VARIATION IN EXTRACELLULAR AND INTRACELLULAR ENZYMATIC ACTIVITIES IN THE GENERA PHIALOPHORA, FONSECAEA AND CLADOSPORIUM

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ABSTRACT

VARIATION IN EXTRACELLULAR AND INTRACELLULAR ENZYMATIC ACTIVITIES IN THE GENERA *PHIALOPHORA*, *FONSECAEA* AND *CLADOSPORIUM*

By

Sumalee Pichyangkura

Some of the dematiaceous fungi in the genera *Phialophora*, *Fonsecaea* and *Cladosporium* may be etiological agents of chromomycosis while others may be saprobes or plant pathogens. There is a similarity in conidial formation and morphology of these organisms which makes differentiation between pathogens and saprobes difficult. Twenty-two species of human and animal pathogens and forty-five species of saprobes and plant pathogens were studied to determine variations in certain extracellular and certain intracellular enzymatic activities. This group of organisms included five etiological agents of chromomycosis: *Phialophora verrucosa* Medlar, 1915, *Fonsecaea pedrosoi* (Brumpt) Negroni, 1936 comb. nov. Carrion, 1940 emend, *F. compactum* (Carrion) Carrion 1940 comb. nov., *F. dermatitidis* (Kano) Carrion, 1950, and *Cladosporium carrionii* Trejos, 1954.

Paranitrophenol (PNP) derivatives were utilized as substrates to assay for twelve extracellular enzymes in representative strains of these three genera. The fungi

were grown on a medium containing casamino acids, bacto-peptone, yeast extract, glucose and 2.0% of agar at 25°C. After 15 and 25 days of incubation, small agar plugs were removed from beyond the edges of the colonies, and were placed in the PNP substrates with their appropriate buffer. Each one was assayed for extracellular enzyme activities.

There was little or no significant extracellular enzyme activity on the 12 paranitrophenol derivatives by most of the human and animal pathogens. All of the saprophytic species of *Phialophora* had extracellular beta-D-glucosidase and N-acetyl-beta-glucosaminidase activity while the human pathogens had no detectable activity. Similar patterns of these two enzyme reactions were seen with the saprophytic and plant pathogenic species of *Cladosporium* and the human pathogens in the genera *Cladosporium* and *Fonsecaea*. In addition all saprophytic species of *Cladosporium* except *C. carpophilum* have extracellular alpha-D-galactosidase activity while no activity was detected in the human pathogenic species of *Cladosporium*, *Fonsecaea* and *Phialophora*.

There was no difference in extracellular peroxidase activity between pathogens and saprobes. All sixty-seven isolates studied released the enzyme.

Also, no close relationship was detected between adenosine triphosphatase activity and the formation of the sclerotic cells, like the tissue phase of chromomycotic agents which were produced by using West's liquid medium with different nitrogen sources. The ability to release

ATPase by these fungi seemed to be dependent on the organisms per se.

Comparison of soluble protein banding patterns between pathogenic and saprobic fungi was made by electrophoretics on acrylamide gel. A greater number of matching bands of soluble proteins was shown among pathogenic intraspecies than among interspecies of these fungi. *Cladosporium* species showed a close relationship to *Fonsecaea* species, while *Phialophora* species showed a less close relationship to *Fonsecaea* and *Cladosporium*. *Fonsecaea dermatitidis* currently classified in this genus, also exhibited a closer relationship to *Fonsecaea* and to *Cladosporium* than to *Phialophora* species. The electrophoretic patterns of specific enzymes and isoenzymes of *F. dermatitidis* also showed a close relationship to *Fonsecaea* species which further justifies the recent reclassification from *Hormodendrum* to the genus *Fonsecaea*.

Furthermore, it was found that there was a distinctive difference in the number of matching bands of soluble proteins between pathogenic species and saprobes. The number of matching bands was greater among pathogens than among saprobes.

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Sumalee Pichyangkura

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To my Parents

Mr and Mrs Sorn Tosunthorn

and my husband Chart

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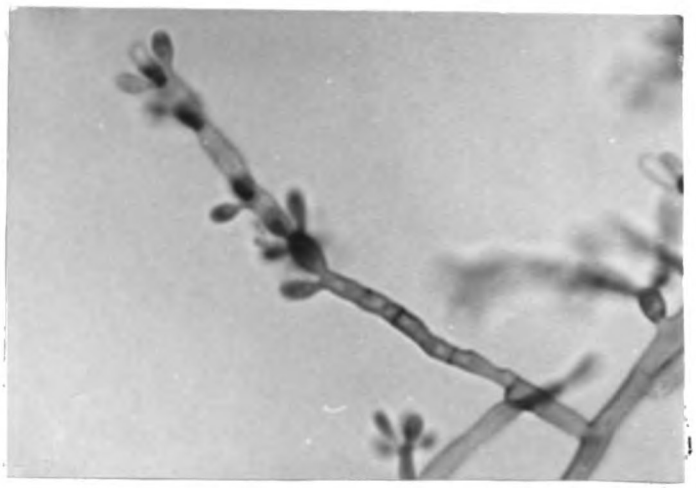
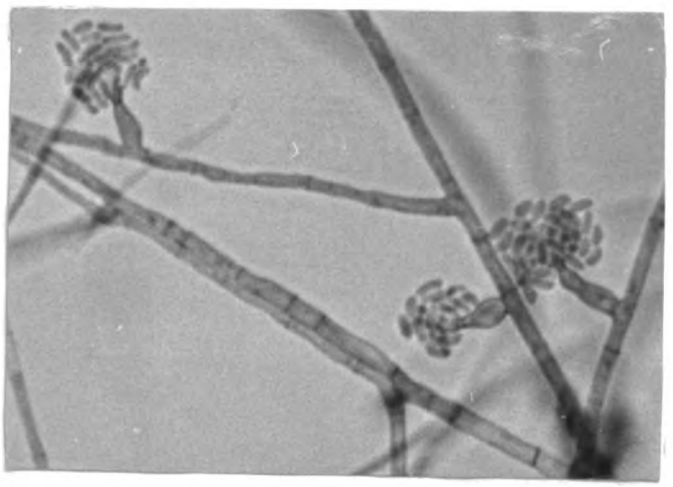
INTRODUCTION

Although much is known about the diagnosis, histopathology and epidemiology of chromomycosis, and about the morphology and immunology of the etiologic agents, the taxonomy of these fungi is still not generally agreed upon. Chromomycosis is commonly caused by any one of five dematiaceous or dark colored fungi, *Phialophora verrucosa* Medlar, 1915, *Fonsecaea pedrosoi* (Brumpt) Negroni, 1936 comb. nov., Carrion, 1940 emend., *F. compactum* (Carrion) Carrion, 1940 comb. nov., *Fonsecaea dermatitidis* (Kano) Carrion, 1950, and *Cladosporium carrionii* Trejos, 1954. These pathogens belong in the form-family Dematiaceae of the Fungi Imperfecti (Deuteromycetes). They are identified to some extent on the basis of three types of conidiophores (see Figure 1), which may vary from one or more types in each culture. The *Phialophora*-type is usually present in *P. verrucosa*; the *Cladosporium*-type is usually present in *Fonsecaea* spp. and always *Cladosporium* spp., and the *Fonsecaea*-type may occur in *Fonsecaea* spp. The genera are closely related and are usually separated by predominance of one or the other of three divergent types of sporulation, but because of multiplicity and variability in details of spore production, they have been placed in many genera, including *Phialophora, Hormodendrum, Acrotheca,*

Cladosporium type

Acrotheca type
(Fonsecaea)





Phialophora type

Figure 1. Types of sporulation found among the agents of chromomycosis (following L. Ajello *et al.*, 1963).

Trichosporium, Gomphinaria, Botrytoides, Phialoconidiophora, Hormodendroides, Rhinocladiella and *Fonsecaea* (Emmons *et al.*, 1970).

The highly complex interrelationships among pathogenic dematiaceous fungi, a cause of identification problems in the routine laboratory procedures and in the literature, is confusing because of the constantly changing terminology and reclassification.

The differentiation of the etiologic agents of chromomycosis from saprophytic members of the dematiaceous is difficult. The pathogens are separated from closely related saprophytic species primarily on the basis of their gross and microscopic morphology. However, the general appearance of the colonies of both pathogenic and saprophytic members of this group is quite similar. In general the saprophytic species grow more rapidly than the pathogens. At any rate, the conidial formation and the similar morphology of human pathogens to plant pathogenic and saprophytic members of the dematiaceous group, makes their identification even more difficult.

Biochemical studies have shown more variation among strains of a single species than between different species, which leads to the lack of reliable biochemical tests to aid in identification (Bindo, 1968; Cooper, 1970; Silva, 1958, 1960). Such physiological characteristics as rate of growth, ability to grow at temperature higher than 30°C, tolerance to cycloheximide, failure to hydrolyze casein, growth stimulation by vitamins, and dimorphism in the

tissue are some of the useful preliminary criteria for separating these fungi from most of the others (Silva, 1960).

Serological and immunological tests have been used by a number of investigators as possible tools for identification and classification of these fungi. The agar gel-diffusion method appears promising for screening of sera (Buckley and Murray, 1966). The fluorescent antibody technique was utilized by Al-Doory and Gordon (1963) to study the serological relationship between members of the dematiaceous fungi. Biguet *et al.* (1965) have utilized immunodiffusion (ID) and immunoelectrophoresis (IEP) tests to compare the antigenic relationships among *P. verrucosa*, *F. pedrosoi* and *C. carrionii*. However, at present serological methods have no important practical application although it is possible to demonstrate the existence of antibodies (Emmons *et al.*, 1963). The agar gel-diffusion test, which may be considered the most practical of all serological methods for this disease, still has limited diagnostic value in advanced cases of chromomycosis.

The purpose of this research was to study the relationship of representative species of pathogenic and saprophytic dematiaceous fungi in the genera *Phialophora*, *Fonsecaea*, and *Cladosporium* on the basis of enzymatic activities. In this study, extracellular enzymatic activities of 22 human and animal pathogenic strains were compared with 45 saprophytic and plant pathogenic strains of dematiaceous fungi. Characteristic electrophoretic patterns of protein

fractions were obtained for comparison by disc gel electrophoresis from each of the pathogenic fungi, and from some isolates of the saprophytic *Cladosporium* and *Phialophora* species. The specificity of intracellular enzymes and isoenzymes, including alkaline phosphatase, acid phosphatase, esterase, peroxidase, amylase, beta-glucosidase and catechol oxidase was identified by using the appropriate substrates on the gels. With each enzyme, the bands for each isolate were compared with those for other isolates of the same species and for isolates of other species.

REVIEW OF LITERATURE

History of the Diseases

The term "Chromoblastomycosis" first used by Terra *et al.* (1922) falsely ascribed the disease to a *Blastomyces* (Carrion, 1950). The term chromomycosis was introduced later by Moore and Almeida in 1935. Since the fungus does not multiply in the tissue by budding but by the formation of septa, the term chromomycosis has been accepted in recent years (Baker, 1971).

The mycological literature for the history of chromomycosis had been published between 1920 and 1940. Many synonyms have been used or suggested for this disease, such as blastomycose negra (Fonseca and Area Leao, 1930), figueira (Rudolph, 1914), verrucous dermatitis (Pedroso and Gomes, 1920), chromomycosis (Moore and Almeida, 1935), dermatite verrucosa cromomicosica (Moore and Almeida, 1935), and Pedroso's, Fonseca's or Gomes' disease (Weidman and Rosenthal, 1941). It was apparent that some cases of this new disease could have been diagnosed in the past as leishmaniasis, syphilis, espundia or mossy foot, which were recorded in Brazil and other South American countries (Al-Doory, 1972).

Chromomycosis generally is accepted as being discovered by Pedroso in Brazil in 1911. Pedroso noticed, as reported

later by Pedroso and Gomes (1920), the presence of large yellowish to dark brown, spherical cells appeared in the skin sections. The disease became known as the black blastomycosis, blastomycose negra, according to Fonseca and Area-Leao (1930). In 1914 Rudolph published his observation on a skin disease popularly known as figueira in the state of Minas Gerais, Brazil. The clinical description and mycologic findings given by Rudolph clearly indicate that he was dealing with the same disease observed by Pedroso in Sao Paulo three years before.

The first chromomycosis report in the United States was in 1915, when two investigators in Boston, Lane and Medlar (Lane, 1915), found a case of verrucosa dermatitis in a young Italian man. Medlar (1915) obtained a fungal isolation from the case. Thaxter in collaboration with the two workers established a new genus and a new species, *Phialophora verrucosa*.

In 1922, Brumpt in his Precis de Parasitologic described the Brazilian isolates as being different from that of the Boston case. He named the Brazilian fungus *Hormodendrum pedrosoi*, based on the branching of the conidia in chains as the characteristic for the genus, and gave the name pedrosoi to the species in honor of the man who discovered the case. In the same year Terra *et al.* (1922) described another Brazilian case of chromomycosis. The isolate displayed dominating terminal conidial clusters characteristic of the genus *Acrotheca* which was established by Fuckel in 1869. They suggested the adoption of the

genus named *Acrotheca* instead of *Hormodendrum*, and named their fungus *Acrotheca pedrosoi* according to the report by Fonseca and Leao (1923). From 1930 on, more cases were reported from the American continent, including a case from Texas caused by *P. verrucosa* (Wilson *et al.*, 1933) and a case from North Carolina caused by *Hormodendrum pedrosoi* (Martin *et al.*, 1936).

Carrion and Emmons, in 1935, reported a third etiologic agent for chromomycosis from Puerto Rico caused by a fungus different from any previous isolates. He described it as a new species called *Hormodendrum compactum*.

