USDA Forest Service
U.S. DEPARTMENT OF AGRICULTURE

Southern Research Station | Research Note SRS-27 | September 2024

Guide to Isolation of *Lecanosticta acicola*, the Causal Agent of Brown Spot Needle Blight of Pines

Tyler Dreaden, Colton Meinecke, Rabiu Olatinwo, Jaesoon Hwang, Isabel Munck, Cady Greenslit, and Caterina Villari

Tyler Dreaden, U.S. Department of Agriculture, Forest Service, Southern Research Station, Lexington, KY 40546
Colton Meinecke, University of Georgia, Daniel B. Warrell School of Forestry and Natural Resources, Athens, GA 30602
Rabiu Olatinwo, U.S. Department of Agriculture, Forest Service, Southern Research Station, Pineville, LA 71360
Jaesoon Hwang, U.S. Department of Agriculture, Forest Service, Forest Health Protection, Pineville, LA 71360
Isabel Munck, U.S. Department of Agriculture, Forest Service, Forest Health Protection, Durham, NH 03824
Cady Greenslit, U.S. Department of Agriculture, Forest Service, Southern Research Station, Lexington, KY 40546
Caterina Villari, University of Georgia, Daniel B. Warrell School of Forestry and Natural Resources, Athens, GA 30602

In the last decade, the pathogen *Lecanosticta acicola* has been reported causing two outbreaks in the Eastern United States on (A) loblolly pine (*Pinus taeda*) and (B) eastern white pine (*P. strobus*), in addition to its ongoing endemic status on (C) longleaf pine (*P. palustris*) across the central Gulf and southeast Atlantic coastal plains.

ABSTRACT

Lecanosticta acicola is a foliar fungal pathogen that affects over 70 conifer species, primarily in the genus *Pinus*, and it has been introduced to much of the Northern Hemisphere. In the last decade, the pathogen has been reported causing two outbreaks in the Eastern United States on loblolly pine (*P. taeda*) and eastern white pine (*P. strobus*), in addition to its ongoing endemic status on longleaf pine (*P. palustris*) across the central Gulf and southeast Atlantic coastal plains. It is often difficult to culture due to its slow growth and poor competitiveness against other fungi on culture media. To remedy this, we developed and describe procedures for *L. acicola* sample collection and methods for isolation that give reproducible results for loblolly, longleaf, and eastern white pines.

Keywords: Brown spot needle blight, white pine needle damage, *Mycosphaerella dearnessii*, single genotype, single spore.

INTRODUCTION

Lecanosticta acicola (Thümen) (formerly *Mycosphaerella dearnessii* or *Scirrhia acicola*) is a well-known and aggressive foliar pathogen that attacks pines, causing brown spot needle blight and white pine needle damage (Sinclair and Lyon 2005, Wyka et al. 2017). Although it is a native pathogen of longleaf pine (*Pinus palustris*) in the Southeastern United States and an important pathogen of Scots pine (*P. sylvestris*) in the Northeastern United States, its behavior on many other *Pinus* species is poorly understood. *Lecanosticta acicola* is increasingly reported in association with outbreak-scale damage on native pines (van der Nest et al. 2019) throughout eastern North America, including loblolly (*P. taeda*), longleaf, and eastern white (*P. strobus*) pines. The fungus is notoriously difficult to isolate in culture due to its slow growth and poor ability to compete with saprophytic microorganisms on nutrient-rich culture media. The protocols in this guide describe reproducible methods to obtain *L. acicola* isolates from loblolly, longleaf, and eastern white pine foliage for further study.

SAMPLE COLLECTION

Symptomatic needles should be obtained from trees that display visible brown spot needle blight or white pine needle damage symptoms. In two- and three-needle pines, lesions typically appear as either straw yellow spots becoming light brown with a dark border or as a brown spot on an amber-yellow band (Sinclair and Lyon 2005) (figs. 1, 2). With time, lesions may grow and coalesce, girdling the needle and resulting in death of the entire needle tip. In five-needle pines (i.e., white pines), needles first turn yellow around June with a spot at the initial infection remaining green. The needles are then shed around July, leaving trees defoliated and the understory covered in needles (fig. 3). Needles selected for sampling need to be collected fresh and kept cool with best results obtained from needles with green bases and discreet spots or coalescing spots with brown tips.

