AUBURN UNIVERSITY
Forest Health Cooperative

RESEARCH REPORT 25-01

ISOLATION AND IDENTIFICATION OF *LECANOSTICTA ACICOLA* AND OTHER FOLIAR PATHOGENS ASSOCIATED WITH NEEDLE BLIGHT ON LOBLOLLY PINE

by
Emmanuel Nyarko and Lori Eckhardt

ABSTRACT

Brown spot needle blight is a pine foliar disease caused primarily by the fungus, *Lecanosticta acicola*. It impacts loblolly pine plantation in the southeast U.S. causing premature defoliation and mortality of loblolly pines. In this study, we surveyed 22 plots (14 privately owned and 8 National Forest (NFs) sites) from March to November in 2023 (private plots) and in 2024 (National Forests), to characterize the community of needle-infecting fungi associated with needle blight. Using sporulation chambers, media plating and molecular techniques on needle samples obtained within the period, we quantified incidences of *L. acicola*, *Pestalotiopsis* spp., *Hendersonia* spp., *Trichoderma* spp., and *Coleosporium* spp. as the predominant fungi associated with this disease. Across the study plots, the North AL sites exhibited the highest detection rates for all pathogens except *Hendersonia* (which peaked in the South AL sites), with *Pestalotiopsis* dominating followed by *L. acicola*. The North AL sites's bimodal peaks in May and August contrasted with the South AL sites's later maximum peak in September to October, reflecting site-specific microclimatic influences on sporulation dynamics. The National Forests (NFs) showed similar seasonal trends: Tuskegee NF registered the greatest *L. acicola* loads, Bankhead NF was intermediate, and Conecuh NF had the lowest. *Trichoderma* proliferation in midsummer suggested warm, humid conditions favor both pathogens and their antagonists. Our findings emphasize the need for effective monitoring and management, especially in pathogen peak months. It further highlights the value of integrating phenological data with molecular diagnostics to inform targeted management of Brown spot needle blight and its co-occurring foliar fungi.

Keywords: Foliar pathogens, Brown spot needle blight

INTRODUCTION

Foliar pathogens play a critical role in the development of plant diseases, often leading to significant economic losses in agriculture and forestry. The infection of leaves can disrupt photosynthesis, reduce plant vigor, and ultimately lead to decreased yield and quality of crops. In many cases, foliar diseases can also predispose plants to secondary infections by other pathogens, compounding the effects of the initial infection (Zellner et al., 2011; Jarecki et al., 2014). The severity of foliar diseases can be influenced by various factors, including environmental conditions, host susceptibility, and pathogen virulence. Effective identification and management of foliar pathogens are essential for maintaining plant health and ensuring sustainable agricultural practices (Deng et al., 2021).

Brown spot needle blight (BSNB) is a destructive foliar disease primarily affecting various pine species in temperate regions of North America, Europe, and Asia (Barnard & Blakeslee, 1980; Smith, 2015). Among the fungal pathogens associated with this disease, *L. acicola* has long been recognized as one of the primary causal agents, characterized by its ability to induce necrotic lesions on needles that often lead to premature defoliation and, in severe cases, mortality

of infected trees (Crous et al., 2019). However, BSNB can involve a complex of foliar pathogens, including species within the genera *Pestalotiopsis* and *Phyllosticta*, which can act synergistically or opportunistically under favorable environmental conditions (Smith & Jones, 2020). Effective isolation and identification of *L. acicola* and other co-occurring foliar fungi are crucial for accurate disease diagnosis, the development of targeted management strategies, and the implementation of breeding programs aimed at disease resistance (Barnard & Blakeslee, 1980).

Molecular methods like polymerase chain reaction (PCR) and DNA sequencing have been used as a more advanced approach to enhance pathogen identification and confirmation (Crous et al., 2015; Smith & Jones, 2020). By these integrated approaches, forest pathologists can more precisely characterize the pathogen community involved in BSNB, thereby improving our understanding of disease epidemiology and informing more robust disease management practices. The objective of this study was to identify and isolate *L. acicola* and other foliar pathogens that are co-occurring in the brown spot needle blight disease.

MATERIALS and METHODS

Study Area Description

A total of twenty-two plots were used for the study (Table 1). Fourteen plots were set up in Cullman (Osko Forest -Glover Property) and Chatom both in Alabama, between January and February 2023. In January 2024, three National Forests were included in the study, out of which eight more plots were set up. The National Forests used were Tuskegee National Forest, Conecuh National Forest, and Bankhead National Forest (Figure 1).

