Quantification of Conidia of *Diplodia* spp. Extracted from Red and Jack Pine Cones

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ABSTRACT

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Frequency of detection and inoculum production by the conifer shoot blight and canker pathogens *Diplodia pinea* and *D. scrobiculata* on cones of red pine (*Pinus resinosa*) and jack pine (*P. banksiana*) were studied. Cones were collected from the ground and from canopies of red and jack pine trees in mixed stands at three sites in each of two different locations during two consecutive summers in Wisconsin. Conidia were extracted in water, quantified, germination tested, and the *Diplodia* species present was determined using molecular methods. At least one pathogen was detected from each tree at each site in both years. Overall, more conidia were extracted from cones from canopies than cones from the ground and from red pine cones than jack pine cones. Both total numbers of conidia extracted and proportions of cones yielding *D. pinea* or *D. scrobiculata* varied by location and pine species. Cones from either the ground or canopies can be used for surveys to detect *Diplodia* spp. at a given site but cones from canopies may be more useful to determine the relative abundance of potentially available inoculum of these pathogens.

Additional keywords: sporulation, *Sphaeropsis sapinea*

Two native pine species, jack pine (*Pinus banksiana* Lamb.) and red pine (*P. resinosa* Ait.), are ecologically important components of conifer forests in northern portions of eastern North America. Red and jack pine grow in sandy, low-fertility soils (20,21). Red pine commonly grows on level to gently rolling sand plains or on low ridges adjacent to lakes and swamps (20). Jack pine usually grows in less fertile and drier soils than those associated with other pine species (21). Each species is shade intolerant and jack pine, with serotinous cones, often invades recently burned areas (21). The ranges of both species overlap in central and eastern portions of the northern United States and Canada. However, jack pine is more widely distributed than red pine and its range in Canada extends farther north-west. Both pine species are at the southern edge of their range in Wisconsin.

Compared with presettlement forests, conifer stands in Wisconsin have been lost to poor regeneration following early logging in the 1800s (35). Although most jack pine stands are naturally regenerated, today red pine is the most planted tree spe-

cies in Wisconsin. Six and a half million hectares are forested in Wisconsin, of which the red and jack pine forest types occupy 243,945 and 156,653 ha, respectively (36). Both species are harvested for pulpwood, and large red pine trees are also used for lumber (36).

Among the pathogens of red and jack pine are two recently differentiated asexual fungi: *Diplodia pinea* (Desmaz.) J. Kickx f. (syn. *Sphaeropsis sapinea*) and *D. scrobiculata* J. de Wet, Slippers & M.J. Wingf. *D. pinea* is the more frequently reported and widely distributed pathogen, causing damages including shoot blight, stem cankers, crown wilt, bluestaining of the sapwood, and seedling collar rot (18). Both native and exotic conifers in nurseries, plantations (27), windbreaks (13), forests, and those planted as ornamentals are affected. *Diplodia* spp. are frequently found sporulating on dead needles, bark, and especially mature seed cones (13,34). Conidia are dispersed by rain throughout the growing season (15) and can germinate within a few hours (5). The fungus infects through wounds and needle stomata (5) and by direct penetration of young stems (6). Shoots are particularly susceptible to shoot blight in early spring during bud break and shoot elongation (15,17). Many publications that describe disease development by *D. pinea* were written before *D. scrobiculata* was recognized as a distinct species (8), but the disease cycles are presumed to be similar. Like *D. pinea*, *D. scrobiculata* is a pathogen of red and

jack pine and the two fungi co-occur in the same forests, trees, and tissues (24).

Information regarding occurrence and inoculum production of *D. pinea* and *D. scrobiculata* on jack and red pine cones is very limited. Vujanovic et al. (33) reported *D. pinea* sensu lato from a collection of red pine cones and seed from a botanical garden but neither the number of trees or cones examined nor the number yielding the fungus is clearly indicated, and jack pine was not studied. Nicholls and Ostry (13) reported that 84% of 300 2-year-old or older red pine cones from two windbreaks bore pycnidia of *D. pinea* sensu lato but no information on sampling design or numbers of conidia present were provided, nor were jack pine trees sampled. Additionally, an attempt was made to study conidial dispersal from individual red pine cones collected from windbreaks of a single nursery (15). Spores were trapped on slides beneath cones placed on screens and the authors stated that the numbers of conidia trapped varied among and within windbreaks. However, data from only five cones from one red pine tree were shown and, again, jack pine was not studied. Palmer (14) also indicated the presence of both *D. pinea* and *D. scrobiculata* among collections of cones and dying or dead red or jack pine branches in four stands in Minnesota or Wisconsin. However, no information on the frequency of detection of either fungus was provided, nor was it stated whether cones (and not branches) were the source of the particular isolates obtained.

