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Characterisation of the 'C' morphotype of the pine pathogen *Sphaeropsis sapinea*

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Abstract

Sphaeropsis sapinea is an opportunistic pathogen of *Pinus* spp. and other conifers. Infection occurs when host trees are predisposed by adverse environmental conditions or mechanical damage. Initially two morphotypes, A and B, were described for *S. sapinea* and these were defined based on cultural characteristics, texture of conidial walls and virulence. The existence of these two morphotypes has, furthermore, been confirmed through the use of RAPDs and DNA sequencing of the ITS region of the rRNA operon. A third RAPD group, including isolates from Indonesia and Mexico, has recently been reported and was designated as a C morphotype based on differences in conidial size. The objective of this study was to characterise isolates of the C morphotype of *S. sapinea* based on cultural characteristics, conidial morphology, growth rate and pathogenicity. Cultural and conidial characteristics of isolates belonging to the A and C morphotypes were more similar to each other than to those of the B morphotype. The growth rates of isolates belonging to the three different morphotypes were not significantly different. Artificial inoculation of both Granny Smith apples and *Pinus patula* seedlings revealed that isolates of the C morphotype were considerably more virulent than those of the A and B morphotypes. The discovery of the more virulent C morphotype of *S. sapinea* could have serious implications for management of this pathogen, as well as quarantine practises in countries that import pine seeds. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: *Sphaeropsis sapinea*; Morphotypes; RAPD marker groups

1. Introduction

Sphaeropsis sapinea (Fr.:Fr.) Dyko & Sutton (=*Diplodia pinea* (Desm.) Kickx.) is an opportunistic pathogen with a cosmopolitan distribution and extensive host range on conifers (Birch, 1937; Eldridge, 1961). The fungus requires stress in order to initiate a pathogenic response which can lead to disease. Symptoms include post-hail associated die-back, cankers, shoot blight, a root disease, collar rot and blue stain (Millikan and Anderson, 1957; Lückhoff, 1964; Marks and Minko, 1969; Wingfield and Knox-Davies, 1980). Factors that predispose trees include conducive environmental conditions, mechanical damage and insect feeding (Lückhoff, 1964; Bega et al., 1978).

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Two morphotypes have been described for *S. sapinea*, and these have been designated A and B (Wang et al., 1985; Palmer et al., 1987). These two morphotypes represent distinct groups, distinguishable based on differences in cultural and conidial characteristics, growth rate, growth requirements, as well as pathogenicity and host range among isolates from the north central United States. In culture, A morphotype isolates have white to grey-green aerial mycelium and isolates of the B morphotype have black to white mycelium appressed to the surface of the agar. The conidia of A morphotype isolates are smooth-walled and slightly longer and wider than the pitted conidia of B morphotype isolates. Generally, isolates of the A morphotype do not require light for sporulation. They also grow more rapidly than the B morphotype isolates that require light to sporulate. Isolates of the A morphotype are more virulent on a wider range of hosts than those of the B morphotype, that was until recently, restricted to *P. banksiana* Lamb. and *P. resinosa* Aiton. from the north central United States (Wang et al., 1985; Smith and Stanosz, 1995). B morphotype isolates are, however, now being reported from other parts of the world and on hosts other than *P. banksiana* and *P. resinosa* (Stanosz et al., 1999; De Wet et al., 2000).

The distinction between the A and B morphotypes of *S. sapinea* has been confirmed using RAPD markers (random amplified polymorphic DNA) and designated as belonging to the A and B RAPD marker groups (Smith and Stanosz, 1995; Stanosz et al., 1996). In a recent study, a third RAPD group, containing seven *S. sapinea* isolates from Indonesia and one isolate from Mexico, was identified (De Wet et al., 2000). Sequences from the ITS region of the rRNA operon of representative isolates of all three RAPD groups resolved only the A and B morphotypes (De Wet et al., 2000). Isolates of the third RAPD group were indistinguishable from those of the A RAPD group based on ITS sequence homology.

Sphaeropsis sapinea isolates in the third RAPD group have been designated as belonging to a C morphotype, based on conidial size. Conidia of the C morphotype isolates are significantly longer than those of both the A and B morphotype isolates. No significant differences in the widths of the conidia between the three morphotypes were observed, although the A morphotype has previously been distinguished from the B morphotype based on its slightly longer and wider conidia (Wang et al., 1985; Palmer et al., 1987).

