Interaction Between *Diplodia pinea* and *D. scrobiculata* in Red and Jack Pine Seedlings

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Accepted for publication 9 October 2010.

ABSTRACT

Santamaría, O., Smith, D. R., and Stanosz, G. R. 2011. Interaction between *Diplodia pinea* and *D. scrobiculata* in red and jack pine seedlings. *Phytopathology* 101:334-339.

Sphaeropsis sapinea sensu lato is a conifer fungal pathogen that causes shoot blight and stem cankers. Recently, the former *S. sapinea* has been divided into two species, *Diplodia pinea* and *D. scrobiculata*. The aims of the study were to determine the contribution of each species in disease development on red and jack pines by means of co-inoculations and molecular identifications, and to evaluate how the presence of each species affects the development and aggressiveness of the other. Symptom severity (distance below the inoculation site at which necrotic needles were observed) and identification length (the maximum distance from inoculation site from which either *D. pinea* or *D. scrobiculata* was identified

using molecular methods) were recorded 4 weeks after inoculating wounded seedlings with agar plugs colonized by these pathogens. The results suggested that *D. pinea* was much more aggressive on both hosts than *D. scrobiculata*. When a seedling was co-inoculated with these pathogens, the symptom development appeared to be mainly due to *D. pinea*. The presence of *D. pinea* also interfered with the establishment of *D. scrobiculata* in the plant tissue. However, *D. scrobiculata* showed antagonism toward *D. pinea*. When both pathogens co-occurred in a single seedling, symptom severity caused by *D. pinea* was less than when *D. pinea* alone was present.

Additional keywords: *Pinus banksiana*, *P. resinosa*, shoot blight, tip blight.

Sphaeropsis sapinea sensu lato is a conifer fungal pathogen of worldwide distribution and importance. It has been associated with significant economic damage in nurseries, plantations, Christmas trees and ornamental plantings, and natural stands (11, 16,21). Hosts include species in at least eight coniferous genera, including many species of pines (16,26). Red pine (*Pinus resinosa* Aiton) and jack pine (*P. banksiana* Lamb.), tree species with a great economical and ecological importance in the central and eastern United States, have been severely damaged in recent decades (21,23). Most parts of host plants can be affected, to result in a range of disease symptoms: shoot blight, stem cankers, branch dieback, dead tops, death, and blue staining of cut wood (21,31).

Two groups, A and B, were distinguished within *S. sapinea sensu lato* (24). These were defined based on conidial morphology, cultural characteristics and virulence (24,37), and molecular markers (8,12,27,38). Recently, the B group has been recognized as a distinct species and assigned the name *Diplodia scrobiculata* J. de Wet, B. Slippers & M. J. Wingfield (13). At the same time, the A group was named *Diplodia pinea* (Desmaz.) J. Kickx fil. (syn. *Sphaeropsis sapinea* (Fr.:Fr.) Dyko & Sutton in Sutton) (13). *D. pinea* produces abundant white to gray-green aerial mycelium in culture and grows more quickly on potato dextrose agar (PDA) than *D. scrobiculata*, which produces white or black mycelium appressed to the agar surface. Identification and detection from colonized host organs is facilitated by use of species-specific PCR primers that allow differentiation of these pathogens from each other and closely related fungi (28). In terms of symptom development, *D. pinea* has been shown to be more aggressive than *D. scrobiculata* (2,5).

Both *D. pinea* and *D. scrobiculata* have been frequently isolated from asymptomatic tissues of several hosts, including red and jack pines (15,18,32,33,34). This persistence in healthy tissues without causing any visible damage has been associated with a latent state of the pathogen (33). Latency may explain pathogen survival in the apparently healthy tree and rapid disease development under conditions that induce host stress (29). Environmental stress from a variety of sources has been shown to induce or to aggravate the damage caused by *Diplodia* species. Among these, water stress appears to be the most important (4,6,30), although other predisposing factors, such as poor site, hail, snow, frost damage, and insects can enhance colonization by *D. pinea* and *D. scrobiculata* (22,29,36).

