Differences in Aggressiveness of *Sphaeropsis sapinea* RAPD Marker Group Isolates on Several Conifers

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ABSTRACT

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Seedlings of Scot's pine varieties East Anglia and Austrian Hills, red pine, mugho pine variety Pumileo, Colorado blue spruce, Douglas-fir, and balsam fir were wounded and inoculated with water agar plugs colonized by isolates of the two random amplified polymorphic DNA (RAPD) marker groups (A and B) of *Sphaeropsis sapinea*. Isolates were obtained from hosts in Michigan, Minnesota, and Wisconsin. Symptom severity (distance from the inoculation site at which necrotic needles were present) resulting from inoculations with each group A isolate exceeded that from inoculations with each group B isolate on all hosts except Colorado blue spruce. Hosts varied considerably in their responses to group A isolates. Based on symptom severity, East Anglia Scot's pine was most susceptible and balsam fir was least susceptible when inoculated with group A isolates. The pathogen was recovered from both symptomatic and asymptomatic seedlings inoculated with isolates of either group. Results emphasize the importance of characterizing a RAPD marker group(s) of *S. sapinea* encountered in the field or used in research; the need for comparative evaluations of resistance among coniferous genera, species, and varieties to *S. sapinea* of both groups; and the potential for asymptomatic persistence of *S. sapinea* from both groups in or on several coniferous hosts.

Additional keywords: *Abies balsamea, Diplodia pinea, Picea pungens, Pinus mugo, Pinus resinosa, Pinus sylvestris, Pseudotsuga menziesii*

Diseases caused by *Sphaeropsis sapinea* (syn. *Diplodia pinea*) can result in extensive losses of native and exotic conifers throughout the world. Damage can occur in nurseries, Christmas tree and ornamental plantings, and forest stands (8,11,12,14,23). Colonization by *S. sapinea* can result in shoot blight, cankers, crown wilt, collar rot, and root disease (5,8,11,18,23,29).

S. sapinea affects hosts in at least eight coniferous genera, including many species in *Pinus* (6,8). Red pine (*Pinus resinosa*) is the most economically important host in the north central United States, and the relationship of *S. sapinea* with this species has been extensively studied (1–3,11,12, 18,21). Other reported hosts with particular value as ornamentals or Christmas trees in this region include Scot's (*P. sylvestris*) and mugho (*P. mugo*) pines, Colorado blue spruce (*Picea pungens*), Douglas-fir (*Pseudotsuga menziesii*), balsam fir (*Abies balsamea*), and white fir (*Abies concolor*) (6–8,10,15,30). However, the relative susceptibility of these hosts cannot be determined from the literature because of the lack of comparative trials under controlled conditions using well-characterized isolates of this pathogen.

Differences in colony appearance and growth rate on potato dextrose agar (PDA) were reported for isolates of *S. sapinea* from pine hosts in the north central United States (13). These two colony types were later referred to as morphotypes (3). Isolates of the A morphotype were described as having fluffy white to gray-green mycelium and as growing more quickly on PDA than B isolates, which were described as having white or black mycelium that was closely appressed to the agar surface (13). Other differences between A and B isolates have been suggested, including differences in radial growth rate, conidial size, texture of the conidial wall, production of spermatia or microconidia, and ability to infect nonwounded hosts (13,27,28). Each of these characters, however, either has been shown to vary substantially within these groups or is now known to be similar for isolates of both groups (3,25,26,31). Swart et al. (25) noted that *S. sapinea* is a highly variable species and concluded that there may be more than two distinct types or that variation might occur within a continuum.

Smith and Stanosz (17) examined random amplified polymorphic DNA (RAPD) markers of *S. sapinea* isolates obtained from pine hosts in the north central United States. They found that selected A and B morphotype isolates could be placed into two corresponding RAPD marker groups. In addition, other isolates that had been difficult to characterize morphologically could be consistently and unambiguously placed into discrete A and B RAPD marker groups. We will subsequently refer to isolates differentiated on the basis of RAPD markers as members of group A or group B. The same techniques were later used to characterize numerous additional isolates of this pathogen obtained from hosts of several coniferous genera in the central United States (19) and many other countries (22).

In our earlier studies (1–3), inoculation of wounded red pine seedlings with group A isolates resulted in greater incidence of symptoms and recovery of *S. sapinea* farther from the inoculation site than inoculations with group B isolates. The objective of the study described in this paper was to compare the aggressiveness of *S. sapinea* isolates of these two groups on several coniferous host species. The null hypothesis that these two RAPD marker groups do not differ in their aggressiveness, as expressed by incidence and severity of symptoms on each of these hosts, was tested in a greenhouse experiment. At the same time, we were able to evaluate the potential for our methods to quantify differences in resistance to each group among a variety of coniferous hosts.

