GA (Woodward)	Sizzling Pink	landscape	2017
LR-4	Columbus, GA (Clinic #46957)	Ruby	landscape	2017
LR-5	Loxley, AL	Purple Diamond®	nursery	2017
LR-6	Loxley, AL	Purple Pixie®	nursery	2017
LR-7	Loxley, AL	Ruby	nursery	2017
LR-8	Loxley, AL	Purple Daydream®	nursery	2017
LR-9	Watkinsville, GA (Griffith)	Crimson Fire™	nursery	2017
LR-10	Watkinsville, GA (Griffith)	Crimson Fire™	nursery	2017
LR-11	Watkinsville, GA (Griffith)	Ruby	nursery	2017
LR-12	Watkinsville, GA (Griffith)	Ruby	nursery	2017
LR-13	Thomson, GA	unknown	landscape	2018
LR-14	Thomson, GA	unknown	landscape	2018
LR-15	Thomson, GA	unknown	landscape	2018
LR-16	Dearing, GA	Zhuzhou Fuchsia	nursery	2018
LR-17	Dearing, GA	Zhuzhou Fuchsia	nursery	2018
LR-18	Dearing, GA	Zhuzhou Fuchsia	nursery	2018
LR-19	Dearing, GA	Emerald Snow®	nursery	2018
LR-20	Dearing, GA	Emerald Snow®	nursery	2018
LR-21	Dearing, GA	Emerald Snow®	nursery	2018
LR-22	Dearing, GA	Purple Diamond®	nursery	2018
LR-23	Dearing, GA	Purple Diamond®	nursery	2018
LR-24	Dearing, GA	Ever Red®	nursery	2018
LR-25	Dearing, GA	Ever Red®	nursery	2018

TABLE 3.3 Length and width of conidia from *Liquidambar styraciflua* leaf spot isolates.

Isolate (Sample Location)	Min. & Max. Length (µm)	Avg. Length (µm)	Min. & Max. Width (µm)	Avg. Width (µm)
SG-1 (Greensboro, GA)	35 - 126[a]	95.6	2 - 5	3.5
SG-2 (Greensboro, GA)	46 - 133[a]	88.9	4 - 6	4.7
SG-5 (Greensboro, GA)	82 - 111[a]	96.2	2 - 6	4
SG-16 (Greensboro, GA)	52 - 126[a]	98.3	3 - 6	4.5
SG-18 (Greensboro, GA)	82 - 134[a]	101.3	3 - 5	4.4
SG-3 (Athens, GA)	100 - 122[a]	105.7	3 - 5	3.8
SG-4 (Athens, GA)	89 - 129[a]	102.3	2 - 5	3.3
SG-6 (Athens, GA)	53 - 113[a]	94.4	3 - 5	3.5
SG-7 (Athens, GA)	74 - 136[a]	95.6	3 - 4	3.4
SG-8 (Athens, GA)	68 - 114[a]	90.5	3 - 4	3.3
SG-9 (Nashville, TN)	47 - 150[b]	92.4	3 - 4	3.28
SG-10 (Nashville,TN)	62 - 129[b]	96.88	2 - 5	3.52
SG-11 (Ellijay, GA)	66 - 133[a]	93.3	3 - 4	3.3
SG-12 (Ellijay, GA)	60 - 116[a]	99.2	3 - 4	3.6
SG-13 (Ellijay, GA)	73 - 137[a]	107.8	2 - 4	3.3
SG-15 (Ellijay, GA)	71 - 127[a]	103.5	3 - 5	3.6
SG-17 (Ellijay, GA)	82 - 117[a]	105.2	3 - 5	3.7
SG-19 (Watkinsville, GA)	60 - 120[a]	101.9	3 - 4	3.6
SG-20 (Watkinsville, GA)	58 - 121[a]	98.7	2 - 4	3.6
SG-21 (Watkinsville, GA)	90 - 140[a]	105.8	3 - 5	4
SG-22 (Watkinsville, GA)	53 - 120[a]	96.7	3 - 5	3.6
SG-39 (Watkinsville, GA)	79 - 140[a]	106.8	4 - 5	4.3
SG-25 (Athens, GA)	52 - 131[a]	92.2	2 - 4	3.1
SG-26 (Athens, GA)	37 - 115[a]	89.5	3 - 4	3.7
SG-28 (Athens, GA)	68 - 143[a]	105.5	3 - 4	3.7
SG-36 (Athens, GA)	46 - 103[a]	81.6	3 - 5	4.1
SG-38 (Athens, GA)	57 - 136[a]	88.1	3 - 4	3.2

SG-29 (Atlanta, GA)	49 - 126[b]	84.56	3 - 5	3.52
SG-30 (Atlanta, GA)	68 - 139[b]	99.64	2 - 4	3.24
SG-40 (Athens, GA)	38 - 156[c]	96.38	2 - 5	3.34
Results	35 - 156[d]	96.5	2 - 6	3.6

[a] Screened and measured 10 conidia from isolate.

[b] Screened and measured 25 spores from isolate.

[c] Screened and measured 50 spores from isolate.

[d] Screened and measured total 400 spores from isolate.

TABLE 3.4 Length and width of conidia from *Loropetalum chinense* leaf spot isolates.

Isolate (Sample Location)	Min. & Max. Length (µm)	Mean Length (µm)	Min. & Max. Width (µm)	Mean Width (µm)
LR-1 (Dearing, GA)	72 – 166[a]	107.2	2 - 4	3.2
LR-16 (Dearing, GA)	71 - 109[a]	95	2 - 4	3
LR-19 (Dearing, GA)	79 - 123[a]	97.9	3 - 4	3.4
LR-21 (Dearing, GA)	60 - 172[a]	116.7	3 - 4	3.6
LR-24 (Dearing, GA)	53 - 128[a]	98.7	2 - 4	2.9
LR-2 (Watkinsville, GA)	58 – 219[b]	129.48	3 - 5	3.52
LR-3 (Watkinsville, GA)	65 - 202[b]	124.04	2 - 4	3.24
LR-4 (Columbus, GA)	40 - 185[c]	112.86	2 - 4	2.9
LR-5 (Loxley, AL)	28 - 152[d]	89.75	2 - 3	2.87
LR-6 (Loxley, AL)	54 - 122[d]	92.45	2 - 3	2.8
LR-7 (Loxley, AL)	77 - 129[d]	99.57	2 - 4	3.07
LR-8 (Loxley, AL)	48 - 177[c]	99.48	2 - 5	3.66
LR-9 (Watkinsville, GA).	56 - 132[e]	93.66	2 - 4	2.93
LR-10 (Watkinsville, GA)	62 - 149[e]	110	2 - 4	3.2
LR-11 (Watkinsville, GA)	82 - 156[e]	118.8	3 - 4	3.4
LR-12 (Watkinsville, GA)	72 - 181[e]	124.9	3 - 5	3.26
LR-13 (Thomson, GA)	48 - 136[d]	90.9	2 - 4	2.95
LR-14 (Thomson, GA)	52 - 127[d]	92	2 - 4	2.72
LR-15 (Thomson, GA)	35 - 138[d]	87.27	2 - 4	3
Mean	28 - 219[f]	103.6	2 - 5	3.11

[a] Screened and measured 10 conidia from isolate.