The objective of this study was to fully characterise isolates of the C morphotype of *S. sapinea* based on cultural and conidial characteristics. In vitro growth studies and pathogenicity tests were also conducted to compare isolates of the three morphotypes.

2. Materials and methods

2.1. Fungal isolates

A collection of 14 *S. sapinea* isolates (Table 1) from the United States, South Africa, Mexico and Indonesia was studied based on morphological characteristics traditionally used to define the A and B morphotypes. These isolates included three A, three B and eight C morphotype isolates and were randomly selected as representatives of each morphotype based on RAPD banding profiles, ITS sequences and conidial size as defined by De Wet et al. (2000).

Sphaeropsis sapinea isolates from South Africa and Mexico were obtained by direct isolation from the pith tissue of *P. radiata* and *P. greggii* cones, respectively, while the Indonesian isolates were obtained from pycnidia on *Pinus. patula* shoots with die-back symptoms. Dark-coloured fungal colonies, obtained after primary isolations, were transferred to 2% Water Agar (WA) (2% m/v Biolab agar) (Biolab Diagnostics, Midrand) supplemented with sterile pine needles to induce sporulation. Identification of *S. sapinea* isolates was based on conidial and pycnidial morphology (Sutton, 1980).

Single conidial cultures were generated by spreading conidia, released from excised pycnidia, onto the surface of 2% WA in Petri dishes. The plates were then incubated at 25 °C under mixed white and near-ultraviolet light, set to provide 12 h light/dark cycles, for at least 16 h to allow for germination to take place. Four germinating conidia for each isolate were transferred to 2% Malt Extract Agar (MEA) (2% m/v Biolab malt extract; 2% m/v Biolab agar) in Petri dishes and incubated at 25 °C. All the single conidial cultures were transferred to 2% MEA slants in McCartney bottles and stored at 4 °C. All isolates are maintained in the Culture Collection of the Tree

Table 1
Culture characteristics, conidial dimensions and morphology of *S. sapinea* isolates used in this study

Isolates	Type[a]	Origin	Host	Colony morphology[b]	No. of septa	Texture of conidial walls[c]
CMW190	A	United States	*P. banksiana*	Fluffy, dark mouse grey–mouse grey	0–1	Smooth
CMW1185	A	South Africa	*P. radiata*	Fluffy, dark mouse grey–mouse grey	0–1	Smooth
CMW4329	A	South Africa	*P. ponderosa*	Fluffy, dark mouse grey–mouse grey	0–1	Smooth
CMW189	B	United States	*P. resinosa*	Suppressed, mouse grey–pale mouse grey	1–3	Pitted
CMW4898	B	Mexico	*P. greggii*	Suppressed, mouse grey	1–3	Pitted
CMW4333	B	Mexico	*P. resinosa*	Suppressed, mouse grey–pale mouse grey	1–3	Pitted
CMW4876	C	Indonesia	*P. patula*	Fluffy, fuscous black–pale mouse grey	0–1	Rough surface
CMW4877	C	Indonesia	*P. patula*	Fluffy, fuscous black–pale mouse grey	0–1	Smooth
CMW4878	C	Indonesia	*P. patula*	Fluffy, dark mouse grey–pale mouse grey	0–1	Rough surface
CMW4879	C	Indonesia	*P. patula*	Fluffy, fuscous black–pale mouse grey	0–1	Smooth
CMW4880	C	Indonesia	*P. patula*	Fluffy, fuscous black–pale mouse grey	0–1	Rough surface
CMW4881	C	Indonesia	*P. patula*	Fluffy, fuscous black–pale mouse grey	0–1	Smooth
CMW4883	C	Indonesia	*P. patula*	Fluffy, fuscous black–pale mouse grey	0–1	Smooth
CMW4899	C	Mexico	*P. greggii*	Suppressed, mouse grey–pale mouse grey	0–1	Smooth

[a] A, B and C refers to the described A, B and C morphotypes of *S. sapinea* (Wang et al., 1985, 1986; Palmer et al., 1987; De Wet et al., 2000).

[b] Cultural characteristics are based on the morphology and colour of mycelium on 2% MEA after 15 days of incubation at 25 °C in the dark. Colony colour was assessed according to the mycological colour charts of Rayner (1970). "Fluffy" indicates to aerial mycelia with a "wooly" appearance while "suppressed" refers to mycelium growing close to the surface or within the growth medium.

[c] Texture of the conidial walls as observed when using scanning electron microscopy.

