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EVALUATION OF THE PRESENCE OF DOUBLE-STRANDED RNA IN SPHAEROPSIS SAPINEA AND DESCRIPTION OF A NEW VARIETY OF THE SPECIES

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Nyan-Tsz Wu

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Gerard C. Adams
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EVALUATION OF THE PRESENCE OF DOUBLE-STRANDED RNA IN *SPHAEROPSIS SAPINEA* AND DESCRIPTION OF A NEW VARIETY OF THE SPECIES

By

Nyan-Tsz Wu

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ABSTRACT

EVALUATION OF THE PRESENCE OF DOUBLE-STRANDED RNA IN SPHAEROPSIS SAPINEA AND DESCRIPTION OF A NEW VARIETY OF THE SPECIES

By

Nyan-Tsz Wu

Forty isolates of Sphaeropsis sapinea were examined and grouped into two types, type A and type B. The number of septations (0-3) and reduced size of conidia of type B isolates was correlated with the appressed cultural morphology and slower relative growth rate compared to type A isolates. A new variety of S. sapinea, S. sapinea var. breviphragmospora var. nov., is described based on septation and length of conidia of the type B isolates. Thirteen out of 20 type A and nine out of 20 type B isolates contained detectable double-stranded RNA (dsRNA). The dsRNA freed subcultures retained the parents' group-specific characteristics. Pathogenicity tests confirmed that wounding was necessary for infection by all type B isolates and subcultures but not type A isolates. As a group, type A isolates were more virulent than type B isolates, particularly on P. nigra trees. DsRNA was not associated with low virulence.

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INTRODUCTION

Sphaeropsis sapinea (Fr.:Fr.) Dyko & Sutton [=Diplodia pinea (Desm.) Kickx] is the causal organism of the disease known by the common name of Diplodia tip blight of pine. Distribution of the fungus is world wide, including Africa, Asia, Australia, New Zealand, Europe, North and South America and the West Indies (36). The fungus is pathogenic on many Pinus species and on some species of Larix, Abies, Chamaecyparis, Cupressus, Picea, Pseudotsuga and Thuja (36, 41). It causes shoot and twig blight (8, 12, 23, 52), branch canker (12, 21, 26, 52), tree and seedling collar rot (11, 31, 49), seed storage rot (39), pre- and post-emergence damping off (15, 39) and it infects seed cones. In addition, the fungus may cause blue-stain of sap-wood of fallen or freshly cut timbers (14, 27), and may saprophytically colonize plant debris on the forest floor (36).

In the North Central States, the pathogen yearly damages ornamental and Christmas trees and woodland pines through the repeated destruction of buds and shoots. It has become a frequent problem in the nurseries and forest causing death and dieback of seedlings (31).

Two forms of S. sapinea type A and type B have been described by Palmer based on morphological and biological differences among 8 isolates (30). Isolates of type A were reported to grow relatively faster than type B, forming aerial flocculent white to grey-green colonies in culture whereas type B colonies are white to black and grow closely appressed to the agar

surface. Type A conidia were longer and wider than type B conidia and had 0-1 septum. Type B conidia had 0-3 septa. The type B isolates also formed spermatia-like spores within the pycnidium (32).

In a study of wall ornamentation of conidia, 19 isolates with characteristics of type A and 7 of type B differed in conidial wall ornamentation (46). The conidia of type A isolates had smooth surfaces and the type B had pitted surface when viewed by scanning electron microscopy (SEM). The pitted surfaces were found to be electron opaque areas on a smooth spore surface when viewed by transmission electron microscopy (47). The areas corresponding to the pits were found to lack electron dense substances in the outer cell wall and apparently these areas collapsed when observed with SEM.

Based on a study of two isolates, the type A isolate of S. sapinea did not require wounding to infect pine seedlings in contrast to the type B strain (32). Researchers have long debated whether S. sapinea can infect uninjured tissues (3, 5, 37, 48). Severe infections of S. sapinea in nature often follow wounding by insects (12, 21, 23, 51) or hail (34, 37, 42, 50, 52) and generally occur on stressed trees (10, 25, 49) and usually on exotic species of pine (20). In 1976, Chou (9) compiled much of the methods and results of previous inoculations studies by others. He suggested that variability in virulence or cultural characteristics among isolates of D. pinea (= S. sapinea) may account for some of the discrepancies in inoculation results. However, in his studies no differences in virulence were evident and wounding was not required for infection. Other researchers have reported differences in cultural morphology (2, 32, 39, 42), spore surface ornamentation (45, 46) and

differences in growth rate and dimensions of the conidia (32, 42) that were correlated with differences in virulence.

Differences in virulence among isolates of fungi also have been correlated with the presence of double-stranded RNA (dsRNA) (7, 13, 17, 22, 24, 29, 38). Although the presence of dsRNA is not always associated with hypovirulence (16, 53), it may effect virulence as well as cause degenerative disease and decline of fungal cultures (1, 6, 7, 19, 24, 44). Slower growth rate, appressed culture morphology, and reduced virulence are common characteristics of declining fungal cultures. Type B isolates of S. sapinea share these characteristics, if compared to type A isolates (32). Slight differences in dimensions of the conidia, in conidial-wall ornamentation and in production of spermatia (32, 46) may overlap among individual isolates of type A and B. Preliminary studies provided an indication that dsRNA might have a role in type B phenotype (Palmer and Mcdonald, personal communication).

The primary objective of this research was to investigate the role of dsRNA in S. sapinea in regard to growth rate, appressed colony morphology, spore dimensions, the requirement of wounding for infection, and the relative virulence following infection; and to determine if dsRNA infection is correlated with the type B phenotype. Secondary objectives are to reconfirm whether type B isolates required wounding for infection in contrast to type A and reconfirm whether the two types differ in spore dimensions and septation and production of spermatia using a large collection of isolates from the North Central States.

MATERIALS AND METHODS

Isolates and cultural characteristics. The cultures used in this research were mostly isolated from pines in the North Central United States. The geographic and host origin for each isolate are listed in Table 1. The cultures were maintained on potato-dextrose agar (PDA, Difco Laboratories, Detroit, MI) at room temperature.

The growth rate of each isolate was determined at 17C, 22C, 27C, and 37C on two media, PDA and 2% malt extract agar (MA, Difco Laboratories, Detroit, MI) by measuring the diameter of the colony every 6-12 hr until the colony reached the periphery of the 9 cm petri plate. Three replicate plates were measured for each isolate. The growth curve of each isolate was then drawn and the slope of the linear phase of growth was calculated (mm/day).

Conidia were produced by inoculating 2% water agar (WA, Bacto) plates with an isolate and placing autoclaved needles of Pinus nigra Arnold on the surface of the cultures. The cultures were incubated at room temperature in diffuse light for 1 to 3 months to induce pycnidia. Needles with pycnidia were cut into pieces and suspended in sterile distilled water to induce exudation of conidia. Conidia were collected by filtering the suspension through a 60 m nylon mesh. The number of septation and the dimensions of 50 conidia/isolate were determined by light microscopy.

TABLE 1. Geographic and host origin of the isolates of *Sphaeropsis sapinea*.

