Variation Among South African Isolates of *Sphaeropsis sapinea*

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ABSTRACT

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Ten isolates of *Sphaeropsis sapinea* (syn. *Diplodia pinea*) obtained from various naturally infected *Pinus* spp. in South Africa were screened for differences in cultural characteristics, virulence, and isozyme profiles of five enzymes. Considerable variation among isolates existed in the length and width of conidia, but this did not allow for the separation of isolates into specific groups. Scanning electron microscopy revealed that two isolates had distinct pits over the entire outer surface of 30% of the conidia examined. Three isolates consistently had smooth conidia, and five isolates were considered intermediate. The latter had some entirely smooth conidia and some with small indentations that were not distinct pits and occurred on only parts of individual conidia. Three isolates grew more slowly ($P = 0.01$) on five culture media tested and at 20 and 25 C on malt extract agar. There was a significant positive correlation between in vitro growth rate and virulence of the 10 isolates in growth chamber and field inoculations. The isozyme banding patterns of the three isolates with smooth-walled conidia and four intermediate isolates were identical to those of a typical smooth-spored isolate of *S. sapinea* from the north central United States for each of the five enzymes tested. The banding patterns of the two isolates with pitted conidia and one intermediate isolate were identical to those of isolates that had smooth conidia for all enzymes except malic dehydrogenase. None of the 10 local isolates displayed banding patterns associated with a typical isolate of *S. sapinea* having pitted conidia from the north central United States.

Additional keywords: isozyme analysis, *Pinus radiata*.

The fungus *Sphaeropsis sapinea* (Fr.:Fr.) Dyko & Sutton is an important pathogen of *Pinus* spp. in many countries in both the Northern and Southern hemispheres (9,13). It has been associated with damping-off, shoot blight, branch and bole canker, sap stain, and root disease (4,7,9,13,16,20,21). A number of disease situations associated with *S. sapinea* in South Africa are either unique to that country or occur only rarely in other parts of the world. The association of *S. sapinea* with a root disease of pines has been reported only in South Africa (16,21). The notoriety of the pathogen may also be attributed largely to the South African experience. Dieback of *P. radiata* D. Don and *P. patula* Sclechtend. & Cham. in South Africa is the best example in the world of large-scale infection by *S. sapinea* following hail damage (9,16).

Conflicting reports regarding the necessity of wounds for infection by *S. sapinea* (7,10,11,14,20) and of significant differences in cultural characteristics (1) and conidial size (2) of isolates of *S. sapinea* suggest that different strains of the fungus exist. Two groups of isolates of *S. sapinea* (designated type A and type B) have been distinguished in the north central United States on the basis of cultural characteristics, isozyme patterns (12), and conidial morphology (18,21). These isolates were obtained from various *Pinus* spp. and locations in the north central United States. Type A isolates have fluffy white to gray-green mycelium, produce conidia with smooth surfaces, and can infect unwounded pine tissue. Type B isolates have white to black mycelium closely appressed to the agar surface, produce conidia with pitted surfaces, and require wounds to infect pine tissue.

Evidence of variation among isolates of *S. sapinea* from other parts of the world is less definitive. No differences in pathogenicity could be found among 18 isolates of *S. sapinea* from New Zealand (4). In contrast, variation in pathogenicity among South African isolates of *S. sapinea* was first mentioned by Laughton in 1937 (10) and has since been confirmed by Swart et al (16,17). The

aim of our study was to determine the range of differences in cultural and morphological characteristics and virulence among isolates of *S. sapinea* from South Africa. Isozyme analyses were used to determine whether the South African isolates could be separated into the type A and B groups recognized in the north central United States.

MATERIALS AND METHODS

Isolates. Isolations were made on 2% malt extract agar (MEA; Difco Laboratories, Detroit, MI) from *Pinus* spp. displaying shoot blight or root disease symptoms in various locations in South Africa. The presence of *S. sapinea* in diseased material was confirmed by identification according to the description of Sutton (15). Monoconidial isolates were maintained at 18 C on MEA slants. Ten isolates (Table 1) with marked differences in cultural appearance were used in all experiments unless otherwise indicated. All chosen isolates were deposited in the National Fungus Collection, Pretoria (PREM).

