Variation in conidial morphology among geographic isolates of *Sphaeropsis sapinea*

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In the U.S.A., *Sphaeropsis sapinea* is believed to occur in at least two distinct forms (type A and B) that can be distinguished by their conidial wall ornamentation. In this study, mature conidia of 50 monoconidial isolates of *S. sapinea* from a worldwide collection were examined using SEM to determine whether these two forms occurred more widely. Twenty *S. sapinea* isolates consistently had conidia with smooth outer surfaces typical of type A. The remaining 30 isolates had typical pits or similar indentations occurring on between 6 and 38% of conidia examined. Pits, or indentations, either occurred uniformly on the outer surface or only on some parts of individual conidia. Authentic *S. sapinea* isolates, categorized as type A and type B in previous studies, were chosen for detailed examination of the effect of spore age, nutrition, and pigmentation on surface ornamentation. No distinct differences in conidial morphology between the two types were discernible between conidia of different ages, or those produced on media containing different carbon or nitrogen sources. The melanin inhibitor, tricyclazole, was used to compare melanin-deficient conidia with pigmented conidia. Although mature, melanin-deficient conidia were hyaline, this did not appear to influence the occurrence of pits on conidia. The results of these studies strongly suggest that conidial morphology of *S. sapinea* is a variable characteristic and, therefore, a poor taxonomic criterion.

Sphaeropsis sapinea (Fr.: Fr.) Dyko & Sutton is a pathogen of cosmopolitan distribution causing a wide range of disease symptoms on conifer species (Punithalingam & Waterston, 1970; Gibson, 1979; Swart, Knox-Davies & Wingfield, 1985). Symptoms associated with *S. sapinea* include damping-off and collar rot of seedlings, and shoot blight, canker, sap-stain and root disease of mature trees (Punithalingam & Waterston, 1970; Gibson, 1979; Wingfield & Knox-Davies, 1980; Palmer & Nicholls, 1985; Chou, 1987). Infection by the pathogen usually occurs in trees that are physiologically stressed or that have been wounded (Bachi & Peterson, 1985; Chou & Mackenzie, 1988; Swart, Wingfield & Knox-Davies, 1987; Nicholls & Ostry, 1990).

There is considerable evidence for variation in morphology and pathogenicity among isolates of *S. sapinea*. At least two distinct forms (designated as type A and type B) have been distinguished in the north central United States on the basis of cultural characteristics, conidial size, pathogenicity and isozyme patterns (Palmer, Stewart & Wingfield, 1987). A study of conidium morphology of 30 isolates of *S. sapinea* from 10 countries, including the United States, substantiated the existence of these two forms (Wang *et al.*, 1985). Mature conidia from all isolates with type A cultural characteristics had conidia with smooth surfaces while isolates that were similar to type B in culture, had conidia with pitted surfaces.

Significant variation in cultural characteristics, conidial size and morphology and pathogenicity have recently been found in 10 South African isolates of *S. sapinea* (Swart *et al.*, 1991). There was, however, no indication that these isolates could be classified into distinct groups based on the above criteria. The reliability of conidial pitting as a taxonomic characteristic to separate isolates of *S. sapinea* was thus questioned.

The pitted appearance of spores of some fungi has been attributed to the absence of pigmented substances such as melanin in the cell walls (Henderson, Eudall & Prentice, 1972; Durrieu, 1974; Ellis & Griffiths, 1974; Griffiths & Swart, 1974). The presence of pigment in spores of *Diplodia*- and *Sphaeropsis*-like fungi is dependent on the maturity of conidia and various nutritional factors of which carbon and nitrogen sources are particularly important (Satour, Webster & Hewitt, 1969*a*, *b*; Webster, Hewitt & Bolstad, 1974). It follows that these and other factors could possibly influence the presence of pits on conidia of *S. sapinea*.

The objectives of the study reported here were twofold: firstly, to determine the extent of variation in conidial morphology among isolates of *S. sapinea* from worldwide collections and secondly, to investigate the effect of physiological factors such as nutrition, pigmentation and maturity on the surface morphology of *S. sapinea* conidia.