Because the distribution and relative abundance of *D. pinea* and *D. scrobiculata* in the forests of jack and red pine in which both the pathogens and hosts co-occur has not been studied, our objective was to use cones as a tool to explore the influence of location and host species on frequency of detection and inoculum production by these fungi. Cones were collected from red and jack pine trees in mixed stands. Conidia were extracted in water and quantified, and the *Diplodia* sp. present was determined using molecular methods. The null hypotheses were (i) there is no difference between hosts in the number of conidia extracted from cones, (ii) there is no difference among sites in the number of conidia extracted from cones, and (iii) there is no difference in the frequency with which *D. pinea* and *D. scrobiculata* are detected from cones.

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MATERIALS AND METHODS

Study area. Two similar locations in central Wisconsin approximately 70 km apart were sampled, one in Adams County and the other in Jackson County. The climate is continental characterized by cool-to-warm summers, cold winters, and low-to-moderate precipitation. The soils are well drained to excessively drained sands common on the glacial outwash and former glacial lakebeds of central Wisconsin. Historically, xeric pine forests primarily made up of red pine, jack pine, and white pine (*P. strobus* L.) commonly occurred on such sites in nearly pure stands of a single species or in mixtures of two or all three (7). Today, red and jack pine are commercially managed in these locations.

Cone collection. During July of two consecutive summers in three sites at each of the two locations, jack and red pine cones that had matured the previous fall were collected where these species co-occurred in the overstory. The sampled sites at each location were separated by at least 680 m. Shoot blight symptoms typical of those caused by *D. scrobiculata* and *D. pinea* were not apparent on trees at these sites and cones were not examined for pycnidial production at the time of collection. In 2004, cones were collected from five jack and five red pine trees at each site. Because of variability in the data in 2004, cones were collected from 10 trees of each species per site in 2005. In Jackson County (in an area between 44.34 and 44.38°N latitude and 90.68 and 90.75°W longitude), the same three sites were visited in 2004 and 2005. In Adams County (in an area between 44.10 and 44.25°N latitude and 89.82 and 89.90°W longitude), two new sites were used in 2005 due to harvesting or inaccessibility of two sites sampled in 2004. Cones were collected from arbitrarily selected pairs of trees (one from each species) growing next to each other. From each tree, five cones were collected from the middle or lower portion of the crown (canopy) and five cones were collected from the ground directly beneath the canopy. The age of the cones collected from the canopy was determined by their position on the branch. Mature open cones closest to the branch tips were collected for each pine species. The most recently fallen, unweathered, opened cones from the ground were collected. In 2004 and 2005, a total of 300 and 600 cones, respectively, of each pine species were collected. Cones were bagged and placed on ice in coolers for transportation to the laboratory where they were stored at -20°C until they were processed.

Conidial extraction and enumeration. Cones were processed individually in the laboratory to extract conidia. Each cone was placed in a 100-ml plastic beaker containing 80 ml of deionized water with Tween 80 (Fisher Scientific Company, Fair Lawn, NJ) at 2 drops/liter and washed for

