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Site-related influences on cone-borne inoculum and asymptomatic persistence of Diplodia shoot blight fungi on or in mature red pines

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ABSTRACT

The shoot blight and canker pathogens *Diplodia pinea* and *D. scrobiculata* sporulate abundantly on cones of many pine hosts. Variation in incidence and abundance of potential inoculum from cones and frequency of asymptomatic persistence on or in shoots was examined for mature red pines in sites differing in dominant presettlement vegetation and soil type in Bayfield and Douglas counties in northern Wisconsin. Collections were made in each county from 6 plantations, 3 each in areas historically vegetated with jack pine and soils mapped as sands and three in areas historically vegetated with red pine with soils mapped as loamy sands. At each site, 5 cones were collected from each of 5 red pines and 10 shoots were collected from up to 5 red pines. Conidia from cones were quantified with a water wash and filtration technique. *Diplodia* species were cultured from surface-disinfested asymptomatic shoots. A species-specific PCR assay was used to identify the *Diplodia* species from cones and shoots. Although cones and asymptomatic shoots from each county yielded *D. pinea* and *D. scrobiculata*, *D. pinea* was detected more frequently. More conidia were obtained from cones from Douglas Co., where there is a history of severe shoot blight damage, than cones from Bayfield Co. In Douglas Co., more conidia were obtained from cones from plantations in areas of more sandy soil and presettlement jack pine dominance than cones from plantations in areas of less sandy soil and presettlement red pine dominance. The numbers of conidia and frequencies of cultural detection of *Diplodia* species from asymptomatic shoots at a site were positively correlated. These results provide evidence for site-related influences on abundance of pathogen inoculum and asymptomatic persistence on or in red pine crowns that may contribute to differences in frequency and severity of damage from Diplodia shoot blight.

1. Introduction

Jack pine (*Pinus banksiana* Lamb.) and red pine (*P. resinosa* Ait.) are two- and three-needle pines of the *Pinus* subgenus Diploxylon. Jack pine grows further north than any other North American pine and is the most widely distributed pine in Canada (Rudolf, 1990). The geographic range of red pine is more limited, overlapping with that of jack pine in central and eastern portions of the northern USA and Canada. Although in Wisconsin, both jack and red pine are at the southern edge of their ranges, both species can be economically important sources of pulpwood and lumber (Rudolf, 1990; Rudolph and Laidley, 1990).

In the Great Lakes region difference in soil type is one of the most important factors in the variation of climax vegetation (Kotar et al., 2002). Soils of this region promoted a vegetation type that was prone to fire, which in turn maintained the region's pine forests (Whitney, 1986) by creating disturbances that favored pioneer species such as jack and red pine. Jack pine readily regenerates naturally, but due in part to unreliable natural regeneration, red pine is the most planted tree species in the Great Lakes region (Rudolph and Laidley, 1990). The variety of sites on which red pine has been extensively planted includes those dominated by jack pine before European settlement.

Presettlement vegetation offers forest managers a baseline framework to evaluate current conditions and a goal for forest restoration (Fule et al., 1997). For example, Radeloff et al. (2000) used presettlement vegetation data to examine possibilities for landscape restoration in the Bayfield sand plains of northwestern Wisconsin. Similarly, forest managers could consider presettlement vegetation when making management decisions related to forest diseases and insect pests. For example, Perkins and Matlack (2002) explored the consequences of current timber management

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practices on the epidemic spread of insect pests and pathogens in southern Mississippi. By comparing presettlement vegetation to current forest composition, the authors concluded that intensive forest management has increased the connectivity of the landscape thereby increasing the potential spread of southern pine beetle (*Dendroctonus frontalis* Zimm.) and fusiform rust (*Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme*) (Perkins and Matlack, 2002). Presettlement vegetation also could be used as an integrative indicator of the environmental conditions present at a site such as soil type, solar radiation, depth to water table, or rainfall, that could directly or indirectly affect pathogen and insect pest activity.

Among the potentially serious diseases of red pine and jack pine are those caused by *Diplodia pinea sensu lato*. Symptoms reported in red and jack pine include shoot blight, stem cankers, branch dieback, dead tops, death, and blue staining of cut wood (Nicholls and Ostry, 1990; Palmer, 1991). Recently, it has been learned that two distinct pathogens, *D. pinea* (Desmaz.) J. Kickx fil. and *D. scrobiculata* J. de Wet, B. Slippers & M. J. Wingfield (De Wet et al., 2003) can be associated with damage in the Great Lakes region. Inoculation with *D. pinea* resulted in greater incidence and severity of shoot blight on potted red pine seedlings than inoculation with *D. scrobiculata* in greenhouse experiments with potted seedlings (Blodgett and Stanosz, 1997) and also in a study using established red pine plantation trees (Blodgett et al., 1997b). Other aspects of the biology of these pathogens appear to be similar. Both fungi infect wounded and nonwounded shoots, sporulate on killed needles and stems, and also sporulate on or in red and jack pine cones from trees that do not exhibit other disease symptoms (Munck, 2008). Such cones could be a point of entry for the pathogens into the pine hosts (Smith et al., 2002). In addition, both *D. pinea* and *D. scrobiculata* are able to persist on or in host tissues without causing visible symptoms. For example, Stanosz et al. (1997) isolated virulent strains of these fungi from shoots of asymptomatic red pines from plantations and forest tree nurseries. Later, Stanosz et al. (2001) also provided experimental evidence suggesting that release from latency might explain rapid disease development after stressful conditions.

