Variation Among Isolates of *Sphaeropsis sapinea* in the North Central United States

M. A. Palmer, E. L. Stewart, and M. J. Wingfield

Research plant pathologist, North Central Forest Experiment Station, St. Paul, MN 55108; professor, Department of Plant Pathology, University of Minnesota, St. Paul 55108; plant pathologist, Plant Protection Research Institute, Private Bag X5017 Stellenbosch, 7600 South Africa.

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ABSTRACT

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Isolates of *Sphaeropsis sapinea* (= *Diplodia pinea*) from naturally infected *Pinus* spp. in the north central United States differed in cultural characteristics and virulence. Isolates designated as type A produced fluffy white to gray-green mycelia on a variety of media. Conidia of these isolates produced in culture were 34.3–39.4 × 12.6–12.8 μm. Isolates designated as type B produced white to black mycelia closely appressed to the agar surface with conidia 33.5–34.3 × 11.6–12.1 μm. Type B isolates produced conidia on sterile pine needles incubated in the dark at 25 C, whereas type A isolates sporulated only in light. Type B isolates also produced spermatia-like spores in dark and light. Type B isolates generally grew more slowly than type A isolates at 20 and 25 C, although optimum growth for most type A and B isolates occurred at 25 C. Type A and B isolates had identical isozyme banding patterns for four of six enzymes. Greenhouse inoculations demonstrated that a representative type B isolate required wounds to infect young shoots, whereas the type A isolate did not. Once wounded, host tissue showed no difference in the extent of discoloration between isolates, as demonstrated by field inoculations. In the north central United States, type B isolates are apparently opportunistic and colonize wounded or declining host tissues.

Additional key word: isozyme analysis.

Sphaeropsis sapinea (Fr.) Dyko & Sutton (= *Diplodia pinea* (Desm.) Kickx.) is worldwide in distribution and importance. It has an extensive host range including *Abies*, *Larix*, *Picea*, *Thuja*, *Pseudotsuga*, and 33 species of *Pinus* (9,19). Symptoms associated with *S. sapinea* include shoot (tip) blight of mature trees and seedlings, wood staining, stem infections, and a root disease (4,7,13,15,17,18,28,29).

Infection by *S. sapinea* is usually favored by drought, poor site, hail damage, or insect attack (2,11,12,27). Wounds have been reported as necessary for shoot infection (12,20), although several researchers have demonstrated infection of nonwounded shoots (5,15,28). Age of host tissue at the time of inoculation (5) and the possibility of pathogenic variation among strains of the fungus (9) have been suggested to explain these conflicting results. Variation in cultural characteristics among isolates has been reported (1), but pathogenic variability has not been demonstrated.

In the north central United States, *S. sapinea* damages native and exotic conifers in plantations, shelterbelts, and forest tree nurseries (15,21). During our investigations of these problems, isolates of *S. sapinea* differing in culture morphology were obtained. All isolates were grown on potato-dextrose agar (PDA). Isolates with fluffy, white to gray-green mycelia were designated type A, whereas isolates with dark gray mycelia closely appressed to the agar surface were designated type B. This research was conducted to determine if these types represented two distinct forms of *S. sapinea*. To accomplish this, cultural characteristics of type A and B isolates were examined and relative virulence of selected isolates was determined. Isozyme analysis was used to determine if isolates differed at the molecular level.

MATERIALS AND METHODS

Isolates. *S. sapinea* was isolated on PDA (Difco, Detroit, MI) from various *Pinus* spp. and locations in the north central United States. Monoconidial isolates were maintained at 5–10 C on PDA slants. All isolates corresponded to the description given by Sutton (25) for *S. sapinea*. The seven isolates listed in Table 1 were used in all experiments unless otherwise indicated.

Effect of media on growth. Isolates were grown on PDA, malt, Czapek, lima bean (Difco), *P. banksiana* Lamb. needle extract (PBEA), and *P. resinosa* Ait. needle extract (PREA) media. Each isolate was grown in five standard plastic petri plates (100 × 15 mm) containing approximately 25 ml of each medium. Plates were incubated until the temperature of the media was 20 C. A 5-mm plug from an actively growing culture on PDA was then placed fungus-side-down in the center of each plate. Plates were enclosed in plastic bags and incubated in the dark at 20 C. After 3 days, two horizontal perpendicular measurements of colony diameter were made. The experiment was replicated twice. An analysis of variance was performed on the average diameter measurement of each colony. Treatment means were compared using the Student-Newman-Keuls' test. Observations of culture morphology were made 7 days after plates were inoculated.