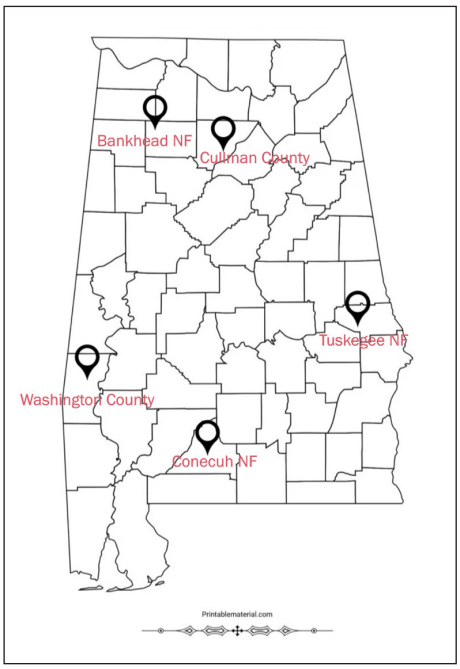

Figure 1. State map of Alabama showing the study locations.

Cullman county is located in North-Central Alabama. Its average temperature in summer often reaches the upper 80s °F (about 31-32 °C), and winters commonly drop into the lower 30s °F (around 0-2 °C). Annual rainfall typically ranges from about 50-55 inches (1270-1400 mm). Rainfall is relatively well-distributed throughout the year, though slightly heavier in late winter and spring months. Chatom is in Washington county (southwestern Alabama). Summers are hot and humid, with average high temperatures commonly reaching the lower to mid-90s °F (around 33-35 °C). Winters tend to be mild, with average lows typically in the upper 30s to low 40s °F (about 3-6 °C). Annual rainfall is relatively high, often around 60 to 65 inches (about 1,520-1,650 mm) and is distributed throughout the year. Slight heavier rainfall can occur in late spring and summer due to Gulf Coast weather patterns and occasional tropical systems. Conecuh county is in South-Central Alabama. Its summers are similarly warm and humid, usually featuring daytime highs in the lower 90s °F (about 32-34 °C), while winter lows commonly settle in the upper 30s to low 40s °F (around 3-6 °C). Annual precipitation totals generally range from about 55 to 60 inches (roughly 1,400-1,520 mm), with rainfall evenly spread throughout the year but often peaking during the late spring and summer months (Weatherspark.com). Information for each stand such as average height (in feet), average diameter at breast height (DBH), average basal area and average disease rating are shown in Table 1.

Information on weather data obtained from the nearest weather stations to the study sites and used to determine the monthly averages for temperature, relative humidity and rainfall is shown in Table 2.

Study Plot Layout

The research plots were designed and established and based on Dunn (1999), comprised of one central plot and three sub-plots identical to it (Figure 2). The subplots are located 120 feet from the central plot and at bearings of 120°, 240°, and 360°. All trees within each center and sub-plot were tagged and flagged.

Sample Collection

Needle samples were taken monthly from one tagged tree in every plot from March to November. For tall trees, the needle samples were collected with a 22-magnum rifle (Figure 3), while for relatively shorter trees, a pole pruner was used. The needle samples from each tree were subdivided into three portions (one for sporulating chambers, plating, and PCR) and labeled. The samples were kept in brown paper bags and stored at 4°C in ice coolers until they were