MATERIALS AND METHODS

Dormant nursery seedlings of 2-year-old Scot's pine varieties East Anglia and Austrian Hills, 2-year-old red pine, 4-year-old mugho pine variety Pumileo, 3-year-old Colorado blue spruce, 2-year-old Douglas-fir, and 3-year-old balsam fir (West Wisconsin Nursery and Christmas Trees, Sparta, WI) were lifted 29 April 1996. These trees were 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 14-year-old red pine plantation in central Wisconsin and Fafard growing mix no. 2 (Conrad Fafard Inc., Inkerman, New Brunswick, Canada). Scot's pine varieties East Anglia and Austrian Hills had mean stem heights of 11.1 cm ± 0.3 cm (standard error) and 12.3 cm ± 0.3 cm, respectively; red pine had a mean stem

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height of 9.6 cm ± 0.2 cm; mugho pine had a mean stem height of 16.7 cm ± 0.4 cm; Colorado blue spruce had a mean stem height of 15.5 cm ± 0.3 cm; Douglas-fir had a mean stem height of 15.1 cm ± 0.3 cm; and balsam fir had a mean stem height of 13.6 cm ± 0.2 cm at the time of transplanting. The mugho pine seedlings were root and shoot pruned to allow transplanting into the Deepot cones.

Seedlings were placed in a greenhouse supplemented with artificial light (maximum recorded ambient greenhouse photon flux density was 1,287 $\mu E \cdot s^{-1} \cdot m^{-2}$; supplemental photon flux density averaged 118 $\mu E \cdot s^{-1} \cdot m^{-2}$) to provide a 16-h photoperiod. The seedlings were watered to field capacity every 2 to 3 days. The average greenhouse temperature was 25.0°C ± 0.8°C, and the average relative humidity was 67% ± 1.9%.

The elongating, asymptomatic terminal shoot of each seedling was inoculated 6 weeks after transplanting. On each shoot, a single wound was made by removing a needle or needle fascicle (by a scalpel cut flush to the stem) approximately 2 cm below the shoot apex. A 4-mm-diameter plug was cut from the margin of an actively growing culture on 1.5% water agar (WA; Difco Laboratories, Detroit, MI) and placed mycelium-side-down on the wound. Parafilm (American National Can Co., Chicago, IL) was wrapped around the plug and shoot and removed after 3 days. Wounded and nonwounded controls were included for each host. A noncolonized WA plug was applied to wounded controls. Four monoconidial isolates of each group were used (Table 1). Six seedlings per isolate, six wounded control seedlings, and six nonwounded control seedlings were used for each tree species or variety in each of two trials, except for mugho pine for which four seedlings were used for each isolate (and for each of the two control treatments) in each of the two trials (800 seedlings total). Inoculations in the second trial followed those in the first by 1 week. All treatments were assigned randomly.

Data were collected and recovery of *S. sapinea* was attempted from each seedling 4 weeks after inoculation. The presence or absence of necrotic needles was recorded, and the distance below the inoculation site at which necrotic needles were present was measured along the stem. After needles were removed, apical-shoot segments (including the site of inoculation) were surface-disinfested for 10 s in 95% ethanol followed by 4 min in 1.05% NaOCl solution with 2 drops/liter of Tween 80 (Fisher Scientific Co., Toronto, Ontario, Canada). The apical 5-cm-long segment of each shoot was placed in a 2.0% WA slant and incubated for 15 weeks at ambient laboratory temperature (approximately 24°C) and light. Identification of *S. sapinea* was based on examination of mycelia, pycnidia, and conidia.

Statistical analyses. The distances below the inoculation site at which necrotic needles were present were analyzed by three-factor analysis of variance with all interactions. Factors used as main effects were tree species or variety, isolate, and trial. The response by RAPD marker group for the distances below the inoculation site at which necrotic needles were present was compared by a contrast. Chi-square two-way cross tabulation analyses were used to analyze numbers of seedlings with symptoms and numbers of seedlings from which *S. sapinea* was recovered. Analyses of variance (using general linear model procedure) and chi-square two-way cross tabulation analyses were performed with the Minitab for Windows program (release 10.2; Minitab Inc., State College, PA).