Effect of temperature on growth. Petri plates containing PDA were incubated until the media was 5, 10, 15, 20, 25, or 30 C. Then, a 5-mm plug of each isolate from actively growing cultures on PDA was placed fungus-side-down in the center of each plate. Five plates of each isolate were enclosed in plastic bags and incubated in the dark at each temperature. After 3 days, three diameter measurements, approximately 120° apart, were made of each colony. The experiment was replicated three times. An analysis of variance was performed on the average colony diameter of each isolate. Treatment means were compared using the Student-Newman-Keuls' test.

Effect of light and substrate on sporulation. Isolates were grown on PDA in the dark or continuous light (Sylvania Gro-lux 14W, GTE Corp., Springfield, VA) at 25 C with or without sterile *P. resinosa* needles placed on the agar surface. Three plates of each treatment were used. Cultures were examined at 4, 5, 6, 14, and 30 days, and presence of conidia noted.

Conidial characteristics. Isolates were grown in continuous light

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at 25 C on PDA with sterile *P. resinosa* needles on the agar surface. The length, width, and number of septa of 100 conidia of each isolate from pycnidia on needles were recorded. An analysis of variance was performed on the conidial measurements. The Student-Newman-Keuls' test was used to compare mean conidial width and length of isolates.

One hundred spores from the naturally infected pine tissue that had yielded isolates A123 and B124 (Table 1) were also measured. Mean conidial length and width of isolates were compared using Student's *t* test.

Virulence. Greenhouse inoculations. Elongating shoots of 5–6-mo-old greenhouse-grown *P. resinosa* and *P. banksiana* seedlings were inoculated with isolates A123, B124, or sterile distilled water using one of the following methods: three drops of inoculum were placed on an intact shoot; three drops of inoculum were placed on a shoot wounded by removing one needle fascicle; or 2 cc of inoculum was injected into a shoot using a 20-gauge syringe. Fungal inoculum consisted of a suspension of spores and mycelium produced by blending two culture plates of mycelium and spores with 200 ml of sterile distilled water. Seedlings were incubated in plastic bags for 48 hr. Isolations were made from all symptomatic seedlings to verify presence of *S. sapinea*. The experiment was replicated three times with 30 seedlings per treatment. An analysis of variance was performed on the number of diseased seedlings in each treatment.

Field inoculations. Saplings of *P. banksiana*, *P. resinosa*, and *P. strobus* L. in the Jackson County Forest, WI, were inoculated on 17 May 1983. Symptoms of *S. sapinea* were not observed on the trees used for inoculations; however, *S. sapinea* has been reported from other areas of this forest (21). The main stem (approximately 2–4 cm in diameter) of each tree was girdled to create stress approximately 1 m below the terminal bud or left intact. A sterile toothpick, or one colonized by isolate A123 or B124, was inserted into a slit made in the stem approximately 80 cm from the terminal bud and wrapped with Parafilm. For purposes of the analysis, inoculation of each tree species was considered as a separate experiment, although each species received the same six treatments. A completely randomized design was used with 20 trees per treatment for a total of 120 trees per host.

On 19 October 1983, the length of the inoculation wound and additional discoloration (if present) of 10 trees in each treatment were measured. The remaining 60 trees per host were examined on 22 October 1984. Isolations were made from each stem to verify the presence of *S. sapinea*. Each year, an analysis of variance was performed on the length of discoloration (excluding length of inoculation wound) of each stem. Trees affected by insects or pathogens other than *S. sapinea* were not included in the analysis.

Isozyme analysis. Isolates were grown on liquid V-8 media (24) at 20 C in the dark for 10 days. Mycelium was harvested on Miracloth and rinsed twice with sterile distilled water. Approximately 3 cc of mycelium was placed in a glass shaker flask containing 1-mm glass beads and 2 ml of amine citrate (AC) buffer (6) and homogenized with a Braun cell homogenizer. The homogenate was absorbed onto five rectangles (3 × 15 mm) of Whatman 3-mm chromatography paper and placed in a horizontal starch gel (Lot 392 Electrostarch Co., Madison, WI) slab. The electrophoresis procedures followed were those of May et al (14) at 50 mA for 2 hr or until the marker dye of dilute red food coloring reached the opposite end of the gel. Gels were sliced horizontally 1.6 mm thick, stained for one of six enzymes, and incubated at 37.5 C until bands became visible. Twenty-five enzyme stains and two buffer systems were tested. Only six of these stains showed activity. The six enzymes used were: acid phosphatase (ACP), alcohol dehydrogenase (ADH), fluorescent esterase (FLE), β-glucosidase (GLU), malic dehydrogenase (MDH), and sorbitol dehydrogenase (SDH). The stain recipes used were those of O'Malley et al (16). An AC buffer system was used with all stains. Visual inspections and diagrammatic representations of isozymes were made based on the mobility of the bands relative to the marker dye. The electrophoresis procedure was repeated six times.