Environmental factors such as drought and unfavorable site conditions has been associated with severe outbreaks of Diplodia shoot blight and canker in red pine plantations in *P. radiata* D. Don. plantations in South Africa (Zwolinski et al., 1990), Australia (Wright and Marks, 1970), and in red and jack pine plantations in Wisconsin (Nicholls and Ostry, 1990). In these studies, trees exposed to drought, poor site conditions, or hail were more frequently damaged. Later, Palmer (1991) isolated both *D. pinea* and *D. scrobiculata* from dead or recovering trees predisposed to disease by drought, hail or frost damage in five red pine plantations in Wisconsin and Minnesota. Later studies conducted in a growth chamber (Blodgett and Stanosz, 1998), a greenhouse (Blodgett et al., 1997a), and in a plantation (Blodgett et al., 1997b) confirmed that water stress enhanced shoot colonization of red pine by *D. pinea* and *D. scrobiculata* isolates. However, the possible effects of site conditions on frequency of cone infection, or inoculum production on cones, or a relationship with the frequency of asymptomatic persistence of these pathogens has not been examined.

The first objective of this study was to investigate the possible influence of site conditions associated with presettlement vegetation and/or soil on the frequency of detection and inoculum production of *D. pinea* and *D. scrobiculata* on red pine cones. A second objective was to examine the possible relationship between frequencies of detection or inoculum production on cones and frequency of asymptomatic persistence of these fungi on or in shoots. The related null hypotheses were: (i) there are no differences related to site conditions in the frequency of detection and inoculum production of these fungi on cones; and (ii) the frequencies of detection or inoculum production on cones are not related to frequency of asymptomatic persistence on shoots.

2. Materials and methods

2.1. Study area

This study was conducted in the Bayfield sand plains of northwestern Wisconsin. The Bayfield sand plains extend across Bayfield and Douglas counties from northeast to southwest. These consist of a strip of pitted glacial outwash dominated by podzolized sands and gravels (Kotar et al., 2002). There is also a soil moisture and nutrient gradient across the Bayfield sand plains, with each tending to be higher in the northeast to lower in the southwest (Kotar et al., 2002). Historically, jack pine was the most widely occurring species in the central portion of the Bayfield sand plains, whereas red and white pines were predominant in the northern and southern extremes (Kotar et al., 2002). East and west of the Bayfield sands the soils are podzolized stony loams over till, outwash, and bedrock. Thus, Bayfield and Douglas counties are favorable locations to investigate the presence and abundance of *D. pinea* and *D. scrobiculata* in red pine plantations under differing site conditions that are related to historical forest composition.

The climate in this region is continental. Thirty-year mean daily temperatures (and mean minima and maxima) recorded at weather stations in the vicinities of the study sites in Bayfield and Douglas counties in January are −11.1 °C (−16.0 °C, −6.2 °C) and −13.7 °C (−20.5 °C, −7.0 °C), respectively, and in July are 19.1 °C (13.1 °C, 25 °C) and 19.9 °C (12.3 °C, 27.4 °C) (National Climatic Data Center). Thirty-year means of annual precipitation recorded at these stations, respectively, are 850 mm and 791 mm, respectively (National Climatic Data Center, 2002).

2.2. Site selection and characteristics

Using GIS software, current stand data were overlaid with historical vegetation cover maps to select mature red pine plantations with the desired characteristics. Public Land Survey (PLS) point data were used to create a GIS database to reconstruct the vegetation cover of the Bayfield sand plains in the 1850s (Radeloff et al., 1999). Staff members of the Douglas County Forestry Office (Solon Springs, WI) and the Washburn Ranger District of the Chequamegon-Nicolet National Forest (Washburn, WI) provided current stand data for Douglas Co. and Bayfield Co., respectively. Current stand data included compartment number, stand number, year of origin, stand location, tree species, mean stand diameter at breast height (dbh), site index, and mapped soil type. For each county, three red pine plantations on loamy sands, vegetated predominately (>70% cover) with red pine prior to European settlement and three red pine plantations on sandy soils, vegetated predominately (>70% cover) with jack pine prior to European settlement were selected (12 stands total) (Figs. 1 and 2). These red pine plantations had been established during the 1940s and 1950s. Three prism plots were established per stand, their plot centers at least 31 m apart, to calculate current basal area (Table 1). A 10 basal-area-factor prism was used to determine which trees were included in each plot and the dbh of all trees in each plot was measured. Descriptive stand data are summarized in Table 1. Typical disease symptoms caused by *D. scrobiculata* and *D. pinea* were not apparent on mature trees at these stands during the time of the study.