Isolate No.	Geographic origin	Host	Isolation of the plant part
120	Michigan	*P. resinosa*	shoot blight
123	Wisconsin	*Pinus resinosa*	shoot blight
147	Wisconsin	*Larix decidua*	
155	Texas	unidentified vine	IMI 264965[1]
183	Montana	*P. ponderosa*	
189	Hawaii	*P. radiata*	
411	Minnesota	*P. resinosa*	stem canker from a healing tree
420	Minnesota	*P. resinosa*	stem canker from a healing tree
461	Minnesota	*P. resinosa*	blue-stained wood in a dead tree
463	Minnesota	*P. resinosa*	stem canker from a healing tree
464	Minnesota	*P. resinosa*	stem canker of a healing tree
467	Minnesota	*P. resinosa*	pycnidium on cone
470	Minnesota	*P. resinosa*	stem canker from a healed tree
472	Minnesota	*P. resinosa*	stem canker from a healed tree
473	Minnesota	*P. resinosa*	stem canker from a dead tree
820	Michigan	*P. nigra*	shoot blight
810	Michigan	*P. mugo*	shoot blight
811	Michigan	*P. mugo*	shoot blight
812	Michigan	*P. mugo*	shoot blight
813	Michigan	*P. mugo*	shoot blight
113	Michigan	*P. banksiana*	shoot blight
124	Wisconsin	*P. banksiana*	shoot blight
131	Michigan	*P. banksiana*	
201	Illinois	*P. strobus*	
215	Wisconsin	*P. resinosa*	
410	Minnesota	*P. resinosa*	pycnidium on a dead branch
412	Minnesota	*P. resinosa*	hail wound
414	Minnesota	*P. resinosa*	pycnidium from a canker on a healed tree
415	Wisconsin	*P. resinosa*	stem canker from a healed tree
419	Wisconsin	*P. resinosa*	stem canker from a healing tree
439	Wisconsin	*P. banksiana*	pycnidia on needle
457	Wisconsin	*P. resinosa*	stem canker from a healing tree
458	Wisconsin	*P. resinosa*	stem canker from a healing tree
462	Minnesota	*P. resinosa*	blue stained wood in a dead tree
465	Wisconsin	*P. resinosa*	stem canker from a healing tree
466	Wisconsin	*P. banksiana*	pycnidium on cone
468	Wisconsin	*P. resinosa*	stem canker from a healing tree
469	Wisconsin	*P. resinosa*	stem canker from a dead tree
471	Minnesota	*P. resinosa*	stem canker from a dead tree
474	Minnesota	*P. resinosa*	stem canker from a healed tree

[1]From C.M.I. (Commonwealth Mycological Institute) culture collection, UK.

Morphological studies were based on examination of conidiomata on inoculated autoclaved pine needles.

In attempts to obtain dsRNA-freed subcultures of the dsRNA-containing isolates, conidia from one pycnidium from each of the 12 dsRNA-containing isolates were separately suspended in sterilized water and spread on a water agar plate. Twenty germinating single conidia were isolated with a fine-pointed scalpel for each strain and transferred onto PDA for incubation. Seven to 19 single spore subcultures from each isolate were screened for the existence of dsRNA by the method described below.

Isolation of double-stranded RNA. The dsRNA was isolated from fungal mycelium essentially as described by Morris & Dodds (28) and Paul & Fulbright (33). Mycelia were grown for 2 wk at room temperature in stationary liquid culture in Endothia complete medium (35) modified by the omission of glucose. The culture broth was adjusted to pH 7.4 before harvesting. Mycelium was then harvested on cheese cloth by vacuum filtration and pressed between layers of paper towel to blot dry, and store until use at -20C. Mycelium (1.5-3 g) was ground into powder in liquid nitrogen with glass beads (490 m) and incubated at 0C for 45 min in 10 ml 2X STE buffer (0.05 M Tris, o.1 M NaCl, and 0.001 M EDTA, pH 7.0), 15 ml phenol (saturated with STE buffer), 1.5 ml 10% sodium dodecyl sulfate, and 0.1% bentonite. After incubation, the solution was centrifuged at 7719 g for 15 min and the aqueous phase was pipetted into a small beaker and adjusted to 15% ethanol/STE buffer by adding ethanol. The solution containing dsRNA was then passed through a CF-11 cellulose (Whatman) chromatography column (2.5 g, pre-saturated by 15% ethanol/STE buffer). The column was then washed by 80 ml 15% ethanol/STE buffer to

remove the other nucleic acids except dsRNA, and the dsRNA was eluted by adding 1, 1, 6, 6, and 6 ml STE buffer to the column step by step. The extract were further purified by repeating the column process twice. The dsRNA was then precipitated by adding ethanol to the extract to make a final concentration of 15% ethanol/STE buffer, and stored in a freezer for a minimum of 4 hr. The precipitated dsRNA was collected as a pellet by centrifugation at 7719 g for 30 min. The pellet was air-dried and the dsRNA was washed into a microcentrifuge tube with 0.5 ml STE buffer. One ml of 95% ethanol was added to further precipitate the dsRNA. The precipitate and solution was incubated at -20C for a minimum of 4 hours and then centrifuged at 8832 g for 4 min. The pellet of dsRNA was air-dried, then resuspended in 100 1 electrophoresis sample buffer (0.04 M Tris, 0.02 M sodium acetate, 0.001 M EDTA, pH 7.8, plus 20% glycerol). Mycelium of a Leucostoma persoonii (isolate 14.4A) and a Cryphonectria parasitica (isolate GH2) containing dsRNA (18,24) were grown and extracted in the same manner as S. sapinea and used as controls for dsRNA isolation and a standard marker for molecular weights respectively. The dsRNA extracts were loaded on a 5% polyacrylamide gel, electrophoresed at 40 mA for 12 hr, stained in 0.5 ppm ethidium bromide for 30 minutes and destained in water for 15 min as described by Paul & Fulbright (33). Ethidium bromide segments on gels were identified as dsRNA based on their resistance to RNase A at high ionic strength (0.3 M NaCl) sensitivity to RNaseA in H_2O (18). Molecular weights of the segments were estimated by the method described by Bozarth and Harley (4).

Virulence tests. Five landscape trees of Pinus nigra (approx. 10-year-old) and five of Pinus sylvestris L. (approx. 7-year-old) were used in the first field test. Each tree was inoculated with the same 30 isolates of S. sapinea and six dsRNA-free subcultures. Inoculation was done in early June when the shoot buds were opening but before shoot elongation. The buds were wounded by removing a bud scale from the young shoot bud and inoculated by placing a PDA plug with mycelium of an isolate on a wounded shoot bud, then wrapping the bud with parafilm. Controls were inoculated similarly with a PDA plug without mycelium. The inoculated buds were checked daily, the dates of bud death were recorded and the dying shoots were harvested for reisolation. The bud was considered to be dead when the bud was girdled, discolored brown and/or wilting. Virulence was rated as the ratio of successful inoculation times 1000 divided by the number of days from inoculation till the death of the buds.

$$\text{Virulence Index} = \frac{\text{ratio of successful infection}}{\text{Days from inoculation till bud death}} \times 1000$$

The greater the number, the more virulent the pathogen.