Cluster analysis. Cluster analysis was used to determine if type A and type B *S. sapinea* could be distinguished on the basis of measured characteristics. Cluster analysis was not performed on the discrete variables (e.g., isozyme patterns, number of septa, ability to sporulate in darkness), because for these variables all type A isolates were identical as were all type B isolates. The clustering method for the continuous variables was the unweighted pair group method with arithmetic averages for standardized unitless variables as described in Romesburg (22). The measure between cluster similarity was the average weighted Euclidean distance with the six growth media variables collectively weighted 1/6, the four temperature variables collectively weighted 1/6, and the two spore dimension variables collectively weighted 2/3.

RESULTS

Effect of media on growth. On malt, lima bean, PDA, PREA, and Czapek media, type A isolates were characterized by fluffy, white to gray-green mycelium and type B isolates by white or black mycelium closely appressed to the agar surface. On PBEA, both types of isolates produced a sparse mycelial mat on the agar surface. There were significant differences ($P = 0.01$) in colony diameter among isolates on three of the six media (Table 2). All isolates showed a similar amount of growth on malt, lima bean, and PBEA media. In general the greatest radial growth by type A isolates occurred on Czapek, PBEA, and PREA media and by type B isolates on PBEA and PREA.

Effect of temperature on growth. No discernible growth was observed after 3 days on plates incubated at 5 and 10 C. Optimum growth for most isolates occurred at 25 C (Table 3); however, colony diameter of isolate A167 from Missouri was greater at 30 than 25 C. Isolates B113 and B124 had significantly ($P = 0.01$) slower growth than the other isolates at 20 and 25 C.

Effect of light and substrate on sporulation. All isolates

TABLE 1. Cultural characteristics of seven isolates of *Sphaeropsis sapinea* isolated from *Pinus* spp.

Type[x]	Isolate identification number	*Pinus* host	Geographic origin	Associated symptoms	Spore characteristics: Length × width (μm)[y]	No. septa	Spermatia-like spores present
A	120	*P. resinosa*	Gogebic County, MI	shoot blight	36.0 c × 12.8 a	0–1	No
A	123	*P. resinosa*	Jackson County, WI	shoot blight	36.6 c × 12.9 a	0–1	No
A	128	*P. resinosa*	Grant County, WI	shoot blight	36.3 c × 12.7 a	0–1	No
A	167	*P. mugo*	Storey County, IA	shoot blight	39.4 a × 12.6 a	0–1	No
A	170	*P. nigra*	Jackson County, MO	shoot blight	37.6 b × 12.6 a	0–1	No
B	113	*P. banksiana*	Gogebic County, MI	shoot blight assoc. w/ *Sirococcus strobilinus*	33.5 d × 11.6 c	0–3	Yes
B	124	*P. banksiana*	Jackson County, WI	shoot blight w/insect damage	34.3 d × 12.1 b	0–3	Yes

[x] Type descriptions given in text.
[y] Average of 100 spores. Values followed by different letters are significantly different ($P = 0.01$) according to the Student-Newman-Keuls' test. Mean width S.E. = 0.1, mean length S.E. = 0.3.

incubated in light produced spores in 6 days on sterile pine needles and in plates without plant tissue, although isolate B113 produced spores within 4 days on plant tissue. In all cultures, pycnidia were produced more abundantly on pine needles than on the agar surface. Pycnidia of type A isolates were formed beneath the mycelium on the agar surface in plates without pine needles. Pycnidia of type B isolates were interspersed with the mycelium on the agar surface. Isolates B113 and B124 produced spores between 14 and 30 days on pine needles when incubated in the dark. Hyaline, cylindrical spores averaging 2.5 × 1.0 μm, similar to those described as microconidia in cultures of *S. malorum* Pk. (23) and as spermatia of *D. pinea* (29) were observed in cultures of isolates B113 and B124 incubated in dark and light. These spores were streaked on PBEA and PDA and incubated in the dark at 15, 25, and 30 C and in continuous light at 25 C. Plates were observed periodically for 30 days. Germination was not observed.

