Identification and Distribution of Fungal Pathogens Associated with Loblolly Pine Defoliation and Tree Mortality in the Southeastern United States

by

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Keywords: Loblolly pine, Needle Defoliation, Fungal Pathogens, Climate Change

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Abstract

Loblolly pine (*Pinus taeda* L.) is a predominant timber species native to the southeastern United States. Since 2016, the species has been experiencing needle defoliation and tree mortality. Symptoms appear as yellowing to progressively browning needles. However, it is uncertain if this disease results from a single or a combination of several fungal pathogens. This study investigated the potential causal agent(s) of this disease as well as pathogen impacts on loblolly pine foliage and foliar chemistry. Climatic regression models were developed to predict loblolly pine defoliation severity in following years.

Based on their colony morphology and molecular ITS-rDNA sequence data, a total of 28 species of fungi representing 17 families were recovered from symptomatic loblolly pine needles from five States. *Lecanosticta acicola*, was repeatedly recovered and identified as the predominant pathogen in Alabama. No sexual stage was observed for *L. acicola* and only the single mating type MAT-1-1 was recovered which suggests low genetic variation. Other pathogens recovered in the study were *Lophodermium* spp., *Coleoporium* spp., *Rhizophaera* spp., and *Diplodia* spp.

Needle pathogen, *L. acicola* had substantial effects on loblolly pine foliage and foliar chemistry. Trees with high incidence produced statistically significantly shorter shoots and needles compared to low incidence trees. Needle nutrient contents of N, S, Na, and B showed a positive correlation with *L. acicola* severity. High incidence trees had an increased level of total phenolics in their needles that correlated to *L. acicola* infection and severity. Seventy trees at

seven long-term monitoring plots showed progressively chlorotic and defoliated crown in Chatom, Alabama, Washington County from 2019 to 2021.

The best 5-factor regression model predicted that the previous year's February, May and June temperature and July and fall precipitation as the best predictors of loblolly pine defoliation severity in following years in Alabama. Increasing summer months temperature and precipitation and decreasing fall months precipitation are expected to favor loblolly pine defoliation severity and spread in following years. Climatic models were developed to aid private landowners and forest managers to monitor, plan and adjust their management strategies accordingly. Brown spot needle blight is an emerging and potentially devastating disease in loblolly pine plantations in Alabama.

Keywords: Loblolly pine, needle defoliation, fungal pathogens, climate change

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Chapter One

Introduction and Review of Literature

1.1 Southern Forestry and Timber Production

The southern timberland covers about 40% of the country's total 521 million acres of timberland. This is the highest share of timberland for any region for which the south is often referred as the "woodbasket" of the United States (Oswalt et al., 2014). Wear (1996) stated that approximately 95% of all forested land in this region is timberland and the average growing stock volume was estimated in 2012 at 41.8 metric tons per acre (Oswalt et al., 2014). Timberland has risen in the south since 1987 and meets 13% of the country's total volume of timber (Smith et al., 2001). Favorable edaphic and climatic characteristics of the southeast promote forest growth very rapidly to reach stand financial maturity in 30-50 years. Timber productivity (60-69%) is mostly concentrated in the coastal plain states from Virginia to Alabama (Wear, 1996). Based on Resources Planning Act Assessment (2012), only 4 million acres of the southern land is reserved forest and 92.9% of land is owned by private industrial owner who manage lands for timber (Oswalt et al., 2014). Primarily, the southeastern United States is a softwood-producing region which comprises about 58% of the growing stock of forest industry land. *Pinus taeda* L. (loblolly pine) and *Pinus echinata* Mill. (shortleaf pine) are the key softwood species in this region and consist of 71% of the softwood growing stock (Smith et al., 2001).

1.2 Loblolly Pine (*Pinus taeda* L.)

1.2.1 Background and biology

Pinus taeda L., commonly known as loblolly pine, is the most productive timber species native to the southeastern United States (Schultz, 1999; Rauscher, 2004). It grows on

approximately 13.4 million ha (29 million acres) in 15 southern and Mid-Atlantic States where it grows naturally along the coastal plain and piedmont zone (Schultz, 1997). Prior to European settlement, loblolly pine was randomly distributed on less than 2 million ha in the southeastern forests. Those forests were converted into agricultural cotton (*Gossypium* spp.) fields during the 1800's after European settlement. But the cotton industry failed to persist in the long term and collapsed in the 1880's due to the introduction of the boll weevil (*Anthonomus grandis grandis* Boheman), which made nearly half of the piedmont a wasteland of abandoned fields. Those open fields were then quickly converted to forests by light-seeded loblolly from the nearby loblolly mixed hardwood stands. Loblolly pine trees were further expanded on the sites where fire control efforts were done in the 1990s (Schultz, 1997).

Loblolly pine is a resilient and productive southern pine. It can easily reproduce and grow rapidly in diverse site conditions and regarded as an ideal species for site restoration and forest management (Schultz, 1997). The species grows with other conifers such as shortleaf pine (*P. echinata* Mill.), longleaf pine (*P. palustris* Mill.), slash pine (*P. elliottii* Engelm.) and with most southern hardwoods. Improved genetics such as easy, quick regeneration and fast-growing performance made loblolly pine dominant on many sites throughout its native range. They can grow in moderately acidic soils with poor surface drainage and thick medium-textured to fine-textured subsoil. They can also grow in moist sites but are prone to hurricanes, and other natural disturbances. Within its physiographic range, they consistently produce prolific seeds along the coastal Atlantic plain. Though growth rate varies between natural and plantation stands, rapid and consistent growth is found for trees in suitable micro-climatic conditions (Baker & Langdon, 1990). However, genetically improved loblolly seedlings tend to have uniform growth in plantation stands. It is one of the most adaptable and successful pine species and has shown its

successful survival, growth, and yield in other continents such as Asia and Africa because of its unique biology (Baker & Langdon, 1990).

1.2.2 Fungal susceptibility and tolerance

Loblolly pine stands have varying degrees of susceptibility to root fungal pathogens (Gandhi et al., 2017). Host-pathogen interactions vary with tree age and site conditions. Older trees (more than 40 years old) are prone to littleleaf disease caused by *Phytophthora cinnamomi* Rands. *Phytopthora cinnamomi* is mostly found in the soil with high clay content, poor drainage capacity and low nutrient availability, specifically phosphorus deficiencies. Consequently, tree growth is hindered by shortening of tree needles, abundant sterile cone production and root death (Roth, 1954). Stands growing on well-drained and coarse-textured soils are more likely to be affected by the fungus *Heterobasidion irregulare* Garbel. & Otrosina which causes Heterobasidion root disease. *Heterobasidion irregulare* produces peak spores during winter period and frequently infects stumps following thinning, then enters into the tree root system causing tree mortality (Stambaugh, 1989). Though loblolly pine is a host to *Cronartium quercuum* f. sp. *fusiforme*, certain families of loblolly pine are resistant to this fungus.

Southern pine decline is a decline disease of loblolly pine caused by a cumulative interaction of factors such as weather stress, inter-specific competition, insects, fungi, anthropogenic disturbances, and previous land management (Eckhardt et al., 2010; Brown & McDowell, 1968), was first described in 1959 in Oakmulgee and Tuscaloosa Ranger Districts in Talladega National Forest in Alabama (Brown & McDowell, 1968). Symptomology of loblolly pine decline includes sparse and chlorotic crowns, low annual stemwood production following mortality of trees within 3 years of symptoms (Hess et al., 1999; Lorio, 1966). Since then, the decline has been documented from Alabama to South Carolina and most notably, several

counties of central Alabama and Georgia (Coyle et al., 2015; Eckhardt et al., 2010). In an inoculation study, Mensah et al., (2020) reported that *Leptographium terebrantis* S. J. Barras and T. J. Perry, a pathogen frequently recovered from pine roots was contributed to tree xylem function reduction, this inoculated phloem lesions, sapwood occlusions and a reduction of tree specific hydraulic conductivity. These physiological and morphological changes coupled with moderate drought exposure were found to cause significant growth decline and tree mortality when inoculated with high levels of *L. terebrantis* when compared to low inoculum levels of *L. terebrantis* (Mensah at el., 2021).

1.3 Tree Foliar Diseases

Foliar diseases can be a serious problem in forest regeneration and nurseries (Singh et al., 2017). Diseases that occur on conifer foliage (Table 1) include leaf spots, blights, needle cast, etc. (Tainter & Baker, 1996). Foliar diseases are principally caused by Ascomycetes. Over 40 species from Ascomycetes cause needle casts and blights in North America (Agrios, 1997). Only the rust fungi are known to cause foliar diseases from Basidiomycetes (Manion, 1991) and species including *Phytophthora* spp. from Oomycetes have been shown to infect foliage (Duran et al., 2008; Dick et al., 2014). Injuries to foliage caused by other biotic agents are possible foliage such as bacteria, viruses, insects, nematodes, mites, phytoplasmas and abiotic agents such as low and high temperatures and air pollutants. (Manion, 1991).

Fungal infection in or on the leaf surface causes reduced photosynthesis and growth resulting in less vigor and increased susceptibility to attack by insects and other fungi. They hinder the normal function of leaves by extracting carbon and nutrients through intercellular haustoria or by the direct penetration of mesophyll and parenchyma cells (Agrios, 1997). Foliar fungal diseases can alter plant competition, which affects their natural assembly. Thus, they

ultimately influence evolution, speciation, and extinction of a species (Parker et al., 2015; Bever et al., 2015). Conifer trees are more seriously damaged by foliar diseases than hardwoods. Long-term defoliation of needles can decrease tree growth because they can't refoliate like hardwoods. In addition, trees become more susceptible when planted out of their native range (e.g., *Pinus radiata* in Chile, New Zealand, and Ecuador) and planted in pure, dense stands (Hansen & Lewis, 1997; Duran et al., 2008; Dick et al., 2014). Symptoms vary from simple localized to total necrosis, discoloration, shriveling of leaves and defoliation (Sinclair et al., 1987). In conifers, heavy infection results in premature defoliation and a thin crown. Fungal fruiting bodies usually develop in necrotic areas.

Weather plays an essential role in the development of foliar disease. Moist and cool weather conditions favor fungal spore release, germination, and infection (Woods et al., 2005). Foliar fungal fitness and spore dispersal are strongly influenced by warming and precipitation (Siebold & Tiedemann, 2013). Temperature and moisture promote fungal growth through increasing mycelium growth, rate of reproduction and dispersal of spores through air currents and rain-splash (Hervell et al., 2002). Warming has a larger effect on fungal diseases than precipitation.

Table 1.1 Foliage diseases of conifers

(Scharpf, 1993; Tainter & Baker, 1996; Agrios, 1997; Hansen & Lewis, 1997; Barnes et al., 2004; Duran et al., 2008; Dick et al., 2014)

Common name	Fungal species	Hosts
Rhabdocline needle cast of Douglas-fir	*Rhabdocline pseudotsugae* *R. weirii*	Douglas-fir
Swiss needle cast of Douglas-fir	*Phaeocryptopus gaeumanii*	Douglas-fir
Lophodermium needle cast	*Lophodermium seditiosum*	Pines
Red Needle Cast (Dano Foliar del Pino)	*Phytopthora pluvialis* *P. pinifolia*	Monterey pine
Larch needle cast	*Meria laricis*	Western & alpine larch, tamarack
Brown spot needle blight	*Lecanosticta acicola*	*Pinus echinata* & 25 other species
Diplodia tip blight	*Sphaeropsis sapinea*	Pines, Douglas-fir rarely
Dothistroma needle blight (Red band needle blight)	*Dothistroma septosporum* *D. pini*	Pines (30 species, especially *P. radiata* & *P. nigra*), hybrids or varieties
Elytrodema needle blight	*Elytroderma deformans*	Ponderosa & Jeffrey pines
Larch needle blight	*Hypodermella laricis*	Western larch
Cedar leaf blight	*Didymascella thujina*	Cedars & incense-cedars
Snow blight	*Phacidium abietis*	True firs & Douglas-fir
Snow molds and brown felt blights	*Herpotrichia coulteri* *H. juniperi*	Pines & other conifers

1.4 Major Conifer Needle Diseases

1.4.1 Brown Spot Needle Blight

Lecanosticta acicola (Thümen) A Sydow (formerly *Mycosphaerella dearnessii*; *Scirrhia acicola*) is a needle pathogen responsible for brown spot needle blight (BSNB) disease of several

conifer species. This pathogen was first described by F. Thümen (1878) in South Carolina, USA (Tainter & Baker, 1996; Adamson et al., 2018). According to Nest et al., (2019), Mesoamerica (Mexico, Guatemala) is a center of diversity for the genus *Lecanosticta* but the origin of *Lecanosticta acicola* is believed to be North America. This fungus is a cosmopolitan pathogen and has been reported in other continents such as Asia (Suto & Ougi, 1998), Africa (Patton, 1997) and Europe (Lavy & Lafaurie, 1994; Jankovsky et al., 2009; Cleary et al., 2019). In Europe and Columbia, this fungus is considered as an A2 quarantine pathogen. Conversely, *L. acicola* has been maintained A1 quarantine status throughout South America, Africa, and Eurasian Economic Union countries (EPPO, 2008).

Lecanosticta acicola has been reported on *P. nigra* (Schwarzkiefer)*, P. sylvestris* L.*, P. halepensis* Mill.*, P. ponderosa* Dougl. ex Laws.*, P. mugo* (bergkiefer)*, P. rotundata* Link. and *P. uncinate* (Ramond) Domin. (Holdenrieder & Sieber 1995; La Porta & Capretti 2000; Diekmann et al. 2002; Jankovský et al. 2009). In Europe, *L. acicola* has jumped into new hosts recently and has been reported from *P. mugo, P. mugo* var. *pumilio, P. ponderosa, P. uncinate* (Adamson, 2015; Cleary et al., 2019; Jankovsky et al., 2009). Symptoms are first expressed as yellowish lesions with a clear border sometimes resin-soaked on infected needles. Round black stromata develop under the epidermis of the needle and later form conidiomata. As the infection progresses, lesions increase in size resulting in necrosis and premature defoliation (Adamson, 2015). Successive defoliation may cause branch death and tree mortality. (Anonymous 2005, 2008, 2015). Mature conidiomata release spores (conidia) causing new infections. Temperature and high moisture such as rain, fog, and dew facilitate long-distance spore dispersal through air currents (Kais, 1975; Tainter & Baker, 1996). Skilling & Nicholls, (1974) reported that the critical lowest temperature for spore release of the BSNB pathogen is 2-3° C. In North America,

BSNB infection can take place throughout the year but the highest sporulation of *L. acicola* occurs in June and August (Kais, 1975).

1.4.2 Dothistroma Needle Blight

Dothistroma septosporum (Dorog.) M. Morelet and *Dothistroma pini* Hulbary are two distinct fungal species associated with Dothistroma Needle Blight (DNB) also known as red-band needle bight (Barnes et al., 2004). *Dothistroma septosporum* has a long taxonomic history arising from two independent origins. In Europe, this fungus was described using two names, *Cytosporina septosporum* Dorog. (Dorogin, 1911), *Septoriella septosporum* (Dorog.) Sacc. (Trotter, 1931). Confusion in the USA occurred when brown spot needle blight fungus, *Lecanosticta acicola* was considered red-band fungus. Gremmen (1968) and M. Morelet (1968) first reported that *D. septosporum* was the pathogen responsible for red-band needle blight both in Europe and the USA. The ongoing debate was resolved by Barnes et al., (2004) who stated that DNB can be caused by two fungal pathogens namely, *D. pini* and *D. septosporum*.

The two Dothistroma needle blight pathogens have a cosmopolitan distribution reported in 76 countries in Eurasia, Oceania, Africa, and the Americas (Drenkhan et al., 2016; Woods et al., 2016). *D. pini* has spread in central and southern Europe as well as North America and occurs in British Columbia, Indiana, North Dakota, South Dakota, Michigan, Minnesota, and Nebraska (Barnes et al., 2004; Barnes et al., 2014). The distribution of *D. septosporum* is wider than *D. pini* and found in Asia, Africa, North America, South America, Oceania and almost every European country (Drenkhan et al., 2016; Barnes et al., 2004). The pathogen was reported to cause damage on more than 82 different conifer species (Watt et al., 2009).

Globally, DNB has devastated many pine plantations across its pine growing region (Barnes et al., 2004). In New Zealand, cost to forest industry due to DNB infection to radiata pines was estimated to be $19.8 million during the first decade of the 21st century (Watt et al., 2011). The fungus enters through stomata and water-soaked lesions start developing on the needles. Black conidiomata surrounded by red band develops at the infection sites and needles become progressively necrotic and cast (Barnes et al., 2004). Infection can cause complete defoliation resulting in stunted growth and mortality (Gibson et al., 1964). The fungus sporulates from April to October and the developing fruiting bodies and sporulation depends on warm and humid weather. Average 15–20°C daily temperature and above 90% relative humidity is optimal for its sporulation, and the fungus can't sporulate when the temperature is below 18^{0}C and relative humidity under 75% (Dvořak *et al.*, 2012). Pine species are highly susceptible to these fungi when they are planted in high rainfall zones. Welsh et al., (2014) reported that August minimum temperature was the most influential climate variable among monthly and seasonally averaged temperature and precipitation variables.

1.4.3 Phytophthora Needle Disease

Phytophthora de Bary (1887) is a genus of plant damaging oomycetes and can cause large-scale economic losses in forestry, agriculture, and horticulture (Erwin & Rebeiro, 1996). According to Brasier (2009), there have been approximately 170 described species in the *Phytophthora* genus. Most of them are destructive plant pathogens causing root rots, collar rots, leaf blight and stem cankers (Goodwin, 1997). *Phytophthora* infections on forest trees were first reported in 1876 in Europe for root and collar rot disease of chestnut trees. Severe mortality of chestnut trees was observed throughout southern Europe due to the spread of *P. cambivora* Petri Buisman and *P. cinnamomi* (Crandall et al., 1945; Peace, 1962). *Phytophthora cinnamomi*, a

serious pathogen associated with dying Chestnut trees, was silently spread across the southeastern United States and isolated from 20 different trees species in nurseries ranging from Pennsylvania and Delaware to Louisiana (Crandall et al., 1945). In 1923, an introduced pathogen *P. lateralis* Tucker and Milbrath was found infecting Port-Orford-Cedar (*Chamaecyparis lawsoniana*) in Washington, USA (Zobel & Hawk, 1980). The pathogen dispersed onto streams, and roads where susceptible Port-Orford-Cedar trees were grown (Hansen et al., 1999).

Since the 1930s, *P. cinammoni* has been reported to cause littleleaf diseases of pine (Hepting et al., 1945). Tree death occurs within 2 to 16 years of first symptoms in the southeastern United States (Cambell, 1948). Poorly drained eroded soil is beneficial for *Phytophthora* spp. and drought invoke their effects. For example, *P. gonapodyides* Hansen is a weak pathogen but exposure to drought allowed the oomycete to kill many bog trees in Alaska. Williams et al., (2014) stated that *Phytophthora* species were not responsible for causing any foliage diseases on pine trees. However, today conifers are at increased risk for new foliage pathogen *P. pluvialis* Reeser, Sutton & Hansen and *P. pinifolia* Duran. In Chili, a needle blight disease caused by *P. pinifolia* is locally known as Daño Foliar del Pino (DFP) resulted in rapid death of needles and subsequent defoliation of trees after successive infection. Mature trees to seedlings and needles to stems are susceptible to *P. pinifolia* (Duran et al., 2008). Similarly, *P. pluvialis* has caused red needle cast disease of *P. radiata* resulted in chlorosis, stunted needles, and reduced needle retention (Dick et al., 2014). Similar symptoms have been reported from the Pacific Northwest where the fungus has been isolated from Douglas fir (Brar et al., 2018). About 55 species in the *Phytophthora* genus has been recognized from 1876 to 1999. Additional 50+ species were recognized and described between 2000 to 2007 (Brasier, 2009). Continued discovery of *Phytophthora* species has increased up to 150 species in *Phytophthora* genus due to

continuous survey of forests and natural ecosystems focusing on distribution and occurrence of exotic species as well as increased knowledge about phylogenetic relationships (Hansen et al., 2012).

1.4.4 Lophodermium Needle Cast

Lophodermium Chevall. is a complex genus comprised of both needle casts pathogens and foliar endophytes on a diverse group of plant hosts (Ortiz-Garcia et al., 2003; Sieber, 1988; Müller & Hallaksela, 1998). In the *Rhytismatacea* family, *Lophodermium* genus is unique for its filiform ascospores and ascocarps that open through a longitudinal slit (Ortiz-Garcia et al., 2003). Globally, approximately 145 *Lophodermium* spp. have been recovered from different plant families (Rajkovic et al., 2013). Over 20 *Lophodermium* species colonize conifer needles and shrubs (Ortiz-Garcia et al., 2003). Sweden, Serbia, Estonia, Finland, Germany, the United States, and Great Britain, are affected by *Lophodermium* disease (Lilja et al., 2010; Nita, 2013; Ostry & Nicholls, 1989; Stenstrom and Ihrmark, 2005; von Rack and Scheidemann, 1987). Some of these fungi are economically important plant pathogens that cause needle cast diseases in nurseries and Christmas tree plantations. The European Black Pine, Red Pine, and Scots Pine are seriously affected by *Lophodemium pinastri* (Shrad.: Fr.) Chev. and *L. seditiosum* Minter, Staley and Millar. Both fungi are pathogenic on *Pinus* spp. and cause browning of needles, premature defoliation eventually disruption of pine tree growth and physiology (Rajkovic et al., 2013).

Until 1978, only *L. pinastri* was thought to be a pathogenic fungus to *Pinus* spp. in *Lophodermium* genus (Minter et al., 1978). However, *L. seditiosum* was also found to be pathogenic in this genus to pine species later and described by (Dewani & Millar, 1990). In Serbia, *Lophodermium* spp. impacted *P. nigra* and *P. sylvestris* seedlings (Rajkovic et al., 2013). *Lophodermium seditiosum* is a serious needle pathogen of pines worldwide, found in both

nurseries and plantations (Bentele et al., 2014). Since the mid-1960s, this pathogen has caused severe damage on millions of red pine species and scotch pine species seedlings as well as Christmas tree plantations in many areas of the United States (Ostry et al., 1989).

1.4.5 Coleosporium Needle Rust

The genus *Coleosporium* Lév. is composed of rust fungi in the *Coleosporiaceae* family (*Pucciniales, Pucciniomycotina*) that includes approximately 100 fungal species that alternate phases between conifers and angiosperms to complete their life cycle (Cummins & Hiratsuka, 2003). This genus is usually macrocyclic and have pycnia, aecia, uredinia, telia and basidia which are subepidermal in the plant tissue. *Colesporium asterum* (Dietel) Syd. & P. Syd. has been reported on *Solidago* (Robert) K. in Korea, Canada, and the United States though their morphological characteristics and 28S rDNA sequences are different (Back et al., 2014). Another rust fungus *Colesporium solidaginis* (Schwein.) Thüm is also found associated with North American *Solidago* spp. (McTaggart & Aime, 2018). Generally, 2 and 3-needled pines are susceptible to *Coleosporium* with the pycnia and aecia appearing on *Pinus* (Pinaceae) (Weir, 1925) and the uredinial and telial stages appear on woody and herbaceous angiosperms mostly infect members of the Asteraceae (Cummins & Hiratsuka, 2003).

Taxonomically, the *Coleosorium* genus is challenging as one species has multiple names based on their host associations. For example, more than 50 synonyms are found for *C. tussilaginis* (Pers.) Lev. based on different hosts in several plant families. Furthermore, urediniospores and aeciospores are morphologically identical even under electron microscopy within and between closely related species (Hiratsuka et al., 1992; Mims and Richardson, 2005). Molecular analysis of the 28S rRNA region and more specific ITS2 region are therefore more

successful in identifying the variation between closely related *Coleosporium* species than using host associations (McTaggart & Aime, 2018).

1.4.6 Sydowia Needle Cast

Sydowia polyspora (Bref. & Tavel) E. Müll. is a foliar endophyte of conifers belonging to Dothideomycetes (Pan et al., 2018) and is considered a pathogen on several conifer hosts (Pan et al., 2018; Ridout & Newcombe, 2018). This fungus can change its role from endophytic to pathogenic under climatic and biotic stressors (Pan et al., 2018). *Sydowia polyspora* was found associated with current season needle necrosis and causing needle necrosis and tan to yellow-colored bands after approximately 2-4 weeks of bud break resulted in significant negative impacts on tree growth and marketability of Christmas trees (Talgø et al., 2010). It also plays a pre-emergent seed pathogen role in forest nurseries and regeneration plantings and affects seedlings performance and the emergence of seedlings of *P. ponderosa* trees (Ridout & Newcombe, 2018). The fungus has a wide geographical range in Europe and North America where it is pathogenic to *Thuja*, *Abies*, *Tsuga*, and *Latrix* families (Talgø et al., 2010). Current season needle necrosis, a serious foliar disease of *Abies* spp. has been reported from Europe (Austria, Germany, Norway, and Denmark) and North America (USA).