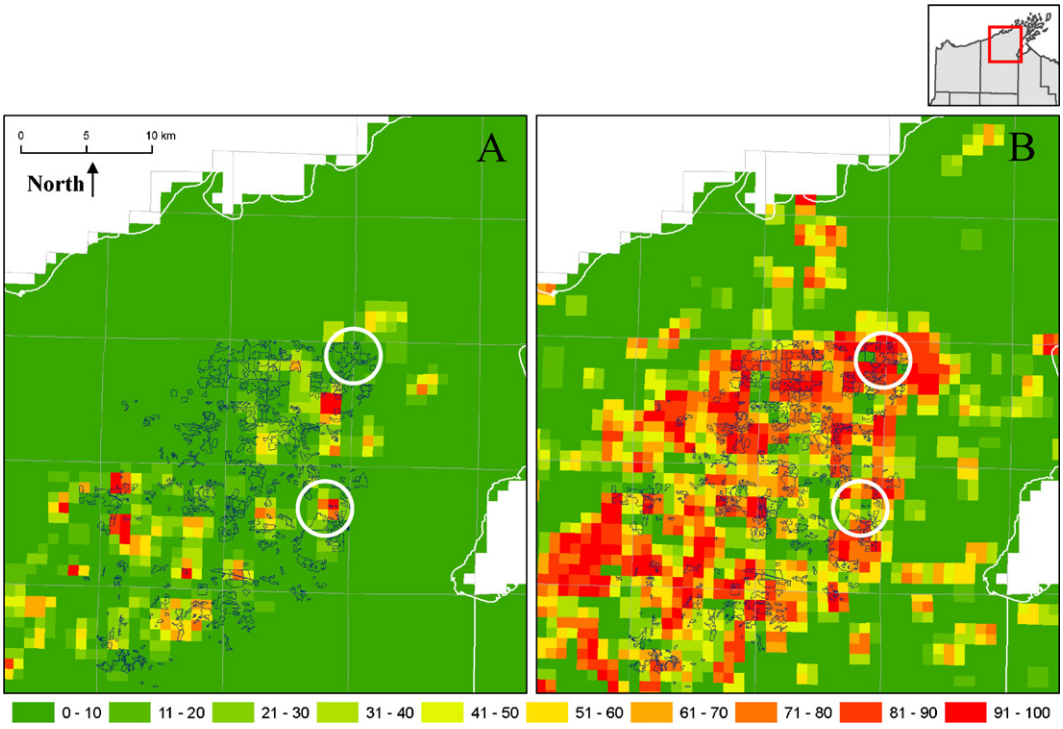

Fig. 1. Relative presettlement jack pine (A) and red pine (B) dominance of red pine stands sampled in Bayfield County. The location of sampled stands in areas historically vegetated with jack pine (south) or red pine (north) are circled in white. Current red pine stand (outlined in blue) data was provided by the US Forest Service. Presettlement dominance data processed from Public Land Survey bearing tree records, by PLS quarter section. The square on the top right is a map of Bayfield and Douglas Counties in northwestern Wisconsin; the enlarged area is highlighted in red. The values in the legend of the map correspond to the percent (%) cover of jack pine (A) and red pine (B) in this area in the 1850s.

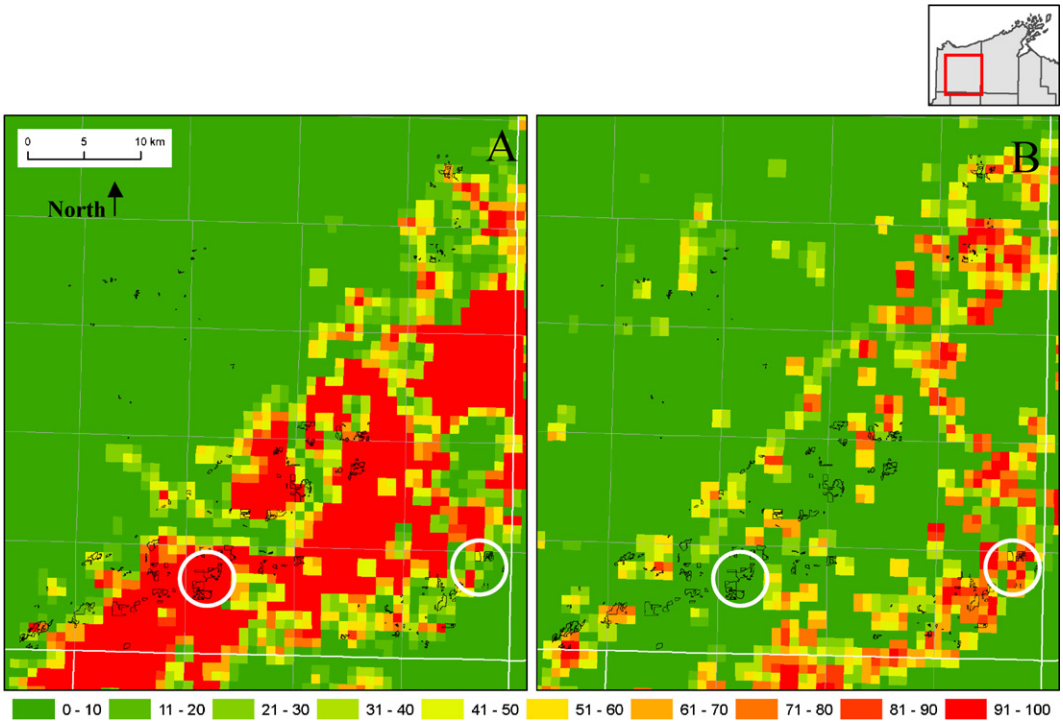

Fig. 2. Relative presettlement jack pine (A) and red pine (B) dominance of red pine stands sampled in Douglas County. The location of sampled stands in areas historically vegetated with jack pine (west) or red pine (east) are circled in white. Current red pine stand (outlined in black) data was provided by the Douglas County Forestry Department. Presettlement dominance data processed from Public Land Survey bearing tree records, by PLS quarter section. The square on the top right is a map of Bayfield and Douglas Counties in northwestern Wisconsin; the enlarged area is highlighted in red. The values in the legend of the map correspond to the percent (%) cover of jack pine (A) and red pine (B) in this area in the 1850s.

Table 1
Descriptive data for 12 red pine stands in Wisconsin sampled to determine the persistence of *Diplodia pinea* and *D. scrobiculata* on mature red pine cones and asymptomatic current year shoots.