In a second field test, 2-year-old nursery seedlings of Pinus resinosa Ait. (Lake States seed source), P. nigra (Caucasus mountains seed source), P. sylvestris (Guadaramma seed source), and 3-year-old seedlings of P. banksiana Lamb. (Michigan seed source) were transplanted into soil beds in early June. When buds began to elongate, they were inoculated with a 5 mm diam. plug of mycelium from a 3-day-old culture of S. sapinea on PDA. Each pine species was inoculated with 40 isolates and six dsRNA-free subcultures. Four buds per seedling were treated; two buds were wounded and two were not. One wounded and one unwounded bud were inoculated and

the other two buds served as separate controls. Each treated bud (including controls) was wrapped in cloth tape in one of four colors to indicated the type of treatment. Buds were wounded by stabbing with a flame-sterilized needle. Agar plugs were held in place with the strand of cloth tape and each seedling was then bagged in a clear plastic bag and shaded for three days by a 80% shade cloth screen. Ten seedlings of each of the four pine species, 4 buds/seedling, were inoculated for each of the 46 strains. All the 7360 inoculations were done in three days to reduce the experimental error due to environmental fluctuation and bud elongation. The buds were checked daily to record the number of days until bud death and the number of successful infections. Virulence was rated as described above.

The virulence of dsRNA(+) isolates of S. sapinea was compared to dsRNA(-) isolates in two ways. All dsRNA(+) isolates were grouped and compared statistically to the group of dsRNA(-) isolates on the host plants. Separately, the virulence of dsRNA-free subcultures were compared to that of their dsRNA containing parental strains on the host plants.

RESULTS

Cultural characteristics. The colony type, spermatia formation, spore surface pitting, spore septation, isozyme patterns, and culture type classification for the forty isolates were listed in Table 2. Data on the pitting ornamentation on spore surfaces and on isozyme patterns were provided by Drs. Blanchette and Palmer (unpublished). For several of the isolates the information has been determined and published previously (46, 32). All of the isolates classified as type A formed a flocculent colony on PDA (Fig. 1A) and most of them produced conidia with smooth surfaces and 0 or 0-1 septum in pycnidia except isolate 470 with 0-2 septa and isolates 420 and 473 with pitted surfaces. All of the cultures classified as type B formed appressed colonies on PDA (Fig. 1B) and produced pitted spores with 0-3 septa in pycnidia except isolate 410 with 0-4 septa, and isolate 201 with a smooth spore surface. Septation of isolates 147, 467, and 201 were not determined because of the difficulties of inducing pycnidia in culture. Most of the type A spores had an average size of 37 x 13 m whereas type B spores usually had an average size of 31 x 12 m. Spermatia were found mostly within the pycnidia of type B isolates with rather than in type A isolates. However, exceptions were present in this character. Generally spermatia averaged 1.4 x 4 m and were hyaline and aseptate.

TABLE 2. Listed for each isolate of Sphaeropsis sapinea are the characteristics of colony morphology, spermatia formation, conidia septation, conidia surface ornamentation, and isozyme pattern, on which the type classifications are designated.

Isolate No.	Colony type on PDA	Spermatia formation	Conidia characteristics: Conidia septation	Conidia characteristics: Conidia wall ornamentation	Isozyme pattern	Type classification
120	Aerial(A)[1]	no(no)[2]	0(0-1)[2]	(Smooth)[1]	(A)[2]	A(A)[2]
123	Aerial(A)[1]	no(no)2	0(0-1)[2]	(Smooth)[1]	(A)[2]	A(A)[2]
147	Aerial(A)[1]	ND[4]	ND	(Smooth)[1]	ND	A
155	Aerial(A)[1]	ND	0	(Smooth)[1]	ND	A
183	Aerial(A)[1]	yes	0	(Smooth)[1]	ND	A
189	Aerial(A)[1]	no	0	(Smooth)[1]	ND	A
411	Aerial	no	0	Smooth[3]	A[3]	A
420	Aerial	no	0	Pitted[3]	A[3]	A
461	Aerial	no	0	Smooth[3]	A[3]	A
463	Aerial	no	0-1	Smooth[3]	A[3]	A
464	Aerial	no	0	Smooth[3]	A[3]	A
467	Aerial	ND	ND	Smooth[3]	B[3]	A
470	Aerial	yes	0-2	Smooth[3]	A[3]	A
472	Aerial	no	0	Smooth[3]	A[3]	A
473	Aerial	no	0	Pitted[3]	A[3]	A
820	Aerial	no	0	ND	ND	A
810	Aerial	no	0	ND	ND	A
811	Aerial	no	0	ND	ND	A
812	Aerial	no	0	ND	ND	A
813	Aerial	no	0	ND	ND	A
113	Appressed(B)[1]	ND(yes)[2]	0-3(0-3)[2]	(Pitted)[1]	(B)[2]	B(B)[2]
124	Appressed(B)[1]	ND(yes)[2]	0-3(0-3)[2]	(Pitted)[1]	(B)[2]	B(B)[2]
131	Appressed(B)[1]	ND	0-3	(Pitted)[1]	ND	B
201	Appressed(I)[1]	ND	ND	(Smooth)[1]	ND	I
215	Appressed(B)[1]	yes	0-3	(Pitted)[1]	ND	B
410	Appressed	yes	0-4	ND	ND	B
412	Appressed	no	0-3	ND	ND	B
414	Appressed	no	0-3	Pitted[3]	B[3]	B
415	Appressed	yes	0-3	ND	ND	B
419	Appressed	yes	0-3	Pitted[3]	B[3]	B
439	Appressed	ND	0-3	ND	ND	B
457	Appressed	yes	0-3	Pitted[3]	B[3]	B
458	Appressed	yes	0-3	Pitted[3]	B[3]	B
462	Appressed	no	0-3	Pitted[3]	B[3]	B
465	Appressed	yes	0-3	Pitted[3]	B[3]	B
466	Appressed	yes	0-3	Pitted[3]	B[3]	B
468	Appressed	yes	0-3	Pitted[3]	B[3]	B
469	Appressed	yes	0-3	Pitted[3]	B[3]	B
471	Appressed	yes	0-3	Pitted[3]	B[3]	B
474	Appressed	yes	0-3	Pitted[3]	B[3]	B

[1]Data in the brackets are borrowed from the paper of Wang et. al., 1985. A: type A colony growth pattern; B: type B colony growth pattern; I: intermediate type colony pattern with some characteristics of both types.

[2]Data in the brackets are borrowed from the paper of Palmer et. al., 1987.

[3]Data supplied by Drs. Marguerita A. Palmer and Robert Blanchette, not published.

[4]ND = Not Determined.

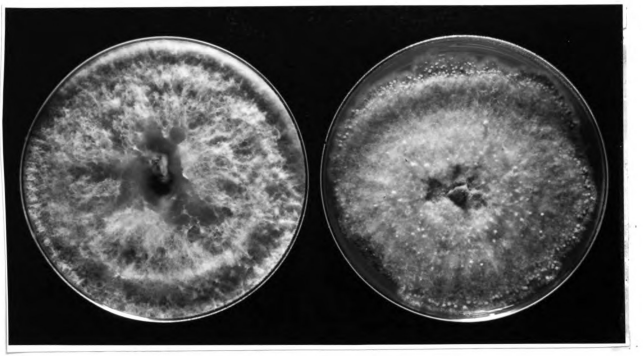


Figure 1. Mycelial growth characteristics of the type A compared to the type B Sphaeropsis sapinea. (A) Type A isolate 812 growing on PDA at room temperature for 14 days, producing the aerial, fluffy colony type. (B) Type B isolate 415 growing on PDA at room temperature for 14 days, producing the appressed colony type and stromata in the medium.

