AN ASSESSMENT OF THE TAXONOMIC RELATIONSHIP BETWEEN SPOROTHRIX SCHENCKII HEKTOEN AND PERKINS AND CERATOCYSTIS STENOCERAS (ROBAK) C. MOREAU

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ABSTRACT

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By

Ronald Anton Heinrichs

Recently, researchers have noted marked similarities between conidial states of members of the genus Ceratocystis, pathogenic on plants, and Sporothrix schenckii Hektoen and Perkins pathogenic on humans and animals. It was hypothesized that S. schenckii is an asexual relative of the genus Ceratocystis. This relationship was tested using four strains of S. schenckii, isolates of a perfect and imperfect stage of Ceratocystis stenoceras (Robak) C. Moreau, its pathogenic mutant, strain of C. fagacearum, and a strain of Calcarisporium arbuscula.

Acrylamide gel electrophoresis was used to analyze the soluble proteins extracted from the fungi. The gels were stained to show the protein bands and duplicate gels were treated to locate acid and alkaline phosphatase activities. The liquid culture medium and mycelial extracts were also assayed for acid and alkaline

phosphatase activity. Sugars hydrolyzed from the cell wall polysaccharides of the fungi were analyzed by paper chromatography. The effect of thiamine on the growth of C. stenoceras was also tested.

Results from electrophoresis indicated, from the number of matching protein bands and sites of acid phosphatase activity, that S. schenckii was similar to C. stenoceras. The level of acid phosphatase activity was much higher than the alkaline phosphatase activity in the culture liquid at 24 C as well as in the protein extracts for most of the strains. The protein extracts showed similar results for enzyme activity. Both fungi showed the presence of mannose and rhamnose in the cell wall polysaccharides. Ceratocystis stenoceras and S. schenckii appeared to require thiamine for growth.

The hypothesis that S. schenckii is a relative of the genus Ceratocystis is supported by the results of this study.

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Ronald Anton Heinrichs

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TO MY WIFE KATHLEEN

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INTRODUCTION

Sporothrix schenckii Hektoen and Perkins 1900 produces a chronic disease, sporotrichosis, involving the subcutaneous tissue in man and animals. The relationship of *Ceratocystis* sp. to *Sporothrix schenckii* was studied by Mariat *et al.* (1968) and Mariat (1969, 1970), who proposed the working hypothesis that the conidial form of *Ceratocystis* sp. represents the ancestral form of *S. schenckii*. This hypothesis was tested by Taylor (1970) who compared cultural, morphological, and serological characteristics and mouse virulence of several *Ceratocystis* species in the conidial state with several strains of *S. schenckii*. His results supported the hypothesis of Mariat.

The objective of this investigation was to compare the relationship between selected strains of *Ceratocystis* and *Sporothrix* and *Calcarisporium*, a genus closely related to *Sporothrix*, using polyacrylamide gel electrophoresis, acid and alkaline phosphatase enzyme activity assays, paper chromatography of sugars hydrolyzed from mannan containing polysaccharides, and growth requirements for thiamine.

REVIEW OF LITERATURE

Sporothrix schenckii Hektoen and Perkins 1900 is a dimorphic fungus able to grow in culture at 24 C as the mycelial phase, which converts to the yeast phase at 37 C on media supplemented with an organic nitrogen source and a CO_2 tension of five percent. The organism at both phases requires the addition of thiamine to the medium for growth. The yeast phase produces the disease sporotrichosis which occurs in man and animals and is characterized by nodular lesions in the lymph system and skin or subcutaneous tissues of the hands, arms or legs. A disseminating form of the disease may attack the skeletal structure or internal organs. The mycelial phase at 24 C has branching, septate hyphae about 2μ in diameter. The hyaline, non-septate conidia range in shape from spherical to pyriform and rarely triangular, appearing on short sterigmata, singly along the hyphae or more often as several conidia clustered on the end of a conidiophore (Beneke and Rogers, 1970).

The species was first isolated from a human case and described by Schenck (1898) and tentatively placed in the genus Sporotrichum by E. F. Smith. Later, Hektoen and Perkins (1900) isolated the same organism from a human case and assigned it to a new genus calling it Sporothrix schenckii. This new classification was ignored by other

workers in the field who continued to use the previous generic name, Sporotrichum, and added different species names.

A revision of the nomenclature of Sporotrichum schenckii began with the effort of Hughes (1958) in regard to the classification of the Hyphomycetes. Part of his work dealt with the genus Sporotrichum as described by Link (1809). Eleven of the 12 type species of Sporotrichum were found to be unrelated and could be classified elsewhere leaving only S. aureum as the lectotype species. This finding was supported by the views of Carrion and Silva (1955) using 1821 as the starting point for nomenclature of the Hyphomycetes. Carmichael (1962) then found Sporotrichum schenckii to be unrelated to the lectotype species of the genus and placed it in the genus Sporothrix as originally proposed by Hektoen and Perkins as S. schenckii Hektoen and Perkins 1900. He also noted that S. schenckii bears its closest resemblance to Calcarisporium Preuss. Barron (1968) mentioned that C. pallidum bears a closer resemblance to S. schenckii than it does to C. arbuscula, the type species of the genus. The organism also appears similar to the "Sporotrichum" states found in some species of Ceratocystis.

Recent developments have put the present classification in question again. Mariat et al. (1968) isolated seven strains of Ceratocystis sp. which had morphological

and physiological characteristics similar to nonpigmented strains of S. schenckii. Perithecia were produced in culture after one month, enabling positive identification of the genus of these fungi. These strains were not pathogenic for the mouse or hamster. Mariat (1968) observed that members of the two genera were found in identical habitats, isolates of both genera formed identical mycelial structures, which in the case of Ceratocystis isolates went on to form perithecia, and Sporothrix occasionally formed nonpigmented conidia on synnemata the same as Ceratocystis in its form-genus Graphium. Mariat (1969) later discovered one of the isolates Ceratocystis stenoceras (Robak) C. Moreau, had developed pathogenicity after one passage through a hamster but it no longer produced perithecia. On this basis, Mariat (1970) developed a working hypothesis that the Sporothrix-like conidial form of Ceratocystis is an ancestral form of S. schenckii.