Stand ID	County	Presettlement vegetation[a]	Latitude	Longitude	Average diameter sampled trees (cm)[b]	Site index (m)[c]	Basal area (m^2/h)[d]	Soil type
162-02	Bayfield	Jack pine	46.65°N	91.067°W	17.5	16	16	Rubicon sand
163-10	Bayfield	Jack pine	46.65°N	91.071°W	25.9	18	24	Rubicon sand
162-03	Bayfield	Jack pine	46.64°N	91.067°W	27.7	21	19	Rubicon sand
222-03	Bayfield	Red pine	46.75°N	91.075°W	41.7	17	20	Vilas loamy sand
207-04	Bayfield	Red pine	46.73°N	91.063°W	37.3	18	14	Vilas loamy sand
223-06	Bayfield	Red pine	46.75°N	91.060°W	36.3	17	22	Vilas loamy sand
216-10	Douglas	Jack pine	46.20°N	91.912°W	26.8	17	28	Grayling sand
216-11	Douglas	Jack pine	46.20°N	91.903°W	22.8	17	29	Grayling sand
217-02	Douglas	Jack pine	46.21°N	91.902°W	30.9	17	20	Grayling sand
235-04	Douglas	Red pine	46.24°N	91.576°W	29.7	18	25	Menahga loamy sand
244-17	Douglas	Red pine	46.21°N	91.634°W	25.9	17	18	Menahga loamy sand
243-26	Douglas	Red pine	46.21°N	91.651°W	35.2	18	25	Menahga loamy sand

[a] Dominant vegetation (>70% cover) at these stands in the 1850s.
[b] Diameter at breast height (1.3 m) of five trees per stand from which cones and asymptomatic shoots were collected.
[c] Average tree height 50 years after planting.
[d] Three prism plots (10 BAF) were established per stand to calculate current basal area.

2.3. Cone tests

During July of 2006 red pine cones that had matured the previous fall were collected from five arbitrarily selected trees in each stand (60 trees total). Selected trees were almost always at the edge of the stand or otherwise had portions of their crown exposed to sunlight, as sunlight favors cone production. From each tree, five third year cones were collected from the middle or lower portion of the crown (300 cones total). The age of the cones collected was determined by their position on the branch. Cones were bagged and placed on ice in coolers for transportation to the laboratory where they were stored at −20 °C until conidia were extracted (all cones were processed by August 8th 2006).

Cones were processed individually in the laboratory to extract conidia. Each cone was placed in a 100 ml plastic cup containing 80 ml of sterile deionized water with 2 drops l^{-1} of Tween 80 (Fisher Scientific Company, Fair Lawn, NJ) and washed for 3 h on a rotary shaker at 110 rpm. Cones were removed from cups and then the volume in each cup was adjusted to 100 ml with deionized water to account for differences in the absorbance of water by each cone. Twenty-five milliliters of the resulting suspension were pipetted out of each cup while stirred at low speed and then filtered through 0.8 μm pore size membrane filter printed with a grid to delineate 3 mm × 3 mm squares (Cat. No.: AAWG047SP, Millipore Corporation, Billerica, MA). Conidia recognized as those of either *D. pinea* or *D. scrobiculata* based on morphological characteristics, were counted on five randomly chosen squares of the membrane filter with the aid of a dissecting microscope at up to 30×. The mean number of conidia from five squares was multiplied by a factor (427.6) to adjust for total area of membrane filter and the volume of water in which the cone was washed to estimate the number of conidia extracted per cone. The oven-dried weight (odw) of each cone was obtained. The mean number of conidia extracted per cone was divided by the odw of the cone to obtain an estimate of the number of conidia extracted g^{-1} odw.

Germination of conidia extracted from two arbitrarily selected cones from each stand was tested. 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 per plate. If the germ tube of a spore was at least equal to the spore length then the spore was considered to have germinated.

A molecular assay was used to determine which species of *Diplodia* was present on cones from each stand. Two cultures were started for each cone 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 100 mg l^{-1} streptomycin sulfate. The cultures were incubated in the dark at room temperature for 7–10 days and then frozen at −20 °C. DNA from cultures from at least four arbitrarily selected cones per stand was extracted by the method described in Smith and Stanosz (1995). Briefly, the fungal cultures were ground in extraction buffer (500 mM NaCl, 100 mM Tris, pH 8.0, 10 mM EDTA, 10 mM β-mercaptoethanol) with Kontes pestles and incubated for 10 min at 60 °C with 20% sodium dodecyl sulfate, proteins were removed with potassium acetate, and DNA precipitated by isopropanol. The extracted DNA was amplified using mt SSU rDNA primers that allow differentiation of *D. pinea* from *D. scrobiculata*, other fungi in the genus *Botrysphaeria*, and related anamorphic fungi (Smith and Stanosz, 2006), and presence of amplified DNA fragments was verified on 0.7% agarose gels in TBE buffer. 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.

Positive and negative controls were used to ensure that conidia could be quantified if available and that no false positives occurred due to contaminated laboratory equipment. Negative controls consisted of cups with 80 ml of the Tween–water solution and positive controls consisted of cups with 80 ml of the Tween–water solution and 1000 conidia of either a known *D. pinea* or *D. scrobiculata* isolate. The liquid in these cups was filtered and as described above, 2 ml were pipetted from the bottom each cup, cultured, and then assayed using molecular methods.