MATERIALS AND METHODS

Scanning electron microscopy of world isolates

Fifty single-conidial isolates of *S. sapinea* from 10 countries (Table 1) were grown in 90 mm diameter Petri dishes containing water agar (WA) (Difco Bacto agar 20 g; distilled water 1000 ml) on which sterile pine needles (*Pinus radiata* D. Don) had been placed. Plates were incubated for approximately

Table 1. Collection data and spore morphology of 50 isolates of *Sphaeropsis sapinea*

Geographic origin	Collector	Isolate no.*	Alternative ref. no.	Host	% conidia with pits
S. Africa	W. Swart	CWS 1	PREM 48859†	*Pinus radiata* D. Don	0
		CWS 5	PREM 49116		24
		CWS 7	—		22
		CWS 8	PREM 48892		10
		CWS 10	PREM 49117		12
		CWS 11	—		6
		CWS 15	PREM 49118		8
		CWS 21	—		0
		CWS 22	—		0
		CWS 23	—		0
		CWS 6	PREM 48860	*P. taeda* L.	30
		CWS 14	—	*P. patula* Schlecht. & Cham.	0
		CWS 27	PREM 49119		12
		CWS 29	PREM 49120		8
		CWS 28	—	*P. radiata*	0
		CWS 30	PREM 49121	*P. taeda*	0
		CWS 31	—	*P. halepensis* Mill.	0
		CWS 32	PREM 49122	*P. elliottii* Engelm.	0
		CWS 33	PREM 49123	*P. virginiana* Mill.	10
Zambia	J. Gibbs	CWS 57	270	*P. oocarpa* Schiede	0
Britain		CWS 52	345	*P. nigra* Arnold	14
		CWS 53	346		20
		CWS 55	348	*P. muricata* D. Don	18
		CWS 58	0902	*P. sylvestris* L.	12
		CWS 78	CC.183	*P. nigra*	8
		CWS 79	CC.185	*P. nigra*	12
		CWS 80	CC.189	*P. nigra*	22
		CWS 81	CC.190	*P. nigra*	0
		CWS 82	CC.191	*P. nigra*	32
Honduras		CWS 56	216	*P. oocarpa*	12
Spain	J. Luque	CWS 44	—	*Cedrus atlantica* (Endl.) Carr.	0
N. Zealand	S. Chou	CWS 47	D5	*P. radiata*	0
		CWS 49	D7	*P. muricata*	0
Australia	E. Davison	CWS 66	DCE 404	*P. radiata*	0
		CWS 67	DCE 402		10
Chile	H. Butin	CWS 40	D 262		0
		CWS 41	D 261		0
U.S.A.					
Michigan	M. Palmer	CWS 59	113	*P. banksiana* Lamb.	38
		CWS 60	120	*P. resinosa* Ait	12
Missouri		CWS 61	170	*P. nigra*	0
Wisconsin		CWS 62	215	*P. resinosa*	18
		CWS 63	411		0
		CWS 64	414		16
		CWS 65	463		28
Florida	S. Fraedrich	CWS 68	ASD/11	*P. elliottii*	12
		CWS 69	SD3/13		32
		CWS 70	SW3/17		12
		CWS 71	SW#/8		12
		CWS 72	SW3-1/24		0
		CWS 73	SW3-2/25		8

* Numbers having a CWS prefix represent isolates of *S. sapinea* that are maintained at the Department of Plant Pathology, University of the Orange Free State, Bloemfontein, South Africa.

† Numbers having a PREM prefix represent isolates of *S. sapinea* that are deposited at the National Collection of Fungi, Pretoria, South Africa.

four weeks at 25 °C under one nuv fluorescent lamp ('black-light') and one cool white fluorescent lamp on a 12 h cycle.

Once sporulation had occurred, plates were flooded with water to allow for the release of mature conidia. The conidial suspension of each isolate was vacuum-filtered through a 5·0 µm polycarbonate membrane filter (Nucleopore Corp., Pleasanton, CA 94566) and a circular portion of filter containing a high concentration of conidia was mounted on an aluminium stub. Specimens were vapour fixed at room temperature for 24–48 h by inverting each stub in a 20 ml McCartney bottle containing 2 ml 2% osmium tetroxide (OsO_4). Samples were allowed to air-dry for 3–5 d and then coated with gold-palladium before being examined with a Jeol JSM 6400 SEM at 15 kV. Fixation by immersion in aqueous fixative solutions, dehydration in graduated acetone series and critical-point drying were substituted, in this work, by vapour

fixation and air-drying to prevent unnecessary disturbance and distortion of spores on membrane filters. Vapour fixation also eliminates the need for continual attendance during sample preparation.