[b] Screened and measured 25 spores from isolate.

[c] Screened and measured 50 spores from isolate.

[d] Screened and measured 13 or 14 spores of isolate.

[e] Screened and measured 12 or 13 spores from isolate.

[f] Screened and measured total 350 spores from isolate.

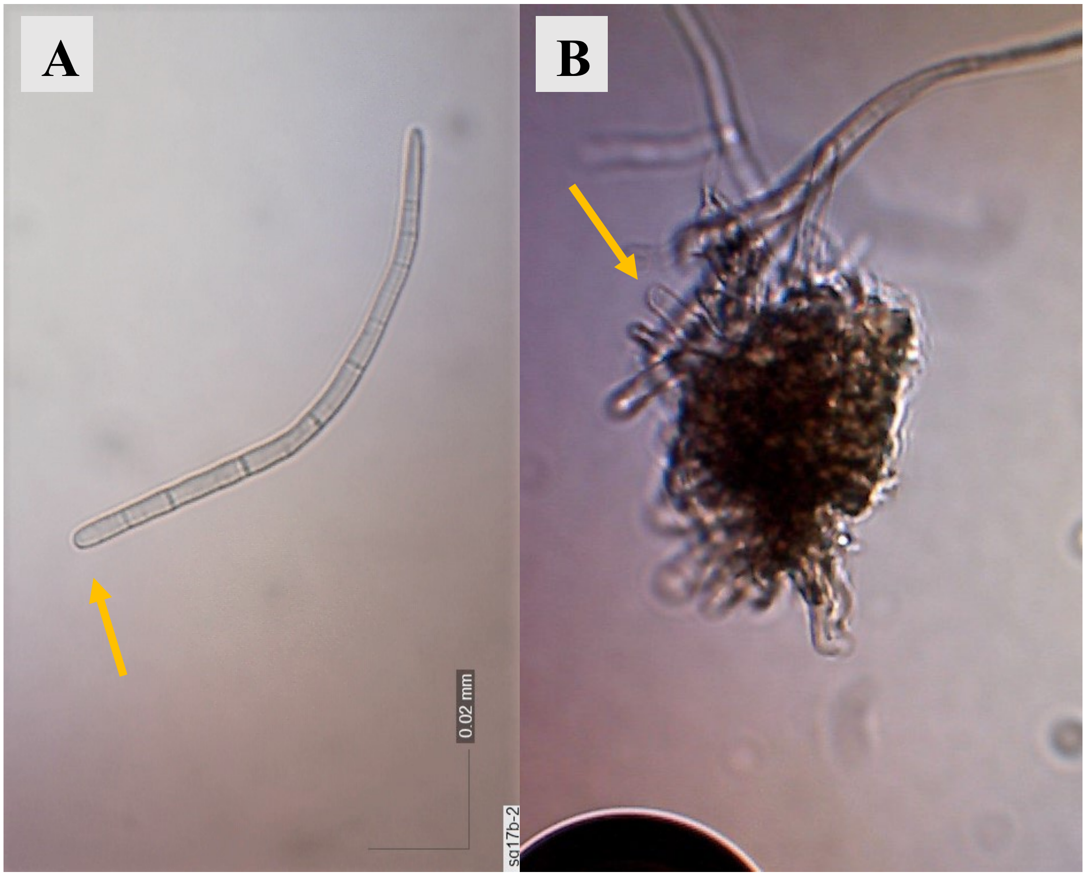

Figure 3.2. Conidium and conidiophores of *Pseudocercospora liquidambaricola* recovered from American sweetgum (*Liquidambaris styraciflua* L.). A) Conidium is cylindrical, slightly curved, 35-156 x 2-6 µm (average 96.5 x 3.6 µm) in size with up to 10 septations, pale olivaceous in color, smooth-walled, with an unthickened hilum (yellow arrow). B) Conidiophores are in small fascicles (yellow arrow), loose to dense, arising from a stroma (brown structure). Conidiophores are 5-25 µm long, olivaceous to olivaceous-brown, conidiogenous loci are inconspicuous or truncated, unthickened and pale.

