Storage Conditions Influence Cultural Detection of the Shoot Blight Pathogen *Diplodia pinea* From Asymptomatic Red Pine Nursery Seedlings

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Abstract

The pine shoot blight, canker, and collar rot pathogen *Diplodia pinea* can persist on or in asymptomatic red pine nursery seedlings, and it can proliferate after outplanting to cause disease and mortality. After lifting from nursery beds, seedlings are routinely kept in cold storage at nurseries. During and after shipment to customers, however, seedlings may be stored without refrigeration. In each of 2 years, we assayed seedlings from a bareroot nursery before and after storage for presence of this pathogen. Each trial included a storage treatment in which seedlings were kept at room temperature for 1 week after cold storage. Results demonstrated the effectiveness of nursery cultural practices and protective fungicide applications, as well as cold storage, to reduce the frequency of association of the pathogen with asymptomatic seedlings. We recommend that seedlings be kept in cold storage, even at moderately cool temperatures, before, and especially after, delivery to customers.

Introduction

Red pine (*Pinus resinosa* Aiton) is the most planted tree in the North-Central region of the United States (Gilmore and Palik 2006), and most contemporary red pine stands are plantations of this single species (USDA Forest Service 2002). Seedlings are planted after clearcut harvests of mature plantations. Most of these seedlings are 2- or 3-year-old bareroot seedlings produced in State and Federal nurseries in Michigan, Wisconsin, and Minnesota.

Shoot blight, canker, and collar rot caused by *Diplodia pinea* (syn. *Sphaeropsis sapinea*) frequently damages red pine nursery seedlings. For example, Palmer and others (1986) reported a 42-percent disease incidence in 2-0 seedlings. In plots located in proximity to red pine windbreaks, which are a source of inoculum, the frequency of shoot blight can be even greater (Stanosz and others 2005). *D. pinea* survives in dead colonized needles, stems, and cones on which it bears asexual fruiting bodies (pycnidia) (figure 1) that release spores (conidia). Spores are disseminated by rain splash and are abundant during spring and early summer (Palmer and others 1988), when young shoots are most susceptible. The pathogen infects through stomata, directly through the surface of young stems, or through fresh wounds (Brookhouser and Peterson 1971, Chou 1976). Pycnidia with conidia can develop within a few weeks after infection on dead seedlings, killed organs of living seedlings, and shoots excised from top-pruned seedlings (Munck and Stanosz 2008, Palmer and others 1988), so that multiple cycles of disease within a single growing season are possible. The similar fungus *D. scrobiculata* also can damage red pines, but it has been less often associated with red pine nursery seedlings (Stanosz and others 2005).

Red pine seedlings of all age classes may be rendered unmerchantable because of Diplodia shoot blight, canker, and collar rot, all of which lead to deformity or death (Palmer and Nicholls 1985). Infection of young seedlings during the first season of growth can result in rapid mortality, with retention of reddish to brown dead needles (figure 2). Colonization of elongating shoots on older seedlings can lead to shoot

Figure 1. Pycnidia of *Diplodia pinea* emerging from the base of a red pine needle. (Photo by Glen R. Stanosz)

death before full needle elongation and result in curling or crooking of the stem (figure 3). Needles of diseased shoots often turn yellow, then red to brown, or gray. Cankers on seedling stems begin as discrete, purplish, resinous lesions that result from direct infection or pathogen growth into stems from diseased needles. Collar rot symptoms include relatively rapid desiccation of needles and seedling death (figure 4), with blackening of the lower stem and root collar inner bark, and with dark staining of the underlying wood (figure 5). Although obviously symptomatic seedlings can be discarded during sorting and grading before packing, shipments of bulk-lifted seedlings (those that are packed immediately after lifting without sorting or grading) may include blighted or dead seedlings that bear the pathogen.