1.4.7 Rhizosphaera Needle Cast

Rhizosphaera needle cast (RNC), is a foliar disorder of *Picea* spp. caused by the fungus, *Rhizosphaera kalkhoffii* Bubak (Peace, 1962). This fungus is found in forest plantations, landscapes and natural stands causing needle damage on Colorado blue spruce (*P. pungens* Engelm.), Norway spruce (*P. abies* (L.) H. Karst.) and White spruce (*P. glauca* (Moench) Voss). In Japan, the fungus is considered a weak pathogen of *P. densflora* Siebold & Zucc. especially in areas subjected to drought and SO_2 injury (Chiba & Tanaka, 1968). In Europe, the fungus

considered as a secondary invader is associated with the top dying of Norway spruce trees, a disease primarily due to water shortage in the crown (Diamandis, 1979). However, in the United States, this is a primary pathogen causing premature needle loss, branch mortality and whole tree defoliation (Blake e al., 1990). A recent study found its association with needle damage of white pine in the New England States and reported it as a secondary invader (Broders et al., 2015). Rhizosphaera needle cast was also reported from *P. virginiana* Mill. alongside *Lophodermium* sp. and *Ploioderma* sp. (Blake et al., 1990). Moreover, it was reported to cause needle blight of *Pinus* spp. by Tanaka & Chiba, (1971).

1.5 Detection and Identification of Needle Fungi

1.5.1 Morphological identification

Accurate identification of fungi to the species level is key to basic (ecology, taxonomy) and applied (genomics, bioprospecting) applications of scientific research (Raja et al., 2017). Invasive fungal pathogens have caused large ecological and economic damage to forests. For example, *Cronartium ribicola* J. C. Fisch., a white pine blister rust fungus on *Pinus* spp., was introduced to Canada and the United States in the early 1990s and caused a decline in the North American five-needle pine species (Fisher et al., 2012). Therefore, rapid and robust identification of pathogens is important in biosecurity so as to help prevent the accidental introduction and spread of pathogens around the world (Bergeron et al., 2019).

Various methods are employed for the detection and identification of fungi. One of the more common methods is histological observation, staining the host tissue and directly examining under a microscope to observe fungal structures (Johnston, 2016). A limitation of the histological observation is its sensitivity when fungi colonization is localized and the failure to recognize small fungal structures in pine needle tissue (Schulz & Boyle, 2005). In the past, fungi

have been identified based on their gross morphological features. For example, the Saccardo system, identification of fungi was based on the morphology of sporulation structures and pigmentation of conidia and conidiophores (Barnett and Hunter, 1972).

For studying endophytic fungal diversity, cultivation-dependent techniques have been regularly employed (Rodrigues and Samuels 1990; Guo et al. 2000; Vieira et al. 2011). The method involves a process of surface sterilization of host tissue, isolation of fungi growing from samples, purification, isolation of fungi under various incubation conditions and the subsequent observation of morphological characteristics in culture (Sun & Guo, 2012).

Identification of fungi through morphological features is sometimes challenging due to (1) some pathogens are slow-growing and are outcompeted by fast-growing species (Sun & Guo, 2102) (2) some pathogens can live on plant material as asymptomatic colonizers (3) some pathogens have a lack of discriminant morphological characteristics and (4) the great taxonomic diversity of fungi (Bergeron et al., 2019). Molecular biology brings a new dimension to apply molecular techniques and overcome many of the obstacles of traditional cultivation-dependent methods.

1.5.2 Molecular identification

Since the beginning of the 21st century, fungal identification has become more reliable, quick, and precise with the inclusion of molecular techniques. Starting from DNA extraction to quantitative PCR technologies, effective amplification platforms and prove development assay have added a new route on fungal detection and identification (Tsui et al., 2011). Molecular methods are required for those fungi that are not easily culturable or where slow-growing fungi are easily outcompeted by fast-growing saprophytic or endophytic fungi yielding false inaccurate results (Ioos et al., 2009; Bakys et al., 2009).

Conventional Polymerase Chain Reaction (PCR) is used with single oligonucleotide probes to detect the presence or absence of a specific organism in a sample (White et al., 1990). PCR is a rapid, specific, and sensitive detection tool; thus, it has been widely used in diagnostic laboratories and clinics for identifying both plant and animal pathogens (Reischl & Lohmann, 1997; Ioos et al., 2009). DNA sequences provide ample evidence of genetic information for phylogenetic analysis and have been often used for this purpose. However, DNA sequencing depends on successful genomic DNA extraction from the fungi which is crucial for all molecular methods (Tsui et al., 2011). DNA fragments (e.g., ITS, 28S, and 18S) are amplified by PCR with fungal-specific primers and then run in the denaturing gradient gel electrophoresis for separating PCR products. Subsequent sequencing and phylogenetic analysis enable investigators to identify fungal species colonizing the plant tissue (Sun & Guo, 2012).

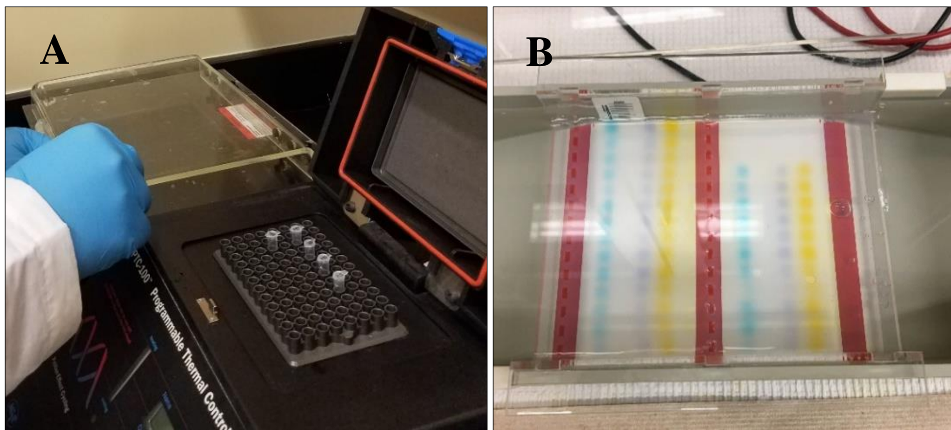


Figure 1.1 Molecular analysis such as (A) Polymerase Chain Reaction & (B) Gel Electrophoresis

1.6 Summary

Loblolly pine defoliation and mortality have become a concern to private landowners and forest managers. Symptomology of loblolly pines mimics needle disease which might emergence

from a single fungal pathogen or a combination of fungal pathogens. However, there is still uncertainty of the causal agents of this disease. This literature is about global major needle pathogens, their damage, life cycle, biology, and ecology with pine trees. This study focus was to identify causal agent(s) of loblolly pine defoliation and mortality with an emphasis on Brown spot needle blight, Phytophthora needle disease, Dothistroma needle blight, Coleosporium needle rust, Lophodermium needle cast, Sydowia needle cast and Rhizosphaera needle cast. Identification of causal agent(s) is crucial to implement disease-based management in the affected stands and take quarantine measurements to prevent the further spread of the disease. This would minimize economic losses associated with loblolly pine defoliation and tree mortality in the affected stands.

Chapter Two

Isolation and identification of fungi associated with loblolly pine defoliation and mortality in the southeastern United States

Abstract

Loblolly pine is an economically important timber species in the southeastern United States. Over the past five years, loblolly pine stands have been observed with defoliation followed by stunted growth and tree mortality. Symptoms first appear as chlorosis following rapid necrosis of needles eventually resulting in complete tree defoliation and death. In an effort to determine the cause, this study investigated the potential causal agent(s) of loblolly pine defoliation and tree mortality in the southeastern United States. A total of 47 stands was sampled in an attempt to isolate and identify the causal agents of loblolly pine defoliation. Based on colony morphology and ITS-rDNA sequence data, a total of twenty-eight fungal species representing seventeen families were recovered from symptomatic loblolly pine needles. *Lecanosticta acicola* was repeatedly recovered (64%) from infected stands in Alabama. Other common needle cast, rust and tip blight fungi such as *Sydowia* spp. (60%), *Rhizosphaera* spp. (36%), *Coleosporium* spp. (9%), *Lophodermium* spp. (28%), and *Diplodia* spp. (6%) were recovered at one or few sites or recovered in a combination with *L. acicola* pathogen. *Lecanosticta acicola* was confirmed using morphological similarity and amplification of the internal transcribed spacer (ITS) region and the translation elongation factor (TEF) 1-alpha gene of interest. No sexual stage was observed for *L. acicola* and only the single mating type MAT-1-1 was tested positive in Alabama. This suggests that there is a less genetic diversity of *L. acicola* in Alabama. Brown spot needle blight is an emerging disease in Alabama.

Keywords: Loblolly pine, needle defoliation, fungal pathogens, brown-spot, needle blight, needle cast

2.1 Introduction

Climatic factors such as increasing temperature and changing precipitation have potential influences on fungal disease emergence and spread in plant communities (Launay et al., 2014; Wood at el., 2005). Warming and precipitation are expected to increase pathogen fitness and transmission by increasing growth rates and spore production, promoting mycelium growth, and extending the lengths of growth and reproduction times (Harvell et al., 2002). Changing interactions between the environmental factors and biotic agents can drive disease outbreaks (Wyka et al., 2017; Agrios, 2005) and may result in changing disease impacts. For example, water-stressed trees have been shown to be affected in the region where precipitation is reduced (Sturrock et al., 2011a).

Environmental conditions affect pathogen virulence, host susceptibility, and can modify interactions between the host and the pathogen (Agrios, 2005). For instance, the number of rainfall days between summer to winter and rainfall intensity have favored *Phytophthora* spp. in Central Europe and are expected to have bigger impacts on forest ecosystems that are dominated by susceptible *Fagus*, *Quercus*, *Alnus*, *Abies*, *Acer* and *Pinus* species (Jung, 2009). Yet, the relationship between climate change and its impact on disease intensity and severity depends on the pathosystem and the environment it happens (Sturrock et al., 2011b).

Fungi associated with needle diseases tend to be more sensitive to moisture as water promotes the rate of reproduction, fungal spread, and infection (Stone et al., 2007). Similarly, increasing temperatures alters fungal virulence by affecting growth and reproduction regardless

of their host (Harvell, 2002). Since 1960s, large-scale mortality of trees reported in Chile, New Zealand, and Africa were found to be associated with *Dothistroma septosporum*, a fungus responsible for Dothistroma needle blight, with increasing temperatures and shifting precipitation patterns (Brown & Webber, 2008; Sturrock et al., 2011b; Woods et al., 2005). Similarly, Swiss needle cast, caused by the fungus *Nothophaeocryptopus gaeumannii* (T. Rohde), occurrence in the Pacific Northwest was found to be positively correlated with increasing average winter temperatures and spring precipitation (Manter et al., 2005). Increasing *Phytophthora* foliar disease has been observed on water-stress forest trees (Jactel et al., 2012). Climatic factors favor needle pathogens, which in turn, limits water and nutrient availability of trees followed by less carbon stocks and forest productivity (Hicke et al., 2012).

In plantation forestry, threats are often imposed by the unintentional introduction of pests and pathogens (Wingfield et al., 2001). For example, *Phytophthora pluvialis* appeared suddenly in New Zealand in 2005 ((Dick et al., 2014) and *P. pinifolia* in Chile since February 2004 (Durán et al., 2008). Both fungi have caused large scale mortalities in exotic *Pinus radiata* (D Don) plantations in Chile and New Zealand and thought to be introduced from North America. Since 2016, loblolly pine has been experiencing repeated defoliation in Chatom, Alabama, Washington County. Symptoms first appear as chlorosis following rapid necrosis of needles eventually full crown defoliation and death. Second-and third-year needles are infected which resulted in tree crowns looking sparse after repeated defoliation. The objective of this study was to isolate and identify the causal agent(s) associated with loblolly pine defoliation and tree mortality (LPDM) in Alabama, Georgia, South Carolina, Mississippi and Louisiana.

2.2 Materials and Methods

2.2.1 Study area and plant material collection

Seven permanent plots in Chatom, Alabama, Washington County were established in 2019 (Table 2.2). The climate of Chatom has an average air temperature from 61°F to 70°F and is rarely below 24°F or above 97°F. The average annual precipitation is 57 to 69 inches (Weatherspark.com). Poorly drained stands composed of Tibbie soils (40%), Pinebarren soils (35%) and 5% of other minor components. (Web Soil Survey). From 2019 to 2021, needle sampling was done once per month from March to November based on the sporulation period of five needle pathogens (Table 2.1). From seven experimental plots, five trees were sampled each time based on the expression of symptoms such as chlorosis, rapid necrosis and defoliation. GPS coordinates were also collected to locate the stands. Permanent plots were composed of 3 to 16 year old trees including 2004, 2009, 2010 and 2012 plantations. Destructive sampling was performed to collect needle samples from trees. To obtain upper and lower crown needles, a 0.22 mag caliber rifle was used to shoot foliage samples out of the tree. Among them, only 2004 plantations receive fertilization after 2-3 years of planting. Otherwise, no silvicultural treatments were applied to the rest of the sampled plots. Genetics of the seedlings were unknown.

Table 2.1 Sporulation period of five needle pathogens

Needle Disease	Causal Agent	Host	Sporulation Period
Phytophthora needle blight	*Phytophthora* spp.	Pines, Oak, Douglas fir,	May to November
Brown spot needle blight	*L. acicola*	Over 53 different pine species	March to October

Dothistroma needle blight	*D. septosporum* *D. Pini*	82 *Pinus* taxa	Late summer to fall (August-October)
Lophodermium needle cast	*L. seditiosum* *L.* spp.	Scots, Austrian and Red pine	Late summer (August-September)
Coleosporium needle rust	*Coleosporium* spp.	2 or 3-needled Pines	Spring (March-May)

2.2.2 Survey area and plant material collection

In addition to permanent sample plots, plots of various site conditions ranging from 3 to 32 year old plantations were assessed for defoliation and mortality and sampled. Private landowners and industry collaborators were sent a one-page information sheet about needle mortality and a collection guide (Appendix B) and instructed to send in infected needle samples (Munck & Burns, 2012). The study also scheduled a sampling visit to the infected stands based on needle disease incidence reports and collected needle samples and information about the stands. The sampling was conducted during the sporulation period of needle pathogens based on the disease similarity such as Dothistroma needle blight, Brown spot needle blight, Phytophthora needle blight, Lophodermium needle cast or Coleosporium needle rust (Table 2.1). Needle samples were collected from infected stands between March and November when landowners recognized symptoms and contacted the Forest Health Dynamics Laboratory. Information for each stand such as GPS coordinates (latitude and longitude of the stand), height (in meter), DBH (diameter at breast height in cm), stand type, site description, soil properties and visual crown rating was intended for later analyses.

Table 2.2 Descriptive data for sampled stands surveyed from 2019 to 2021 in the southeastern United States.

State	County	Stand type	Site description	Soil Properties	Latitude	Longitude
*Alabama	Washington	Plantation	Wet area	Sandy loam	31.2729	-88.3094
*Alabama	Washington	Plantation	Edge of water	Sandy loam	31.2725	-88.3094
*Alabama	Washington	Plantation	Wet area	Sandy loam	31.2719	-88.3098
*Alabama	Washington	Plantation	Wet area	Sandy clay loam	31.2649	-88.3169
*Alabama	Washington	Plantation	Wet area	Sandy clay loam	31.2607	-88.2937
*Alabama	Washington	Plantation	Wet area	Sandy clay loam	31.2395	-88.2807
*Alabama	Washington	Plantation	Wet area	Sandy clay loam	31.2422	-88.2769
South Carolina	Hampton	Natural	N/A	Silty clay	33.8361	-81.1637
Alabama	Bibb	Plantation	N/A	Sandy	33.1388	-87.1820
Alabama	Butler	Plantation	Flat Plain land	Sandy clay loam	34.8526	-82.3941
Alabama	Colbert	Plantation	Edge of water	Sandy	34.8267	-87.6128
Alabama	Crenshaw	Plantation	Edge of water	Sandy	31.6671	-86.2641
Alabama	Macon	Plantation	Flat plain land	Sandy	32.3731	-85.6846
Alabama	Walker	Plantation	N/A	Sandy	33.0154	-87.0099
Alabama	Bullock	Plantation	N/A	Sandy	32.0574	-85.7256
Alabama	Colbert	Natural	Edge of water	Clay loam	34.8192	-87.6119
Alabama	Elmore	Plantation	Steep slope	Sandy	32.6153	-86.0498
Alabama	Madison	Plantation	Flat plain land	Sandy	34.5672	-86.3605
Alabama	Crenshaw	Plantation	Wet area	Clay loam	31.6671	-86.2641
Alabama	Lamar	Plantation	Wet area	Sandy loam	34.0469	-88.1839

Alabama	Lamar	Natural	Flat plain land	Sandy loam	33.8179	-88.1429
Alabama	Lamar	Natural	Dry steep slope	Sandy loam	33.8737	-88.1808
Alabama	Cullman	Plantation	Flat plain land	Sandy loam	34.1301	-87.0793
Alabama	Cullman	Plantation	Edge of water	Sandy loam	34.1292	-87.0782
Alabama	Cullman	Plantation	Edge of water	Sandy loam	34.1139	-87.0783
Alabama	Cullman	Plantation	Steep slope	Sandy loam	34.1263	-87.0816
Alabama	Cullman	Plantation	Flat plain land	Sandy loam	34.1279	-87.0774
Alabama	Pickens	Plantation	Edge of water	Sandy	33.3341	-88.0901
Alabama	Wilcox	Plantation	Wet area	Sandy loam	32.1282	-87.4203
Alabama	St. Clair	Plantation	N/A	Silty loamy	33.8338	-86.2124
Alabama	Butler	Plantation	Wet area	Sandy Clay Loamy	31.7115	-86.4233
Alabama	Butler	Plantation	Wet area	Sandy Clay Loamy	31.7118	-86.4441
Alabama	Butler	Plantation	Wet area	Sandy Clay Loamy	31.7116	-86.4444
Alabama	Greene	Plantation	Wet area	Sandy clay loamy	31.7114	-86.4446
Georgia	Upson	Plantation	Steep slope	Sandy	32.8029	-84.3105
Georgia	Upson	Plantation	Steep slope	Sandy	32.8001	-84.1547
Georgia	Ware	Plantation	Steep slope	Sandy	31.1344	-82.4753
Georgia	Camden	Plantation	Flat plain land	Sandy	30.8983	-81.6035
Mississippi	Noxubee	Plantation	Flat plain land	Sandy loam	33.2451	-88.5642
Mississippi	Noxubee	Plantation	Wet area	Clay loam	33.1331	-88.1931
Mississippi	Kemper	Plantation	Flat plain land	Clay loam	33.2481	-88.3346
Mississippi	Kemper	Plantation	Flat plain land	Silty loam	33.2481	-88.3346
Mississippi	Lauderdale	Plantation	Steep slope	N/A	32.5738	-88.8298

Mississippi	Clarke	Plantation	Steep slope	Sandy	31.9654	-88.6579
Mississippi	Clarke	Plantation	Flat plain land	Sandy loam	32.0042	-88.6979
Mississippi	Greene	Plantation	Edge of water	Sandy	31.1979	-88.4847
Mississippi	Greene	Plantation	Edge of water	Sandy	31.3153	-88.4441
Louisiana	Natchitoches Perishes	Plantation	Dry steep slope	N/A	31.6801	-93.1780

N.B. Asterisk (*) sites are permanent sample plots and others are survey plots based on the random reports from private landowners and industry collaborators.

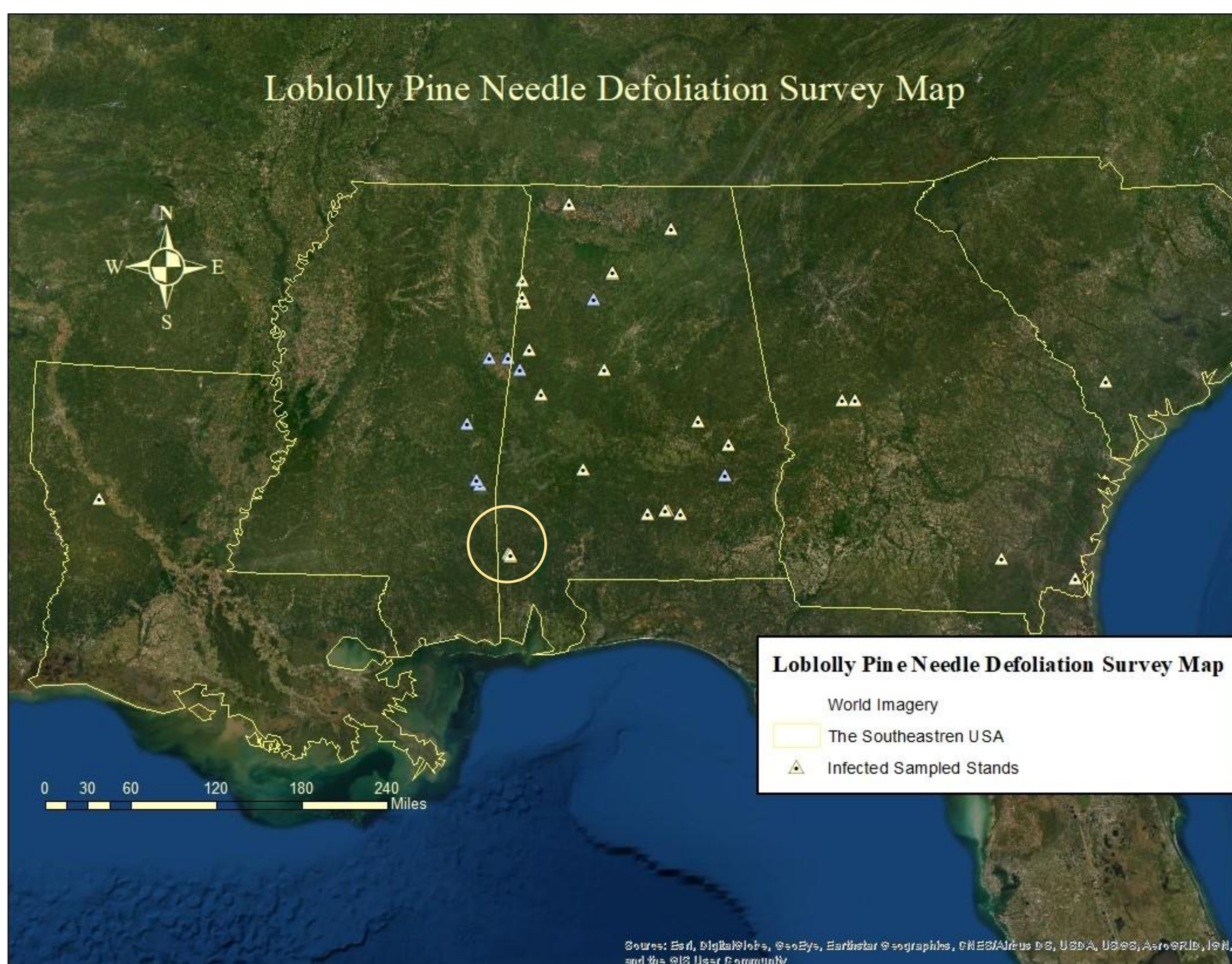


Figure 2.1 Map showing sampled stands in South Carolina, Georgia, Alabama, Mississippi, and Louisiana from 2019 to 2021 from surveys and the permanent study area. Circled stands indicate permanent sampled plots in Chatom, Washington County, Alabama.

2.2.3 Media and fungi isolation

To recover fungi from needle samples and induce sporulation from those samples, there were eight agar media were used in the study. Modified cornmeal agar (CMA-PARP) and V8 agar were used to recover *Phytophthora* species, dothistroma media was selected to recover *dothistroma* species, 2% malt extract and acid potato dextrose agar were used to recover *Lecanosticta* species in the study (Table 2.3).