2.4. Asymptomatic shoot assays

In early October of 2006, 10 asymptomatic current-year shoots were collected from the same trees in each stand from which cones were collected earlier. Healthy-appearing branches from the lower to middle crown (6–9 m above ground) were removed from each tree. Occasionally, lengths of current year's shoots were insufficient to yield segments for processing as described below, and in those cases fewer than five trees were sampled. Samples (530 total) were separately bagged and kept cold until they were processed.

Asymptomatic persistence of *D. pinea* and *D. scrobiculata* was determined culturally using methods similar to those of Stanosz et al. (2007). A stem segment approximately 5 cm long was cut from the center of the current year's growth of shoots and the needles were removed. In some cases (due to relatively little shoot growth) two or more shorter segments totaling 5 cm in length from different

shoots were pooled and processed together as one composite "stem segment." If multiple shoots were combined to make one sample, 2–3 cm was taken from the center of the current year's growth of each shoot. The segments were then surface-disinfested by immersion for 30 s in 95% ethanol, and then two immersions for 2 min each in 1.05% NaClO plus two drops Tween 80 l^{-1}. Stem segments were then placed on tannic acid agar (TAA) medium (Blodgett et al., 2003) in Petri plates. Twice-autoclaved red pine needles were placed on the agar opposite the stem segments, and dishes were incubated in the dark at ambient laboratory temperatures for 10–14 days. Petri plates were then incubated at ambient laboratory temperatures approximately 30 cm below a fluorescent light for at least 2 weeks. Detection of the pathogen was based on characteristic pycnidia and conidia that developed on the stem segment or (more commonly) on red pine needles after the mycelium grew across the medium to them (Stanosz et al., 2007).

In addition, the identity of the pathogen detected from asymptomatic but culturally positive shoots was confirmed using molecular methods. This was done for four randomly chosen positive shoots from each tree or, if less than four per tree were positive, all positive shoots. DNA was extracted from small pieces of *Diplodia*-colonized red pine needles from the TAA medium in Petri plates used in the cultural assay of asymptomatic shoots. The extraction was performed using the slightly modified method of Cubero et al. (1999) outlined in Smith and Stanosz (2006). This method uses an extraction buffer containing hexadecyltrimethylammonium bromide (CTAB) to remove polysaccharides and polyvinylpyrolidone (PVP) to remove polyphenols, and employs two chloroform extractions to remove proteins. The presence of *D. pinea* and *D. scrobiculata* DNA were identified by PCR specific primers (Smith and Stanosz, 2006). If this method failed to yield results from the PCR, transfers were made from the pycnidia produced on the *Diplodia*-colonized red pine needles to potato dextrose broth and after approximately 7 days DNA from these subcultures was extracted using procedures of Smith and Stanosz (1995). The pathogen was then again identified using PCR primers specific to these two *Diplodia* species.

2.5. Soil analyses

In each stand, five soil cores were obtained under the canopy of each tree from which cones had been obtained. Forest floor material was removed and soil was collected to a depth of 15 cm. A subsample was taken from each tree and then these were pooled to provide a stand sample that was analyzed. Soil samples were transported to the laboratory and stored at −20 °C until they were dried prior to analysis by the Soil and Plant Analysis Laboratory (University of Wisconsin-Madison, Verona, USA). Soil pH and organic matter content were determined and the proportion of sand, silt, and clay in the inorganic portion of each soil sample was measured with a hydrometer.

2.6. Statistical analyses

Fisher's Exact Tests (Statistical Analysis Software v. 9.1.3, SAS Institute Inc., Cary, NC) with the Bonferroni correction were used to determine if the number of cones and trees with at least one cone positive for *D. pinea* and *D. scrobiculata* was related to county or presettlement vegetation. Similar tests were conducted to determine if the number of asymptomatic shoots and trees with at least one asymptomatic shoot positive for *D. pinea* and *D. scrobiculata* was related to county or presettlement vegetation.

Statistical analyses were performed with SAS to determine if there was a relationship between county, or presettlement vegetation and the number of conidia extracted g^{-1} odw. The data were analyzed by a two-way analysis of variance (two-way ANOVA) for main effects of county and presettlement vegetation and their interactions. The dependent variables were county and presettlement vegetation. The independent variable was the mean number of conidia g^{-1} odw. When main effects were significant at ($p \leq 0.05$), differences between means were identified using Tukey's LSMEANS comparisons at $p = 0.05$. Two-way ANOVAs were also performed to determine if there was a relationship between county or presettlement vegetation and soil characteristics which included pH and proportion of organic matter, sand, silt, and clay.

Correlations and single linear regression analyses were conducted with R (v. 2.7.2, The R Foundation for Statistical Computing) to explore the relationships between site characteristics and the frequency of detection on asymptomatic shoots and cones and inoculum production of *Diplodia* species on cones at a site. The independent variables for regression analyses were: number of conidia extracted g^{-1} odw, proportion of cones positive for *Diplodia* species, or proportion of asymptomatic shoots positive for *Diplodia* species of a site. The dependent variables for regression analyses were: number of conidia extracted g^{-1} odw, proportion of cones positive for *Diplodia* species, or proportion of asymptomatic shoots positive for *Diplodia* species, soil pH, soil organic matter, proportion (%) of soil sand, silt, and clay, basal area, or site index of a site.

3. Results

Most trees (≥60%) sampled in the two counties yielded cones and asymptomatic shoots that were positive for *D. pinea*, *D. scrobiculata*, or both (Table 2). Red pine cones from Douglas Co.