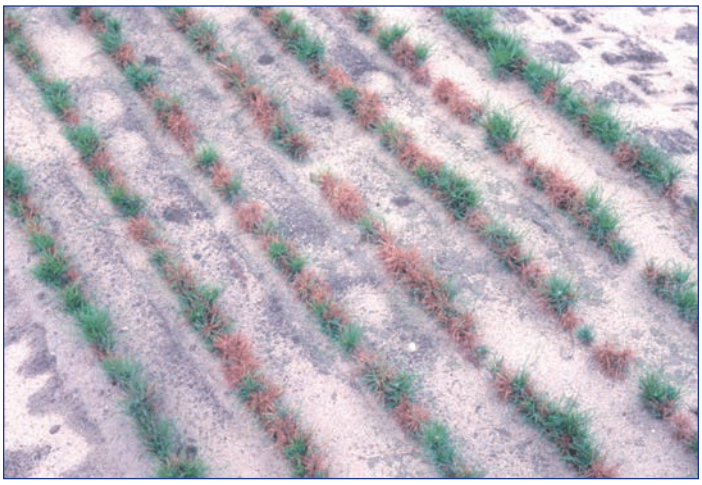

Figure 2. Dead red pine seedlings killed by *Diplodia pinea* in the first season of growth. (Photo by Glen R. Stanosz)

Figure 3. Distorted red pine shoot killed by *Diplodia pinea* during elongation. (Photo by Glen R. Stanosz)

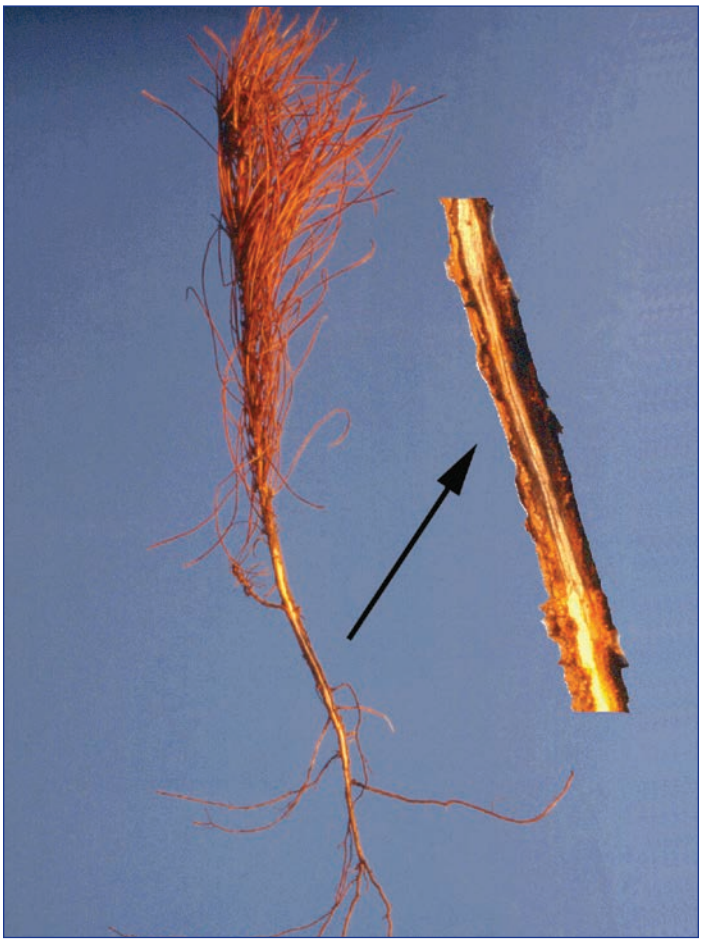

Figure 4. Red pine seedling that was rapidly killed by Diplodia collar rot (inset) shortly after outplanting. (Photo by Glen R. Stanosz)