Table 2.3 List of agar growth media used in the study

Growth media	Basal Ingredients	Amount	Amendments	Amount	References
CMA-PARP	Difco Cornmeal Agar	17 g	Pimaricin (50%)	10 mg	Jeffers and Martin, 1986 Ferguson and Jeffers, 1999)
	Distilled Water	1000 ml	Rifamycin-Sodium Salt	66.7 mg	
			PCNB (75%)	50 mg	
			Hymexazol (70%)	71.4 mg	
V8 Agar	Clarified V8 Concentrate	50 ml	β-sitosterol	0.03 mg/ml	Jeffers, 2006
	Difco Bacto Agar	15 g			
	Distilled Water	950 ml			
2% Malt Extract Agar	Malt Extract	20 g			Barnes et al., 2004
	Agar	15 g			
	Distilled Water	1000 ml	Streptomycin	100 g/L	
Dothistroma Medium	Malt Extract	50 g			Bradshaw et al., 2000
	Nutrient Agar	23 g			
	Distilled Water	1000 ml			

2% Dothistroma Sporulating Medium	Malt Extract	20 g	Streptomycin	100 g/L	Bradshaw et al., 2000 Nest et al., 2019
	Yeast Extract	5 g			
	Agar	15 g			
	Distilled Water	1000 ml			
Fresh Pine Needle Agar	Pine Needle Extract	500 ml			Luchi et al., 2007
	Agar	30 g			
	Distilled Water	1000 ml			
Modified Ground Pine Needle Agar	Ground pine needle	100 g			Luchi et al., 2007
	Agar	30 g			
	Distilled Water	1000 ml			
Acid Potato Dextrose Agar	Potato Dextrose Agar	39 g			Wyka, et al., 2015
	Concentrated Lactic Acid (85%)	1 ml			
	Distilled Water	1000 ml			

2.2.4 Isolation of fungi from loblolly pine needles

Needle samples collected from the plots and submitted to the lab were split into 3 subsamples: one for plating on growth media, one for moisture chamber sporulation and one for DNA extraction. These samples were processed and analyzed in the Forest Health Dynamics Laboratory following above mentioned fungal recovery methods for fungal identification and confirmation.

Plating: Needles were cut into 1-2 cm pieces and surface sterilized with 10% NaOCl (sodium hypochlorite) solution for 1 minute followed by 70% ethanol for another 1 minute and 2 subsequent washes for 1 minute each with distilled water. Surface-sterilized needles were

transferred to a sterile filter paper and blotted dried under a laminar flow hood for 20 minutes (Aboshosha et al., 2007). Needles (four needle pieces per Petri plates) were then placed onto the range of growth media (Table 2.3) and 5 replications per media per tree sample were followed. Petri plates were incubated at ambient temperature and light and regularly checked for fungal growth. Once growth was observed, hyphal tips and conidial heads were transferred to fresh 2% MEA media plates (Guo et al., 1998; Drenkhan et al., 2016). For long-term storage, pure cultures were maintained on MEA slants and stored at -4°C in the Forest Health Dynamics Laboratory collection in the School of Forestry and Wildlife Sciences at Auburn University.

Moist chamber: Symptomatic needles were placed in a moist chamber made up of a glass Petri plate, filter paper (Whatman) and ~500 uL of distilled H_2O. In every moist chamber, 2-3 fascicles were incubated and total five moist chamber were incubated for each sample tree. Spores recovered on the needles were examined under a compound microscope and transferred onto a 2% MEA media for DNA extraction (Broders et al., 2015).

Direct DNA Extraction: Third subsample was cut into 2-3 mm pieces and kept in a refrigerator at 4°C until freeze drying. DNA extraction process from freeze dried needles is described in section 2.2.5.

2.2.5 DNA extraction

Hyphal tips from each of the pure cultures recovered from symptomatic needles were transferred into liquid MEA media and incubated at 25°C for 2 to 3 weeks. Liquid media were centrifuged (Thermo Scientific Sorvall BIOS 16 centrifuge) at maximum speed (≤16,000 rpm) for 10 minutes. The liquid suspension was discarded and fungal pellets were transferred to a lyophilizer and kept overnight at -55^{0}C. The genomic DNA was extracted from dried mycelium using a Phenol-chloroform method (Comey et al., 1994).

Fungal tissues were homogenized using liquid nitrogen followed by lysis buffer. Suspension was transferred into a new 1.5 mL microcentrifuge tube, proteinase K (2-3 µL, 100 ng/µL) added, and kept at 55ºC in a water bath for 15 minutes. Ammonium acetate (NH_4AC, 250 µL) was added to the microtube which was then placed into ice to precipitate histone proteins for DNA denaturation. Next 750 µL phenol: chloroform: isoamyl alcohol (25:24:1) was added to the suspension and vortexed for 5 minutes. Suspensions were added to 2-3 uL (100 ng/ µL) RNase A. Two subsequent additions of 750 µL chloroform: isoamyl alcohol (24:1) and separation of suspensions followed. Finally, sodium acetate NaAc (120 mL) and 100% Ethanol (880 mL) were added to the suspension and kept in the refrigerator at 4ºC for 30 minutes to precipitate the fungal DNA. The supernatant was discarded and 50 µL of molecular water was added to suspend DNA (Comey et al., 1994).

Total DNA was also extracted directly from symptomatic needle samples collected from loblolly pine. Needles cut at 2-3 mm were ground with liquid nitrogen. Each sample consisted of 5-100 mg of ground needles to extract DNA. DNA extraction was followed by DNeasy Plant Pro Mini Kit instructions. As per DNeasy Plant Pro Mini Kit, CD1 buffer was decreased from 500 uL to 400 uL and 50-100 uL PS buffer was added for highly symptomatic samples (Barnes et al., 2008). The rest of the procedures followed as described by DNeasy Mini Kit's instructions without any modification.

2.2.6 Polymerase Chain Reaction (PCR)

Internal transcribed spacer (ITS) regions were amplified using universal primers ITS1 and ITS4 (White et al., 1990). The thermal cycling reaction was carried out in an MJ Research PTC-100. PCR amplification was run at 25 uL reaction volume made up of 1uL of template DNA, 1uL of each primer pair, 12.5 uL Green Master Mix (GMM) and 9.5 uL nuclease-free water. The

reactions were as follows; initial denaturation of 95°C for 2 minutes, annealing 56°C for the 30S of primer pairs, 72°C for 2 minutes, and 39 cycles were performed each time for maximum amplification. PCR products were kept in a molecular refrigerator for further analysis. However, PCR cycling conditions were varied at least for annealing temperature for other sets of primers used in the study (Table 2.4). PCR purification was conducted using E.Z.N.A purification kit. DNA concentration was maintained each time between 18 ng/uL to 100 ng/mL (Drenkhan et al., 2016).

Table 2.4 List of primers used in this study

Marker name	Forward or Reverse	Sequence (5' to 3')	References
ITS1	Forward	TCCGTAGGTGAACCTGCGG	White et al., 1990
ITS4	Reverse	TCCTCCGCTTATTGATATGC	White et al., 1990
LAtef-F	Forward	GCAAATTTTCGCCGTTTATC	Ioos et al., 2009
LAtef-R	Reverse	TGTGTTCCAAGAGTGCTTGC	Ioos et al., 2009
Rust ITS1-F	Forward	GAAGTAAAAGACGTAACAAGGT	McTaggart & Aime, 2018
Rust ITS2-R	Reverse	CACCTGATTTGAGGTCTTAAAA	McTaggart & Aime, 2018
LRust1R	Forward	TAAGACCTCAAATCAGGT	Beenken et al., 2017
LR6	Reverse	CGCCAGTTCTGCTTACC	Beenken et al., 2017
FM35	Forward	CAGAACCTTGGCAATTAGG	Martin, 2000; Durán et al., 2008
FM58	Reverse	CCACAAATTTCACTACATTGA	Martin, 2000; Durán et al., 2008
DStub2	Forward	CGAACATGGACTGAGCAAAAC	Ioos et al., 2009
DStub2	Reverse	GCACGGCTCTTTCAAATGAC	Ioos et al., 2009
DPtef	Forward	ATTTTTCGCTGCTCGTCACT	Ioos et al., 2009
Dtef	Reverse	CAATGTGAGATGTTCGTCGTG	Ioos et al., 2009
MdMAT1-1F	Forward	CGCATTCGCACATCCCTTTGT	Janoušek et al. 2014

MdMAT1-1R	Reverse	ATGACGCCGATGAGTGGTGCG	Janoušek et al. 2014
MdMAT1-2F	Forward	GCATTCCTGATCTACCGTCT	Janoušek et al. 2014
MdMAT1-2R	Reverse	TTCTTCTCGGATGGCTTGCG	Janoušek et al. 2014

2.2.7 DNA sequencing and phylogenetic analyses

Purified PCR products were sent to Laragen Inc. Biotechnology Company (Virginia Ave, Culver City, California) to sequence fungi. Raw sequence editing was done manually in BioEdit software where forward and reverse sequences were adjusted. Fungal sequences were identified based on their sequence similarity with fungi stored in NCBI GenBank. Edited sequences were deposited at Gen Bank and BankIt for future reference. Other closely related species and outgroups of fungi were imported from NCBI GenBank to serve as known reference sequences for comparison to the distance and character-based relation of fungi recovered in the study. MEGA version 4.0 software was used to develop phylogenetic trees and subsequent molecular analyses. Sequence alignments were created using the ClustalW tool in MEGA where ambiguous sites were ignored, and gaps were counted as missing data. Both neighbor-joining (NJ) and maximum likelihood (ML) methods were performed to construct phylogenetic trees. Following that, 1000 bootstrap applications were performed for NJ analysis to increase statistical confidence and the relative support for the branches (Broders et al., 2015).

2.3 Results

2.3.1 Morphologic and genetic diversity of fungi associated with loblolly pine defoliation and tree mortality

A total of 7 permanent plots and 40 private stands were sampled to identify pathogens associated with loblolly pine defoliation and tree mortality in Alabama, Georgia, South Carolina, Louisiana and Mississippi. Most (43 of the stands) were privately owned plantation forests. Soil

conditions of the sites were sandy to sandy loam and sandy clay loam to silty loam (Table 2.2). Stands were located at wet areas or near the edges of water bodies (within 1/2 mile) and at deep steep slopes. Needle samples were taken from a total of 282 trees and overall 2820 incubated needles and 1410 branch tips were examined from 2019 to 2021.

The fungal pathogens *L. acicola*, *S. polyspora*, *R. kalkhoffii*, *Lophodermium* spp., *D. sapinea*, and *Coleosporium* spp. were recovered from stands at 64%, 60%, 36%, 28%, 6%, and 9% respectively (Figure 2.2). *Lecanosticta acicola* was recovered at eleven sites in Alabama (Washington, Wilcox, Elmore, Butler, Crenshaw, Pickens, Lamar, Cullman, Colbert, Madison, and Greene) and one site in Mississippi (Greene). *Sydowia Polyspora* and *R. kalkhoffii* were recovered in Alabama in combination with *L. acicola*. *Lophodermium* spp. were recovered in Alabama (Bibb, Walker) and Georgia (Ware and Camden). *Diplodia sapinea* was recovered in South Carolina (Hampton) and Georgia (Upson). *Coleosporium* spp. was recovered in Louisiana (Natchitoches perishes) and Alabama (Colbert and Macon) (Figure 2.5).

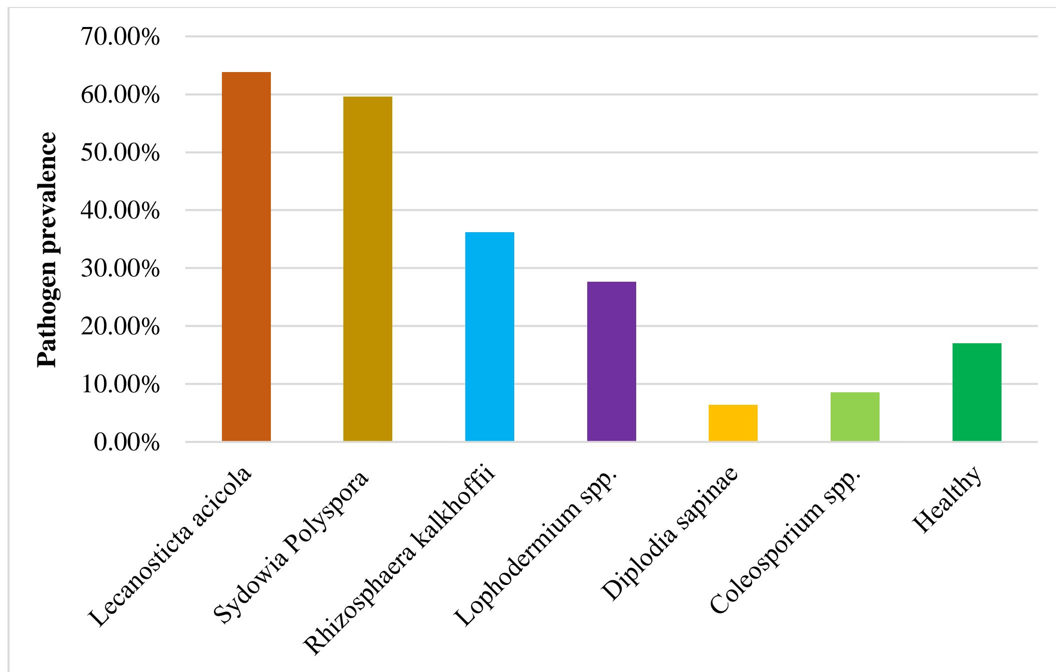


Figure 2.2 Pathogen prevalence by stands of *Lecanosticta acicola*, *Sydowia polyspora, Rhizosphaera kalkhoffii*, *Lophodermium* spp., *D. sapinae*, *Coleosporium* spp. and healthy sites.

Signs and symptoms for *L. acicola* were most frequently observed in a combination with *S. polyspora, R. kalkhoffii, Lophodermium* spp., and *Coleosporium* spp. in Alabama. *Lecanosticta acicola* produced hard small black fruiting bodies consisting of brown and banana-shaped spores. *Lophodermium* fungi were identified by their football-shaped black conidiomata that developed on the upper and lower portions of the infected needles. *Diplodia sapinea* formed brown to black circular spots and irregular sizes fruiting bodies which were densely recovered at the tip and base of the needles. Only *Coleosporium* spp. produced white aecia in the diseased needles (Figure 2.3).

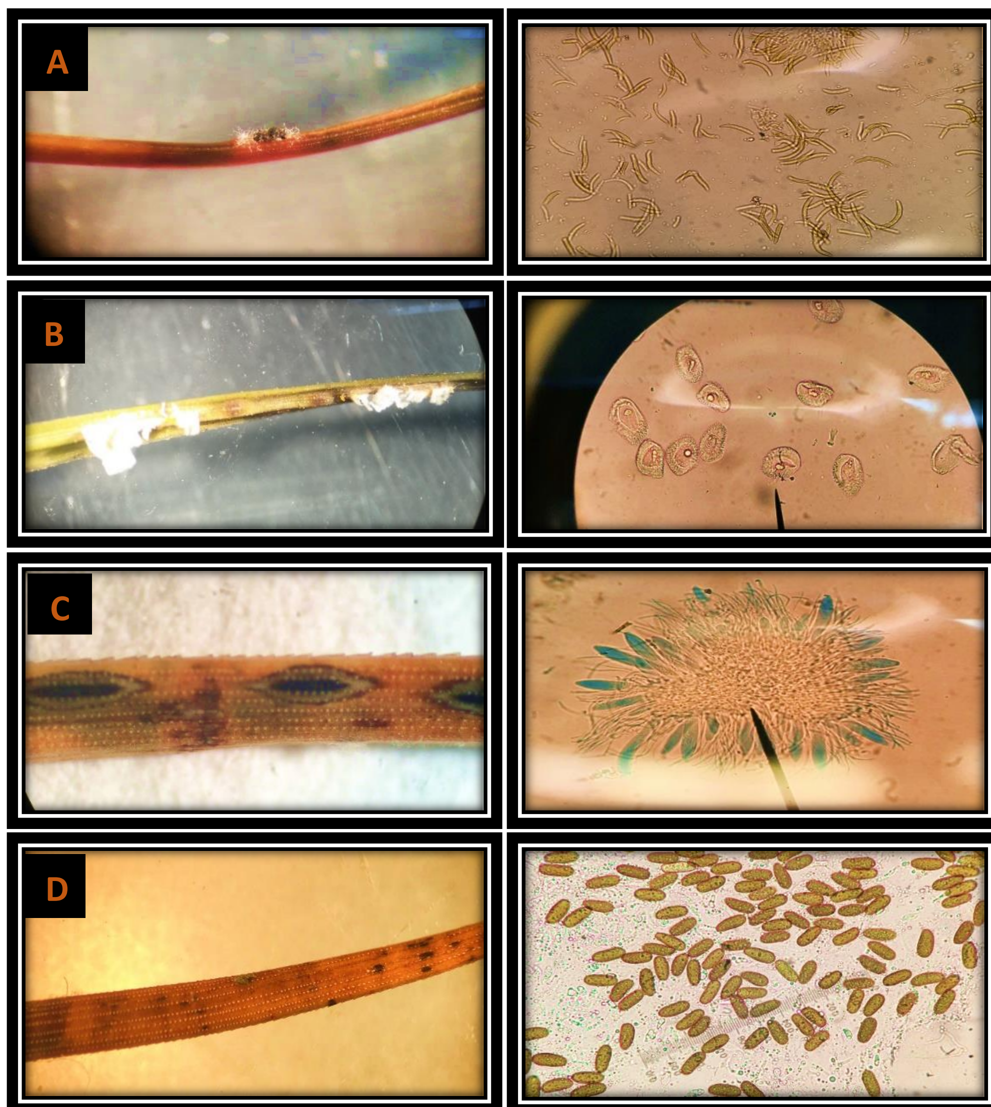


Figure 2.3 Expression of disease symptoms and reproductive structures of dominant fungal pathogens such as (A) *Lecanosticta acicola* (B) *Coleosporium* sp. (C) *Lophodermium* sp. and (D) *Diplodia sapinea* in the infected loblolly pine needles.

A diverse group of fungi were isolated from symptomatic needles and identified based on colony morphology and ITS-rDNA sequence data. A total of six pathogens were recovered from symptomatic needles (Figure 2.2). *Lophodermium* spp. and *Coleosporium* spp. were recovered at 6% and 2% stands respectively. Otherwise, pathogens were recovered in the study as a combination of two or three fungal species. For example, *L. acicola* and *S. polyspora* were

recovered at 19% of stands followed by *L. acicola* and *Lophodermium* spp. at 13% trees. *Lecanosticta acicola* and *Coleosporium* spp. combinations were found at 4% of stands and *Lophodermium* spp. and *Coleosporium* spp. from 3% of stands. *Diplodia sapinea* and *Lophodermium* spp. were recovered at 9% of stands in the study. Based on stand prevalence i.e., disease severity, pathogens were occasionally recovered as a combination of three fungal species such as *L. acicola*, *S. polyspora* and *R. kalkhoffii* were recovered at 25% of stands followed by *L. acicola*, *S. polyspora* and *Lophodermium* spp. were recovered at 13% of stands (Figure 2.4). Additionally, other endophytic and saprophytic fungi were recovered in the study. The distribution map demonstrates the fungal pathogen distribution in Alabama, Georgia, Mississippi, South Carolina and Louisiana (Figure 2.5).

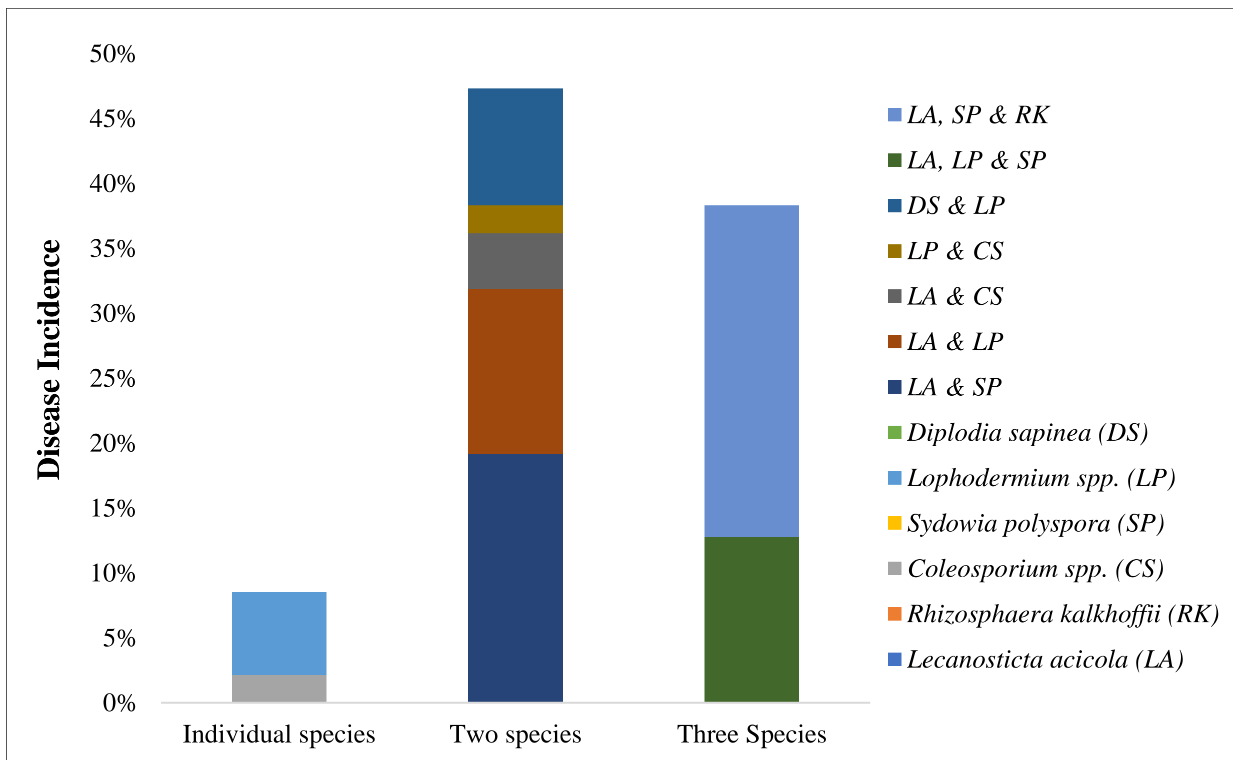


Figure 2.4 Fungal prevalence of individual species and species in combinations (one to three species present) of *Lecanosticta acicola* (LA), *Sydowia Polyspora* (SP) *Lophodermium sp.* (LP), *Rhizosphaera kalkhoffii* (RK), *Coleosporium* sp. (CS), and *Diplodia sapinae* (DS), as a percent of a total of 47 infected stands in the southeastern United States.