The reported geographic and host ranges of *D. pinea* and *D. scrobiculata* are broad and overlapping. Both species also have been isolated from individual red pine plantations (22,32), from a single tree (19), and even detected using molecular methods from the same small bark and wood samples (28). Those experiments indicated the potential for intimate association between *D. pinea* and *D. scrobiculata* within host tissues. However, relatively little is known about their local co-occurrence and the implications of this fact for the production of inoculum, disease development, and subsequent survival. Therefore, the objectives of the present study were (i) to determine the contribution of each species to disease development on red and jack pine seedlings by means of co-inoculations and identification using molecular methods, and (ii) to evaluate how the presence of each species affected symptom development by the other.

MATERIALS AND METHODS

Plant material. Dormant, 1-year-old jack pine and 2-year-old red pine nursery seedlings (Wisconsin Department of Natural Resources Wilson State Nursery, Boscobel, WI) were lifted on 27

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doi:10.1094/PHYTO-07-10-0180

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November 2007 and transplanted into Deepot cones (conical tubes, 6.4 cm wide × 25.4 cm deep; Stuewe & Sons Inc., Corvallis, OR) in a soil mix (1:1, vol/vol) of Plainfield sand (containing 89% sand and 7% silt) from a 24-year-old red pine plantation in central Wisconsin and Fafard Growing Mix no. 2 (Conrad Fafard Inc., Inkerman, New Brunswick, Canada). Red pine seedlings had a mean stem height of 16.9 cm ± 0.3 standard error (SE) and jack pine seedlings had a mean stem height of 26.8 cm ± 0.6 SE at the time transplanted. Seedlings were placed in a greenhouse supplemented with artificial light (maximum recorded ambient greenhouse photon flux density was 1,560 μmol·s⁻¹·m⁻²; supplemental photon flux density averaged 132 μmol·s⁻¹·m⁻²) to provide a 16-h photoperiod. The seedlings were watered to field capacity every 2 to 3 days. The temperature and humidity in the greenhouse were: mean temperature (T) 25.8°C ± 0.6 SE, mean relative humidity (RH) 30.1% ± 1.3 SE during trial 1 of the jack pine experiment; T = 27.9°C ± 0.9 SE, RH = 27% ± 1.7 SE during trial 2 in jack pine; T = 33.1°C ± 0.9 SE, RH = 14.3% ± 1.8 SE during trial 1 in red pine; and T = 35.7°C ± 0.6 SE, RH = 12.2% ± 1.2 SE during trial 2 in red pine experiment.

Inoculations. Mycelial inoculum was produced for two monoclinal isolates of each species collected from various pine species and locations in the northcentral United States (Table 1). The elongating, asymptomatic terminal shoot of each seedling was inoculated in the greenhouse approximately 11 weeks after transplanting in the case of red pine, and 6 weeks for jack pine. Five seedlings per treatment combination (isolate and fungal species) were used in each of two trials separated by 2 weeks. Thus, in each trial five seedling of each tree species were inoculated in each of the following treatments: (i) P1+S1, (ii) P1+S2, (iii) P2+S1, (iv) P2+S2, (v) P1+P1, (vi) P2+P2, (vii) S1+S1, (viii) S2+S2, and (ix) agar A+A (or 45 seedlings per tree species per trial). All seedlings were assigned randomly to a particular treatment.

On each shoot, two wounds (in the opposite sides of the shoot) were made by removing a needle fascicle per side (by a sterile scalpel cut flush to the stem) approximately 2 cm below the shoot apex. Inoculum plugs 4 mm in diameter were cut from the margin of an actively growing culture on PDA (Becton, Dickinson and Co., Sparks, MD) and placed on each of the two wounds. Another five nonwounded seedlings for each host were incorporated in the experiments as additional controls but those were not included in the statistical analyses. Parafilm (Pechiney Plastic Packaging, Menasha, WI) was wrapped around the shoots for 7 days.

Four weeks after inoculation, the distances below the inoculation site at which necrotic needles and cankers were present were measured (symptom severity). After needles were removed, 1-cm-long segments, centered at 0, 3, 6, 9, 12, and 15 cm below the inoculation site, were aseptically removed and stored at -20°C for later molecular detection of pathogen species. Additionally, 10% of seedlings that had not been inoculated with either pathogen were similarly analyzed for the presence of *Diplodia* species.