Effect of maturity on spore ornamentation

The effect of age on spore surface ornamentation was examined using isolates of *S. sapinea* from the north central U.S.A. supplied and categorized as type A and type B by Dr M. A. Palmer (c/o Dept of Plant Pathology, University of Minnesota, St Paul, MN 55108). Sporulation of the two type-A isolates (CWS 60 and CWS 61) and two type-B isolates (CWS 59 and CWS 64) (Table 1) was induced as described above. Pycnidia were removed from pine needles using a fine scalpel, placed on a membrane filter and broken in a drop of water to liberate both juvenile and mature conidia. Five aluminium stubs were prepared for each isolate. One stub of each isolate was immediately vapour-fixed in OsO_4, coated with gold-palladium and examined using SEM. The remaining four stubs of each isolate were stored in closed Petri plates at room temperature. Every fortnight, a single stub of each isolate was vapour-fixed, coated with gold-palladium and examined using SEM.

Effect of nutrition on spore morphology

A 2% WA medium was supplemented, individually with 30 g l^{-1} of maltose, glucose, xylose and sorbose as sources of carbon. Four amino acids, cystine, lysine, arginine and alanine, as sources of nitrogen, were added individually in amounts of 20 g l^{-1} of WA medium. Each compound was added before sterilizing the medium at 121° for 15 min. WA that did not contain sugars or amino acids, served as a control. Media were poured into 90 mm diam. Petri dishes and allowed to cool, whereupon sterile cellophane (Visking tubing) was placed over the agar. Plugs of WA on which the two type-A and two type-B isolates had been cultured were transferred to each of the above media and sporulation was induced as described previously. Pycnidia were then removed from the cellophane using a fine scalpel, broken in a drop of water on a membrane filter and prepared for SEM. Fifty conidia of each isolate were examined.

Effect of melanin inhibition on conidial morphology

A culture medium was prepared comprising 2% malt extract agar (MEA) and 100 ppm of the melanin inhibitor, tricyclazole (5-methyl-1,2,4-triazole (3,4)-benzo-thiazole) (Hyakumachi, Yokoyama & Ui, 1987). A layer of sterile cellophane or autoclaved pine needles was placed directly on the agar medium and small agar plugs from cultures of the authenticated type A and B isolates were transferred to the medium. Plates were incubated as described above to induce sporulation. Isolates grown on 2% MEA without tricyclazole served as controls. After sporulation had occurred, conidia were prepared for SEM and 50 conidia of each isolate, cultured with and without tricyclazole, were examined.

Pycnidia were prepared for TEM by fixing 2 mm lengths of pine needles bearing sporulating pycnidia in 3% glutaraldehyde in 0·1 M phosphate buffer (pH 7·0) for 5 h and 2% OsO_4 in 0·1 M phosphate buffer for an addition 4 h. The material was then dehydrated in a graduated acetone series and embedded in Spurr's embedding medium (Spurr, 1969). Thin sections were cut through pycnidia with glass knives, poststained with 0·5% aqueous uranyl acetate (15 min) and Reynolds' (1963) lead citrate (7 min) and observed with a Philips EM300 TEM.

RESULTS

Scanning electron microscopy of world isolates

Twenty isolates of *S. sapinea* consistently had conidia with smooth outer surfaces (Fig. 1) typical of those reported for the type-A form (Table 1). The remaining thirty isolates had between 6 and 38% of conidia with small indentations, or pits, distributed either over the entire outer surface, or parts of individual conidia (Figs 2, 3).

Effect of maturity on conidial ornamentation

The age of conidia did not appear to affect the occurrence of pits in any of the four isolates examined (Table 2). Over the entire 56 d period, the two type-A isolates, CWS 60 and CWS 61, respectively had means of 19·6 and 18·4% pitted conidia. The two type-B isolates, CWS 59 and CWS 64, respectively had means of 23·6 and 18·4% of pitted conidia over the same period.