Table 1. Origin of *Sphaeropsis sapinea* isolates used to inoculate several conifer species

Isolate[a]	Isolate no.[b]	Pine host[c]	Geographic origin
A1	411	Red	Clearwater Co., MN
A2	128	Red	Grant Co., WI
A4	92-14-A	Austrian	Dane Co., WI
A9	92-66-A	Scot's	Kalamazoo Co., MI
B1	124	Jack	Jackson Co., WI
B2	215	Red	Douglas Co., WI
B3	113	Jack	Gogebic Co., MI
B6	462	Red	Clearwater Co., MN

[a] Random amplified polymorphic DNA (RAPD) marker group and isolate number used in our previous study (3). Representative A and B group isolates used in this study were deposited at the Canadian Collection of Fungal Cultures (CCFC/DAOM), Ottawa, Ontario, Canada. The accession numbers for isolates A2 and B1 are DAOM 222530 and DAOM 222531, respectively.
[b] Culture collection numbers of M. A. Palmer (3-digit number) or G. R. Stanosz (92-xx-A).
[c] Host collected from red (*Pinus resinosa*), Austrian (*P. nigra*), Scot's (*P. sylvestris*), and jack (*P. banksiana*) pines.

Table 2. Incidence of symptoms on wound-inoculated conifers inoculated with random amplified polymorphic DNA (RAPD) marker group A and B isolates of *Sphaeropsis sapinea*

	Percentage (range) with symptoms		
Host species/variety	Group A	Group B	*P*[a]
Scot's pine, East Anglia	100 (all 100)[b]	17 (0-33)	<0.001
Scot's pine, Austrian Hills	100 (all 100)	15 (0-33)	<0.001
Red pine	100 (all 100)	6 (0-14)	<0.001
Colorado blue spruce	90 (75-100)	46 (10-100)	<0.001
Mugho pine, Pumileo	91 (75-100)	16 (0-25)	<0.001
Douglas-fir	90 (75-100)	25 (0-50)	<0.001
Balsam fir	83 (67-92)	0 (all 0)	<0.001
P[c]	0.002	<0.001	

[a] Probability that there is no difference between groups within a row, based on chi-square cross tabulation analyses. Probabilities are based on the number of seedlings with symptoms.
[b] Mean percentage (range) of seedlings with symptoms 4 weeks after wound inoculation with group A or B isolates. Data were pooled from two greenhouse trials, each having four isolates per group and six seedlings per combined treatment (tree species or variety and isolate, N = 48), except for mugho pine for which four seedlings per combined treatment were used (N = 32).
[c] Probability that there is no difference among hosts within a column, based on chi-square cross tabulation analyses. Probabilities are based on number of seedlings with symptoms.

RESULTS

Inoculated seedlings produced symptoms similar to those reported for seedlings in field and nursery studies, including necrotic needles, stem cankers, and crooked and dead shoot tips. Blue spruce, Douglas-fir, and balsam fir lost their needles as shoots died, but the pine species retained their necrotic needles. Initial symptoms included necrotic needles and stem cankers at the wound sites. These were first observed at the following intervals after inoculation: 2 days for blue spruce; 3 days for Scot's pine and Douglas-fir; and 5 days for red pine, mugho pine, and balsam fir. No symptoms developed on wounded or nonwounded controls of any host species or variety.

Incidence of symptoms (the percentage of seedlings that exhibited any symptoms) differed between pathogen groups and among host species and varieties (Table 2). Isolates of group A caused greater mean incidence of symptoms than isolates of group B ($P < 0.001$) on all hosts. There was consistent separation of incidence of symptoms between groups for all isolates on all hosts except blue spruce, in which ranges for isolates overlapped between the groups (Table 2). Significant differences in incidence of symptoms occurred among hosts inoculated with either the A ($P = 0.002$) or B ($P < 0.001$) isolates. For seedlings inoculated with isolates of group A, the highest incidence of symptoms occurred on the Scot's pine varieties and red pine, followed by mugho pine, blue spruce, Douglas-fir, and balsam fir. For seedlings inoculated with isolates of group B, the

highest incidence of symptoms occurred on blue spruce, followed by Douglas-fir, the Scot's pine varieties, and mugho pine. The incidence of symptoms was low for red pine seedlings inoculated with isolates of group B, and no symptoms were detected on balsam fir inoculated with isolates of this group.

Symptom severity (distance below the inoculation site at which necrotic needles were present) also differed between isolates and among host species or variety (Table 3). Three-factor analysis of variance of the distance below the inoculation site at which necrotic needles were present indicated significant effects of the isolate used ($P < 0.001$) and host species or variety ($P < 0.001$), but not of trial ($P = 0.471$). There was also interaction between isolate used and tree species or variety ($P < 0.001$), indicating that the hosts responded differently to the different isolates. The interactions of trial with isolate used ($P = 0.635$) and tree species or variety ($P = 0.953$) were not significant.