Table 2
Proportion of mature cones and asymptomatic shoots collected from trees in 12 red pine stands in Wisconsin positive[a] for either *Diplodia pinea* or *D. scrobiculata*.

County	Presettlement vegetation[b]	Proportion (%) of trees with at least one positive cone[c,d,f]	Proportion (%) of cones positive for *Diplodia* species[c,d,f]	Number of conidia per gram of oven dry weight cone tissue[c,e,f]	Proportion (%) of trees with at least one positive shoot[c,d,f]	Proportion (%) of asymptomatic shoots positive for *Diplodia* species[c,d,f]
Bayfield	Jack pine	73 ± 12 a	37 ± 6 c	1298 ± 648 b	67 ± 17 a	24 ± 8 c
Bayfield	Red pine	60 ±13 a	47 ± 6 ab	1759 ± 1344 b	100 ± 0 a	61 ± 7 b
Douglas	Jack pine	100 ± 0 a	95 ± 3 a	21227 ± 1861 a	100 ± 0 a	92 ± 4 a
Douglas	Red pine	80 ± 11 a	64 ± 6 b	8584 ± 2933 b	78 ± 11 a	56 ± 11 b

[a] Only 3% of the cones and 5% of the asymptomatic shoots were positive for *D. scrobiculata*.
[b] Dominant vegetation (>70% cover) at these stands in the 1850s.
[c] Mean ± standard error (S.E.) for three stands. Cones and shoots were collected from five trees at each stand. Five cones per tree were collected the summer after they had matured. Ten asymptomatic current-year shoots were collected from the same trees in each stand from which cones were collected earlier. Occasionally lengths of current year's shoots were insufficient to yield segments for processing and in those cases fewer than five trees were sampled.
[d] Fisher's Exact Tests with the Bonferroni correction were used to determine if the number of cones, shoots, and trees with at least one cone or asymptomatic shoot positive for *D. pinea* and *D. scrobiculata* was related to county or presettlement vegetation.
[e] Data were analyzed by a mixed linear model using a two-way analysis of variance, which indicated an effect of county ($p \leq 0.01$), presettlement vegetation ($p \leq 0.01$), and their interaction ($p \leq 0.01$) on the number conidia extracted per gram of cone oven dry weight.
[f] Values within a column that are followed by the same letter are not significantly different at $p \leq 0.05$.

Table 3
Summary of soil[a] characteristics by stand for 12 red pine stands in Wisconsin sampled to determine the persistence of *Diplodia pinea* and *D. scrobiculata* on mature red pine cones and asymptomatic current year shoots.

Stand ID	County	Presettlement vegetation[b]	pH	Mean ± S.E., pH[c]	Organic matter (%)	Mean ± S.E., organic matter (%)[c]	Sand–silt–clay (%)	Mean ± S.E., sand–silt–clay (%)[c]
162-02	Bayfield	Jack pine	5.0	4.9 ± 0.2 b	2.4	2.7 ± 0.3 a	88–18–5	81 ± 3.4 a–16 ± 1.2 a–6 ± 0.7 a
163-10	Bayfield	Jack pine	4.8		2.4		77–16–7	
162-03	Bayfield	Jack pine	5.0		3.4		79–14–7	
222-03	Bayfield	Red pine	5.2	5.1 ± 0.1 ab	2.5	2.7 ± 0.1 a	77–14–9	78 ± 1.3 a–13 ± 0.7 a–8 ± 0.7 a
207-04	Bayfield	Red pine	5.3		2.7		77–14–9	
223-06	Bayfield	Red pine	4.9		2.9		81–12–7	
216-10	Douglas	Jack pine	5.3	5.4 ± 0.1 a	2.2	2.5 ± 0.3 a	89–4–7	88 ± 1.3 a–6 ± 1.2 b–6 ± 0.7 a
216-11	Douglas	Jack pine	5.7		2.3		89–6–5	
217-02	Douglas	Jack pine	5.3		3.1		85–8–7	
235-04	Douglas	Red pine	5.2	5.2 ± 0 ab	3.3	3.2 ± 0.1 a	79–14–7	78 ± 1.8 a–15 ± 1.8 a–7 ± 0 a
244-17	Douglas	Red pine	5.3		3.1		81–12–7	
243-26	Douglas	Red pine	5.2		3.3		76–18–7	

[a] A sample consisting of five soil cores was collected at each stand.
[b] Dominant vegetation (>70% cover) at these stands in the 1850s.
[c] Mean ± standard error (S.E.) for three stands. Data were analyzed by a mixed linear model using a two-way analysis of variance. Values within a column followed by the same letter are not significantly different at $p \leq 0.05$ using Tukey Simultaneous Tests.

were more frequently positive for *Diplodia* species than cones from Bayfield Co. (Table 2). In addition more conidia were extracted from cones from Douglas Co. than cones from Bayfield Co. (Table 2). Cones and asymptomatic shoots collected at sites in Douglas Co. that had been historically vegetated with jack pine were most frequently positive (95% and 92%, respectively) for *D. pinea* (Table 2). Cones collected from these sites also yielded the most conidia (21,227 g^{-1} odw) (Table 2). The main effects of county, presettlement vegetation, and their interaction on the number of conidia extracted from cones were all significant ($p \leq 0.01$).