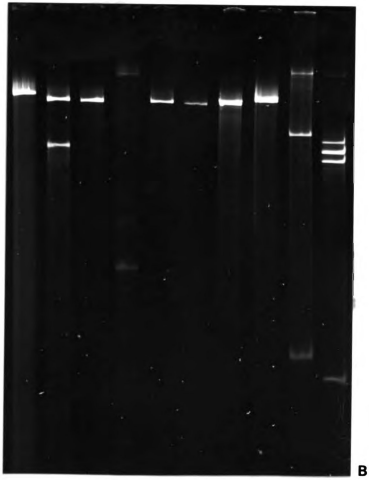
The optimum growth rate of S. sapinea occurred on PDA at 27C for all of the cultures. There was little difference in growth rate of the cultures on PDA or on malt extract agar. However, type A isolates grew faster than type B on PDA at 17C, 22C and 27C and on 2% malt agar at 22C. No isolate grew at 37C, although the temperature was not lethal. Our results (Table 2) confirmed most of the earlier works of Wang (45) and Palmer, et. al (32).

Presence of dsRNA and its effects on morphology and growth rate. Ethidium bromide staining bands on acrylamide gels were identified as segments of dsRNA based on their resistance to RNase A at high ionic strength (0.3 M NaCl), and sensitivity to RNase A at low ionic strength (in water). Thirteen out of 20 isolates of type A and nine out of 20 isolates of type B were found to contain dsRNA. The dsRNA varied in the number and molecular weight of the segments among isolates (Fig. 2). The molecular weight of the dsRNA in both type A and type B isolates that could be estimated by comparison to standards ranged from 0.6×10^5 to 6×10^6. Three isolates each contained several segments of dsRNA that had molecular weights smaller than 0.5×10^6 (Table 3).

Five to 29% of conidia from six dsRNA-containing isolates were found to be dsRNA-free when subcultured; however six other isolates were not successfully freed of dsRNA by this method (Table 4).

Figure 2. Patterns of double-stranded RNA segments found in Sphaeropsis sapinea. (A) Type A isolates from left to right: 813, 812. 811, 810, 820, 473, 472, 463, 461, 189, 183, and 123. The end well contains molecular weight standards from Cryphonectria parasitica GH2. (B) Type B isolates from left to right: 131, 469, 468, 462, 415, 215 and 113. Well 9 contains molecular weight standards from Cryphonectria parasitica GH2 and well 10 contains dsRNA from Leucostoma persoonii 14.4A.

A



B

Figure2

TABLE 3. Estimated molecular weights of double-stranded RNA segments found in type A and type B isolates of Sphaeropsis sapinea.

Type A	Molecular weight(10^6)	Type B	Molecular weight(10^6)
123	2	113	3
147	3	131	3
183	4	215	3
189	4	201	5
461	4	412	3
	0.9	415	3
463	4	462	6
472	4		2
	4		1
	3		1
	2		ND[1]
473	6	468	3
	0.7	469	3
820	5		1
	0.7		
810	5		
811	5		
	0.6		
812	0.7		
	0.7		
	0.7		
	ND[1]		
	ND[1]		
813	5		
	0.7		
	0.7		
	0.7		
	ND[1]		
	ND[1]		
	ND[1]		

[1]The molecular weight is roughly estimated or not determined (ND) because the mobilities of the standards used did not bracket the dsRNA segments being compared.

TABLE 4. Ratio of Sphaeropsis sapinea single spore subcultures free of double-stranded RNA (dsRNA) that were obtained from parents having dsRNA infection.

Isolate No.	Ratio[1]
123	2/7
189	1/9
463	0/15
472	0/14
473	1/8
820	1/10
810	0/10
113	1/19
215	0/19
415	1/17
468	0/19
469	1/19

[1]Ratio = dsRNA-free subculture/single spore subcultures tested.

The dsRNA-free isolates were found to maintain the culture characters of their dsRNA(+) parent strains in colony morphology and spore septation. The relationship of the size of the conidia in dsRNA(+) and dsRNA(-) isolates of type A and type B is represented in Fig. 3. The presence or absence of dsRNA did not influence the size of the conidia.

The presence of dsRNA on the average did not affect the growth rate of either type A or B cultures (Fig. 4). Single-spored dsRNA-free subcultures of type A grew as fast as, or faster, than their infected parental cultures. In type B, dsRNA-free isolate 415 grew slower than the dsRNA(+) parent culture but dsRNA-free isolate 469 grew faster than the dsRNA(+) parent culture (Fig. 5).

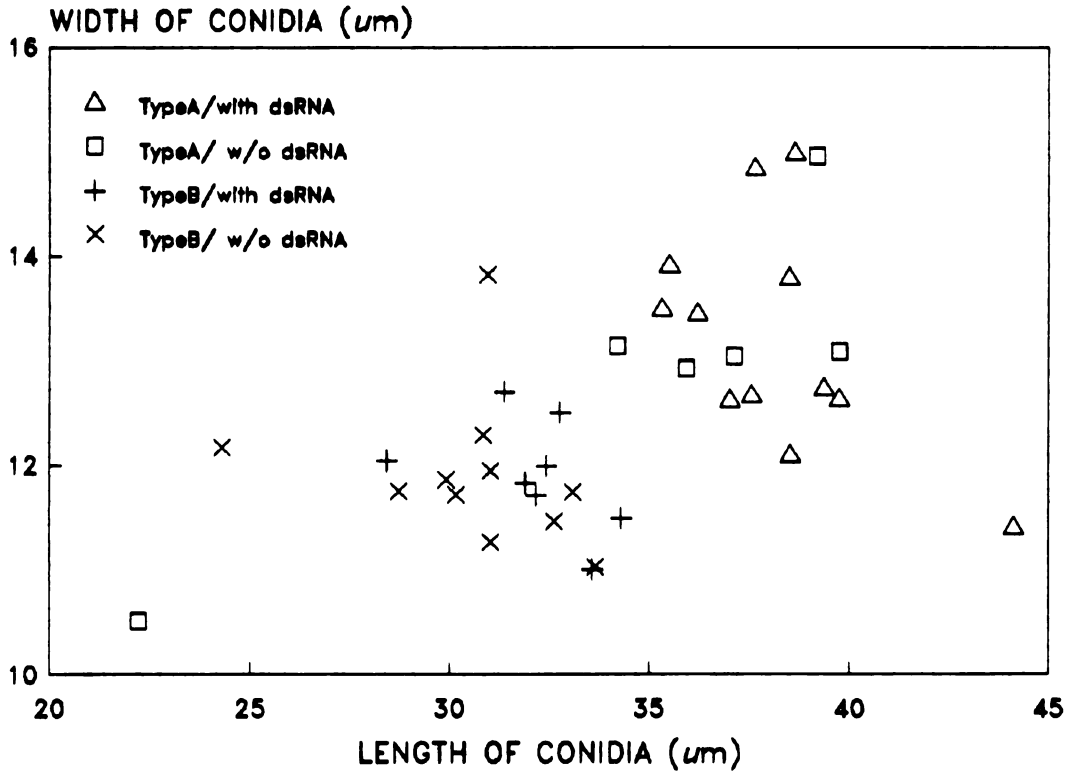


Figure 3. Distribution of the sizes of conidia in type A and type B isolates of Sphaeropsis sapinea including isolates with or without double-stranded RNA (dsRNA).