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2.2. Culture and conidial characteristics

Colony colour and morphology of all *S. sapinea* isolates were noted on 2% MEA in Petri dishes (9 cm). After 15 days of incubation at 25 °C in the dark, colony colour was assessed using the mycological colour charts of Rayner (1970). Conidial morphology was examined using bright field microscopy after sporulation was induced on 2% WA supplemented with sterile pine needles and incubation at 25 °C under mixed white and near-ultraviolet light, set to provide 12 h light/dark cycles. The texture of conidial walls was also examined using a JEOL 840 scanning electron microscope at 5 kV and sample preparation methods were similar to those used by Wang et al., 1985. Excised pycnidia on sterile pine needles were fixed with 2.5% glutaraldehyde in a 0.075 M phosphate buffer (pH 7.4–7.6), followed by osmium fixation using 1% osmium textroxide (OsO_4). The material was dehydrated in 70% ethanol, followed by three steps in 100% ethanol. Material was then critical-point dried using a Bio-Rad E3000 critical point dryer and CO_2, mounted on aluminium stubs and plated with a mixture of gold–palladium.

2.3. Growth rate in culture

An in vitro growth study was conducted on the 14 *S. sapinea* isolates. Mycelial plugs (10 mm diameter) from the margins of 1-week-old, single conidial cultures were transferred to Petri dishes (9 cm) containing 2% (MEA). Four Petri dishes for each isolate were incubated at each of the four temperatures (10, 15, 25 and 30 °C), in the dark. The growth rates of these isolates were determined by measuring colony diameter after 2, 4, 8 and 15 days for the four temperatures. The growth rate (milimeter per day) of all the *S. sapinea* isolates included in this study was statistically analysed using mean separation procedures (SYSTAT, version 7.0.1). The Analysis of Variance and the Tukey pairwise comparisons test (SYSTAT, version 7.0.1) were used to analyse data.

2.4. Pathogenicity tests

All *S. sapinea* isolates were included in the artificial inoculations on both Granny Smith apples and 1-year-old *P. patula* seedlings. Inoculations of apples have, in the past, been effectively used to predict the virulence of isolates of *Cryphonectria parasitica* (Murr.) Barr (Elliston, 1985), as well as *S. sapinea* isolates (Steenkamp, 1996). The inoculum was prepared by growing single conidial isolates on 2% MEA at 25 °C a week prior to inoculation. Sterile 2% MEA plugs were inoculated as controls. A completely randomised block design was used and all tests were repeated once.

2.5. Tests on Granny Smith apples

Prior to inoculation, Granny Smith apples were surface sterilised using 70% ethanol and 1% NaClO. A wound (10 mm wide and 5 mm deep) was made in the surface of the apple using a cork borer. Mycelial plugs were taken from the margins of 1-week-old, actively growing *S. sapinea* cultures and placed mycelium-surface down, in the wounds made in the apples. Four apples were inoculated for each isolate. The wounds were sealed with masking tape to prevent desiccation and incubated at room temperature with a 12 h day/night light regime. After 2 weeks lesion lengths, widths and surface areas were measured and means compiled.

2.6. Tests on P. patula seedlings

One hundred and fifty, 1-year-old *P. patula* seedlings (main stem ca. 10 mm diameter) were inoculated with the same *S. sapinea* isolates used in tests on apples. A superficial wound (ca. 3 mm diameter) to the cambium, was made on the stems of seedlings using a scalpel. Mycelial plugs were taken from the margins of 1-week-old, actively growing *S. sapinea* cultures and placed mycelium-surface down, in the wounds made on the stems. Ten seedlings were inoculated for each isolate. The wounds were sealed with masking tape to prevent desiccation. The tests were carried out in a controlled greenhouse environment (average day temperature of 25 °C and normal daylight conditions in September). Lesion lengths were measured after 2 weeks.

Results for both pathogenicity tests, on apples and pine seedlings, were separately analysed using mean separation procedures (SYSTAT, version 7.0.1). The analysis of variance and the Tukey pairwise comparisons test (SYSTAT, version 7.0.1) were used to analyse data.

3. Results

3.1. Culture and conidial characteristics

The A morphotype isolates had fluffy, dark mouse grey to mouse grey (Rayner, 1970) mycelium and the B morphotype isolates displayed mouse grey to pale mouse grey (Rayner, 1970) mycelium apprised to the surface of the medium (Table 1). These cultural characteristics are typical of the two morphotypes (Wang et al., 1985; Palmer et al., 1987). Isolates of the C morphotype had cultural characteristics that included those of both the A and B morphotypes. It was, thus, impossible to distinguish the C morphotype isolates from those representing the A and B morphotypes based on cultural characteristics.

