Double-stranded RNA and associated virulence in South African isolates of *Sphaeropsis sapinea*

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Abstract: *Sphaeropsis sapinea* (Fr.) Dyko & Sutton is an opportunistic pathogen of various *Pinus* spp., causing severe shoot blight and dieback. Some isolates of *S. sapinea* display characteristics such as reduced virulence, reduced growth rate, lack of pigmentation, altered colony morphology, and suppressed conidiation. South African isolates of *S. sapinea* displaying a range of growth patterns, including reduced growth rate and atypical morphology, were screened for the presence of double-stranded RNA (dsRNA). They were also tested for relative virulence in pathogenicity tests. Double-stranded RNA was isolated by means of phenol extraction and cellulose chromatographic purification. A single species of dsRNA (± 4.3 kilobase pairs in size) was obtained from two slow-growing isolates and two isolates with more regular growth. The virulence of these dsRNA-containing isolates was tested on mature *Pinus patula* Schlecht. et Cham. trees. Although reduced virulence was positively correlated with slower growth in vitro, the presence of dsRNA could not be linked to either of these characteristics.

Key words: double-stranded RNA, hypovirulence, *Sphaeropsis sapinea*.

Résumé : Le *Sphaeropsis sapinea* (Fr.) Dyko & Sutton est un champignon pathogène opportuniste chez les *Pinus* spp. causant des brûlures sévères des tiges et la mort en cime. Certains isolats du *S. sapinea* montrent des caractéristiques telles que virulence réduite, taux de croissance faible, absence de pigmentation, déformation des colonies et absence de conidiation. Les auteurs ont examiné des isolats sud-africains du *S. sapinea* montrant une gamme de patrons de croissance, incluant un taux de croissance réduit et une morphologie atypique, afin d'y déceler la présence d'ARN doubles brins (ARNdb). Ils les ont également testés quand à leur virulence relative par essais de pathogénicité. Ils ont isolé l'ARNdb par extraction phénolique et purification par chromatographie sur cellulose. Ils ont obtenu une seule espèce d'ARNdb (± 4.3 paires kilobase en dimension) à partir de deux isolats à croissance lente et deux isolats à croissance plus régulière. Ils ont testé la virulence de ces isolats contenant de l'ARNdb sur des *Pinus patula* Schlecht. et Cham. matures. Bien que la virulence réduite soit positivement corrélée avec la croissance plus lente in vitro, la présence d'ARNdb n'a pu être reliée à aucune de ces caractéristiques.

Mots clés : ARN doubles brins, hypovirulence, *Sphaeropsis sapinea*.

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Introduction

Sphaeropsis sapinea (Fr.) Dyko & Sutton [*Diplodia pinea* (Desm.) Kickx] is an opportunistic pathogen of *Pinus* spp. with a cosmopolitan distribution (Waterman 1943; Punithalingam and Waterston 1970). The fungus is commonly associated with disease symptoms such as shoot blight and collar rot of seedlings (Buchanan 1967; Palmer and Nicholls 1985), root disease (Wingfield and Knox-Davies 1980), cankers accompanied by resinosis (Waterman 1943; Marks and Minko 1969), crown wilt (Chou 1987), and blue stain (Eldridge 1957). In South Africa, the most important losses to *Pinus radiata* D. Don and *Pinus patula* Schlecht. & Cham. plantations, results from the post-hail infection of shoots by *S. sapinea* (Lückhoff 1964; Evans 1987; Swart and Wingfield 1991). In Australia and New Zealand, *S. sapinea* associated dieback also results in a major reduction in increment and the potential marketable volume of *P. radiata* timber (Wright and Marks 1970; Currie and Toes 1978). Zwolinski et al. (1990*a*, 1990*b*) estimated an average loss of US$4 million per year (1986 values) due to *S. sapinea* associated die-back, following hail damage in South African pine plantations.