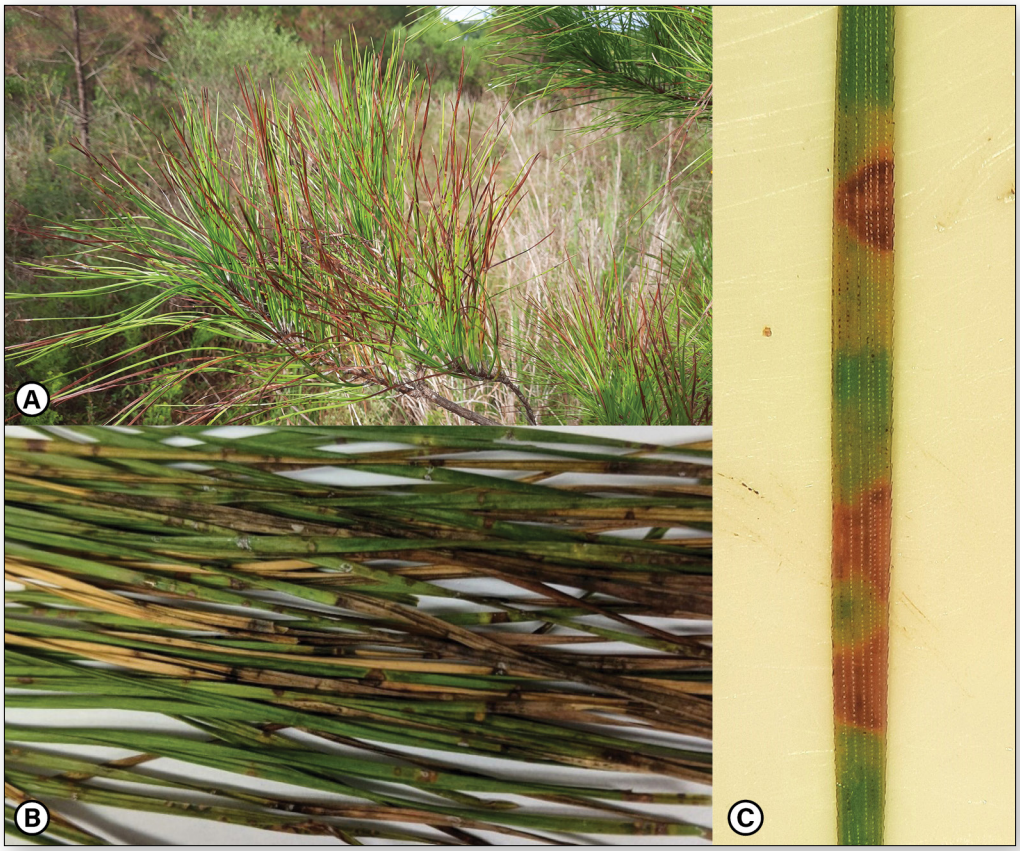

Figure 1—Loblolly pine (*Pinus taeda*) foliage displaying brown spot needle blight symptoms, including the disease namesake necrotic spots. (A) branch tip with symptomatic needles, (B) view of needles with light brown spots with a dark border, and (C) needle with yellow halo surrounding the typical brown spot symptom.