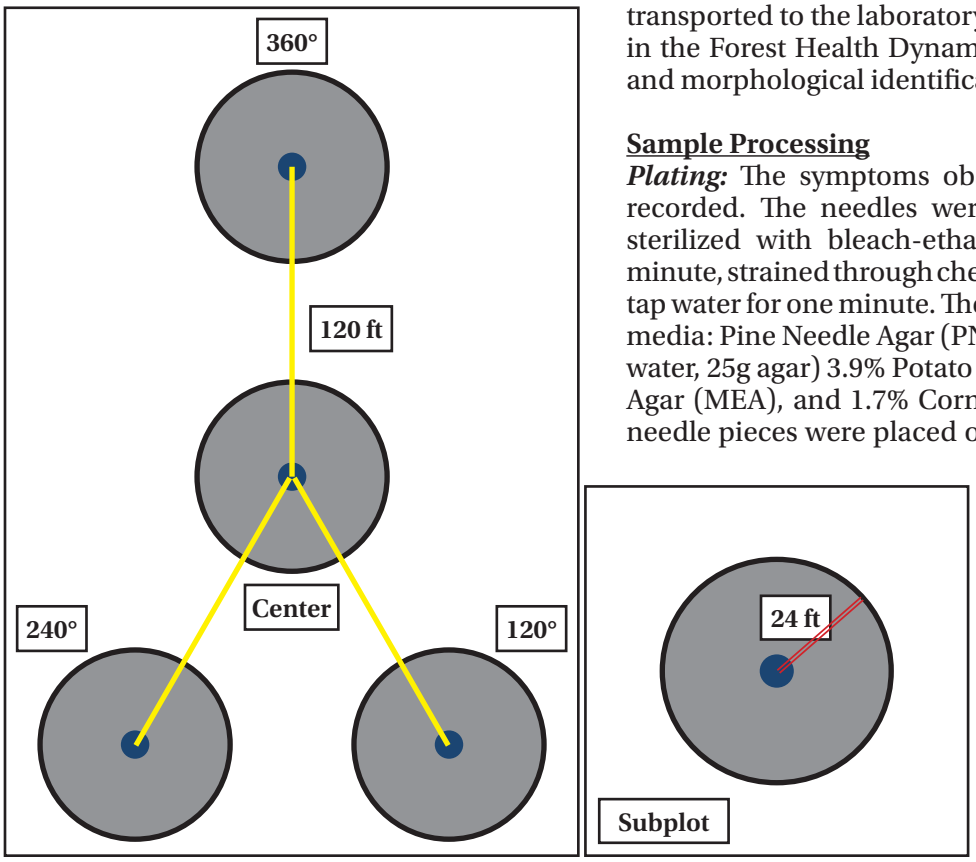

Figure 2. Description of plot layout (Dunn, 1999)

transported to the laboratory. The needle samples were processed in the Forest Health Dynamics Laboratory, where fungi isolation and morphological identification were carried out.

Sample Processing

Plating: The symptoms observed on the needle samples were recorded. The needles were cut into 1-2 cm pieces and then sterilized with bleach-ethanol solution (10:10:80 v/v) for one minute, strained through cheesecloth and then rinsed with running tap water for one minute. The needles were plated on four different media: Pine Needle Agar (PNA) (55g fresh pine needles/L distilled water, 25g agar) 3.9% Potato Dextrose Agar (PDA), 2% Malt Extract Agar (MEA), and 1.7% Corn Meal Agar-PARP (CMA-PARP). Four needle pieces were placed on each media plate. Each media type was replicated four times for each sample. The plates were incubated at 20-25 °C for 5 days to 2 weeks and monitored for mycelial growth daily. When mycelial growth was observed, it was sub-cultured onto 2% Malt Extract Agar to obtain a pure culture of growing fungal mycelium for easy identification.

Sporulation chambers: The sporulation chamber is made up of a 10mm glass petri dish, 90mm Whatman filter paper and moistened with 500µL of distilled water. One fascicle was placed in each sporulation chamber. This was done in five replicates for every sample tree. The symptoms observed on the needles were recorded. Five whole fascicles were sterilized with bleach-ethanol solution (10:10:80 v/v) for one minute, strained through cheesecloth and rinsed with running tap water for one minute. The chambers were incubated for 3-5 days at 25°C. Spores recovered on the needles were observed under a compound microscope.

Molecular analysis: The third portion of the needle samples was ground in liquid nitrogen and stored at -4°C until DNA extraction was carried out. Whole DNA was extracted directly from symptomatic needle samples collected from the plots. 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 without any modifications (Barnes et al., 2008). The extracted DNA was stored at -4°C until PCR was done.

Figure 3. Needle sample collection (A) Shooting to collect needle samples from tall trees (B) Symptomatic pine needle samples

Polymerase chain reaction (PCR) was carried out first by thermal cycling reaction was done 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 8.5 uL nuclease-free water. The 29 reactions were as follows; initial denaturation of 95°C for 10 minutes, annealing 59°C for the 30S of primer pairs, 72°C for 1 minute, and 39 cycles were performed each time for maximum amplification. This was followed by a final extension at 72^0C for 10 minutes. PCR products were run through agarose gel electrophoresis and subsequently analyzed using gel illuminator. PCR cycling conditions were varied at least for annealing temperature for other sets of primers used in the study (Table 3). 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).

Gel electrophoresis was carried out by preparing 2% gel (2.0g of agarose to 100ml of 1X TAE buffer) into a tray taped at both ends and properly sealed to prevent leaking. 10 uL of GelRed (10,000X) was added to the gel. Two combs were placed in the tray and the molten gel was poured into the tray and left briefly to solidify after which the tapes were removed, and the plate was placed in an electrophoresis tray. The tray was filled with 1X TAE buffer to cover the gel and the combs were removed. Extracted DNA samples were loaded into the well and a record chart was made to track each sample to their corresponding lanes. A mini ladder VWR (100-500 bp) was used to serve as a reference point to estimate the sizes of the DNA molecules in the sample.

RESULTS

Morphologic and molecular identification of *L. acicola* and associated pathogens

A total of 22 plots (14 privately owned and 8 National Forests) in Alabama were sampled for the study. The private plots were sampled from March to November 2023, and the National Forests were sampled from March to November 2024.