Conidial characteristics. Isolates were grown under continuous black light at 18 C on 2% water agar (WA; Difco) with sterile needles of *P. radiata* on the agar surface. For each isolate, the length and width of 100 conidia from pycnidia on pine needles were measured (Table 1) after 14 days. A one-way analysis of variance (ANOVA) was performed on conidial measurements. Tukey's HSD procedure was applied to compare mean conidial width and length. Pycnidia were prepared for scanning electron microscopy (SEM) according to the methods of Wang et al (19), and 50 conidia of each isolate were observed for pitting.

Effect of media on growth. Isolates were grown on the following media: potato-dextrose agar (PDA; Difco), MEA, Czapek-Dox agar (CD; Merck), WA, and cornmeal agar (CMA; Difco). A plug of each isolate, 5 mm in diameter, from an actively growing MEA culture was placed fungus-side down in the center of a petri dish, 90 mm in diameter, containing 25 ml of medium. Each treatment was replicated four times. The dishes were sealed with Parafilm (American Can, Greenwich, CT) and incubated in the

dark at 25 C. Colony diameters were measured along two perpendicular lines after 96 hr. The experiment was arranged as a randomized complete block design and conducted four times. Variances among trials were tested for homogeneity by Bartlett's test, and a two-way ANOVA was performed on the pooled data. Tukey's HSD procedure for comparison of means was applied where analyses of variance showed significant variation. Observations of culture morphology were made 7 and 14 days after isolates were transferred to test media.

Growth at different temperatures. Isolates were transferred as above to petri dishes, 90 mm in diameter, containing 15 ml of MEA and incubated at 10, 15, 20, 25, 30, and 35 C. Each treatment was replicated three times. Colony diameters were measured after 96 hr. The experiment was arranged as a randomized complete block design and conducted three times. Variances among trials were tested for homogeneity, and a two-way ANOVA was performed on the pooled data. Tukey's HSD procedure was applied for comparison of means.

Growth chamber inoculations. Seedlings of *P. radiata* were raised on steam-pasteurized soil in plastic sleeves for 12 mo. Seedlings were preconditioned (6) 5 wk before inoculation by transferring them to a growth chamber maintained at 22–25 C and 75% relative humidity, with cool white fluorescent lighting (10^3 lx, 12 hr/day).

Ten monoconidial isolates of *S. sapinea* (Table 1) were grown on 2% WA covered with autoclaved needles of *P. radiata* and incubated at 25 C under near ultraviolet light (12 hr/day) for 3 wk. Needles bearing pycnidia were then agitated in a 0.5% gelatin solution at 10 C for 30 min to release conidia. After the needles were removed, the suspension was adjusted to 1×10^4–1.5×10^4 conidia per milliliter.

Seedlings were wounded by puncturing the terminal shoot, 25 mm from the tip and approximately 3 mm deep, with three 12-gauge hypodermic needles set 5 mm apart. Both wounded and unwounded shoots were inoculated by applying inoculum to the shoots with a soft camel-hair brush (5). During inoculation, the conidial suspension was kept in an ice bath to prevent premature spore germination. Control plants were treated similarly but with a sterile 0.5% gelatin solution. After inoculation, all plants were covered for 48 hr with clear plastic bags and held in the growth chamber. The number of seedlings with dead tops was recorded after 3 wk, and isolations were made from all seedlings with disease symptoms. Each of three trials was arranged as a randomized complete block design with 10 seedlings per treatment. Data were expressed as percentages and transformed by the arcsine square root transformation. Homogeneity of variances among trials was tested via Bartlett's test, and a two-way ANOVA was performed on the pooled data (Table 2).

Field inoculations. Four-year-old trees of *P. radiata* were inoculated by the following procedure. Trees were wounded on each of 11 lateral branches by removing cambial disks with a cork borer, 8 mm in diameter. Disks of 4-day-old monoconidial cultures of 10 isolates of *S. sapinea* (Table 1) grown on 1% MEA

were placed in the wounds, one isolate per wound per tree. A sterile MEA disk was placed in the 11th wound of each tree. The wounds were covered with Parafilm. After 6 mo, bark and phloem surrounding the inoculation points were removed with a scalpel, and the length of the discolored cambium was measured. Isolations were made from the discolored cambium to confirm pathogenicity. Each of three trials was arranged as a randomized complete block design with 20 wounds per isolate. Variances among trials were tested for homogeneity, and a two-way ANOVA was performed on the pooled data. Tukey's HSD procedure was applied to separate treatment means among isolates (Table 2).