In 1936, Negroni made a thorough mycologic study on a fungus he isolated from a case in Argentina. He reported this fungus had both "*Hormodendrum* type" (*Cladosporium* type) and "*Acrotheca* type" (*Fonsecaea* type) sporulation, which made it unsuitable for inclusion in either of the two genera. He created a new genus named "*Fonsecaea*" to include fungi possessing the combined method of sporulation. He named his fungal isolate *Fonsecaea pedrosoi*.

In the following year, Kano (1937) isolated a fungus from the first Japanese case of chromomycosis. He described it as a new agent under the name *Hormiscium dermatitidis*.

Another new fungal agent for chromomycosis was established by Simson in 1946 from a case in South Africa. Carrion compared Simson's isolate, and found it to be similar to a previous fungus isolated three years earlier by O'Daly (1943) from a case in Venezuela. Simson named

the new agent *Fonsecaea pedrosoi* var. *cladosporium*. This fungus was later renamed by Trejos (1954) as a new species, *Cladosporium carrionii*.

Cladosporiosis was first reported in 1911 by Guido Banti (1911) in a woman who died with symptoms of brain tumor which had brown septate hyphae and spherical forms of a fungus in the lesions. In 1912 Saccardo studied and published the name of this organism as *bantiana*. Cladosporiosis had been largely ignored until 1952, when Binford and associates found the fungus in the brain tumor of a man. They named this organism *Cladosporium trichoides*. There can be little doubt today that both the cases of Banti and Binford were caused by the same fungus as both illustrations are similar. Borelli (1960) proposed a new combination to designate the species, *Cladosporium bantinum*.

Phialophora jenselmei , a pathological agent of maduromycosis, has been isolated in the United States and in Europe (Emmons, 1945). The synonyms are: *Torula jeanselmei* Langeron, 1928; and *Pullularia jeanselmei*, Dodge, 1935. The fungus was first isolated from a mycetoma by Jeanselme, Huett and Lott and named *Torula jeanselmei* by Langeron (1928). The fungus was studied and renamed *Phialophora jeanselmei* by Emmons (1945).

Schwartz and Emmons (1968) reported a new pathogenic *Phialophora richardsiae* caused by a subcutaneous cystic granuloma in a man. This organism was originally cited in 1934 by Melin and Nannfeldt as one of the several fungi responsible for the discoloration of ground woodpulp. No

known human infection with this fungus has been reported.

The presence of large, dark-brown to yellowish, spherical bodies, sclerotic cells, in a biopsy is the characteristic appearance in cases of chromomycosis. The sclerotic cells divide by splitting or forming septa in cells. The physiology of sclerotic cells is obscure (Silva, 1957).

Source and Geographical Distribution of the Disease

The existence of chromomycosis has been established throughout the world. Although *Fonsecaea pedrosoi* is infrequently found in soil, Carrion (1950) found the widespread distribution of its victims has established its ubiquity. It is the most common etiologic agent of chromomycosis. Actually Conant (1937) and Conant and Martin (1937) presented the first evidence that the etiologic agent of chromomycosis was found in nature in soil, plant debris or wood fragments. Three hundred thirty-six samples of soil, wood and plants from several areas in the State of Merida, Venezuela, and adjacent areas were studied by Salfelder *et al.* (1968). Six strains of *Fonsecaea pedrosoi* were obtained from samples. Gezuele *et al.* reported in 1972 that thirty-one strains of *P. verrucosa* and 34 of *P. pedrosoi* were isolated from 329 samples of plant debris, soil and other materials by utilizing inoculated animals and by direct cultures. The determination of the pathogenic strains recovered from natural sources is presumably based on the mycological and biological similarity between

them and those isolated from man. The climatic conditions probably play a role in the location of the fungi. This is indicated by Carrion (1950) in his survey of 90 cases of chromomycosis. He found 83 per cent of the isolates from these patients were *F. pedrosoi*, as these patients seemed to have contracted the disease in tropical or subtropical regions, and only 17 per cent were from the temperate zone. It appeared that 5 out of 6 existing isolates of *P. verrucosa* were from patients in colder climates. Cole and Kendrick (1973) presented a classification of six common wood-inhibiting species of *Phialophora* associated with bleeding of softwoods and some hardwoods in North America, which included *P. verrucosa*. Chromomycosis is more common in the tropical and subtropical regions. The disease is considered rare in some areas and even absent in some countries. Al-Doory (1972) has compiled an extensive list showing the distribution of case reports of chromomycosis in the world. He listed the following countries with the greatest number of cases in decreasing numbers: Brazil, Costa Rica, Madagascar, Dominican Republic, Australia, Cuba, Japan, Colombia, Venezuela, Mexico, South Africa, Paraguay, India, Honduras, and United States.

Nutritional and Biochemical Studies

The most recent studies on nutrition of chromomycotic agents were those of Silva (1957, 1958, 1960). Silva (1958) observed that alterations in basal medium (Czapek-Dox) by increasing the concentration of inorganic nitrogen,

substituting ammonium chloride for sodium nitrate, or adding yeast extract would increase the pseudo-*Acrotheca* type of sporulation of *Fonsecaea pedrosoi*. No substance was found that consistently stimulated either the *Phialophora* or *Cladosporium* type of sporulation. Silva (1960) found organic nitrogen essential for the growth of a strain of *P. jeanselmei*, both organic nitrogen and the vitamin supplement stimulated the growth of strains of *P. verrucosa* and *P. obscura*, but had no effect on growth of any other strains studied. The amount of carbohydrate was the factor that influenced the rate of growth, rather than the amount of nitrogen. Substitution of glucose for sucrose did not significantly alter the sporulation ratio of *Cladosporium* and pseudo-*Acrotheca* types (Silva, 1958).

The vitamin requirement study by Area Leao and Cury (1950) indicated that only thiamine is required by *P. pedrosoi*, *P. verrucosa*, *F. compactum* and *P. jeanselmei*. A species identified by Area Leao and Cury (1950) as *Cladosporium wernecki* requires no vitamins for growth. Gilardi (1965) in a study of vitamin and nitrogen requirements found that inorganic ammonium salts are required for *F. pedrosoi*, *F. compactum*, *H. dermatitidis*, *C. carrionii*, *C. sphaerospermum*, and *P. richardsiae*, and organic nitrogen salts are required for *P. jeanselmei*, *P. verrucosa* and *P. obscura*. Silva (1960) observed the superiority of bactopeptone over neopeptone in Sabouraud's agar for cultivation of the etiological agents of chromomycosis.

Montemayor (1949) reported an optimal temperature range of 20 to 25°C for saprophytic *Cladosporium* spp, 30°C for one of the agents of mycetoma, *P. jeanselmei*, and 37°C for agents of chromomycosis. Silva (1958, 1960) reported the optimal temperature for growth of agents of chromomycosis ranged between 25 and 35°C. She concluded that the faster growth rate at 25°C for saprophytic Cladosporia does not provide a reliable differentiation.

The physiological activity of *F. compactum*, *F. pedrosoi*, *P. jeanselmei* and *P. verrucosa* was examined by Montemayor (1949). None of these species liquified gelatin, Loeffler's serum or coagulated milk, in contrast to the positive activity of two saprophytic *Cladosporium* isolates. Trejos (1954) states that *C. carrionii* and *C. trichoides* did not exert proteolytic activity on Loeffler's serum, contrary to the activity of saprophytic species of this genus. De Vries (1952) studied the enzymatic activity of 24 saprophytic species of *Cladosporium*. He found that production of tributyrine hydrolyzing lipases seemed to be a constant characteristic of these species since all decomposed tributyrine in varying degrees. Fuentes and Bosch (1960) studied biochemical activities for distinguishing etiologic agents of chromomymycosis, brain abscess, and maduromycosis and certain related non-pathogenic species. None of the strains recognized as etiologic agents of chromomycosis had the ability to liquify gelatin and Loeffler's serum, to coagulate milk, to digest starch, or to utilize tributyrine and cellulose. On the other

hand saprophytic *Cladosporium* spp. all are able to utilize tributyrine or cellulose. Rosenthal (1964) reported *F. pedrosoi*, *F. compactum*, *F. dermatitidis*, *P. verrucosa* and *H. capsulatum* utilized tyrosine and urea but have no activity on casein and gelatin, and hydrolysis of starch was flexible.

West (1967) attempted without success to induce fertile perithecia in *P. verrucosa* A.126, isolated from a patient with chromoblastomycosis by varying the amounts of 19 different carbon and 44 nitrogen sources.

The parasitic phase of these agents, which are spherical, brown with thick-walled cells, is of the sclerotic type. Moore (1941) used the surface of the chorioallantoic membrane of the fertile egg for the cultivation of *P. verrucosa*. The fungal growth reversed to the parasitic phase morphology within 5 to 10 days.

Silva (1957) tried to elicit the parasitic phase in many artificial media that would imitate the habitat of these fungi both *in vitro* and *in vivo*. The conversion of vegetative mycelium to sclerotic cells was clearly seen from the experiments, but the factors required for the induction of the parasitic phase of chromomycosis are unknown. Later on West (1967) used L-proline in a synthetic medium to induce the formation of the sclerotic cells in *P. verrucosa*.

Szaniszlo *et al.* (1972) attempted to differentiate the three chromomycosis agents on the basis of chemical composition of the hyphal walls. They demonstrated the similarity of the unfractionated hyphal wall composition

for *P. verrucosa*, *F. pedrosoi* and *Cladosporium carrionii*, which consisted of large amounts of glucose (17-31%) and protein components (29-42%), small amounts of mannose (8-14%) and glucosamine (6-8%). Alkali-soluble wall fractions consisted predominantly of glucose and mannose, while alkali-insoluble wall fractions consisted mainly of glucosamine and protein components. Chitin was identified as a wall component by release of N-acetyl glucosamine during enzymatic digestion of the wall with *Streptomyces griseus* chitinase. They concluded that the hyphal forms of human pathogenic fungi have wall compositions intermediate between those of Euascomycetes and Hemiascomycetes.

Serology and Immunity

Stone (1930) first studied the dematiaceous fungi serologically by the use of the complement fixation test. Conant and Martin (1937) and Martin *et al.* (1936) were the first to employ the complement fixation test for the identification and classification of the various fungi of chromomycosis and some other members of the dematiaceous groups. They used fungal extracts as antigen. Conant and Martin (1937) noted that sera of rabbits immunized with *F. pedrosoi* and *F. compactum* have a high complement fixation titer for their homologous antigen and for each other. Meanwhile sera from rabbits immunized with *P. verrucosa* produced a positive reaction with their homologous antigen only. Martin (1938) determined the antigenic similarity between *Cladophora americana*, isolated from wood pulp, and

a strain of *P. verrucosa* isolated from a chromomycosis patient in Uruguay.

Martin *et al.* (1936) and Conant *et al.* (1937) reported that complement-fixing antibodies have been demonstrated in sera of patients with chromomycosis. They found that serum from a North Carolina patient infected with *F. pedrosoi*: a) fixed complement with strains of the same fungus isolated from the patient as well as with isolates from South American and Puerto Rican strains; b) cross reacted with a strain of *P. verrucosa*; and c) failed to obtain a complement fixation with other fungi, such as *Blastomyces dermatitidis, Sporotrichum schenckii,* saprophytic *Cladosporium* spp. and other pigment-producing fungi.

Wilson and Plunkett (1965) found no correlation between a certain serological titer and the clinical course of the disease, which would aid in diagnosis or prognosis.

Seeliger reported (1968) that *P. verrucosa, F. compactum, F. pedrosoi* and *C. carrionii* produce cross precipitin reaction with antisera against *Pullularia pullulans, P. bergeri (Torula bergeri), P. werneckii (Cladosporium werneckii), Sporotrichum gougerotii (Cladosporium gougerotii)* and *S. schenckii.*

Nielson and Conant (1967) evaluated the relationship of the yeast-like dematiaceous fungi utilizing the agglutination tests. With few exceptions, they obtained good correlation between the micromorphological differences in fungi and their antigenic properties. Isolates from chromomycosis showed antigenic similarities to *S. gougerotii, P.*

jeanselmei and *F. dermatitidis*. However, isolates from brain abscesses produced various patterns of agglutination.

Agar gel-diffusion appears to produce the most promising results for identifying the organisms in this disease of any tests. Recently Buckley and Murray (1966) obtained precipitating bands in 12 of their 13 cases tested. The 13th patient was under treatment with amphotericin B and had no reactive precipitating bands. This is probably due to the diminishing of antibodies during the treatment. There was more cross reactivity between *F. compactum* and *F. pedrosoi* than between either of them and *P. verrucosa*.

Biguet *et al.* (1965) reported on the common antigen factors among dematiaceous pathogens from a study of water soluble antigens extracted from mycelia and extracellular antigens from culture filtrates. Immunodiffusion (ID) and immunoelectrophoresis (IEP) tests were used to study antigenic relationships among *P. verrucosa*, *F. pedrosoi* and *C. carrionii*. They reported more common antigens appeared between *P. verrucosa* and *C. carrionii* than between either species and *F. pedrosoi*, and concluded that antigenically, *P. verrucosa* and *C. carrionii* are more closely related to one another than to *F. pedrosoi*. In 1970, Cooper and Schneideu used the immunodiffusion test and obtained fewer numbers of antigens from the filtrate (F antigen) than from mycelial growth (M-1 and M-2 antigens) of *P. verrucosa*, *F. pedrosoi* and *C. carrionii*. Common antigens were found among all three species, indicating some degree of relationship. The overall results were similar to that obtained by Biguet *et al.* (1965).