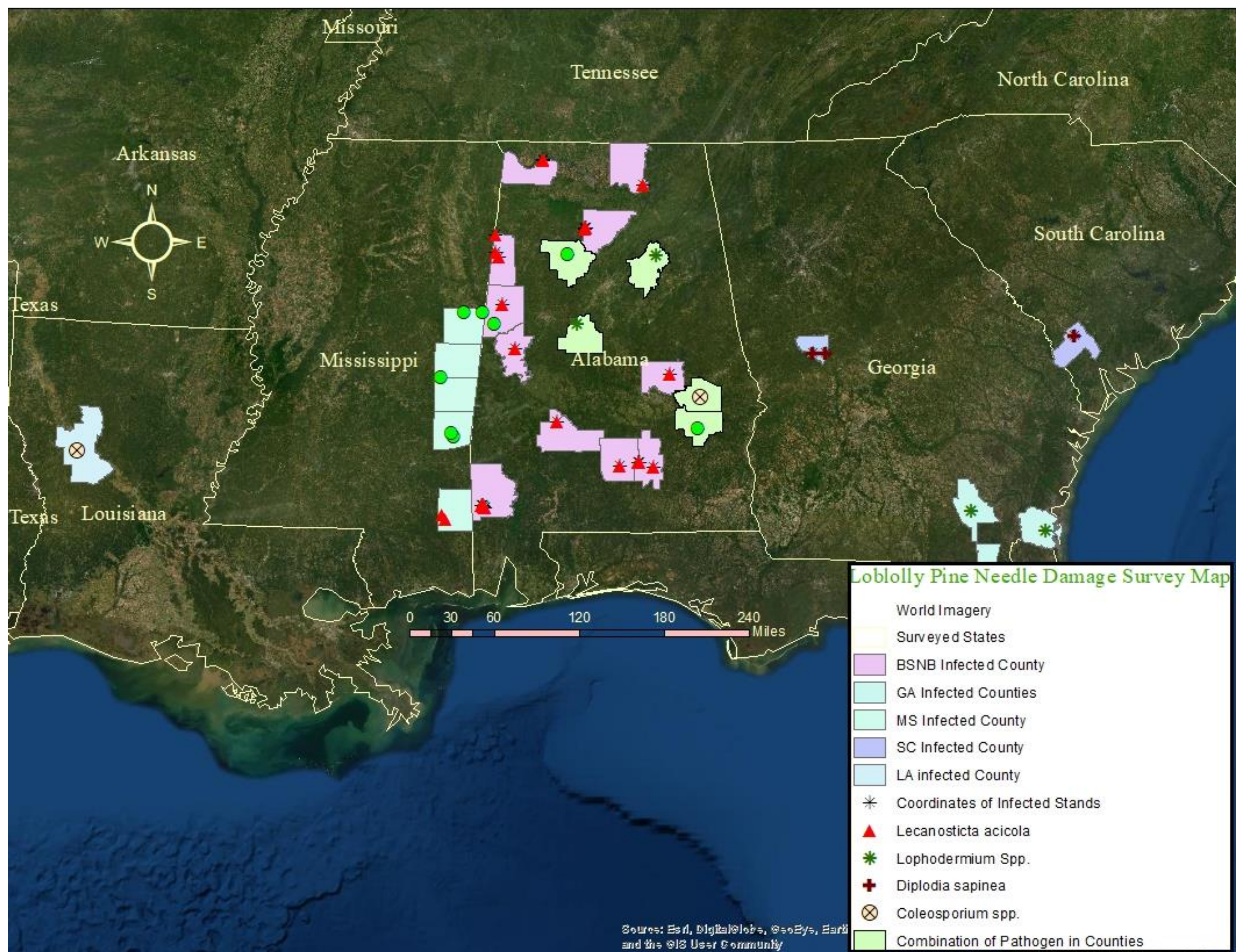


Figure 2.5 Map of pathogen distribution and loblolly pine foliar defoliation observed in stands in the southeastern United States

Fungi recovered in the media from plated needles and fungal spores recovered in the moist chambers from formed fruiting bodies in the symptomatic needles making it total 904 fungal cultures in the study. Pure cultures were divided into 58 groups based on colony color, shape, height, marginal growth, and surface characteristics. Each morphological group was examined and identified based on their distinct hyphal shape and spore structures (Webster & Weber, 2007). Twenty-eight species of fungi representing seventeen families were identified and confirmed based on morphological and molecular analysis. Twelve species of fungi appeared to be pathogens or weak parasites and they were *L. acicola, R. kalkhoffii, Lophodermium* spp., *D. sapinea, D. seriata*,

D. scrobiculata, *Coleopsorium* spp., *S. polyspora*, *Ramularia weberiana, Meyerozyma caribbica*, *Meyerozyma carpophila* and *Hormonema macrosporum*. Eight species were likely to be associated as endophytes such as *Hendersonia pinicola*, *Epicoccum nigrum*, *Alternaria tenuissima, Alternaria alternata*, *Preussia isomera*, *Penicillium spp., Sardiniella celtidis*, and *Pestalotiopsis spp*. Eight additional species appeared to be saprophytes such as *Sordaria fimicola*, *Myrmaecium rubricosum*, *Talaromyces amestolkaie, Talaromyces purpureogenus, Cladosporium anthropophilum, Cladosporium cladosporioides, Trichoderma caerulescens*, and *Paraconiothyrium brasiliense* in the study (Figure 2.4). *Pestalotiospsis* spp. were the most common endophyte recovered (100%) from both asymptomatic and symptomatic loblolly pine needles at all sites in Alabama, Georgia, Mississippi, Louisiana and South Carolina.

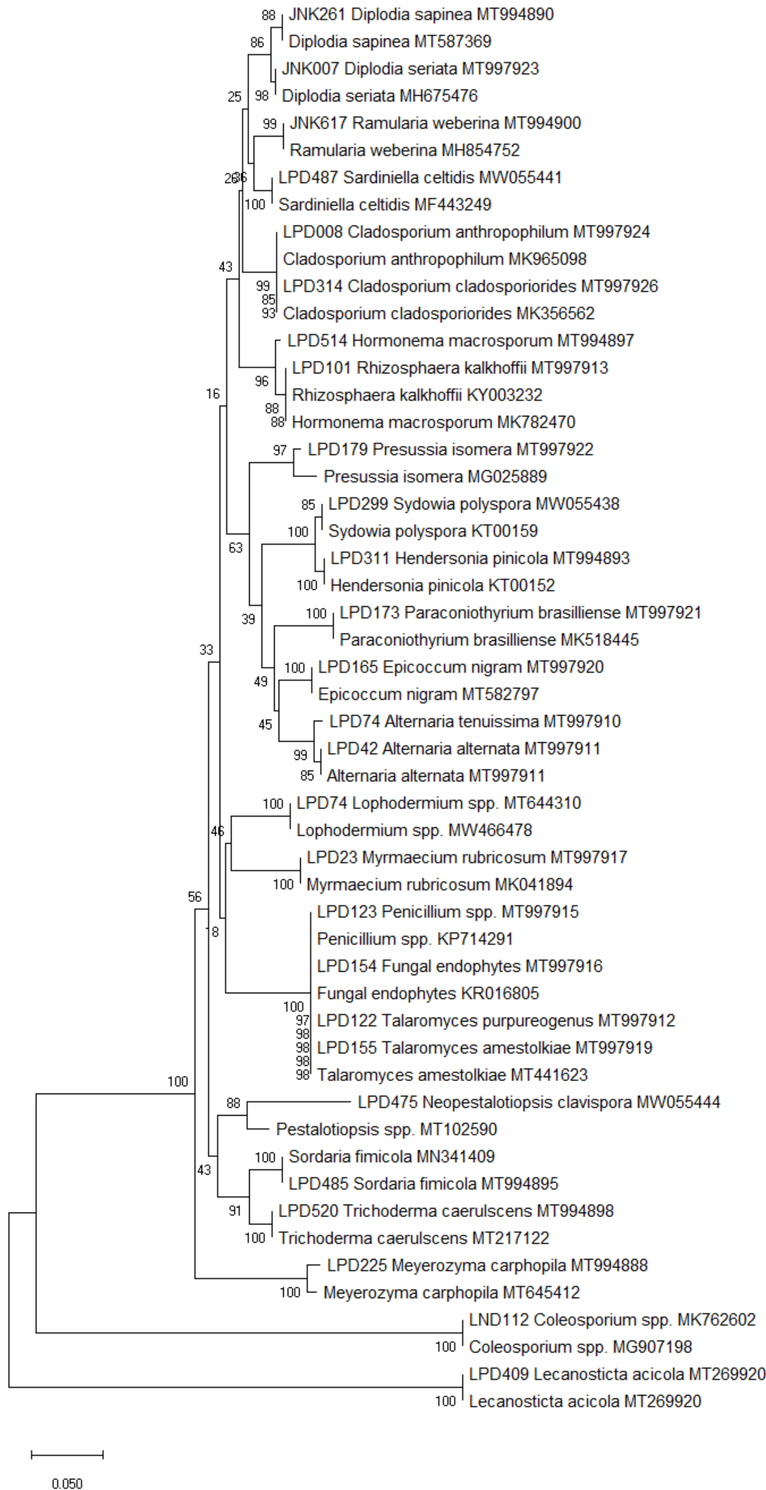


Figure 2.6 Neighbor-joining phylogenetic tree based on ITS1 and ITS4 sequences of fungi recovered from loblolly pine needles in the study.

2.3.2 *Lecanosticta acicola* associated with loblolly pine defoliation and mortality in Alabama

Lecanosticta acicola was recovered from needles collected from 32 diseased stands at 11 different sites in Alabama and one site in Mississippi including Washington (7 stands), Colbert (2 stands), Crenshaw (2 stands), Elmore (1 stand), Madison (2 stands), Pickens (1 stand), Greene (AL, 1 stand), Lamar (3 stands), Cullman (5 stands), Wilcox (1 stand), Butler (5 stands) and Greene (MS, 2 stands) counties. A foliar endophyte and pathogen, *Sydowia polyspora* was frequently recovered along with *L. acicola* in Alabama (Washington, Elmore, Butler, Madison, Greene, Lamar, Cullman, Wilcox and Pickens) where nearly 80% of trees were infected and tree mortality was observed due to defoliation.

The desired length of PCR products for fungal identification were confirmed by gel electrophoresis. For universal ITS1/ITS4 primers and species-specific LAtef-F/LAtef-R primers, successful amplification was about 500 (Figure 2.5) and 230 base pairs (Figure 2.6) respectively.

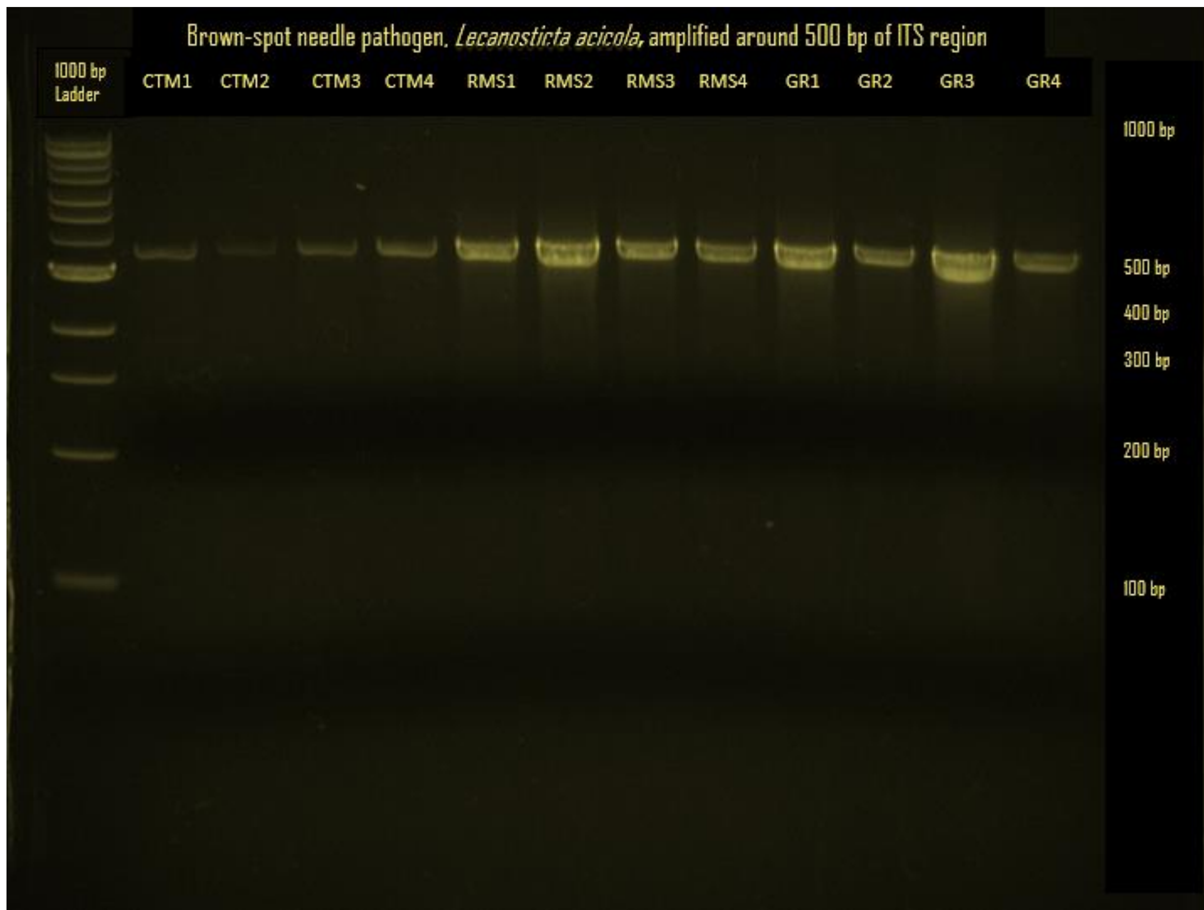


Figure 2.7 Gel electrophoresis results of PCR products showing amplification of internal transcribed spacer (ITS) region of *L. acicola* isolates recovered in the study. The top of the gel indicates *L. acicola* isolates recovered from Chatom, Butler, and Greene counties.

The sequences recovered in the study were identified to have three distinct lineages of *L. acicola* globally (Figure 2.6). The first lineage was related to a *L. acicola* population in Spain where it was reported to infect *Pinus radiata* stands (Janoušek et al. 2016). The second lineage was associated with fungus populations in France where *L. acicola* infects *P. radiata* plantations (Alvère et al. 2010). Finally, the third relationship was related to a *L. acicola* population in the northeastern United States where it infects *Pinus strobus* (eastern white pine) (Broders et al., 2015).

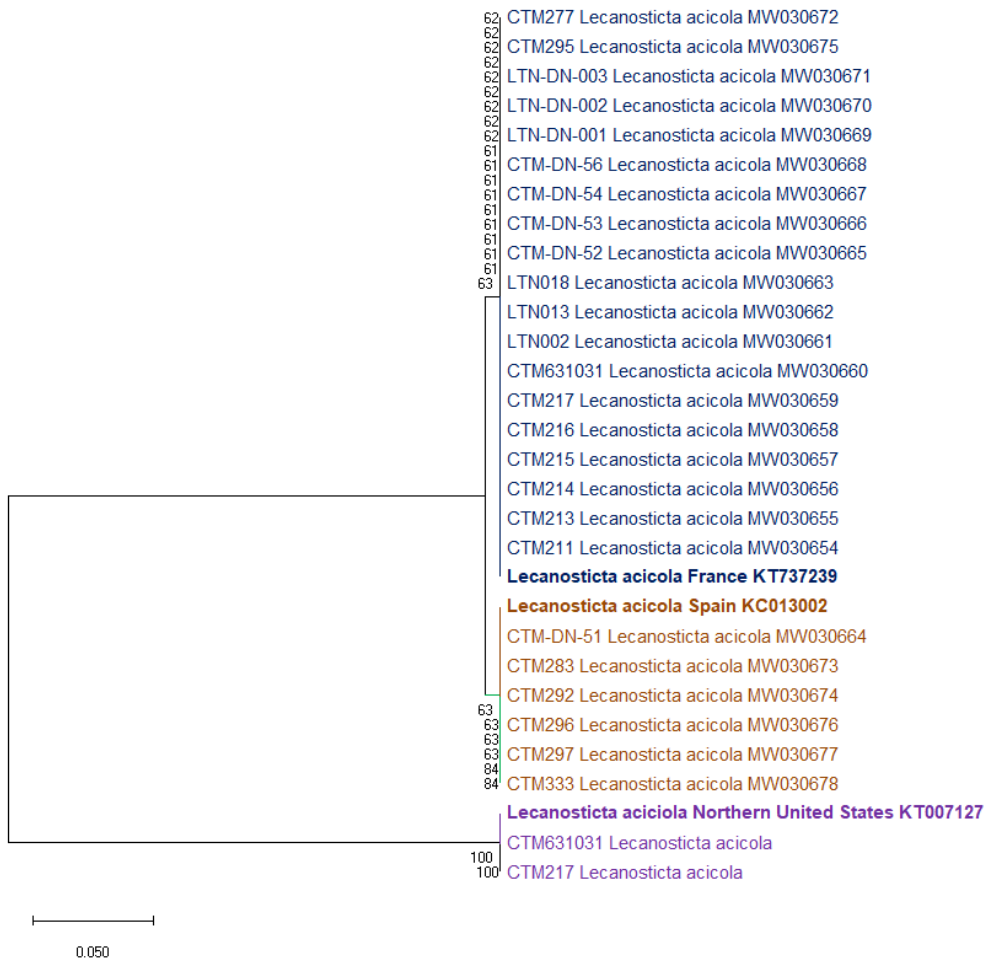


Figure 2.8 Maximum likelihood (ML) phylogenetic tree representing *L. acicola* and its associated lineages globally.

Mating type analyses of *L. acicola* were confirmed using mating-type primers Md MAT1-1F, Md MAT1-1R, and Md MAT1-2F, Md MAT1-2R (Janousik et al., 2016). Positive *L. acicola* isolates were amplified for a single mating type, MAT-1-1. However, amplification did not successfully detect *L. acicola* mating-type 2 loci, MAT-1-2. Microscopic examination of fruiting bodies recovered from needles in Alabama and Mississippi did not result in the formation of a sexual stage in those infected stands. Morphological examination of fungal isolates of *L. acicola* black to olive green mucilaginous conidiomata was recovered from

symptomatic loblolly pine needles. Conidia were light green to olivaceous to pale brown in color and rough verruculose thick walls. Spore shapes varied from fusiform to cylindrical with straight to curved conidia ranging from 2 to 4 septation with truncate base and rounded apex. Dimensions of the conidia varied between 22.15 - (33.34) - 38.38 × 3.59 - (4.84) - 7.32 µm (Figure 2.3A).

2.4 Discussion

The loblolly pine defoliation and mortality (LPDM) were predominantly due to brown-spot needle blight fungus, *L. acicola* in Alabama. This pathogen was recovered from Central (1), Southern (3), Northern (6), and Southwestern (1) counties of Alabama and indicates that this fungal presence is ubiquitous in Alabama. The mating-type analysis of *L. acicola* confirmed that a single mating type MAT-1-1 was present in the samples in Alabama. No sexual state was observed during the study indicating that there is less possibility of sexual outcrossing (Janoušek et al. 2016). A similar study of mating-type loci analysis of *L. acicola* from *Pinus palustris* Mill. seedlings in Mississippi was negative also supporting that only one mating types was present in Alabama and Mississippi (Bartlett, 2015).

Lecanosticta acicola wasn't recovered at infected stands in South Carolina, Georgia or Louisiana where infected trees showed varied symptomology compared to Alabama. Trees sampled on those sites did not mimic *L. acicola* symptomology (chlorosis followed by rapid necrosis leading to premature defoliation) which supported the study results. In South Carolina (Hampton) and Georgia (Upson), infected trees showed tip blight of the needles and were located at deep steep slopes. In Louisiana (Natchitoches Perishes), yellow to orange spots on the green needles, yellowing over time and defoliation was observed. Other needle casts were also associated with LPDM but their recovery was only at a site or in combination with *L. acicola* pathogen in Alabama. Several stands in Alabama and Mississippi were considered healthy

because no needle pathogens were recovered from those stands. They were located at Noxubee, Lauderdale, Clark counties in Mississippi, and Walker and Bullock counties in Alabama (Figure 2.2). Infected trees on those sites showed symptoms such as chlorotic needles, shortened internodes and sparse crown which is indicative of a root disease such as pine decline (Eckhardt et al., 2010; Hess et al., 1999; Lorio, 1966) or littleleaf disease (Crandall et al., 1945).

One of the more intensifying recovery of the fungus was *Sydowia polyspora*, a foliar endophyte (Ridout & Newcombe, 2018) and pathogen (Talgø et al., 2010). *Sydowia polyspora* was recovered at more than 80% of brown spot needle blight affected stands in Alabama. Nearly all loblolly trees were affected and one-third trees have started dying due to defoliation. Frequent recovery of this *S. polyspora* along with *L. acicola* indicates *S. polypora* may have a potential role associated with increasing disease severity. Other studies reported that *S. polypora* fungus changes its role from endophytic to pathogenic when exposed to warm climate and/or if the host tree is under stress (Muñoz-Adalia et al., 2017; Pan et al., 2018). Most of the positive stands were situated in the wet areas and/or close to a water body and in poor drainage conditions. Although loblolly pine grows and survives in poor soil conditions, it is also possible that trees are stressed due to site conditions and inoculum pressure. Spore survival might have increased in those sites (Gadgil, 1970). Edaphic factors such as soil types, slope, aspect, depth of soil to impermeable layer and nutrient availability are thought to be associated with disease incidence and occurrence. Average infection of dothistroma needle blight is positively correlated with sulfur-deficient basalt parent materials and infection levels are related to soil and topographic factors (Eldridge et al., 2013).

Based on the study, needle pathogens are increasing in appearance in Alabama. Most notably, brown spot needle blight, *L. acicola* has resulted in tree mortality of loblolly pine trees

in Alabama. This pathogen has been a major problem for grass-stage longleaf pine (*P. palustris* Mill.) in the southeastern United States (Siggers, 1944) and Scotch pine Christmas tree plantations in Minnesota and Wisconsin (Skilling & Nicholls, 1974). However, this is the first report of loblolly pine defoliation and mortality due to this pathogen in Alabama. In the context of climate change, this disease is likely to continue if the pathogen is favored by changes in temperature and moisture conditions. Since loblolly pine is one of the most productive timber species native to the southern United States, it is crucial to manage this disease for the sustainability of the species. Current research is underway to attempt to predict loblolly pine defoliation severity in the coming years (see Chapter V).

2.5 Conclusion

A total of twenty-eight different fungi were recovered from symptomatic loblolly pine needles in Alabama, Mississippi, South Carolina, Georgia, and Louisiana. The brown-spot needle blight fungus, *L. acicola*, was the predominant needle pathogen associated with loblolly pine needle defoliation and tree mortality in Alabama. Only mating type 1, MAT-1-1 was present in Alabama indicating a less genetically diverse population and reduced recombination. Other needle cast, rust, and tip blight pathogens were recovered at one or few sites or in combination with *L. acicola*. Their role in LPDM was secondary and might be associated with increasing disease severity in the *L. acicola* infected stands. A foliar endophyte and pathogen *S. polyspora* was also recovered with *L. acicola* in Alabama where the disease was most severe. Based on the study, *L. acicola* has become a problem to forest managers in Alabama. Due to the nature of the disease, stand characteristics, and other factors, an understanding of LPDM is critical to guiding management and policy recommendations.

Chapter Three

Needle pathogen, *Lecanosticta acicola*, effects on *Pinus taeda* shoot and needle lengths

Abstract

Loblolly pine is a crucial economic component of plantation forestry in the southeastern United States. Loblolly pine has been experiencing repeated defoliation due to brown-spot needle blight fungus, *L. acicola* in Alabama. The study objectives were to assess the repeated infection of *L. acicola* on loblolly pine (1) needle growth and (2) shoot growth as well as (3) to monitor and record the annual progression of this disease in loblolly pine stands. Results indicate that high incidence trees produced shorter shoots and needles compared to low incidence trees. Shoot length was reduced by 3.42 cm and 1.61 cm at lower whorl and upper whorl heights respectively of high incidence trees than low incidence trees. Moreover, high incidence trees were found to have 3.16 cm and 2.59 cm (lower whorl and upper whorl, respectively) shorter needles compared to low incidence trees. High incidence and low incidence trees were increasingly chlorotic and defoliated from 2019 to 2021 indicating that disease is progressing both temporally and spatially. Apart from needle necrosis and premature defoliation, *L. acicola* can additionally reduce the tree's photosynthetic ability through the reduction of needle area. The pathogen is likely to spread in the coming years and cause significant volume loss of infected loblolly pine stands.

Keywords: Loblolly pine health, needle length, shoot length, long-term monitoring

3.1 Introduction

Brown spot needle blight is a conifer disease caused by the fungus *Lecanosticta acicola* (*Mycosphaerella dearnessii* (syn. *Scirrhia acicola*)). This fungal pathogen was first reported by Thümen in South Carolina in 1878 and has been found to be a destructive pathogen of over 50 *Pinus* species in North and Central America, Eastern Asia and the Central European and Mediterranean Plant Protection Organization (EPPO) region (Quaedvlieg et al., 2012). Genetic analyses of this pathogen suggests that *L. acicola* is a native pathogen of Central America specifically in the mountain areas of Guatemala and Honduras (Bednářová et al., 2013). This pathogen is well-known for its potential of killing foliage and retarding the growth of trees, most notably, longleaf pine seedlings in the southern United States (Siggers, 1932, 1944).

The first symptoms of this disease appear as small yellow, sometimes reddish-brown, or grey-green, irregular circular spots which turn brown over time and are often surrounded by a yellow halo at the point of infection (Hedgcock 1929; Skilling & Nicholls, 1974). Infection is usually more severe on the lower crown and moves upward as the disease progresses (Sinclair & Lyon, 2005). Depending on the host and fungal prevalence, infections can occur on several parts of the needles leading to rapid necrosis resulting in severe defoliation which may lead to the death of branches and whole trees (Kais, 1975). Generally, second and third-year needles are infected only leaving new growth needles at the tip of the branches. Current year needles become infected in the next year because of conidia spreading from infected old needles (Siggers, 1944; Skilling & Nicholls, 1974). Development of disease symptoms and damage depend on the fungal strain and favorable climatic conditions (Kais, 1975).

Lecanosticta acicola overwinters as vegetative mycelium or asexual acervuli or sexual ascostromata in the infected needles (Siggers, 1974). When light, temperature, and humidity are

favorable, *L. acicola* can spread through its mucilaginous conidia or sexual ascospores (Kais, 1975; Tainter & Baker, 1996). Conidial dispersal to adjacent trees is mostly triggered by rainfall patterns and contributes to rapid disease build-up in pine stands. Conidia germinate on the needle surfaces, penetrate germ tubes, enter stomatal antechamber, and then increase in diameter and become thick-walled and melanized (Van der Nest et al., 2019; Kais, 1975). Light indirectly plays a role as it stimulates the opening of the guard cell of the stomata. Similarly, the wound helps conidia to penetrate needles (Kais, 1975). Asci form on necrotic parts of the infected living needles or dead needles, and ascospores are forcibly expelled and disseminated by wind currents and/or by rain (Henry, 1974). Based on the strain of *L. acicola*, conidia and ascospores both can survive, spread, and germinate between -5°C to 35°C. High levels of infection occur with warm temperatures and high rainfall after a long period of dryness. Conidial dispersals were recorded the highest in the United States between late spring and summer. Abundant ascospores were recorded between late summer to autumn in the United States when the temperature is above 15°C (Kais, 1975). Insects, animal movement, and anthropogenic movement of infected materials could be other mechanisms of conidial spread and dispersal (Skilling & Nicholls, 1974; Tainter & Baker, 1996; Wingfield et al., 2015). However, these are deemed to be unlikely based on the biology of the pathogen, *L. acicola*.