Molecular identifications. The technique used allowed determination of the presence of either or both *Diplodia* species in the same plant segment. Fungal DNA was extracted directly from the plant cross sections by using the slightly modified method of Cubero et al. (10) outlined in Smith and Stanosz (28). This method uses an extraction buffer containing hexadecyltrimethylammonium bromide (CTAB) to remove polysaccharides and polyvinylpyrrolidone (PVPP) to remove polyphenols, and employs two chloroform extractions to remove proteins. The extracted DNA was amplified using species-specific mitochondrial small subunit rDNA primers (28), and presence of amplified DNA fragments was verified on 0.7% agarose gels in TBE buffer. Primer sequences and detailed procedures were provided by Smith and Stanosz (28). Positive and negative controls were used in the amplifications to ensure that *Diplodia* DNA could be detected if present and that no false positives occurred due to con-

tamination. Sections from each seedling were processed in order starting with the inoculation site and proceeding downward. If the pathogen was not detected in two consecutive sections, no further sections were processed.

Statistical analysis. Symptom severity was analyzed by two-factor analysis of variance (ANOVA) with interaction. Factors used as main effects were treatment and trial. Fisher's least significant difference was used for multiple comparison among treatments by means of the general linear model procedure of SAS (Statistical Analysis Software version 9.1.3, SAS Institute Inc., Cary, NC) when significant differences were found in the analysis of variance table. The ln(x + 1) transformation of the response variable was used to stabilize the residual variance, although back-transformed values are presented in tables and figures for clarification. Assumptions of normality and homoscedasticity were assured by Kolmogorov-Smirnov and Levene's tests, respectively.

Spearman's coefficient of rank correlation was used to examine relationships between symptom severity and the farthest cross section from inoculation site from which either *D. pinea* or *D. scrobiculata* was identified (identification length). In the case of *D. pinea*, the identification length was also used as a response variable to make comparisons among treatments by using the Kruskal-Wallis test of equality of medians. A chi-square goodness-of-fit test was used to compare treatments by analyzing the frequency variable (percentage of seedlings from which *D. scrobiculata* was identified). All the nonparametric analyses were performed using Minitab for Windows version 14 (Minitab Inc., State College, PA). All the analyses were carried out separately for both red and jack pine since those were separate experiments.

RESULTS

Inoculated seedlings of both tree species produced symptoms similar to those reported for seedlings in field and nursery studies, including necrotic needles, stem cankers, and crooked and dead shoot tips. Symptoms were observed in 100% of the seedlings inoculated with *D. pinea*, in 60% of the seedlings inoculated with *D. scrobiculata*, and in 95% of the seedlings inoculated with both pathogens. No symptoms developed on wounded or nonwounded control seedlings of either host.

In jack pine, the two-factor analyses of variance of the symptom severity (distance below the inoculation site at which necrotic needles were present) indicated significant effect of the treatment ($F = 50.75$, $P < 0.01$), but not of the trial ($F = 1.92$, $P = 0.170$). Thus, the multiple comparison of the treatments was performed with the pooled data of the two trials. Symptom severity in jack pine was greater on seedlings inoculated with isolates of *D. pinea* than on seedlings inoculated with isolates of *D. scrobiculata* (Fig. 1). Symptom severity on seedlings inoculated with the isolate S2 of *D. scrobiculata* was not significantly different from that on control seedlings. It was observed that symptom severity also differed between the two isolates of *D. pinea*. On seedlings inoculated with the most aggressive isolate (P1), there were not

TABLE 1. Origin of *Diplodia* isolates used to inoculate red and jack pine seedlings

Isolate ^a	Isolate no. ^b	Pine host	Geographic origin
P1	411	<i>Pinus resinosa</i>	Clearwater Co., MN
P2	04-126	<i>Pinus banksiana</i>	Wood Co., WI
S1	124	<i>Pinus banksiana</i>	Jackson Co., WI
S2	462	<i>Pinus resinosa</i>	Clearwater Co., MN

^a Species and isolate number: P (*Diplodia pinea*), S (*Diplodia scrobiculata*). The isolates were previously characterized according to the specific primers given by Smith and Stanosz (28).