3 h on a rotary shaker at 110 rpm. Cones were removed from beakers and then the volume in each beaker was adjusted to 100 ml with deionized water to account for differences in the absorbance of water by each cone. The resulting liquid (25 ml) was pipetted out of each cup while being stirred at low speed and then filtered through a membrane filter (pore size: 0.8 µm) printed with a grid to delineate 3-by-3-mm squares (cat. no.: AAWG047SP; Millipore Corporation, Billerica, MA). Conidia recognized as those of *D. pinea* and *D. scrobiculata* were counted on five randomly chosen squares of the filter paper with the aid of a dissecting microscope at up to ×30. Because conidia of *D. pinea* and *D. scrobiculata* have very similar morphology, these two species were differentiated with molecular methods described in the next section. The mean number of conidia from five squares was multiplied by a factor to adjust for total area of filter paper and the volume of water in which the cone was washed to estimate the number of conidia extracted per cone. Because red pine cones are typically heavier than jack pine cones, the number of conidia extracted per gram of oven dry weight of each cone also was calculated. Weight was considered to be an adequate substitute for surface area because Sutton et al. (31) found that red pine cone fresh mass was highly correlated with cone area (correlation coefficient, $r = 0.95$).

Conidial germination tests. In 2004, five additional cones were collected at each site from both the canopy and the ground for one of the sampled trees. These cones were utilized to conduct germination tests and identify the pathogen species present. Cones were washed as described above and 0.5 ml of debris from the bottom of each cup was pipetted into petri plates containing 2% water agar (WA), which were then incubated at 24°C in continuous light for at least 5 h and no more than 10 h. Germination was quantified for plates with at least 50 conidia. If the germ tube of a spore was at least equal to the spore length, then the spore was considered to have germinated.

Confirmation of pathogen identity. Molecular methods described by Smith and Stanosz (24) were utilized to determine the identity of 10 single-spore isolates per site obtained from 2% WA germination plates and then transferred to potato dextrose agar (PDA) slants. In all, 51 of these isolates were obtained from red pine canopy cones and 9 were obtained from jack pine canopy cones. A small piece of a colony of each isolate was placed in a 1.5-ml microcentrifuge tube containing 500 µl of potato dextrose broth and streptomycin sulfate (100 mg/liter). The cultures were incubated in the dark at room temperature for 7 to 10 days and then DNA was extracted from the cultures by the method described in Smith and Stanosz (23).

Briefly, the fungal cultures were ground in extraction buffer (500 mM NaCl; 100 mM Tris, pH 8.0; 10 mM EDTA; and 10 mM β-mercaptoethanol) with Kontes pestles (Kimble/Kontes, Vineland, NJ) and incubated for 10 min at 60°C with 20% sodium dodecyl sulfate. Then, proteins were removed with potassium acetate and DNA precipitated by isopropanol. The extracted DNA was amplified using primers specific to each *Diplodia* sp. (24) and presence of amplified DNA fragments was verified on 0.7% agarose gels in Tris-borate-EDTA buffer.

These methods were altered for the cones collected in 2005. Two arbitrarily selected cones from each species from both the canopy and the ground at each site from which conidia were successfully extracted by methods described above were utilized to conduct germination tests. After the cones were washed, 0.5 ml of debris from the bottom of each cup was pipetted onto petri plates of 2% WA, which were then incubated at 24°C in continuous light conditions for 6 h. Germination was quantified for at least 50 conidia/plate. For the molecular assay, two cultures were started for each of the two selected cones by transferring 100 µl of liquid and debris from the bottom of each cup into a 1.5-ml microcentrifuge tube containing 500 µl of potato dextrose broth and streptomycin sulfate at 100 mg/liter. DNA was extracted and amplified from the first culture by the methods described above. If the DNA extraction and amplification for the first culture per cone failed, a second attempt was conducted for the second culture obtained from the same cone.

Statistical analyses. The data for each year were analyzed separately. Either χ^2 tests (Excel; Microsoft, Redmond, WA) or Fisher's Exact Tests (Statistical Analysis Software v. 9.1.3; SAS Institute Inc., Cary, NC), if sample sizes were too small, were used to determine whether the number of trees and cones positive for *D. pinea* and *D. scrobiculata* was related to cone position (collected from the canopy or from the ground) for both pine host species or pine host species for both cone positions.

Statistical analyses performed with SAS were conducted to determine whether there was a relationship between pine host species, site, or cone position and the number of conidia extracted per cone or per gram of oven dry weight. The data were analyzed by a three-way analysis of variance for main effects of pine host species, site, cone position, and their interactions. The dependent variables were pine host species, site, and cone position. The independent variables were the mean number of conidia per cone or the mean number of conidia per gram of oven-dried weight. Before data analysis, the individual conidial count values per cone or per gram of oven dry weight (x) were transformed [$\log(x + 1)$] to meet the assumptions of nor-

mality. When main effects were significant at ($P \leq 0.05$), differences between means were identified using Tukey's LSMEANS comparisons at $P = 0.05$.