Taylor (1970) expanded on the investigations of Mariat. He compared the conidial states of several species of Ceratocystis with strains of S. schenckii and found the cultural, morphological, serological characteristics, and virulence for mice to be essentially the same. Certain Ceratocystis species in the conidial states appeared identical to S. schenckii and could only be identified properly by the formation of the perfect stage.

The technique of polyacrylamide gel disc electrophoresis was used by deBievre (1967) to study the yeast and mycelial phases of S. schenckii. This method has also been used by Schechter et al. (1966), Stipes (1970), and Sorenson et al. (1971) in their efforts to determine the taxonomic status of fungi. The value of this procedure as a taxonomic tool, however, has been questioned by Sorenson et al. (1971) because of variability of protein patterns between strains of the same species. They have used mathematically similar coefficients to determine the degree of relationship between fungi based on the results of disc electrophoresis. In addition to the use of strains (to detect proteins in the gels), Stipes (1970) detected sites of alkaline phosphatase activity utilizing the methods of Davenport (1960) and Jensen (1962). The enzyme results provided an additional comparative basis for the classification of organisms.

On the basis of the enzyme work by Beneke et al. (1969), and Rogers and Beneke (1971), demonstrated that the level of enzyme activity in culture filtrates differed in the strains of organisms tested. This may help to differentiate strains of fungi.

Proton magnetic resonance spectra of the mannose-containing polysaccharides and their component sugars identified by paper chromatography were used by Spencer and Gorin (1971) as a means of grouping various species of Ceratocystis and the form genus Graphium.

MATERIALS AND METHODS

Culture Media

Stock cultures were obtained from the following sources: four strains of *S. schenckii* (Spar, Flint, Pulmo, and Belo), and one of *Ceratocystis fagacearum* (fag) from the Michigan State University stock culture collection; *Ceratocystis stenoceras* strains 1013 and 1020 from Dr. F. Mariat, Pasteur Institute, Paris; and *Calcarisporium arbuscula* Preuss (Arb) from Dr. H. L. Barnett, West Virginia University. The organisms were cultured in 250 ml. Erlenmeyer flasks with 100 ml. of liquid medium. The medium of deBievre (1967) was used with a modification in the trace element solution. The composition of the medium is: potassium phosphate, 0.91 g; sodium phosphate·7 H_2O, 1.80 g; magnesium sulfate, 0.60 g; potassium chloride, 1.00 g; l-arginine monohydrochloride, 1.00 g; glucose, 20. g; $1x10^{-5}$M thiamine hydrochloride; $1x10^{-9}$M biotin; and 1 ml. of a trace element solution composed of ferric chloride·6 H_2O, 1.0 µg.; 0.5 µg. each of manganese chloride·4 H_2O and cobalt sulfate·7 H_2O; and zinc chloride, 0.8 µg.; made up to a liter of water. For solid medium 15 g of agar was added. This medium was then inoculated with the fungi. The inoculum consisted of 5 ml. of a cell

suspension made by taking three plugs, one cm. in diameter from the edge of an actively growing colony, on solid medium at 24 C; adding the plugs to 50 ml. of liquid medium and run in a Waring blender for 30 seconds. The medium was of the same composition as noted above. Each fungus was cultured in two flasks at 24 C on a rotary shaker at 160 rpm.

Extraction of Soluble Protein

After seven days the flasks were checked microscopically for contamination, the mycelium collected by filtration on two sheets of Whatman No. 1 filter paper in a Buchner funnel under vacuum and washed three times with distilled water. The mycelial mats were added to 40 ml. of acetone at -20 C and 3 gm. of acid washed sand in a Servall Omni-mixer which was run for three minutes at 15,000 rpm with the container immersed in an acetone-dry ice bath. The resulting slurry was filtered on Whatman No. 1 filter paper in a Buchner funnel under reduced pressure, washed with 50 ml. of acetone at -20 C, and worked to a dry powder with a spatula. The powder was stored at -20 C. In preparation for electrophorsis, 50 mg. of powder were added to 1 ml. of 0.004 M sodium bicarbonate solution and stored overnight at 4 C. The suspension was centrifuged at 4500 rpm for 25 minutes at 4 C and the supernatant solution collected and analyzed for the amount of protein following the Lowry method

(Lowry et al., 1951, as modified by Miller, 1959). The protein extract was either used immediately or stored at -20 C in 750 µg portions with 0.1 ml. of 40% sucrose solution.

Disc Electrophoresis Procedure

The method of disc electrophoresis described by Davis (1964) was followed, except for the use of a 12% lower gel and TRIS-glycine buffer diluted three times. Polyacrylamide gel columns were made in 5 x 100 mm. cylindrical glass tubes. The gels were composed of two layers. The lower layer was a small pore gel where separation of the proteins took place. The upper layer was a large pore gel where the proteins became stacked according to size and charge before being separated in the lower gel. Materials used in making the gels were acrylamide, N-N'-methylenebisacrylamide (BIS), tris aminomethane (TRIS), N,N,N'-tetramethylethylenediamine (TEMED), riboflavin, hydrochloric acid, and ammonium persulfate. Six stock solutions were prepared as follows:

Stock A		Stock B	
1 N HCl	48 ml.	1 N HCl	approx. 48 ml.
TRIS	36.6 g.	TRIS	5.98 g.
TEMED	0.23 ml.	TEMED	0.46 ml.
water to	100 ml.	water to	100 ml.
(pH 8.9)		(pH 6.7)	

Stock C		Stock D	
Acrylamide	28.0 g.	Acrylamide	10 g.
BIS	0.735 g.	BIS	2.5 g
water	to 100 ml.	water	to 100 ml.

Stock E		Stock F	
Riboflavin	4 mg.	Sucrose	40 g.
water	to 100 ml.	water	to 100 ml.