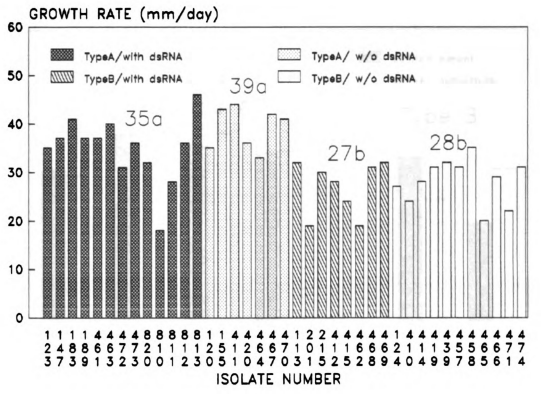


Figure 4. Growth rates (mm/day) in log phase of type A and type B isolates of Sphaeropsis sapinea with or without double-stranded RNA infection. Growth rates were measured at the optimum temperature (27C) on potato-dextrose agar. The number above each group of bars is the mean growth rate for the group. Means followed by different letters are significantly different (P=0.05), as determined by Duncan's multiple range test. (SD value=6.38).

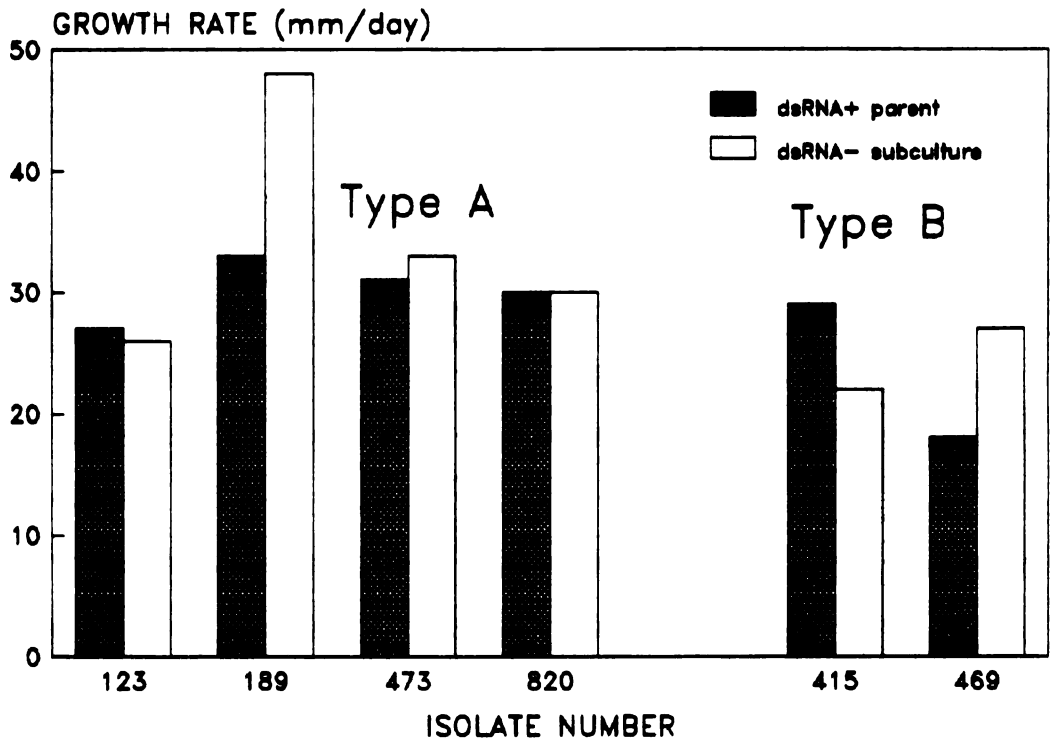


Figure 5. Growth rate of parent strains of Sphaeropsis sapinea containing double-stranded RNA (dsRNA) compared to single-conidial subcultures freed of dsRNA at room temperature on potato dextrose agar.

Virulence on mature trees. The landscape trees of P. sylvestris appeared more susceptible than those of P. nigra (Table 5). The wounded P. nigra buds were apparently resistant to type B S. sapinea.

Isolates of S. sapinea could be separated into ten to thirteen pathogenic groups on the mature trees by Duncan's multiple range test (Table 6). Most of the type A isolates were more virulent than the type B isolates on P. nigra or P. sylvestris regardless of whether the pathogen did or did not contain a dsRNA infection. On P. nigra, both type B dsRNA(+) and dsRNA(-) were avirulent, and dsRNA(-) type A isolates were not more virulent than dsRNA(+) isolates. On P. sylvestris, dsRNA(-) isolates in both groups also were not more virulent than dsRNA(+) isolates (Table 7).

The dsRNA-free subcultures were either similar to the parent strains in virulence or were more virulent than the parents on P. nigra and P. sylvestris (Fig. 6).

TABLE 5. Comparisons of mean virulence of type A versus type B Sphaeropsis sapinea following inoculation of wounded buds of 10-year-old Pinus nigra and 7-year-old P. sylvestris in the field.

Host	Type of the culture	Virulence[1] Index
Pinus sylvestris	A	109 a[2]
	B	56 b
	average	86
Pinus nigra	A	46 b
	B	5 c
	average	28

[1]Virulence Index= (ratio of successful infection/ days from inoculation till bud death) x 1000.
[2]Values followed by different letters are significantly different (P=0.05), as determined by Duncan's multiple range test.

TABLE 6. Relative rating of virulence among type A and type B isolates of Sphaeropsis sapinea with or without double-stranded RNA inoculated on wounded buds of 10-year-old P. nigra or 7-year-old P. sylvestris in the field.

Pinus nigra				Pinus sylvestris			
Isolate No.	Culture type	dsRNA +/-	Virulence Index[1]	Isolate No.	Culture type	dsRNA +/-	Virulence Index
411	A	-	108 a[2]	470	A	-	158 a
461	A	+	102 a	461	A	+	157 a
813	A	+	99 ab	813	A	+	152 ab
810	A	+	76 abc	810	A	+	151 abc
470	A	-	66 bcd	812	A	+	149 abc
464	A	-	55 cde	411	A	-	140 abcd
812	A	+	54 cde	464	A	+	128 abcde
463	A	+	45 cdef	463	A	+	122 abcdef
820	A	+	41 defg	471	B	-	106 bcdefg
189	A	+	37 defgh	472	B	+	104 bcdefgh
120	A	-	34 defghi	469	B	+	103 bcdefgh
183	A	+	27 efghij	123	A	+	102 cdefgh
472	A	+	20 fghij	468	B	+	101 defgh
469	B	+	14 fghij	466	B	-	99 efghi
468	B	+	13 fghij	120	A	-	98 efghi
123	A	+	12 ghij	183	A	+	98 efghi
473	A	+	12 ghij	414	B	-	96 efghi
201	B	+	9 ghij	820	A	+	95 efghi
462	B	+	6 hij	473	A	+	75 fghij
415	B	+	5 hij	201	B	+	64 ghij
124	B	-	5 hij	811	A	+	61 ghijk
457	B	-	4 ij	189	A	+	57 hijk
471	B	-	3 ij	457	B	-	51 ijkl
113	B	+	3 ij	462	B	+	45 jklm
147	A	+	0 j	124	B	-	38 jklm
131	B	+	0 j	113	B	+	16 klm
414	B	-	0 j	147	A	+	7 lm
466	B	-	0 j	215	B	+	4 lm
215	B	+	0 j	415	B	+	0 m
811	A	+	0 j	131	B	+	0 m

[1]Virulence Index= (ratio of successful infection/days from inoculation till bud death)x1000.
[2]Values followed by different letters are significantly different (P=0.05), as determined by Duncan's multiple range test.