Al-Doory and Gordon (1963) used the fluorescent antibody technique to differentiate *C. carrionii* from *C. bantianum*, as well as to differentiate these two species from all other species tested, including *C. guogerotii*, *C. mansonii*, *C. werneckii*, *F. dermatitidis*, *F. compactum*, *F. pedrosoi*, *P. jeanselmei*, *P. verrucosa*, *T. bergeri* and nonpathogenic *Cladosporium* spp.

In 1965 the fluorescent antibody technique was reported again by Gordon and Al-Doory. Ninety-two strains belonging to 39 species of fungi related or unrelated in the dematiaceous group were tested with conjugates of *F. pedrosoi*, *F. compactum* and *F. dermatitidis*. The genera *Cladosporium* and *F. compactum* were found to be closely related serologically to *F. dermatitidis*. There was little relationship shown serologically between either of these species and *P. verrucosa*. Under the same condition conjugates of two strains of *P. verrucosa* failed to react with any of the three species of *Fonsecaea*.

The hypersensitivity test was done by Martin *et al.* (1936) in pathogenesis of chromomycosis. The test showed only a slight delayed reaction in the site of the intracutaneous injection with an autogenous heat killed antigen.

MATERIALS AND METHODS

Collection of Cultures

Twenty-two isolates of pathogenic dematiaceous fungi, including *Phialophora verrucosa, Fonsecaea pedrosoi, Fonsecaea compactum, Hormodendrum dermatitidis, Cladosporium carrionii, Cladosporium trichoides* and *Phialophora jeanselmei*, and forty-six isolates of non-pathogenic fungi and plant pathogens were obtained from three different sources.

A. Department of Health, Education, and Welfare, Center for Disease Control, Mycology Section, Atlanta, Georgia:

1. A835 *Cladosporium carrionii*
   Record from Dr. A. Trejos (#27, 1954)
   Original from Emmons #8619 "Hormodendrum from Australia"

2. A984 *Cladosporium carrionii*
   Record from Dr. Borelli 1955
   Borelli #943 Isolated from skin scraping in Venezuela

3. A980 *Cladosporium trichoides*
   Record from Dr. Emmons, 1955
   Original from Emmons #8590 Isolated from Barnola case

B. Department of the Army, U.S. Army Natick Laboratories, Natick, Massachusetts:

1. QM 260 *Phialophora compactum* (NIH 8605; ATCC 10222; CBS 285.47)
Isolated by A. L. Carrion in Puerto Rico in 1935 from a case clinically diagnosed as chromoblastomycosis

2. QM 265 *Phialophora fastigiata* (NIH 8705)
Isolated by E. Melin in Sweden in 1937 from wood pulp

3. QM 8008 *Phialophora fastigiata*
IMI 86,982;
Culture received October 1961 from D. Eveleigh. Isolated and identified by D. Brewer in Quebec, Canada, in 1958 from paper-mill slime

4. QM 267 *Phialophora lagerbergii* (NIH 8707)
Isolated by E. Melin in Sweden in 1937 from wood pulp

5. QM 270 *Phialophora jeanselmei*
NIH 8724; ATCC 10,224;
Culture from C. W. Emmons
Isolated by D. Symmers in New York in 1944 from hand; clinical diagnosis mycetoma

6. QM 1487 *Phialophora malorum*
No isolation data. Obtained from L. P. McColloch, Beltsville, Maryland, 1949

7. QM 266 *Phialophora melinii* (NIH 8706)
Isolated by E. Melin in Sweden in 1937 from wood pulp

8. QM 268 *Phialophora obscura* (NIH 8708)
Isolated by E. Melin in Sweden in 1937 from wood pulp

9. QM 259 *Phialophora pedrosoi* (NIH 8603)
Isolated by A. L. Carrion in Puerto Rico in 1935 from a case clinically diagnosed as chromoblastomycosis

10. QM 261 *Phialophora pedrosoi*
NIH 8610;
Culture received from C. W. Emmons 8 May 1947. Isolated by H. Hailey in Georgia in 1939 from a case clinically diagnosed as chromoblastomycosis

11. QM 262 *Phialophora pedrosoi*
NIH 8615: ATCC 10,221
Culture from C. W. Emmons
Isolated by C. Binford in New Orleans in 1943 from a case clinically diagnosed as chromoblastomycosis

12. QM 263 *Phialophora richardsiae* (NIH 8703)
Isolated by E. Melin in Sweden in 1937 from wood pulp

13. QM 6808 *Phialophora richardsiae*
WB 1630; NRRL 1630; Conant 330;
Culture received July 1955 from K. B. Raper, University of Wisconsin. Sent to NRRL from Harvard in June 1940 as Harvard 260. Isolated by Conant, June 10, 1937

14. QM 264 *Phialophora verrucosa* (NIH 8704)
Isolated by E. Melin in Sweden in 1937 from wood pulp

15. QM 269 *Phialophora verrucosa*
NIH 8723; ATCC 10,223;
Culture from C. W. Emmons
Isolated by M. Moore in St. Louis in 1943 from a case clinically diagnosed as chromoblastomycosis

16. QM 489 *Cladosporium cladosporioides*
(ATCC 16,022; CMI 45,534)
Isolated by E. Sigel from caulking compound in 1948. Determined by G. A. deVries

17. QM 9485 *Cladosporium cladosporioides*
Isolated by E. G. Simmons in Bogor, Indonesia, in 1969 from decaying leaf. Determined by M. B. Ellis

18. QM 3167 *Cladosporium herbarum*
Isolated from a collapsible canteen in 1945. Det. de Vries

19. QM 9466 *Cladosporium oxysporum*
Isolated by E. G. Simmons in Bogor, Indonesia, in 1969 from dead leaf of Palmae. Det. M. B. Ellis

20. QM 9481 *Cladosporium oxysporum*
Isolated by E. G. Simmons in Bogor, Indonesia, in 1969 from decaying petiole of *Pangium edule*. Det. M. B. Ellis

21. QM 9489 *Cladosporium oxysporum*
Isolated by E. G. Simmons in Bogor, Indonesia, in 1969 from rotting leaf of dicot. Det. M. B. Ellis

22. QM 9495 *Cladosporium oxysporum*
Isolated by E. G. Simmons in Bogor, Indonesia, in 1969 from decaying woody male inflorescence of *Arenga* sp. Det. M. B. Ellis

23. QM 9496 *Cladosporium oxysporum*
Isolated by E. G. Simmons in Bogor, Indonesia, in 1969 from decaying woody male inflorescence of *Arenga* sp. Det. M. B. Ellis

24. QM 8013 *Cladosporium resinae* (ATCC 18215)
Isolated by G. A. Atkins in Victoria, Australia, in 1961 from Avtur fuel, P20

25. QM 7998 *Cladosporium resinae* f. *avellaneum*
Isolated in 1961 in Victoria, Australia, from Avtur fuel. Det. de Vries

26. QM 9257 *Cladosporium resinae* f. *avellaneum* (*Amorphotheca resinae* st. perf.)
Isolated by D. G. Parbery in Melbourne, Australia (in 1968?), from soil. Det. Parbery

27. QM 9258 *Cladosporium resinae* f. *avellaneum* (*Amorphotheca resinae* st. perf.) Isolated by D. G. Parbery in Melbourne, Australia (in 1966-67?), from grassland soil. Det. Parbery

28. QM 55b *Cladosporium sphaerospermum* Isolated from leather band, collected Finschafen, New Guinea, in 1944. Det. de Vries

29. QM 8050 *Cladosporium sphaerospermum* Isolated from marine habitat. Received from S. Meyers, Marine Laboratory, Miami, Florida, in 1961. Det. E. G. Simmons and D. I. Fennell

30. QM 9494 *Cladosporium sphaerospermum* Isolated by E. G. Simmons in Bogor, Indonesia, in 1969 from hard decaying fruit of *Alsomitra macrocarpa*. Det. M. B. Ellis

31. QM 9516 *Cladosporium sphaerospermum* Isolated by E. G. Simmons in Bogor, Indonesia, in 1969 from decaying spadix of *Oncosperma horrida*. Det. E. B. Ellis

32. QM 9517 *Cladosporium sphaerospermum* Isolated by E. G. Simmons in Bogor, Indonesia, in 1969 from decaying spadix of *Oncosperma horrida*. Det. M. B. Ellis

C. Michigan State University Culture Collection:

a. Mycology Laboratory, Dr. E. S. Beneke

1. *Phialophora verrucosa* G. S. Bulmer, University of Oklahoma Med. Sch. Oklahoma City, Oklahoma
2. *Phialophora verrucosa* H. D. MacCurdy, University of Windsor, Windsor, Ontario, Canada
3. *Phialophora jeanselmei*, Michigan State University
4. *Fonsecaea pedrosoi* Patient in Belo Horizonte, Brazil
5. *Fonsecaea pedrosoi*, Duke University

6. *Fonsecaea compactum*, Duke University
7. *Hormodendrum dermatitidis*, University of Michigan
8. *Cladosporium trichoides*, Michigan State University
9. *Phialophora* sp. (unk.), Duke University

b. Plant Pathology Laboratory, Dr. Edward Klos

1. *Cladosporium carpophylum* causes peach scab disease, identification based on U.S.D.A. Bureau Plant Industry Bulletin No. 395
2. *Cladosporium carpophylum* causes apricot scab

c. Laboratory of Dr. Donald J. Dezeeuw

1. *Cladosporium cucumerinum* (F-26) Isolate I from Dept. of Plant Pathology, University of Wisconsin
2. *Cladosporium cucumerinum* (A-I) Isolated from a cucumber fruit obtained from Arenac County, Michigan, in 1967
3. *Cladosporium cucumerinum* (St. clair) Isolated from a cucumber fruit obtained in St. Clair County, Michigan, in 1957

d. Author's collection

Twenty isolates of unidentified species *Cladosporium*, collected from air and soil around M.S.U. campus

D. University of Michigan Culture Collection:

Unidentified fungus, caused amphibian chromomycosis (*Rana pipiens*, RAP; Leopard frog) E-286 *Phialophora* (or possibly *Fonsecaea*)

Extracellular Enzyme Procedures

1. Survey for extracellular enzymatic activity in solid medium

The CPYG solid medium introduced by Beneke *et al.* (1969) was used to grow the organisms for the study of extracellular enzymes. The CPYG solid medium is composed of 4 grams of casein, 4 grams of bactopeptone, 4 grams of yeast extract, 10 grams of glucose and 20 grams of agar per

liter with the final pH being 5.8. Twelve-day-old stock cultures of the fungi, which had been grown on Sabouraud's medium, were used as inocula. A plug 2 mm. in diameter was removed from each fungal culture for inoculum and placed on the center of a CPYG medium plate. After incubation at 25°C for 15 and 25 days, small uniform-sized plugs of the agar were removed from beyond the growing fungal colony with a 0.5 cm. diameter of cork borer. These plugs were then used directly in the enzyme assays.

Twelve paranitrophenol (PNP) derivatives were chosen as substrates for the various enzymes investigated. The substrates and buffers were: PNP-alpha-D-glucoside 0.5 mg. per ml., PNP-beta-D-glucoside 0.5 mg. per ml., PNP-N-acetyl-beta-glucosaminide 0.15 mg. per ml., all in 0.005 M, pH 5.4, sodium acetate buffer; PNP-alpha-D-galactoside, PNP-beta-D-galactoside, PNP-butyrate, PNP-caprylate, PNP-laurate, PNP-palmitate, PNP-stearate, all 1.0 mg. per ml. in 0.1 M, pH 7.0, citrate-phosphate buffer; PNP-phosphate 1.0 mg. per ml. in 0.1 M, pH 8.6, Tris HCL buffer for alkaline phosphatase, and in 0.1 M, pH 5.0, sodium acetate buffer for acid phosphatase.

The assay for the hydrolysis of the above substrates was performed by using a plug of agar as the enzyme source and adding 1.0 ml. of substrate solution into tubes. The assay mixtures were then incubated at 37°C for two hours. At the end of incubation period 2.0 ml. of a 0.5 M Tris buffer, pH 9.8, was added to stop the reaction and develop

the yellow color of the liberated PNP. The activity of the enzymes is expressed as O.D. (optical density) units by spectrophotometer at 410 nanometers.

The organisms were grown in liquid CPYG medium to study the extracellular enzymes with these substrates listed above, but showed no satisfactory results.

2. Survey for extracellular enzymatic activity in liquid medium

a. Liquid CPYG medium for culture of organisms for detecting determination of peroxidase activity

Suspensions of conidia and mycelial fragments were made from twelve-day-old stock culture tubes growing on Sabouraud's medium, with 5 ml. physiological saline (0.85% NaCl). Then 1 ml. of the cell suspension, which consisted of approximately 45×10^4 to 50×10^4 cells was inoculated into 150 ml. Erlenmeyer flasks with 100 ml. of the liquid CPYG medium. The flasks were inoculated and placed on a rotary shaker at 150 rpm at 25°C. The liquid cultures with any extracellular enzymes were filtered at 5, 10, and 15 days through a Seitz sterile filter. Then 1 ml. of each filtrate was used in the extracellular enzyme assays.

The method of Lück (1963) was used to determine peroxidase activity. Stock solutions and buffers were: hydrogen peroxide 0.03 M (0.66 ml. of H_2O_2 or 30% w/v, in 200 ml. of double distilled water), p-phenylenediamine 1% w/v, and 0.067 M, pH 7, phosphate buffer.

The assay of peroxidase is based on the principle that P-phenylenediamine is the hydrogen donor. It is oxidized by H_2O_2 and peroxidase to a colored derivative which is a molecule formed from diamine and diimine. The O.D. activity is measured by the increase in the purple color per unit of time at 485 nanometers.