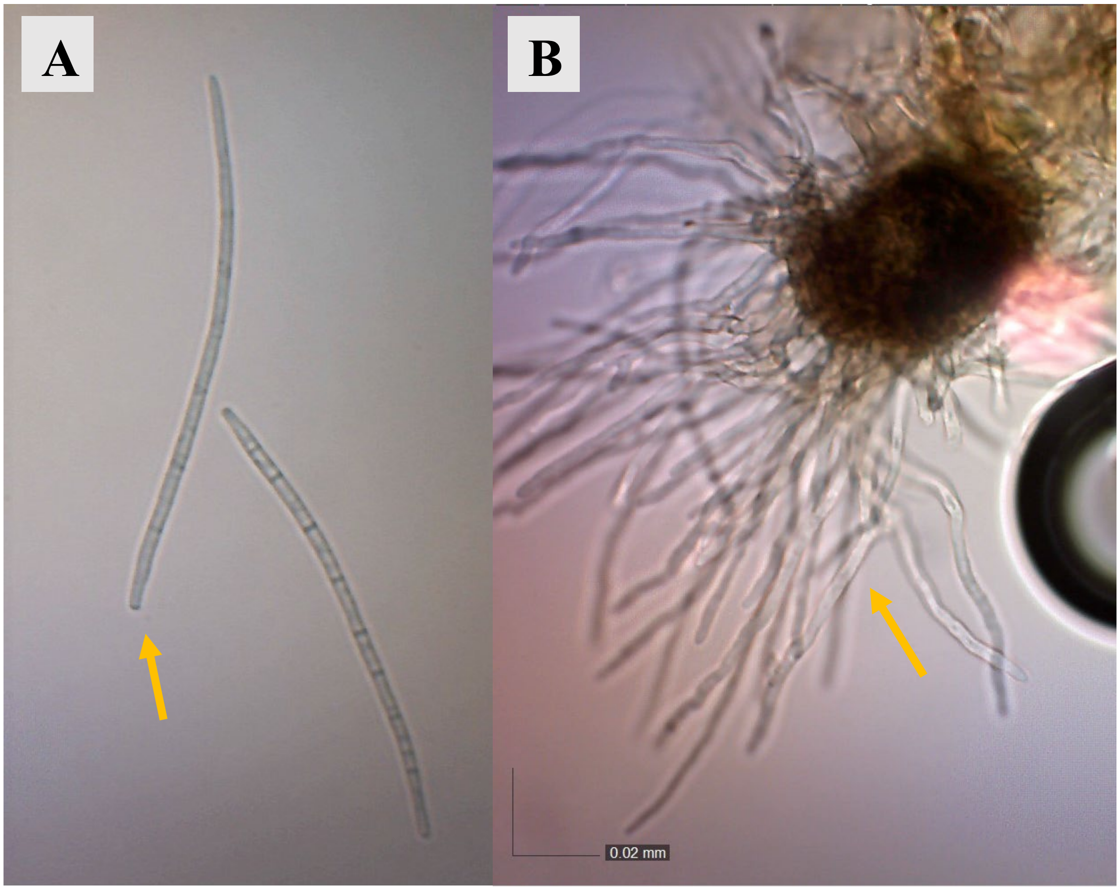

Figure 3.3. Conidium and conidiophores of *Pseudocercospora liquidambaricola* (possibly *Cercospora liquidambaris*) recovered from Chinese fringe flower (*Loropetalum chinense* Oliv.). A) Conidia are cylindrical to filiform, straight to slightly curved, 28-219 x 2-5 µm (average 103.6 x 3.1 µm) in size with 3-12 septations, subhyaline to pale olivaceous, smooth-walled, with an often-truncated base, hila are unthickened to slightly thickened, somewhat darkened or refractive (yellow arrow). B) Conidiophores are in small to large fascicles (yellow arrow) arising from substomatal hyphae or stromata (brown structure). Conidiophores (arrow) are 25-100 µm long with several septations, subcylindrical or attenuated towards the apex, pale brown and paler toward the tip, conidiogenous loci are conspicuous or somewhat refractive.

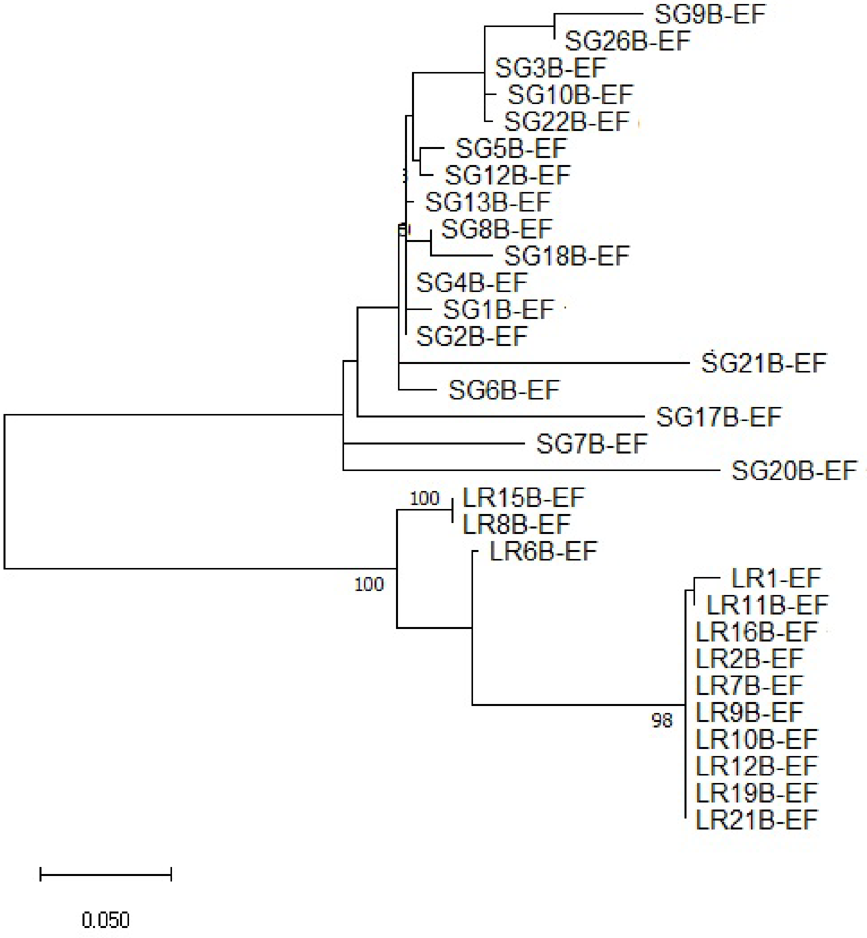

Figure 3.4. Phylogram obtained from the elongation factor 1-alpha (EF-1α) DNA sequence alignment of 31 *Loropetalum chinense* (LR) and *Liquidambar sytraciflua* (SG) isolates. Created by using MEGA X 10.1.5.