Isolates of *S. sapinea* display characteristics such as variable colony morphology (Palmer and Stewart 1982), differences in conidial size and morphology (Slagg and Wright 1943; Barker 1979; Palmer and Stewart 1982; Wang et al. 1985; Wang and Blanchette 1986), differences in virulence (Palmer and Stewart 1982), and differences in growth rate (Swart et al. 1991). These characteristics have previously

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been linked to hypovirulence and the presence of double-stranded RNA (dsRNA) in fungi such as *Cryphonectria parasitica* (Murr.) Barr (Day et al. 1977; Anagnostakis and Day 1979; Dodds 1980; Elliston 1981; McManus et al. 1989).

Naturally occurring hypovirulent strains of *C. parasitica*, containing dsRNA, have resulted in the effective biological control of chestnut blight in parts of Europe (Elliston 1981; Heiniger and Rigling 1994). Preliminary studies have revealed that a *C. parasitica*-like hypovirulence, associated with the presence of dsRNA, occurs in certain isolates of *S. sapinea* (Steenkamp et al. 1995). It could, however, not be determined whether reduced virulence was due to the presence of dsRNA. The objective of this study was to ascertain whether these preliminary findings on hypovirulence-associated characteristics, are consistent with those from a larger group of South African isolates of *S. sapinea*.

Materials and methods

Fungal strains

The isolates of *S. sapinea* used in this study were collected from a wide range of *Pinus* spp. and geographic regions in South Africa (Table 1). Two isolates from *Pinus banksiana* Lamb. and *Pinus resinosa* Ait. from the United States were also included for comparative purposes. These isolates were maintained on 2% (w/v) malt extract agar (MEA) (Biolab Diagnostics Ltd., Fedlife Park, Midrand, South Africa) in the culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Table 1. Origin and hosts of Southern African isolates of *S. sapinea* used in this study.

Isolate No.*	Origin†	Host
CMW 1184	Western Cape	*P. radiata*
CMW 1185	Western Cape	*P. radiata*
CMW 4235	Western Cape	*P. radiata*
CMW 4241	Western Cape	*P. radiata*
CMW 1187	Western Cape	*P. radiata*
CMW 4240	Western Cape	*P. radiata*
CMW 1188	Eastern Cape	*P. radiata*
CMW 4243	Eastern Cape	*P. radiata*
CMW 4244	Eastern Cape	*P. radiata*
CMW 4247	Eastern Cape	*P. pinaster*
CMW 4246	Kwazulu Natal	*P. patula*
CMW 1189	Lesotho	*P. patula*
CMW 4239	Western Cape	*P. patula*
CMW 1191	Western Cape	*P. patula*
CMW 4232	Western Cape	*P. patula*
CMW 4236	Western Cape	*P. patula*
CMW 4242	Western Cape	*P. patula*
CMW 4251	Western Cape	*P. patula*
CMW 4252	Western Cape	*P. patula*
CMW 1186	Western Cape	*P. taeda*
CMW 1192	Swaziland	*P. taeda*
CMW 4253	Gauteng	*P. halepensis*
CMW 1193	Mpumalanga	*P. elliottii*
CMW 4254	Gauteng	*P. roxburghii*
CMW 4233	South Africa	*Pinus* sp. ‡
CMW 4238	South Africa	*Pinus* sp. ‡
CMW 4245	South Africa	*Pinus* sp. ‡
CMW 4248	South Africa	*Pinus* sp. ‡
CMW 4249	South Africa	*Pinus* sp. ‡
CMW 4234	North America	*P. banksiana*
CMW 4250	North America	*P. resinosa*

*All isolates are maintained in the culture collection of Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

†Western Cape, Eastern Cape, Kwazulu Natal, Gauteng, and Mpumalanga are provinces of South Africa. In all other cases the only location known relates to country.

‡Hosts are all species of *Pinus*.

Comparison of growth in cultures

Mycelial plugs, 5 mm in diameter, cut from the edges of actively growing cultures, were transferred to 90 mm Petri dishes, containing MEA. Nine plates were prepared for each isolate at each temperature to be tested, i.e., 20, 25, and 30°C. After 52 h of incubation, growth was determined by measuring the diameter of growth of each colony, along three axes, at 120° intervals to each other. Means were calculated for each set of three measurements. For statistical analysis, only the results obtained from growth at 25°C were selected. Analysis of variance was performed to determine the effect of isolate on diameter growth. Tukey's procedure for comparison of means was applied to test the significance of differences (Winer 1971).