A tube with a mixture of 1 ml. of the filtrate, 3 ml. of 6.7×10^{-2} M, phosphate buffer, pH 7, 0.3 ml. of hydrogen peroxide and 0.3 ml. of p-phenylenediamine was incubated at 25°C on the shaker for 20 minutes. The control tube contained the same mixture except for the p-phenylenediamine was added at the end of incubation period. The control tube was set at time zero, and then the incubated tube was read after 20 minutes.

b. West's liquid medium for culture of organisms for detecting extracellular adenosine triphosphatase activity

The various agents of chromomycosis and saprobes were grown in West's Basal liquid medium (1967) for determination of adenosine triphosphatase. The medium consists of:

Carbon source containing C in amount of	48.00 g.
Nitrogen source containing N in amount of	0.285 g.
$MgSO_4 \cdot 7H_2O$	0.5 g.
$KHPO_4$	1.3 g.
$FeCl_3$	0.005 g.
Thiamine HCL	50.0 µg.
Distilled water to adjust total volume to	1000 ml.

The pH was adjusted to 6.2 with either HCL or NaOH, then autoclaved for 10 minutes at 121°C. West's basal medium had glucose and DL-isoleucine or glucose and l-proline as carbon and nitrogen sources, respectively. The sterile filtrate of thiamine HCL was added after the medium was autoclaved.

The inoculum was prepared by adding 5 ml. of 0.85% NaCl to a 14-day-old potato dextrose agar slant stock culture, then gently scraping to remove spores. A 1 ml. conidial suspension containing approximately 15×10^4 to 20×10^4 cells/ml. was inoculated into 50 ml. of West's liquid medium in 125 ml. Erlenmeyer flask. After incubation at 25°C for the period of 7, 14, 21 and 28 days on a rotary shaker at 150 rpm, liquid cultures were filtered through sterilized Seitz filters. The filtrates were then used for determination of ATPase activity.

The assay method was based on the determination of inorganic phosphate which is released from the hydrolytic reaction of the enzyme (Ames, 1960; Pollard and Singh, 1968). The inorganic phosphate is released from the molecule of adenosine triphosphate by the hydrolytic enzyme adenosine triphosphatase, which will combine with ammonium molybdate to form phosphomolybdate complex. Further reduction of this complex by ascorbic acid will develop a bluish purple colored complex which could be measured colorimetrically. The reagents for the determination of the enzyme consisted of two parts: A) 10% ascorbic acid, B) 0.42%

ammonium molybdate, $4H_2O$ in 1 N H_2SO_4. Mixture of one part of A and 6 parts of B had to be freshly made just before the assay.

Enzymatic assays were done in a clean test tube. The mixture of 0.5 ml. of growth media filtrate, 0.5 ml. of 0.1 micromole of adenosine triphosphate substrate, 1 ml. of citrate buffer pH 4.8 and 1.5 ml. of mixture reagent of A and B were mixed thoroughly and incubated at 37°C for 2 hours. A control tube in which substrate was added at the end of incubation period was utilized. The optical density was read at 680 nanometers.

Gel Electrophoresis of Intracellular Soluble Proteins, Enzymes and Isoenzymes

1. Culture preparation

Twenty-two human pathogenic fungi and eight saprobic dematiaceous fungi were subjected to gel electrophoresis. Fourteen-day-old stock cultures of the fungi grown on potato dextrose agar were used to prepare spore and hyphal suspensions with 5 ml. of 0.85% NaCl (physiological saline). Four milliliters of this suspension was inoculated into each 250 ml. Erlenmeyer flask containing 100 ml. of CPYG liquid medium (Beneke *et al.*, 1969). The flasks were then incubated and placed on a rotary shaker at 150 rpm at 25°C for two weeks.

2. Extraction of soluble proteins

After two weeks all of the cultures in the flasks were checked for contamination before extraction. The extraction of soluble proteins was based on Gunsalus' method (1962) by using acetone dried preparation. The mycelia collected by filtration on Whatman No. 1 filter paper in a Buchner funnel under vacuum were thoroughly washed three times with sterile double distilled water. Five grams of the mycelial mats were then added to 15 ml. of acetone at -20°C. Three grams of acid-washed sand and the acetone suspension were put in a Waring micro-mixer for approximately five minutes at 15,000 rpm with the mixer container covered with acetone dry ice. Approximately 30 ml. of acetone at -20°C was slowly added into the container. After stirring, the aqueous portion was allowed to settle, and was filtered through a Whatman filter paper No. 1 in a Buchner funnel under vacuum. The powder on the paper was washed with 20 ml. of acetone at -20°C, sucked dry on the filter, and ground to a dry powder with a spatula. The powder was stored at -20°C in a dried bottle.

The dried acetone powder was added to 1 ml. of 0.004 M sodium bicarbonate solution and was stored overnight at 4°C. The suspension was centrifuged at 4,500 rpm for 25 minutes at 25°C. The supernatant, collected by using a micropipette, was kept at 4°C for further determination of the protein concentration.

Protein concentrations were estimated by Lowry's Folin method (Lowry *et al.*, 1951). Bovine serum albumin was used as a standard. An estimated amount of 300 µg./ 0.1 ml. of protein in each fungus was used for disc electrophoresis. The protein extracts with 0.1 ml. of 40% sucrose were either used immediately or stored at -20°C.

3. Disc electrophoresis procedure

The theory and method of disc electrophoresis described by Ornstein (1964) and Davis (1964), respectively, was followed, except for the use of Tris-glycine buffer diluted two times and a 11% small pore gel (Frank and Berry, 1972). Polyacrylamide gel columns were made in 5 x 100 mm. cylindrical glass tubes. The gels were composed of two parts. The lower part was a small pore gel where the fractionation of proteins took place. The upper part was a large pore gel where the protein became stacked according to their sizes and charges before being separated in the small pore gel. Reagents used in separation of gels were: acrylamide, N,N'-methylenebisacrylamide (BIS), 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIZMA Base or TRIS), N,N,N',N'-tetramethylethylene diamine (TEMED), riboflavine hydrochloric acid and ammonium persulfate. Stock solutions were prepared as follows:

Stock A			Stock B		
1N HCL	48	ml.	1N HCL	approximately 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	44.0	g.	Acrylamide	10	g.
BIS	0.197	g.	BIS	2.5	g.
Water to	100	ml.	Water to	100	ml.

Stock E			Stock F		
Riboflavine	4	mg.	Sucrose	40	g.
Water to	100	ml.	Water to	100	ml.

Working solutions were prepared every time before using.

Working Solutions

Small pore Solution #1	Small pore Solution #2	Large pore Solution
1 part A 2 parts C 1 part water pH 8.9 (8.8-9.0)	Ammonium persulfate 0.14 ml. water to 100 ml.	1 part B 2 parts D 1 part E 4 parts F pH 6.7 (6.6-6.8)

The stock buffer solution for the reservoirs consisted of TRIS 6.0 g., glycine 28.8 g., water to 1 liter, pH 8.3, and a dilution of 1 part of buffer to 2 parts of water. The

buffer in the upper reservoirs was kept separate in another container from the lower one until used again.

The gels were prepared by placing clean glass tubes upright into the rubber holder rack. The lower gel solution was made by combination of 1 part A, 2 parts C, 1 part water and 4 parts of small-pore solution #2. A special long needle and 30 ml. syringe was used to place the mixture of gel up to 7 cm. into the glass tube. A thin layer of water was gently added on the surface edge of the tube to flatten the surface of the gel. Polymerization of the gel was complete after exposure under fluorescent light for 20 minutes. Then the water on the surface was blotted away. The large pore gel, the upper gel, was made of 1 part B, 2 parts D, 1 part E and 3 parts F. A height of 1 cm. of upper gel was laid on top of the polymerized lower gel, and two drops of water added to flatten the surface. The water was removed when polymerization was completed.

The gel tubes were placed in the holes of the upper tank. Both tanks were filled with TRIS-glycine buffer pH 8.3, and diluted 1:2 with double distilled water. The tubes were promptly connected by the upper and lower buffer tanks. A few drops of 0.5 g./ml. of bromephenol blue (crystal) were added to the buffer in the upper tank for tracing the progression of electrophoresis. Fifteen microliter aliquot (300 µg.) of soluble protein extract combined with 0.1 ml. of 40% sucrose was gently placed on top of the gel column by a micropipette.

A Heathkit Model ZP-32 Power supply was connected to an electrophoresis apparatus by an anionic system. The negative charge protein molecules will move along the gel toward the anode (+) which was located in the buffer of the lower tank; the cathode was in the upper one. A current of 3.5 milliamperes per tube and 120 volts/cm. was applied on the system (Bloemendal, 1967). The time required as indicated by the dye to move about 60 mm. in the separated gel was usually about 120 minutes.

At the completion of electrophoresis, the gel tubes were removed from the upper reservoir and put on crushed ice for a few minutes. The gels were removed from the glass tube by rimming under cold water and injecting water between the gel and tube wall with a long needle syringe. The wire was then withdrawn. The gels were removed with a slight pressure against the gel. The gel columns were stained with amido Schwarz dye in order to determine the general soluble protein pattern. The others were incubated with the specific substrates for determination of enzymes and isoenzymes.

4. Gel staining

a. Staining for general soluble proteins

Fractionated soluble protein gels were stained 0.5% (w/v) in amido Schwarz dye in 7% acetic acid for 30 minutes, and transferred into 7% acetic acid destaining solution overnight until the sharp dark bands appeared.

The individual gel was kept in a tube with 2% acetic acid. Reading of protein bands was made by using a Gilford 2400 spectrophotometer with a gel transport attachment at 680 nanometer, a chart speed of 1 minute/inch, and a scan rate of 2 cm./min. Diagrammatic interpretations were made of the bands in addition to photographs.

b. Specific soluble protein staining for intracellular enzyme and isoenzyme locations

The gels were removed from the glass tubes immediately and were incubated with specific enzyme substrates. There were eight enzymes, including peroxidase, acid phosphatase, alkaline phosphatase, alpha-D-glucosidase, beta-D-glucosidase, esterase, catechol oxidase and alpha amylase, which were located within the gels after electrophoresis.

Peroxidase enzymatic activity was located by the method used by Macko *et al.* (Macko *et al.*, 1967, modified by Webb *et al.*, 1972). Catechol was used as H_2 donor. The gels were immersed after electrophoresis in 0.02 M solution of catechol in 0.02 M phosphate buffer, pH 5.8, for 30 minutes at room temperature. Gels were rinsed with distilled water and then transferred to a 0.3% H_2O_2 solution for band development. Bands developed within 10 minutes and rapidly faded. So immediate recording of their patterns was necessary.

Acid phosphatase was located following Jensen's method (1962). The gels were immersed directly into a substrate

solution which was composed of 0.6 g. lead nitrate in 500 ml. of 0.05 M acetate buffer at pH 4.5 to which was added sodium beta-glycerophosphate $5H_2O$ in 0.10 M concentration, and the final pH was adjusted to 5.0. After incubation at 37°C for 20 minutes, gels were rinsed in 2% acetic acid and washed with distilled water. The bands were developed to indicate where enzyme activity was located by the use of 1% sodium sulfite solution at room temperature. The gels were stored in 1% sodium sulfite for measurement records.

Alkaline phosphatase was located following Gerhardt *et al.* (Gerhardt *et al.*, 1963), which has been modified by Webb *et al.* (1972). The gels were pre-incubated in a 0.05 M borate buffer for 10 minutes at room temperature, and then immersed in a solution containing 25 mg. of alpha-naphthyl phosphate (sodium salt), 100 mg. Fast Red TR salt and 100 ml. 0.05 M borate buffer for 2 hours at 37°C. The orange brown bands developed if alkaline phosphatases were present. Their locations were then recorded.

Alpha and beta D-glucosidases were located by using a method described by Beneke *et al.* (Beneke *et al.*, 1969). After electrophoresis, the gels were immersed in 30 ml. of sodium acetate buffer pH 5.4 containing 1.5 mg./ml. PNP-alpha-D-glucoside for 1/2 hour at 37°C and then transferred to 0.5 M buffer at pH 9.8 to stop the reaction and to develop the yellow colored bands of liberated PNP at room temperature. Beta-D-glucosidase assay was based on the same

method but had PNP-beta-D-glucoside as the substrate. The distancesof bands were recorded immediately after their movements.

Esterase was located by the method of Desborough and Peloquin (1967). Immediately after electrophoresis, gels were transferred to phosphate buffer for 10 minutes, before being placed in the substrate which consisted of 50 mg. alpha-naphthyl acetate dissolved in 2 ml. acetone and 75 mg. fast blue 2R salt, in 100 ml. of phosphate buffer at pH 7.4, and filtered, and then incubated at 37°C for 25 minutes. The purplish blue bands were developed within the gels. The gels were kept in phosphate buffer pH 7.4 for further measurement.

Catechol oxidase localization was based on the color products resulting from the oxidation of catechol. The method followed that given by Shannon *et al.* (1973). The substrate was prepared by adding 165 mg. of catechol to 15 ml. of sodium phosphate buffer at pH 4.2 with the addition of water up to 135 ml. Gels were incubated overnight at room temperature and were rinsed with distilled water, and the locations of the bands were recorded.

Amylase localization followed the technique given by Brewbaker *et al.* (1968), which has been modified by Webb *et al.* (1972). Gels were made up by using ammonium persulfate dissolved in a 1% starch solution in place of small pore solution (Ornstein, 1964; Davis, 1964). After electrophoresis, the gels were incubated in 0.01 M phosphate buffer at pH 5.8, for 40 minutes at room temperature and

then quickly immersed in Gram's iodine solution. The gels were removed and recorded for the development of the clear bands. The existence of unstained regions, where the blue color indicative of the presence of starch failed to appear, was taken as an indication of amylase activity.