^b Culture collection numbers of M. A. Palmer (3-digit number) or G. R. Stanosz (04-xxx).

statistical differences in the symptom severity between seedlings inoculated only with P1 and those inoculated with P1 in combination with either isolate of *D. scrobiculata* (Fig. 1). However, on seedlings inoculated with the less aggressive isolate of *D. pinea* (P2), the co-inoculation of these fungal species into the seedling significantly reduced symptom severity in comparison to that caused by P2 when it was inoculated alone (Fig. 1).

In the case of red pine seedlings, two-way ANOVA of the symptom severity showed a significant effect of the treatment ($F = 15.89$, $P < 0.01$), and trial ($F = 16.29$, $P < 0.01$), but not of their interaction ($F = 0.34$, $P = 0.88$). Therefore, a multiple comparison was performed separately for each trial (Fig. 2). As in the case of jack pine, isolates ranked in the same order in relation to their aggressiveness, although in general terms, symptom severity was lower in red pine than in jack pine. In red pine, it was also observed that the presence of either isolate of *D. scrobiculata* in the seedling reduced the symptom severity caused by either isolate of *D. pinea* (except in trial 1 for P2 isolate), although in red pine, the differences between treatments were not as great as for jack pine (Fig. 2).

Data from the farthest segments from inoculation site from which *D. pinea* was identified using molecular methods (identi-

fication length) are summarized in Table 2. *D. pinea* was identified in 98% of the seedlings inoculated with *D. pinea*. Additionally, it was also recorded in three seedlings (from a total of 12 seedlings analyzed) which had not been inoculated with *D. pinea*. In 15 seedlings (from a total of 120), *D. pinea* was identified in a segment farther than the distance below the inoculation site at which necrotic needles were present, although the correlation between identification length and symptom severity was very high (Pearson's coefficient = 0.87; P value < 0.001). *D. scrobiculata* was identified in 72% of the seedlings inoculated with *D. scrobiculata*, although, it was detected in a segment beyond the segment centered at the inoculation site only once. Therefore, the correlation between identification length and symptom severity was very low for *D. scrobiculata* (Pearson's coefficient = 0.12; P value < 0.419). Additionally, it was also detected in one control seedling (from a total of 12 seedlings analyzed) which had not been inoculated with *D. scrobiculata*.

The Kruskal-Wallis test performed on the identification length for *D. pinea* revealed significant differences between isolates of *D. pinea*, but not between treatments for each *D. pinea* isolate when analyzed separately (Table 3). When the frequency variable (percentage of seedlings from which *D. scrobiculata* was identi-

Fig. 1. Mean symptom severity (distance below the inoculation site at which necrotic needles were present) caused by isolates of *Diplodia pinea* (p1, p2), *D. scrobiculata* (s1, s2), both (p1s1...), or controls (c) for each treatment on jack pine seedlings. Averages with the same letter are not significantly different according to Fisher's least significant difference test at a significant level of 0.05. Vertical bars indicate standard error.

Fig. 2. Mean symptom severity (distance below the inoculation site at which necrotic needles were present) caused by isolates of *Diplodia pinea* (p1, p2), *D. scrobiculata* (s1, s2), both (p1s1...), or controls (c) for each treatment and for each trial on red pine seedlings. Averages with the same letter are not significantly different according to Fisher's least significant difference test at a significant level of 0.05. Vertical bars indicate standard error.

fied) was analyzed by means of a chi-square goodness-of-fit test (Table 4), it was observed that there were significant differences between trials. In trial 1, *D. scrobiculata* was identified on both hosts with the same statistical frequency in seedlings inoculated only with *D. scrobiculata* as in those inoculated with *D. scrobiculata* in combination with *D. pinea*. However in trial 2, it was observed that the presence of *D. pinea* in seedlings of both hosts reduced the percentage of seedlings from which *D. scrobiculata* was identified (Table 4).

DISCUSSION

Inoculations with *D. pinea* isolates resulted in greater severity of symptoms than inoculations with *D. scrobiculata* isolates on seedlings of red and jack pine. This result agrees well with previous studies (5,7) which used a similar inoculation technique on seedlings of a variety of hosts. However in Blodgett and Stanosz (5), *D. scrobiculata* was more aggressive in jack pine and *D. pinea* in red pine. In our study, although results are not statistically comparable since they were obtained in separate experiments, jack pine appeared to be the most susceptible host for both pathogen species.