Morphological identification results were based on the fungi recovered from needle plating and sporulation chambers. A total of 684 trees were sampled, resulting in 3,420 sporulation chambers and 2,736 cultures. Twenty-one fungal genera were identified using an identification manual by Barnett & Hunter (1998,4th Edition). The fungi genera were *Alternaria, Cladosporium, Flagellospora, Epicoccum, Penicillium, Trichoderma, Coleosporium Articulospora, Tilletiopsis, Aspergillus, Ceratosporium, Geotrichum, Thallospora, Bispora, Cephalosporium, Dendrographium, Papulospora, Hendersonia, Pestalotiopsis, Diplodia* and *L. acicola*. Among these genera, *Pestalotiopsis, Hendersonia, Trichoderma* and *Coleosporium* were the predominant fungi in association with *L. acicola* on the private forests. This diversity of fungi did not vary across samples from the National Forests (Figure 4). Cullman county recorded the highest overall detections for every pathogen except for *Hendersonia*. *Pestalotiopsis* was the most recovered pathogen (105 detections in Cullman county and 95 detections in Washington county) (Figure 4). *Lecanosticta acicola* had a higher prevalence in Cullman county (70 detections) than in Washington county (40 detections). *Trichoderma* (usually a saprophyte/antagonist) was four times more frequent in Cullman county than in Washington county.

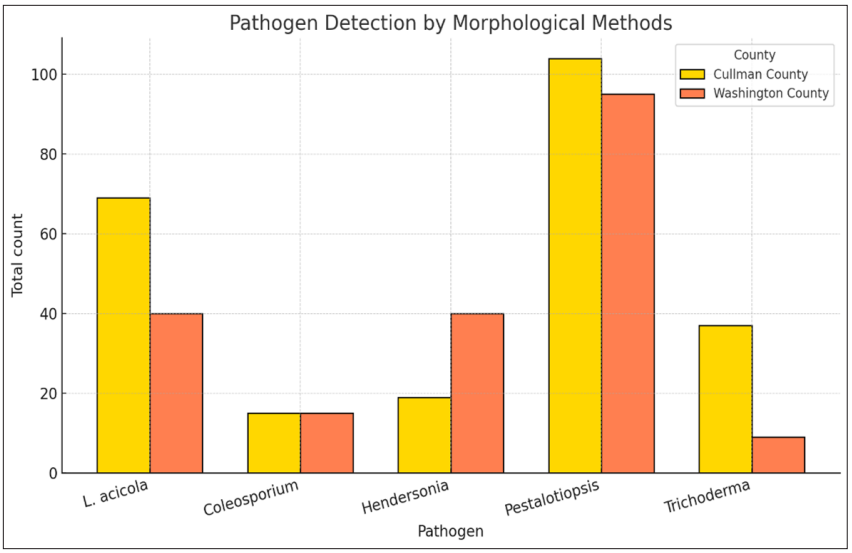

Figure 4. Fungal pathogen detection by morphological methods.

Temporal analysis of the monthly pathogen detections revealed distinct patterns in the two counties. In Cullman county, pathogen incidence exhibited a bimodal distribution, with an early season increase in May, where *Lecanosticta acicola* reached 15 detections (highest of the period). This was followed by 18 *Pestalotiopsis* detections and a pronounced mid-summer outbreak in August, when *Pestalotiopsis* increased to 28 detections and *Trichoderma* soared to 17 detections. *Lecanosticta acicola* detections in Cullman county also showed a secondary rebound in September and October (12 and 10 detections, respectively) (Figure 5).

By contrast, Washington county's overall pathogen burden peaked later, during September to October with *Pestalotiopsis* detections at 19 in September, while *Hendersonia* increased steadily from 2 detections in May to 10

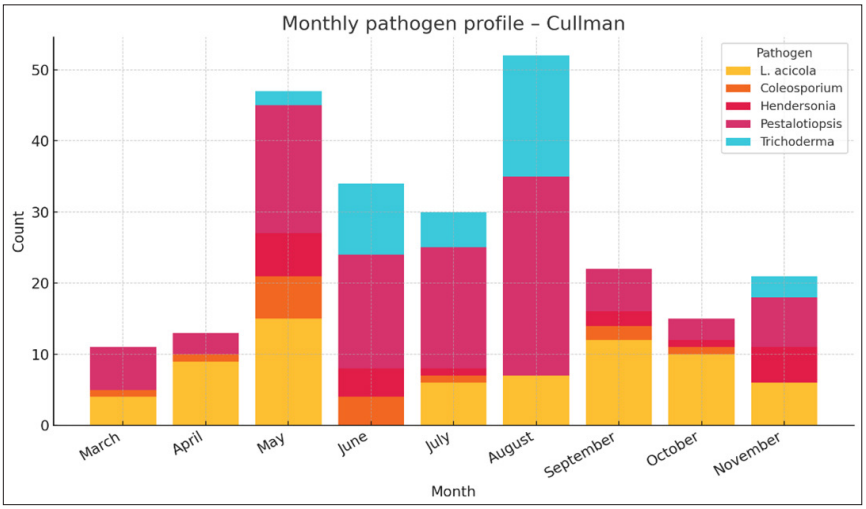

Figure 5. Monthly pathogen profile in Cullman county in 2023

detections in October, indicating a late season shift in community composition. *Lecanosticta acicola* in Washington county rose more gradually to a maximum of 8 detections in August before declining (Figure 6). The mid-summer proliferation of both *Pestalotiopsis* and *Trichoderma* in Cullman county suggests that warm, humid conditions favored not only the primary foliar pathogen but also its mycoparasitic antagonists.