Extraction and purification of dsRNA

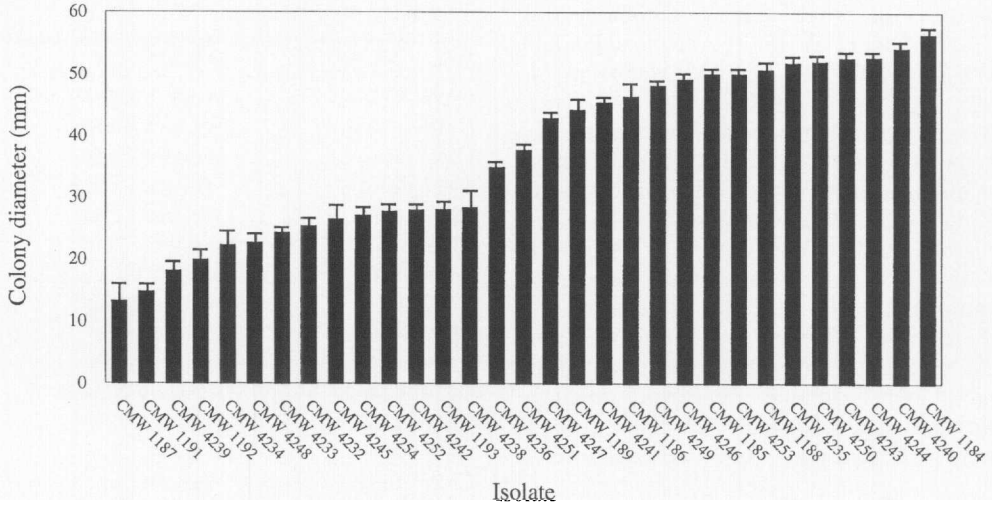
Isolates were grown in the dark in 1-L Erlenmeyer flasks containing 500 mL of 2% (w/v) malt extract broth (Biolab Diagnostics Ltd., Fedlife Park, Midrand, South Africa). Cultures were incubated at room temperature (20–25°C) for 2 weeks. Mycelial tissue was harvested by filtration (Whatman BioSystems Ltd., Maidstone, Kent, U.K.: No. 1 filter paper). A modification of the procedure described by Morris and Dodds (1979) and Valverde et al. (1990) was employed to isolate dsRNA. Harvested mycelium was ground to a powder in the presence of liquid nitrogen by using a mortar and pestle. Ground mycelium (1.5 g) was suspended in 10 mL of 1 × STE buffer, containing 0.1 M NaCl, 0.05 M Tris-HCl (pH 8.0), and 0.001 M EDTA (pH 8.0). For extraction of the nucleic acid, this suspension was added to an equal volume of a SDS–phenol mixture (1:9), containing 10% (w/v) sodium dodecyl sulphate and phenol equilibrated with 0.5 M Tris-HCl (pH 8.0). This mixture was shaken (30 min) and centrifuged (16 000 × *g*) at room temperature. Ethanol was added to the aqueous phase to a final concentration of 16.5% (v/v) and subjected to cellulose (Whatman BioSystems Ltd., Maidstone, Kent, U.K.; CF-11 cellulose) column chromatography for dsRNA purification.

Columns were prepared as described by Valverde and Fontenot (1991) but modified to include the use of 60-mL, glass wool plugged, plastic syringes. After application of the ethanolic aqueous phase to columns, each column was washed with at least 100 mL of 1 × STE buffer, containing 16.5% (v/v) ethanol, to remove contaminating DNA and single-stranded RNA. Purified dsRNA was eluted with 1 × STE and precipitated with 0.1 volume of 3 M sodium acetate (pH 5.2) and 0.6 volumes of 2-propanol.