The correlation analysis performed between symptom severity and the farthest segment from inoculation site from which either *D. pinea* or *D. scrobiculata* was identified using molecular methods suggests that, when both pathogens were inoculated in the same seedling, *D. pinea* was mainly responsible for the necrosis length observed. This was consistent with the greater aggressiveness showed by *D. pinea*. The lower symptom severity when both species were inoculated compared with when *D. pinea* alone was inoculated suggests a potential antagonism between both pathogens. This may have very important implications, given the co-occurrence of *D. pinea* and *D. scrobiculata* in nature (19, 22,28,32). Even in the present study, both pathogens were identified from a 1-cm-long shoot segment obtained from a non-inoculated jack pine seedling. It would be very interesting to develop further studies to investigate whether disease incidence or severity is lower in plantations in which both pathogens occur compared with those where just one pathogen is present.

This result should be considered cautiously, however, because the reduction of the symptom severity caused by *D. pinea* when *D. scrobiculata* was also present was not always statistically significant. One possible explanation for this lack of significance could be related to the variability recorded among the repetitions. This great variation could in part be a consequence of the natural presence of both pathogens, in a latent state, in the seedlings used. Detection of *S. sapinea sensu lato* from 20 to 85% of asymptomatic samples has previously been reported (14,15, 18,32,33). Even in the present study, *D. pinea* occurred asymptotically in 25% of the noninoculated seedlings and *D. scrobiculata* in 8%. Despite this natural presence of the pathogen in the seedlings of our experiments, control seedlings did not show any symptoms. Lack of disease development in these seedlings may have been due to the favorable conditions provided for seedling growth, in contrast to stressful conditions that have been demonstrated to release *D. pinea* from latency to cause disease (29,30). Proliferation of the previously latent pathogen in seedlings which we also inoculated could result in overestimation of symptom severity due to the stress caused by inoculations to the plants.

This natural latency also may have affected the molecular identifications of both pathogens in the stem segments and may explain several unexpected findings. For example, in several

TABLE 4. Percentage of seedlings from which *Diplodia scrobiculata* was identified by using molecular methods^a

Host species	Trial	Percentage (range)		
		<i>D. scrobiculata</i> + <i>D. pinea</i>	<i>D. scrobiculata</i>	<i>P</i>
Jack pine	1	85 (60–100)	90 (80–100)	0.7055
	2	55 (0–100)	80 (60–100)	0.0314
	<i>P</i>	0.0112	0.4431	
Red pine	1	80 (60–100)	100 (all 100)	0.136
	2	45 (20–60)	70 (40–100)	0.0197
	<i>P</i>	0.0017	0.0184	

^a Values of *P* for comparisons between those percentages by using a chi-square goodness-of-fit test.

TABLE 2. Number of seedlings from which *Diplodia pinea* was identified using molecular methods from stem segments centered at 0, 3, 6, 9, 12, or 15 cm from the site of inoculation

Treatment	Trial	Distance from site of inoculation (cm)											
		Jack pine segments						Red pine segments					
		0	3	6	9	12	15	0	3	6	9	12	15
P1S1	1	5	4	3	3	1	1	5	2	2	0	0	0
	2	5	5	4	4	3	1	5	3	0	0	0	0
P1S2	1	5	5	5	4	2	2	5	1	0	0	0	0
	2	5	4	5	2	1	0	4	3	1	0	0	0
P1P1	1	5	4	2	1	1	0	5	3	0	0	0	0
	2	4	5	4	3	1	0	5	4	2	0	0	0
P2S1	1	5	0	0	0	0	0	5	1	1	0	0	0
	2	5	1	2	1	0	0	4	1	0	0	0	0
P2S2	1	5	0	0	0	0	0	5	0	0	0	0	0
	2	5	2	0	2	0	0	5	0	0	0	0	0
P2P2	1	5	1	1	1	1	1	5	0	0	0	0	0
	2	5	4	2	1	0	0	5	1	0	1	0	0