The distance below the inoculation site at which necrotic needles were present differed by group on all host species or varieties based on contrast analysis ($P < 0.001$ for all comparisons). Symptom severity was greater on seedlings inoculated with isolates of group A than on seedlings inoculated with isolates of group B on all hosts (Table 3). There was consistent separation in symptom severity between groups for all isolates on all hosts except blue spruce, in which the ranges of isolates overlapped between the groups (Table 3).

Significant differences in symptom severity occurred among hosts inoculated with either the A or B isolates ($P < 0.001$ for both comparisons). For seedlings inoculated with isolates of group A, the greatest disease severities occurred on the Scot's pine varieties, followed by red pine, blue spruce, mugho pine, Douglas-fir, and balsam fir. For seedlings inoculated with isolates of group B, the greatest symptom severity occurred on blue spruce, followed by the Scot's pine variety Austrian Hills. The distance below the inoculation site at which necrotic needles were present was always less than 1 cm for all other hosts inoculated with isolates of group B.

S. sapinea was frequently recovered from each host species and variety inoculated with isolates of either group. When symptomatic and asymptomatic seedlings are considered together, the pathogen was recovered more often ($P < 0.001$) from seedlings inoculated with isolates of group A (93% ± 0.01%) than from seedlings inoculated with isolates of group B (76% ± 0.02%). These figures were not influenced, however, by host species or variety ($P = 0.707$) and did not differ between trials ($P = 0.764$). The pathogen was recovered from asymptomatic blue spruce, mugho pine, Douglas-fir, and balsam fir seedlings that were inoculated with group A isolates and from asymptomatic seedlings of each host species and variety inoculated with group B isolates (Table 4). *S. sapinea* was seldom isolated from the controls (1 of 80 from asymptomatic wounded and 2 of 80 from asymptomatic nonwounded controls).

DISCUSSION

Although group A isolates were relatively more aggressive on the majority of hosts, the ability of group B isolates to cause disease should not be overlooked. Using similar techniques, inoculations with A isolates usually resulted in greater incidence and severity of symptoms than inoculations with B isolates on seedlings of red and jack (*P. banksiana*) pines, American larch (or tamarack, *Larix laricina*), and European larch (*L. decidua*) (3,20). However, some group B isolates were more aggressive than some A isolates on jack pine seedlings (3). The ability of a group B isolate to colonize and kill shoots of American and European larch seedlings also was demonstrated. In our current study, inoculation with group B isolates resulted in measurable symptom development on blue spruce seedlings. Consequently, both groups should be considered when determining the cause of symptoms attributed to *S. sapinea* in regions where each might be present. Our demonstration of host species or variety and isolate interaction indicates that caution should be used in extrapolating conclusions from previous reports of variation in host susceptibility when the RAPD marker group(s) of this pathogen is not known.

By inclusion of hosts other than pines, our results considerably strengthen and broaden experimental evidence for the generalization that conifer hosts vary in susceptibility to *S. sapinea* (8,29). Rees and Webber (16) previously demonstrated differences in susceptibility among seeds, seedling, and saplings of *Pinus caribaea*, *P. oocarpa*, and *P. pseudostrobus* to this pathogen. Swart et al. (24) showed differences in susceptibility to *S. sapinea* of pine species cultivated in South Africa, including *P. elliottii*, *P. kesiya*, *P. patula*, *P. pinaster*, *P. radiata*, and *P. taeda*. We have previously reported differences between responses of red and jack pine to inoculation with *S. sapinea* (3). Likewise, Burdon et al. (4) working with progenies of Monterey pine and Gerhold et al. (7) working with cultivars of Scot's pine demonstrated variation in responses to inoculation with *S. sapinea*. Experimental data demonstrating the relative susceptibility of hosts in other coniferous genera are scarce. Several

Table 3. Symptom severity for wound-inoculated conifers inoculated with random amplified polymorphic DNA (RAPD) marker group A and B isolates of *Sphaeropsis sapinea*