Conidia of all 14 *S. sapinea* isolates included in this study were dark brown with thick walls (Table 1). Conidia of the A and C morphotype isolates rarely had septa and never had more than one septum per conidium. At least one and frequently more septa were, however, observed in the conidia of the B morphotype isolates.

The A morphotype isolates had conidial walls that were generally smooth and B morphotype isolates had pitted conidial walls (Table 1). Conidia of isolates belonging to the C morphotype all had smooth walls and this made them indistinguishable from those of the A morphotype. The conidial walls of three of the C morphotype isolates (CMW4876, CMW4878 and CMW4880), although smooth, had a rougher texture than those of the other C morphotype isolates (CMW4877, CMW4879, CMW4881, CMW4883 and CMW4899).

3.2. Growth rate in culture

All *S. sapinea* isolates included in this study had optimal growth at 25 °C and slower growth at 10, 15 and 30 °C. After 8 days, all the isolates incubated at

Table 2
Lesion lengths, widths and areas on Granny Smith apples, as well as lesion lengths on *P. patula* seedlings after inoculation with isolates representing the three morphotypes of *S. sapinea*

Isolates	Type	Granny Smith apples[a]				*P. patula* seedlings[b], mean lesion length (mm)
		Mean growth rate (mm)[c]	Mean lesion length (mm)	Mean lesion width (mm)	Area of lesion (mm^2)	
CMW190	A	20.0 ab	11.5 a	10.5 a	24.6 a	29.1 b
CMW4332	A	18.9 ab	11.2 a	10.6 a	24.8 a	23.3 b
CMW1185	A	18.6 ab	49.5 d	49.8 c	553.8 c	43.2 c
CMW189	B	13.7 c	10.8 a	10.2 a	21.5 a	9.0 a
CMW4334	B	13.0 c	17.8 b	13.6 a	42.0 a	14.9 a
CMW4898	B	13.8 c	25.1 c	23.1 b	116.0 b	22.3 b
CMW4876	C	21.8 ab	31.6 c	30.1 b	228.3 b	68.3 c
CMW4877	C	15.0 c	47.2 d	45.1 c	491.3 c	91.1 c
CMW4878	C	20.6 ab	25.9 c	23.9 b	114.8 b	24.1 b
CMW4879	C	17.0 ab	31.5 c	29.4 b	193.8 b	68.0 c
CMW4880	C	23.5 a	36.1 c	31.1 b	268.8 b	64.0 c
CMW4881	C	17.8 ab	49.6 d	46.4 c	577.5 c	40.8 c
CMW4883	C	18.4 ab	53.7 d	49.1 c	655.5 c	74.2 c
CMW4899	C	20.0 ab	11.4 a	10.5 a	24.1 a	78.0 c
Control		0	0	0	0	0

[a] Mean lesion lengths, widths and areas were determined after two independent inoculation trials in which four Granny Smith apples per isolate were inoculated. The area of the lesion was determined by calculating the number of 1 mm × 1 mm squares represented by the infected area. Values within a column followed by the same letters (a, b, c, d) do not differ significantly ($P < 0.05$).

[b] Mean lesion lengths were determined after two independent inoculation trails in which ten *P. patula* seedlings per isolate were inoculated. Values within a column (a, b, c) followed by the same letter do not differ significantly ($P < 0.05$).

[c] The mean growth rate of the isolates was calculated after 8 days of incubation on MEA at 25 °C. Values within a column with the same letters (a, b, c, d) do not differ significantly with the Tukey pairwise comparisons test ($P < 0.05$).

25 °C had completely covered the surface of the medium in Petri dishes. No significant differences were observed in mean growth rates (milimeter per day) for isolates of the three morphotypes of *S. sapinea* at 10 and 15 °C. At 25 and 30 °C, the mean growth rates for isolates belonging to the A and C morphotypes were, however, significantly greater than those of isolates belonging to the B morphotype (Table 2).