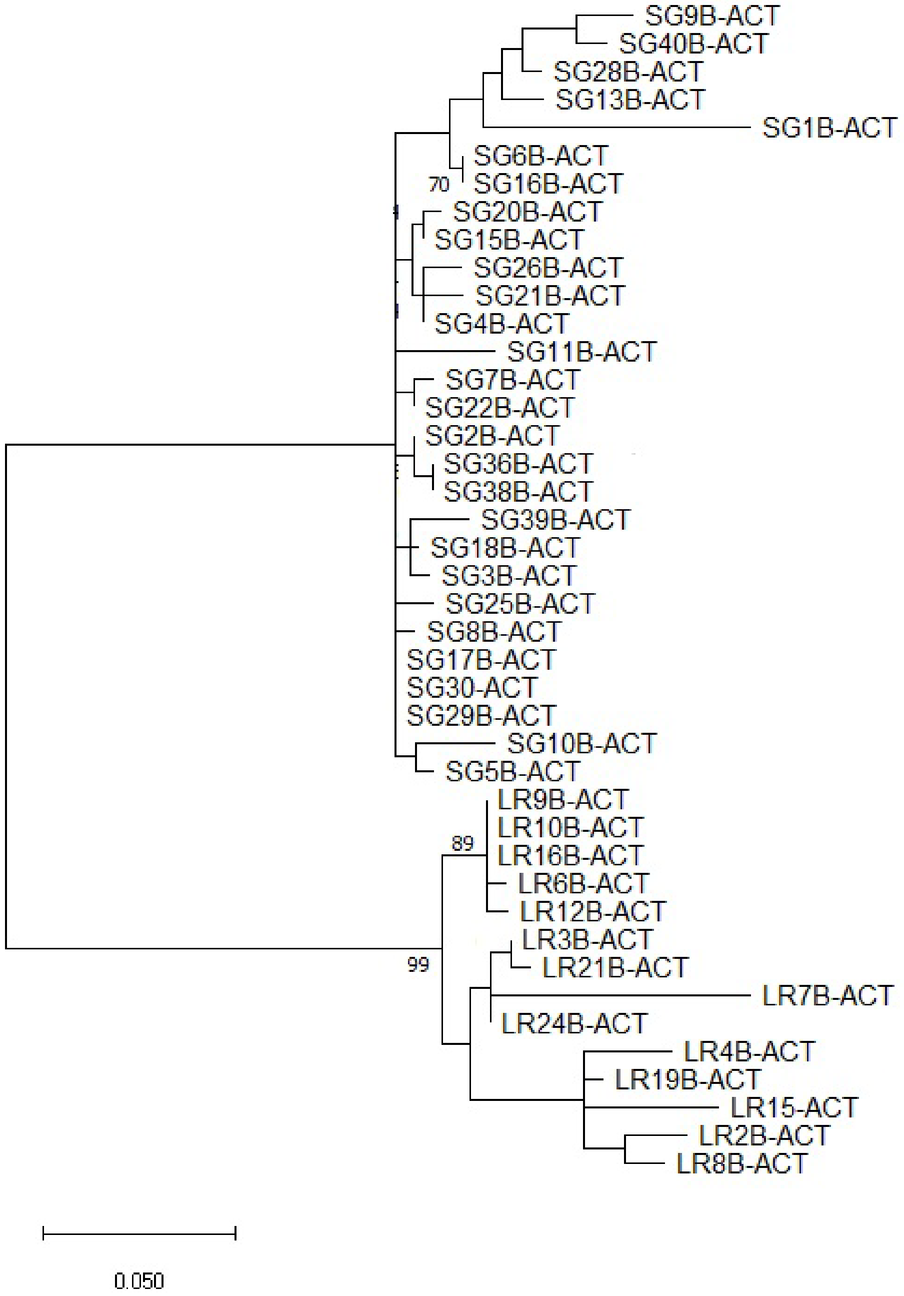

Figure 3.5. Phylogram obtained from the ACT DNA sequence alignment of 42 *Loropetalum chinense* (LR) and *Liquidambar sytraciflua* (SG) isolates. Created by using MEGA X 10.1.5.

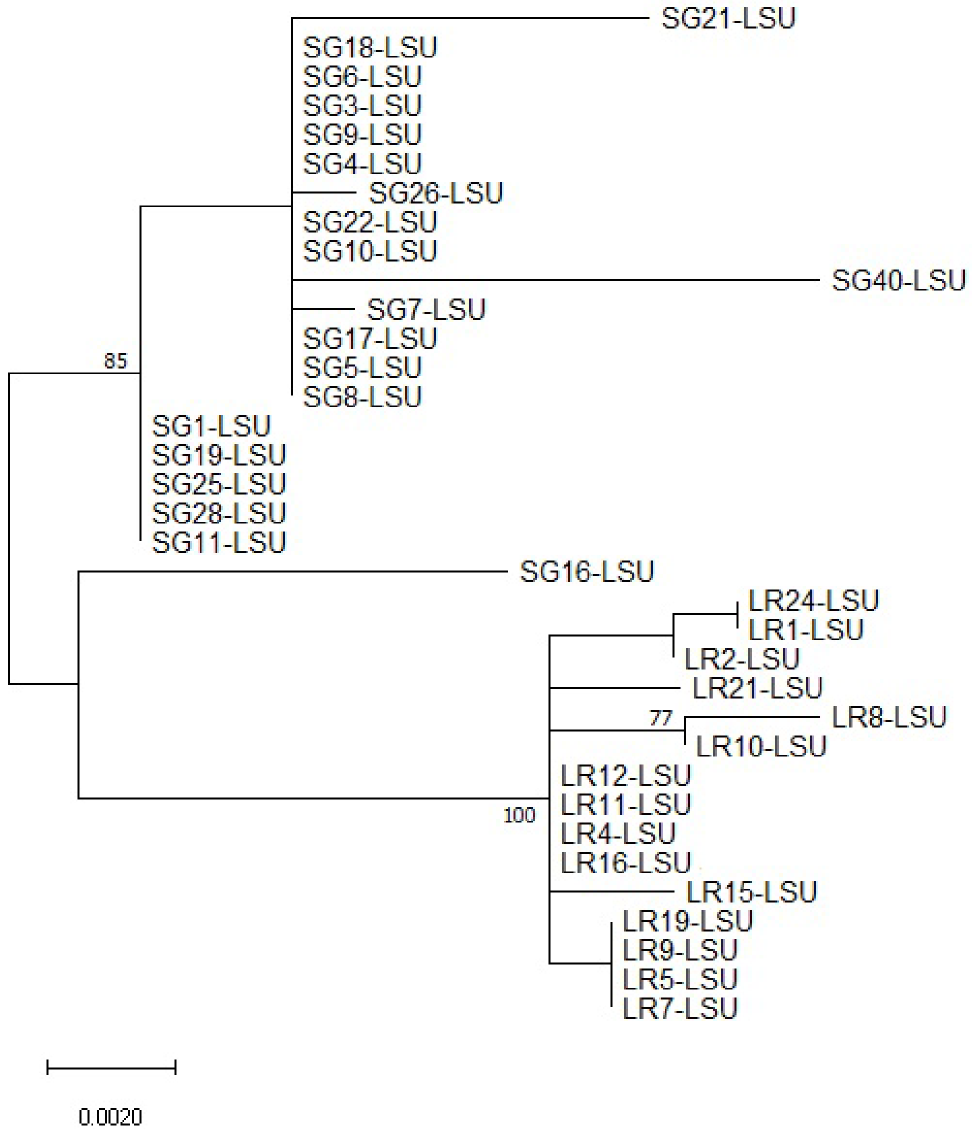

Figure 3.6. Phylogram obtained from the LSU DNA sequence alignment of 35 *Loropetalum chinense* (LR) and *Liquidambar sytraciflua* (SG) isolates. Created by using MEGA X 10.1.5.