Separation of dsRNA was performed on 0.7% gels (Promega Corp., Madison, Wis.: Molecular Biology Grade agarose) containing ethidium bromide (0.2 µg/mL). Electrophoresis was performed at 3 V/cm (room temperature) and in 0.5 × TBE electrophoresis buffer, containing 4.5 mM Tris, 4.5 mM boric acid, and 1 mM EDTA (pH 8.0). The dsRNA was visualized in transmitted ultraviolet (UV) light and photographed.

To confirm the RNA nature of the extracted dsRNA, enzymatic digestions of nucleic acid were attempted by treatment with

Fig. 1. Mean colony diameters of 31 *S. sapinea* isolates on Petri dishes containing MEA measured after 52 h at 25°C. Error bars are SD.



7.5 U/μL of deoxyribonuclease I (DNase I; Boehringer Mannheim, Mannheim, Germany) in 6 mM $MgCl_2$ and 40 mM Tris-HCl (pH 7.5) at 37°C for 1 h. To confirm the double-stranded nature of the extracted dsRNA, samples were treated with 25 μg/mL ribonuclease A (RNase A; Boehringer Mannheim, Mannheim, Germany) in 30 mM and 300 mM NaCl, respectively, at 30°C for 1 h. Nucleic acids hydrolysed by RNase A at the lower salt concentration and resistant to DNase I and RNase A at the higher salt concentration were considered to be dsRNA (Hansen et al. 1985).

Pathogenicity tests

During October 1995, 20 four-year-old *P. patula* trees at the Ngodwana plantation, Mpumalanga, South Africa, were inoculated with selected isolates of *S. sapinea*. These pathogenicity tests were repeated during November of the same year. Inoculum of *S. sapinea* was prepared by culturing isolates CMW 1184, CMW 4235, CMW 4236, CMW 1191, CMW 1192, and CMW 4254 (Table 1) on MEA plates at 25°C for a period of 48 h. Mechanical wounds were made on seven lateral branches of each tree using a cork borer (5 mm in diameter). Agar disks (5 mm in diameter) overgrown with mycelium were placed on the artificial wounds and covered with masking tape to prevent contamination and desiccation. Six of the seven wounded branches were inoculated with the selected *S. sapinea* isolates. For the control treatment, a sterile disk of MEA was placed in the wound, on the remaining branch and covered with masking tape. After inoculation (5 weeks), the masking tape and bark was removed and the lesion lengths were measured. Re-isolations were made from these lesions, to show that the inoculated fungi remained present. A randomized block experimental design was used, with the 20 trees representing 20 blocks. An ANOVA was used to detect differences. Tukey's procedure for comparison of means was applied to test the significance of differences (Winer 1971).

Results

Comparison of growth rates

Analysis of variance indicated that isolates of *S. sapinea* varied considerably in growth rate ($p = 0.05$). Faster growing isolates were consistently fast growing at all temperatures tested. The slower growing isolates were also not affected by the temperature of incubation. Most of the *S. sapinea* isolates displayed an optimum temperature for growth at 25°C, with the exception of three isolates (CMW 4239, CMW 4236, and CMW 4245). These isolates grew optimally at 30°C. All the isolates displayed least growth at 20°C (results not shown). At 25°C, the mean diameter of growth ranged from 13.1 mm for CMW 1187 to 56.3 mm for CMW 1184 (Fig. 1).

Table 2. Mean growth rates in vitro and lesion lengths on branches of *P. patula* trees, caused by dsRNA-free and dsRNA-containing isolates of *S. sapinea*.

Isolate*	Mean growth rate (mm)†	Mean lesion length (cm) ‡	Presence of dsRNA
CMW 1191	14.6*a*	5.5*a*	+
CMW 1192	19.7*b*	6.0*ab*	–
CMW 4254	26.9*c*	7.5*abc*	+
CMW 4236	34.7*d*	10.7*cd*	+
CMW 4235	51.7*e*	11.5*cde*	+
CMW 1184	56.3*f*	16.4*f*	–

*All isolates are maintained in the culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

†Mean of colony diameters (mm) on MEA plates measured after 52 h at 25°C. Values within the column followed by different letters differ significantly ($p = 0.05$; Tukey's test) (Winer 1971).

‡Mean lesion lengths measured on 4-year-old *P. patula* trees after 5 weeks. Values within the column followed by different letters differ significantly ($p = 0.05$; Tukey's test) (Winer 1971).