The monthly *L. acicola* detections in the samples from Cullman county plots showed that there were 4 *L. acicola* spores in March, which increased to 15 spores in, as the highest count throughout the period. No spores were detected in June; however, 6 spores were recorded for July and then it increased from 7 spores to 12 spores, from August to September. It declined to 10 spores in October and then 6 spores in November (Figure 7).

The Washington county plots showed 3 *L. acicola* spore counts in March and 4 spores in April through May. In June and July, 6 spores were recovered, and the number increased to 8 spores in August, but it declined to 3 spores and a single count in September and October respectively. The spore detections finally increased to 5 spores by November (Figure 7).

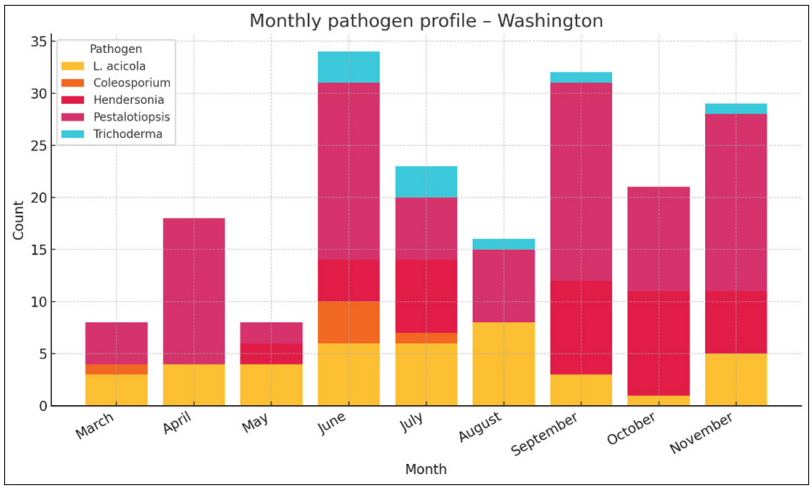

Figure 6. Monthly pathogen profile in Washington county in 2023

The monthly detection counts of *L. acicola* across the three National Forests from March through November showed that, Bankhead NF consistently showed the highest inoculum levels, beginning at 8 spores in March, peaking at 12 spores in April, and maintaining elevated counts (10-11 spores) through June and July before a gradual decline to 6 spores by September and 0 in November. Tuskegee NF followed a similar pattern; 6 spores in March, a peak of 12 spores in April, a mid-summer plateau around 6 to 8 spores from May to July, and a tapering to 4 spores in October and 0 by November. Conecuh NF exhibited the lowest spore counts overall. It rose from 2 spores in March to 4 spores in April, fell to 0 in May, then increased to 7 spore detections in July before declining to 2 spores by October and 0 in November (Figure 8).

The overall pathogen detections across all the sampled sites, using morphological and molecular means, showed that *Pestalotiopsis* and *L. acicola* were the most frequent fungi. *Hendersonia* detection levels were moderate with the other less common, slow-growing or opportunistic saprobes that were detected. *Trichoderma* and *Coleosporium* were detected at relatively lower levels (Figure 9).

Expression of reproductive structures of some predominant fungi recovered from the study

Lecanosticta acicola conidia are generally oval or cylindrical in shape, with rounded ends. Conidia are hyaline and small measuring about 10 to 20 micrometers in length. *Pestalotiopsis* is characterized by dark acervuli, discoid or cushion-shaped; dark conidia, several-celled with hyaline, pointed end cells, ellipsoid to fusoid. *Hendersonia* has black pycnidia, separate, globose, dark conidia, several-celled, elongate to fusoid. *Alternaria* has conidiophores which are dark, mostly simple with both cross and longitudinal septa, usually elliptical or ovoid in shape (Figure 10).