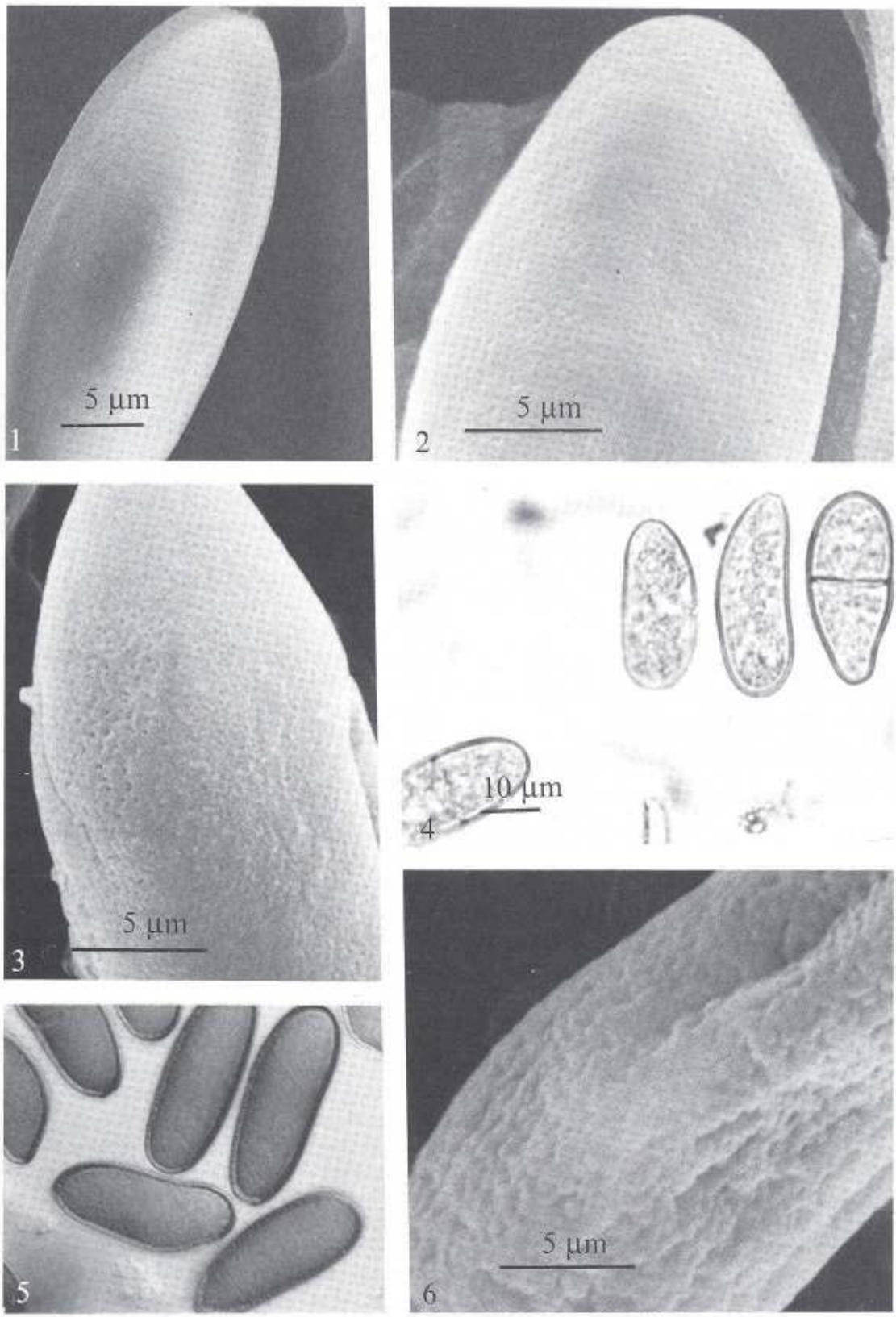
Effect of nutrition on spore ornamentation

No distinct differences in conidial morphology between typical A-type and type-B isolates were discernible following sporulation on media supplemented with specific sugars or amino acids (Table 2). On the four sugars, means of 13 and 13·5% of conidia were pitted for isolates CWS 60 and CWS 61, respectively. Isolates CWS 59 and CWS 64, respectively, had means of 26·5 and 19% of pitted conidia. On the four amino acids, means of 8·5 and 12·5% of conidia were pitted for isolates CWS 60 and CWS 61, respectively. Isolates CWS 59 and CWS 64, respectively, had means of 20·5 and 19% of pitted conidia on the four amino acids.