TABLE 3. Values of *P* for pairwise comparisons using the Kruskal-Wallis test of equality of medians for farthest stem segment from the inoculation site from which *Diplodia pinea* was identified using molecular methods: jack pine data (below the diagonal) and red pine data (above the diagonal)

Treatment	P1S1	P1S2	P1P1	P2S1	P2S2	P2P2
P1S1	...	0.460	0.328	0.144	0.013	0.102
P1S2	0.969	...	0.088	0.492	0.135	0.427
P1P1	0.313	0.229	...	0.018	0.001	0.009
P2S1	0.002	0.001	0.005	...	0.543	0.957
P2S2	0.003	0.002	0.008	0.914	...	0.317
P2P2	0.037	0.010	0.095	0.214	0.248	...

samples, *D. pinea* was detected in segments below other negative segments, indicating a possible latent *Diplodia* in those farther sections. In other samples, *D. pinea* was identified farther from the inoculation site than the necroses length recorded in the corresponding seedling. This result could be also explained by latent infections since it has been demonstrated in other studies (5) that symptom development, expressed as mean distance below the inoculation site with necrotic needles, was very positively correlated with distance of detection. A certain antagonistic effect between *D. pinea* and *D. scrobiculata* appeared to be reflected in the results of detection using molecular methods, because the presence of *D. scrobiculata* in the samples was clearly less frequent in seedlings also inoculated with *D. pinea* compared with those seedlings inoculated only with *D. scrobiculata*.

Among the three types of interactions between antagonistic organisms proposed by Adams (1), competition, antibiosis, and hyperparasitism, the former could be the most important mechanism between both pathogens. However, antibiosis could also be acting since *S. sapinea sensu lato* has been shown to produce metabolites with antifungal activity (9). With the experimental methodology followed to perform the present study it was not possible to determine if hyperparasitism was or was not an important mechanism in this antagonistic interaction, although it may be unlikely since it has not been described for *Diplodia* species so far. Another possibility could be hypovirulence, which has been described to take place for several other fungal pathogens like *Cryphonectria parasitica* (Murril) Barr. (17). Hypovirulence is due to a double-stranded (ds)RNA virus which infects fungal cells. Although hypovirulence has not been described for *Diplodia* species, dsRNA of several virus families has been found infecting *Diplodia* isolates (25) at a high frequency. Steenkamp et al. (35) identified viral dsRNA in 32% of the *Diplodia* isolates analyzed. Therefore, the frequency and the type of dsRNA virus present in *D. pinea* and *D. scrobiculata* and their relationship with virulence may deserve further investigations.

Systemic induced resistance (SIR) is a host defense mechanism which has also been proposed by several authors (3,20) to explain why inoculations with *D. scrobiculata* reduced the symptom severity on seedlings inoculated later with *D. pinea*. However in such studies, *D. scrobiculata* was inoculated several weeks before inoculating with *D. pinea*, because plants need some time to produce the defensive action. Therefore, the reduction of symptom severity caused by *D. pinea* when *D. scrobiculata* is also inoculated into the same seedling at the same time, as it is our case, could not be explained by this mechanism of SIR. The effect caused by *D. scrobiculata* in the defense system of the plant could only be used preventively on healthy trees, since it has no curative properties (20). In the same sense, Palmer (22) affirmed that no evidence suggested that *D. scrobiculata* was related to tree recovery.

In conclusion, the results presented here suggested that, when both *D. pinea* and *D. scrobiculata* co-occurred in a single plant, the symptom development was mainly due to *D. pinea*. Furthermore, *D. scrobiculata* showed some antagonism toward *D. pinea*, since when both pathogens co-occurred in a single seedling, symptom severity caused by *D. pinea* was lower than that caused when *D. pinea* was acting alone. The presence of *D. pinea* made difficult the establishment of *D. scrobiculata* in the plant tissue.

ACKNOWLEDGMENTS

This research was partially supported by a grant provided by the Ministry of Culture and Science of Spain (Program: José Castillejo) and by the Department of Plant Pathology of the University of Wisconsin-Madison. We thank the Wisconsin Department of Natural Resources for seedlings, and J. A. Stanosz and L. Gardiner for technical assistance.

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