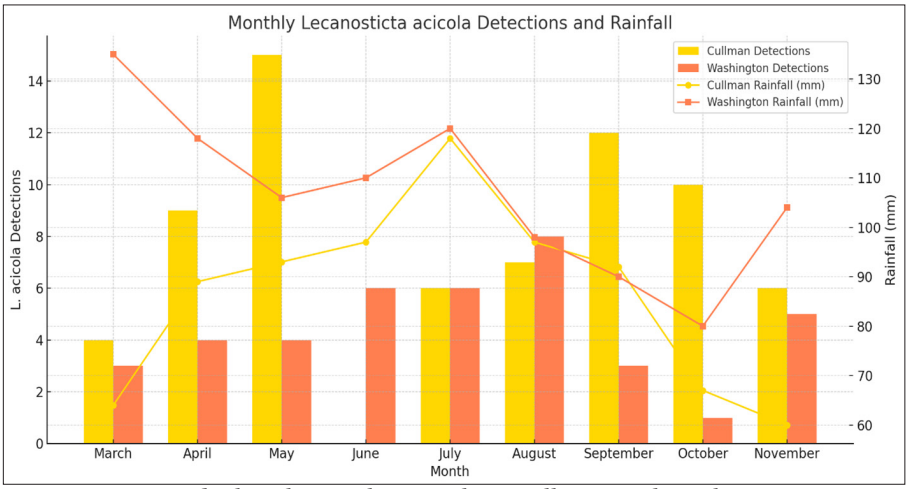

Figure 7. *L. acicola* distribution by month in Cullman and Washington counties in 2023

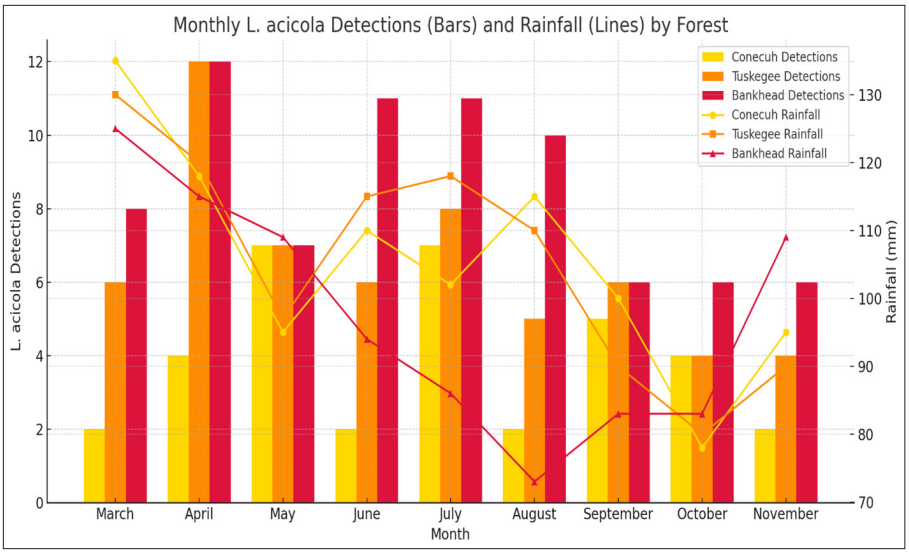

Figure 8. Monthly *L. acicola* detections by National Forests in 2024

DISCUSSION

Sampling across 22 plots in Alabama (14 private, 8 National Forests) revealed a consistent community of foliar fungi *L. acicola*, *Pestalotiopsis* spp., *Hendersonia* spp., *Trichoderma* spp., and *Coleosporium* spp., with little variation among the National Forest sites. This stability suggests that regional climate and host availability, as well as stand ownership or management history, structure needle-blight complexes (Stone & Roberts, 2002).

In the private plots, Cullman county exhibited the highest overall pathogen detections, except for *Hendersonia*, which peaked in Washington county. *Pestalotiopsis* was the dominant species in both counties (105 detections in Cullman county; 95 detections in Washington county), followed by *L. acicola* (~70 detections vs. 40 detections). The saprophytic antagonist *Trichoderma* was four times more abundant in Cullman county, perhaps reflecting warmer, more humid microclimates that favor both pathogen proliferation and antagonist responses (Wyka et al., 2018).

Temporal dynamics diverged notably between counties. Cullman county experienced two surges: a spring peak in May driven by *L. acicola* and *Pestalotiopsis*, and an August spike dominated by *Pestalotiopsis* and *Trichoderma*. In contrast, Washington's highest disease pressure arrived later September to October marked by rising *Pestalotiopsis* and a pronounced *Hendersonia* increase. *Lecanosticta acicola* detections in Cullman county peaked at 15 spores in May, dipped to zero in June, then rebounded in autumn; Washington county's more gradual rise peaked at 8 spores in August, indicating a more extended but less intense sporulation window. These bimodal patterns align with known rainfall-driven conidial release in BSNB (Sinclair & Lyon, 2005) but also highlight inter-site microclimatic variation. Among National Forests, Bankhead displayed the highest *L. acicola* counts (8-12 spores from March-July), followed by Tuskegee NF and Conecuh NF, reflecting local differences in stand age, density, and canopy moisture (Barnes et al., 2019). The uniform absence of *Diplodia* across all sites underscores its minor role in these pine needle communities. Collectively, these results emphasize the need for site-specific timing of monitoring and interventions. In areas like