Fisher's Exact Tests with the Bonferroni correction were used to determine whether the proportion of cones positive for *D. pinea*, *D. scrobiculata*, or both was related to pine host species. The data for each cone position were analyzed separately for each location (Adams and Jackson Counties).

RESULTS

Cones yielded conidia characteristic of *D. pinea* or *D. scrobiculata* in both years and from $\geq 63\%$ of the trees sampled at each of the three sites at each of the two locations. Red pine cones were more frequently positive for *Diplodia* spp. than jack pine cones (Table 1). In general, cones collected from the canopy yielded conidia more frequently than cones collected from the ground (Table 1). Percentages of positive red pine cones were similar each year but relatively fewer jack pine cones were positive in 2005 than in 2004. In 2004 and 2005, the main effects of species, cone position, and site on the amount of conidia extracted from cones were significant ($P \leq 0.01$). Except for an interaction between site and position ($P \leq 0.02$) for the 2004 data, other two-way interactions between variables were not significant ($P \geq 0.22$). Three-way interactions between variables also were not significant ($P \geq 0.60$).

The number of conidia extracted from cones varied between trees species and cone positions, among inoculum sources among sites, and from year to year. Typically, more conidia were extracted from red pine cones than jack pine cones (Tables 2 and 3). Differences between pine species in the numbers of conidia extracted from cones were observed regardless of cone weight (Table 3). Typically, fewer conidia were extracted from cones collected from the ground than cones collected from the canopy (Tables 2 and 3). Differences in the numbers of conidia extracted from jack and red pine cones were more frequently observed in cones collected from the canopy (two sites in 2004 and five sites in 2005) than cones collected from the ground (no sites in 2004 and three sites in 2005). Correlations in the number of conidia extracted from cones from a tree and cones collected from the ground under the same tree were weak (correlation coefficient, $r \leq 0.26$) for each species and each year. The numbers of conidia extracted from red pine cones differed among sites, particularly for red pine cones collected from the canopy (Tables 2 and 3). Also, in general, more conidia were extracted from cones in 2005 than in 2004.

D. pinea and *D. scrobiculata* were detected from cones collected at all sites of each of the two locations. However, the proportion of cones for which molecular

methods identified *D. pinea* or *D. scrobiculata* varied by location (Table 4). In Adams County, *D. pinea* was more frequently detected than *D. scrobiculata* from red pine cones collected from both the canopy and the ground, whereas *D. scrobiculata* was more frequently detected than *D. pinea* from jack pine cones collected from both the canopy and the ground ($P \leq 0.01$). In Jackson County, *D. pinea* was more frequently detected from red and jack pine cones collected from both the canopy and the ground than *D. scrobiculata*. A small number of cones of each pine species were positive for both *D. pinea* and *D. scrobiculata*. Germinability was relatively high, with more than half (mean $\geq 67\%$, range = 8 to 100%) of the conidia germinated in each pine species and cone position categories.

DISCUSSION

Sporulation by *D. pinea* sensu lato has been observed frequently on mature cones of many pine species (33,34), including cones of red pine (10,13,15). However, relatively little has been reported about the frequency of cone colonization or the abundance of conidia that might be obtained from cones. The ability to assay cones for the presence of *D. pinea* and *D. scrobiculata* and to estimate the relative abundance of potential inoculum can contribute to knowledge of the distribution of these pathogens and their interactions with host trees and facilitate efforts to estimate the risk of disease development.