Effect of melanin inhibition on conidial morphology

Isolates grown on MEA containing tricyclazole were white to creamy without showing any tendency to become darker in colour. This was in contrast to isolates grown on the control plates that turned dark olive brown and eventually black after approximately 1 wk of incubation. Examination of mature conidia using light microscopy revealed that conidia were virtually hyaline and not the typical brown colour of those formed on control plates (Figs 4, 5).

Examination of conidia belonging to each of the four isolates using SEM did not provide any evidence of pitting being influenced by the lack of melanin in conidial walls (Table 2). The general appearance of conidia having normal pigmentation did, however, differ from those lacking melanin in their walls. Approximately 50% of the latter group had an irregular form owing to a collapse of the cell walls (Fig. 6).



Figs 1–6. SEM and light micrographs of mature conidia of *Sphaeropsis sapinea*. **Fig. 1.** Part of conidium wall with smooth outer surface. **Fig. 2.** Part of conidium wall with pits on entire outer surface. **Fig. 3.** Part of conidium wall with indentations on part of outer surface. **Fig. 4.** Conidia formed on 2% MEA containing the melanin inhibitor, tricyclazole. **Fig. 5.** Conidia formed on 2% MEA without tricyclazole. **Fig. 6.** Part of conidium formed on MEA containing tricyclazole.

Table 2. Effect of carbon and nitrogen sources, conidial age and tricyclazole on conidial morphology of authenticated type-A and type-B isolates of *Sphaeropsis sapinea* from the north-central United States*

	% conidia with pits or indentations†			
	Type A		Type B	
	CWS 60	CWS 61	CWS 59	CWS 64
Carbon source				
Maltose	12	10	26	12
Glucose	10	2	24	18
Xylose	22	30	32	28
Sorbose	8	12	24	18
Control	20	28	32	26
Nitrogen source				
Cystine	0	24	14	8
Lysine	10	12	26	22
Arginine	8	16	18	22
Alanine	16	18	24	14
Control	16	22	24	26
Age of conidia‡				
0	20	16	20	32
14	12	12	14	12
28	16	22	26	14
42	24	26	34	16
56	26	16	24	18
Tricyclazole				
Present	16	24	26	28
Absent	24	32	28	34

* Type-A isolates are reported to have smooth conidial walls whereas type-B isolates have pitted walls (Wang *et al.*, 1986).

† A total of 50 conidia was examined for each treatment.

‡ Number of days since conidia were released from pycnidia and stored at room temperature.

These misformed conidia were, therefore, not examined for the appearance of pits.

Examination of type-A and type-B conidia with normal pigmentation using TEM revealed two distinct layers within conidial cell walls and numerous lipid bodies in the cytoplasm (Figs 7, 8). The outer layer of walls in both isolate types had considerably darker pigmentation than the inner zone. The pigmentation in the outer zone of both type-A and type-B conidia was, however, not uniform but interspersed with areas lacking pigment (Fig. 9). The inner zone was approximately 3–4 times wider than the outer zone in both isolate types. The inner zone of the type-B conidial walls appeared to have a smoother texture than those of type-A.

There were distinct differences in wall structure of pigmented and non-pigmented conidia. The definition of two distinct wall layers and the presence of lipid bodies in the cytoplasm was considerably less distinct than that observed in pigmented conidia. Cell walls of non-pigmented conidia were significantly more granular in appearance with irregularly sized, pigmented granules densely deposited in the outer zone but sparse in the inner zone (Fig. 10).