The soil properties also varied between sites. The proportion of silt was lower (6%) in sites that had been historically vegetated with jack pine in Douglas Co. than in all other sampled stands (13–16%) (Table 3). Conversely, there was a higher proportion of sand at $p = 0.06$ in these stands (88%) compared to other sampled stands (78–81%). The main effects of county, presettlement vegetation and their interaction on the soil proportion of silt were all significant ($p < 0.05$). The main effect of presettlement vegetation on the soil proportion of sand was significant ($p = 0.02$), but the main effects of county and the interaction between presettlement vegetation and county on the soil proportion of sand were not ($p > 0.17$).

Table 4
Pearson's correlation coefficients (r) and adjusted coefficients of determination (adj.-R^2) for the correlations and regressions of the following: number of conidia per gram of oven dry weight (odw), the proportion of cones and asymptomatic shoots positive for *Diplodia* species with soil and stand parameters for 12 red pine stands in northern Wisconsin ($n = 12$).

Parameter	r, adj.-R^2		
	Conidia g^{-1} odw	Cones (%) for *Diplodia* species	Shoots (%) for *Diplodia* species
Conidia g^{-1} odw	–	–	–
Cones (%) for *Diplodia* species	078, 0.58**	–	–
Shoots (%) for *Diplodia* species	0.73, 0.49**	0.88, 0.75***	–
Soil pH	0.74, 0.50**	0.62, 0.33*	0.62, 0.32*
Soil organic matter (%)	ns	ns	ns
Sand (%)	0.62, 0.33*	ns	ns
Silt (%)	−0.75, 0.52**	ns	ns
Clay (%)	ns	ns	ns
Site index (m)	ns	ns	ns
Site basal area (m^2/h)	0.63, 0.34*	ns	ns

Significance of correlations and regressions: ns is used if $p > 0.10$, *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$.

The proportion of asymptomatic shoots culturally positive for *Diplodia* species at a site was highly positively correlated with the proportion of cones positive for *Diplodia* species at the same site (Table 4). Similarly, the proportion of asymptomatic shoots culturally positive for *Diplodia* species at a site was also highly positively correlated with the number of conidia extracted from cones at the same site (Table 4). Finally, the number of shoots culturally positive for *Diplodia* species from a given tree was correlated with the number of positive cones (Pearson's correlation coefficient, $r = 0.74$) and the number of conidia extracted from cones ($r = 0.55$) from the same tree.

Soil pH was positively correlated with, and explained 32%, 33% and 50%, respectively, of the variation in the proportion of asymptomatic shoots culturally positive for *Diplodia* species, the number of positive cones, and the number of conidia extracted from cones at a site, respectively (Table 4). The proportion of sand (%) in the soil and basal area (m^2/h) were positively correlated with, and explained 33% and 34%, respectively, of the variation in the number of conidia extracted from cones at a site (Table 4). In contrast, the proportion of silt (%) in the soil was negatively correlated with, and explained 52% of the variation in the number of conidia extracted from cones at a site (Table 4).

Both *D. pinea* and *D. scrobiculata* were detected from cones and shoots collected in each of the two counties, but with different frequencies. *Diplodia pinea* was detected from 95% (58 of 61) of the cones and 94% (163 of 173) of the asymptomatic shoots tested. In contrast, only 3% (2 of 61) of the cones and 5% (8 of 173) of the asymptomatic shoots were positive for *D. scrobiculata*. Only one cone and one asymptomatic shoot were positive for both *D. pinea* and *D. scrobiculata*. Negative controls were never positive for either *Diplodia* species. Positive *D. pinea* controls were only positive for *D. pinea* and positive *D. scrobiculata* controls were only positive for *D. scrobiculata*. More than half (mean ≥65% ± 3 (S.E.), range 10–98%) of the 2305 examined conidia germinated.

4. Discussion

The incidence of *D. pinea* on cones and the number of conidia extracted from red pine cones in the current study were comparable to data obtained from previous sampling in other regions of Wisconsin (Jackson Co., approximately 250 km distant and Adams Co., approximately 300 km distant) (Munck, 2008). In each study the incidence of *D. pinea* or *D. scrobiculata* on at least one tree (i.e., at least one positive cone) exceeded 60%. In that study, the mean number of conidia extracted per cone was

12,601 g^{-1} odw (ranging from 3494 to 23,981 between different sites and from 0 to 44,511 for individual cones). In this study, the mean number of conidia extracted per cone was 8217 conidia g^{-1} odw (ranging from 1 to 23,216 between different sites and from 0 to 103,294 for individual cones). Therefore, it is likely that data collected in the current study are representative of other red pine stands in similar sites in Wisconsin.

Not only were conidia abundantly available on cones but also they were viable as most of them (64%) germinated. This result is consistent with that of a study by Santini et al. (2008) of cones of *P. pinea* collected from the forest floor. Conidia were extracted from cones and germination tests were conducted. In that study more than half of the conidia also germinated (Santini et al., 2008).

In the current study and also previously (Munck, 2008) *D. pinea* was much more frequently detected from red pine cones than *D. scrobiculata*. Similarly, *D. pinea* was detected more frequently than *D. scrobiculata* from symptomatic bark and wood sampled from red pine seedlings with Diplodia collar rot symptoms in central Wisconsin (Smith and Stanosz, 2006). One possible explanation for the more frequent detection of *D. pinea* from red pine tissues is greater aggressiveness of this pathogen than *D. scrobiculata*. In addition to the differences in aggressiveness between *D. pinea* and *D. scrobiculata*, there appears to be a difference in host preference. *Diplodia pinea* has been more frequently associated with red pine cones, asymptomatic shoots, and seedlings with collar rot than *D. scrobiculata* (Munck, 2008; Smith and Stanosz, 2006). Conversely, *D. scrobiculata* was detected more frequently than *D. pinea* from jack pine cones (Munck, 2008) and jack pine seedlings with collar rot (Smith and Stanosz, 2006). Differences between *D. pinea* and *D. scrobiculata* in aggressiveness on conifer hosts and in the prevalence of each *Diplodia* species indicate the importance of identifying the pathogen(s) present at a particular site in areas where either might occur. Cones offer a convenient, accessible sample unit for this purpose.