Knowledge of the very abundant inoculum available from cones of jack and red pine, even in apparently healthy forests sampled for this study, is important because of the role of conidia in the initiation of the *Diplodia* shoot blight disease cycle. Details of infection following conidial inoculation of needles and young stems of other pine species have been described (5,6). In addition, inoculation with conidia of *D. pinea* and *D. scrobiculata* has resulted in shoot blight symptoms on both jack pine and red pine seedlings (3). The relationship between abundance of inoculum and shoot blight incidence also has been examined. Palmer et al. (15) placed red pine litter, seedlings, and cones colonized by *D. pinea* on screens over young red pine seedlings. Inoculum was monitored by trapping spores deposited below the screens. In general, the greatest numbers of seedlings that became diseased were below colonized host material from which the greatest numbers of conidia were trapped (i.e., cones). Palmer et al. (15) also established that gradients in *Diplodia* shoot blight incidence occurred in red pine seedling nursery beds, showing that the numbers of diseased seedlings decreased with increasing distance from red pine windbreaks that were sources of inoculum. The relationship of proximity to an inoculum source with both the inci-

dence of shoot blight and the frequency of persistence of virulent strains of *D. pinea* on or in asymptomatic red pine nursery seedlings was confirmed in later studies (28,29).

Because results indicated differences between pine hosts and sampling locations in the frequencies with which *D. pinea* and *D. scrobiculata* were detected, and because of reported differences in aggressiveness of these pathogens on jack pine and red pine, identification of the pathogen or pathogens present at a particular site may be important in considering the potential for damage. In greenhouse experiments with potted seedlings, Blodgett and Stanosz (3) found that inoculation with *D.*

Table 1. Incidence of conidia of *Diplodia pinea* or *D. scrobiculata* extracted from jack and red pine cones sampled during two consecutive summers in six mixed pine stands

Year, species, position ^x	Incidence of DP-DS (%) ^w	
	By tree	By cone
2004 ^y		
Jack pine, ground	96	60
Jack pine, canopy	100	68
Red pine, ground	97	73
Red pine, canopy	100	90
2005 ^z		
Jack pine, ground	63	27
Jack pine, canopy	77	42
Red pine, ground	92	70
Red pine, canopy	95	84

^w A tree was considered positive if any of the five cones collected from it were positive. If *D. pinea* or *D. scrobiculata* conidia were successfully extracted from a cone, then that cone was considered to be positive.

^x Collection year, pine species, cone position. Cones were collected the summer after they matured from three sites at each of two locations. In 2004, five trees of each species were sampled per site. In 2005, 10 trees of each species were sampled per site. For each sampled tree, five cones were collected from the canopy and five cones were collected from the ground directly beneath the canopy of each tree. Collection sites sometimes differed between years due to harvesting or road inaccessibility.

^y For the data collected in 2004, Fisher's Exact Tests indicated that the number of positive trees was not related to pine host species for either cone position or to cone position for either tree species ($P \geq 0.48$). However, χ^2 analysis indicated that the number of positive cones was related to pine species for both cone positions ($P \leq 0.03$). The number of positive jack pine cones was not related to cone position ($P = 0.21$) but the number of positive red pine cones was related to cone position ($P \leq 0.01$).

^z For the data collected in 2005, χ^2 analysis or Fisher's Exact Tests indicated that the number of positive trees was not related to cone position for either pine species ($P \geq 0.11$) but it was related to pine species for both cone positions ($P \leq 0.01$). In addition, χ^2 analysis indicated that the number of positive cones was related to pine species for both cone positions and to cone position for both pine species ($P \leq 0.01$).

pineae resulted in greater incidence and severity of shoot blight on red pine and jack pine than inoculation with *D. scrobiculata*. Similar differences in severity of symptoms resulted from inoculation of potted seedlings of other *Pinus*, *Picea*, and *Abies* spp. (4) and also from inoculation of established red pine plantation trees (2). Site-to-site and host differences in relative frequency of occurrence of *D. pineae* and *D. scrobiculata* indicated by our data have been noted elsewhere in the Great Lakes region of the United States where both are known to occur. Palmer (14) found differences among sites in the pathogen species isolated from diseased trees in Minnesota and Wisconsin red pine plantations, and attributed these differences to the occurrence of *D. pineae* or *D. scrobiculata* in overstory trees or adjacent stands. Simi-

larly, Smith and Stanosz (24) found differences between Wisconsin locations in the frequencies of detection of *D. pineae* and *D. scrobiculata* in red and jack pine seedlings exhibiting symptoms of Diplodia collar rot. Therefore, because pathogen species can be readily determined from cones collected from the ground and because these cones can be easily obtained, a survey using cones collected from the ground might be a useful first step in assessing risk.