Isozyme analysis. Isolates were prepared for starch-gel electrophoresis according to the method of Palmer et al (12). The enzyme stains screened for activity of *S. sapinea* were alcohol dehydrogenase, fluorescent esterase, β-glucosidase, malic dehydrogenase (MDH), and sorbitol dehydrogenase. The entire electrophoresis procedure was repeated three times for each isolate. For the purpose of making comparisons, a type A isolate (A123) and a type B isolate (B124) (12) were included in each gel run.

RESULTS

Conidial characteristics. Significant differences were found in the sizes of conidia of the various isolates (Table 1). The variation in the length of isolates was no greater than the range given for *S. sapinea* by Punithalingam and Waterston (13), but widths were generally greater.

Isolates PREM 49116 and 48860 had distinct pits over the entire surface of approximately 30% of the conidia examined by SEM (Fig. 1A and B). Several isolates had some conidia with various degrees of indentation on parts of the conidium. These included PREM 48892, 49117, 49118, 49119, and 49120 (Fig. 1C and D). These conidia had pits unlike those of PREM 49116 and 48860. However, isolates PREM 49121, 49122, and 49123 always had smooth conidia (Fig. 1E and F).

Effect of media on growth. All 10 isolates had either fluffy white or black mycelium on MEA, PDA, CMA, and CD. On WA, the mycelium of all isolates was closely appressed to the agar surface and varied from black to almost transparent. Growth varied significantly ($P = 0.01$) among isolates and media (data not shown). The isolate × medium interaction was also significant ($P = 0.01$), and thus inferences about main-effect treatments cannot be made. Interactions were of both change-in-rate and

TABLE 1. Conidial characteristics of 10 isolates of *Sphaeropsis sapinea* from *Pinus* spp. in South Africa

Isolate	*Pinus* host	Associated symptoms	Conidial size (μm)[z] Length	Width
PREM 48860	*P. taeda*	Root disease	42.0 b	18.8 ab
PREM 48892	*P. radiata*	Canker	39.7 bc	17.0 d
PREM 49116	*P. radiata*	Shoot blight	39.7 bc	17.3 bc
PREM 49117	*P. radiata*	Shoot blight	40.9 bc	15.0 f
PREM 49118	*P. radiata*	Root disease	38.7 c	17.2 cd
PREM 49119	*P. patula*	Root disease	38.2 c	18.2 abc
PREM 49120	*P. patula*	Shoot blight	35.0 d	16.7 de
PREM 49121	*P. taeda*	Root disease	40.0 bc	15.5 ef
PREM 49122	*P. elliottii*	Root disease	45.2 a	19.0 a
PREM 49123	*P. virginiana*	Root disease	44.8 a	18.7 abc

[z] Average of 100 conidia. Values followed by different letters within columns differ significantly ($P < 0.05$) according to Tukey's HSD procedure. Mean length SE = 0.8; mean width SE = 0.5.

TABLE 2. Susceptibility of *Pinus radiata* seedlings (expressed as mean percentage of dead tops) and trees (expressed as mean length of cambial discoloration) to 10 isolates of *Sphaeropsis sapinea*

Isolate	Dead tops (%)[y] Wounded	Unwounded	Length of discoloration (mm)[z] Mean	Range
PREM 48860	50 bc	30 b	24.5 bc	12–49
PREM 48892	100 d	40 b	31.7 cde	16–52
PREM 49116	100 d	60 bc	30.3 bcde	20–55
PREM 49117	90 d	40 b	32.7 de	20–51
PREM 49118	100 d	90 d	31.2 cde	21–41
PREM 49119	90 d	40 b	35.2 de	16–60
PREM 49120	40 b	0 a	23.1 b	15–35
PREM 49121	60 bc	10 a	29.3 bcd	12–49
PREM 49122	100 d	30 b	34.6 e	23–55
PREM 49123	80 cd	50 bc	37.2 e	21–91
Control	0 a	0 a	11.6 a	...
Mean	73.6 B	35.4 A	...	...

[y] Values are the means of three growth chamber inoculation trials, each having 10 seedlings per treatment. The number of seedlings with dead tops was recorded 3 wk after inoculation. Values followed by different lowercase letters and means followed by different uppercase letters differ significantly ($P < 0.05$) according to Tukey's HSD procedure.