3.3. Pathogenicity

3.3.1. Tests on Granny Smith apples

All inoculations of *S. sapinea* isolates into Granny Smith apples resulted in lesions, whereas control inoculations with sterile MEA plugs showed no lesion development (Table 2, Fig. 1). The surface areas of lesions produced on apples by the A and B morphotype isolates were small and generally did not differ significantly from each other (Table 3). However, the South African A morphotype isolate (CMW1185) and the Mexican B morphotype isolate (CMW4898) produced larger areas of infection than other isolates of those particular morphotypes. Generally, the C morphotype isolates produced significantly larger areas of infection than either the A and B morphotype isolates. One exception was the Mexican C morphotype isolate (CMW4899), that resulted in a smaller area of infection, similar to those of the B morphotype. Based on mean surface area of infection, isolates of the C morphotype were significantly different to isolates of either the A and B morphotype.

3.3.2. Tests on P. patula seedlings

Inoculation of pine seedlings with *S. sapinea* isolates resulted in lesions, whereas control inoculations with sterile MEA plugs resulted in no lesion development (Table 2). Lesions produced by A and B morphotype isolates were generally very small, with those of the B morphotype smallest (Table 3, Fig. 2).

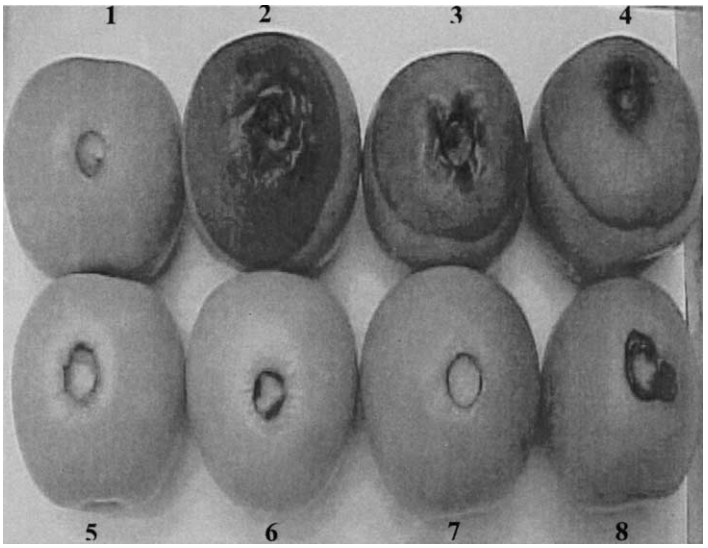

Fig. 1. Lesions formed on Granny Smith apples after inoculation with isolates representing the three morphotypes of *S. sapinea.* (1) Sterile control; (2–4) isolates representing the C morphotype (CMW4877, CMW4880, CMW4883); (5–6) isolates representing the A morphotype (CMW4332 and CMW190); and (7–8) isolates representing the B morphotype (CMW189 and CMW4334).

Table 3
Analysis of variance for the pathogenicity of isolates representing the three morphotypes *S. sapinea* after inoculation of Granny Smith apples and *P. patula* seedlings

	Granny Smith apples[a]			*P. patula* seedlings[b]		
	A	B	C	A	B	C
Least squares means (L.S.M.)[c]	1.4 a	1.4 a	2.4 b	1.4 a	1.0 b	1.7 c
Standard error (S.E.)	0.09	0.1	0.06	0.2	0.2	0.1
Population size (N)	3	3	8	3	3	8

[a] Mean surface area on Granny Smith apples for the three *S. sapinea* morphotypes.
[b] Mean lesion length on *P. patula* seedlings for the three *S. sapinea* morphotypes.
[c] Values followed by the same letter (a, b, c)do not differ significantly ($P < 0.05$).

Isolates of the C morphotype however, produced significantly longer lesions than those of either the A and B morphotypes.

4. Discussion

In this study, isolates of the three morphotypes of *S. sapinea* were found to be very similar, based on cultural and conidial characteristics. The A and B morphotypes were initially described based on cultural or conidial differences (Wang et al., 1985; Palmer et al., 1987). Conidial size is, thus, far the only distinguishable morphological characteristic between *S. sapinea* isolates belonging to the C morphotype and those belonging to the A and B morphotypes (De Wet et al., 2000). Other conidial characteristics considered in this study were insufficient to distinguish between isolates of the three morphotypes. The conidial walls of the A morphotype isolates were found to be generally smooth and those of the B morphotype isolates were pitted, which is consistent with previous observations (Wang et al., 1985; Palmer et al., 1987). The conidia of most C morphotype isolates had smooth walls making them indistinguishable from those of the A morphotype. The conidia of some C