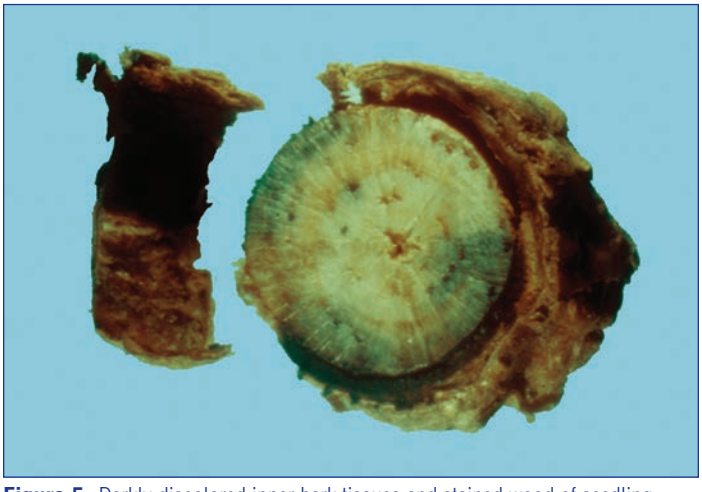

Figure 5. Darkly discolored inner bark tissues and stained wood of seedling killed by Diplodia collar rot. (Photo by Glen R. Stanosz)

Application of protectant chemicals to reduce losses caused by *D. pinea* has produced mixed results. Palmer and others (1986) reported that only 2.7 percent of 2-0 red pine seedlings were diseased when treated with benomyl during both growing seasons. In one nursery, however, Stanosz and others (2005) found that, in spite of benomyl application, the average disease incidence, based on visible symptoms, was 43 percent in plots of 2-0 seedlings in close proximity to a windbreak inoculum source. In addition, fungicide applications may not prevent persistence of *D. pinea* on or in seedlings in the absence of disease development. Stanosz and others (2005) culturally assayed surface-disinfested lower stem segments from healthy-appearing seedlings. The pathogen was detected on 63 percent (Wilson State Nursery, Wisconsin) and 88 percent (Badoura State Nursery, Minnesota) of asymptomatic seedlings in beds that were in close proximity to a windbreak inoculum source and in which symptomatic seedlings also were common. In addition, *D. pinea* can subsequently proliferate and kill previously asymptomatic seedlings under conditions that induce host stress (Stanosz and others 1997, Stanosz and others 2001). This ability of *D. pinea* to act as a latent pathogen may explain the frequent mortality associated with collar rot of recently outplanted red pine seedlings (Stanosz and Cummings Carlson 1996).

After dormant bareroot red pine seedlings are lifted and packed in early spring, they usually are stored until delivery to customers. For example, at the Minnesota Department of Natural Resources General Andrew Nursery, seedlings are placed in plastic bags and then into shipping cartons and maintained in a cold room at 3.3 to 4.4 °C (38 to 40 °F) for as long as 3 weeks. After seedlings are transferred to customers, however, conditions during transport and storage for days or even weeks until seedlings are planted are highly variable and often do not include cold storage.

Nurseries in which Diplodia shoot blight, canker, and collar rot have caused serious losses have implemented practices intended to reduce both the incidence of these diseases in nursery beds and the persistence of *D. pinea* on healthy-appearing seedlings. The influence of storage conditions on the activity of *D. pinea* on or in the asymptomatic seedlings, however, has not been explored. The objectives of this study were to (1) quantify the effectiveness of disease management practices on the persistence of *D. pinea* on or in asymptomatic red pine nursery seedlings and (2) determine the influence of storage, including a period of nonrefrigerated storage, on asymptomatic persistence of the pathogen on red pine nursery seedlings. Studies were conducted in each of 2 years, using cultural methods to detect the pathogen and molecular methods to confirm pathogen identity.

Methods

Experiments With Noninoculated Seedlings

Experiment 1 was designed to compare the frequency of cultural detection of *D. pinea* among seedlings assayed (1) upon receipt from the nursery (without extended storage), (2) after storage for 3 weeks in a cold room, or (3) after storage for 3 weeks in a cold room and then 1 additional week at a room temperature. The third treatment was intended to simulate proper cold storage of seedlings after lifting, followed by storage at a warmer temperature during delivery or after receipt by a customer.

Asymptomatic, dormant red pine seedlings were lifted from two nursery beds in late April 2009 and 2010 from the Minnesota Department of Natural Resources General Andrews State Nursery, Willow River, MN (46.32° N., 92.84° W.). Seedlings from each nursery bed were packaged 10 per plastic bag (a replicate), with these bags placed within a larger plastic bag and corrugated cardboard box normally used for seedling shipment. The two boxes were shipped overnight to the laboratory at the University of Wisconsin-Madison, where five replicate bags of seedlings from each nursery bed were randomly assigned to each of the three treatments, and then replaced in the larger plastic bag in the shipping boxes.