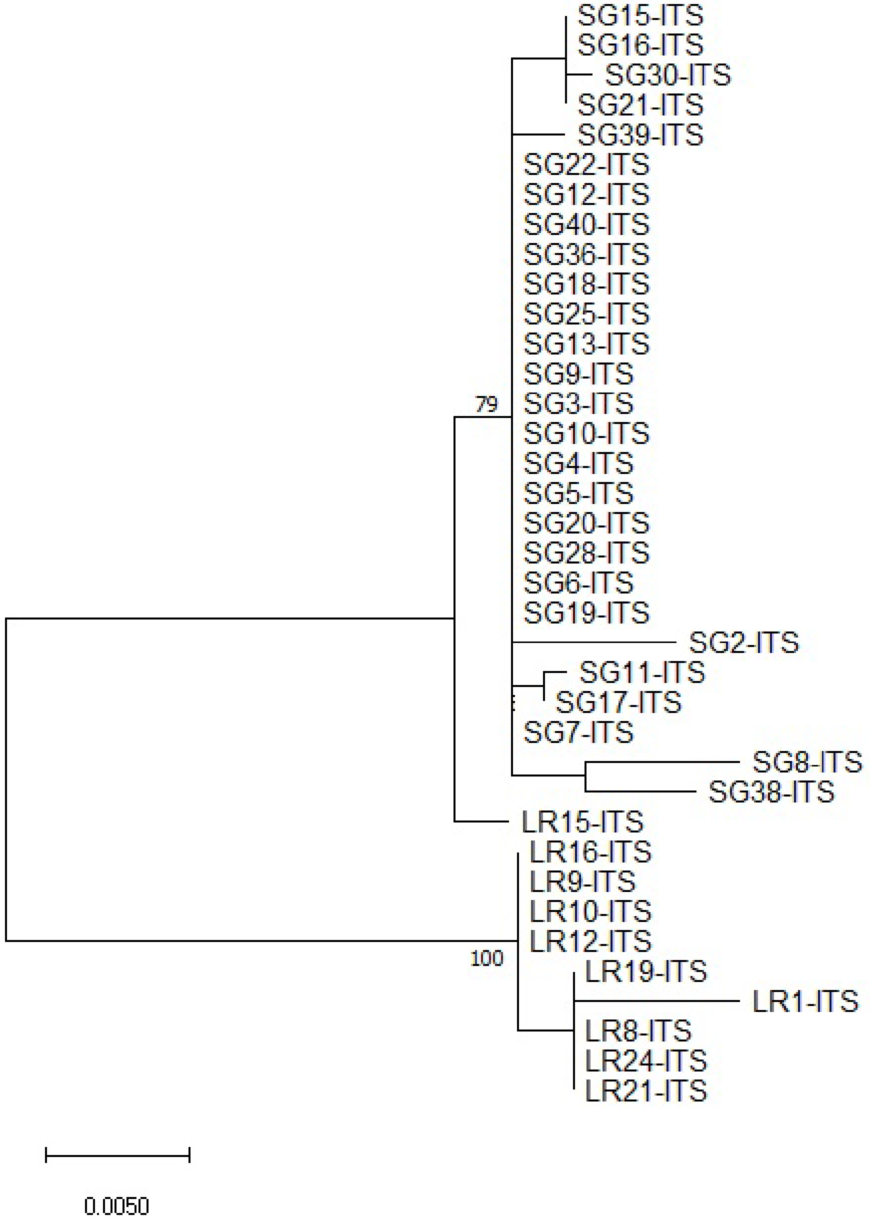

Figure 3.7. Phylogram obtained from the ITS DNA sequence alignment of 37 *Loropetalum chinense* (LR) and *Liquidambar sytraciflua* (SG) isolates. Created by using MEGA X 10.1.5.

CHAPTER 4

IN-VITRO FUNGICIDE EFFICACY TO CONTROL LOROPETALUM AND SWEETGUM LEAF SPOT PATHOGENS

INTRODUCTION

A fungal leaf spot disease affecting Chinese fringe flower (*Loropetalum chinense* Oliv.) has been identified on cultivars of loropetalum in ornamental plant nurseries in the southeastern USA since at least 2014 based upon plant disease clinic records from the University of Georgia Plant Disease Clinic (J. Williams-Woodward, personal communication). Very little is known about this disease. A published report of this disease within North America has not been completed. The pathogen is currently identified as *Pseudocercospora liquidambaricola* (J.-M. Yen) U. Braun (Braun et al., 2015), which is also described as a pathogen infecting American sweetgum (*Liquidambaris sytraciflua* L.).

The leaf spot pathogen causes angular, purplish to brown leaf spots mostly within the lower canopy of *L. chinense* cultivars. Infection causes premature leaf drop that can significantly affect plant marketability for ornamental plant producers. Loropetalum sales are not ranked nationally (Chappell et al., 2017), and economic value of this host is not known. However, loropetalum sales is increasing as many of the branded ornamental plant collections, such as Southern Living Collection, Proven Winners, and Gardener's Confidence Collection, contain one or more loropetalum cultivars. Most fungal leaf spot diseases are managed in a similar manner within nurseries. Management recommendations include growing less susceptible cultivars, changing environmental conditions to avoid prolonged periods of leaf wetness and overhead sprinkler irrigation, and often preventative fungicide applications. Fungicide efficacy information for the leaf spot pathogen on loropetalum or sweet gum is unavailable.

Fungicides efficacy trials are often conducted on containerized plants using natural inoculum. When trials are artificially inoculated, one or a mixture of several fungal isolates are sprayed in a water-based suspension onto plants to induce infection and symptom development. Inoculation of *L. chinense* and *L. styraciflua* was shown to be difficult and inconsistent (Chapter 3). Therefore, an in vitro assay using fungicide-amended agar may provide more usable results. In vitro assays are routinely used for fungicide screening, in particular for fungicide resistance screening of fungal populations (Olsen et al., 2013; Secor et al., 2010). The objective of this study was to evaluate the in vitro efficacy of the most commonly used fungicides against leaf spot diseases in ornamental plant nurseries.

MATERIAL AND METHODS

Flasks of V8 juice agar media [200 ml clarified V8® juice (Campbell Soup Co., Camden, NJ), 17 g Bacto Agar, 800 ml deionized water] were prepared and autoclaved at 121°C for 30 minutes. Molten agar was then cooled in a water bath to 45°C prior to fungicide amendment. Six fungicides (Table 4.1): azoxystrobin (Heritage®; Syngenta, Greensboro, NC), chlorothalonil (Daconil Weatherstik®; Sygenta, Greensboro, NC), fludioxonil (Medallion® WDG; Syngenta, Greensboro, NC), mancozeb (Dithane® 75DF; Corteva Agrisciences, Indianapolis, IN); tebuconazole (Torque™; Cleary Chemical LLC, Alsip, IL), thiophanate methyl (3336 WP; Cleary Chemical LLC, Dayton, NJ) were individually mixed into the molten agar and immediately poured into 100 x 15 mm plastic disposable petri plates. Non-amended V8 juice agar plates served as a control. All agar media were stored at 4°C until use. The higher labeled rate for *Cercospora* leaf spot control was used for each fungicide (Table 4.1).

Four isolates of *Pseudocercospora* sp. recovered from *L. chinense* were used in this assay. Three of the isolates originated from ornamental plant production nurseries in GA or AL, and one isolate originated from a landscape planting established in 2004 (Table 4.2). Four isolates of *P. liquidambricola* recovered from American Sweetgum (*L. styraciflua*) from four landscape or forest locations in GA were also used in this assay. Isolates were grown on V8 juice agar medium (200 ml clarified V8® juice, 17 g Bacto Agar, 800 ml deionized water) at 24°C under 12 hr diurnal light for 18 to 24 days. One 4-mm diameter agar disc containing superficial hyphae and conidia was extracted from the isolate colony and placed into sterile 1.5 ml microcentrifuge tubes containing 1 ml of sterile deionized water. The agar disc was crushed in the water and vortexed to release conidia. A 100 µl conidial suspension was pipetted onto non-amended and fungicide-amended V8 juice agar plates for each fungal isolate and spread using a sterilized, bent glass rod. The assay was repeated with three replicate plates per media. Agar plates were incubated at 24°C under 12 hr diurnal light for 18 days at which time the number of colonies per replicate plate were counted.