Loblolly pine is a commercial timber species native to the southeastern United States. According to Boyce (1958), all ages of loblolly pines affected by *L. acicola* which caused die-back of the needles. However, no needle casting resulted in defoliation leading to reduced growth and tree mortality was documented for loblolly pine because of brown spot needle blight infection. Siggers (1932, 1944) stated that loblolly pine is a common associate of longleaf pine and thus a frequent host of brown spot needle blight fungus, *L. acicola*. Histological analyses of

symptomatic loblolly pine needles indicated that mesophyll tissues were collapsed, and resin ducts exhibited cellular necrosis and dissolution as a result of *L. aciola* infection (Jewel, 1993).

Lecanosticta acicola is the causal agent of loblolly pine defoliation and tree mortality in the southeastern United States. Based on chapter II, this is the first study that reported defoliation causing loblolly pine mortality in the region due to this *L. acicola* pathogen (see Chapter I). As a primary infection, symptoms appear as chlorosis, needle necrosis, and premature defoliation (Figure 3.1) resulting in a thin sparse crown. Anecdotal evidence suggests that loblolly pine with high levels of infection have shorter needles and shoots compared to trees with low or no levels of infection. An epidemiology study of *Dothistroma septosporum*, a fungus mimicking a similar life cycle and biology as *L. acicola* was found to affect needle and shoot growth of *Pinus nigra* subsp. *laricio* (Corsican pine) trees in Britain. *Dothistroma septosporum* was able to cause shorter needles and shoots in the years following infection which resulted in the reduction of photosynthetic ability and growth of the trees (Mullet, 2014). However, additional influence on needle and shoot growth due to repeated infection by *L. acicola* remains unknown. The study objectives were to assess the repeated infection of *L. acicola* on loblolly pine (1) needle growth and (2) shoot growth as well as (3) to monitor and record the annual progression of this disease in loblolly pine stands.

3.2 Materials and Methods

3.2.1 Stand characteristics and symptomology of trees

In the permanent study plots, plots were composed of 16 years, 11 years, 10 years and 8 years old plantations. Symptoms were showing yellowing to progressively browning needles, defoliation, thinned crown, and tree mortality across stands (Figure 3.1). The disease was first expressed in 2004 plot and eventually discovered in all other adjacent stands. Only 2004 plot

received fertilization after three years of plantation establishment. Other plots didn't receive any silvicultural treatments. Soils consisted of Tibbie soils (40%), Pinebarren soils (35%) and 5% of other minor components. (Web Soil Survey).

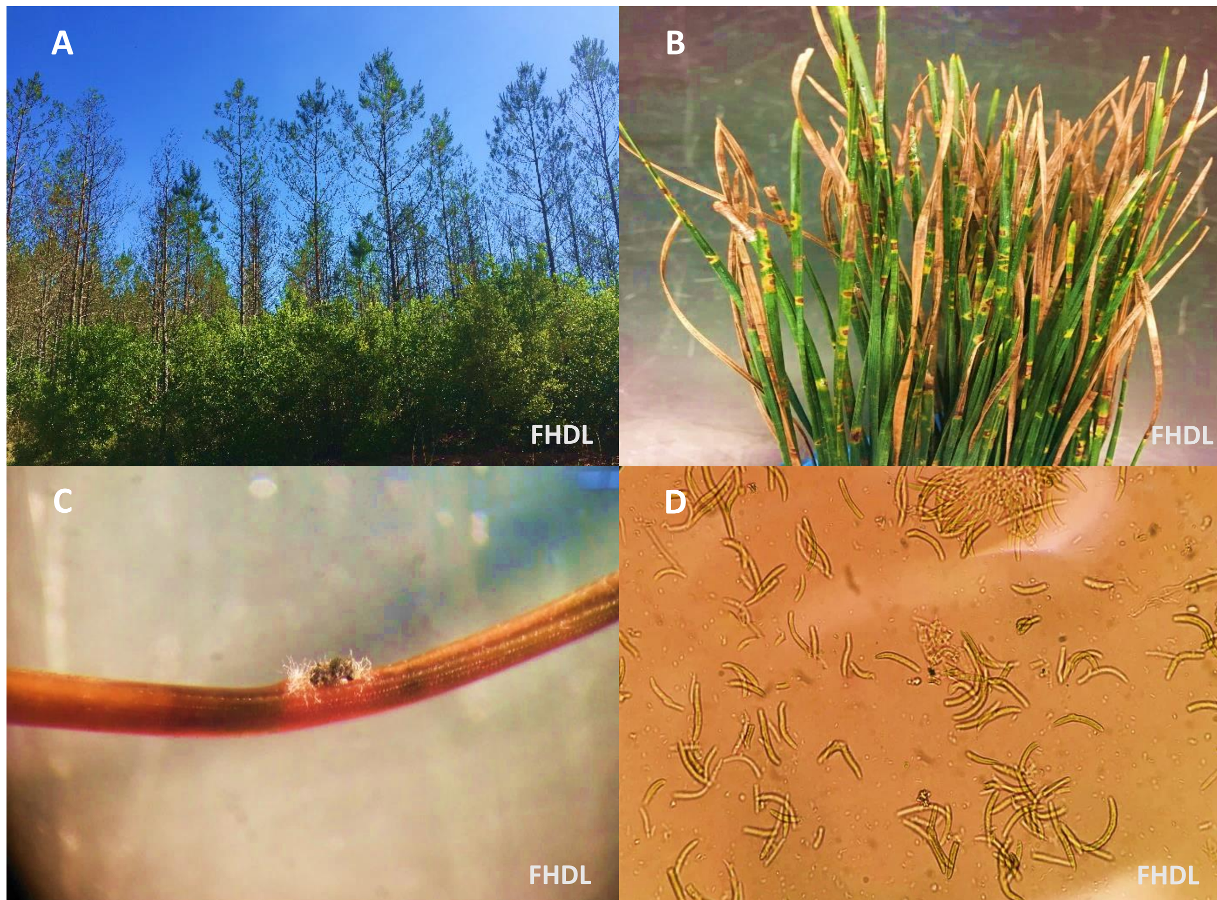


Figure 3.1 Disease symptoms and reproductive structures showing (A) stands infected by brown-spot needle blight, note the thinning canopies (B) irregular frequent brown-spots surrounded by a yellow halo (C) black shiny fruiting body protruding needles & (D) microscopic banana-shaped septate conidia.

3.2.2 Study design and data collection

The study was conducted in one of the *Pinus taeda* infected permanent plots in Chatom, Alabama, Washington County (-88.31687 N 31.26497 W). This plantation plot was established in 2012 and no silvicultural treatments were applied after establishment. To analyze *L. acicola* disease severity on shoot and needle length, *L. acicola* infected trees were assessed at the end of

the growing season to ensure maximum retention of needle and shoot growth. Two infection levels [low infection level (10-20% crown infection) and high infection level (>50% crown infection)] were chosen to evaluate comparative impacts in needle and shoot lengths. Since whorl height has effects on the shoot and needle lengths due to light availability, two whorl heights were chosen. From the base of the tree, the first whorl height at 2-5 m and the second whorl height at 5-8 m were chosen. A total of twenty-eight low infection trees and thirty-three high infection trees were measured in 2019 and 2020. From each tree, nine to eleven side apical shoots were assessed at each whorl height. Shoot length and needle length were measured in centimeters. A total of ten random fascicles were chosen per shoot to obtain the average needle length. The experiment was conducted in October 2019 and November 2020. 2018 growth was measured for 2018 in 2019 and 2019 growth was measured for 2019 in 2020 (Mullett, 2014).

3.2.2 Three-year loblolly pine health monitoring

To monitor loblolly pine health, seven permanent monitoring plots were established at Chatom, Alabama, Washington County in summer 2019. Those plots were of eight- to sixteen-year-old plantations and were suffering from needle damage at least since 2018. At each experimental plot, 6-8 high incidence trees (>1/3 tree crown is affected), and 2-4 low incidence trees (<1/3 tree crown are infected) were tagged in summer 2019. It was very difficult to find a completely healthy tree in a diseased stand; therefore, trees with less than 1/3 crown infections were chosen and considered as low incidence trees. Detailed information of tagged trees such as height (m), DBH (cm), GPS coordinates (latitude and longitude), location of damage (upper, middle, or lower canopy) was collected.

Crown rating conditions of trees were ranked and recorded in the summer of 2019, 2020, and 2021. The crown rating was based on visual inspection of a proportion of tree crowns

affected (Figure 3.2) and categorized as (1) less than one-third of tree crown infected (<1/3), (2) one-third to two-third of crown infected (1/3 to 2/3), and (3) more than two-thirds crown infected (>2/3) followed by Broders et al., (2015). Crown ratings were recorded as "chlorosis per tree" and "defoliation per tree".

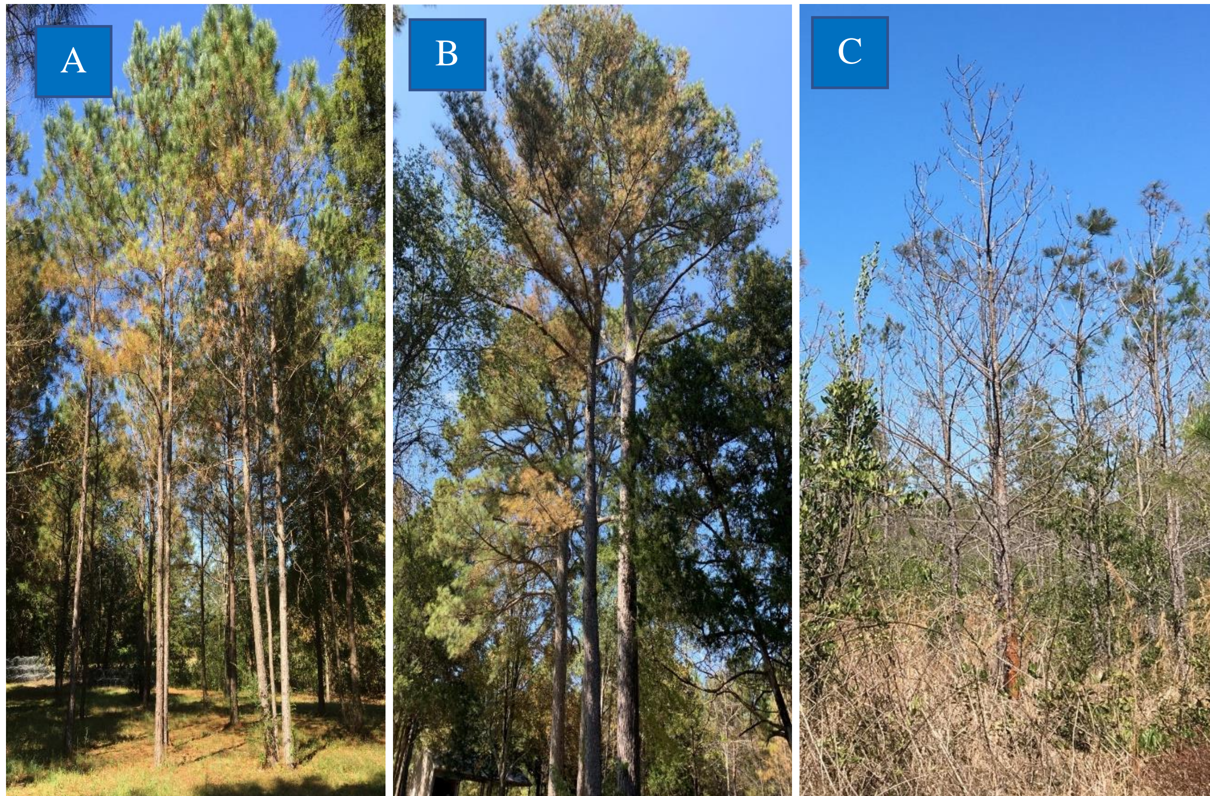


Figure 3.2 Crown severity rating such as (A) One-third of crown infected (B) One-third to two-third of crown defoliated/infected (C) More than two-third to full crown defoliated/infected.

3.2.3 Statistical analyses

To evaluate the effect of "infection level" and "whorl height" on the shoot and needle lengths, a restricted maximum likelihood model was fitted. Statistical software SAS version 9.4 was used to conduct data analysis. Growth year and individual trees were considered as random effects whereas infection level and whorl height were considered as fixed effects.

Loblolly pine health was analyzed using linear mixed-effect models where the “stand” was considered as a random effect and “year” and “initial health status” were treated as fixed effects. In both cases, normality and homogeneity of data were checked. No transformations were required. Goodness fit test was performed, and no assumptions of the linear regression model were violated.

3.3 Results

3.3.1 Measurement of shoot and needle lengths

High incidence trees produced significantly shorter shoots than low incidence trees. Both infection level and whorl height had impacts on shoot length (F statistics = 27.31; $d.f._{4,115}$, $P<0.0001$; F statistics = 33.70; $d.f._{4,115}$, $P<0.0001$ respectively) as shown in Table 3.1. Infection level and whorl height independently influenced the tree shoot length and therefore, did not have any additive effects on the model (F statistics = 3.51; $d.f._{4,115}$, $P<0.06$). High incidence trees had 3.42 cm and 1.61 cm short shoots compared to low incidence trees at their lower whorl and upper whorls respectively (Figure 3.3).

Table 3.1 Probabilities of a greater *F*-value ($P > F$) of *P. taeda* trees to *L. acicola* infection on needle length, shoot length, and fascicle density.

Effect	[a]DF	[b]Den DF	Infection Level (IL)		Whorl Height (WH)		IL*WH	
			F Value	**P>F**	**F Value**	**P>F**	**F Value**	**P>F**
Shoot Length	4	115	27.31	<.0001	33.70	<.0001	3.51	0.06
Needle Length	4	115	73.07	<.0001	38.06	<.0001	0.00	0.96
Fascicle Density	4	115	42.19	<.0001	8.87	.004	3.98	0.05

[a]DF, numerator degrees of freedom; [b]Den DF, denominator degrees of freedom; $P > F$, probability of a greater *F*-value

Needle length was affected by *L. acicola* infection. High incidence trees produced on average 3.16 cm and 2.59 cm (lower whorl and upper whorl, respectively) than low incidence trees (Figure 3.3). There were nearly 24.8% and 17.7% of needle length reduction of lower branches and upper branches, respectively. Infection level (F statistics = 73.07; $d.f._{4,115}$, $P<.0001$) and whorl height (F statistics = 38.06; $d.f._{4,115}$, $P<.0001$) were found inversely related to needle length (Table 3.1). The study found that shoot length and needle length were positively correlated (Pearson's correlation coefficient = 0.312277, p = 0.0005).

The total number of needles per shoot and fascicle density were positively correlated to shoot length (Pearson's correlation coefficient = 0.6243, p<0.05). However, no significant interactions between infection level and whorl height was detected for total number of needles (F statistics = 38.79; $d.f._{1, 118}$, $P<0.05$; F statistics = 6.48; $d.f._{1, 118}$, $P=0.01$; F statistics = 0.31; $d.f._{1, 118}$, $P=0.58$;) and fascicle density (F statistics = 42.19; $d.f._{1, 118}$, $P<0.001$; F statistics = 8.87; $d.f._{1, 118}$, $P=0.04$; F statistics = 3.98; $d.f._{1, 118}$, $P=0.05$;) as shown in Table 3.1.

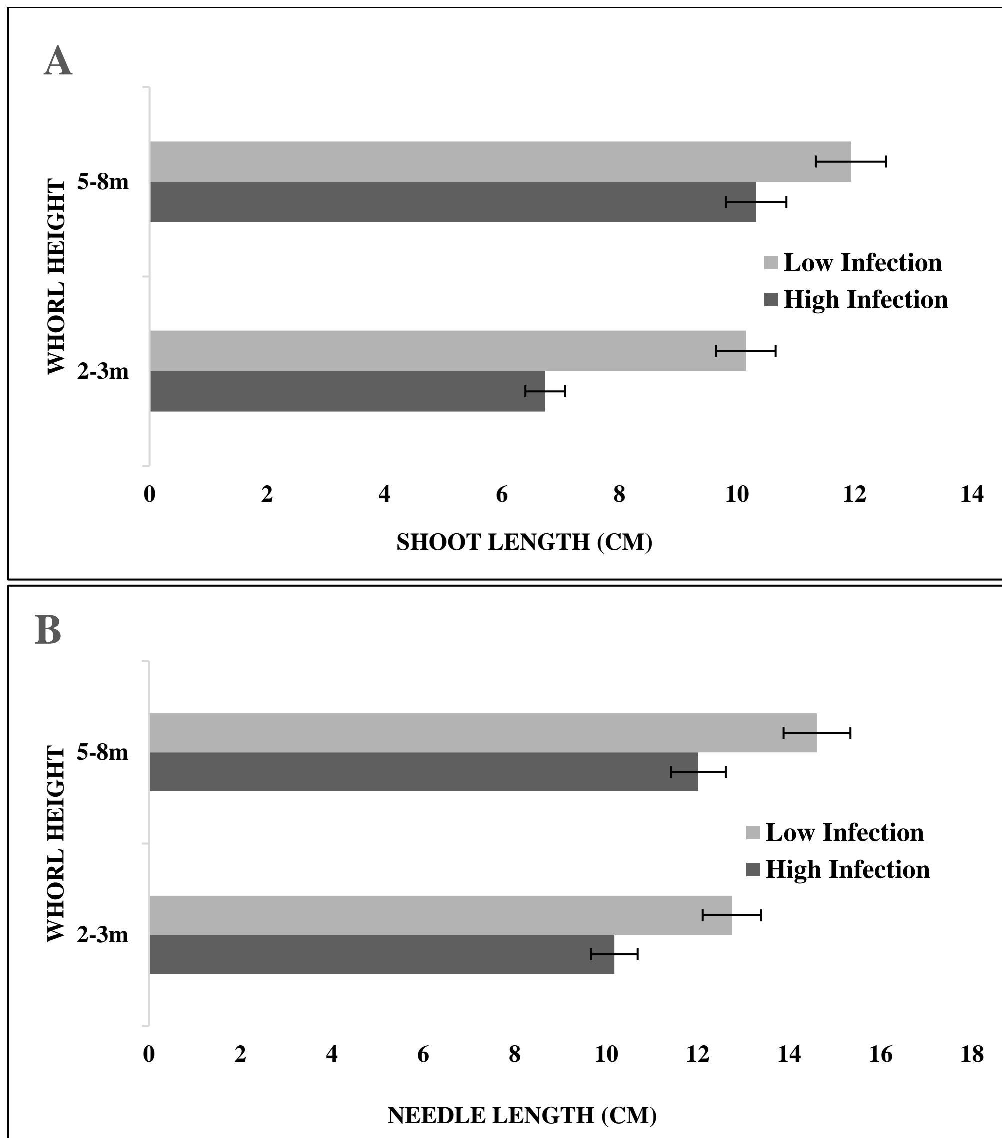


Figure 3.3 Observed means and standard errors of (A) shoot length and (B) needle length

3.3.2 Three-year loblolly pine health monitoring in Chatom, Alabama

Seventy trees were tagged and assessed for chlorosis and defoliation rating. Based on visual observation, the crown rating was conducted in the summer of 2019, 2020, and 2021. Trees displayed chlorosis and defoliation in the summer. Initially considered low incidence trees became significantly chlorotic and defoliated from 2019 to 2021 (Figure 3.4 & Figure 3.5). High incidence trees did not recover from crown damage. Furthermore, the proportion of tree crowns

ranked chlorotic and defoliated in 2019 considerably increased in 2021 for both low incidence and high incidence trees. Crown damage progressed from bottom to top of the tree crown and high incidence trees to low incidence trees.

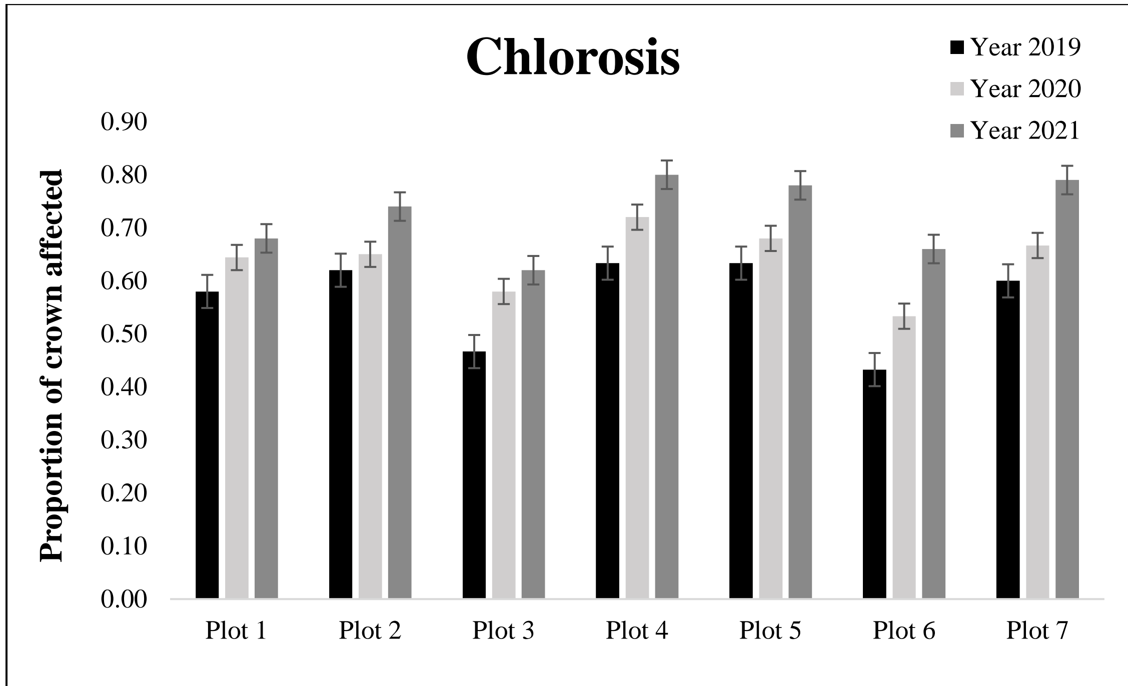


Figure 3.4 Bar represents the mean chlorosis of seventy tagged trees at seven long-term monitoring plots in Chatom, Washington County, Alabama in the summer of 2019, 2020 & 2021.

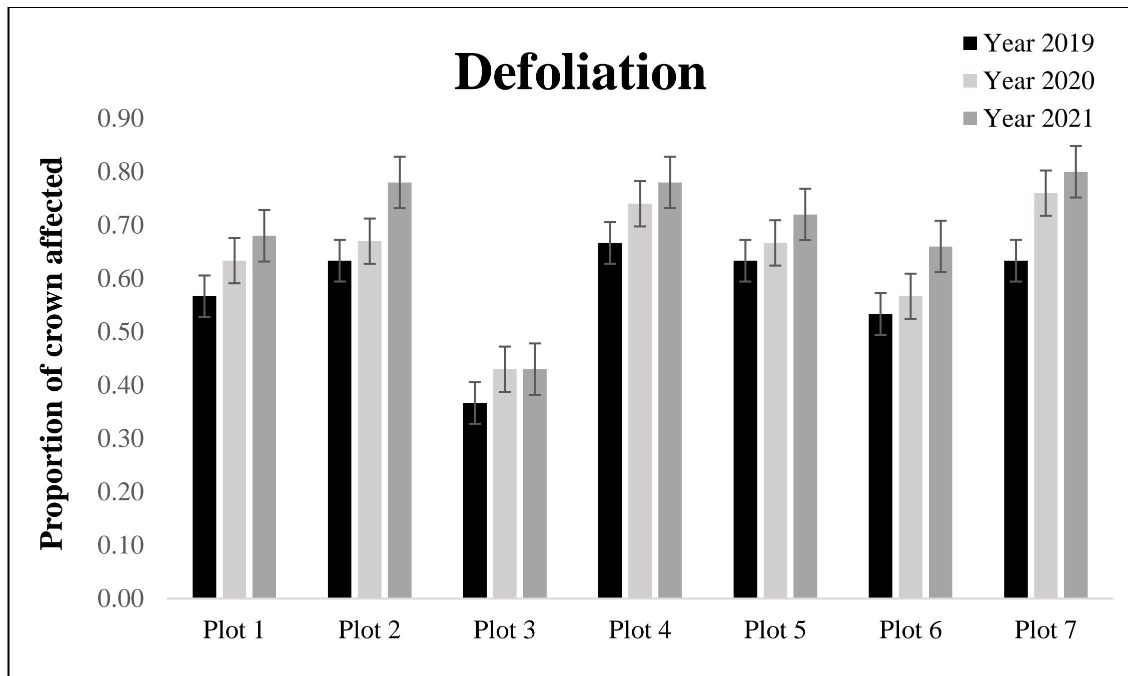


Figure 3.5 Bar represents the mean defoliation of seventy tagged trees at seven long-term monitoring plots in Chatom, Washington County, Alabama in the summer of 2019, 2020, & 2021.