Data on numbers of conidia extracted from cones collected from the ground, however, indicates that these cones might not be as useful for estimation of the amount of potentially available inoculum. Relatively lower numbers of conidia extracted from cones from the ground than cones collected from tree canopies is con-

sistent with previous reports. Excised shoots, resulting from top-pruning red pine seedlings at a Wisconsin tree nursery, that were suspended in the seedling canopy yielded more *D. pineae* conidia than shoots collected from the adjacent alleyway soil (12). Palmer et al. (15) trapped spores naturally released from red pine cones collected from nursery windbreak trees and cones from the ground and found that many more conidia were trapped below the former than below the latter. In addition, the lack of correlation between numbers of conidia extracted from cones from the ground and cones from tree canopies in the current study indicates that data from ground cones is not predictive of potentially available inoculum. The location of cones also probably affects the efficiency of dissemination of conidia dispersed dur-

Table 2. Numbers of conidia of *Diplodia pineae* or *D. scrobiculata* extracted from jack and red pine cones sampled during two consecutive summers in six mixed pine stands^y

Collection year, site ^z	Cones from the ground		Cones from the canopy	
	Jack pine	Red pine	Jack pine	Red pine
2004				
A	106 (17–188) a A	174 (0–787) a A	89 (68–120) a A	811 (17–1,847) a A
B	436 (103–1,340) a A	1,536 (34–3,301) a AB	2,579 (68–9,236) a A	4,153 (120–13,239) a AB
F	265 (256–274) a A	322 (222–411) a A	1,871 (120–4,498) a A	1,189 (641–3,164) a A
C	226 (68–393) a A	777 (205–1,282) a AB	981 (85–2,172) a A	11,409 (5,080–15,513) b B
K	318 (17–616) a A	1,533 (479–2,429) a B	729 (17–2,429) a A	5,200 (342–8,449) a B
S	787 (34–3,096) a A	1,888 (34–5,542) ab AB	322 (17–1,129) a A	6,113 (547–16,135) b A
2005				
D	368 (0–2,668) x X	489 (0–3,968) x X	5,762 (0–17,480) x X	18,064 (0–81,810) x X
B	257 (0–1,864) x X	16,786 (85–76,285) x Y	82 (0–291) x X	34,764 (1,522–14,8193) y XY
Z	5,502 (0–40,281) x X	15,974 (154–40,024) y Y	5,215 (0–22,423) x X	47,541 (1,266–118,225) y Y
C	15 (0–120) x X	11,060 (0–51,911) x XY	11,780 (0–48,200) x X	119,844 (0–208,776) y Y
K	1,208 (0–10,416) x X	7,661 (376–33,918) y Y	5,184 (17–23,860) x X	102,992 (33,935–230,123) z Y
S	19 (0–103) x X	24,998 (51–115,865) y Y	55 (0–205) x X	67,679 (667–142,120) z Y

^y Cones were collected the summer after they matured in July. Data for each year were analyzed separately. Mean and range for the number of conidia per cone collected from 5 trees (replicates) in 2004 and 10 trees in 2005; five cones (subsamples) from both the canopy and the ground directly beneath the canopy of each tree were collected. Data were analyzed by a mixed linear model using a three-way analysis of variance after applying the $[\log(x + 1)]$ transformation. Values across a row followed by the same lowercase letter are not significantly different at $P \leq 0.05$, and values within a column for a given year followed by the same uppercase letter are not significantly different at $P \leq 0.05$ using Tukey Simultaneous Tests.

^z Sites A, B, D, F, and Z are located in Adams County, WI and sites C, K, and S are located in Jackson County, WI.