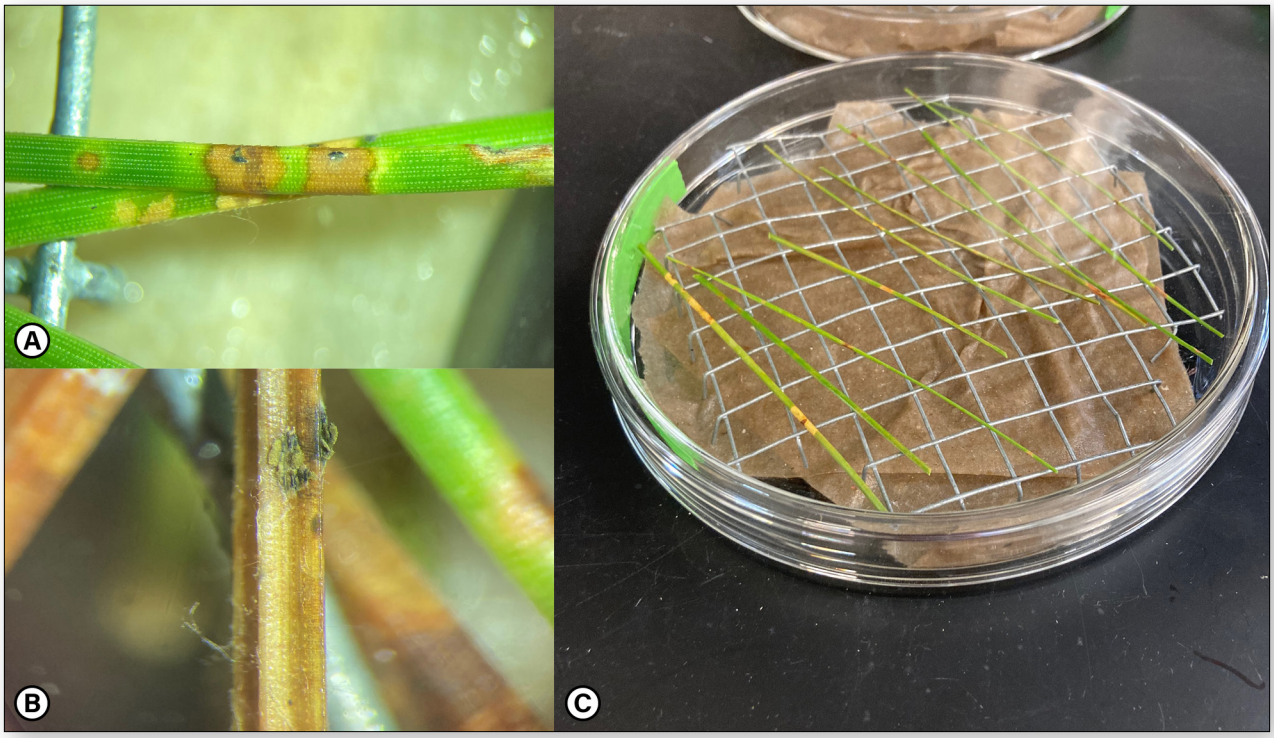

Figure 2—Longleaf pine (*Pinus palustris*) foliage with symptoms of brown spot needle blight. (A) yellow halo surrounding the typical brown spot symptom, (B) greenish black spores erupting from fruiting body, and (C) moist chamber used to promote spore production.