3.4 Discussion

These studies demonstrate that *L. acicola* had substantial impacts on tree shoot and needle lengths. Infection level contributed to shoot and needle lengths reduction. In the pathosystem, needle size was reduced due to a needle pathogen *D. septosporum* infection in *P. nigra* subsp. *laricio* (Corsican pine) infected stands in Britain (Mullett, 2014). Needle length reduction could be related to the previous year needle damage such as chlorosis, necrosis, and premature defoliation caused by *L. acicola*. Needle damage resulted in (1) a reduced photosynthesizing area, (2) a reduction of the photosynthesizing ability, and (3) fewer carbohydrate reserves that feed new shoot and needle growth. Consequently, needle damage in 2018 reduced needle growth in 2019 which further cumulatively led to fewer energy sources for trees for shoot and needle growth in 2020.

Needle size was positively correlated with tree whorl height indicating that the lower crown of infected trees was more impacted compared to the upper crown and experienced greater needle length reduction. Other studies supported the findings that brown spot needle blight disease is more detected in the lower crown in comparison to the upper crown (Siggers, 1944; Skilling & Nicholls, 1974; van der Nest et al., 2019). Needle pathogen, *L. acicola* usually starts infection in the lower crown and stay there until get favorable conditions because of high moisture availability which resulted in greater needle damage in the lower crown. Pallardy (2008) stated that the growth and development of needles are influenced by shoot locations on the stems. In the healthy tree, larger and heavier fascicles, longer flushes of shoots, and more leaf area are positively correlated to crown position i.e., vertical light gradient within the canopy (Pensa & Jalkanen, 2005). A study by Wang et al., (2019) found that loblolly pine needle length is positively correlated to leaf mechanical support and physiological capacity. Therefore, reduction of needle length significantly diminished leaf mechanical support and physiological functions indicating that repeated *L. acicola* infection on tree foliage such as chlorosis, needle necrosis, premature defoliation, and needle length reduction supported tree mortality and substantial volume loss in loblolly pine infected stands.

Shoot length also similarly reduced at lower whorl and upper whorl heights of high incidence tees compared to low incidence trees. Tanga et al., (1998) found that upper crown shoots were significantly longer than lower crown shoots as the effect of thinning and fertilizer application. Since no silvicultural treatments were applied on the experiment plot, the study safely excluded fertilizer and thinning effects on the seasonal shoot and needle growth. Shoot length of irrigated trees versus drought trees are also varied almost double based on water availability (Garrett & Zahner, 1973). Since the study plot didn't vary significantly from tree to

tree, other factors such as water availability, temperature variation and light accessibility were not related to making such a difference. This supported that comparative effects of *L. acicola* caused significant differences in shoot length variations based on infection severity.

Long-term monitoring of loblolly pine health confirmed that *L. acicola* was progressing both temporally and spatially on every experimental plot. Within three years of observation, both infection and mortality increased. Only the 2010 experimental plot showed little variation. This variation may result from either genetic variability or soil conditions (Eldridge & Lambert, 1980). Disease progression indicates that either air currents or rain-splash spores of *L. acicola* were spreading from high incidence to low incidence trees and lower crown to upper crown (Siggers, 1944) and causing chlorosis, needle necrosis, repeated defoliation, needle length reduction leading to rapid death of the infected trees (Broders et al., 2015). Tree mortality is likely to increase in the coming years, which in turn, may cause large-scale volume loss in loblolly pine stands.

3.5 Conclusion

The current emergence of brown spot needle blight in loblolly pine stands has impacted both shoot and needle lengths. The study found that infection level is inversely related to tree shoot and needle lengths. High incidence trees significantly reduced shoot and needle lengths compared to low incidence trees. High levels of infection caused 3.16 cm and 2.59 cm of lower whorl and upper whorl needle growth reduction compared to low levels of infection. Similarly, shoot length was reduced by 3.42 cm and 1.61 cm at lower whorl and upper whorl height of high incidence trees respectively. Whorl height was positively correlated with the shoot and needle lengths. During the study, low incidence and high incidence tagged trees were ranked as increasingly chlorotic and defoliated indicating that *L. acicola* is progressing from lower crown

to upper crown and trees to trees. To conclude, *L. acicola* can reduce shoot and needle lengths which can result in an additional reduction of the photosynthetic ability of infected trees leading to carbon deficit and rapid death of the infected trees.

Chapter Four

Lecanosticta acicola impacts on foliar nutrient contents and total phenolics in *P. taeda* needles

Abstract

Brown-spot needle blight fungus, *L. acicola*, has been reported from loblolly pine stands in Alabama. Needle sampling to measure macro and micro-nutrient contents and total phenolics was conducted at seven permanent plots in Chatom, Alabama, Washington County. Infection of *L. acicola* increased the total phenolics concentrations (($p < 0.005$). Higher concentrations were positively correlated with disease severity. Foliar concentrations of N, Na, S and B ($p \leq 0.05$) were also correlated with *L. acicola* infection severity. Higher concentrations of foliar nutrients could be a result of leaf area loss and concentration effects in those remaining needles.

Keywords: Brown spot needle blight, foliar nutrients, total phenolics, loblolly pine

4.1 Introduction

Pinus taeda is an economically valuable timber species native to the southeastern United States. Commercially managed plantations account for nearly half of the pine growing stock in the south (McKeand et al., 2003) as intensive management practices (mechanical and chemical site preparation, fertilizer application) and improved genetics have increased stand productivity (Wear & Gries, 2002; McKeand et al., 2003).

Nutrient availability is essential to determine tree growth and productivity (Fox et al., 2007). Tree growth is affected if the nutrient level is below or above the threshold level. Sayer et al., (2009) confirmed that elevated Mn concentrations contributed to reduced vigor of longleaf (*P. palustris*) plantations in Georgia and Florida. However, growth is often limited by soil

nutrient availability in southern pine plantations. Soil nutrients such as nitrogen (N) and phosphorus (P) promote the expansion of leaf area, which in turn, contributes to wood production. Therefore, nutrient-deficient stands respond quickly following N and P fertilization and increase their radial growth (Fox et al., 2007).

Foliar nutrient contents can be used as an indirect indicator of soil nutritional status. Content and relative abundance of macronutrients in the needles can be used to determine the nutritional status of pine forests across varied habitats (Ballard and Carter, 1986; Braeke and Sahin, 2002). Nutrient elements are also interrelated as excess or deficiency of one mineral can affect the function of other elements and alter normal physiological processes. Nutrient deficiency of an element can indicate available soil supply and stands demand for nutrients (Marschner, 2011).

Host-pathogen interactions have been reported to cause changes in foliage nutrition status in many conifers (Singh & Bhure, 1974; Lambert et al., 1986; Lan et al., 2019). Infetstation of *Armillaria mellea* (Vahl ex Fr.) Kummer, a root pathogen on coniferous species induced reduction of foliar N, P, K, Mg, and Na concentrations and caused an increase in Mn, Ca, Fe, and Zn concentrations (Singh & Bhure, 1974). Disease severity and nutrient relations were examined on Douglas fir infected with *Pseudotsuga menziesii* (Mirb.) Franco, Swiss Needle Cast. Disease severity correlated with an increase of C, N, Na, K and S concentrations and a reduction of P and Mn (Lan et al., 2019). In addition, correlation was observed between sulfur decrease and infection level of Dothistroma needle cast fungus on *Pinus radiata* species (Lambert et al., 1896).

Many conifers including loblolly pine develop carbon-based soluble compounds such as total phenolics in their foliage as defensive compounds (Aspinwall et al., 2011). These phenolics

play a role in herbivore deterrence, nutrient cycling, litter decomposition, and soil carbon sequestration (Chung & Barnes, 1977). Increased total phenolic response has been observed in response to insects (Holopainen et al., 2006; Williams et al., 1994), or fungal infestation (Bahnweg et al., 2006) and foliar injury (Booker et al., 1996). Needles high in phenolic compounds have a reduce rate of litter decomposition that results in nutrients accumulating and decreasing the tree productivity. Therefore, it may be possible by assessing total phenolics can shed a light the overall tree productivity.

Brown spot needle blight (BSNB) has been observed in loblolly pine plantations in Alabama, causing repeated needle defoliation and tree mortality. Base on needle cast disease, it is possible that *L. acicola* would decrease loblolly pine foliar nutrient contents and increase defensive chemicals such as total phenolics response as a defensive mechanism against *L. acicola* infection and severity. To determine the effects of *L. acicola* on tree growth and physiological functions, this study examined the available nutrient contents and total phenolics in the foliage at BSNB infected plots.

4.2 Materials and Methods

4.2.1 Study area and sample collection

The study was conducted at seven permanent infected plots located in Chatom, Alabama, Washington County (31°27′42″N 88°14′53″W). Infected plots were of 2004, 2009, 2010, and 2012 plantations. Those plots were categorized as low, moderate, high or very high incidence stands based on the scoring of visual crown severity of infected trees and scoring of needle chlorosis and necrosis. Only 2004 plot received fertilizer treatment within three years of planting. No silvicultural treatments were applied on other experimental stands. A 0.22 mag caliber rifle was used to collect symptomatic foliage from the trees (Lan et al., 2019). To

determine baseline nutrient nutrients, a healthy plot with no symptomology was chosen nearly 1.5 km away from the infected sites and used to collect needles. Approximately 50 fascicles were collected per tree with 50 trees sampled in the study. Needle samples were oven-dried at 70°C for up to three days and then passed through a 0.5 mm mesh screen in a Wiley mill. Nutritional analyses were conducted by Waypoint Analytical Laboratory in Memphis, Tennessee, USA. Phosphorus (P) concentration was analyzed by combustion (Bryson et al., 2014) while nitrogen (N), potassium (K), magnesium (Mg), calcium (Ca), sulfur (S), sodium (Na), boron (B), zinc (Zn), manganese (Mn), iron (Fe), copper (Cu), and aluminum (Al) were determined by a wet digestion standard procedure (Bryson et al., 2014).

Total phenolic were determined from 40 needle samples collected from high incidence and low incidence plots as discussed above. Symptomatic needle samples were collected from both the upper and lower crown of sampled trees. Tree age, DBH and height were collected. Trees were ranked visually based on their severity of chlorosis and necrosis. Needle samples were kept in dry ice during field collection and stored at -20^{0}C when brought back in the laboratory. Samples were placed in a mortar along with liquid nitrogen and ground up with a pestle. Ground samples were then placed in a desiccator (Aspinwall et al., 2011) and total phenolic extractions were followed by 4.2.2.

4.2.2 Determination of soluble components

Two extraction procedures were used to ensure total phenolics recovery. The first extraction used 70% acetone and the second extraction used 250 ml sodium citrate in combination with 0.04% sodium bisulfite (pH 7) (Blum, 1997). Needle tissue was extracted 4 times with each 1ml solvent, then vortexed and incubated for 5 minutes at room temperature. Total phenolic extractions following both procedures were examined and compared. It was

observed that 70% acetone was 100% efficient compared to 250 ml sodium citrate with 0.04% sodium bisulfite which was 97-98% efficient.

Two 50 mg needle tissue per sample by whorl height were extracted 3 times with 70% acetone. The mixture was vortexed for 5 minutes at room temperature and centrifuged (max 16,000g) for another 5 minutes to pellet insoluble materials. Both sample supernatants were combined and the Folin-Ciocalteu method was used to determine total phenolics concentrations in the foliage (Booker and Maier, 2001). Supernatants were diluted at 1:10 with 70% acetone and duplicate 10 µl aliquots were mixed with 495µl of 0.25 N Folin-Ciocalteu reagent and 495µl of 1 M Na_2CO_3. With two aliquots and two 50 mg samples extracted, a total of four samples were analyzed for each sampled tree. Samples were vortexed and incubated at 25^0C for 30 min. The solution absorbance wavelength was recorded at 724 nm on spectrometer. Absorbance was based on a calculated catechin concentration equivalent (Booker & Maier, 2001) for which a standard curve was produced. Lastly, total phenolic concentrations were determined for the foliage and expressed as catechin equivalents i.e., mg g^{-1} dry needle mass.

4.2.4 Statistical analyses

Normality and homogeneity of data were verified using Shapiro-Wilk and Levene's tests. Two sample welch t-tests were performed to evaluate total phenolics differences between low and high infection needles. Simple linear regression models were performed to determine relationships of total phenolic concentrations with infection level, disease severity, tree age, whorl height, DBH and whorl height. Similarly, needle nutrients were analyzed using the ANOVA test. Data analyses were conducted by statistical R version 4.0.3 software. A simple linear regression was performed to determine the relationship between infection level and needle nutrient concentrations.

4.3 Results

Foliar Ca was the greatest (coefficient of variance [CV] > 38%) and Na was the least variable (CV < 0%) macronutrients in the asymptomatic trees (Table 4.1). Macronutrients in asymptomatic trees were ranked from most to least variable as follows Ca > N > S > Mg > P > K > Na. Conversely, foliar Na was the greatest (CV >70%) and foliar N was the least variable (CV<15%) macronutrients in the symptomatic trees. Macronutrient ranking from most to least variable as follows Na > K > Ca > P > Mg > S > N in the symptomatic trees (Table 4.2).

Nutrient content Al (CV > 130%) and Fe (CV > 68%) were the greatest variable micronutrients in the asymptomatic and symptomatic trees, respectively. However, asymptomatic and symptomatic trees showed Zn was the least variable macronutrient in their needles. Ranking of micronutrients followed as Al > Mn > Fe > Cu > B > Zn in the asymptomatic trees and Fe > Mn > B > Al > Cu> Zn in the symptomatic trees (Table 4.2).

Nitrogen content showed significant positive correlation (*p*-value=0.02; R^2=31%) with infection level. Marginal relationship was obtained for S concentration (*p*-value=0.05; R^2=14%) and *L. acicola* infection. A statistically significantly increased amount of Na (*p*-value=0.004; R^2=27%) concentration was detected for the infected trees when compared to asymptomatic trees (Figure 4.1). No statistically significant relationship was observed for other macronutrients such as S, K, Ca, and Mg contents with *L. acicola* infection severity. Macronutrients such as Al, Cu, Fe, Mn, and Zn were not correlated with *L. acicola* infection (Table 4.1 & Table 4.2). Only macronutrient B showed positive correlation (*p*-value=0.009; R^2=28%) with infection level (Figure 4.1).

Table 4.1: Descriptive statistics of foliar nutrition contents in loblolly pine needles (asymptomatic trees) collected in spring 2020.

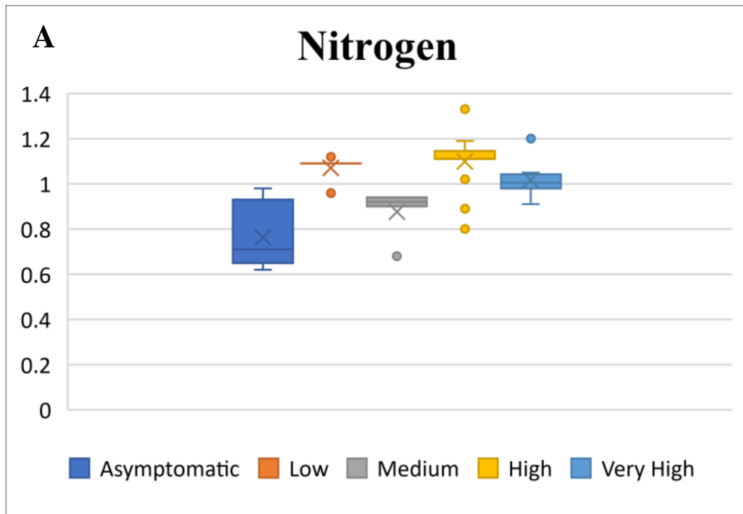
Nutrient element	[1]N	Mean	SD	CV (%)	Minimum	Maximum
Macronutrients (%)						
[2]N	15	0.77	0.15	19.97	0.65	0.94
S	15	0.09	0.01	13.32	0.08	0.10
P	15	0.08	0.01	6.93	0.08	0.09
K	15	0.40	0.02	3.85	0.38	0.41
Mg	15	0.16	0.02	10.83	0.15	0.18
Ca	15	0.32	0.13	39.28	0.25	0.47
Na	15	0.01	0.00	0.00	0.01	0.01
Micronutrients (mg/Kg)						
B	15	7.00	1.73	24.74	6.00	9.00
Zn	15	49.33	5.51	11.16	44.00	55.00
Mn	15	108.67	58.07	53.44	67.00	175.00
Fe	15	68.00	30.79	45.28	42.00	102.00
Cu	15	4.67	1.53	32.73	3.00	6.00
Al	15	97.67	132.00	135.15	17.00	250.00

[1]N, number of samples; Mean, an average of samples; SD, standard deviation; CV, coefficient of variation; Minimum, minimum value of samples; Maximum, maximum value of samples. [2]N, nitrogen; S, sulfur; P, phosphorus; K, potassium; Mg, magnesium; Ca, calcium; Na, sodium; B, boron; Zn, zinc; Mn, manganese; Fe, iron; Cu, copper; Al, aluminum. Needle samples were collected from both lower branches and upper branches of control and infected trees.

Table 4.2: Descriptive statistics of the foliar nutrition contents in loblolly pine needles (symptomatic trees) collected in spring 2020.

Nutrient element	[a]N	Mean	SD	CV (%)	Minimum	Maximum
Macronutrients (%)						
[b]N	35	1.03	0.15	14.18	0.68	1.33
S	35	0.10	0.01	14.39	0.08	0.13
P	35	0.08	0.01	18.74	0.04	0.09
K	35	0.47	0.18	38.14	0.28	1.01
Mg	35	0.14	0.02	16.64	0.10	0.20
Ca	35	0.40	0.13	32.04	0.20	0.81
Na	35	0.03	0.02	72.07	0.01	0.09
Micronutrients (mg/Kg)						
B	35	15.59	6.05	38.82	6.00	32.00
Zn	35	38.50	10.42	27.07	23.00	61.00
Mn	35	242.36	138.53	57.16	57.00	576.00
Fe	35	73.18	51.09	69.81	35.00	255.00
Cu	35	4.45	1.26	28.33	3.00	9.00
Al	35	354.86	134.93	38.02	151.00	733.00

[a]N, number of samples; Mean, an average of samples; SD, standard deviation; CV, coefficient of variation; Minimum, minimum value of samples; Maximum, maximum value of samples. [b]N, nitrogen; S, sulfur; P, phosphorus; K, potassium; Mg, magnesium; Ca, calcium; Na, sodium; B, boron; Zn, zinc; Mn, manganese; Fe, iron; Cu, copper; Al, aluminum. Needle samples were collected from both lower branches and upper branches of control and infected trees.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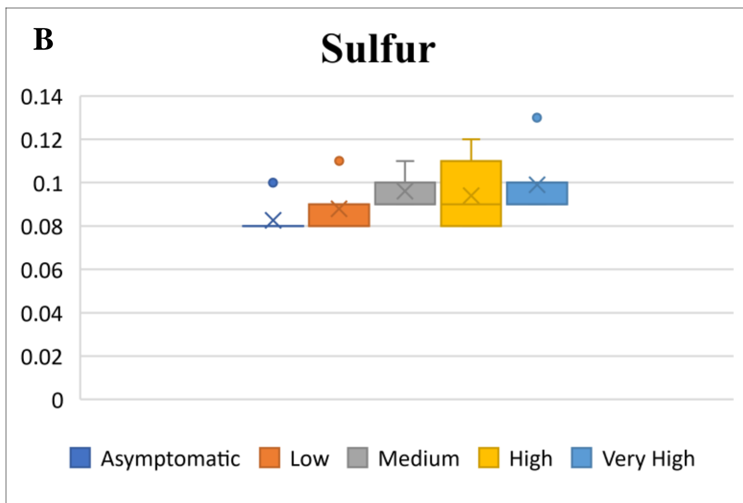
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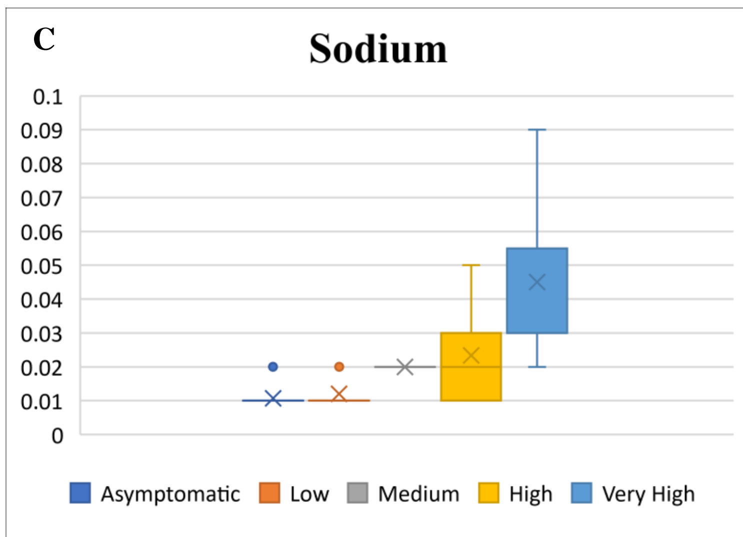
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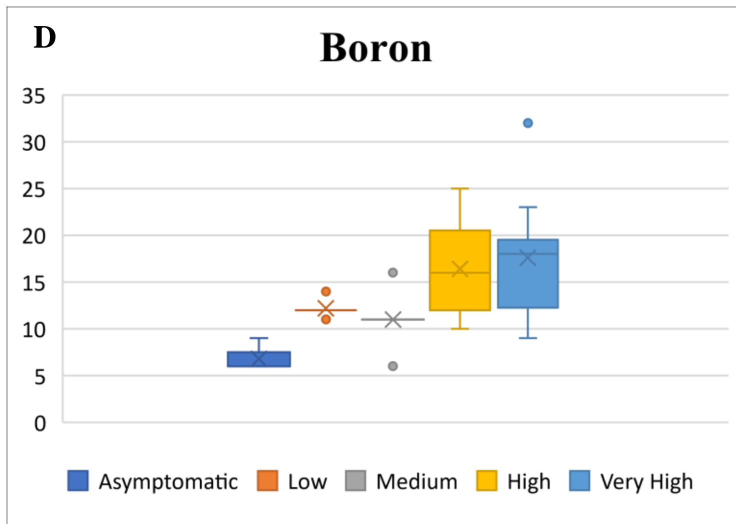

Figure 4.1 Relationships of foliar macronutrients
(A) N (*p*-value=0.02; R^2=31%)
(B) S (*p*-value=0.05; R^2=14%)
(C) Na (*p*-value=0.004; R^2=27%) and micronutrient
(D) B (*p*-value=0.009; R^2=28%) with BSNB severity.

No relationships were detected for nutrient ratios such as N/S, N/K, P/S, P/Zn, K/Mg, K/Mn, Ca/K, Fe/Mn, and Ca/Mg with the infection level. Only Ca/B showed significant negative correlation (*p*-value = 0.002; R^2=41%) with infection level (Figure 4.2). The foliar ratios showed a wide range of variation in the foliage that were not statistically significantly affected by the brown spot needle blight fungus, *L. acicola*. Thus, the range is not due to the presence of the fungus, *L. acicola*.

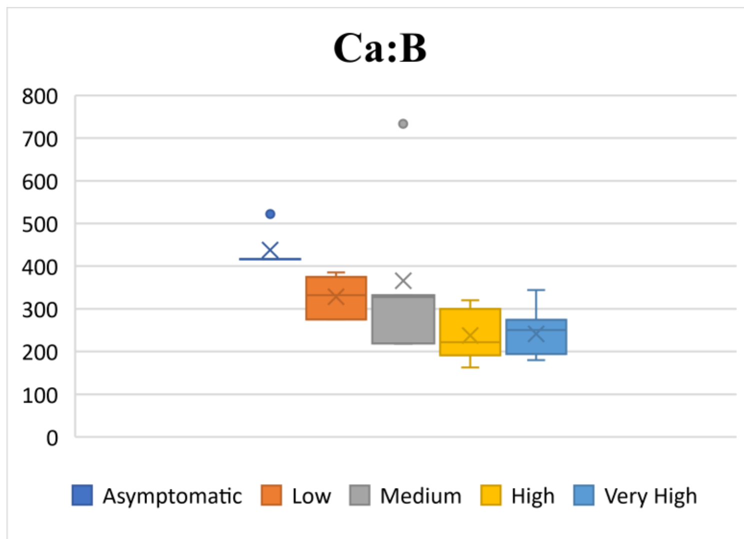

Figure 4.2 Relationship of foliar nutrient ratio Ca/B with BSNB severity.