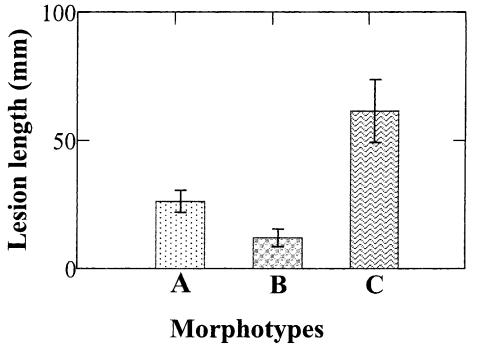

Fig. 2. Mean lesion length (±S.E.M.) on *P. patula* seedlings after inoculation with isolates representing the three morphotypes of *S. sapinea*. Repeated one-way ANOVAs based on the least squares means were used to analyse data (SYSTAT version 7.0.1).

morphotype isolates, however, had a rougher texture but no obvious pits, such as those described for the B morphotype. The septation of A and C morphotype conidia was the same but different to that of the B morphotype conidia. Thus, both the A and C morphotype conidia usually have no or only one septum, while the B morphotype conidia can be multi-septate. The fact that C morphotype conidia are longer than those of both the A and B morphotypes (De Wet et al., 2000), is thus, the only useful morphological characteristic that can be used to distinguish between all three morphotypes of *S. sapinea*.

Morphological characteristics in *S. sapinea* are highly variable and dependant on many factors, apart from the genetic composition of the fungus. These include external factors, such as environmental conditions, the nutrient composition of the growth medium and sample preparation methods for microscopy (Wang et al., 1985, 1986; Swart and Wingfield, 1991; Swart et al., 1991). Reports of isolates of *S. sapinea* that are neither typical of the A or B morphotypes have been made (Wang et al., 1985; Swart et al., 1991; Stanosz et al., 1999). Results of this study suggest that the C morphotype isolates have morphological characteristics that are most like those of A morphotype isolates. This is consistent with recently published sequence data that also show the C morphotype to be more similar to the A than to the B morphotype (De Wet et al., 2000). The C morphotype was first recognised based on RAPD markers and this technique allows reliable recognition of the three forms of *S. sapinea*.

No differences were detected in vitro growth rate of isolates representing the A and C morphotypes of *S. sapinea,* but they grow more rapidly than isolates belonging to the B morphotype. However, isolates belonging to the C morphotype of the fungus were obviously more pathogenic than those isolates of the A and B morphotypes. Lesions produced by the A morphotype isolates were larger than those of the B morphotype and this is consistent with previously published results (Wang et al., 1985, 1986; Palmer et al., 1987; Smith and Stanosz, 1995). The B morphotype isolates produced small or even no lesions when inoculated into *P. patula* seedlings. This could be due to host specificity, as this form of *S. sapinea* was, until recently, only known on *P. resinosa* Aiton. and *P. banksiana* Lamb. (Wang et al., 1985; Smith and Stanosz, 1995; Stanosz et al., 1999). Results of pathogenicity tests on both Granny Smith apples and *P. patula* seedlings were similar and it might be possible to use apples for rapid screening of isolates for relative pathogenicity.

The discovery of a third form of *S. sapinea*, now known as the C morphotype of the fungus, has important quarantine implications. Prior to its discovery, the A morphotype of the fungus was recognised as considerably more pathogenic than the B morphotype (Wang et al., 1985, 1986; Palmer et al., 1987; Smith and Stanosz, 1995). The A morphotype is also the form of the fungus that has assumed a wide distribution in countries of the world, such as South Africa, Australia and New Zealand, where pines are grown as exotics (Lückhoff, 1964; Marks and Minko, 1969; Currie and Toes, 1978; Swart et al., 1991). This wide distribution might be attributed to the higher level of pathogenicity of this form of the fungus compared to isolates belonging to the B morphotype. The fact that isolates of the C morphotype of *S. sapinea* are considerably more virulent than those of the A morphotype is important. *Sphaeropsis sapinea* is well recognised to be seed-borne and it has apparently been widely distributed in countries that grow pines as exotics, together with seed (Currie and Toes, 1978; Smith et al., 1996, 2000). The high level of pathogenicity of C morphotype isolates suggests that great care should be taken when importing seed from Indonesia and Mexico. The fact that the fungal species is already in a country should not be used to imply reduced risk. The danger of introducing additional and

more virulent genotypes of *S. sapinea* should be carefully considered.

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