[z] Values are the means of three field inoculation trials, each consisting of 20 trees, with 11 treatments per tree. The length of the discolored cambium was recorded 6 mo after inoculation. Values followed by different letters differ significantly ($P < 0.05$) according to Tukey's HSD procedure; SE = 2.3.
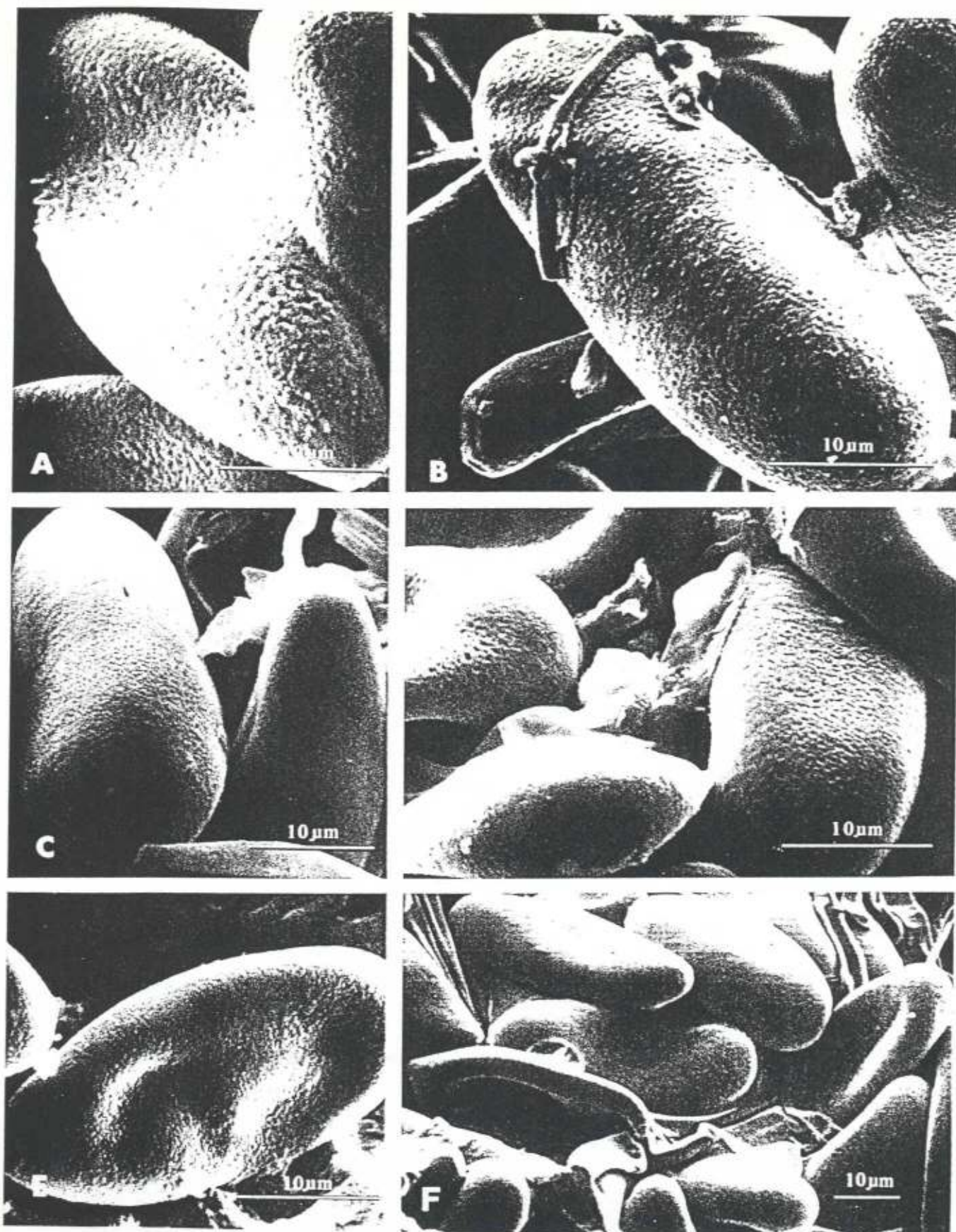
Fig. 1. Scanning electron micrographs of conidia of *Sphaeropsis sapinea*. A and B, Pitted conidial walls of isolates PREM 49116 and 48860, respectively. C and D, Conidia of isolates PREM 49119 and 48892, respectively, showing various degrees of indentation. E and F, Smooth conidial walls of isolates PREM 49122 and 49121, respectively.

reversal types. A general trend, however, was that PREM 48860, 49120, and 49121 had less growth than the remaining seven isolates on all five media. Other trends were that PREM 49117 had the fastest mean radial growth on all five media (74.5 mm), and PREM 48860 the slowest (32.9 mm). In general, mean radial growth of all isolates was greatest on PDA. Growth on MEA and CMA was similar but generally greater than on either CD or WA.