Total phenolic concentrations in the needles were measured as a catechin equivalent (mg/ml) (Figure 4.3). There was a significant increase in total phenolic concentrations (*p*-value 0.006) on high incidence trees when compared to low incidence trees. Similarly, total phenolics increased in response to disease severity (*p*-value 0.002). Tree whorl height, height, and age were negatively associated with total phenolics. However, tree DBH was not significantly correlated with infection severity (*P*-value 0.218) (Figure 4.4).

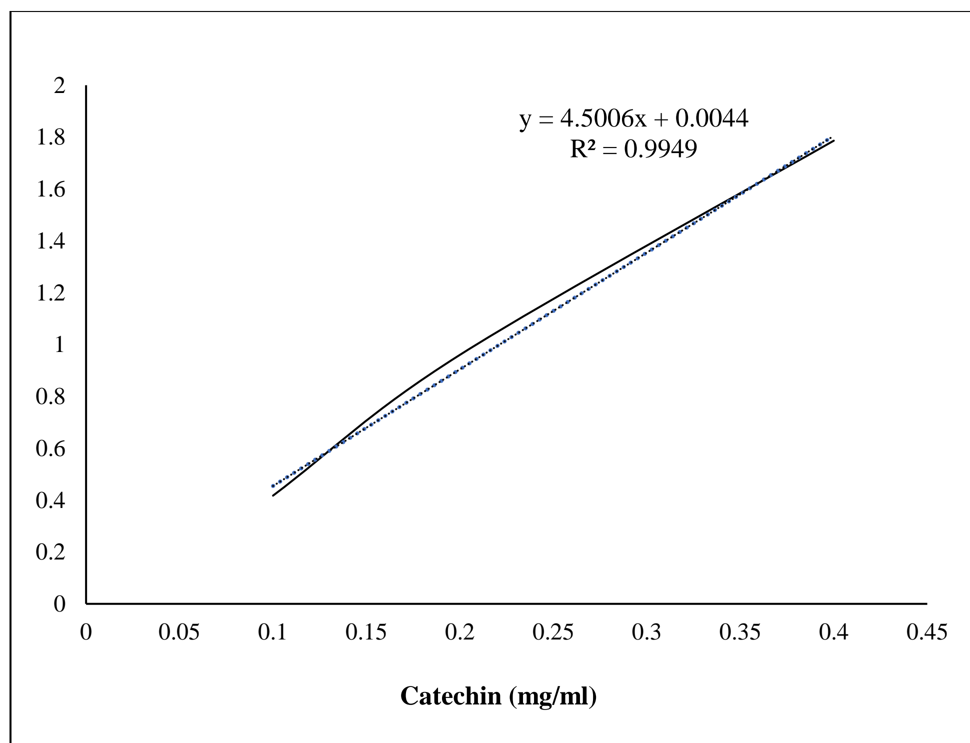

Figure 4.3 Catechin calibration curve for total phenolics contents

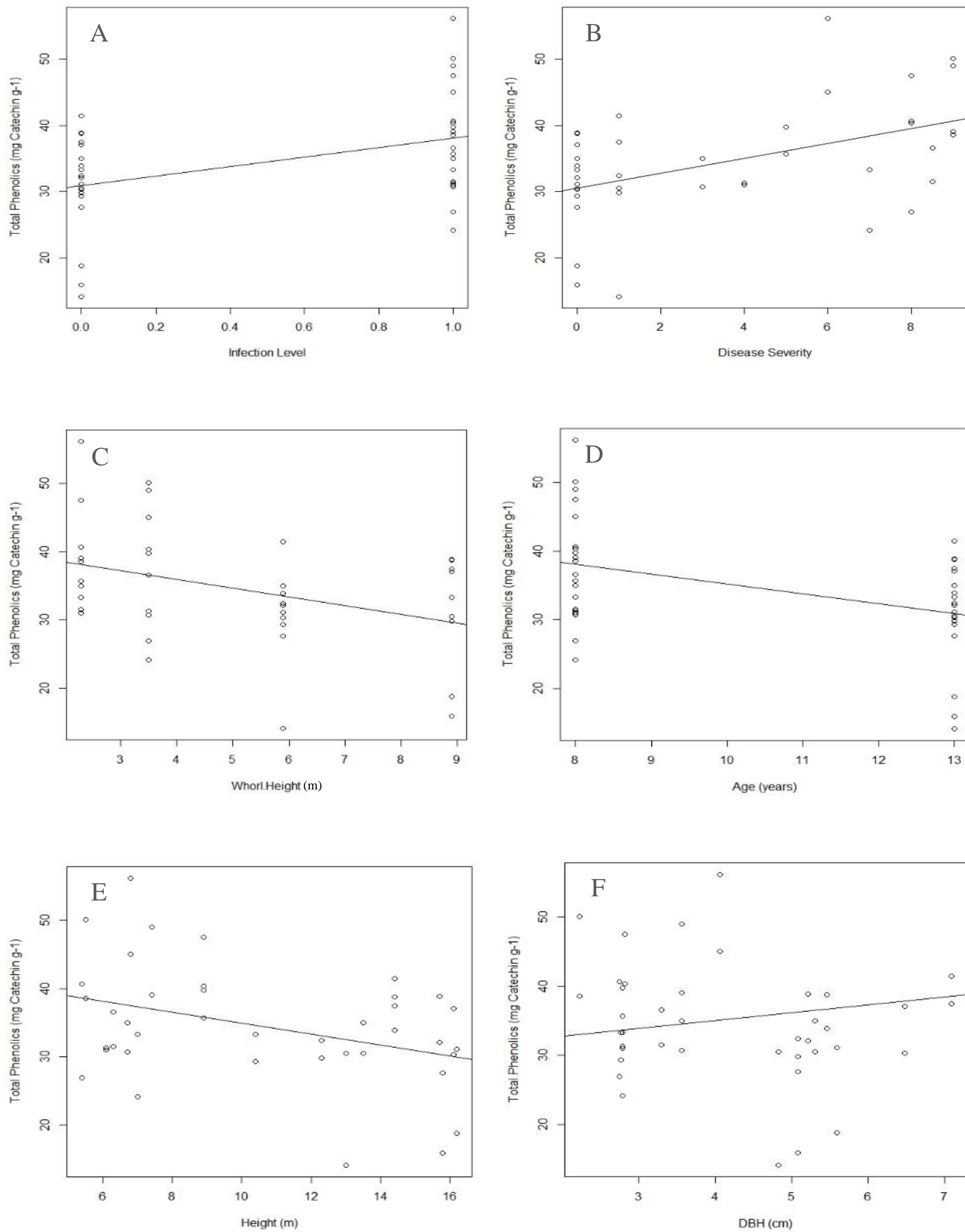

Figure 4.4 Relationships between total phenolics concentration and (A) infection level (*p*-value: 0.005954) (B) disease severity (*p*-value: 0.00208) (C) whorl height (*p*-value: 0.01414) (D) age (*p*-value: 0.005954) (E) height (*p*-value: 0.01759) and (F) dbh (*p*-value: 0.218).

4.4 Discussion

Needle nutrient contents of N, Na, B and P were positively correlated to infection severity. Previous studies examined responses of foliar nutrient concentrations either with fertilization application or as an effect of thinning treatment (Fox et al., 2007; Gurlevik et al., 2003). In the study, one experimental plot was fertilized which was planted in 2004 and fertilized after three years of planting. Other six experimental plots didn't receive any fertilization or silvicultural treatments. Therefore, it is difficult to extrapolate fertilizer application relation to BSNB disease severity. However, needle disease impacts are varied with plantation attributes such as foliar nutrient application (Lan et al., 2019). Therefore, foliar nutrients may influence BSNB severity and interacts with *L. acicola* severity which requires further investigation.

Other nutrient content P, K, Mg, Ca, Zn, Mn, Fe, Cu and Al showed variations among infection levels. Foliar nutrient deficiency or abundance are related to soil nutrient availability (Pietrzykowski et al., 2013). Therefore, those variations of nutrient contents might be related to spatial variations of the plots. Albaugh et al., (2010) reported that loblolly plantations showed highly variable foliar micronutrients comparative to foliar macronutrients across the southeastern United States. Variations also could be due to the severity of infection or interaction between severity of the infection and some other variable related to tree vigor (Vose et al., 1994; Sayer et al., 2009). For example, crown class (dominant/codominant vs intermediate/suppressed) could affect light availability to foliage, C-fixation, and C allocation to defense (Niinemets et al., 2002; Vose et al., 1994). Variability of N might be due to one of these factors or a combination of these factors. A high amount of infection is going to be more detrimental under poor light conditions because the defense would be compromised by available C. In a similar manner, tree leaf area, live crown ratio, and crown condition are other variables that could interact with

infection severity to affect vigor. Higher concentrations could be driven by a loss of leaf area before sampling that resulted in a concentration effect in the foliage. High variability of the "affected" trees suggests that these trees might be in varying stages of vigor loss.

Some of the variability could be attributed to the age class of foliage sampled- especially for immobile nutrients like Ca, B, and Mn. In loblolly pine trees, variations of needle nutrient contents are driven by season, needle age, soil, and crown position. Older needles contain more nutrients than young needles. Seasonal variations such as spring and winter also contribute to nutrient variations. Soil pH also changes the form of nutrients in the soil and affects their nutrient availability to, and uptake by the trees (Schultz, 1997). Percentage of N, Ca and Mg are greater in upper crown foliage and percentage of P and K are greater in lower crown foliage (Wells & Metz, 1963). Apart from that, needle morphology (needle weight and length), and growth of tree stands (average height and DBH of the stands) are related to needle nutrient variability. Since the study has found lots of variation of foliar nutrition data in loblolly pine needles, the study requires further investigation to make a conclusion about foliar nutrients and BSNB severity.

The study detected a positive correlation of total phenolic in loblolly pine foliage with infection level and disease severity. Total soluble phenolics in the needles are linked to plant defense capacity against fungi, insects, or microbial infestation. It is expected that infected trees could increase defensive chemicals in the foliage to defense against *L. acicola*. Total phenolic decreased with increasing tree height, whorl height, and age. Although *L. acicola* infection progressed from lower canopy to higher canopy (see chapter III), needle pathogens are likely to stay more on the lower canopy due to high relative moisture availability. Higher concentrations of total phenolics in the lower canopy needles might be in response to severe *L. acicola* infection in the lower crown.

4.5 Conclusion

Foliar concentrations such as N, B, Na and S and nutrient ratios such as Ca/B were affected by *L. acicola* infection. High incidence trees had higher total phenolic when compared to low incidence trees perhaps as a mechanism to defend against *L. acicola* infection. The higher concentration of foliar nutrients in samples are most probably a function of loss of leaf area due to the defoliation. Variability among foliar concentrations could also be influenced by other factors such as soil pH, tree vigor and crown condition.

Chapter Five

Prediction of loblolly pine defoliation severity associated with changes in pathogen pressure in response to climate change in Alabama

Abstract

Loblolly pine defoliation was first noticed in 2013 and currently has been observed on more than 25,000 hectares of loblolly plantations in Alabama. *Lecanosticta acicola* is the predominant pathogen causing loblolly pine defoliation in combination with common needle cast pathogens in Alabama (see Chapter II). Understanding abiotic factors such as temperature, precipitation, and moisture influence on loblolly pine defoliation is important to predict defoliation severity in following years in Alabama. Weather data were collected from 14 land-based weather stations located within a 10 m radius of the infected stands. Forty years of long-term regional weather data indicates that there was an increase temperature and a decrease precipitation in this region from 1981 to 2019. Data confirmed that the previous year's Max. February, Max. June and Min. May temperature and increasing July and decreasing fall precipitation are the best predictors of defoliation severity in Alabama. Increasing summer months precipitation and temperature are expected to favor loblolly pine defoliation the following years. Climatic models were developed to aid private landowners and forest managers to adjust their management strategies accordingly.

Keywords: Loblolly pine, defoliation, climatic change, *Lecanosticta acicola*

5.1 Introduction

Lecanosticta acicola is a predominant pathogen associated with brown spot needle blight in both natural and plantation loblolly pine stands in Alabama. From the first half of the 20th

century, *L. acicola* has been a persistent problem for grass stage longleaf pine seedlings in the southeastern United States (Siggers, 1944). Later this disease was found to cause serious damage to scotch pine Christmas tree plantations in Wisconsin and Minnesota (Skilling & Nicholls, 1974).

Historically, this fungal pathogen was found to be associated with loblolly pine trees in its native range (Siggers, 1944; Hedgcock, 1929) but its impacts on tree health were never assessed as damage and tree mortality were not observed until recently. *Diplodia sapinea*, *Lophodermium* spp., and *Coleosporium* spp. pathogens have been found associated with loblolly pine defoliation. Two other fungi *Rhizosphaera kalkhoffii* and *Sydowia polyspora* have also been recovered in association with *L. acicola.* These fungi may affect disease severity in the infected stands. The understanding of abiotic factors such as temperature, precipitation and relative humidity is important as they may drive the emergence and spread of disease.

Needle disease and host susceptibility are correlated to climatic factors such as temperature and moisture (Wyka et al., 2017; Broders et al., 2015; Munck & Burns, 2012). Changing environmental conditions such as increasing temperature, precipitation, and humidity favors foliar fungal disease development by altering fungal virulence and behavior (Skilling and Nicholls, 1974). Moreover, temperature and moisture directly influence pathogen distribution and movement into area where regional climates are conducive for spore reproduction and survival (Sturrock et al., 2011).

Increasing overnight minimum temperature, summer and spring rainfall following needle wetness were found positively correlated to *Dothistroma septosporum* infection (Woods et al., 2005). Similarly, increasing winter mean temperature and spring precipitation was projected to increase Swiss needle cast disease in the Pacific Northwest (Manter et al., 2005). In the northern

United States, white pine needle damage resulting from multi-fungi interactions were expected to become worsen with increasing spring and summer rainfall and winter temperature (Wyka et al., 2017). A study tested climatic influence on the distribution of *Fusarium* spp. and found that differences in *Fusarium* communities were resulted from differences in temperature which mimicked the natural communities of *Fusarium* spp. found in similar temperature gradients (Saremi et al., 1999).

Predictions of pathogen behavior to changing climate conditions are challenging and constitute a high degree of uncertainty. In forest settings, it is even more complex because of long-lasting tree life versus the short life span of pathogens (Burdon et al., 2021; Gray et al., 2013). The objectives of this study were to (a) determine if climatic patterns drive the recent emergence and spread of loblolly pine defoliation and (b) develop a climatic regression model to predict disease severity of loblolly pine to aid forest managers to adjust management strategies accordingly.

5.2 Materials and Methods

5.2.1 Visual rating, and mapping of pathogen distribution

Total 32 brown spot needle blight infected loblolly plots were sampled from March to November. Needle samples were collected from the number of 212 symptomatic trees using a 0.22 mag caliber rifle. Samples were placed in a cooler and brought back to the laboratory in Auburn. Rating of the crown infection was scored as follows (a) less than one-third of the crown infected (<1/3) (b) one-third to two-thirds of the crown infected (1/3 to 2/3) and (c) more than two-thirds of the crown infected (>2/3). Tree and site information such as aspect, slope, recent silviculture were also collected (Wyka et al., 2017).

5.2.2 LPND infected sites and climatic records

Climate data available online in National Climatic Data Center were collected to obtain regional historical weather data and station history. Daily summary observations of temperature, precipitation and relative humidity around infected stands were collected. Since preceding year temperature, moisture and precipitation have been shown to be the best indicators of following years defoliation (Munck & Burns, 2012; Wyka et al., 2017), daily maximum and minimum temperature, the sum of seasonal precipitation in the year preceding scoring defoliation ratings of infected trees were obtained from NOAA online data. Counties with available weather stations were identified and measured the distance from the infected stands using an interactive mapping tool. Weather data were collected within 10 miles radius of infected stands except for two stations due to data availability. To collect relative humidity data, POWER Data Access Viewers were used. Data points were selected based on the longitude and latitude of infected stand. Missing data were adjusted, maximum and minimum temperature were averaged, and the sum of precipitation was estimated.

5.2.3 Statistical analyses

To build climatic regression models, forty-nine climatic variables were included in the model. Average maximum and minimum temperature of the following: spring (March to May), summer (June to July), modified spring (May to August), and winter (December to February). Average relative humidity and cumulative precipitation were included as follows: spring, summer, modified spring and winter. Individual months within these seasons were also collected. To predict the best-fitted regression model, the stepwise selection was performed to choose variables in the model. Final linear regression models were performed with eleven variables in the model since they explained relationships with loblolly pine defoliation severity.

Several statistical analyses such as Akaike Information Criterion (AIC) were used to verify small sample correlation, Variation Inflation Factor (VIF) was used to check collinearity of the variables, adjusted R^2, root-mean-square error, and model parsimony were tested to develop confidence on the models.

5.3 Results

5.3.1 Long-term regional weather data

Forty years of regional long-term weather data revealed that increasing temperature and decreasing precipitation in the region during loblolly pine growing season. About 1^0C shifts of mean temperature from 1980 to 2019 (Figure 5.3). Rainfall patterns decreased in infected sites. Cumulative rainfall decreased from 1400 mm to 1300 mm from 1981 to 2019 (Figure 5.4). However, no changes in relative humidity from 1981 to 2019 (Figure 5.5).

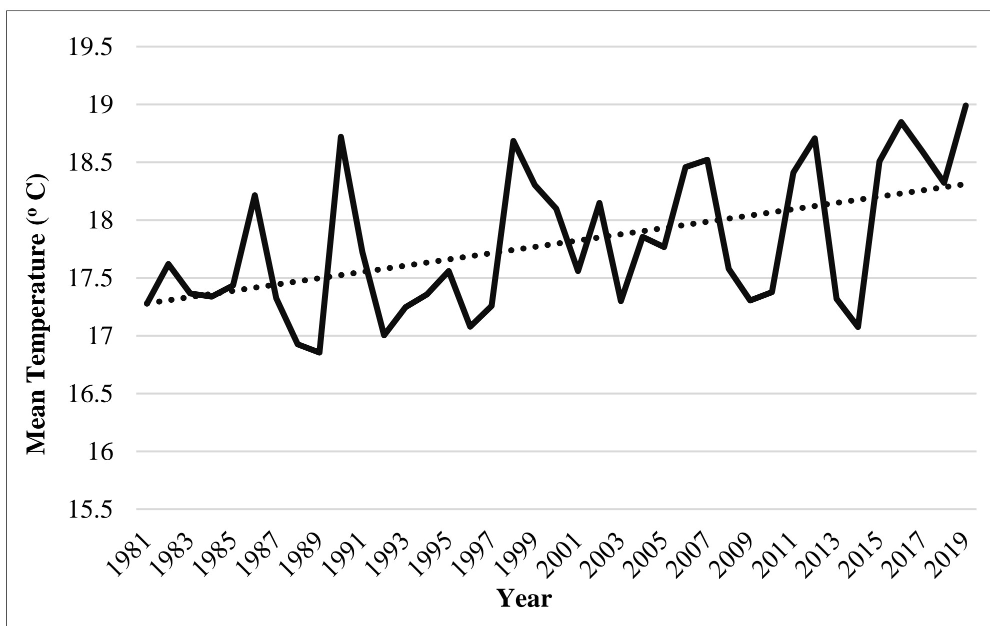

Figure 5.1 Average temperature during loblolly pine growing season at 11 infected sites in Alabama

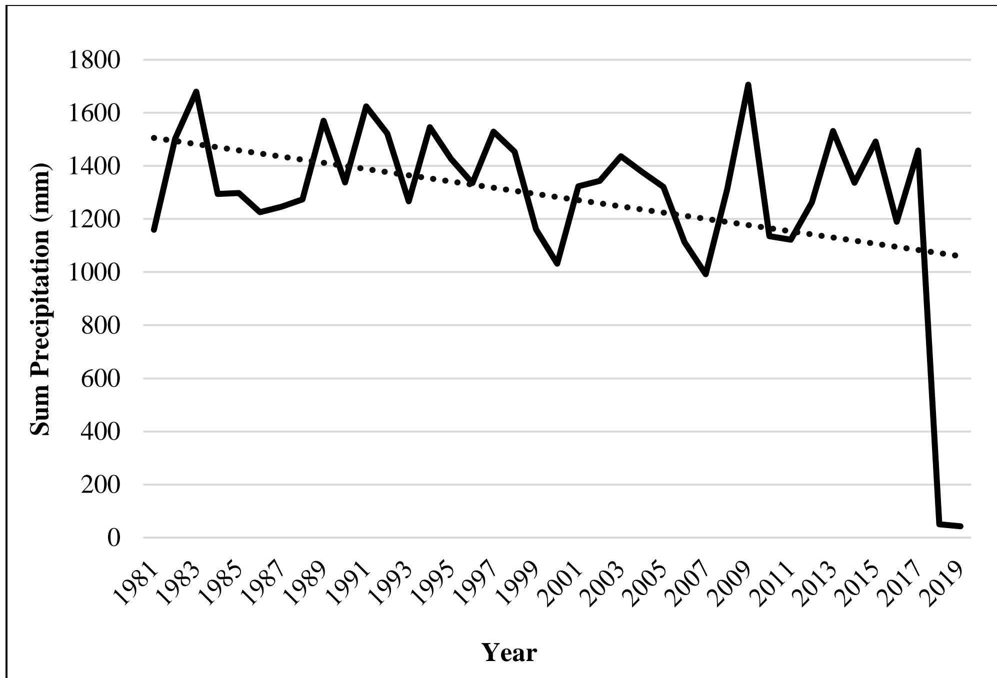

Figure 5.2 Cumulative precipitation during loblolly pine growing season at 11 infected sites in Alabama

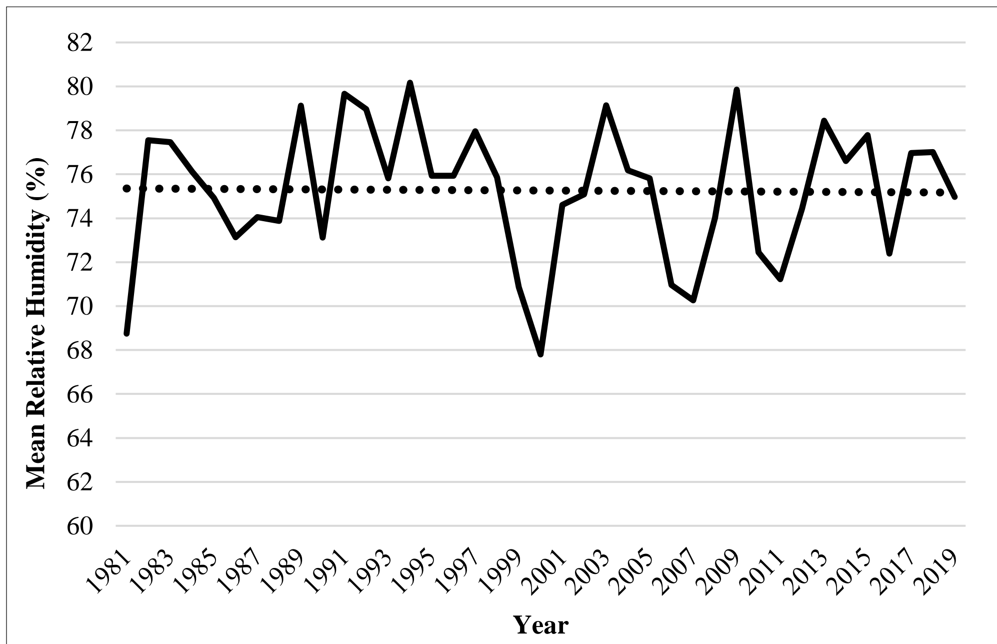

Figure 5.3 Mean relative humidity during loblolly pine growing season at 11 infected sites in Alabama

5.3.2 Climatic regression model to predict loblolly pine defoliation severity in future

One factor model predicted that decreasing May minimum temperature was negatively correlated to loblolly pine defoliation severity in the coming year. May minimum temperature was added with February maximum temperature in the 2-factor model and model significance was improved from 37% to 66%. The 2-factor model found that May minimum temperature and February maximum temperature are the best predictors of loblolly pine defoliation severity.