Conidial characteristics. Conidia of type A isolates produced in culture were significantly ($P = 0.01$) longer and wider than those of type B isolates (Table 1). Spore size among type A isolates also varied. Most spores were aseptate; however, when septa were present, type A isolates had a single septum, whereas the number of septa for type B isolates ranged from zero to three. Spores of the type A isolate (A123) from naturally infected tissues averaged 39.3 ± 0.04 × 13.1 ± 0.02 μm and were longer and narrower ($P = 0.01$) than the spores of the type B isolate (B124), which averaged 30.8 ± 0.3 × 15.0 ± 0.02 μm. Number of septa of both isolate types ranged from zero to one.

Virulence. Greenhouse inoculations. The number of seedlings infected by the two isolates varied among replicates of the experiment. There were no significant differences among treatments for either host. However, in all replicates, isolate A123 infected more *P. resinosa* and *P. banksiana* seedlings than isolate B124 (Table 4). Isolate B124 infected *P. resinosa* only when injected into the shoot and infected considerably more *P. banksiana* seedlings by injection than with the other inoculation methods. Isolate A123 infected seedlings regardless of inoculation method. Symptoms of *S. sapinea* were not observed on seedlings inoculated with sterile water.

Field inoculations. In both years, the extent of host discoloration caused by *S. sapinea* varied widely among treatments. In 1983, there were no significant differences in the

TABLE 2. Growth of *Sphaeropsis sapinea* on six agar media at 20 C in the dark

	Colony diameter after 3 days (mm)[x]					
Isolate	Czapek	Malt	Lima bean	*Pinus banksiana* extract	Potato dextrose	*P. resinosa* extract
A120	41.9 a	30.1	26.9	35.2	38.7 a	38.9 ab
A123	41.6 a	31.2	27.3	34.4	38.7 a	36.8 b
A128	43.2 a	31.4	27.6	36.2	43.0 a	40.5 a
A167	37.3 ab	31.3	27.2	35.3	40.5 a	38.7 ab
A170	42.9 a	31.6	26.6	33.5	41.3 a	38.2 ab
B113	33.5 bc	30.7	26.8	37.0	34.2 b	38.6 ab
B124	28.7 c	31.1	24.7	34.6	31.5 b	37.5 ab

[x] Values are averages of two replications. Values within columns followed by the same letter or without letters are not significantly different ($P = 0.01$) according to the Student-Newman-Keuls' test.

TABLE 3. Growth of seven isolates of *Sphaeropsis sapinea* on potato dextrose agar at six temperatures in the dark

	Colony diameter after 3 days (mm)[x]					
Isolate	5 C	10 C	15 C	20 C	25 C	30 C
A120	0	0	10.2	37.8 a	57.8 ab	52.4 ab
A123	0	0	8.8	37.6 a	57.1 ab	54.1 ab
A128	0	0	9.6	36.2 a	55.7 ab	54.2 ab
A167	0	0	12.5	40.4 a	55.5 ab	63.8 a
A170	0	0	8.6	42.7 a	62.6 a	53.3 ab
B113	0	0	8.4	31.1 b	42.3 bc	44.2 b
B124	0	0	9.1	29.5 b	44.5 bc	44.6 b

[x] Values are means of three replications. Means within columns followed by the same letter or without letters are not significantly different ($P = 0.01$) according to the Student-Newman-Keuls' test.

TABLE 4. Virulence of isolates A123 and B124 of *Sphaeropsis sapinea* to *Pinus resinosa* and *P. banksiana* seedlings in greenhouse inoculations

		Diseased seedlings (no.)			
		P. resinosa		*P. banksiana*	
Inoculum[x]	Inoculation method	Avg.[y]	Range	Avg.	Range
Isolate A123	D/NW[z]	5.7	0–6	8.3	0–22
	D/W	13.7	1–17	10.7	2–22
	INJ	23.0	15–28	12.3	4–19
Isolate B124	D/NW	0	0	0.7	0–1
	D/W	0	0	1.3	0–1
	INJ	8.3	3–14	12.7	6–15
Sterile water	D/NW	0	0	0	0
	D/W	0	0	0	0
	INJ	0	0	0	0

[x] Isolate A123 from *P. resinosa*; isolate B124 from *P. banksiana*.
[y] Values are averages of three replications of 30 trees per treatment.
[z] D/NW = Three drops of inoculum placed on nonwounded shoot; D/W = three drops of inoculum placed on wounded shoot; INJ = 2 cc of inoculum injected into shoot.