Extraction and purification of dsRNA

Of the 31 isolates of *S. sapinea* tested, only four contained detectable amounts of dsRNA (Table 2). Each of these strains harboured a single, high molecular weight fragment of dsRNA, of similar size, based on mobility in agarose gels (Fig. 2). Comparison with a *Eco*RI–*Hin*dIII digestion of the DNA lambda (λ) phage revealed that these dsRNAs are approximately 4.3 kilobase pairs in size. The nature of the dsRNA was confirmed by its resistance to digestion by DNase I and RNase A at the higher salt concentration and sensitivity to RNase A at the lower salt concentration. The isolated nucleic acid from all four of the dsRNA-containing isolates were tested with RNase and DNase and in each case the 4.3 kilobase pair fragment were found to be dsRNA. In Fig. 2 the results of the RNase and

Fig. 2. Agarose gel (0.7%) showing dsRNA extracted from *S. sapinea* CMW 4254, CMW 1192, CMW 1191, CMW 4236, CMW 4235, and CMW 1184. Lane 1, molecular weight marker [*Eco*RI–*Hin*dIII digestion of the lambda (λ) phage]; lane 2, dsRNA from CMW 1191, 4.3 kilobase pairs in size; lane 3, dsRNA from CMW 4254, 4.3 kilobase pairs in size; lane 4, dsRNA from CMW 4236, 4.3 kilobase pairs in size; lane 5, dsRNA from CMW 4235, 4.3 kb in size; lane 6, CMW 1184 did not contain double-stranded RNA; lane 7, CMW 1192 did not contain detectable amounts of dsRNA; lane 8, nucleic acid isolated from CMW 4235 that were digested with RNase A at 300 mM NaCl; lane 9, nucleic acid isolated from CMW 4235, digested with DNase I; lane 10, nucleic acid isolated from CMW 4235, digested with RNase A at 30 mM NaCl.

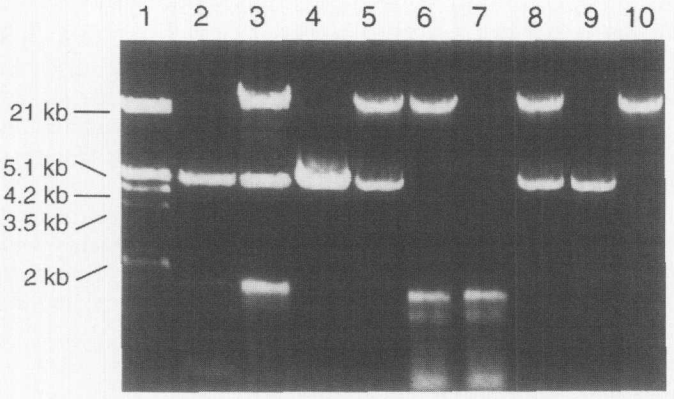
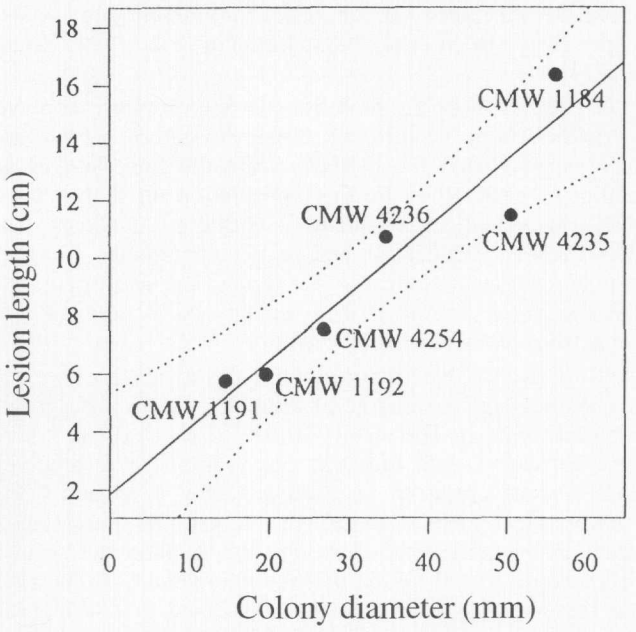


Fig. 3. Mean lesion lengths (mm) on the branches of 4-year-old *P. patula* trees, associated with inoculation of six selected isolates of *S. sapinea*, plotted against the mean colony diameters of these isolates. The correlation coefficient (r = 0.95) indicates a positive correlation between lesion length and growth. The 95% confidence intervals are indicated with broken lines.