Table 7. Comparisons of mean virulence of type A versus type B Sphaeropsis sapinea, with or without double-stranded RNA, following inoculation of wounded buds of 10-year-old Pinus nigra and 7-year-old P. sylvestris in the field.

Type of culture	dsRNA +/-	Virulence Index[1] Pinus nigra	Pinus sylvestris
A	-	66 a[2]	131 a
A	+	40 ab	102 ab
B	-	2 c	78 bc
B	+	6 c	42 cd

[1]Virulence Index= (ratio of successful infection/ days from inoculation till bud death) x 1000.
[2]Values followed by different letters are significantly different (P=0.05), as determined by Duncan's multiple range test.

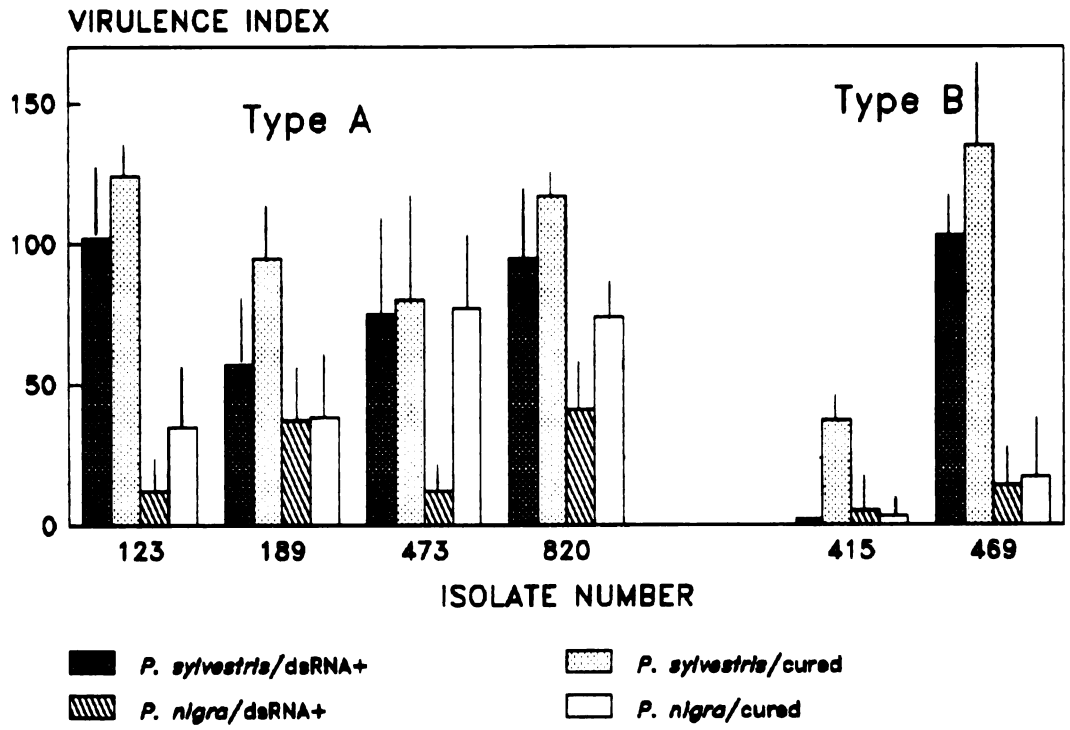


Figure 6. Comparisons of the relative virulence of isolates of type A and type B Sphaeropsis sapinea containing double-stranded RNA versus their dsRNA-free subcultures when inoculated onto buds of 10-year-old Pinus nigra and 7-year-old P. sylvestris. The lines above the bars represent the standard deviation in virulence for each isolate.

Virulence on seedlings. Wounding was found to be required for all type B isolates to infect pine seedlings but not for type A cultures, except isolates 812 which was a low virulent isolate on seedlings. However, wounding did enhance infectivity and virulence of type A cultures (Table 8). DsRNA-free subcultures from type B strains, isolate 415 and 469, also required wounding to infect the seedlings, but not in type A strains.

The majority of type A isolates were more virulent than the majority of type B on wounded buds of seedlings of the three pine species (Table 9). When evaluated as a group, the type A dsRNA(+) isolates were not significantly less virulent than the group of type A dsRNA(-) isolates. Similarly, the type B dsRNA(+) isolates were not less virulent than the type B dsRNA(-) isolates (Table 10). Also, the dsRNA-free subcultures were not significantly more virulent on the seedlings than their parents (Figure 7).

P. sylvestris and P. nigra were not significantly different in susceptibility in these tests whereas P. banksiana was less susceptible than P. sylvestris (Table 11). Few buds of P. resinosa became infected following inoculation with the 46 isolates of S. sapinea despite wounding. The relative susceptibility of the seedlings to infection by S. sapinea was in order of the most to the least susceptible species: P. sylvestris, P. nigra, P. banksiana and P. resinosa.

TABLE 8. The effect of wounding on the mean virulence of type A and type B Sphaeropsis sapinea on buds of 2- to 3-year-old seedlings of Pinus sylvestris, P. nigra, and P. banksiana.

Type of culture	Wounding[1] or not	Ratio of successful infection	Virulence[2] Index
A	+	0.52	45 a[3]
A	-	0.33	30 ab
B	+	0.33	37 ab
B	-	0.03	3 c

[1]The bud were wounded with a sterilized needle before inoculation.
[2]Virulence Index = (ratio of successful infection/days from inoculation till bud death) x 1000.
[3]Values followed by different letters are significantly different (P=0.05), as determined by Duncan's multiple range test.

TABLE 9. Relative rating of virulence among type A and type B isolates of *Sphaeropsis sapinea* with or without double-stranded RNA inoculated on wounded buds of 2 to 3-year-old seedlings. (Combined results on *Pinus sylvestris*, *P. nigra*, and *P. banksiana*.)