Growth at different temperatures. Growth varied significantly ($P = 0.01$) among isolates and temperatures (data not shown). The isolate $\times$ temperature interaction was significant ($P = 0.01$). Interactions were of both change-in-rate and reversal types. PREM 48860, 49120, and 49121, generally, had slower growth than the other seven isolates at 20 and 25 C. PREM 49117, generally, was the fastest-growing isolate, and PREM 49120 the slowest. The optimum growth for all 10 isolates occurred at 25 C.

Growth chamber inoculations. PREM 48860, 49120, and 49121 infected fewer seedlings ($P < 0.05$) than the remaining seven isolates (Table 2). All 10 isolates infected wounded seedlings more readily than unwounded seedlings. No symptoms developed on control seedlings treated with the gelatin solution.

Field inoculations. The length of cambial discoloration on branches inoculated with PREM 48860 and 49120 was less ($P < 0.05$) than that on branches inoculated with other isolates (Table 2). PREM 49119, 49122, and 49123 induced longer lesions ($P < 0.05$) than the remaining isolates.

There was a significant positive linear correlation between the mean length of cambial discoloration in inoculated trees and the radial growth of the 10 isolates on each medium tested. The r values for MEA and CMA were 0.846 and 0.823, respectively (8 df; $P < 0.01$); the values for PDA, CD, and WA were 0.753, 0.711, and 0.725, respectively (8 df; $P < 0.05$). Cambial discoloration was also positively correlated ($r = 0.739$ with 8 df; $P < 0.05$) with the mean percentage of dead shoots caused by the 10 isolates following wound inoculation in the growth chamber.

Isozyme analysis. Isozyme patterns are presented as composites of the banding patterns observed (Fig. 2). No attempt was made to interpret the genetic origin of the bands, and each isozyme, therefore, is considered an electrophoretic phenotype.

The isozyme patterns of PREM 49117, 49118, 49119, 49120, 49121, 49122, and 49123 were identical to those of isolate A123 for all five enzymes (Fig. 2A). The isozyme patterns of PREM 48860, 48892, and 49116 were identical to those of A123 for all enzymes except MDH, in which case the bands differed from those of isolates A123 and B124 (Fig. 2B). None of the South African isolates displayed a banding pattern similar to that of B124 (Fig. 2C).

DISCUSSION

Significant differences in conidial dimensions and morphology, cultural morphology, virulence, growth at different temperatures and on different media, and isozyme patterns were demonstrated for the 10 South African isolates of *S. sapinea* examined in this study. There was, however, no clear indication that isolates could be classified into two or more distinct groups based on these criteria.

Considerable variation in the length and width of conidia existed among the 10 isolates, and spore dimensions differed greatly from those given for type A and B isolates (12). The use of conidial dimensions as a criterion for distinguishing between groups within the species is, therefore, not possible.

Three isolates, PREM 49121, 49122, and 49123, consistently displayed smooth conidia like those typical of type A isolates (12). PREM 49121 was significantly less pathogenic and grew slower than type A isolates, although its isozyme pattern was that of a typical type A isolate (12). The only two isolates that fit the description of type A isolates with regard to spore surface morphology, pathogenicity, and isozyme pattern were PREM 49122 and 49123.

Only two isolates, PREM 49116 and 48860, had conidia that were pitted like those of type B isolates from the north central United States (19). The relatively low percentage of these conidia

with pits is probably related to the maturity of the conidia examined in the present study. Wang et al (19) found that pits only became visible in mature conidia. In its growth and pathogenicity, however, PREM 49116 was similar to type A isolates, although its banding patterns for MDH were unlike those of type A or B. Although PREM 48860 had more type B characteristics than any other isolate, it could not be considered a true type B isolate because of the banding pattern for MDH. Eight isolates of *S. sapinea*, therefore, showed characteristics distinct from those of type A and type B isolates.