DISCUSSION

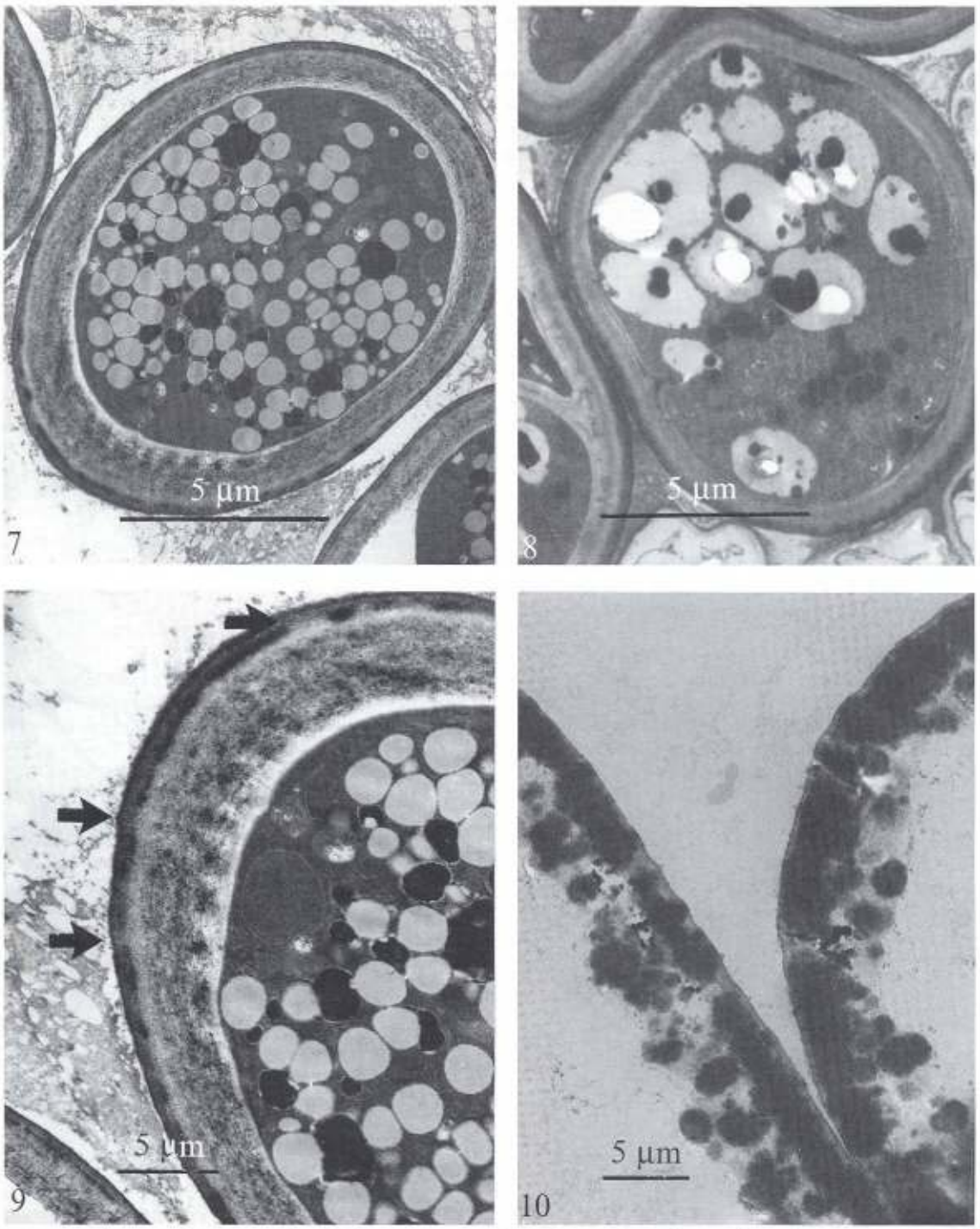
Examination of 50 isolates of *S. sapinea* from a worldwide collection showed that the majority of isolates had mature conidia that were both pitted and smooth. The results of the current study are generally consistent with a previous study conducted with 10 South African isolates of *S. sapinea* (Swart *et al.*, 1991). Results of both studies are, however, in contrast to those of Wang *et al.* (1985) who reported that thirty isolates of *S. sapinea* from 10 countries could be separated into two distinct forms of isolates that had either exclusively smooth or pitted conidia.