Isolate No.	dsRNA +/-	Type of culture	Virulence[1] Index
464	-	A	93 a[2]
463	+	A	90 ab
472	+	A	70 bc
411	-	A	70 bc
470	-	A	60 cd
120	-	A	53 cde
466	-	B	53 cde
415	+	B	52 cdef
473	+	A	50 cdefg
439	-	B	50 cdefg
124	-	B	50 cdefg
123	+	A	47 defgh
189	+	A	47 defgh
461	+	A	47 defgh
813	+	A	47 defgh
183	+	A	40 defghi
420	-	A	37 efghij
811	+	A	37 efghij
820	+	A	37 efghij
468	+	B	37 efghij
414	-	B	33 efghijk
215	+	B	33 efghijk
465	-	B	33 efghijk
457	-	B	33 efghijk
412	+	B	30 ghijkl
462	+	B	30 ghijkl
810	+	A	27 hijklm
458	-	B	27 hijklm
471	-	B	27 hijklm
474	-	B	27 hijklm
410	-	B	27 hijklm
113	+	B	23 ijklm
419	-	B	23 ijklm
201	+	B	23 ijklm
812	+	A	17 jklm
147	+	A	13 klm
155	-	A	10 lm
131	+	B	10 lm
467	-	A	7 m
469	+	B	7 m

[1]Virulence Index = (ratio of successful infection/mean days from inoculation till bud death) x 1000.

[2]Values followed by different letters are significantly different (P=0.05), as determined by Duncan's multiple range test.

TABLE 10. Comparisons of mean virulence of type A versus type B *Sphaeropsis sapinea* with or without double-stranded RNA following inoculation of wounded buds of 2- to 3-year-old seedlings of *Pinus sylvestris*, *P. nigra*, and *P. banksiana* in the field.

Type of culture	dsRNA	Virulence[1] Index
A	-	47 a[2]
A	+	44 ab
B	+	35 abc
B	-	27 bc

[1]Virulence Index= (ratio of successful infection/ days from inoculation till bud death) x 1000.
[2]Values followed by different letters are significantly different (P=0.05), as determined by Duncan's multiple range test.

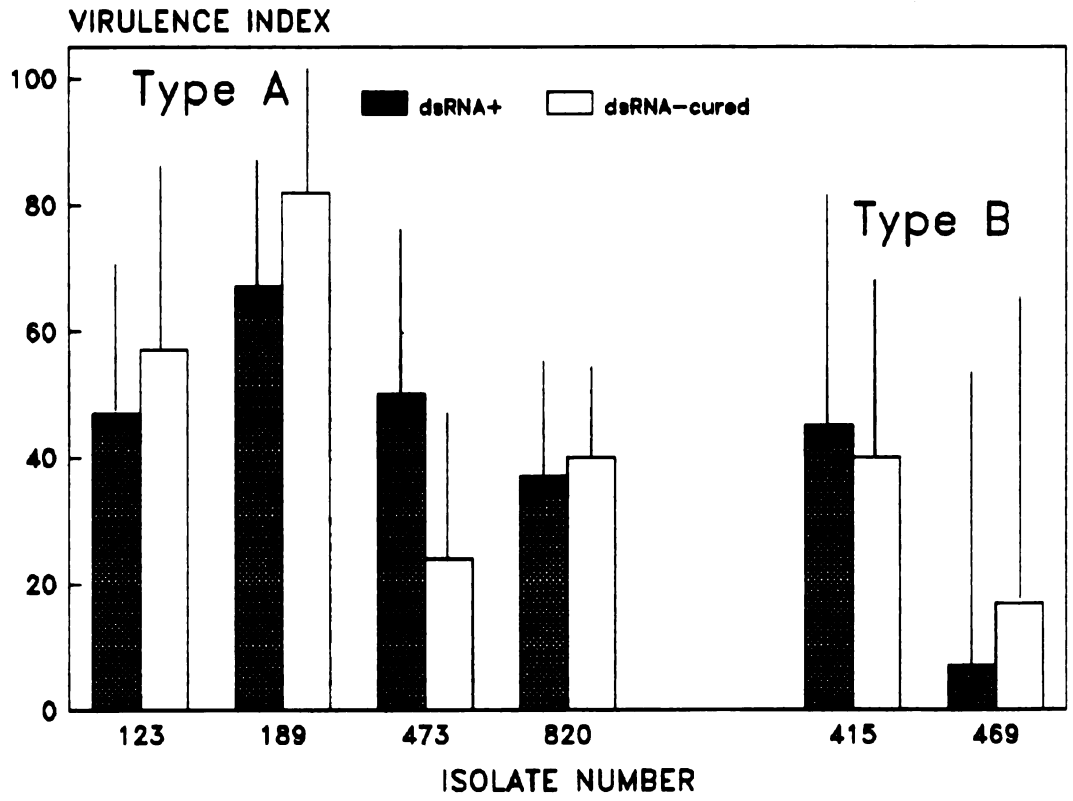


Figure 7. Virulence of parent strains of Sphaeropsis sapinea containing double-stranded RNA (dsRNA) compared to single-conidial subcultures freed of dsRNA when inoculated on buds of seedlings of three pine species (Pinus sylvestris, P. nigra, and P. banksiana). The lines above the bars represent the standard deviation in virulence for each isolate.

TABLE 11. Comparisons of susceptibility of 2- to 3-year-old seedlings of *Pinus sylvestris*, *P. nigra* and *P. banksiana* infected by *Sphaeropsis sapinea* following inoculation of wounded buds in the field.

Seedling type	Virulence Index[1]
P. sylvestris	37 a[2]
P. nigra	31 ab
P. banksiana	17 b
P. resinosa	0 c

[1]Virulence Index= (ratio of successful infection/mean days from inoculation till bud death) x 1000.

[2]Values followed by different letters are significantly different (P=0.05), as determined by Duncan's multiple range test.

Description of the new variety:

The distinct morphological characteristics of the type B isolates (Table 2) were correlated with important pathogenic characteristics of aggressiveness (Table 8) and virulence (Table 6 & 9). The two groups of isolates are common and coexist in the same regions on the same hosts in the north central United States (Table 1). The type B isolates are distinguishable from type A isolates in morphological differences in spore septation and spore length and ecological differences in aggressiveness and virulence. Therefore, a new variety of S. sapinea is here proposed.

Sphaeropsis sapinea (Fr.:Fr.) Dyko & Sutton var. breviphragmospora Wu, Palmer & Adams, var. nov.

Type: Collected from an annual canker on a branch of Pinus resinosa Ait. near Lake Itasca, Becker County, T. 27 N., R. 3 W., Sec. 30, Minnesota, M. A. Palmer n. XXX, 7/10/1987 (FH).

Habitat: Occurring on stem cankers, cones or blighted shoots of Pinus resinosa and P. banksiana.

Geographic Distribution: Northern Great Lakes region including Minnesota, Wisconsin and Michigan.

Etymology: The variety epithet breviphragmospora is from Latin, referring to the conidia that are shorter and narrower, and a portion of which will have 0, 1, 2, and 3[4] septation when mature.

Ecology: Collections are associated with locations near or on Pinus banksiana Lamb. The variety occurs on P. resinosa where the overstory or adjacent stand is P. banksiana and is a facultative pathogen causing

annual cankers on pine trees and seedlings that have been predisposed by frost, hail, drought or similar injury.