The best 3-factor model included sum fall precipitation as well as other two predictors i.e., May minimum temperature and February maximum temperature in the model. Lowering cumulative fall precipitation in the preceding year predicted increasing loblolly pine defoliation severity in the following year. Following that, the best 4-factor model added cumulative July precipitation and improved the model significance by 4% and predicted loblolly pine defoliation severity related to the previous year's decreased cumulative fall precipitation. The best 5-factor model included May minimum temperature, February maximum temperature, June maximum temperature, cumulative July precipitation and cumulative fall precipitation to predict loblolly pine defoliation events in following year (Table 5.1). The final model revealed that increasing summer precipitation and temperature are the best climatic factors that would affect LPD in following years.

Table 5.1 Best-fit regression models to predict loblolly pine defoliation severity.

Model, variables	**Parameter estimate**	**SE£**	**Prob. > t**	**VIF¥**	**Model Prob. > F**	**Adj. R^2**	**RMSE ¢**	**AIC€**
5-Factor								
Intercept	0.45597	1.31881	0.7355					

Max February T	0.01921	0.00500	0.0023	4.21				
Max June T	0.03203	0.01507	0.0550	3.76				
Min May T	-0.06964	0.00802	<.0001	3.10				
Sum July P	0.03583	0.00941	0.0025	4.64				
Sum Fall P	-0.00838	0.00557	0.1579	4.27				
Total model					<.0001	0.8874	0.00113	-62.6345
4-Factor								
Intercept	3.14165	0.42606	<.0001					
Max February T	0.02228	0.00539	0.0012	4.21				
Min May T	-0.06716	0.00894	<.0001	3.10				
Sum July P	0.02585	0.00920	0.0147	4.64				
Sum Fall P	-0.01535	0.00507	0.0097	4.27				
Total model					<.0001	0.8450	0.00224	-74.789
3-Factor								
Intercept	3.07400	0.51974	<.0001					
Max February T	0.02529	0.00646	0.0016	4.21				
Min May T	-0.06785	0.01092	<.0001	3.10				
Sum Fall P	-0.01305	0.00612	0.0511	4.27				
Total model					0.0002	0.7508	0.00845	-66.5523
2-Factor								
Intercept	2.81150	0.56158	0.0002					
Max February T	0.02623	0.00717	0.0023	4.21				
Min May T	-0.06697	0.01214	<.0001	3.10				
Total model					0.0002	0.6698	0.01132	-57.6458

1-Factor								
Intercept	2.75236	0.74780	0.0020					
Min May T	-0.03746	0.01209	0.0069	3.10				
Total model					0.0069	0.3749	0.01852	-42.4364

P=sum precipitation, T=average temperature; £SE=Standard error; ¢RMSE=Root Mean Square Error; ¥VIF=variance inflation factor; €AICc=Akaike information criterion. (Seasonal temperatures were averaged and precipitation was summed up in the year before defoliation ratings of the trees were conducted such as winter (December, January, February); spring (March, April, May); mod. spring (May, June, July); summer (June, July, August).

5.4 Discussion

Lecanosticta acicola was the primary pathogen causing loblolly pine defoliation in Alabama. This pathogen wasn't recovered from other infected sites of Louisiana, Georgia, and South Carolina and Mississippi (except one site in Greene). Infected loblolly pine trees in Louisiana, Georgia, Mississippi and South Carolina didn't mimic the symptomology of diseased loblolly trees in Alabama. Therefore, those sites were not included in the model to predict loblolly pine defoliation severity. Temperature has increased and precipitation has decreased in infected sites in Alabama. The results indicate that changing climatic conditions in Alabama are likely to have an impact on loblolly pine defoliation in Alabama since fungal growth, reproduction and spread are associated with increasing temperature (Wyka et al., 2017; Tainter and Baker, 1976). The study developed a model and identified that February, May and June temperature and July and fall months precipitation are the best predictors of loblolly pine defoliation severity in following years. Wyka et al., (2017) reported that increasing temperature and rainfall have been shown to drive emergence of white pine needle disease in the northeastern United States. Similarly, a local increase in spring precipitation and winter mean temperature are the best predictors to determine increase Swiss needle cast severity in western North America (Manter et al., 2005). Moreover, increasing overnight temperature and summer and spring

rainfall are identified as the driving factors to increase *D. septosporum* severity to lodgepole pine plantations across its native range in northeastern British Columbia, Canada (Woods et al., 2005). All these findings supported the study findings and emphasized that increasing summer months temperature and precipitation provide favorable environmental conditions for *L. acicola* pathogen to cause loblolly pine defoliation disease emergence and outbreak.

Changing climatic conditions i.e., temperature and precipitation favored the spore development, dispersal, and infection potential of needle pathogens (Sturrock et al., 2011). Although it is difficult to establish a causal relationship between local biological trends and climate change, a mechanistic relationship exists between an observed climate trend and host-pathogen interactions (Woods et al., 2005). Wet moist site conditions and poor drainage might have posed stress to the trees and increased temperature might have facilitated increased fungal disease by increasing fungal growth and reproduction eventually leading to increased pathogen pressure to loblolly trees (Hansen, 1999). Since fungal loads, dispersal are correlated to changing temperature and precipitation (Manter et al., 2005; Woods et al., 2005; Wyka et al., 2017), temperature and precipitation may modulate *L. acicola* spore development, virulence, and infection potential which might have resulted in a recent emergence and outbreak of loblolly pine defoliation. Inoculum pressure is the most basic requirement to overcome the host defense (Agrios, 2005). Presence of abundant loblolly pine might have allowed the increasing number of *L. acicola* spore development which may have helped the fungus to overcome the defense of host tree. A wide geographic distribution of host species favored the potential distribution of fungi through air-currents and rain-splash spores (Siggers, 1944). The study concluded that the high occurrence of loblolly pine trees coupled with changing seasonal temperature and precipitation has driven the current loblolly pine defoliation emergence and outbreak.

This disease is expected to increase in a broader geographic region and cause tree mortality if there are favorable environmental conditions. The uncertainty related to climate change associated with loblolly pine defoliation requires regular monitoring, planning, and mitigation strategies as well as linking them in forest management policies, and decision making.

5.5 Conclusion

The study identified the role of temperature and precipitation influence on loblolly pine defoliation in Alabama. Warmer and wetter summer and drier spring and fall are likely to favor loblolly pine defoliation severity in following years. The high occurrence of loblolly pine coupled with increasing temperature and precipitation are at the greatest risks in this region. Short-term and long-term changes in climatic conditions can result in a disease outbreak. However, it is established that climate change can make trees more vulnerable to damage by insects, pests, and pathogens and especially, from those which have not been considered a threat due to unfavorable climate. The study requires incorporation of host layer mapping to better predict the disease patterns in Alabama. Southern forest managements are based on an implicit assumption that management will increase yield. However, this could be contributed to high disease occurrence.

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Appendix A-C

Appendix A

Table 1. Fungi recovered from symptomatic loblolly pine needles either from cultured isolates or from fruiting bodies in the study.

Sample ID	DNA[a] Source	Species	GeneBank[b] Accession
LP-11	Mycelia	*Hendersonia pinicola*	MT994893
LP-14	Fruit body	*Lecanosticta acicola*	MW030654
LP-16A	Mycelia	*Meyerozyma carphophila*	MT994890
LP-23	Mycelia	*Myrmaecium rubricosum*	MT997917
CTM-04	Mycelia	*Rhizosphaera kalkhoffii*	MT997918
CTM-12	Mycelia	*Alternaria tenuissima*	MT997910
CTM-13	Fruit body	*Lecanosticta acicola*	MW030655
CTM-24	Mycelia	*Alternaria alternata*	MT997911
CTM-14	Fruit body	*Lecanosticta acicola*	MW030656
CTM-25	Mycelia	*Talaromyces purpureogenus*	MT997912
CTM-26	Mycelia	*Pestalotiopsis* sp.	N/A
LP-06	Mycelia	*Meyerozyma caribbica*	MT994894
LP-10	Mycelia	*Rhizosphaera kalkhoffii*	MT997913
LP-15	Fruit body	*Lecanosticta acicola*	MW030657
LP-16	Fruit body	*Lecanosticta acicola*	MW030658
DS-17	Fruit body	*Lecanosticta acicola*	MW030659
WD-13	Fruit body	*Lophodermium* sp.	N/A
CA-05	Fruit body	*Lophodermium* sp.	N/A
LP-73	Mycelia	*Lophodermium macci*	MF540559
LP-154	Mycelia	*Lophodermium australe*	MK762602
DS-12	Mycelia	*Talaromyces purpureogenus*	MT997914

LP-31	Mycelia	*Lecanosticta acicola*	MW030660
LP-12	Mycelia	*Penicillium* sp.	MT997915
DS-26	Mycelia	*Rhizosphaera kalkhoffii*	MT994892
LP-15	Mycelia	*Talaromyces amestolkaie*	MT997916
LP-18	Mycelia	*Neopestalotiopsis clavispora*	MW055444
CTM-15	Mycelia	*Talaromyces amestolkaie*	MT997919
CTM-16	Mycelia	*Epicoccum nigrum*	MT997920
CTM-17	Mycelia	*Paraconiothyrium brasiliense*	MT997921
CP-12	Fruit body	*Coleosporium* sp.	N/A
CTM-26	Fruit body	*Coleosporium sp.*	N/A
CTM-18	Mycelia	*Preussia isomera*	MT997922
CTM-24	Mycelia	*Cladosporium cladosporioides*	MT994891
CTM-26	Mycelia	*Meyerozyma carphophila*	MT994888
CTM-02	Mycelia	*Sydowia polyspora*	MW055447
DS-01	Fruit body	*Lecanostica acicola*	N/A
DS-02	Fruit body	*Lecanostica acicola*	MW030661
DS-13	Fruitbody	*Lecanostica acicola*	MW030662
LTN-08	Fruit body	*Coleosporium* sp.	N/A
LTN-10	Fruit body	*Coleosporium ipomoeae*	MW147070
LTN-11	Mycelia	*Hormonema macrosporum*	MT994897
LTN-17	Mycelia	*Trichoderma caerulescens*	MT994898
LTN-22	Mycelia	*Kwoniella pini*	MW055445
LTN-23	Mycelia	*Sydowia polyspora*	MW055448
LTN-24	Mycelia	*Hendersonia pinicola*	N/A
LTN-08	Fruit body	*Lecanostica acicola*	MW030663
LTN-12	Fruit body	*Lecanostica acicola*	N/A
LP-12	Mycelia	*Sordaria fimicola*	MT994895

LP-14	Mycelia	*Talaromyces purpureogenus*	MT994896
LP-20	Fruit body	*Hormonema macrosporum*	MT994897
LP-21	Mycelia	*Trichoderma caerulescens*	MT994898
JNK-07	Mycelia	*Diplodia seriata*	MT997923
JNK-08	Mycelia	*Cladosporium anthropophillum*	MT997924
JNK-12	Mycelia	*Diplodia sapinae*	MT994890
JNK-03	Mycelia	*Cladosporium cladosporioides*	MT994899
JNK-17	Mycelia	*Ramularia weberiana*	MT994900
LP-07	Mycelia	*Talaromyces purpureogenus*	N/A
LP-12	Mycelia	*Sardiniella celtidis*	MW055441

[a]Indicates DNA source either from grown pure cultures or fungal fruiting bodies. [b]Denotes identification was conducted by morphology; no molecular analysis was performed for the fungi labelled as N/A. N.B Fungal DNA directly amplified from needle samples was not included in this list.

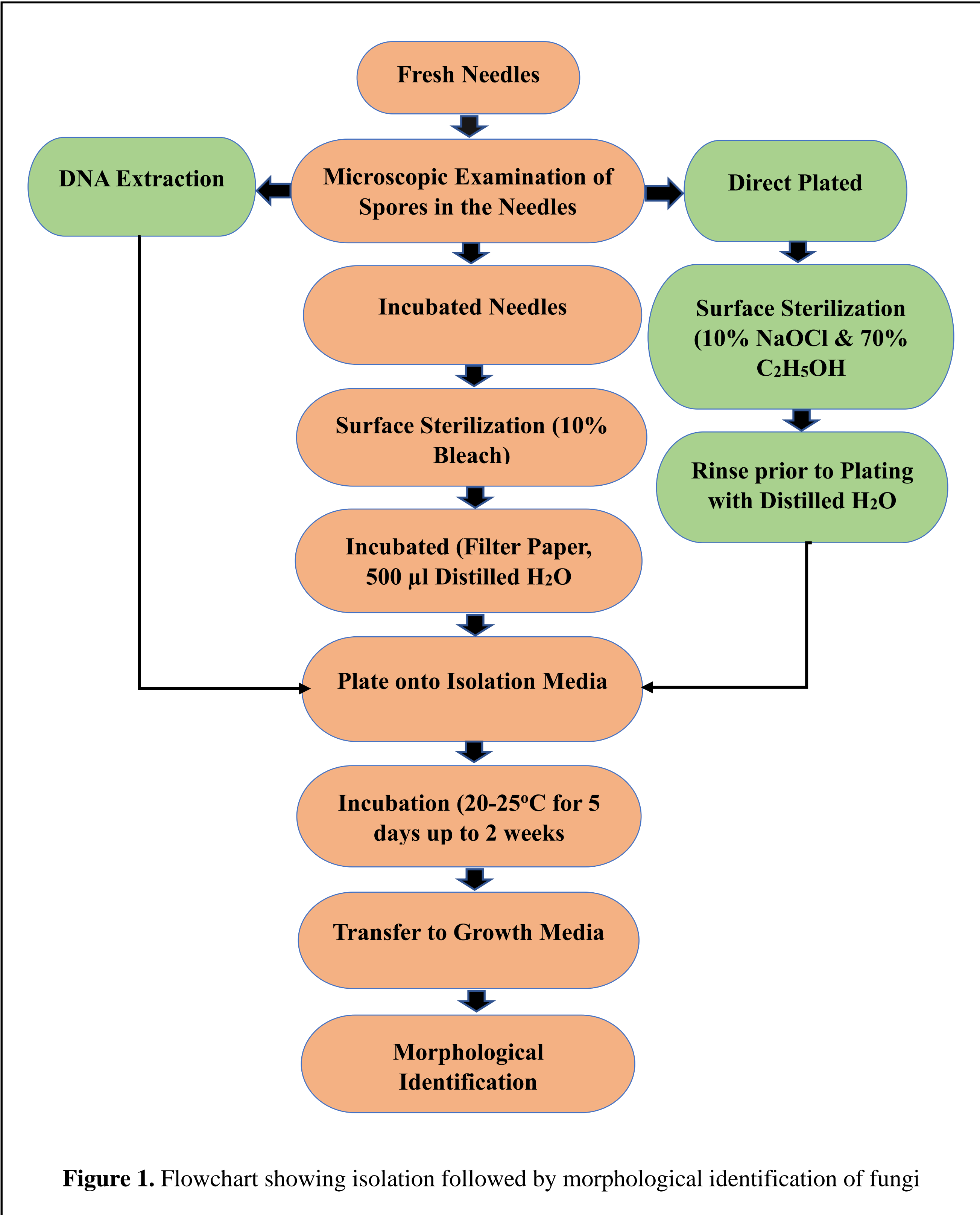

Figure 1. Flowchart showing isolation followed by morphological identification of fungi

Appendix B

Information Sheet

Host: *Pinus* Species (Pine trees)

Alternate Host: Herbaceous plant – *Solidago* (Goldenrod), *Aster* (Aster)

Needle sample: Symptomatic

Pathogen: Unknown

Impact: Growth retardation, tree mortality and sporadic occurrence

Needle damage picture:

Figure 1. Needle symptoms (A) yellow or brown discoloration of needles, die-back of the apex of the needles (B) tree crown looks thin or empty (C) reddening of the needles (D) dead needles on the lower side of a branch (E) exudation of resin from base of the needles (F) affected lower canopy

of a tree (G) black round spots on needles (H) fruiting bodies on the needles & (I) symptomatic leaves of alternate host *Solidago* (Goldenrod)

B. Pine Needle Sample Collection and Submission Guide

Samples are recorded and processed routinely by the date and time in which they are received. All samples will be initially processed within five days of receipt with results available twenty-one days after processing. Some laboratory diagnostic techniques take longer than others, which may affect result punctuality.

Sample Collection

Tree disease diagnosis is largely dependent on the quality of the sample and on the relevant information provided by the submitter. Samples must be of sufficient quality and quantity to allow for proper laboratory testing and pertinent information, such as sample tree identification, is essential.

1. Samples should be collected from symptomatic trees showing thinning/transparent crowns, and foliage discoloration.

2. Samples should NOT be collected from dead trees; determining the causal agent from such trees is highly unlikely.

3. Submit a generous amount of sample material to allow for all required laboratory processes. Remove needles from the branch. Sterilize all tools between trees

4. Keep all samples separated and cool until ready to be shipped, do not expose collected needles to high temperatures. Place samples in an envelope or paper bag

5. Include the following information: Tree species and age, Recent Silviculture, Location (GPS coordinates), Date of Collection, Stand Prevalence and Severity.

6. Samples may be delivered in person or mailed to the Forest Health Dynamics Laboratory, 602 Duncan Drive, Ste 3301, Auburn University, AL 36849.

7. When submitting samples by mail, either mail them early in the week to avoid weekend layovers or use an overnight service. Send us an email letting us know that you have sent us some samples, include pictures of the damage in the email. Dr. Lori Eckhardt (eckhalg@auburn.edu) and/or Luis Mendez (lmm0081@auburn.edu)

8. Samples should be mailed in an appropriately sized box, with padding, or in a padded envelop. Please write on the outside "Refrigerate Upon Arrival".

9. Complete a "**Tree Disease Diagnostic Form**" for each sample.

Contact us if you would to like to become part of a study where we would come to your property to collect monthly samples over the course of a year. This will help us in our studies to solve this problem!

Contact: Professor Dr. Lori Eckhardt

Phone: (334) 332-6462 **Email:** eckhalg@auburn.edu

Tree Diagnostic Form

Forest Health Cooperative
Forest Health Dynamics Laboratory
602 Duncan Drive, Suite 3301
Forestry and Wildlife Sciences Building
Auburn University, Auburn, AL 36849-5418
Luis Mendez (lmm0081@auburn.edu)
334-844-1538

Diagnostic Laboratory Use Only:

Date Received: __________

Received By: __________

Tree Disease Diagnostic Form

Please include ALL relevant data; maintain an office copy: Submit original copy with information

Date Sample Collected: _____________ Date Sample Shipped: ___________ No. of Samples: ______

Sample Location-County, State: ____ Sample ID: ______

______

______

	Submitter Information	**Results Recipient** (If different than submitter)
Name:	________________________	____________________________
Company:	________________________	____________________________
Address:	________________________	____________________________
City/Zip:	________________________	____________________________
Phone No:	________________________	____________________________
Fax No:	________________________	____________________________
Email:	________________________	____________________________

Tree Species: ___Loblolly ___Longleaf ___Shortleaf ___Slash

Site Location: ___Forest ___Nursery ___Greenhouse ___Other

Aspect: ___N ___NE ___E ___SE ___S ___SW ___W ___NW

Slope: ___0-5% ___5-10% ___10-15% ___>15%

Soil Type: ___Sand ___Silt ___Clay ___Loam

Age of Planting: ___0-10 ___11-20 ___21-30 ___31-40 ___>40

Foliage Symptoms: ___Flagging ___Thin ___Wilted ___Yellowed

Root Symptoms: ___Insect Signs ___Resinous ___Rotted ___Stained

Insect Attack: ___BTB ___Hylastes ___Ips ___SPB ___Termites

Insect Damage: ___Boles ___Branches ___Foliage ___Roots

Stand Prevalence: ___Entire ___ Localized ___Scattered ___% Affected

Severity of Damage: ___Low ___Medium ___High ___ Severe

Recent Silviculture: ___Fertilizer ___Fire ___Herbicide ___Insecticide ____Thin/Harvest

Problem Description: ____

____

Appendix C

Brown Spot Needle Blight of Loblolly Pine

Brown spot needle blight is an emerging disease in loblolly pine plantations in Alabama. Identification and control of this fungus are crucial for the survival of this native species.

Figure 1. Loblolly pine is adaptable to many types of soil and growing conditions from central Texas east to Florida and north to Delaware and southern New Jersey.

Brown spot needle blight is a native disease caused by the fungus *Lecanosticta acicola* (*Mycosphaerella dearnessii*, syn. *Scirrhia acicola*). First reported in South Carolina in 1878, this fungal pathogen is known for causing needle shed and stunted growth in many pine species (*Pinus* spp.). Recently the disease has become an important problem of loblolly pine plantations in Alabama.

Loblolly pine (*Pinus* taeda) is one of the most productive pine species native to the southeastern United States. Approximately 29 million acres of loblolly pine plantations

exist in this region. Over the past 3 years, however, premature needle defoliation followed by tree mortality has occurred.

More than 1,000 hectares of loblolly pine plantations in Alabama are currently infected with brown spot needle blight that has caused stunted growth and tree mortality. The identification and control of this emerging disease is an important concern to landowners, forest managers, and forest health state cooperators.

Diagnosis and Identification

Brown spot fungi, *L. acicola*, complete their life cycles on pine needles. Infection causes needle chlorosis (yellowing) followed by necrosis (dead needles), which results in premature shedding of needles, reduced growth, and tree mortality.

Infections and visible symptoms occur from late February to early November. There are two distinct types of necrotic lesions observed on infected needles. The most notable symptom is an irregular brown spot surrounded by a yellow halo. In some cases, the yellow tissue can be infiltrated with resin. The second type of lesion is a spot that is initially yellow but browns over time and is surrounded by a darker border. This symptom is similar to a different fungal disease called “red band” or Dothistroma needle blight. Symptomology additionally can mimic abiotic factors, such as chloroflurocarbon or sulphur dioxide exposure, as well as deficiencies in nutrients such as magnesium and potassium.

Symptoms must be verified with laboratory diagnosis at facilities such as the Forest Health Dynamics Laboratory (FHDL) at Auburn University. Identification is reliable only

when evidence of the characteristic conidia of the anamorphic state (*L. acicola*) is recovered in culture and/or identified using molecular methods.

Figure 2a. Disease symptoms showing stands infected by brown spot needle blight (note the thinning canopies).

Figure 2b. Disease symptoms showing irregular, frequent brown spots surrounded by a yellow halo.

Figure 2c. Reproductive structures showing black, shiny fruiting body protruding from needles.

Figure 2d. Reproductive structures showing microscopic banana-shaped septate conidia.

Control and Management Guidelines

Foliar chemical treatments are available; however, the most successful steps for disease control are based on the host, environmental conditions, and precautions taken during plantation establishment.

Prevention

Not all loblolly pine families are equally susceptible to the disease. Selecting seedlings resistant to brown spot needle blight infection will decrease the chance of infection after outplanting in the field.

Cultural Control and Sanitary Methods in Landscape Settings

The fungus is most active during warm (62 to 82 degrees F) and wet (humidity greater than 80 percent) conditions. Increasing spacing between seedlings allows air circulation to minimize infection. The pruning of lower branches also can be helpful in increasing air circulation.

Monocultures of loblolly pines are at greatest risk. Consider planting a mixture of pines (such as shortleaf or longleaf) to prevent the spread of infection and potential mortality to all landscape trees.

Replace weeds and grasses under the trees with a layer of composted mulch to prevent plant competition and mower damage and also to improve tree vigor. Apply mulch 3 to 4 inches deep, ideally extending to the tree's dripline.

Cultural Control and Sanitary Methods in Forest Settings

Visually inspect tree crowns in forest settings for brown spot needle blight severity. This can be done by visually rating the proportion of tree crown with yellowing, dead needles and/or by examining the extent of the thinning crown. Another visual inspection method is to evaluate the progression of the disease from the pine's lowest branches to the uppermost part of the tree.

Selective removal of infected branches can be a promising solution when less than one-third of the tree crown is infected and the disease is detected only on the lower branches. This

may reduce pathogen pressure or fungal loads of L. acicola of an infected stand. Prune during dry summer periods and sanitize tools after each tree pruning to prevent pathogen spread.

If two-thirds or more of the crown is infected, whole tree removal is recommended. Fell and burn infected trees along with any litter under the trees. If 50 percent of trees in a stand are infected, clear-cutting the stand and regenerating the stand is recommended.

No fungicides are recommended in an established setting due to the cost as well as negative environmental factors associated with the use of chemicals.

Chemical Control

Fungicide treatments are effective in protecting pine seedlings from infection by L. acicola. In nursery settings, chlorothalonil or maneb broad-spectrum fungicides (e.g., Bravo and Daconil) can be sprayed to provide successful control against brown spot infection. Another option is a Bordeaux mixture composed of copper sulfate and slaked lime; this adds a protective barrier on the plant surface that inhibits conidial germination. This product can be used for the protection of landscape seedlings, seed orchard trees, and Christmas tree plantations.

It is best to initiate spraying in the spring when newly emerging needles are 2 to 5 centimeters long. Four to six applications throughout the year are usually sufficient for adequate control. Preventative fungicide treatments also are applicable in the landscape settings of high-value trees. For product rates and recommendations of fungicides, always read and follow product labels.

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