RESULTS

Extracellular Enzyme Studies

1. Survey for extracellular enzymatic activity in CPYG solid medium

The extracellular enzyme activities of twenty-two pathogens and forty-six saprobes were investigated after the colonies had grown on CPYG agar for 5-, 15-, and 25-day periods. The plugs of CPYG agar cut from near the edge of the individual colonies of the isolates were incubated at 37°C for 2 hours with the twelve paranitrophenol (PNP) derivative substrates.

None of the twenty-two human pathogens and forty-six saprobes and plant pathogens had detectable enzyme activities with the twelve PNP derivative substrates at 5 days. However, some of these pathogens and most of the saprobes produced a sufficient quantity of extracellular enzymes, which diffused into the agar medium for positive plug tests at 15 days.

Table 1 shows the extracellular enzyme activities of the twenty-two pathogenic strains of *Phialophora, Fonsecaea* and *Cladosporium* grown on CPYG medium at 25°C for 15 days. There is little or no detectable extracellular enzyme activity with the twelve PNP derivatives used as substrates for enzyme testing on most of the twenty-two human pathogenic

Table 1. Extracellular enzyme activities of human pathogenic species of *Phialophora*, *Fonsecaea* and *Cladosporium* grown at 25°C on solid CPYG medium for 15 days

Organism	Activity* (O.D. 410 nm/agar plug/hour x 1000)								
	Acid PNPPase	Alk. PNPPase	alpha-PNPGase	beta-PNPGase	PNP N-acetyl GL-amine	PNP Caprylic esterase	alpha-PNPG-alase	beta-PNPG-alase	PNP Lauric esterase
P. verrucosa (ok)	0	0	0	0	0	0	0	0	0
P. verrucosa (mac)	0	0	0	0	0	0	0	0	0
P. verrucosa (tex)	0	0	0	0	0	0	0	0	0
P. verrucosa (264)	0	0	0	0	0	45	0	0	0
P. verrucosa (269)	0	0	0	0	5	5	5	0	0
P. jeanselmei (msu)	5	0	10	10	5	20	5	0	5
P. jeanselmei (bon)	0	5	0	0	0	0	0	0	0
F. pedrosoi (belo)	0	0	0	0	0	0	0	0	0
F. pedrosoi (msu)	0	0	0	10	0	40	0	0	10
F. pedrosoi (259)	5	0	0	0	0	0	0	0	5
F. pedrosoi (tex)	10	0	0	0	0	0	0	0	0
F. pedrosoi (261)	0	0	0	5	0	10	0	0	0
F. pedrosoi (262)	0	0	0	0	0	30	0	0	0
F. compactum (msu)	0	0	0	0	0	40	0	0	0
F. compactum (bon)	0	0	0	0	0	50	0	0	0
F. dermatitidis (msu)	0	0	0	0	0	0	0	0	0

Table 1 (Cont'd.)

Organism	Activity* (O.D. 410 nm/agar plug/hour x 1000)								
	Acid PNPPase	Alk. PNPPase	alpha-PNPGase	beta-PNPGase	PNP N-acetyl GL-amine	PNP Caprylic esterase	alpha-PNPG-alase	beta-PNPG-alase	PNP Lauric esterase
C. carrionii (g8)	0	0	10	0	0	15	0	0	0
C. carrionii (g9)	0	0	0	0	0	55	0	0	0
C. carrionii (tex)	0	0	0	0	0	0	0	0	0
C. trichoides (msu)	0	0	0	10	0	40	0	0	0
C. trichoides (g10)	0	0	0	15	0	0	0	0	0
Fonsecaea sp. (E-286)	0	0	0	0	0	0	0	0	0

*Butyric, palmitric and stearic esterases had no detectable activities.

strains. There was more activity toward the PNP derivative of the fatty acid caprylate than toward other substrates especially for one strain of *P. verrucosa*, 2 strains of *F. pedrosoi*, 2 strains of *F. compactum* and 1 strain each of *C. carrionii* and *C. trichoides*. There were no detectable enzyme activities with PNP-stearate, PNP-palmitate and PNP-butyrate. The yellow color of paranitrophenol could be differentiated visibly at about 25 O.D./agar plug/hour X 1000.

The saprobes and plant pathogens at 15 days on CPYG agar showed more extensive extracellular enzyme activity on the twelve substrates (Table 2). No acid phosphatase activity was detected except in two strains of *Phialophora* saprobes, while there was activity in many *Cladosporium* saprobes and most plant pathogens. No alkaline phosphatase activity was detected in *Phialophora* saprobes and *Cladosporium resinae* isolates; however, activity was evident in all of the *Cladosporium oxysporum* strains. The extracellular activity of alpha-D-glucosidase was varied among *Cladosporium* saprobes, while none of the *Phialophora* saprobes showed activity. The extracellular beta-D-glucosidase and N-acetyl-beta-glucosaminidase activities were high (with several exceptions) in the saprobic isolates of *Phialophora* and *Cladosporium*. The extracellular activity of alpha-D-galactosidase of *Phialophora* saprobes was low or not detected when compared with the irregular variations for *Cladosporium*. Table 3 shows all the saprobic

Table 2. Extracellular enzyme activities of saprobes and plant pathogens of *Phialophora* and *Cladosporium* grown at 25°C on solid medium CPYG for 15 days

Organism	Activity (O.D. 410 nm/agar plug/hour x 1000)								
	Acid PNPPase	Alk. PNPPase	alpha-PNPGase	beta-PNPGase	PNP N-acetyl GL-amine	PNP Caprylic esterase	alpha-PNPG-alase	beta-PNPG-alase	PNP Lauric esterase
P. richardsiae (6808)	15	0	0	35	70	0	10	0	0
P. richardsiae (263)	0	0	0	85	175	100	0	0	0
P. fastigiata (8008)	5	0	0	80	15	0	10	0	0
P. fastigiata (265)	0	0	0	180	20	50	0	0	0
P. mellinii (266)	0	0	0	95	0	35	0	0	0
P. lagerbergii (267)	0	0	0	225	20	0	0	0	0
P. obscura (268)	0	0	0	110	45	0	0	0	0
P. malorum (1487)	0	0	0	180	35	0	20	0	0
C. herbarum (pear)	20	0	0	95	265	90	50	0	0
C. herbarum (3167)	95	50	20	405	550	75	0	0	0
C. cladosporioides (489)	0	0	0	0	135	0	0	0	0
C. cladosporioides (9485)	215	0	0	105	265	0	20	0	0
C. oxysporum (9466)	65	40	0	375	205	50	150	0	0
C. oxysporum (9481)	75	30	0	135	210	60	15	0	0
C. oxysporum (9489)	50	35	10	400	260	100	650	0	0
C. oxysporum (9495)	100	130	25	575	675	125	380	35	0

Table 2 (Cont'd.)

Organism	Activity (O.D. 410 nm/agar plug/hour x 1000)								
	Acid PNPPase	Alk. PNPPase	alpha-PNPGase	beta-PNPGase	PNP N-acetyl GL-amine	PNP Caprylic esterase	alpha-PNPG-alase	beta-PNPG-alase	PNP Lauric esterase
C. oxysporum (9496)	50	45	0	200	95	0	5	0	0
C. resinae (8013)	375	0	85	735	425	0	50	0	0
C. resinae f. *avellaneum* (7998)	85	0	0	375	35	80	20	0	0
C. resinae f. *avellaneum* (9257)	20	0	30	600	310	70	175	0	0
C. resinae f. *avellaneum* (9258)	25	0	35	450	155	0	10	0	0
C. sphaerospermum (556)	80	45	0	130	375	225	30	0	0
C. sphaerospermum (8050)	50	30	15	35	185	215	45	0	0
C. sphaerospermum (9494)	35	0	0	0	95	165	0	0	0
C. sphaerospermum (9516)	25	0	0	45	85	0	25	0	0
C. sphaerospermum (9517)	80	100	0	100	45	0	45	0	0
Cladosporium sp. (1)	75	100	125	200	550	0	250	0	0
Cladosporium sp. (2)	60	60	60	110	450	0	50	0	0
Cladosporium sp. (3)	0	0	50	125	50	0	125	0	0
Cladosporium sp. (4)	0	0	55	75	50	50	50	0	0
Cladosporium sp. (5)	0	50	70	50	80	50	50	0	0

Table 2 (Cont'd.)

Organism	Activity (O.D. 410 nm/agar plug/hour x 1000)								
	Acid PNPPase	Alk. PNPPase	alpha-PNPGase	beta-PNPGase	PNP N-acetyl GL-amine	PNP Caprylic esterase	alpha-PNPG-alase	beta-PNPG-alase	PNP Lauric esterase
Cladosporium sp. (6)	0	0	50	0	65	50	75	0	0
Cladosporium sp. (7)	0	50	145	210	350	55	175	0	0
Cladosporium sp. (8)	0	0	0	40	60	25	0	0	0
Cladosporium sp. (9)	35	0	0	100	235	0	60	0	0
Cladosporium sp. (10)	70	25	35	220	305	60	165	0	0
Cladosporium sp. (11)	0	0	0	150	235	75	115	0	0
Cladosporium sp. (12)	0	35	0	235	525	25	80	0	0
Cladosporium sp. (13)	0	0	0	20	45	110	0	0	0
Cladosporium sp. (14)	50	0	0	95	345	90	70	0	0
C. cucumerinum (A-1)	500	65	0	650	500	210	165	0	75
C. cucumerinum (F-26)	145	0	0	365	375	160	45	0	50
C. cucumerinum (St.clair)	660	360	0	660	625	175	385	0	50
C. carpophilum (apricot)	40	0	0	0	105	125	0	0	0
C. carpophilum (peach)	0	0	0	0	105	0	0	0	0

Table 3. Extracellular enzyme activities of saprophytic or plant pathogenic species of *Cladosporium* grown on solid CYPG medium for 25 days at 25°C

Organism	No. of Strains	Acid PNPPase	Alk. PNPPase	alpha-PNPGase	beta-PNPGase	PNP N-acetyl GL-amine	PNP Caprylic esterase	alpha-PNPGalase	beta-PNPGalase	PNP Lauric esterase
C. herbarum	2	-(1) +(1)	-	-	+	+	+	+	-	-
C. cladosporioides	2	-(1) +(1)	-	-	+	+	+	+	-	-
C. oxysporum	5	+	+	-(4) +(1)	+	+	+	+	+(1)	-
C. resinae f. *avellaneum*	4	+	-	+	+	+	+	+	-	-
C. sphaerospermum	5	+	+	-(4) +(1)	+	+	+	+	-	-
C. cucumerinum	3	+	+	-	+	+	+	+	-	+(3)
C. carpophilum	2	-(1) +(1)	-	-	-	+	+	-	-	-
C. sp. (air-borne)	14	-(10) +(4)	-(7) +(7)	-(12) +(2)	+	+	-(5) +(9)	+	-	-

(+) shows positive enzyme activities
(-) shows negative enzyme activities
( ) number of strains

strains of *Cladosporium*. There was only one isolate of *C. oxysporum* (9495) among all the saprobes and plant pathogens that showed beta-D-galactosidase activity. Caprylic esterase activity was varied among *Phialophora* and *Cladosporium* saprobes. Only three strains of the plant pathogen *C. cucumerinum* showed lauric esterase activity out of all the saprobes and plant pathogens tested.

The extracellular enzyme activities were increased for some strains at 25 days of growth. Most of human and animal pathogens still showed low or no extracellular enzyme activities with the twelve PNP derivative substrates, except for two isolates of *Phialophora jeanselmei* which showed an increase in alpha-D-glucosidase, beta-D-glucosidase, alpha-D-galactosidase and caprylase activities. The results of the enzyme activities of twenty-two *Phialophora*, *Fonsecaea* and *Cladosporium* species are shown in Table 4.

The extracellular enzyme activities were compared between the human pathogenic and saprobic strains of *Phialophora* species at 25 days of growth in Table 5. Marked beta-D-glucosidase and N-acetyl-beta-glucosaminidase activities were evident among the saprobe isolates, while these activities were not detected in the human pathogenic strains of *Phialophora* isolates except for two isolates of *P. jeanselmei*. The other enzymes were low or not detectable in general.

Human and animal pathogenic *Fonsecaea* and *Cladosporium* species had little or no enzymatic activities with the

Table 4. Extracellular enzyme activities of human pathogenic species of *Phialophora*, *Fonsecaea* and *Cladosporium* grown at 25°C on solid CPYG medium for 25 days

Organism	Activity (O.D. 410 nm/agar plug/hour x 1000)								
	Acid PNPPase	Alk. PNPPase	alpha-PNPGase	beta-PNPGase	PNP N-acetyl GL-amine	PNP Caprylic esterase	alpha-PNPG-alase	beta-PNPG-alase	PNP Lauric esterase
P. verrucosa (ok)	0	0	0	0	0	0	0	0	0
P. verrucosa (mac)	0	0	0	0	0	0	0	0	0
P. verrucosa (tex)	0	0	0	0	0	0	0	0	0
P. verrucosa (264)	0	0	0	5	10	0	0	0	0
P. verrucosa (269)	0	0	0	5	0	0	0	0	0
P. jeanselmei (msu)	15	5	55	445	10	200	30	0	0
P. jeanselmei (bon)	10	10	15	25	20	35	10	0	0
F. pedrosoi (belo)	10	0	0	0	0	0	0	0	0
F. pedrosoi (msu)	0	0	0	0	0	70	0	0	0
F. pedrosoi (259)	0	0	0	5	0	5	0	0	0
F. pedrosoi (tex)	0	0	0	0	0	0	0	0	0
F. pedrosoi (261)	0	0	0	0	0	0	0	0	0
F. pedrosoi (262)	0	0	0	20	0	95	0	0	0
F. compactum (bon)	0	0	0	0	0	60	0	0	0
F. compactum (msu)	0	0	0	0	0	50	0	0	0
F. dermatitidis (msu)	0	0	0	5	0	35	0	0	0

Table 4 (Cont'd.)