Table 3. Numbers of conidia of *Diplodia pineae* or *D. scrobiculata* extracted from jack and red pine cones (per gram of oven dry weight) sampled during two consecutive summers in six mixed pine stands^y

Collection year, site ^z	Cones from the ground		Cones from the canopy	
	Jack pine	Red pine	Jack pine	Red pine
2004				
A	30 (5–54) a A	25 (0–113) a A	22 (17–29) a A	157 (3–357) a A
B	125 (29–384) a A	221 (5–475) a AB	634 (17–2,269) a A	803 (23–2,561) a AB
F	51 (0–78) a A	46 (32–59) a A	460 (29–1,105) a A	294 (128–612) a A
C	65 (20–113) a A	112 (30–185) a AB	241 (21–534) a A	2,207 (983–3,001) b B
K	91 (5–176) a A	221 (69–349) a B	179 (4–597) a A	1,006 (66–1,634) a B
S	225 (10–887) ab A	272 (5–797) ab AB	79 (4–277) a A	1,182 (106–5,055) b A
2005				
D	105 (0–765) x X	70 (0–571) x X	1,416 (0–4,295) x X	3,494 (0–15,824) x X
B	74 (0–534) x X	2,415 (12–10,976) x Y	20 (0–71) x X	6,724 (294–28,663) y XY
Z	1,577 (0–11,542) x X	2,298 (22–5,759) y Y	1,281 (0–5,510) x X	9,196 (245–22,868) y Y
C	4 (0–34) x X	1,591 (0–7,469) x XY	2,894 (0–11,843) x X	23,181 (0–40,382) y Y
K	346 (0–2,985) x X	1,102 (54–4,880) y Y	1,274 (4–5,863) x X	19,921 (6,564–44,511) z Y
S	5 (0, 29) x X	3,597 (7–16,671) y Y	13 (0–50) x X	13,091 (129–27,489) z Y

^y Cones were collected the summer after they matured in July. Data for each year were analyzed separately. Mean and range for the number of conidia (per gram of oven dry weight) for cones collected from 5 trees (replicates) in 2004 and 10 trees in 2005; five cones (subsamples) from both the canopy and the ground directly beneath the canopy of each tree were collected. Data were analyzed by a mixed linear model using a three-way analysis of variance after applying the $[\log(x + 1)]$ transformation. Values across a row followed by the same lowercase letter are not significantly different at $P \leq 0.05$, and values within a column for a given year followed by the same uppercase letter are not significantly different at $P \leq 0.05$ using Tukey Simultaneous Tests.

^z Sites A, B, D, F, and Z are located in Adams County, WI and sites C, K, and S are located in Jackson County, WI.

ing periods of rainfall (15) to susceptible host organs. Although not studied, it seems likely that conidia from cones within tree canopies are more likely to be splashed to susceptible shoots within the canopy or to drip onto understory trees than conidia released from cones on the ground. Therefore, although greater time and effort is involved, collection of cones from tree canopies for conidial extraction would be recommended for estimation of the relative amount of potentially available inoculum at a site.

In the current study, including many cones from numerous trees from multiple sites, there was a tendency for more frequent detection of conidia of either pathogen from red pine cones than jack pine cones and for extraction of greater numbers of conidia from red pine cones than jack pine cones that may reflect the relative susceptibility of these hosts to *D. pinea*. Different responses of nonwounded, elongating shoots of young red and jack pine seedlings to inoculation with conidia of *D. pinea* were quantified in a greenhouse experiment by Blodgett and Stanosz (3). Symptoms were present 8 weeks after inoculation on 97% of red pine seedlings, compared with 42% of jack pine seedlings. Symptom severity, recorded as the percentage of the seedling crown with necrotic needles, was 38% for red pine seedlings and 17% for jack pine seedlings. Similarly, although just five cones from each of two trees of each of four pine species were examined, Smith et al. (26) reported differences in frequency of cone colonization by *D. pinea*. In that study, the numbers of cones positive for *Diplodia* spp. of *P. elliotii* Engelm., *P. taeda* L., *P. patula* Schldl. et Cham., and *P. radiata* D. Don were 0, 1, 5, and 9 (out of 10), respectively. The authors related these results to relative susceptibility (least to greatest) of these pine species as reported by Swart et al. (32).