Experiment 2 was conducted similarly in 2010 with five replicate bags of seedlings from each nursery bed assigned randomly to (1) storage for 4 weeks in a cold room or (2) storage for 3 weeks in a cold room, followed by 1 week at room temperature. Storage temperatures during each experiment were recorded hourly using Hobo data loggers (Onset Computer Corporation, Bourne, MA) placed among the bags of seedlings.

After storage, seedlings were culturally assayed using procedures similar to those previously developed to evaluate asymptomatic persistence of the pathogen on or in red pine seedlings (Stanosz and others 2005). A segment approximately 5 cm (2 in) long was cut from the lower stem/root collar of each seedling, needles were removed, and then surface-disinfested by 30 sec immersion in a 95-percent ethanol solution followed by two immersions for 2 min each in a solution of 1.05 percent NaClO plus two drops of Tween-80 per liter (8 drops per gallon) deionized water. Each segment was then placed on one side in an 84-mm-diameter (3.3-in-diameter) Petri dish containing tannic acid agar medium (Blodgett and others 2003) and twice-autoclaved red pine needles were placed on the other side (figure 6). The dishes were incubated 30 cm (12 in) beneath one cool white

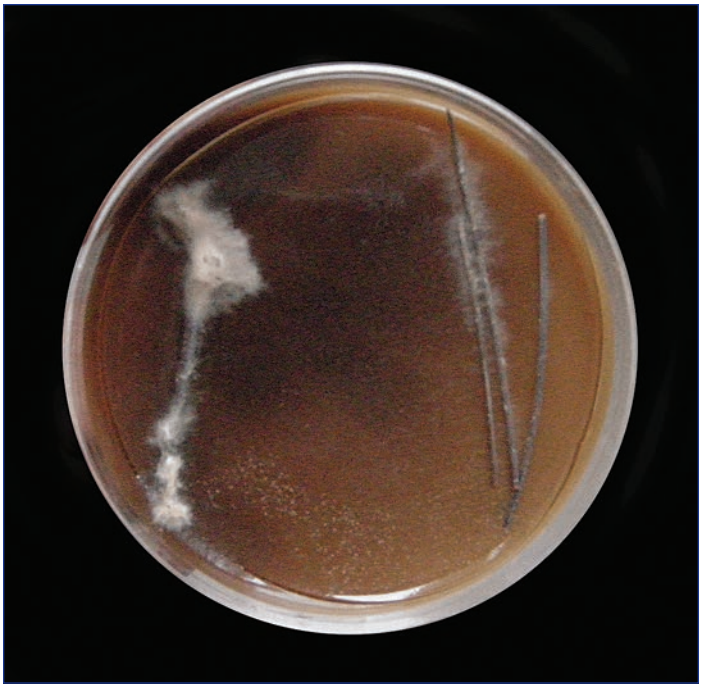

Figure 6. *Diplodia pinea* mycelium that has grown from a surface-disinfested red pine stem segment (left) to red pine needles (right) in a Petri dish containing tannic acid agar medium. (Photo by Glen R. Stanosz)

fluorescent light tube and one ultraviolet light tube for up to 6 weeks at approximately 24 °C (75 °F). Conidia from pycnidia produced on the needles were examined for characteristics consistent with those of *D. pinea* (Punithalingam and Waterston 1970).

To confirm the pathogen species, pycnidia from the Petri dishes were transferred to potato dextrose broth and incubated for approximately 1 week. After incubation, DNA from these subcultures was extracted using the procedures of Smith and Stanosz (1995). The fungus was then identified using specific mt SSU rDNA PCR primers that allow differentiation of *D. pinea* from the similar conifer pathogen *D. scrobiculata* and other related fungi (Smith and Stanosz 2006).