The gels were prepared by placing glass tubes upright into rubber holders. The lower gel solution was made by combining 1 part A, 2 parts C, 1 part water, and 4 parts of 0.14 g. ammonium persulfate made up to 100 ml. with water and put into the glass tube up to 6 cm. high using a syringe and needle. A thin layer of water was put on the top surface to flatten it, then was removed when polymerization was complete. The upper, small pore gel was made of 1 part B, 2 parts D, 1 part E, and 3 parts F. It was put on top of the polymerized lower gel to a height of 1 cm. and the surface flattened with water. A 750 µg portion of protein extract combined with 0.1 ml. of 40% sucrose solution was placed on top of the gel column and the remaining space in the glass tube filled with TRIS-gylcine buffer, pH 8.3, diluted 1:2 with water. In the electrophoresis apparatus, the gel tubes connected an upper and a lower buffer reservoir, each containing TRIS-glycine buffer. Some bromphenol blue crystals were added to the buffer in the upper tank to enable the progress of electrophoresis to be followed. The apparatus was taken

to a cold room at 4C where a direct current power supply, Heathkit Model IP-32, was connected to an electrode positioned in the center of each buffer tank, the anode connected to the lower electrode and the cathode connected to the upper electrode. A current of 3.5 mA per tube was applied for the time required by the indicator dye to travel 40 mm. in the separation gel, usually one hour.

Upon completion of electrophoresis the gel tubes were put into crushed ice. The gels were removed from the glass tubes by injecting water between the gel and the tube wall using a syringe with a 22 gauge, 5 inch needle. They were then put into 12% trichloracetic acid to fix the protein. After 30 minutes the gels were stained for three hours using comassie blue stain solution diluted 1/10 with destain solution (Weber et al., 1969). The gels were rinsed in water and immersed in destaining solution for two days, then stored in 12% TCA. Readings of the protein bands were made using a Gilford 2400 spectrophotometer at 549 nm. with a gel transport attachment. Diagramatic interpretations were made of the bands in addition to photographs.

Location of Acid and Alkaline Phosphatase Activity in the Gels

Gels taken from their glass tubes after electrophoresis were assayed for acid and alkaline phosphatase activity following the methods of Jensen (1962). The gels

for the acid phosphatase assay were immersed directly into a substrate solution composed of: 0.6 g. lead nitrate in 500 ml. of 0.05 M acetate buffer pH 4.5 to which was added sodium-beta-glycerophosphate·5 H_2O in 0.10 M concentration, and the final pH adjusted to 5.0. The gels for the alkaline phosphatase assay were immersed in a substrate solution of: 5 ml. of a 2% calcium chloride solution, 2 ml. of a 2% magnesium chloride solution, 10 ml. of a 0.1 M solution of barbital buffer and 33 ml. of water, 0.1 M sodium-beta-glycerophosphate·5 H_2O; 1 N sodium hydroxide was added to adjust the pH to 9.4. After incubating in their respective solutions at 37 C for 10 minutes the gels were rinsed in several changes of water, and in the case of the acid phosphatase gels, once in 2% acetic acid, and then several more times in water. Dilute sodium sulfide solution at room temperature was used to develop the bands to indicate where enzyme activity was located. The same solution was also used to store the gels.

Survey for Enzymatic Activity of the Organisms in Liquid Media and in Protein Extracts

The liquid culture medium derived as filtrate from shake culture flasks incubated at 24 C for seven days and the soluble protein extracts in 0.004 M sodium bicarbonate were tested for alkaline and acid phosphatase activity. The enzyme substrate was paranitrophenylphosphate at a concentration of 1 mg. per ml. in each of 0.1 M acetate

buffer pH 5.0 and 0.1 M TRIS buffer pH 8.5. Substrate solution (0.8 ml.) was added to each tube plus 0.2 ml. of culture filtrate or protein extract diluted 1:9. The solutions were mixed well and incubated at 30 C for one hour. Enzymatic activity was stopped using 3 ml. of 0.5 M TRIS buffer. Control tubes used for each sample contained 0.8 ml. of water in place of substrate solution with 0.2 ml. of the protein solution. They were treated in the same manner as the other tubes. A Turner model 330 spectrophotometer at a setting of 410 nm was used to read the amount of yellow colored p-nitrophenol derived in proportion to the activity of the enzyme. The activity is expressed as spectrophotometer $O.D._{410}$ (nanometers) Units/one hour at 30 C.

Extracting and Analyzing Mycelial Polysaccharides

The Gorin and Spencer (1968) method of extracting fungal polysaccharides and analyzing the component sugars was used for this investigation. The method of Trevelyan et al. (1950) was used to develop the chromatograms. Polysaccharides were extracted from the mycelium using hot potassium hydroxide. Mannan containing polysaccharides were precipitated out of solution using Fehling solution and then were hydrolyzed into the component sugars by acid hydrolysis. Paper chromatography was used to identify the sugars by running them along with known sugars on the same

Whatman 1 paper and developing them by the method noted previously.

Requirement of Thiamine for Growth of C. stenoceras 1013 and 1020

A total of eighteen 250 ml. Erlenmeyer flasks were used, nine contained 50 ml. each of standard medium with 0.05 mg. of thiamine and nine flasks contained 50 ml. each of standard medium without thiamine were used to study the effect of thiamine on growth of the organisms. Sporothrix schenckii Belo and C. stenoceras 1013 and 1020 growing on standard agar medium were used in the experiment. Inoculum was prepared by washing the plate cultures with sterile buffer solution to bring the fungus spores into suspension. Spore counts were made with a haemacytometer and the concentration adjusted to approximately $50x10^6$/ml. by diluting with sterile buffer solution. For each of the three fungi, 1 ml. of the spore suspension was put into each of six flasks: three containing the standard medium and three containing the medium deficient in thiamine. The flasks were incubated at 24 C for seven days on a rotary shaker at 160 rpm. The mycelium was harvested by filtration on Seitz sterilizing filter pads which were previously dried to constant weight in an over at 96 C for sixteen hours and tared. The pads plus the mycelium were dried to constant weight as above and the weight of the mycelium calculated.

RESULTS

Disc Electrophoresis of Soluble Protein Extracted from the Mycelium of S. schenckii, C. stenoceras, C. fagacearum, and Calcarisporium arbuscula

Comparisons of the electrophoretic patterns of soluble protein from the mycelium of seven day cultures at 24 C were made using the eight strains: four of S. schenckii, two of C. stenoceras, one of C. fagacearum, and one of C. arbuscula. The electrophoresis patterns of protein extract of eight strains of organisms showed characteristic bands varying in thickness, density, and spacing, enabling identification of the fungus source. All eight protein extracts were tested at the same time in six electrophoresis runs and every run resulted in essentially the same characteristic protein pattern. Rf values were measured and calculated from diagramatic drawings made directly from the gels. This method gave Rf values as accurate as those derived from densitometer readings and resulted in recording almost twice as many protein bands since many were close to each other or of low density and could not be detected from densitometer tracings. The Rf values of corresponding bands from gels of all six replications which were essentially the same were totaled and an average Rf value for each band was

calculated, Table 1. Figure 1 shows the results of electrophoresis and diagramatic representations of the average gels for the eight organisms. Average Rf values of each protein in the electrophoretic patterns were compared in all possible paired combinations to determine the number of bands having equal Rf values for the different organisms and strains. The bands which were within one Rf value of each other were considered matching. The results of these band matchings for the organisms are shown in Table 2.