Organism	Activity (O.D. 410 nm/agar plug/hour x 1000)								
	Acid PNPPase	Alk. PNPPase	alpha-PNPGase	beta-PNPGase	PNP N-acetyl GL-amine	PNP Caprylic esterase	alpha-PNPG-alase	beta-PNPG-alase	PNP Lauric esterase
C. carrionii (g8)	0	0	10	0	0	80	0	0	0
C. carrionii (g9)	0	0	0	0	0	60	0	0	0
C. carrionii (tex)	0	10	30	70	0	0	0	0	0
C. trichoides (msu)	0	0	0	25	0	5	0	0	0
C. trichoides (g10)	0	0	0	90	0	125	0	0	0
Phialophora sp. (F-286)	10	10	10	5	5	0	0	0	0

Table 5. Extracellular enzyme activities of human pathogenic and saprobic *Phialophora* species grown at 25°C on solid CPYG medium for 25 days

Organism	Activity (O.D. 410 nm/agar plug/hour x 1000)								
	Acid PNPPase	Alk. PNPPase	alpha-PNPGase	beta-PNPGase	PNP N-acetyl GL-amine	PNP Caprylic esterase	alpha-PNPG-alase	beta-PNPG-alase	PNP Lauric esterase
P. verrucosa (ok)	0	0	0	0	0	0	0	0	0
P. verrucosa (mac)	0	0	0	0	0	0	0	0	0
P. verrucosa (tex)	0	0	0	0	0	0	0	0	0
P. verrucosa (264)	0	0	0	5	10	0	0	0	0
P. verrucosa (269)	0	0	0	5	0	0	0	0	0
P. jeanselmei (msu)	15	5	55	445	10	200	30	0	0
P. jeanselmei (bon)	10	10	15	25	20	35	10	0	0
Phialophora sp. (E-286)	10	10	10	5	5	0	0	0	0
P. richardsiae (6808)	25	40	25	525	355	80	0	0	0
P. richardsiae (263)	0	0	0	215	450	50	0	0	0
P. fastigiata (8008)	30	25	0	290	55	0	0	0	0
P. melinii	0	0	0	575	35	0	0	0	0
P. lagerbergii	0	50	0	350	80	50	0	0	0
P. obscura	0	0	0	675	50	0	0	0	0
P. malonum	0	0	0	375	80	0	0	0	0

twelve substrates at 25 days, except for a few isolates that showed enzyme activity with the PNP fatty acid caprylate (Table 6). One isolate of *C. carrionii* (tex) and two isolates of *C. trichoides* also showed beta-D-glucosidase activity, while the other *Cladosporium* and *Fonsecaea* pathogens showed very little or no activity. Marked enzyme activities for beta-D-glucosidase, N-acetyl-beta-glucosaminidase, and alpha-D-galactosidase were shown in *Cladosporium* saprobes and plant pathogens. A marked difference in N-acetyl-beta-glucosaminidase and alpha-D-galactosidase activities can be noted between the human and animal pathogenic strains of *Fonsecaea* and *Cladosporium* with no detected enzyme activity and the saprobic and plant pathogenic strains with well defined activity.

A comparison of the growth rate of *Phialophora jeanselmei* (msu), a human pathogen, with extracellular enzyme activities on twelve PNP-derivative substrates is shown in Figure 2. The growth rate was determined by the increasing diameter of colonies with time in Table 7. That there was a substantial increase in the activity of enzyme, alpha-D-glucosidase and a marked increase of both beta-D-glucosidase and caprylase (assayed at 5-, 15-, and 25-day colony growth) are shown in Figure 2. A small amount of extracellular enzymes were released from *P. jeanselmei* during the exponential phase of growth, and a rapid increase in beta-D-glucosidase and caprylase was noted at approximately 15 days after inoculation or right at the beginning of the stationary phase of growth. Two

Table 6. Extracellular enzyme activities of human pathogenic and saprobic *Fonsecaea* and *Cladosporium* species at 25°C solid CPYG medium for 25 days

Organism	Activity (O.D. 410 nm/agar plug/hour x 1000)								
	Acid PNPPase	Alk. PNPPase	alpha-PNPGase	beta-PNPGase	PNP N-acetyl GL-amine	PNP Caprylic esterase	alpha-PNPG-alase	beta-PNPG-alase	PNP Lauric esterase
F. pedrosoi (belo)	10	0	0	0	0	0	0	0	0
F. pedrosoi (msu)	0	0	0	0	0	70	0	0	0
F. pedrosoi (259)	0	0	0	5	0	5	0	0	0
F. pedrosoi (tex)	0	0	0	0	0	0	0	0	0
F. pedrosoi (261)	0	0	0	0	0	0	0	0	0
F. pedrosoi (262)	0	0	0	20	0	95	0	0	0
F. compactum (bon)	0	0	0	0	0	50	0	0	0
F. compactum (msu)	0	0	0	0	0	60	0	0	0
F. dermatitidis (msu)	0	0	0	5	0	35	0	0	0
C. carrionii (g8)	0	0	10	0	0	80	0	0	0
C. carrionii (g9)	0	0	0	0	0	60	0	0	0
C. carrionii (tex)	0	10	30	70	0	0	0	0	0
C. trichoides (msu)	0	0	0	25	0	5	0	0	0
C. trichoides (g10)	0	0	0	90	0	125	0	0	0
C. herbarum (msu)	0	0	0	275	430	105	215	0	0
C. herbarum (3167)	280	0	0	625	675	125	425	0	0

Table 6 (Cont'd.)

Organism	Activity (O.D. 410 nm/agar plug/hour x 1000)								
	Acid PNPPase	Alk. PNPPase	alpha-PNPGase	beta-PNPGase	PNP N-acetyl GL-amine	PNP Caprylic esterase	alpha-PNPG-alase	beta-PNPG-alase	PNP Lauric esterase
C. cladosporiodes (489)	0	0	0	55	455	50	135	0	0
C. cladosporiodes (9485)	285	0	0	255	450	35	55	0	0
C. oxysporum (9466)	105	60	0	500	460	60	205	0	0
C. oxysporum (9481)	125	100	0	600	600	110	125	0	0
C. oxysporum (9489)	100	80	0	550	650	125	210	0	0
C. oxysporum (9495)	200	150	40	700	675	175	650	70	0
C. oxysporum (9496)	25	15	0	285	355	75	50	0	0
C. resinae (8013)	70	0	465	255	335	145	320	0	0
C. resinae f. *avallaneum* (7998)	65	0	60	235	70	125	95	0	0
C. resinae f. *avallaneum* (9257)	45	0	65	625	625	135	155	0	0
C. resinae f. *avallaneum* (9258)	95	0	55	650	425	140	50	0	0
C. sphaerospermum (556)	390	305	0	350	675	165	250	0	0
C. sphaerospermum (8050)	305	230	0	215	635	85	165	0	0
C. sphaerospermum (9494)	70	0	0	200	465	130	55	0	0
C. sphaerospermum (9516)	50	0	0	200	375	100	55	0	0

Table 6 (Cont'd.)

Organism	Activity (O.D. 410 nm/agar plug/hour x 1000)								
	Acid PNPPase	Alk. PNPPase	alpha-PNPGase	beta-PNPGase	PNP N-acetyl GL-amine	PNP Caprylic esterase	alpha-PNPG-alase	beta-PNPG-alase	PNP Lauric esterase
C. sphaerospermum (9517)	65	250	75	600	550	150	400	20	0
Cladosporium sp. (1)	80	90	100	550	600	0	410	0	0
Cladosporium sp. (2)	50	60	0	100	400	0	60	0	0
Cladosporium sp. (3)	0	70	0	180	480	50	325	0	0
Cladosporium sp. (4)	0	75	0	130	310	50	305	0	0
Cladosporium sp. (5)	0	50	0	315	475	50	275	0	0
Cladosporium sp. (6)	0	0	0	205	400	50	255	0	0
Cladosporium sp. (7)	0	50	85	180	500	50	395	0	0
Cladosporium sp. (8)	0	0	0	190	335	0	50	0	0
Cladosporium sp. (9)	60	0	0	185	255	0	170	0	0
Cladosporium sp. (10)	130	35	0	305	70	0	180	0	0
Cladosporium sp. (11)	0	0	0	145	230	95	225	0	0
Cladosporium sp. (12)	35	0	0	105	275	0	110	0	0
Cladosporium sp. (13)	0	0	0	175	275	75	95	0	0
Cladosporium sp. (14)	0	0	0	100	250	50	145	0	0
C. cucumerinum (A-1)	225	105	0	560	450	65	300	0	0

Table 6 (Cont'd.)

Organism	Activity (O.D. 410 nm/agar plug/hour x 1000)								
	Acid PNPPase	Alk. PNPPase	alpha-PNPGase	beta-PNPGase	PNP N-acetyl GL-amine	PNP Caprylic esterase	alpha-PNPG-alase	beta-PNPG-alase	PNP Lauric esterase
C. cucumerinum (F-26)	135	65	0	605	370	70	200	0	0
C. cucumerinum (St. clair)	560	465	0	650	500	55	500	0	0
C. carpophilum (apricot)	0	0	0	0	60	255	0	0	0
C. carpophilum (peach)	70	0	0	0	275	100	0	0	0

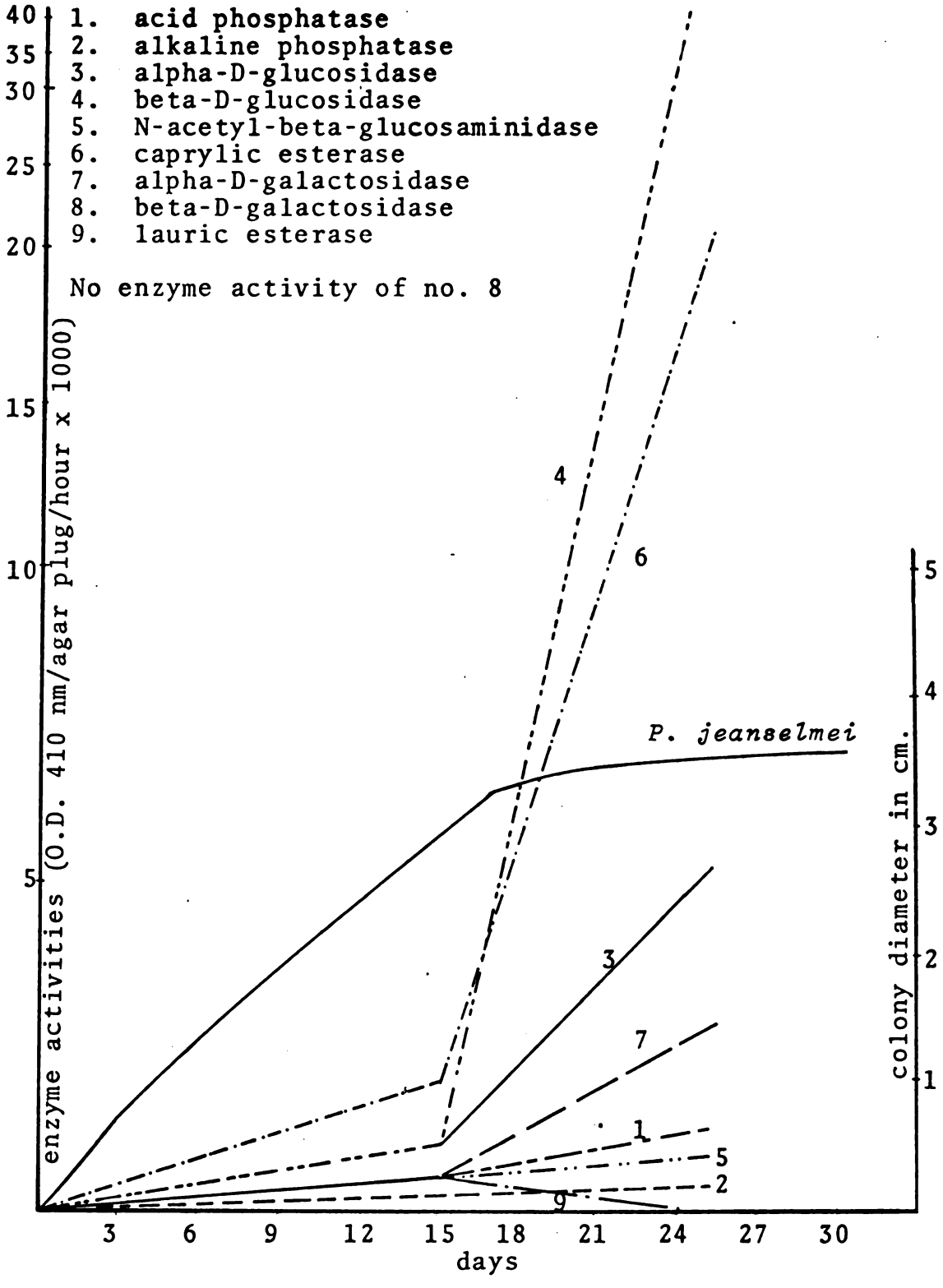


Figure 2. Comparison of extracellular enzyme activities and growth rate of *Phialophora jeanselmei* (msu) human pathogen grown at 25°C on solid CPYG medium.