TABLE 5. Length of stem discoloration resulting from inoculation of three *Pinus* spp. with isolates A123 and B124 of *Sphaeropsis sapinea*

		Length of discoloration (cm)					
		P. resinosa		*P. strobus*		*P. banksiana*	
Year examined[x]	Treatment[y]	Avg.[z]	Range	Avg.	Range	Avg.	Range
1983	Girdled						
	A123	41.3/8	24–63	42.2/9	3–101	58.9/10	12–165
	B124	29.2/10	0–63	38.6/9	0–108	32.1/10	0–54
	Control	0/10	0	2.2/9	0–20	0/10	0
	Not girdled						
	A123	29.1/10	12–60	40.6/10	0–85	25.1/8	10–36
	B124	28.5/10	0–74	37.2/10	0–94	19.8/8	0–40
	Control	0/10	0	13.6/10	0–48	0.8/6	0–3
1984	Not girdled						
	A123	66.8/7	45–83	105.1/9	55–162	70.7/9	33–140
	B124	83.6/8	51–140	86.1/10	65–216	70.5/10	40–130
	Control	47.7/9	21–95	65.7/9	0–120	35.8/9	11–45

[x] Length of stem discoloration in centimeters of 10 trees per treatment was determined on 19 October 1983. The remaining stems were examined on 22 October 1984.
[y] Trees inoculated on 17 May 1983 by placing a sterile toothpick or a toothpick colonized by *S. sapinea* into a slit made in a girdled or nongirdled stem. Isolate A123 from *P. resinosa*; isolate B124 from *P. banksiana*.
[z] Average length of discoloration per number of trees per average. Trees affected by insects or pathogens other than *S. sapinea* are not included. In 1984, only nongirdled trees were examined.

amount of discoloration between fungal isolates or between girdled and nongirdled treatments (Table 5). By 1984, all of the stems of the girdled trees were broken at the girdle and most of these stems had fallen to the ground. Only data from nongirdled treatments were used in the 1984 analysis. No significant differences among treatments were found. Discoloration beyond the inoculation wound was noted in the control inoculations on *P. banksiana* and *P. strobus*. Type B isolates were recovered from 30–80% of the control inoculations of these species and were also isolated from *P. banksiana* inoculated with isolate A123. Type B isolates were recovered from 80–100% of the broken, girdled stems of all hosts.

Isozyme analysis. Isozyme patterns are presented in Figure 1. No attempt was made to interpret the genetic origin of the bands, thus each isozyme is considered an electrophoretic phenotype. Isozyme patterns were identical among type A and type B isolates for MDH, ACP, FLE, and GLU; however, type B isolates produced two bands for ADH and SDH, whereas type A isolates produced only a single band.

Cluster analysis. Results of the cluster analysis (Fig. 2) demonstrate that isolates of a particular type clustered with each other before they clustered with isolates of the other type.

DISCUSSION

Two distinct types of *S. sapinea* were identified in the north central United States. These types were clearly distinguished on the basis of several cultural characteristics and isozyme patterns. Although there was variation in measured characteristics (e.g., spore dimensions, growth at different temperatures) within types, cluster analysis demonstrated that the clustering within each type occurred at much lower levels than the clustering between types. This suggests that the between-type similarity is much less than the within-type similarity. Isolates with type A culture morphology also differed from type B isolates in spore surface morphology (26). The cultural, morphological, and isozyme differences between type A and B isolates are indicative of genetic divergence in *S. sapinea*. We chose not to assign a taxonomic designation to this degree of divergence, however, because differences of similar magnitude have been observed among isolates of several fungi, including *Endothia parasitica* (10), *Rhizoctonia solani* (3), and *Ceratocystis ulmi* (8). The differences in cultural characteristics of these fungi were associated with differences in aggressiveness or virulence.

A relationship between aggressiveness and cultural characteristics cannot be demonstrated for *S. sapinea* because relative virulence was determined with only one representative isolate of each type. However, there were distinct differences in virulence between the two isolates tested. The representative type B isolate used, B124, required wounds to infect shoots. The type A isolate did not. In field inoculations, type B *S. sapinea* colonized wounded stems of many control and inoculated treatments as well as most stems that had fallen to the ground. The type B isolates used in this study were obtained from *P. banksiana* tissues infected by *Sirococcus strobilinus* or wounded by insects (Table 1) and have also been recovered from *P. resinosa* in association with insect damage (30). The apparent association with senescent or wounded tissues might suggest that in the north central United States, type B isolates represent an opportunistic fungus that is less likely to be the primary cause of disease than type A. Therefore, before initiating management strategies for diseases caused by *S. sapinea* in this area, it will be important to identify the isolate type involved.