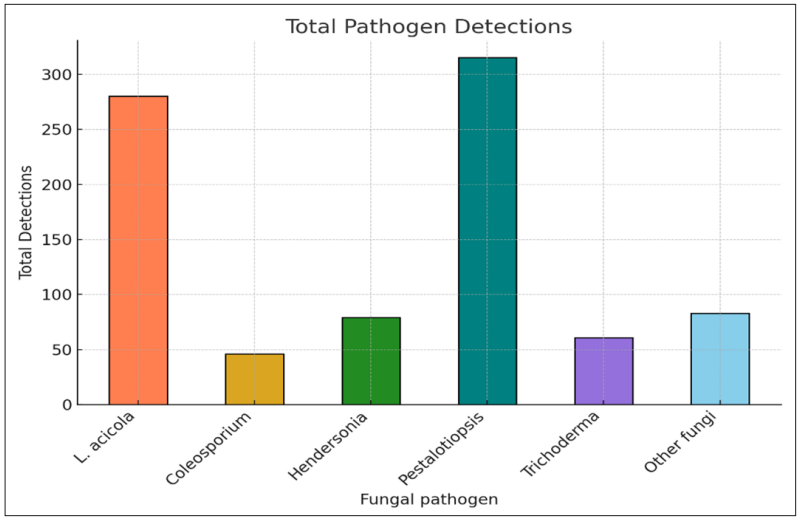

Figure 9. Overall pathogen detections across the study sites

Cullman, cultural treatments (e.g. litter removal through pescribed burning) may best curb initial outbreaks, whereas Washington county might benefit from late-season management targeting *Hendersonia* and *Pestalotiopsis* (Cech & Klepzig, 2001). Integrating these phenological insights with genetic resistance screening could optimize control strategies for BSNB and its associated fungi.

CONCLUSION

This multiple site survey confirms that *L. acicola* co-occurs with a suite of foliar fungi: *Pestalotiopsis*, *Hendersonia*, *Trichoderma*, and *Coleosporium* across the private and National Forest plots in Alabama. Seasonal peaks in spore detections underscores the roles of rainfall, temperature, and humidity in driving sporulation of the pathogen. The dominance of *Pestalotiopsis* and the notable antagonist *Trichoderma* also suggest needle-blight complexes occur when the infection progresses. The presence of *L. acicola* in the various sites aligns with its emerging threat to pine plantations globally while the detection of *Coleosporium* sp. highlights its ongoing epidemiological importance. In summary, these findings emphasize the need for an integrated, and improved management practices to mitigate BSNB and associated pathogens in loblolly pine ecosystems.

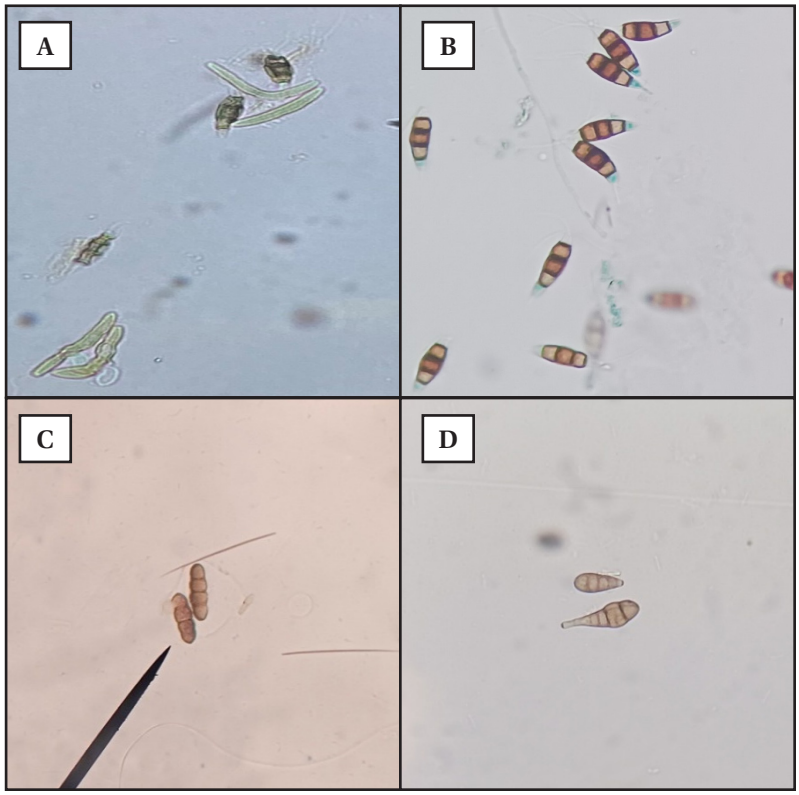

Figure 10. (A) *L. acicola* conidia (B) *Pestalotiopsis* sp. (C) *Hendersonia* (D) *Atlternaria* recovered from the samples in the study.