Experiments With Inoculated Seedlings

Because nursery disease management practices likely reduced *Diplodia* frequency on red pine seedlings, additional experiments were conducted to further evaluate storage effects on the pathogen's persistence and disease. Dormant seedlings from the same nursery were lifted from two nursery beds in late April 2009 (experiment 3) and 2010 (experiment 4), packaged 10 per plastic bag, and shipped to the laboratory as described previously for experiments 1 and 2. Conidial inoculum of *D. pinea* was applied to seedlings after receipt, however, to ensure presence of the pathogen with seedlings during storage treatments. Experiment 3 was conducted in 2009 with 10 bags (replicates) from each nursery bed assigned randomly to each of two treatments: (1) storage for 3 weeks in a cold room or (2) storage for 3 weeks in a cold room, followed by 1 additional week at room temperature. Experiment 4 was conducted in 2010 with 10 bags from each nursery bed assigned randomly to (1) storage for 4 weeks in a cold room or (2) storage for 3 weeks in a cold room, then 1 additional week at room temperature.

Conidial inoculum was obtained from twice-autoclaved red pine needles incubated for several weeks on colonies of *D. pinea* on water agar medium. Needles bearing pycnidia were crushed in sterile, deionized water. The resulting suspension was filtered through two layers of cheesecloth, and more water was added to adjust the concentration of conidia to 5 by 10^4 spores per millimeter. An atomizer was used to apply 1 ml of con-idial suspension to seedlings in each replicate bag and then the bag was resealed. Germination of conidia in the inoculum suspensions was assessed by examination of 50 conidia per trial of experiments 3 and 4 after 4 hours incubation on water agar medium at 24 °C (75 °F) in the dark. For both exper-iments, germination exceeded 80 percent. After storage, seedlings were culturally assayed using procedures described above.

Experimental Design and Data Analysis

Because results were similar, data for seedlings from the two nursery beds were pooled into a single, completely randomized design for each experiment. Means of temp-eratures recorded hourly for each experiment were calculated, and maximum and minimum temperatures were determined. For each experiment, mean percentages of seedlings from which the pathogen was detected were calculated. Because the data lack normality, analyses were performed using a nonparametic method. Differences among storage treatments in each experiment were determined using the Kruskal-Wallis test of equality of medians using Minitab for Windows version 14 (Minitab Inc., State College, PA).

Results and Discussion

Experiments With Noninoculated Seedlings

Use of molecular methods confirmed *D. pinea* as the pathogen cultured from noninoculated seedlings in every case except one, when the similar pathogen *D. scrobiculata* was detected. The detection of *D. pinea* in this study is consistent with prevalence of this pathogen with asymptomatic nursery seedlings at other nurseries, but contrasts with previous results for the General Andrews State Nursery. When surveyed in 2002, 7 of

10 seedlings from the General Andrews State Nursery for which molecular methods were used to confirm pathogen identity yielded *D. scrobiculata* (Stanosz and others 2005). Whether the current result indicates a shift in pathogen population in, or in the vicinity of, this nursery is unknown. These findings, however, underscore the importance of employing methods that allow for unambiguous identification of fungal pathogens.

Noninoculated seedlings were infrequently (0 to 7 percent) culturally positive with or without extended storage in both 2009 and 2010 (table 1). Detection of a *Diplodia* pathogen was rare in these 2 years compared with 2002, when seedlings from this nursery were similarly assayed. At that time, averages of 20 and 26 percent of asymptomatic seedlings from the two locations sampled tested positive for either pathogen, with as many as 40 percent of seedlings positive in one plot (Stanosz and others 2005). At other nurseries sampled that year in Minnesota and Wisconsin, as many as 88 percent of asymptomatic seedlings in proximity to windbreaks bore *D. pinea* or *D. scrobiculata*. The much lower frequency of detection in the current study can be attributed to efficacy of current disease management practices at the General Andrews State Nursery and the other affected nurseries. Removing red pine windbreaks, rouging affected seedlings, avoiding top pruning, and adopting a 2-year production cycle (instead of a 3-year cycle) reduce the exposure of seedlings to inoculum. Coupled with judicious application of fungicidal sprays, these measures have drastically reduced association of the pathogens with seedlings (Minnesota Department of Natural Resources 2009, Wisconsin Department of Natural Resources 2011).