Some conidia of isolates PREM 48892, 49117, 49118, 49119, and 49120 had small indentations on parts of the conidial surface unlike those of other pitted conidia. The indentations became more prominent under the SEM beam and possibly were due to a collapse of the cell wall caused by the beam in areas lacking pigmentation (18). Because these indentations were different from those observed previously, these isolates may also represent an intermediate form of *S. sapinea*. It is important, however, to establish whether pitting of conidia, whatever form this may take, is a consistent characteristic of a specific isolate. The maturity of conidia should be examined as a possible factor that could induce pitting in any isolate of *S. sapinea*, irrespective of other characteristics such as growth, virulence, or isozyme patterns. Other factors, such as temperature and humidity, could also play a role in pitting and should be critically examined.

The relationship existing between in vitro radial growth and virulence has not previously been demonstrated for *S. sapinea*. A similar relationship was found for isolates of *Ophiostoma ulmi* (3,8). In general, linear in vitro growth, therefore, seems to be
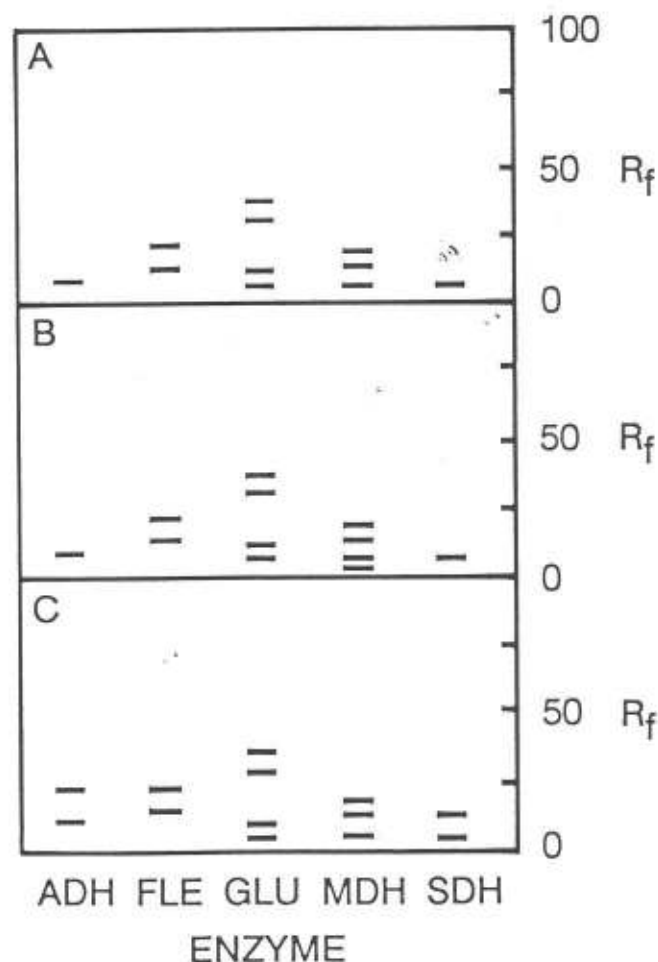

Fig. 2. Isozyme banding patterns of isolates of *Sphaeropsis sapinea*. ADH = alcohol dehydrogenase; FLE = fluorescent esterase; GLU = β-glucosidase; MDH = malic dehydrogenase; SDH = sorbitol dehydrogenase. A, Isolates A123 and PREM 49117, 49118, 49119, 49120, 49121, 49122, and 49123. B, Isolates PREM 48860, 48892, and 49116. C, Isolate B124.

a better parameter than conidial morphology for screening the virulence of South African isolates of *S. sapinea*.

We and others (1,2,18,19) have shown that *S. sapinea* is a highly variable species. Specific characteristics of isolates of *S. sapinea*, such as smooth-walled conidia, virulence, and a specific isozyme pattern, are not invariably correlated with each other in the same way that virulence and growth seem to be. For this reason, it is possible that there are more than two distinct types or that variation within the species occurs in a continuum without any definite types or strains. It is, therefore, important that isolates of *S. sapinea* from many parts of the world be compared to obtain a better understanding of the biology of this pathogen.

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