The number of matching bands for C. stenoceras 1020 and three strains of S. schenckii, Pulmo, Flint, and Sparrow are high, indicating a close relationship. There are a few less for S. schenckii Belo strain. There are nine matching bands between the two strains of C. stenoceras (strains 1013 and 1020) which is less than the number of matching bands with the other seven strains with two exceptions. The number of bands match more closely in the imperfect stage of C. stenoceras strain 1020, to the Pulmo, Flint, and Spar strains of S. schenckii than the perfect stage of C. stenoceras strain 1013.

TABLE 1.--Average Rf values of protein bands (six replicates) in the electrophoretic patterns of protein extracts of eight organisms in standard medium for seven days at 24 C.

Rf	S. schenckii				C. stenoceras		C. fagacearum	C. arbuscula
	Pulmo	Flint	Belo	Spar	1013	1020		
0				2.6		2.5	2.2	
		5.0	4.4	5.8	4,2		6.0	
	8.1	7.8		7.2	7.2			
10	9.3	9.8			9.6	10.1	9.5	10.2
	11.8				11.5	12.4	12.8	12.7
		13.7	12.5	13.6		13.6		15.3
	15.8	16.0	14.7			16.9	16.2	16.8
	17.8	19.4		18.0	18.0			18.8
	19.5		19.1	20.2		19.6	20.9	
20	21.4	22.1		22.6	22.6	22.4		
								24.3
	26.0	26.0		25.8	26.3	25.0	25.0	
	28.1	27.4		28.5	28.9	27.0	27.0	28.3
		28.8				29.6	29.6	
30	30.6	30.0	30.7	31.0	31.3		31.4	
	33.4	33.6				33.6	33.3	33.0
				34.2	34.0		35.0	35.8
	37.3	36.5	36.6	36.2	36.4	36.4	36.7	
		39.0	38.5			38.0	39.1	38.6
40				39.8		40.1		40.2
	42.1	42.4	42.6	42.3	41.7		42.8	42.2
						43.0		
	45.6	46.1	46.5	45.6		46.2		45.6
					47.3		47.4	
		50.4				49.4		
50	52.9	53.2	51.8	52.1		52.1	52.9	
								54.5
	57.2		57.3	56.9	56.2		56.4	
						57.9	59.4	58.3
60		61.2	62.1	61.9				
					63.0	62.6	62.3	62.9
			66.3					
		68.6				67.4	68.9	
70								71.1
	71.3	71.7					73.4	
	74.8	74.4	74.3	75.0	75.5	75.2		74.4
							77.8	
80			80.0			79.0		
	83.5		83.8	83.4				81.2
90								
							94.6	
100								