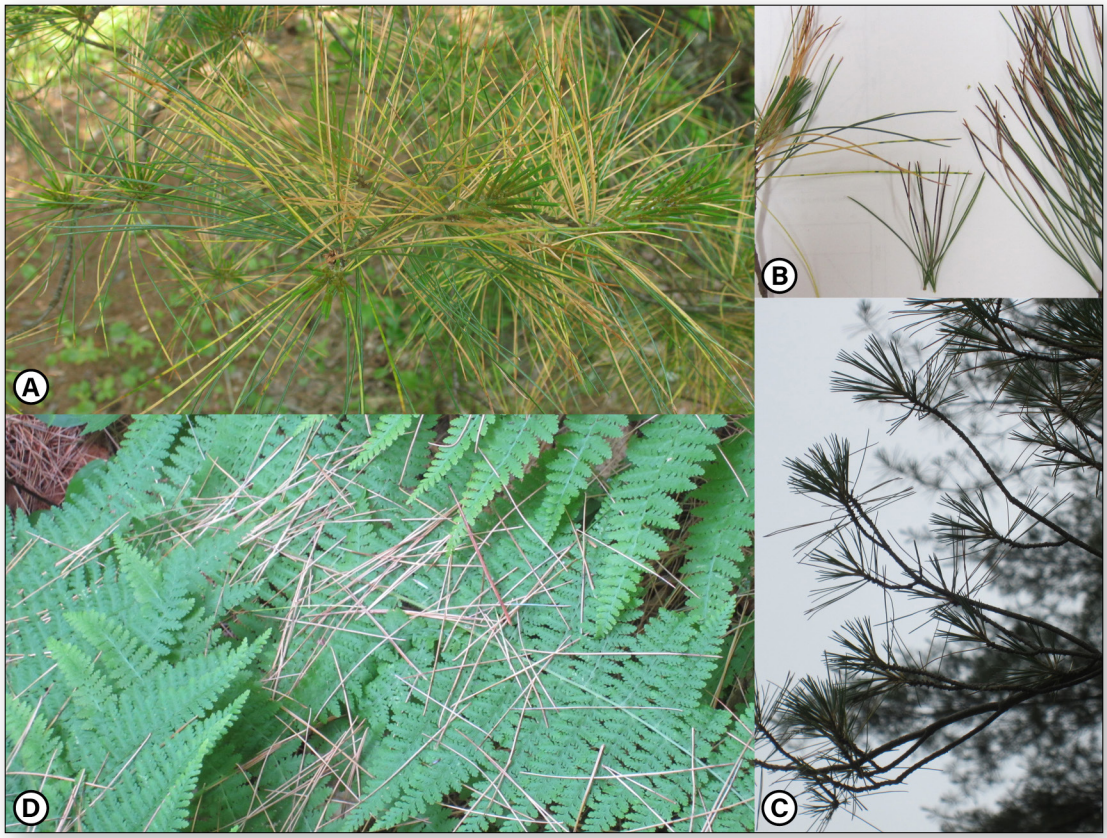

Figure 3—Eastern white pine (*Pinus strobus*) foliage with symptoms of white pine needle damage. (A) infected needles first turn yellow around June and (B, C) are shed around July, leading to a tufted or "bottle brush" appearance, (D) resulting in the understory being covered with shed infected needles.

Needles should be harvested, kept from desiccating, stored cool (ice chest, refrigerator, or air-conditioned building), and sent for processing within 2 days. Needle samples should be placed in individual bags (paper or zipper-top plastic, with a dry paper towel if needles are wet) and placed in a secondary larger plastic bag. Collectors should contact the receiving lab for any specific instructions and for U.S. Department of Agriculture, Animal and Plant Health Inspection Service permits if they are required. Needles can be placed in -80 °C freezers to kill contaminating insects and mites (Barnes et al. 2004), but insect contamination has not been a frequent problem and freezing reduces isolation success.

MOIST CHAMBER

Needles are incubated in a moist chamber at room temperature to promote fruiting body and spore development by using one of three methods, which is selected based on samples, lab setup, and preferences.

Method 1: symptomatic needles are placed in a zipper-top bag with a damp paper towel for at least 24 hours. Samples that are not overgrown with saprophytic fungi (mycelia covering needle surfaces) can be left to incubate for a longer period.

Method 2: symptomatic needles are briefly wiped with a paper towel saturated with 70 percent ethanol (EtOH) on all surfaces, cut into sections surrounding symptomatic tissue, and placed on a wire platform in a Petri dish with a moist paper towel (fig. 2C).

Method 3: symptomatic needles are washed in 10 percent bleach solution for 1 minute and then triple rinsed with sterile water. Needles are then incubated in Petri dish moist chambers (see Wyka et al. [2017] and Broders et al. [2015] for additional information and figures).