Table 1. Descriptive data for stands sampled from 2023 to 2024 in the southeastern U.S.

Location	Plot no.	Age (yrs)	Avg. Height (ft)	Avg. DBH (in)	Avg. Basal Area (ft^2/acre)	Disease Rating
Washington county	1	27	54.21	9.03	0.446	2.5
Washington county	2	22	50.32	7.28	0.289	2.5
Washington county	3	13	23.74	4.48	0.109	2.8
Washington county	4	11	21.00	3.62	0.071	2.9
Washington county	5	11	19.88	3.50	0.067	2.8
Washington county	6	10	20.65	3.47	0.066	2.6
Washington county	7	11	19.62	3.63	0.072	2.4
Washington county	8	15	32.26	5.04	0.138	2.2
Washington county	9	12	23.52	4.10	0.092	2.1
Cullman county	10	34	72.33	11.26	0.691	1.6
Cullman county	11	61	99.44	20.43	2.277	1.4
Cullman county	12	24	59.88	7.90	0.340	0.6
Cullman county	13	35	71.89	11.68	0.744	2.0
Cullman county	14	32	67.67	10.68	0.622	2.2
Conecuh NF	15	17	48.00	5.60	0.171	0.0
Conecuh NF	16	20	56.00	6.69	0.244	1.0
Tuskegee NF	17	26	61.20	8.64	0.407	0.0
Tuskegee NF	18	36	61.10	12.10	0.799	0.0
Tuskegee NF	19	26	79.30	8.69	0.412	0.0
Bankhead NF	20	39	79.60	13.03	0.926	0.0
Bankhead NF	21	41	75.50	13.53	0.998	0.0
Bankhead NF	22	26	69.10	8.69	0.412	0.0

Table 2. Weather data for all the study sites during the sampling period

Location	Weather factor	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov
Cullman county	Rainfall (mm)	91	104	80	73	94	91	67	64	60
	Relative Humidity (%)	78	76	76	75	76	75	74	72	74
	Temperature (^{0}C)	16.8	21.9	26.3	30.5	31.8	31.3	28.6	22.5	15.6
Washington county	Rainfall (mm)	138	119	107	110	119	96	89	80	104
	Relative Humidity (%)	72	72	72	74	75	75	73	73	72
	Temperature (^{0}C)	14.4	18.3	22.7	26.1	26.7	26.7	24.4	18.3	13.3
Conecuh NF	Rainfall (mm)	134	109	88	116	124	104	91	78	96
	Relative Humidity (%)	66	64	61	71	74	71	73	74	72
	Temperature (^{0}C)	16.1	19.4	23.9	26.7	27.8	27.8	25.6	20.0	15.0
Bankhead NF	Rainfall(mm)	124	117	109	94	86	73	83	83	109
	Relative Humidity (%)	64	66	68	71	73	73	72	71	71
	Temperature (^{0}C)	11.1	15.6	20	24.4	26.1	25.6	22.2	16.1	10.6
Tuskegee NF	Rainfall (mm)	130	112	91	118	117	107	83	81	90
	Relative Humidity (%)	61	63	67	72	75	75	72	70	68
	Temperature (^{0}C)	13.3	17.2	21.7	25.0	26.7	26.1	23.9	18.3	12.2

Table 3. List of primers used for the study

Primer	Organism	Sequence (5' to 3')	Reference
LAtef-F	*L. acicola*	5'-GCAAATTTTCGCCGTTTATC -3'	Ioos et al., 2010
LAtef-R	*L. acicola*	5'-TGTGTTCCAAGAGTGCTTGC -3'	Ioos et al., 2010
DpF	*D. sapinea*	5'-CTTATATATCAAACTATGCTTTGTA -3'	Smith and Stanosz, 2006
BotR	Botryosphaericeae	5'-GCTTACACTTTCATTTATAGACC -3'	Smith and Stanosz, 2006
LophActF	*Lophodermium*	5'-GATGCTCCCAGAGCTGTTTTCCG -3'	Stenström et al., 2005
LophActR	*Lophodermium*	5'-CGAGTCCTTCTGGCCCATACC -3'	
CsolF	*Coleosporium*	5'-GCGTACCAGTGAGCCGAA -3'	Stenström et al., 2005
CsolR	*Coleosporium*	5' -ACGAGACTTGAAACTCGAACC -3'	