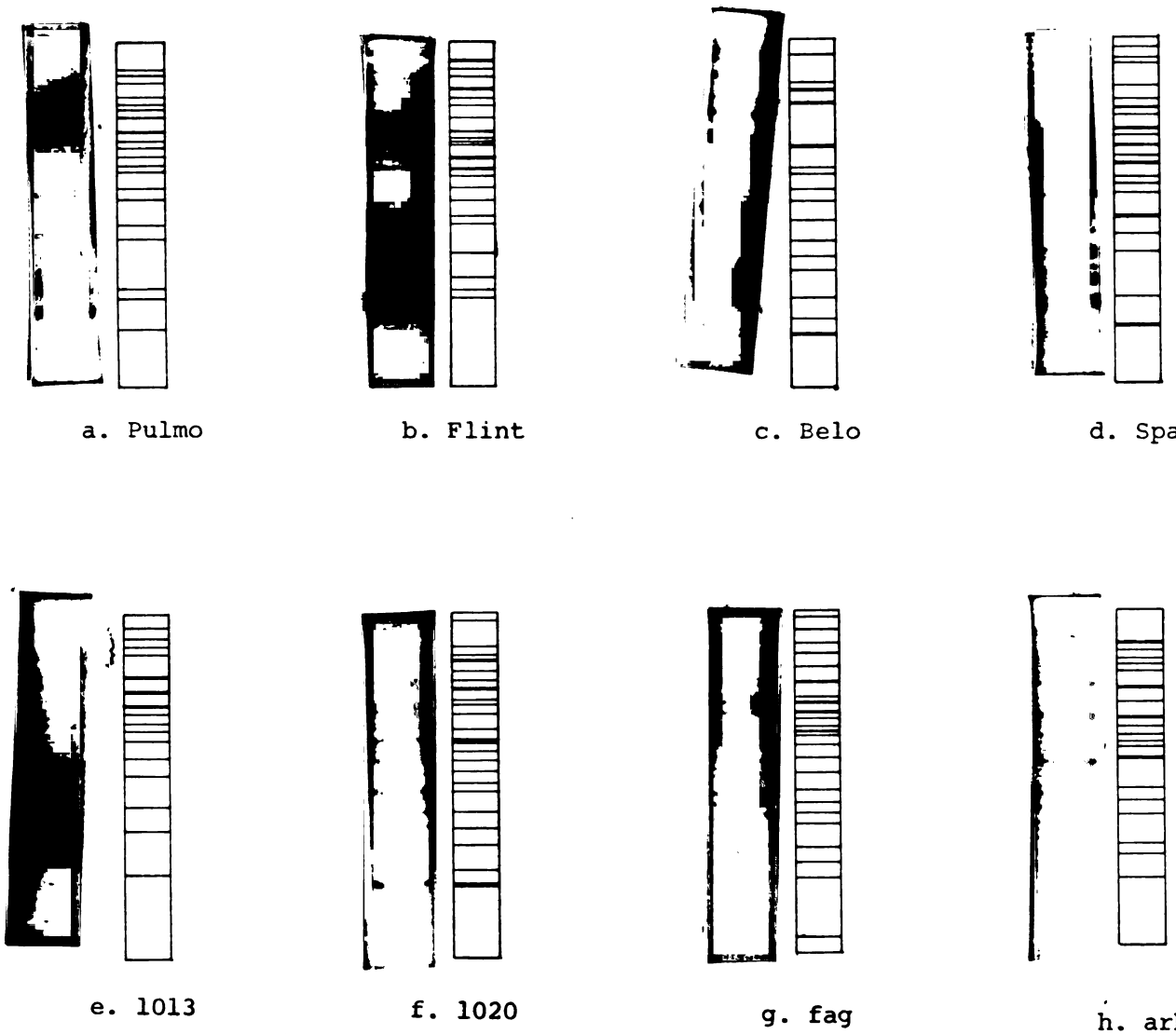

Figure 1.--Electrophoretic patterns in acrylamide gels from soluble protein extracts of S. schenckii strains; (a) Pulmonary, (b) Flint, (c) Belo Horizonte, (d) Sparrow; C. stenoceras strains, (e) 1013, (f) 1020; C. fagacearum, (g) fag; and C. arbuscula, (h) arb.

TABLE 2.--The number of matching protien bands between pairs of gels of four strains of S. schenckii, two strains of C. stenoceras, and one each C. fagacearum, and C. arbuscula.

	arb	fag	1020	1013	Spar	Belo	Flint	Pulmo
Pulmo	13	12	15	12	14	9	15	--
Flint	11	14	14	11	15	10		
Belo	10	9	11	7	9			
Spar	9	13	14	11				
1013	8	9	9					
1020	14	13						
fag	10							
arb	--							

Tests for the Presence of Acid and Alkaline Phosphatase Activity in the Gels

Acid phosphatase activity for the eight strains occurred in six of the eight gels after electrophoresis. Two runs were made to verify the results. The gels containing the proteins of S. schenckii and C. stenoceras formed one to two sites of enzyme activity. All of these gels shared a homologous site of activity while S. schenckii Belo and C. stenoceras 1013 consistently exhibited a second non-homologous band. The gels of C. fagacearum and C. arbuscula had no enzyme activity. There was no evidence of alkaline phosphatase activity. The results are presented in Figure 2. To verify that the bands were the result of

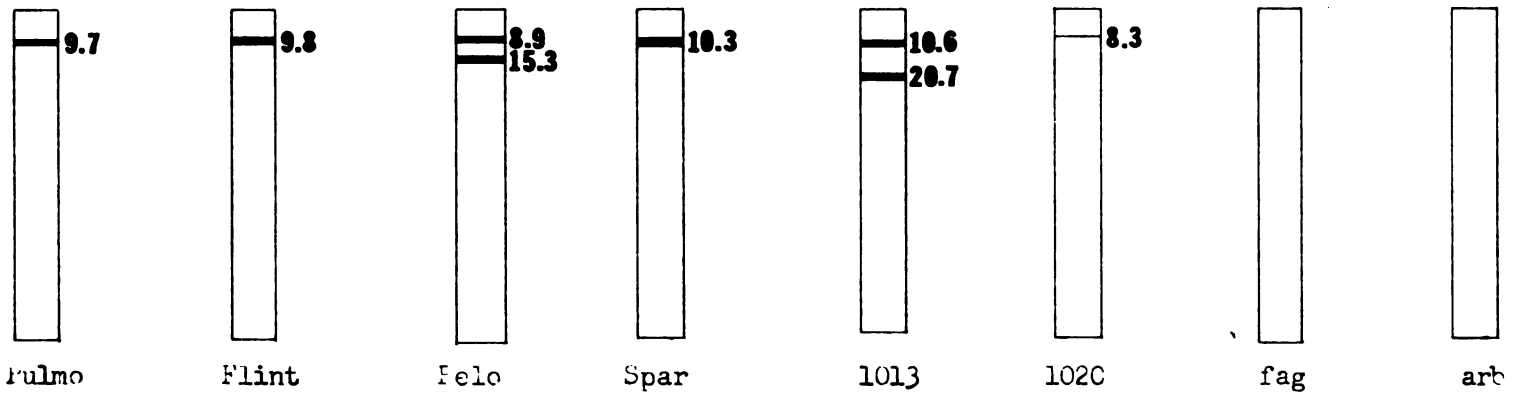

Figure 2.--Electrophoretic gels of S. schenckii (Pulmo, Flint, Belo, Spar), C. stenoceras (1013, 1020), C. fagacearum (fag), C. arbuscula (arb). Sites of acid phosphatase activity with Rf value.

a reaction between the protein and the enzyme substrate solution, the gels from an electrophoretic run were incubated in distilled water rather than the usual substrate solution. No bands were evident after immersing the gels in the sodium sulfide stain solution.

Survey for Acid and Alkaline Phosphatase Enzyme Activities in Culture Filtrates and Protein Extracts

Filtrates, collected after filtering the fungus mycelium grown in liquid standard medium for seven days at 24 C., were tested for the presence of enzyme activity. In Table 3 C. arbuscula filtrate showed the highest acid phosphatase activity while the C. stenoceras 1020 filtrate showed the lowest. The remaining filtrates were at approximately equal levels of activity. Alkaline phosphatase activity was highest for C. fagacearum while C. arbuscula showed a medium amount and the remaining filtrates a low amount of activity. Extracts of soluble protein from the fungus mycelium were also tested for enzyme activity. The level of acid phosphatase activity in the S. schenckii Belo extract was the highest while the C. fagacearum extract showed the lowest. Alkaline phosphatase activity was the highest for C. fagacearum while C. stenoceras 1013 showed the lowest (see Table 4). In general, acid phosphatase activity was higher for most strains in both the culture filtrates and in the protein extracts. The lower acid and alkaline phosphatase activity

TABLE 3.--Acid and alkaline phosphatase activities in the filtrate of eight fungi grown in standard liquid medium for seven days at 24 C.