For each method, needles can be maintained in moist chambers, with water added to remoisten the paper towel as needed, for up to 3 weeks. Chlorotic and necrotic spots and bands of individual needles are inspected for evidence of fungal fruiting bodies erupting from the surface of the needle, which may manifest as black asexual acervuli (fig. 4), or less commonly as sexual ascostromata (EPPO Standards 2015).

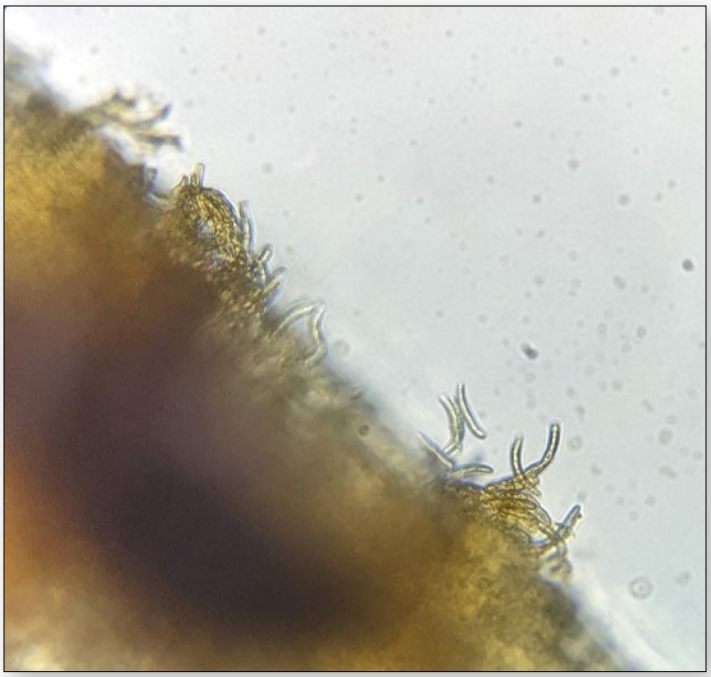

Figure 4—*Lecanosticta acicola* conidia emerging from an acervulus that formed within loblolly pine (*Pinus taeda*) foliage, viewed at 400x magnification.

Isolation Method 1

Fruiting bodies, usually observed emerging from within or just outside old bands, or from dead needle tips and always from necrotic tissue, are excised from the needle with a sterile scalpel and transferred to a glass microscope slide. Slides are prepared by wiping with 70 percent EtOH, drying, and then placing one drop of sterile water in the center. The fruiting bodies are placed in the sterile water and crushed gently with the coverslip. The slides are then examined under a compound microscope. Acervuli and conidia morphologies (fig. 4) are compared to references (EPPO Standards 2015, CABI 2021, van der Nest et al. 2019). Microscope slides that contain fruiting structures similar to *L. acicola* are retained for isolation. The coverslips are carefully removed, leaving the water on the glass slides. A sterile inoculating loop is used to capture the fruiting body and a small amount of water. This suspension is then streaked for isolation (as with yeast or bacteria) on 2 percent malt extract agar (MEA) (modified from Mullett and Barnes 2012). A second loopful can be

streaked on ground pine needle agar (gPNA) (modified from Luchi et al. 2007) prepared with 2 percent weight/volume agar and 8 percent weight/volume ground longleaf pine needles. Growing media may be amended with antibiotics to prevent contamination: streptomycin for gram-negative bacteria and chlortetracycline for gram-positive bacteria.

Isolation Method 2

Symptomatic needles are maintained in the Petri dish moist chambers and inspected under a stereo microscope every 2 to 3 days. When greenish black conidia are found erupting from fruiting bodies typical of *L. acicola* (fig. 2A), the conidia are harvested with a sterile dissecting needle or small scalpel and suspended in 500 µl sterile water, vortexed to mix, and streaked onto thin potato dextrose agar (PDA), or other semitranslucent general medium, with a sterile bacterial inoculation loop.