	Distance (range) from site of inoculation (cm)		
Host species/variety	**Group A**	**Group B**	***P*[a]**
Scot's pine, East Anglia	7.3 (6.3-8.4)[b]	0.2 (0.0-0.3)	<0.001
Scot's pine, Austrian Hills	6.0 (5.1-7.0)	0.4 (0.0-1.2)	<0.001
Red pine	5.2 (4.1-7.0)	0.0 (0.0-0.1)	<0.001
Colorado blue spruce	4.1 (2.2-4.9)	1.3 (0.4-3.3)	<0.001
Mugho pine, Pumileo	2.6 (2.0-2.9)	0.2 (0.0-0.3)	<0.001
Douglas-fir	2.0 (1.3-2.6)	0.3 (0.0-0.5)	<0.001
Balsam fir	1.3 (1.0-1.7)	0.0 (all 0.0)	<0.001
P[c]	<0.001	<0.001	

[a] Probability that there is no difference between groups within a row, based on a contrast.
[b] Mean distance (range) below the inoculation site at which necrotic needles were present 4 weeks after wound inoculation with group A or B isolates. Data were pooled from two greenhouse trials, each having four isolates per group and six seedlings per combined treatment (tree species or variety and isolate, N = 48), except for mugho pine for which four seedlings per combined treatment were used (N = 32).
[c] Probability that there is no difference among hosts within a column, based on one-way analyses of variance with tree species as the factor.

Table 4. Recovery of *Sphaeropsis sapinea* from wound-inoculated conifers inoculated with random amplified polymorphic DNA (RAPD) marker group A and B isolates of *S. sapinea* that remained asymptomatic

	Percentage of recovery (n)[a] from asymptomatic seedlings	
Host species/variety	**Group A**	**Group B**
Scot's pine, East Anglia	… (0)	63 (40)
Scot's pine, Austrian Hills	… (0)	66 (41)
Red pine	… (0)	69 (45)
Colorado blue spruce	80 (5)	58 (26)
Mugho pine, Pumileo	67 (3)	78 (27)
Douglas-fir	80 (5)	69 (36)
Balsam fir	63 (8)	71 (48)

[a] Values are total numbers of asymptomatic seedlings 4 weeks after wound inoculation with group A or B isolates. Data were pooled from two greenhouse trials, each having four isolates per group and six seedlings per combined treatment (tree species or variety and isolate, N = 48), except for mugho pine for which four seedlings per combined treatment were used (N = 32).

of the species we examined were included in two previous studies (15,30). Although it was found that Scot's, red, and mugho pines, Colorado blue spruce, and Douglas-fir are hosts of *S. sapinea*, results in those studies were not statistically analyzed. Substantial differences in symptom incidence and severity were quantified in our study, but further comparative tests with a diversity of sources for each host species and/or variety might improve estimates of the relative host resistance to each *S. sapinea* group.

Greenhouse studies using standardized methods for artificial inoculation of small trees with *S. sapinea* are a convenient method for comparing differences in aggressiveness among isolates and the relative susceptibility among host species or varieties. Differences in aggressiveness between marker groups on red pine demonstrated under greenhouse conditions were reflected by results of inoculation of established plantation trees (1,2). Similarly, Swart et al. (24) found consistency between results of artificial inoculations in the growth chamber and the field with observations by foresters regarding the relative susceptibility of six pine species cultivated in South Africa to *S. sapinea*. Therefore, it seems likely that standardized tests with characterized inoculum will yield information useful for selecting and deploying resistant host material. Wounding and site factors that induce physiological stress, however, are known to influence susceptibility to *S. sapinea* (1,2, 9,11,23). These should be considered in addition to relative host resistance when determining the risk of damage to a particular host.

S. sapinea has been shown to persist asymptomatically on or in shoots of naturally infected red and jack pines (21). Isolates from asymptomatic trees were proven by inoculation to be virulent. Previously, however, all but two of the 46 isolates tested were determined to be group A. The ability of virulent group B isolates to persist asymptomatically for at least 4 weeks following wound-inoculation was confirmed in the current study. This ability is a potential feature of the relationship of both groups with their hosts. Further, asymptomatic persistence on or in *Abies*, *Picea*, and *Pseudotsuga* is reported for the first time. The role of asymptomatic persistence in pathogen survival and subsequent disease development by each of the groups is the subject of current studies.

Planting species or varieties resistant to *S. sapinea* would be desirable where losses caused by *S. sapinea* have been reported in forests, or in ornamental plantings and Christmas tree plantations where there is little tolerance for damage. This study provides experimental evidence of variation in host response to *S. sapinea* and a convenient method for further comparison of susceptibility using other host material. Because differences in aggressiveness between groups occurred on the host species and varieties used in this study, RAPD marker group characterization of *S. sapinea* isolates encountered in nurseries, plantations, and ornamental landscapes also may help estimate risk of damage from this disease.

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