Species or Strain	O.D. (410 nm)/ml./hour x 100					
	Acid Phosphatase			Alkaline Phosphatase		
	I*	II	III	I*	II	III
S. schenckii						
Pulmo	28	16	12	2	4	0
Flint	44	22	0	2	4	0
Belo	32	15	18	0	3	0
Spar	18	8	17	0	2	0
C. stenoceras						
1013	32	6	12	0	3	0
1020	6	4	2	0	3	0
C. fagacearum	22	15	17	--	4	1
C. arbuscula	112	88	81	9	6	1

*Replicate.

TABLE 4.--Acid and alkaline phosphatase activities in the soluble protein extract of the mycelium of the eight fungi in standard liquid medium for seven days at 24 C.

Species or Strain	O.D. (410 nm)/ml./hour x 100					
	Acid Phosphatase			Alkaline Phosphatase		
	I*	II	III	I*	II	III
S. schenckii						
Pulmo	8400	1435	1575	340	130	20
Flint	3480	320	4125	85	135	75
Belo	11400	4380	3930	75	80	20
Spar	1290	910	1250	50	65	20
C. stenoceras						
1013	3400	2100	1700	35	40	15
1020	5775	3125	3200	380	130	120
C. fagacearum	860	635	840	2100	775	450
C. arbuscula	3250	1210	1845	820	275	180

*Replicates.

of C. stenoceras strains 1013 and 1020 are at a level closer to the pathogenic strains of S. schenckii than the high level of enzyme activity in C. fagacearum and C. arbuscula.

Sugars Derived from Fungal Polysaccharides

Sugar derivatives from the mycelium of S. schenckii Pulmo (P) and Belo (B), and C. stenoceras 1013 (13) and 1020 (20) were mannose (man) and rhamnose (rh) with possible traces of galactose (gal) and glucose (glu) while C. fagacearum (fag) yielded mannose and glucose. These results are illustrated in Figures 3 and 4. Results were checked by co-chromatography, Figure 5, running the known and unknown sugars together by mixing each sample with two of the known sugars. Aside from the normal separation pattern, any separation in the known sugar spots would indicate that the sugars were not the same.

Effect of Thiamine Deficiency on Growth

The results of growing S. schenckii Belo and C. stenoceras 1013 and 1020 in standard medium with and without thiamine at 24 C. are presented in Table 5. Sporothrix schenckii was run as a control since it requires thiamine for growth (Drouhet et al., 1952). Thiamine appeared to be required for the growth of C. stenoceras strains 1013 and 1020 similar to the thiamine requirement of S. schenckii.

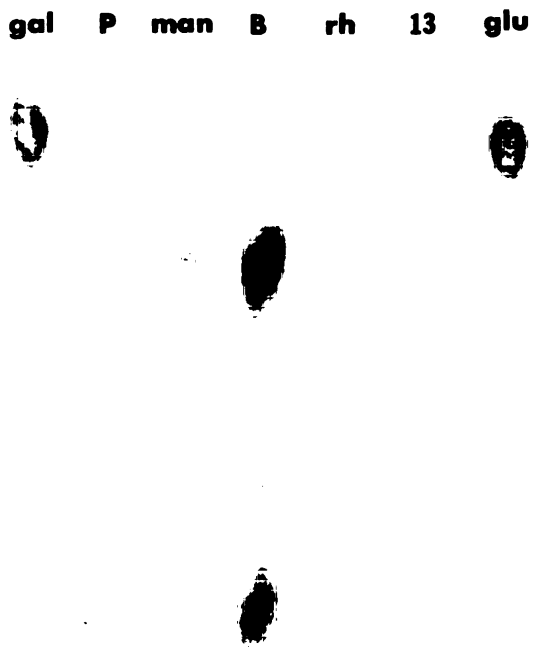

Figure 3.--Paper chromatography of sugars derived from mycelium of S. schenckii strains Pulmo (P) and Belo (B), and C. stenoceras 1013 (13).

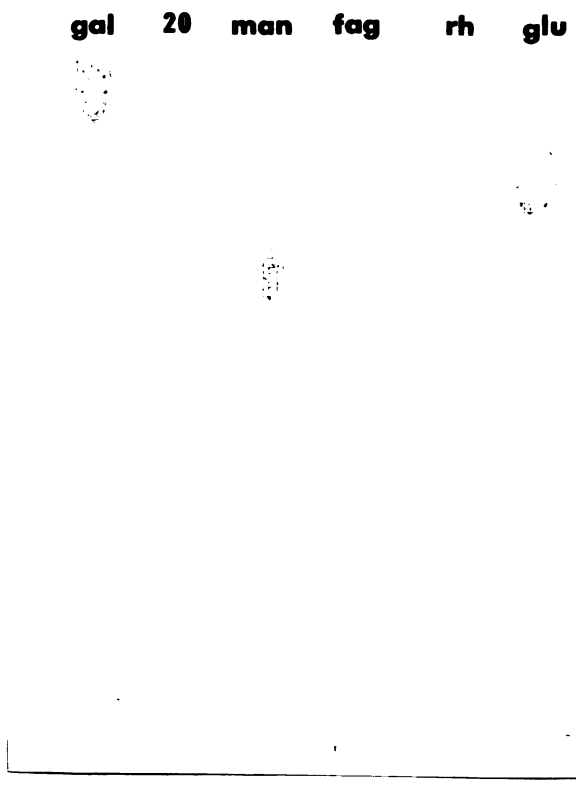

Figure 4.--Paper chromatography of sugars derived from mycelium of C. stenoceras 1020 (20) and C. fagacearum (fag).