Isolation Method 3

Direct isolation methods can be used with or without the moist chamber step when needle samples show an abundant amount of fruiting bodies that are visible under the dissecting microscope. The symptomatic pine needle is gently wiped with a paper towel saturated with 70 percent EtOH. A drop (approximately 1–2 µl) of sterile water is applied to a target fruiting body under the dissecting microscope, carefully excised from the needle with a sterile scalpel and transferred directly to an isolating culture media such as PDA (fig. 5). Alternatively, the excised acervulus may be placed directly onto culture media and rolled or streaked for isolation across the media (Mullett and Barnes 2012). Repeated rolling, using as much of the plate as possible, enhances isolation success. Direct plating of the acervulus is recommended if the needles were recently collected and are free from saprophytic growth.

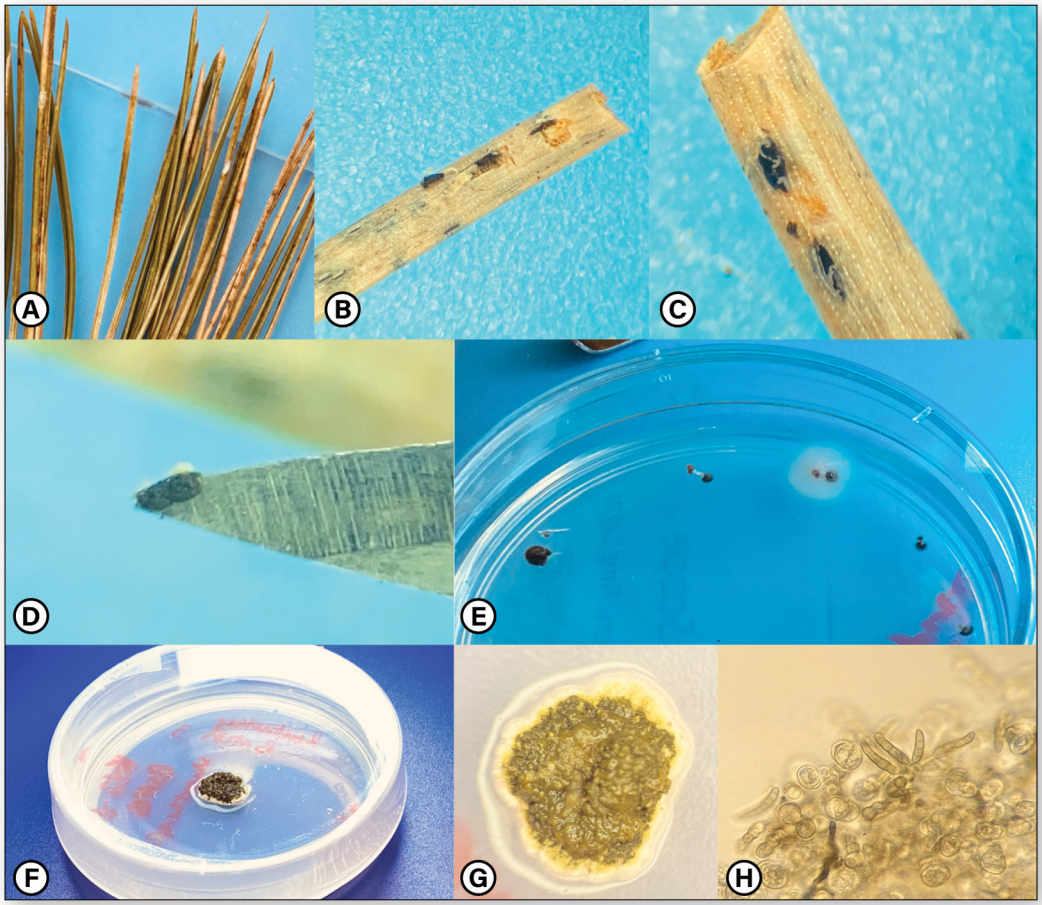

Figure 5—(A, B) *Lecanosticta acicola* fruiting bodies on the surface of sterilized pine needles showing symptoms of brown spot needle blight, (C) a fruiting body exposed and (D) carefully excised with sterile sharp scalpel, (E) germinated spores from transferred fruiting bodies, (F, G) a 4-week-old culture incubated on potato dextrose agar (PDA) at approximately 25 °C, and (H) spores observed under the microscope.