DNase tests are shown only for the dsRNA-containing isolate CMW 4235.

Results of the dsRNA screening, in combination with those obtained from growth studies, assisted in the selection of six isolates for further study. Two dsRNA-containing isolates of *S. sapinea* (CMW 1191 and CMW 4254) were selected on the basis of slower growth. After 52 h at 25°C, these isolates displayed mean colony diameters of 14.6 and 26.9 mm, respectively. Two additional dsRNA-containing isolates of *S. sapinea* (CMW 4236 and CMW 4235) were also selected. These selections were based on relatively faster growth, i.e., 34.7 and 51.7 mm, respectively, after 52 h at 25°C. The fastest growing isolate (CMW 1184) and the one of the slower growing isolates of *S. sapinea* (CMW 1192), which were both free from detectable amounts of dsRNA, were also selected for comparative purposes.

Pathogenicity tests

Analysis of variance indicated that the dsRNA-free and dsRNA-containing *S. sapinea* isolates tested, varied significantly in their virulence to *P. patula* trees (p = 0.01) (Table 2). For example, the dsRNA-free *S. sapinea* isolate, CMW 1184, was significantly more virulent to *P. patula* than the other five isolates used. Although isolates CMW 1184 and CMW 1192 both harboured no detectable amounts of dsRNA, they produced significantly different lesion sizes (16.4 and 6.0 cm, respectively). Isolates CMW 4235, CMW 4236, and CMW 4254 all contained dsRNA but differed markedly in their degree of virulence (Table 2). From this study it was thus impossible to correlate reduced virulence on *P. patula* with the presence of dsRNA.

Comparison of in vitro growth and mean lesion length on 4-year-old *P. patula* trees, revealed a significant (p = 0.004) positive correlation (r = 0.95) between virulence and growth rate (Table 2, Fig. 3). The fastest growing isolate (CMW 1184) produced the largest lesions and the slowest growing dsRNA-containing isolate (CMW 1191) produced the smallest lesions. Isolate CMW 4235, with the second fastest growth rate, produced the second largest lesions. The same result was found with isolate CMW 4236, which was slightly slower growing than CMW 4235, and it also produced slightly shorter lesions than the latter isolate.

Discussion

The discovery of dsRNA in phytopathogenic fungi has introduced a new avenue to biological control of fungal pathogens. One of the best examples is based on the natural occurrence of hypovirulent strains of *C. parasitica* that have prevented the destruction of European chestnuts by this pathogen (Elliston 1981; Heiniger and Rigling 1994). Results of this study of dsRNA in South African isolates of *S. sapinea*, however, suggest that there is no association between the presence of dsRNA and hypovirulence in this fungus. This is in contrast to a previous study (Steenkamp et al. 1995) with a much smaller set of isolates that suggested a possible dsRNA-associated hypovirulence in South African isolates of *S. sapinea*.

Prior to the onset of studies on hypovirulence in South African *S. sapinea*, the presence of dsRNA in this fungus had only been reported once. Wu et al. (1989) reported on dsRNA extracted from North American isolates of *S. sapinea*. The dsRNA obtained from these isolates displayed a range of sizes. The dsRNA patterns for each of these isolates also appeared to be unique (Wu et al. 1989). In

contrast, only one species of dsRNA appears to be present in South African isolates of *S. sapinea*. It is, however, not uncommon for a fungal isolate to harbour only a single species of dsRNA. Certain isolates of *Ceratocystis ulmi* (Buis.) Moreau, *Chalara elegans* Nag Raj & Kendrick, and *Rhizoctonia solani* Kühn, for example, contain single segments of dsRNA that were 3.0, 2.8, and 12.0 kilobase pairs in size, respectively (Hoch et al. 1985; Bottacin et al. 1994; Kousik et al. 1994).