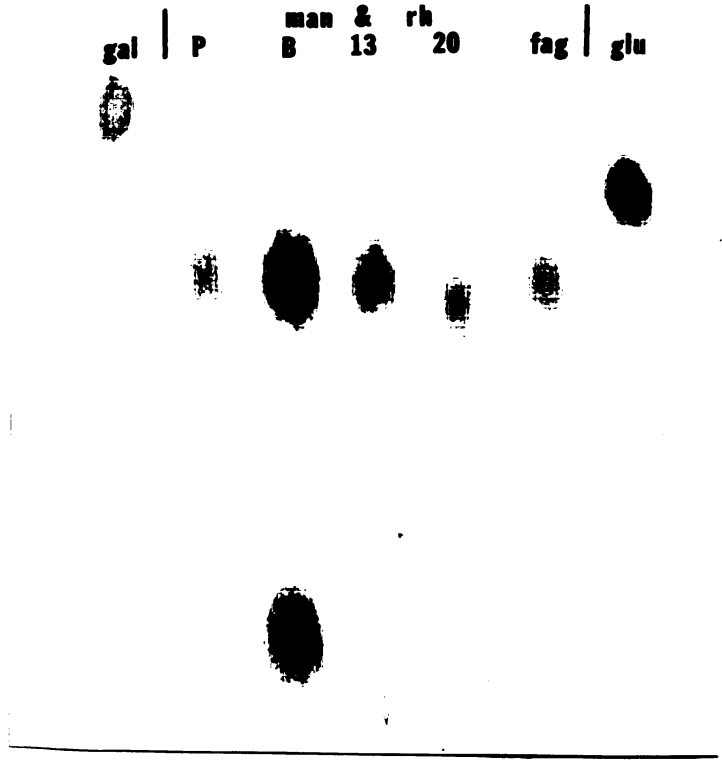

Figure 5.--Paper chromatography of sugars derived from mycelium of S. schenckii strains Pulmo (P) and Belo (B), C. stenoceras 1013 (13) and 1020 (20), and C. fagacearum (fag) and each sample combined with mannose and rhamnose.

TABLE 5.--Requirement of thiamine for growth. Weight (g.) of mycelium from seven day liquid shake culture dried at 96 C.

Medium	*S. schenckii*	*C. stenoceras*	
	Belo	1013	1020
Thiamine Deficient	0.002	0.000	0.031
Thiamine Enriched	0.406	0.347	0.434

DISCUSSION

Electrophoresis

Since the imperfect stage Ceratocystis stenoceras 1020 produces sporotrichosis in laboratory mice, this strain was selected for comparison with four strains of Sporothrix schenckii which also causes sporotrichosis. Several isolates were used to study the extent of strain variations. The perfect stage of C. stenoceras strain 1013 and the imperfect stage 1020 that produces sporotrichosis in mice, were used to compare the relationship to S. schenckii by: disc electrophoresis, acid and alkaline phosphatase activity, thiamine requirement, and analysis of polysaccharides for their mannose contant. Ceratocystis fagacearum was chosen for an intrageneric comparison with C. stenoceras. Carmichael (1962) noted that S. schenckii bears its closest resemblance to Calcarisporium Preuss while C. pallidum, mentioned by Barron (1968), may be more closely related to S. schenckii than C. arbuscula and is like the "Sporotrichum" state of some Ceratocystis species. Since Calcarisporium pallidum was unavailable C. arbuscula was used to study the relationships by the above mentioned methods.

Certain points should be mentioned as a basis for interpreting the results of electrophoresis of fungal proteins. The principle of separation of proteins on polyacrylamide gels is that the size and charge of the protein molecules determined how far down the gel they travel under an electrical potential. In the gels a sieve effect is exerted on proteins due to a network of connected strands leaving holes of a uniform size, varying with the concentration of the acrylamide. Larger proteins then, are retained in the upper portion of the gel while smaller ones are able to penetrate further. The net electrical charge of the protein molecules in a solution varies with the pH. The greater the charge on one of two particles, the faster the movement in an electrical field. Any attempt at reproduction must take into account strict control of the pH. Considering separation of fungal proteins with these two factors in mind, homologous proteins may or may not occupy the same location between gels or a band may be composed of several different proteins. A small protein may be the same size as another, but due to its net charge and a difference in amino acid composition, it will not migrate as fast. It could end up forming a homologous band or else share one with a larger protein of greater net charge compared to other large proteins. There are many possible variations (Ornstein, 1964).

Other factors affecting the results of electrophoresis are culture conditions of the test organism. The age upon harvesting the fungus culture has an effect on the pattern of protein bands (Whitney et al., 1968). The temperature, size of inoculum, and the medium must all be the same if consistent results are to be achieved (Glynn and Reid, 1969). In the case of this experiment all factors appeared to be the same except for possible variation in pH and ionic strength of the TRIS-glycine buffer used in the electrophoresis apparatus during each run. This was thought to have caused the slight differences between homologous protein bands from one electrophoresis run to the next. The results of the experiment could be improved by using as many strains as possible of each fungus, since S. schenckii showed significant differences between strains. The fungi should also be compared at several stages over the culture period and the results averaged because Whitney et al. (1968) found much variation in percent similarity between fungi over the growing period.

The use of electrophoresis to study phylogenetic relationships is based on the theory that the proteins reflect the genotype. Differences in genotypes then are supposedly shown by differences in protein and these can be detected by electrophoresis. The degree of similarity and difference between protein patterns of electrophoretic gels theoretically indicates the closeness of relationship (Dessauer and Fox, 1964).

The probability that proteins in homologous bands are the same, according to Milton et al. (1971), varies directly with the closeness of relationship between the two fungi being compared. Glynn and Reid (1969) mentioned that it may not be valid to compare species of different genera using electrophoresis since a close similarity was found in the protein patterns between Verticillium albo-atrum Reinke and Berth, and Fusarium oxysporum f. sp. cubense. A larger difference was also found by Whitney et al. (1968) between patterns of Verticillium dahliae Kleb. and V. albo-atrum than between V. dahliae and F. oxysporum.

Schechter et al. (1966, 1968) obtained characteristic protein bands from species of fungi offering encouragement that electrophoretic analysis may be used in the identification of fungi. However, Glynn and Reid (1969) concluded that the method is not useful as a taxonomic tool. In their opinion, use of electrophoresis in fungal taxonomic problems was made without enough consideration given towards variations in the protein patterns within a species, the limitations of the technique, and the possible range of interpretation of the results.

Sorenson et al. (1971) found variations of protein patterns between strains of a species and concluded that electrophoresis may not be as valuable a taxonomic tool as was previously thought. They derived an estimate of

the degree of similarity between species using coefficients of similarity (Cs) calculated from the following formula: Cs=a/a+b+c, where a = the number of matching bands between the gels being compared, b = the number of bands in gel 1 which are not matching, and c = the number of bands in gel 2 which are not matching. This method takes into account the number of bands being compared. One of the gels may have more bands than the other gel thereby increasing the chance that there will be more matching bands than a comparison between another strain with fewer bands. This method was used to compare the results. The figures are shown in Table 6.

TABLE 6.--Coefficients of similarity.