Table 7. The diameter measurements of colonies of *P. jeanselmei*, *P. fastigiata*, *C. cucumerinum* (F-26) and *C. oxysporum* (9496) for determination of growth rate, when grown on solid CPYG medium at 25°C for 33 days

Days	Colony diameter in cm.			
	P. jeanselmei (msu)	*P. fastigiata* (8008)	*C. oxysporum* (9496)	*C. cucumerinum* (F-26)
3	0.7	0.3	0.5	0.7
6	1.5	0.8	1.9	2.5
9	1.9	2.1	3.4	4.0
12	2.4	3.3	4.0	4.4
15	3.0	3.8	4.2	4.5
18	3.4	4.7	4.5	4.6
21	3.5	5.4	4.5	4.5
24	3.6	5.6	4.6	4.5
27	3.6	5.8	4.6	4.5
30	3.6	6.0	4.7	4.5
33	3.6	6.0	4.7	4.5

other enzymes, alpha-D-glucosidase and alpha-D-galactosidase, also increased modestly after 15 to 25 days of fungal growth.

Comparisons of the growth rate and changes in enzymatic activities with twelve PNP-derivative substrates for the *Phialophora* and *Cladosporium* saprobes and plant pathogens *P. fastigiata* (8008), *C. oxysporum* (9496) and *C. cucumerinum* (F-26) are demonstrated in Figures 3, 4 and 5, respectively. These fungi were representative of their groups.

Beta-D-glucosidase showed a high rate of activity for *P. fastigiata* (8008) at the beginning of the colony growth, and increased up to 25 days as seen in Figure 3. Acid and alkaline phosphatase and N-acetyl-beta-glucosaminidase increased in amount after exponential phase between 15 and 25 days. The alpha-D-galactosidase gradually decreased in activity after 15 days.

Figures 4 and 5 illustrate the relationships between growth rate and enzymatic activities of *Cladosporium oxysporum* (9496), a saprobe, and *C. cucumerinum* (F-26), a plant pathogen. Both beta-D-glucosidase and N-acetyl-beta-glucosaminidase increased rapidly at the beginning of colony growth and continued through 15 days. Caprylic esterase and acid phosphatase activities were greater in the first 15 days in *C. cucumerinum* (F-26) than in *C. oxysporum* (9496). Both fungi had no detectable alpha-D-glucosidase and beta-D-galactosidase activities. Alkaline phosphatase and lauric esterase were detected on the 25th day only for *C. cucumerinum* (F-26), while only caprilic esterase and alpha-D-galactosidase were

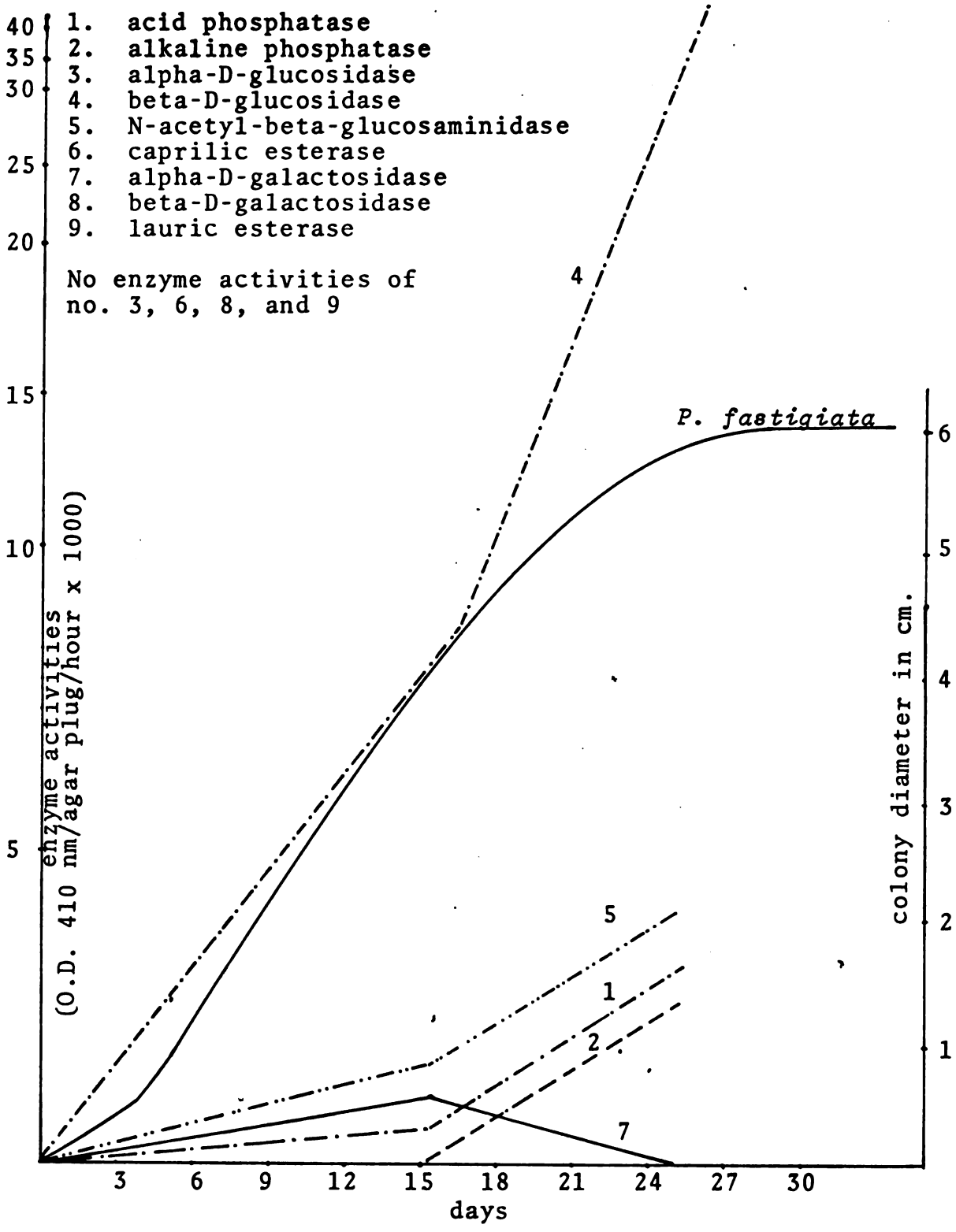


Figure 3. Comparison of extracellular enzyme activities and growth rate of *Phialophora fastigiata* (8008) saprobe grown at 25°C on solid CPYG medium.

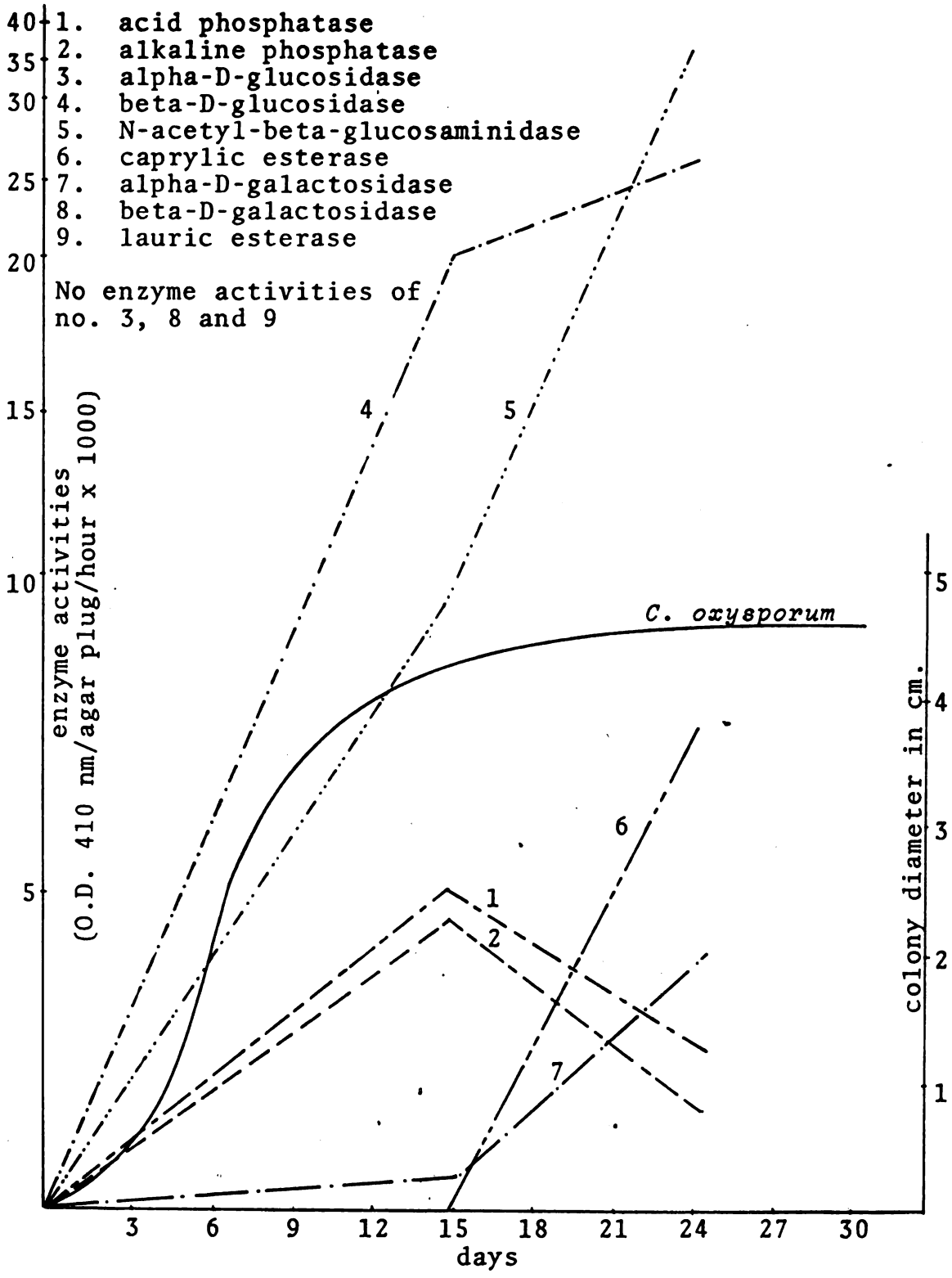


Figure 4. Comparison of extracellular enzyme activities and growth rate of *Cladosporium oxysporum* (9496) saprobe grown at 25°C on solid CPYG medium.

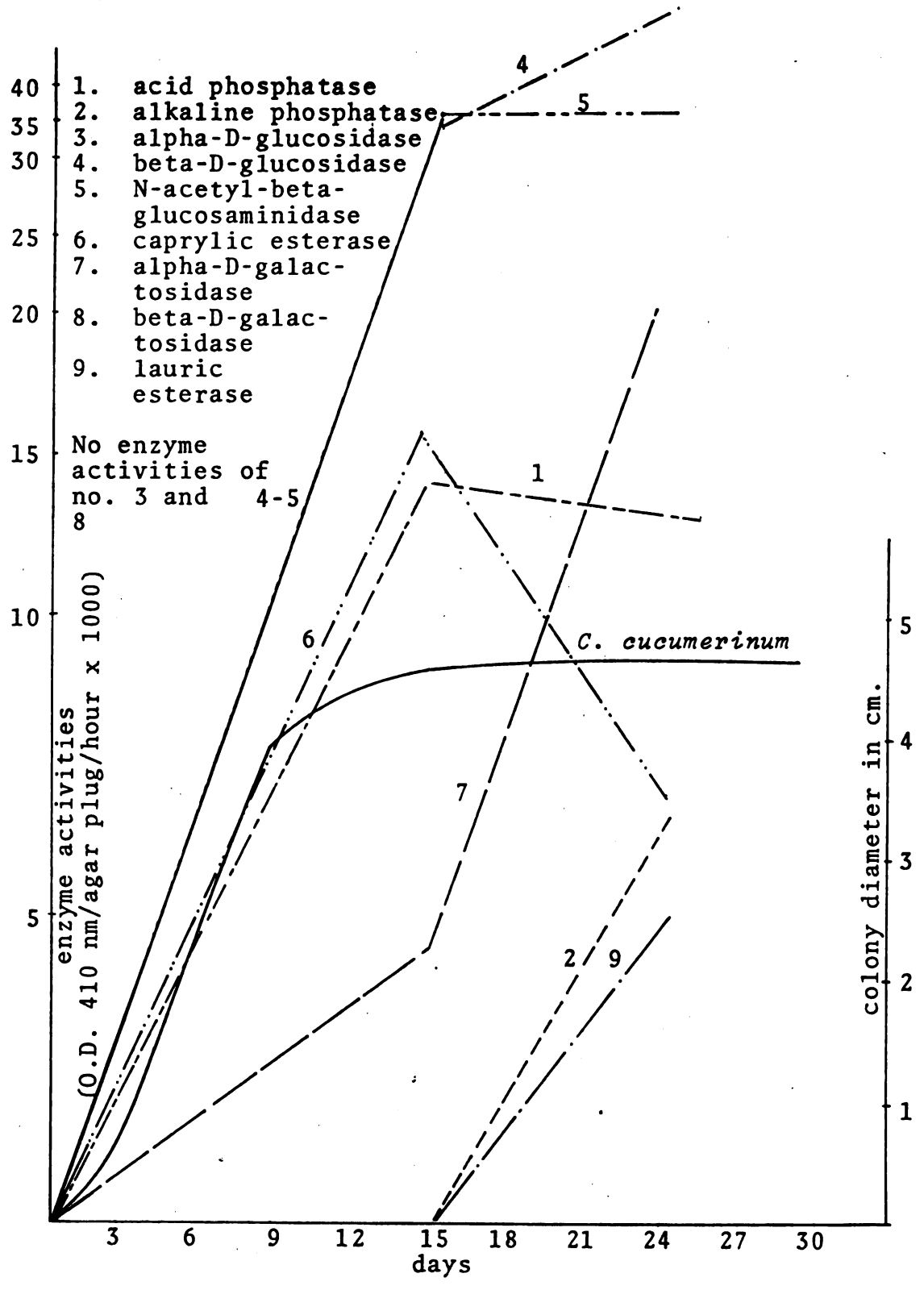


Figure 5. Comparison of extracellular enzyme activities and growth rate of *Cladosporium cucumerinum* (F-26) plant pathogen grown at 25°C on solid CPYG medium.

presented in the assay for the 25-day culture of *C. oxysporum* (9496).