INCUBATION AND CULTURE CHARACTERISTICS

Plates are wrapped in parafilm and incubated on an open shelf under ambient (room temperature) conditions. Streaked plates are examined on an inverted or stereo microscope daily, and individual germinating conidia are harvested with a sterile small scalpel by using a stereo microscope and then placing them on new plates. On MEA, PDA, and gPNA, single spore *L. acicula* colonies may be visible after 2 days and will be apparent within 7 days after inoculation. Colonies should be transferred to new plates as soon as they are visible to avoid overgrowth by potential contaminants. This method is helpful when single genotype (i.e., single spore) colonies are needed. Plates with fruiting bodies are monitored for contamination and *L. acicola* growth. After 2 to 3 weeks the resulting spore mass or hyphal tips of the mycelia growth are subcultured to a clean culture plate and incubated at ambient conditions (approximately 25 °C) (fig. 5). Spore morphology can be observed under the microscope for identification and confirmation (figs. 4, 5) (EPPO Standards 2015). Nascent colonies display hyaline or white aerial hyphae that turn gray or olive in 1 to 2 weeks. Pigmentation occurs in approximately 9 days on gPNA, though it is generally lighter and appears later (within 12 days) on MEA (fig. 6). Notably, colonies on gPNA sometimes appear to release a compound that turns the medium around the colonies an orange-red color (fig. 6B) and to a lesser extent on PDA turning the medium yellow-brown (fig. 6D). Similar results are observed on gPNA plates prepared from loblolly and eastern white pine needles. *Lecanosticta acicola* colonies grow radially and slowly at a rate of 2.5–3.0 mm per week (CABI 2021). Contaminants are often distinguishable from *L. acicola* by their faster growth rate or yeast-like colony morphology. Morphological identification of *L. acicola* is difficult and DNA-based molecular methods are often needed to confirm the identity of isolates (EPPO Standards 2015).

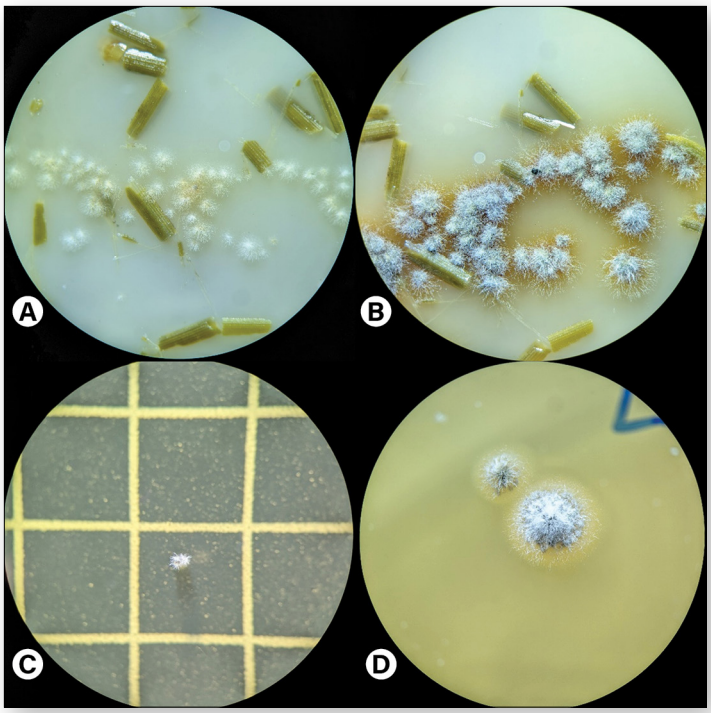

Figure 6—*Lecanosticta acicola* colonies on ground pine needle agar (gPNA) at (A) 5 days and (B) 8 days, highlighting colony pigmentation and discoloration of the surrounding media, and on malt extract agar (MEA) at (C) 4 days and (D) 12 days.