S. sapinea var. breviphragmospora is distinguishable from other S. sapinea by the smaller and multiseptate conidia, a relatively slower growth rate, a more appressed mycelium and darker colony color in culture, pitting of the conidium wall (46, 47), spermatia formation, requirement for wounding or predisposing injury to host for pathogenicity and unique allozyme zymograms (32). Mature conidia, 11-14 x 24-34 m, occur with 0, 1, 2, and 3(4) septations when pigmented, and occasionally when hyaline, in all collections. The hallmarks of the variety are conidia with more than two septation and with pitted surface ornamentation on the walls when viewed by scanning electron microscopy. Also one of the characteristics of the variety is the common occurrence of hyaline and oblong spermatia, 1.4 x 4 m, that do not germinate into hyphae. Mycelium on potato glucose agar differs from other S. sapinea in being more appressed and in its dark green to dark gray or black color after 20 days at 23C. Conidiomata form on or in the agar medium, but not on aerial mycelium as do other S. sapinea. Formation of the conidiomata is not light dependent. The pycnidial initials form on agar in a week in diffuse indoor light or in the dark; other S. sapinea generally form pycnidia in culture in a week only following exposure to bright light. Fertile pycnidia occur on autoclaved pine needles incubated on 2% water agar after 30-90 days at 17-26C.

DISCUSSION

In this work, 55% of the isolates of Sphaeropsis sapinea were found to contain double-stranded RNA. This frequency of dsRNA infection in the population of a fungal species appears unusually high in our current knowledge of other species (22, 24, 29, 38). However frequencies as high as 98% have been reported in populations of Thanatephorus cucumeris (53). Why fungal species vary in the frequency in which they accumulate viral genomes within their populations is poorly understood but three factors are known to influence the incidence of viral transmission and distribution: [1] somatic incompatibility systems, [2] inheritance through mitospores (conidia), and [3] inheritance through sexual transmission to meiospores. It is yet unknown whether S. sapinea has a somatic incompatibility system or it can be sexually reproduced, however dsRNA was found to be transmitted into conidia at a variable frequency (71 to 100%) depending presumably on the dsRNA genome. This high transmission rate might not influence dsRNA incidence in the population because rates of transmission through mitospores also can approach 100% in species with low incidence of viral infection, such as Leucocytospora leucostoma (Jensen and Adams, unpublished data) and Cryphonectria parasitica (40).

Most isolates of S. sapinea were found to have one detectable dsRNA segment, but several contained a minimum of two, four, five or seven segments of variable molecular weights. All dsRNA in fungi is believed to

be of viral origin (43) and often causes fungi to decline in culture, (7, 19, 44) altering colony morphology, growth rate, virulence and survival. The presence of dsRNA in S. sapinea was not well correlated with character traits of type B isolates that we had hypothesized to be symptoms of decline, such as: a more appressed colony morphology, a slower growth rate, smaller spore dimensions, a greater number of septations in mature spores and diminished pathogenicity. When individual type B isolates that contained dsRNA were freed of the infection (by selection of conidia that escaped transmission during development) the phenotype of the isolates did not change into the type A phenotype.

The 20 isolates in this study that had the type B morphological characteristics uniformly failed to infect pine buds unless the buds were predisposed by wounding, in contrast to the type A isolates. Additional comparison tests of dsRNA-free subcultures and their parents, verified that dsRNA was not a contributing factor to the low aggressiveness of the type B cultures. These tests reconfirm that the wounding requirement trait in part differentiates the two groups of S. sapinea, with the A group being more aggressive.

The question of whether prior wounding of the host is necessary or not for infection of pine by S. sapinea has been the subject of several previous studies (3, 5, 37, 48). Chou (9) reviewed this topic listing the methods of inoculation used in many older works. He stressed the importance of inoculating plants at the proper stage of host development, such as bud break, and concluded that wounding of the host was not necessary for infection. Here, while following Chou's recommendation (9), we have found that wounding is necessary for infection by one group of

isolates of S. sapinea but not for the other, reconfirming the studies of Palmer (32). The presence of type A and type B isolates coexisting in the same region or forest particularly might explain many discrepancies in the literature regarding the wounding requirement for the pathogen. Undoubtedly, this variation in aggressiveness in populations of the species has been a major confounding factor in virulence tests and has shaped our interpretation of the natural history of the Sphaeropsis /Pinus pathosystem. For example, is S. sapinea normally a saprophyte and parasite of immature cones only occasionally becoming an opportunistic pathogen of shoots, or is it an invasive and aggressive pathogen?

Isolates of S. sapinea in this study ranged from virtually avirulent (several dsRNA(+) strains) to non-aggressive moderately virulent opportunists (most type B isolates) to aggressive and highly virulent pathogens (most type A isolates). As a group, type A isolates were more virulent than type B when compared on wounded buds. It was also found that type B isolates were avirulent or extremely low in virulence when inoculated onto wounded P. nigra buds (Table 5). Higher virulence appears to be a characteristic trait of the A group, particularly on P. nigra.

For future studies, a thorough characterization of strains of S. sapinea prior to their use in research is warranted.

DsRNA was associated with reduced virulence in some strains (Fig. 6 & 7). Individual dsRNA-free subcultures of type A and type B had either increased virulence or were equally virulent compared to the dsRNA infected parents on trees of P. nigra, and P. sylvestris. Tests on seedlings gave similar results but were less clear and one of the dsRNA-free strains, strain 473, appeared less virulent compared to the parent

when tested on seedlings, but not on trees (see Fig. 6 and 7). Whether any specific dsRNA virus suppressed virulence on a certain pine species but not on other species was not clarified in these tests.

The results might support the contention of Finkler, et al (16) that some dsRNA viruses may suppress whereas others may induce virulence in fungal pathogens, presumably by modifying the regulation of virulence genes in the chromosomal DNA.

In our virulence tests of dsRNA(+) isolates, dsRNA(-) isolates and dsRNA-free subcultures on pine, over 80% of inoculations on the pine landscape trees yielded successful infections, however this success rate varied with species in seedling inoculation trials. Successful infection of wounded buds following inoculation of seedlings of P. nigra, and P. sylvestris yield similar percentages, 72% and 68% respectively, to the tree trial at over 80%. However, successful infection of wounded P. banksiana averaged only 42% and less than 5% of the P. resinosa were successfully infected. This difference in percentage of successful infection might reflect differences in host susceptibility at the species level or reflect genetic variability of many individuals within the seed source. Undoubtedly the recently transplanted seedlings were also under differing levels of environmental stresses that affect susceptibility of species and individuals. Therefore, we place greater trust in the virulence trials conducted on the trees and recognize that more thorough tests of specific strains on established trees are needed.

In summary, we discovered that the dsRNA was not associated with any of the morphological criteria or physiological and pathological differences that have been used to distinguish type A isolates from type B isolates.

We further reconfirmed that these group characteristics were associated with a relatively reduced growth rate, a requirement of host-wounding predisposition for pathogenicity, a reduced virulence on P. nigra and formation of spermatia. A unique pattern of allozyme polymorphism also is apparently associated with the phenotype as well as an unique ecological niche within the natural range of P. banksiana (Palmer, unpublished data). This work and an accumulating body of research (32, 45) have resolved the type B isolates of S. sapinea as unique and deserving of varietal classification. Description and assignment of a new variety name, Sphaeropsis sapinea var. breviphragmospora, encompassing and restricted to the type B phenotype is proposed. This classification of isolates should contribute to clarifying our understanding of the ecology, pathogenicity and epidemiology of Sphaeropsis sapinea.

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