Total colony formation, which corresponds to conidial germination and hyphal growth, for each sweetgum and loropetalum isolate was analyzed across fungicide treatments using ANOVA General Linear Model. Treatment means were separated suing Tukey's HSD means separation test (Minitab Statistical Software, Minitab LLC, State College, PA).

RESULTS

Colony growth was observed for all isolates evaluated in this study. Non-amended control agar plates had mean colony growth ranging from 117.6 to 282 colonies per plate for isolates recovered from loropetalum and 187.3 to 418 colonies per plate for isolates recovered from sweetgum. The fungicide, fludioxonil (Medallion WDG), did significantly reduce colony development compared to the non-amended media by 22-37% and 14-35% for loropetalum and sweetgum isolates, respectively (Tables 4.3 and 4.4). However, colony growth was still high and unacceptable for inclusion in a disease management plan (Figure 4.1 and 4.2).

The fungicides, chlorothalonil (Daconil WeatherStik), mancozeb (Dithane 75DF), and tebuconazole (Torque) completely inhibited conidial germination and colony growth for all the loropetalum isolates. There was very minor colony growth on the azoxystrobin (Heritage) across all four loropetalum isolates. The fungicide thiophanate methyl (3336 WP) gave variable results across the loropetalum isolates (Table 4.4, Figure 4.2). Thiophanate methyl provided complete control for the isolate LR-3D, which was recovered from a plant in a 15-yr-old landscape. The other three isolates originated from plants growing in ornamental plant nurseries. Although thiophanate methyl reduced colony growth compared to the non-amended medium by 70%, it may indicate that resistance may have developed for this fungicide within this GA nursery.

Thiophanate methyl, mancozeb, tebuconazole were very effective in stopping conidial germination and colony growth for all four isolates recovered from sweetgum. There was minor colony growth for isolate SG-25, that was collected from a city park in Athens, GA on chlorothalonil and azoxystrobin amended media. These fungicides effectively reduce or stopped colony growth for the other sweetgum isolates.

DISCUSSION

Fungicides are an integral component of disease management recommendations for fungal leaf spot control in ornamental plant production. The multitude of ornamental plant species and their often host-specific fungal leaf spot pathogen species found within an ornamental plant nursery complicates disease management. Few fungicide efficacy trials are conducted on all host plants against all their specific pathogens. Efficacy information is important to aid fungicide selection and use. Often, if a fungicide is found to effectively reduce a pathogen on one host, then it is assumed that it would also control a similar pathogen on a different host. In addition, ornamental plant producers will most often use broad-spectrum fungicides in rotation or as tank mixes to maximize disease control across all plant species for ease and time constraints. Since fungicides are most effective when applied preventively, and since plants within ornamental nurseries can be found at all stages of plant growth, fungicide applications are often made on a calendar basis with application intervals of 7-14 days for at least 9-10 months in the southeastern US. The large number of applications per year, as well as the use of a limited number of fungicides can increase the risk of fungicide resistance development.

Fungicides are relied on heavily in ornamental nurseries. In 2009, when the last census of agriculture was collected for the ornamental nursery and floriculture industries, it was reported that a total of 406,400 lbs of fungicides were applied to woody ornamental nurseries in the six states included in the census (CA, FL, MI, OR, PA, TX). The fungicides used the most within woody ornamental plant nurseries to manage foliage diseases included chlorothalonil (111,200 lbs), mancozeb (90,400 lbs), thiophanate methyl (56,600 lbs), copper hydroxide (34,700 lbs), iprodione (6,700 lbs), and azoxystrobin (3,400 lbs) (NASS, 2011). Several of these fungicides were evaluated in this study.

Several broad spectrum, non-systemic fungicides, including chlorothalonil and mancozeb, were identified as being very effective at reducing pathogen growth and colony developemnt. These products are some of the most widely used fungicides in ornamental production (NASS, 2011). They both have multisite modes of action (FRAC group M5 and M3, respectively) meaning that the risk of developing fungicide resistance is low. These products are often used as a tank mix partner with fungicides that have a higher risk of resistance development.

The fungicide tebuconazole was also very effective against the leaf spot pathogen on both loropetalum and sweetgum. Tebuconazole is a demethylation inhibitor (DMI; FRAC group 3) fungicide. This group of fungicides do not affect spore germination and their inclusion within in vitro assays evaluating spore germination is inappropriate. However, the assay in this study was not specifically evaluating spore germination, but rather colony development and fungal growth. Tebuconazole was every effective in stopping fungal growth in this study.

Azoxystrobin also provided effective control in limiting fungal growth for both the sweetgum and loropetalum isolates. However, colony development did occur. This could be just that this fungicide is less effective against this pathogen or it could indicate that possible fungicide resistance may be developing, particularly because other than in one sweetgum isolate, there was more colony development within the sweetgum isolates originating from ornamental plant nurseries. Azoxystrobin is a widely used quinone outside inhibitor (QoI; strobilurin, FRAC group 11) fungicide. This fungicide has a single-site mode of action and fungicide resistance has been documented in numerous crops (Kim et al, 2003; Pasche, 2004; Wise et al., 2009).

Of particular interest from this study is how thiophanate methyl fungicide, a benzimidazole fungicide (FRAC group 1), effectively controlled fungal growth in the sweetgum isolates and in one loropetalum isolate (LR-3D) that originated from a landscape setting. Thiophanate methyl was particularly ineffective in reducing colony growth for the loropetalum isolate (LR-16C) that originated from an ornamental nursery. The reduced efficacy of thiophanate methyl against this isolate may indicate that fungicide resistance has developed within the pathogen population at this nursery.

Fungicide resistance has been identified in ornamental plant nurseries in the US for *Phytophthora* and *Pythium* spp. causing root and crown rot diseases (Hwang and Benson, 2005; Lamour et al., 2003; Moorman et al., 2002; Olsen et al., 2013). Resistance has also been identified for *Botrytis cinerea* on greenhouse crops (LaMondia and Douglas, 1997; Rupp et al., 2016; Yourman and Jeffers, 1999) and chrysanthemum white rust (*Puccinia horiana*) (Cook, 2001; Dirkse et al., 1982). Although fungicide resistance has been documented for leaf spot pathogens in agronomic crops, resistance has not been identified within leaf spot pathogens in ornamental crops.

Leaf spot diseases caused by Cercosporoid fungi account for a large percentage of diseases affecting ornamental and agronomic crops. Fungicide resistance has been identified in several Cercospora leaf spot diseases, namely Cercospora leaf spot of sugarbeet (*C. beticola*) (Secor et al., 2010; Weiland and Halloin, 2001) and frogeye leaf spot (*C. sojina*) and Cercospora leaf blight (*C. kikuchii*) in soybean (Price et al., 2015). However, fungicide resistance within Cercosporoid fungi has not been identified nor documented within ornamental plant nurseries. This may be the first report of possible fungicide resistance within a leaf spot pathogen in an ornamental nursery,

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Table 4.1. Fungicides and rates used to test in-vitro fungicide efficacy against *Pseudocercospora* species isolated from *Loropetalum chinense* and *Liquidambar styraciflua*.