LITERATURE CITED

Barnes, I.; Crous, P.W.; Wingfield, B.D.; Wingfield, M.J. 2004. Multigene phylogenies reveal that red band needle blight of Pinus is caused by two distinct species of Dothistroma, *D. septosporum* and *D. pini*. Studies in Mycology. 50: 551–565.

Broders, K.; Munck, I.; Wyka, S. [et al.] 2015. Characterization of Fungal Pathogens Associated with White Pine Needle Damage (WPND) in Northeastern North America. Forests. 6: 4088–4104. https://doi.org/10.3390/f6114088.

CABI Compendium [CABI]. 2021. Datasheet 49057. *Mycosphaerella dearnessii* (brown spot needle blight). www.cabi.org/isc/datasheet/49057. [Date accessed: 5 April 2022].

EPPO Standards. 2015. PM 7/46 (3) *Lecanosticta acicola* (formerly *Mycosphaerella dearnessii*), *Dothistroma septosporum* (formerly *Mycosphaerella pini*) and *Dothistroma pini*. EPPO Bulletin. 45(2): 163–182. https://doi.org/10.1111/epp.12217.

Luchi, N.; Capretti, P.; Bonello, P. 2007. Production of *Diplodia scrobiculata* and *Diplodia pinea* pycnidia on ground Austrian pine needle agar medium. Phytopathologia Mediterranea. 46(2): 230–235.

Mullett, M.; Barnes, I. 2012. *Dothistroma* isolation and molecular identification methods. COST Action FP1102 training school document. https://doi.org/10.13140/RG.2.2.15129.36964.

Sinclair, W.A.; Lyon, H.H. 2005. Diseases of Trees and Shrubs. 2nd ed. Ithaca, NY: Cornell University Press. 660 p.

van der Nest, A.; Wingfield, M.J.; Janoušek, J.; Barnes, I. 2019. *Lecanosticta acicola*: A growing threat to expanding global pine forests and plantations. Molecular Plant Pathology. 20(10): 1327–1364. https://doi.org/10.1111/mpp.12853.

Wyka, S.A.; Smith, C.; Munck, I.A. [et al.] 2017. Emergence of white pine needle damage in the northeastern United States is associated with changes in pathogen pressure in response to climate change. Global Change Biology. 23: 394–405. https://doi.org/10.1111/gcb.13359.

Dreaden, T.; Meinecke, C.; Olatinwo, R. 2024. Guide to Isolation of *Lecanosticta acicola*, the Causal Agent of Brown Spot Needle Blight of Pines. Res. Note SRS-27. Asheville, NC: U.S. Department of Agriculture, Forest Service, Southern Research Station. 6 p. https://doi.org/10.2737/SRS-RN-27.

Lecanosticta acicola is a foliar fungal pathogen that affects over 70 conifer species, primarily in the genus *Pinus*, and it has been introduced to much of the Northern Hemisphere. In the last decade, the pathogen has been reported causing two outbreaks in the Eastern United States on loblolly pine (*P. taeda*) and eastern white pine (*P. strobus*), in addition to its ongoing endemic status on longleaf pine (*P. palustris*) across the central Gulf and southeast Atlantic coastal plains. It is often difficult to culture due to its slow growth and poor competitiveness against other fungi on culture media. To remedy this, we developed and describe procedures for *L. acicola* sample collection and methods for isolation that give reproducible results for loblolly, longleaf, and eastern white pines.

Keywords: Brown spot needle blight, white pine needle damage, *Mycosphaerella dearnessii*, single genotype, single spore.

September 2024

https://doi.org/10.2737/SRS-RN-27

Forest Service
Research & Development
Southern Research Station
Research Note SRS-27

Southern Research Station
200 W.T. Weaver Blvd.
Asheville, NC 28804
https://research.fs.usda.gov/srs