Conidia with pits or indentations were observed in 30 of the isolates examined in the present study. The percentage of conidia having pits varied between 6 and 38% among the different isolates (Table 1). Variation in the degree of pitting present on individual conidia of any particular isolate was also observed. Wang *et al.* (1985) found that only 7 out of 30 isolates had pits and these were thus designated as type-B isolates. All conidia of type-B isolates had distinct pits on the outer surface except when immature, when they appeared smooth. Although these authors did not reveal the number of conidia examined for each isolate, it is unlikely that the low percentage of pitted conidia per isolate, or the degree of pitting on individual conidia, observed in the present study is attributable to examination of a high percentage of immature conidia. Conidia examined in the present study had already been expelled from pycnidia, and thus had a greater chance of being mature than when exposed within pycnidia such as those of Wang, Blanchette & Palmer (1986). The above results suggest that smooth conidia are the norm for *S. sapinea*.

Examination of conidia from authenticated type-A and type-B isolates provided the most convincing evidence for a lack of consistency in the presence or absence of pits among isolates of *S. sapinea* in this study. Although the results are in contrast with those of Wang *et al.* (1985, 1986), they are consistent with the results of an independent study conducted in the Netherlands with 30 isolates of *S. sapinea* including authenticated type-A and type-B isolates from the north central United States (de Kam, 1990; van Dam, 1990). The latter study showed that all 30 isolates produced conidia with and without pits, and that results were influenced by the method of preparation for SEM.

This study could find no proof that the pigmentation of conidia of *S. sapinea* as influenced by nutrition of the fungal colony, or conidial age, played any role in determining the presence or absence of pits in cell walls. The use of tricyclazole was demonstrated here as having a dramatic inhibitory effect on the formation of melanin in conidia of *S. sapinea* when observed with a light microscope. However, no relationship between hyaline conidia and pitting could be demonstrated. Transmission electron micrographs, although showing a distinct loss of structural integrity in melanin-deficient conidia nevertheless still contained dark granules. This might be due to greater penetrability and effectiveness of stains used in TEM on conidia with walls devoid of melanin (Hayat, 1970).

Wang *et al.* (1986) noted the presence of pigmented granules in the cell walls of mature conidia of *S. sapinea*, an observation that is substantiated by the present study. The authors postulated that the lack of these pigmented granules in mature cell walls of *S. sapinea* is responsible for the pitted appearance observed in type-B conidia due to a collapse of the conidium wall in that specific area. This hypothesis is consistent with observations by Durrieu (1974) to explain the



Figs 7–10. TEM of sectioned mature conidia of *Sphaeropsis sapinea*. **Fig. 7.** Section through a conidium of a type-A isolate (CWS 60). **Fig. 8.** Section through a conidium of a type-B isolate (CWS 54). **Fig. 9.** Section through part of a conidium of a type-A isolate (CWS 60) showing areas lacking pigment in outer cell wall (arrows). **Fig. 10.** Two adjacent conidial walls of a type-B isolate (CWS 59) grown on malt extract agar containing tricyclazole.

formation of pitted teliospores in *Puccinia cervariae* Lindr. Wang *et al.* (1986) could not, however, substantiate their hypothesis, because depressions within conidial walls were not discernible in transmission electron micrographs. The authors assumed that TEM preparation techniques might have hidden the depressions. The above hypothesis is, however, also inconsistent with observations of the present study which showed that areas devoid of pigmentation in the outer conidial wall occurred in conidia of both type-A and B (Fig. 9).

The considerable variation in the physical appearance of pits observed in this study, is further cause for scepticism regarding the use of pits as a taxonomic criterion. It suggests, as stated by de Kam (1990), that pits or indentations are artifacts which result from SEM preparation procedures. Preparation procedures for SEM are, for example, known to influence the presence of pits in the outer surface of teliospores of *Tilletia indica* (Mitra) Mund. (Hess & Gardner, 1988). The choice of vapour-fixation in the present study as a preparation

procedure for SEM was deliberately made to avoid the possibility of artifacts.

The inconsistencies observed among isolates of *S. sapinea* with regard to their conidial surface morphology strongly suggests that pitting is a very variable characteristic. It is, therefore, unsuitable as a taxonomic criterion in *S. sapinea* and should be avoided.

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