Experiments With Inoculated Seedlings

Results differed significantly between storage treatments for seedlings to which inoculum had been added in 2009 ($p < 0.01$). The frequency of culturally positive inoculated seedlings was 6 percent when seedlings were cold stored (approximately 3.5 °C) for 3 weeks compared with 33 percent for seedlings that were stored for 1 additional week at room temperature (table 1). This difference demonstrates the potential for pathogen proliferation after removal of seedlings from cold storage. Detection, even after rigorous surface disinfestation, suggests that a pathogen is not merely persisting superficially, but that infection has occurred.

In 2010, temperature in cold storage was not as low as desired, averaging nearly 8 °C (14.4 °F) (table 1). The frequency of cultural detection was 12 percent for seedlings that were cold stored for 4 weeks and 21 percent for seedlings that were removed from the cold room and stored for a 4th week at room temperature ($p = 0.12$). Even though cold storage temperatures were higher than planned, a tendency still existed for more frequent pathogen detection after exposure to a warmer temperature for the final week.

Implications for Nurseries and Customers

Similar to the current study, previous research to examine the effect of temperature on growth of *D. pinea* and *D. scrobiculata* found that temperatures of 20 °C (68 °F), 25 °C (77 °F), and 30 °C (86 °F) were conducive to colony growth after 3 days on potato dextrose agar, whereas no discernable growth was observed for cultures at 5 °C (41 °F) or 10 °C

Table 1. Percentages of asymptomatic red pine seedlings from which cultural detection of *Diplodia pinea* or *D. scrobiculata* occurred.

Treatment	Noninoculated seedlings (%)[a]		Inoculated seedlings (%)[b]	
	2009 (experiment 1)	2010 (experiment 2)	2009 (experiment 3)	2010 (experiment 4)
No storage	3	7	—	—
Stored 3 weeks at 3.5 ± 1.1 °C (38.3 ± 2.0 °F)	1	—	6	—
Stored 3 weeks at 3.5 ± 1.1 °C (38.3 ± 2.0 °F), followed by 1 week at 23.0 ± 1.1 °C (73.4 ± 2.0 °F)	1	—	33	—
Stored 4 weeks at 7.8 ± 1.0 °C (46.0 ± 1.8 °F)	—	0	—	12
Stored 3 weeks at 7.9 ± 0.5 °C (46.2 ± 0.9 °F), followed by 1 week at 24.8 ± 1.6 °C (76.6 ± 2.8 °F)	—	4	—	21
	$p = 0.40$[c]	$p = 0.10$	$p < 0.01$	$p = 0.12$

[a] Experiments 1 and 2: n = 10; 5 replicates from each of two nursery beds.
[b] Experiments 3 and 4, n = 20; 10 replicates from each of two nursery beds.
[c] Values of p for treatment differences using Kruskal-Wallis test of equality of medians.

(50 °F) (Palmer and others 1987). Temperatures of 0 °C to 2 °C (32 °F to 36 °F) are recommended as ideal cold storage temperatures for seedlings for up to 2 months (Landis and others 2010). Many nurseries now have facilities for storage of seedlings at these temperatures, although customers may not. Results of this study and others support the likely benefit of preplanting storage by customers at even moderately cool temperatures (≤ 10 °C [≤ 50 °F]).

In addition to the direct influence of temperature on fungal growth, lengthy cold storage durations or storage without refrigeration could affect seedling physiological condition (Landis and others 2010) and render seedlings susceptible to infection or disease development. For example, a controlled experiment with potted red pine seedlings demonstrated that moisture stress induces more severe Diplodia shoot blight symptoms (Blodgett and others 1997). As mentioned previously, stress can stimulate proliferation of *D. pinea* to kill previously asymptomatic seedlings (Stanosz and others 2001). Storage in sealed plastic bags lessens drying in storage and no visible indications of drying were apparent in the current study.

Conclusions

We infrequently cultured *Diplodia pinea* and *D. scrobiculata* from asymptomatic red pine seedlings grown in a nursery where practices included removal of inoculum sources, chemical protection, and other measures to reduce or eliminate presence of these pathogens. When we inoculated the seedlings with *D. pinea* immediately before storage, however, a period of storage without refrigeration led to more frequent cultural detection of this pathogen. Storage of seedlings at even moderately cool temperatures before, and especially after, delivery to customers is recommended.

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