The similarity in the mobilities in agarose gels, displayed by dsRNA from the different South African *S. sapinea* isolates suggests that the dsRNAs from the four isolates are similar to each other. Further speculation on their relatedness, however, necessitates Northern analyses and cDNA:RNA hybridization and sequencing studies. We hope to undertake such studies in the future and also to compare dsRNAs from South African isolates of *S. sapinea* with those from other parts of the world.

Swart et al. (1991) reported on a significant positive correlation between the degree of virulence and in vitro growth rate in *S. sapinea*. The results of the present study are, therefore, consistent with previous observations. The relatively small lesions produced by isolates CMW 4254 and CMW 1191 are clearly linked to growth rate and not necessarily to infection by dsRNA. This would also explain the reduced virulence displayed by the dsRNA-free isolate, CMW 1192, and the high degree of virulence displayed by CMW 1184. Reduced growth rates and virulence may both be symptoms of some other interruption or dysfunction (Mahanti and Fulbright 1995).

The results of this study are not unusual, because the presence of dsRNA in fungi is not always associated with reduced virulence. Certain isolates of *R. solani* and *Phytophthora infestans* (Mont.) de Bary, for example, contain dsRNA that apparently has no effect on either virulence or growth rate (Tooley et al. 1989; Kousik et al. 1994). Mahanti et al. (1993) also reported on strains of *C. parasitica* that exhibited all the typical dsRNA-associated hypovirulence traits but that harboured no detectable dsRNA (Fulbright 1985). Mitochondrial dysfunction is thought to play a role in this reduced virulence, since modified respiration occurs in these dsRNA-free strains (Mahanti and Fulbright 1995). Such a dysfunction could provide a possible explanation for reduced virulence in some South African isolates of *S. sapinea*.

Although the presence of dsRNA in South African isolates of *S. sapinea* is not linked to either growth or virulence, it could play a possible role in intraspecific variation. The available South African isolates of *S. sapinea* display a considerable degree of variation (Swart et al. 1991, 1993). *S. sapinea* is, however, an introduced fungus in South Africa and might have a limited population structure, with a narrow genetic base. There is no evidence to support the existence of a sexual state and variation does not appear to be linked to sexual recombination. An explanation for the observed variation among *S. sapinea* isolates may be found in the presence of dsRNA. Characteristics such as cultural morphology, pigment, and toxin production have previously been linked to the presence of these extrachromosomal elements (Brazier 1983; Elliston 1985; Ghabrial 1986; Rogers et al. 1986; Wickner 1986; Koltin 1988).

Double-stranded RNA could play an important role in the biology of *S. sapinea*. Its function, therefore, deserves further consideration, since *S. sapinea* is one of the most serious impediments of the South African forestry industry (Swart et al. 1985). Any factor that might be associated with reduced virulence, or a better understanding of the fungus and its biology, is of interest. Elucidation of the role that dsRNA plays in the biology of *S. sapinea*, however, necessitates dsRNA transmission and elimination studies. Fulbright (1984) provided evidence for a cause and effect relationship between the presence of dsRNA and reduced virulence in certain strains of *C. parasitica* by eliminating the dsRNA from a dsRNA-containing isolate, i.e., curing. Cause and effect relationships between the presence of dsRNA and hypovirulence have also been established in fungi such as *C. elegans*, *Sclerotinia sclerotiorum* (Lib.) de Bary, and *Leucostoma persoonii* (Nits.) Hoehn. by curing dsRNA-containing isolates and infecting dsRNA-free isolates, i.e., conversion (Hammar et al. 1989; Boland 1992; Bottacin et al. 1994). Kousik et al. (1994) also employed these curing and conversion experiments to show that dsRNA in some isolates of *R. solani* play no part in its virulence and mycelial growth. Curing and conversion experiments will eventually assist in our understanding of the role that dsRNA plays in South African isolates of *S. sapinea*, and we hope to undertake such studies in the future.

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