Type B isolates are presently known only to occur in the north central United States. However, the report of variation in cultural characteristics (1) and conflicting reports regarding wound requirements for infection by *S. sapinea* (7,12,15,20,28) suggest that different forms of this fungus may exist in other areas. For example, Wang et al (26) examined a collection of 30 isolates of *S. sapinea* and identified isolates with culture morphology intermediate between type A and B. *S. sapinea* is therefore an apparently variable species, and it is possible that more than two distinct types exist.

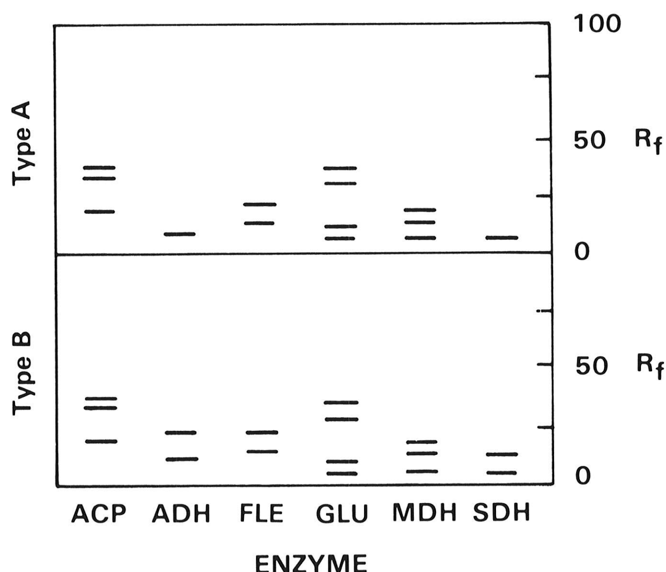

Fig. 1. Diagrammatic representation of isozyme patterns of type A and type B isolates of *Sphaeropsis sapinea*. Type A isolates are A120, A123, A128, A167, and A170; type B isolates are B113 and B124. ACP = acid phosphatase, ADH = alcohol dehydrogenase, FLE = fluorescent esterase, GLU = β-glucosidase, MDH = malic dehydrogenase, and SDH = sorbitol dehydrogenase.

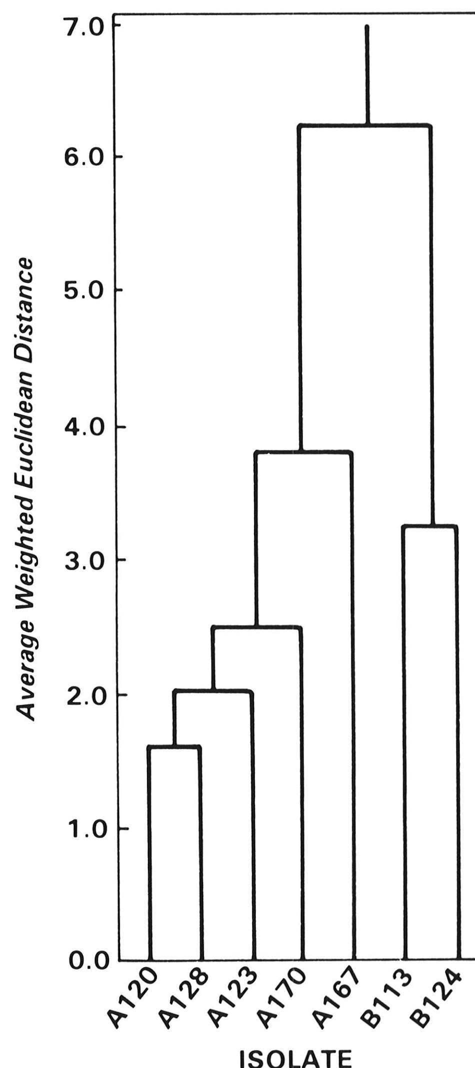

Fig. 2. Dendrogram showing results of cluster analysis on continuous variables. The six growth media variables were collectvely weighted 1/6, the four temperature variables collectively weighted 1/6, and the two spore dimension variables collectively weighted 2/3.

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