55.6	Pulmo-Flint	39.3	Belo-1020
55.6	Flint-Spar	38.2	1020-fag
53.9	Pulmo-Spar	36.5	Pulmo-fag
53.6	Pulmo-1020	35.7	Flint-Belo
50.0	Pulmo-1013	34.6	Pulmo-Belo
48.4	Spar-1020	33.3	Spar-Belo
45.2	Flint-1020	30.0	1013-1020
44.0	Spar-1013	29.1	Belo-fag
43.8	Flint-fag	29.0	1013-fag
41.9	Spar-fag	28.0	Belo-1013
40.7	Flint-1013		

In reference to Table 6, S. schenckii strains Pulmo, Flint, and Sparrow were approximately equal to each other in percent similarity while Belo was not. This may be due to geographic variation since Belo was isolated in Belo

Horizonte, Brazil while all the others were isolated in Michigan. C. stenoceras 1013 and 1020 appeared more similar to the strains of S. schenckii than to each other with the exception of S. schenckii Belo. Strain 1020 was always more similar than 1013, however. These relationships are supported by the general appearance of the fungi in culture. Strain 1020 appears more like S. schenckii than 1013, forming slow growing colonies with dark pigmentation compared to the faster growing, pure white colonies of 1013. Strain 1020, as indicated, is similar in pathogenicity to strains of S. schenckii. C. fagacearum followed next in its similarity to S. schenckii Flint and Sparrow. The closest organism S. schenckii Belo came to in similarity was C. stenoceras 1020. It was then next most similar to the other three strains of S. schenckii followed by C. fagacearum and C. stenoceras 1013. In view of the findings of Glynn and Reid (1969) and Whitney et al. (1968) the use of the results from C. arbuscula in the comparisons of protein bands was deemed not valid and was not undertaken.

Other Enzyme Studies, Sugar Analyses, and Thiamin Requirement Studies

Using acid phosphatase activity in the gels as a criterion, the fungi were divided into two groups: those showing activity and those not showing activity. The gels which showed activity were those of S. schenckii strains Pulmo, Flint, Belo, and Sparrow, and C. stenoceras strains

1013 and 1020. All of the gels shared a common band while S. schenckii Belo and C. stenoceras 1013 consistently exhibited a second non-homologous band. All or part of the protein in each common band must be the same since it exhibits the same function. The lack of alkaline phosphatase activity in the gels may have been due to dissociation of the enzyme during electrophoresis or binding of the enzyme to high molecular weight protein, preventing entry into the gel.

The results of sugar analysis by paper chromatography support those of Spencer and Gorin (1971) except for the presence of mannose derived from C. fagacearum. They were unable to obtain a mannose-containing polysaccharide from this fungus. The reason for this difference is unknown unless it is a strain variation. A rhamnomannan was isolated from S. schenckii cells and used as a skin-active antigen by Aoki et al. (1968). Other polysaccharides in fungi reported by the investigators include: mannan in Candida, xylomannan in Cryptococcus, and galactomannan in Aspergillus. The polysaccharides of each genus are different and may be valuable as a taxonomic aid.

The use of certain enzyme activities as a basis for comparing fungi may be of some value in characterizing each one. Large variations in results between each assay indicates the necessity of several assays to arrive at a more representative figure. Generally, S. schenckii and

C. stenoceras showed similar levels of activity in the protein extracts and culture filtrates, while C. fagacearum and C. arbuscula differed in activity.

Thiamine appears to be required for growth by C. stenoceras 1013 and 1020 as well as for S. schenckii. C. fagacearum and C. arbuscula were not tested for this requirement. A slight increase in growth of the organisms in the medium lacking thiamine was most likely the result of thiamine being present in the fungal spores.

The use of various techniques, including acrylamide gel electrophoresis to analyze the soluble protein extracted from the eight fungi, the assay for and location of acid and alkaline phosphatase activities, and paper chromatography of certain sugars have been useful in showing relationships among the organisms selected which may have taxonomic value. Additional studies, utilizing more strains and species should indicate even more clearly the relationships of this complex group of organisms.

SUMMARY

1. Four strains of Sporothrix schenckii; the isolates of the perfect and imperfect stage of Ceratocystis stenoceras; Ceratocystis fagacearum; and Calcarisporium arbuscula were compared by: disc electrophoresis, enzyme activity, paper chromatography, and nutritional requirement for thiamine in the case of C. stenoceras.

2. The four strains of S. schenckii showed variations in protein bands in the electrophoretic gels. The closeness of relationship between the fungi was evaluated using a percentage of similarity calculated from the number of homologous protein bands and the number of non-homologous protein bands between paired gels. Going from a high to a low degree of similarity: three of the four strains of S. schenckii were similar to each other, the two strains of C. stenoceras similar to S. schenckii Pulmo; C. fagacearum, similar to S. schenckii Flint; S. schenckii Belo, similar to C. stenoceras 1020; and C. stenoceras 1013, similar to 1020. C. arbuscula was not usable as a valid comparison since it was not, or hypothesized not to be, of the same genus as the others.

3. Acid phosphatase activity was present in the same location of the gels containing the protein of the

four strains of S. schenckii and the two strains of C. stenoceras. No activity was detected for C. fagacearum or C. arbuscula. There was no alkaline phosphatase activity in the gels.

4. Acid and alkaline phosphatase enzyme assays in culture filtrates at 24 C. and soluble protein extracts of the four strains of S. schenckii and the two of C. stenoceras showed comparable results. For most strains the acid phosphatase activity was much higher than the alkaline phosphatase activity. Ceratocystis fagacearum and C. arbuscula showed dissimilar levels of enzyme activity.

5. Paper chromatography of the sugars hydrolyzed from mannose containing polysaccharides showed the two strains of S. schenckii and the two strains of C. stenoceras had mannose and rhamnose sugars. Ceratocystis fagacearum yielded mannose and glucose, while C. arbuscula had no recoverable mannose.

6. The two strains of C. stenoceras produced no growth or very little growth in medium deficient in thiamine while heavy growth was evident in medium with thiamine. The same results were obtained with S. schenckii Belo which requires thiamine.

7. The results of this study support the hypothesis of Mariat (1970) that S. schenckii is ancestrally related to members of the genus Ceratocystis.

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