High frequencies of detection of *D. pinea* and *D. scrobiculata* from jack pine and red pine cones examined in this study may also indicate a risk for disease development beyond their role as a substrate for sporulation. In addition to serving as a source of inoculum of *Diplodia* spp., there is evidence that cones may be important as sites of initial infection of trees. In a survey of 35-year-old red pine trees, Nicholls and Ostry (13) observed that branch infection frequently originated at points of cone attachment. Based upon similar observations of 25-year-old *P. patula* in South Africa, Smith et al. (25) expressed the belief that extensive mortality resulted from extensive branch pith colonization which originated from numerous third-year seed cones. Thus, in addition to their role in inoculum production, colonized cones may be important routes for invasion of crowns of cone-bearing trees.

The implications of colonization of red and jack pine cones by *Diplodia* spp. for seed production, seed contamination, and subsequent disease of seedlings of these species are unknown. Numerous fungi associated with conifer cones and seed cause damage in storage, attack germinating seed, cause disease of seedlings within nurseries or when subsequently exported to out-planting sites (1), or even affect natural regeneration (30). *D. pinea* has been frequently associated with seed and damages seedlings of many pine species (18,33). Anderson et al. (1) and Fraedrich and Miller (11) isolated *Diplodia* spp. from both sound and damaged *P. elliotii* seed obtained from seed orchards in the southeastern United States. Smith et al. (26) isolated *D. pinea* from seed of *P. patula* and *P. radiata* in South Africa. However, in none of these studies were effects on seed or subsequently produced seedlings studied. Rees and Webber (19), however, obtained *D. pinea* isolates from seed of tropi-

cal pine which were able to reduce seed germination and cause disease to seedlings of *P. oocarpa* Schiede, *P. caribaea* Morelet, and *P. michoacana* Martínez in laboratory and greenhouse experiments. Given the chronic occurrence of Diplodia shoot blight in some nurseries (16,28,29), current underrepresentation of red pine and jack pine in Great Lakes region forests (compared with forests prior to settlement by Europeans; 22), and the recognized but unexplained unreliability of natural regeneration of red pine (9), study of the potential effects of cone colonization and subsequently produced conidia of *D. pinea* and *D. scrobiculata* on seed and seedlings of these species is justified.

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Table 4. Incidence of *Diplodia pinea*, *D. scrobiculata*, or both in positive jack and red pine cones collected in 2005 from six mixed pine stands^x

Location, pine host species	Proportion (%) of cones on which the indicated pathogens were detected					
	Cones from the ground ^y			Cones from the canopy ^z		
	<i>D. pinea</i> only	<i>D. scrobiculata</i> only	Both	<i>D. pinea</i> only	<i>D. scrobiculata</i> only	Both
Adams County						
Jack	20	80	0	42	58	0
Red	80	13	7	93	0	7
Jackson County						
Jack	71	14	14	75	0	25
Red	100	0	0	81	13	6

^x If *Diplodia* spp. conidia were successfully extracted from a cone, then that cone was considered to be positive. For each stand, we tested two positive cones collected from the canopy and two from the ground directly beneath the canopy of two jack and two red pine trees.

^y Fisher's Exact Tests indicated that the proportion of cones collected from the ground in Adams County positive for *D. pinea*, *D. scrobiculata*, or both was related to pine species ($P \leq 0.01$). Jack pine cones were more frequently positive for *D. scrobiculata* than *D. pinea* whereas red pine cones were more frequently positive for *D. pinea* than *D. scrobiculata*. In contrast, Fisher's Exact Tests indicated that the proportion of cones collected from the ground in Jackson County positive for *D. pinea*, *D. scrobiculata*, or both was not related to pine species ($P = 0.11$). Both jack pine and red pine cones were more frequently positive for *D. pinea* than *D. scrobiculata*.

^z Fisher's Exact Tests indicated that the proportion of cones collected from the canopy in Adams County positive for *D. pinea*, *D. scrobiculata*, or both was related to pine species ($P \leq 0.01$). Jack pine cones were more frequently positive for *D. scrobiculata* than *D. pinea* whereas red pine cones were more frequently positive for *D. pinea* than *D. scrobiculata*. In contrast, Fisher's Exact Tests indicated that the proportion of cones collected from the canopy in Jackson County positive for *D. pinea*, *D. scrobiculata*, or both was not related to pine species ($P = 0.62$). Both jack pine and red pine cones were more frequently positive for *D. pinea* than *D. scrobiculata*.

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