The relationship between presettlement vegetation and numbers of cones positive for *Diplodia* species as well as the numbers of conidia extracted was inconsistent. Although red pine cones from Douglas Co. sites in areas historically dominated by jack pine almost always yielded conidia and very high numbers of conidia were obtained, this was not true for cones from presettlement jack pine sites in Bayfield Co. Differences in soil texture of these sites in the different counties, however, may offer one explanation for these differences. The available potential inoculum from cones was positively correlated with the proportion of sand and negatively correlated with the proportion of silt. Consistent with the association of Diplodia epidemics in this region with drought and unfavorable site conditions (Nicholls and Ostry, 1990; Palmer, 1991) and Wisconsin Department of Natural Resources (WDNR, 1977–1996) reports of shoot blight and canker epidemics in Douglas Co., soils of these presettlement jack pine sites in Douglas Co. had the greatest mean percentage of sand and the least mean percentage of silt. Silt has more available water-holding capacity than sand (Foth, 1990). Therefore, in addition to more available inoculum at these sites, trees at these sites might be more frequently and severely affected by drought stress to increase the risk for damage.

The influence of variation in other site characteristics on the activity of *Diplodia* species in red pine plantations offers potential for study. For example, soil pH was positively correlated with the number of *Diplodia* species conidia extracted from cones, and the proportion of cones and asymptomatic shoots positive for *Diplodia* species. Soil pH can affect plant growth and health by affecting the availability of nutrients for plants. However, the soil pH data for the sites sampled in this study were all within the suitable range for red pine (Foth, 1990). Nicholls and Ostry (1990) reported that cankers caused by *Sphaeropsis sapinea* sensu lato occurred more frequently on trees exposed to environmental stresses such as poor site conditions (site index = 14 m) and drought. In the current study site index was not correlated with potential inoculum abundance, *Diplodia* species or cone and asymptomatic shoot incidence, but site indices in the current study exceeded 16 m. Other differences among sites in these counties that were not explored (such as the effects of latitude, proximity to Lake Superior, solar radiation, other soil factors, etc.) might also offer possible explanations. However, how any of these factors might directly or indirectly influence susceptibility of cones to infection by *Diplodia* species, subsequent proliferation in cones, and abundance of pycnidial and conidial production remains to be investigated.

Host and environmental factors that might influence the frequency of persistence of *D. pinea* in asymptomatic shoots of mature trees have been previously investigated (Flowers et al., 2001; Maresi et al., 2007). Flowers et al. (2001) were able to culture *D. pinea* from asymptomatic shoots from mature Austrian pines (*P. nigra* Arn.) in an urban setting in Kentucky, USA. Relatively greater proportions of asymptomatic shoots positive for *D. pinea* in that study were associated with greater shoot blight severity (percentages of shoots that were blighted), but the quantities of potentially available inoculum were not investigated in that study. Maresi et al. (2007) used both cultural and molecular assays to detect asymptomatic persistence of *D. pinea* on or in shoots from nine Austrian pine plantations in northern Italy in which soil, air temperature, and precipitation were similar. However, plantations differed in the Normalized Insolation index which is a measure of the heat on site based on the solar radiation received (Maresi et al., 2007). In that study the pathogen was more frequently detected from asymptomatic shoots from sites receiving greater solar radiation than from shoots from sites receiving less. Again, possible differences in available inoculum among these sites were not investigated.

There is a relationship between the number of *Diplodia* species conidia extracted from cones and the proportion of cones and asymptomatic shoots positive for *Diplodia* species, but from data of the current study it is impossible to determine if the abundance of potential *Diplodia* species inoculum is the cause or the result of frequency of cone infections or asymptomatic shoot persistence. Although persistence of *D. pinea* and *D. scrobiculata* on or in asymptomatic shoots has not yet been directly related to subsequent disease development in the field, virulence of isolates from asymptomatic shoots was demonstrated by Stanosz et al. (1997) and Flowers et al. (2001). In these studies isolates from asymptomatic shoots caused disease to red pine, jack pine (Stanosz et al., 1997), and Austrian pine (Flowers et al., 2001) seedlings in the greenhouse. Furthermore, Stanosz et al. (2001) demonstrated that red pine seedlings on or in which *D. pinea* persisted asymptomatically subsequently became diseased when subjected to water stress. Therefore, shoots from trees sampled in this study on or in which *D. pinea* and *D. scrobiculata* persisted asymptomatically may have the potential to become diseased if other conditions favorable for disease development occur.

Results from this study add to previous evidence that *D. pinea* and *D. scrobiculata* are able to persist in the overstory as conidia in cones of mature trees, maintaining an abundant source of inoculum to threaten current stands, and natural and artificial regeneration in the understory or in adjacent areas. It is not known at what distance from matured red pines serving as an inoculum source it is safe to plant red pine seedlings. Given the economic and ecological importance of red pine in the north central and eastern United States and Canada (Rudolf, 1990), and losses due to Diplodia shoot blight, canker, and collar rot diseases in nurseries, plantations, and natural forests, these topics deserve further investigation.

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