Trade Name	Active Ingredient	FRAC Code[1]	Labeled Rate[2] (per 100 gal.)	Amended Conc. (per liter)
3336 WP	Thiophanate methyl	1	16 oz	1.20 g
Torque™	Tebuconazole	3	10 fl.oz	0.80 ml
Heritage®	Azoxystrobin	11	4 oz	0.30 g
Medallion® WDG	Fludioxonil	12	2 oz	0.15 g
Dithane® 75DF	Mancozeb	M3	1.5 lb	1.80 g
Daconil WeatherStik®	Chlorothalonil	M5	1.4 lb	1.68 g

[1] Numerical classification of fungicide mode of action groups based upon the Fungicide Resistance Action Committee (FRAC) designation.

[2] The higher labeled rate for Cercospora leaf spot control was used when a concentration range was provided on the product label.

Table 4.2. Isolates of *Pseudocercospora* species used in in-vitro fungicide efficacy assay.

Isolate	Host	City/State of Sample Origin	Location
LR-3D	*Loropetalum chinense*	Watkinsville, GA	landscape
LR-7A	*Loropetalum chinense*	Loxley, AL	nursery
LR-8B	*Loropetalum chinense*	Loxley, AL	nursery
LR-16C	*Loropetalum chinense*	Dearing, GA	nursery
SG-10B	*Liquidambar styraciflua*	Nashville, TN	landscape
SG-17A	*Liquidambar styraciflua*	Ellijay, GA	landscape
SG-25A	*Liquidambar styraciflua*	Athens, GA	landscape
SG-30B	*Liquidambar styraciflua*	Atlanta, GA	landscape

Table 4.3. Mean number of colonies growing on fungicide amended agar plants for four *Pseudocercospora sp.* isolates recovered from *Loropetalum chinense*.

	Mean number of colonies per Loropetalum isolate[1]			
Fungicide (active ingredient)	LR-3D	LR-7A	LR-8B	LR-16C
Non-amended control	146.6 a[2]	117.6 a	241.6 a	282.0 a
Medallion (fludioxonil)	91.6 b	88.3 b	183 b	218.3 b
3336 WP (thiophanate methyl)	0 c	9.6 c	1.3 c	83.3 c
Heritage (azoxystrobin)	1.3 c	5.3 c	1.6 c	6.3 d
Daconil (chlorothalonil)	0 c	0 c	0 c	0 d
Dithane (mancozeb)	0 c	0 c	0 c	0 d
Torque (tebuconazole)	0 c	0 c	0 c	0 d

[1] Mean number of isolates from three replicate fungicide-amended agar plates.

[2] Numbers followed by the sample letter are not significantly different from each other at $P<0.05$ using Tukey's HSD means separation test.

Table 4.4. Mean number of colonies growing on fungicide amended agar plants for four *Pseudocercospora liquidambaricola* isolates recovered from American Sweetgum.

	Mean number of colonies per Sweetgum isolate[1]			
Fungicide (active ingredient)	SG-10B	SG-17A	SG-25A	SG-30B
Non-amended control	187.3 a[2]	372.7 a	412.7 a	418.0 a
Medallion (fludioxonil)	161.3 b	239.7 b	306.0 b	263.7 b
3336 WP (thiophanate methyl)	0 c	0 c	0 c	0 c
Heritage (azoxystrobin)	0 c	0 c	46.0 c	1.3 c
Daconil (chlorothalonil)	0 c	0 c	3.0 c	0 c
Dithane (mancozeb)	0 c	0 c	0 c	0 c
Torque (tebuconazole)	0 c	0 c	0 c	0 c

[1] Mean number of isolates from three replicate fungicide-amended agar plates.

[2] Numbers followed by the sample letter are not significantly different from each other at $P<0.05$ using Tukey's HSD means separation test.

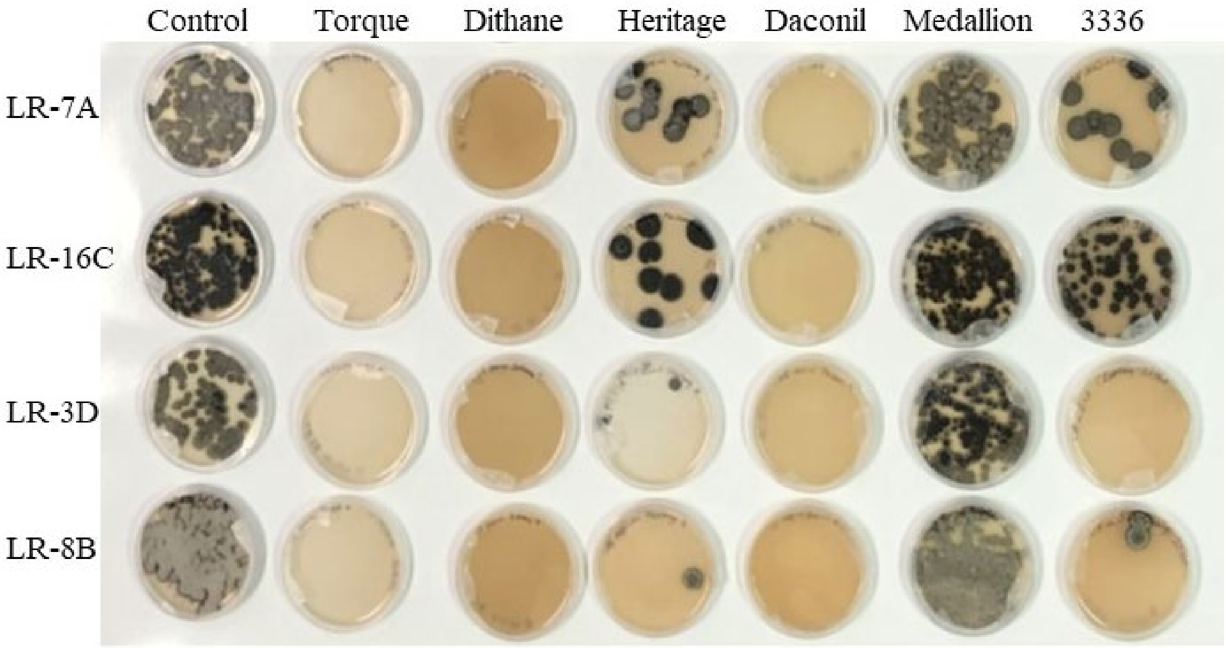

Figure 4.1. Colony growth after incubating 18 days at 25°C under 12 hr. light/dark diurnal light cycle for four *Pseudocercospora* sp. isolates recovered from *Loropetalum chinense* (LR-7A, LR-16C, LR-3D, LR-8B) on fungicide-amended and non-amended (control) V8 juice agar media. Three isolates (LR-7A, LR-8B, LR-16C) were recovered from Loropetalum grown in ornamental plant nurseries in GA and AL. Isolate LR-3D was recovered from Loropetalum in a 15-yr-old landscape.

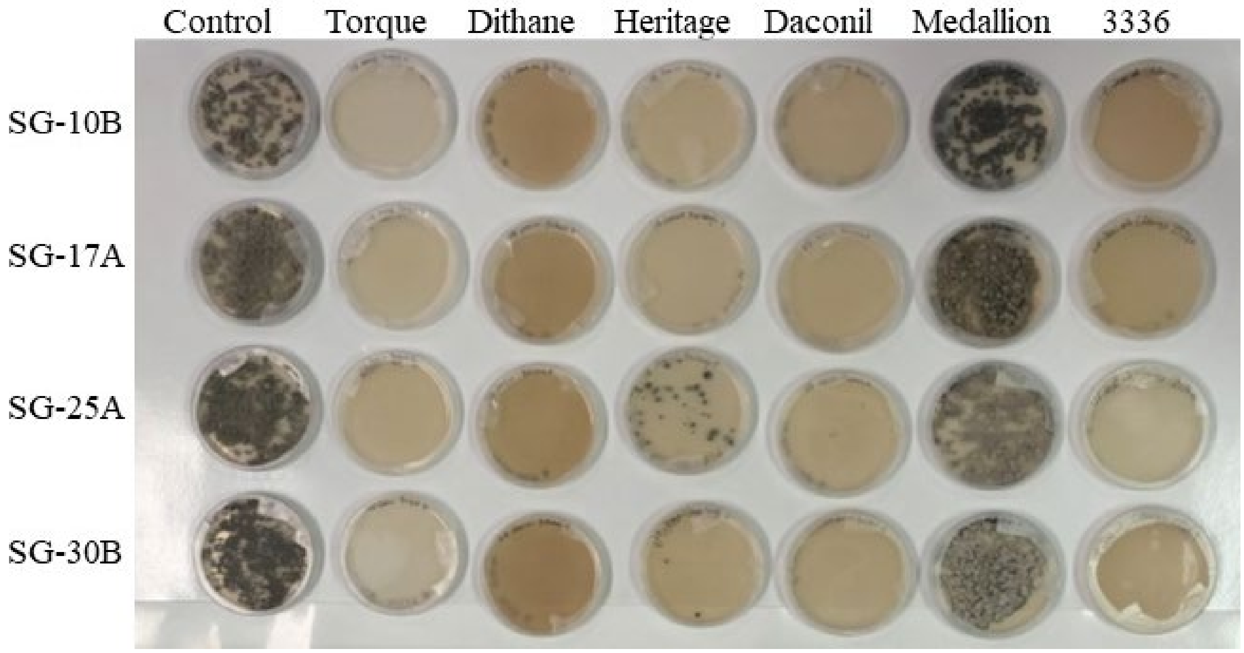

Figure 4.2. Colony growth after incubating 18 days at 25°C under 12 hr light/dark diurnal light cycle for four *Pseudocercospora* sp. isolates recovered from *Liquidambar sytraciflua* (SG-10B, SG-17A, SG-25A, SG-30B) on fungicide-amended and non-amended (control) V8 juice agar media. Isolates were collected from four roadside/landscape areas in TN and GA. Isolate SG-25A was from a city park in Athens, GA.

CHAPTER 5

CONCLUSION

Two main conclusions can be drawn from this study. First, the pathogen causing leaf spots on *Loropetalum chinense* is not the same pathogen causing leaf spots on *Liquidambar styraciflua* in the southeastern US. Based upon the morphology of the isolates recovered from *L. styraciflua* leaf spot symptoms, this pathogen most closely resembles the description for *Pseudocercospora liquidambaricola* (J.-M. Yen) U. Braun. Sequences for five genetic loci for this pathogen will be deposited within the National Center for Biotechnology Information (NCBI) database for use in future evaluations. The leaf spot pathogen recovered from *L. chinense* was morphologically different from the *L. styraciflua* isolates. These isolates more closely resembled the original description for *Cercospora liquidambaris*. However, taxonomically, this species is no longer valid as it has been reclassified to *P. liquidambaricola*. It has been tentatively retained as a separate species until additional evaluations and phylogenetic relationships can be determined for North American *L. styraciflua* herbaria specimens that have conidial characteristics more similar to *Cercospora* or *Passalora* complex than to *Pseudocercospora*. Therefore, additional evaluations are needed to determine if *C. liquidambaris* should be retained as the genus and species for the *L. chinense* leaf spot pathogen, or since the conidia are slightly pigmented with a visible hilum that the *L. chinense* pathogen should be named a distinct species within the genus *Passalora*.

Genetically, the isolates from the two hosts separated into two clades. Additional evaluation is needed to clarify some isolate groupings; however, this should not affect the conclusion that the two isolates are different phylogenetically. Cross-inoculation studies using isolates from each host failed to cause symptoms on the alternative host. Isolates from *L. styraciflua* only caused symptoms when spray-inoculated onto *L. styraciflua* seedlings. Symptoms on *L. chinense* did not develop when plants were spray-inoculated with isolates from either host. Infection of *L. chinense* was only seen when a droplet of conidial suspension from *L. chinense* isolates was applied directly to a detached leaf and incubated for 10-14 days. Additional inoculation trials are needed to confirm the cross-inoculation results from this study.

The second main conclusion from this study was the possible documentation of fungicide resistance development for a foliar pathogen in ornamental plant nurseries. Fungicide resistance has been documented for root pathogens within nurseries in the southeastern US. This study is to our knowledge the first documentation of fungicide resistance to a foliar pathogen in nurseries. Four isolates from *L. chinense* and four from *L. styraciflua* were evaluated in-vitro against six fungicides commonly used against Cercospora leaf spot diseases. All isolates from *L. styraciflua* and one isolate from *L. chinense* that was collected from a 15 year old landscape were sensitive to the fungicides evaluated in this study. Three *L. chinense* isolates recovered from plants growing in nurseries in GA and AL showed reduced sensitivity to the fungicide thiophanate methyl. This was particularly evident for one isolate from a GA nursery. Thiophanate methyl is widely used within ornamental plant nurseries. Resistance to this fungicide has been documented in fruit and agronomic crops. Additional studies are needed to determine the level of sensitivity (EC50 value) for this fungicide since only one fungicide concentration was used in this study. Additional isolates from within the GA nursery and among the other landscape and nursery isolates collected in this study need to be evaluated to determine how widespread thiophanate methyl resistance may be in nurseries. This also could lead to additional studies screening other leaf spot pathogens in ornamental plant nurseries for fungicide resistance.