2. Survey for extracellular enzymatic activity in CPYG liquid medium

Peroxidase activity

The results of extracellular peroxidase activity of human pathogenic *Phialophora*, *Fonsecaea* and *Cladosporium* species are shown in Table 8. The difference in peroxidase activity was varied, with no distinctive enzymatic pattern among these genera. *Phialophora jeanselmei* (msu) showed a high activity in 15 days, while *C. carrionii* had high activity at all times. Great variation of peroxidase activity was also found in individual strains of the fungi. The pattern of enzymatic activity was somewhat similar for all strains of *Phialophora* and *Cladosporium* at 5, 10 and 15 days, as seen in Table 9. Two strains of *Cladosporium* sp., number 5 and 6, had high enzymatic activity in 5 days with decreasing activities in 10 and 15 days. A third isolate, *C. cucumerinum* (St. Clair), had the highest peroxidase activity in 5-day cultures.

Adenosine triphosphatase activity

Glucose was used as a carbon and DL-Isoleucine and L-proline were used as nitrogen sources for West's liquid medium.

The extracellular adenosine triphosphatase activity in 7-day cultures showed variation from no activity to high enzyme activity for some strains, as shown in Tables 10

Table 8. Extracellular peroxidase activity of *Phialophora*, *Fonsecaea* and *Cladosporium* human and animal pathogenic species grown at 25°C in liquid CPYG medium

Organism	Enzyme activity (O.D. 485 nm/ml./hour x 1000) 5 days	10 days	15 days
P. verrucosa (ok)	210	330	660
P. verrucosa (mac)	330	210	270
P. verrucosa (tex)	510	210	480
P. verrucosa (264)	390	240	360
P. verrucosa (269)	450	540	150
P. jeanselmei (msu)	450	540	1350
P. jeanselmei (bon)	540	660	450
F. pedrosoi (belo)	300	260	450
F. pedrosoi (msu)	300	570	540
F. pedrosoi (259)	360	510	780
F. pedrosoi (tex)	260	390	570
F. pedrosoi (261)	270	240	270
F. pedrosoi (262)	390	900	150
F. compactum (msu)	270	510	510
F. compactum (bon)	210	360	90
F. dermatitidis (msu)	240	360	660
C. carrionii (g8)	180	660	270
C. carrionii (g9)	1200	1680	3600
C. carrionii (tex)	300	510	270
C. trichoides (msu)	60	360	690
C. trichoides (g10)	90	330	450
Phialophora sp. (E-286)	510	420	780

Table 9. Extracellular peroxidase activity of *Phialophora* and *Cladosporium* saprobic and plant pathogenic species grown at 25°C in liquid CPYG medium

Organism	Enzyme activity (O.D. 485 nm/ml./hour x 1000) 5 days	10 days	15 days
Cladosporium herbarum (pear)	300	330	150
C. herbarum (3167)	300	810	330
C. cladosporiodes (489)	270	510	600
C. cladosporiodes (9485)	330	480	390
C. oxysporum (9466)	210	210	480
C. oxysporum (9481)	450	390	420
C. oxysporum (9489)	600	900	570
C. oxysporum (9495)	300	420	300
C. oxysporum (9496)	210	900	360
C. resinae f. *avellaneum* (7998)	360	480	300
C. resinae f. *avellaneum* (8013)	360	270	840
C. resinae f. *avellaneum* (9257)	270	270	390
C. resinae f. *avellaneum* (9258)	270	390	390
C. sphaerospermum (556)	420	240	420
C. sphaerospermum (8050)	300	210	360
C. sphaerospermum (9494)	660	1530	360
C. sphaerospermum (9516)	210	360	720
C. sphaerospermum (9517)	420	2400	750
C. cucumerinum A-1	720	510	720
C. cucumerinum (F-26)	450	690	480
C. cucumerinum (St. Clair)	1740	600	840
C. carpophylum (apricot)	240	450	390
C. carpophylum (peach)	270	660	270

Table 9 (Cont'd.)

Organism	Enzyme activity (O.D. 485 nm/ml./hour x 1000) 5 days	10 days	15 days
Cladosporium sp 1	270	270	240
Cladosporium sp 2	570	600	810
Cladosporium sp 3	660	390	330
Cladosporium sp 4	570	570	330
Cladosporium sp 5	1500	780	240
Cladosporium sp 6	1560	360	450
Cladosporium sp 7	990	270	240
Cladosporium sp 8	510	570	330
Cladosporium sp 9	510	330	840
Cladosporium sp 10	330	420	300
Cladosporium sp 11	990	540	300
Cladosporium sp 12	480	210	270
Cladosporium sp 13	600	210	330
Cladosporium sp 14	480	150	210
Cladosporium sp 15	540	570	630
P. richardsiae (263)	270	270	240
P. richardsiae (6808)	30	180	300
P. fastigiata (265)	750	810	420
P. fastigiata (8008)	360	570	720
P. mellinii (266)	510	360	420
P. lagerbergii (267)	420	360	240
P. obscura (268)	450	420	270
P. malorum (1487)	630	570	630

Table 10. Extracellular adenosine triphosphatase of human and animal pathogenic species *Phialophora*, *Fonsecaea* and *Cladosporium* grown at 25°C in West's liquid medium with L-proline as the nitrogen source

Organism	Enzyme activity (O.D. 680 nm/ml./hour x 1000)			
	7 days	14 days	21 days	28 days
P. verrucosa (ok)	150	10	50	0
P. verrucosa (mac)	0	0	0	0
P. verrucosa (tex)	0	190	10	20
P. verrucosa (264)	0	0	0	0
P. verrucosa (269)	0	30	0	0
P. jeanselmei (msu)	0	0	80	0
P. jeanselmei (bon)	0	0	0	0
F. pedrosoi (belo)	0	170	170	0
F. pedrosoi (msu)	0	0	0	0
F. pedrosoi (259)	0	0	0	0
F. pedrosoi (tex)	0	190	10	20
F. pedrosoi (261)	0	0	0	0
F. pedrosoi (262)	0	0	0	0
F. compactum (msu)	70	100	30	0
F. compactum (bon)	0	0	0	0
F. dermatitidis (msu)	0	0	0	0
C. carrionii (g8)	70	0	0	0
C. carrionii (g9)	0	0	0	0
C. carrionii (tex)	30	0	0	0
C. trichoides (msu)	30	0	0	0
C. trichoides (g10)	0	0	0	0
Phialophora sp. (E-286)	0	0	0	0

and 11. The activity of adenosine triphosphatase was detected in a few additional strains at 14, 21 and 28 days of growth. Most of the twenty-two pathogens in the genera *Phialophora*, *Fonsecaea* and *Cladosporium* showed no detectable adenosine triphosphatase activity. In all cases the adenosine triphosphatase activity was lower at 28 days than at 21 days.

Fonsecaea pedrosoi (tex) showed high extracellular activity at day 14 followed by decreased activity through day 28 (see Table 10). Two strains of *P. verrucosa* and two strains of *C. carrionii* had marked increase in enzyme activity by day 21 followed by a decrease on day 28 (see Table 10). *Cladosporium* plant pathogens did show variation in enzymatic activity from day 7 through day 28 with some strains showing no detectable activity and others showing adenosine triphosphatase activity at varied times in the liquid cultures.

Upon changing the nitrogen source from L-proline to DL-isoleucine it was found that there was a difference in the enzymatic activity of the human pathogens. In Tables 10 and 11, for instance, *F. pedrosoi* (259) showed an increase in the adenosine triphosphatase activity when DL-isoleucine was utilized as a nitrogen source. No activity was detected when L-proline was used as the nitrogen source. *Fonsecaea compactum* (msu) showed greater enzymatic activity when L-proline was utilized, and less activity when DL-isoleucine was used. Similar results occurred in several other species (Tables 10 and 11).

Table 11. Extracellular adenosine triphosphatase of human pathogenic species *Phialophora*, *Fonsecaea* and *Cladosporium* grown at 25°C in West's liquid medium with DL-isoleucine as the nitrogen source

Organism	Enzyme activity (O.D. 680 nm/ml./hour x 1000)			
	7 days	14 days	21 days	28 days
P. verrucosa (ok)	0	0	40	20
P. verrucosa (mac)	0	0	40	20
P. verrucosa (tex)	0	0	0	0
P. verrucosa (264)	0	0	0	0
°. verrucosa (269)	0	0	0	0
P. jeanselmei (msu)	0	0	0	0
P. jeanselmei (bon)	0	0	0	0
F. pedrosoi (belo)	0	10	10	0
F. pedrosoi (msu)	0	0	0	0
F. pedrosoi (259)	0	40	80	20
F. pedrosoi (tex)	220	90	110	40
F. pedrosoi (261)	0	0	0	0
F. pedrosoi (262)	0	0	0	0
F. compactum (msu)	10	10	0	0
F. compactum (bon)	0	0	0	0
F. dermatitidis (msu)	0	0	0	0
C. carrionii (g8)	0	0	280	80
C. carrionii (g9)	0	0	30	10
C. carrionii (tex)	0	0	0	0
C. trichoides (msu)	0	20	0	10
C. trichoides (g10)	0	0	0	0
Phialophora sp. (E-286)	0	0	0	0

There was no enzymatic activity toward ATP in the medium of *C. cucumerinum* with DL-isoleucine as a nitrogen source, but high enzymatic activity was observed with L-proline was utilized. Similar results occurred in the other species, including *C. carpophylum* and *C. cladosporioides* (see Tables 11 and 12).

In conclusion, there were no differences seen in the adenosine triphosphatase activity in human pathogens and in saprobes. The results were variable among species of the fungi. Changing the nitrogen source from L-proline to DL-isoleucine in the West's liquid medium did not show any promising results. This will be discussed later.

Comparative Gel Electrophoresis of Intracellular Soluble Protein, Enzymes and Isoenzymes

1. General soluble protein staining

The electrophoretic patterns of soluble proteins extracted from mycelia of fourteen-day-old cultures of twenty-two human pathogens of *Phialophora*, *Fonsecaea* and *Cladosporium* species and eight saprobes were compared. The amount of the extracted proteins from the saprophytic mycelia was found to be lower than that of the extracted proteins obtained from the human pathogens both from 14-day-old cultures. The amount of protein per 1 mg. of dried acetone powder varied from 100 to 200 µg. in the different isolates. A large amount of acetone powder was utilized in order to get the same amount of soluble proteins from these saprobes for running electrophoresis. There

Table 12. Extracellular adenosine triphosphatase activity of some *Phialophora* and *Cladosporium* saprobic species in West's liquid medium with DL-isoleucine or L-proline as nitrogen sources

Organism	Enzyme activity (O.D. 680 nm/ml./hour x 1000) 7 days	14 days	21 days	28 days
DL-isoleucine				
C. resinae f. *avellaneum* (2958)	0	10	0	0
C. cucumerinum (F-26)	0	0	0	0
C. sphaerospermum (8050)	0	0	0	0
C. cladosporiodes (489)	0	30	60	0
C. carpophylum (peach)	0	20	0	0
L-proline				
C. resinae f. *avellaneum* (2958)	10	0	0	0
C. cucumerinum (F-26)	0	190	100	80
C. sphaerospermum (8050)	0	0	0	0
C. cladosporiodes (489)	0	0	270	100
C. carpophylum (peach)	120	10	40	0
P. obscura (268)	0	50	80	10
P. mellinii (266)	0	0	0	0
P. molorum (1487)	0	0	0	0
P. richardsiae (6808)	50	0	150	10
P. lagerbergii (267)	30	0	20	0
P. fastigiata (265)	0	0	0	0
P. fastigiata (8008)	0	0	0	0

was enough protein for detecting enzymatic activity with the specific substrates. The saprobes used for comparison with pathogens included *C. resinae* (7898), *C. spherosproum* (9494), *P. richardsiae* (6808), *P. richardsiae* (236), *P. fastigiata* (265), *P. lagerbergii* (276), *C. cucumerinum* (F-26) and *Phialophora* species (msu).

The migration patterns, migration distance from the origin to the front, of each extracted protein were measured and used for calculation of Rf values. The calculation was based on the migration distance of bands divided by the distance of front and multiplied times 10. The electrophoretic patterns of these fungi showed characteristic bands varying in density, thickness and spacing enabling identification of the individual isolate. The Rf values were measured and calculated directly from distance on gels. The number of bands were determined by Rf values and also read out from the densitometer records. This Rf value method gave number of bands almost as accurate as those derived from densitometer records, even though many bands were close to each other. However, not all isolates were run by electrophoresis at the same time, so the use of both methods helped in determination of the number of bands of soluble proteins.

The Rf values of the twenty-two pathogenic fungi and eight saprobes are shown in Table 13. The electrophoretic patterns of all thirty isolates were drawn by using the Rf values and are seen in Figure 6. Photographs of some of the gel electrophoresis protein bands are shown in Figure 7.

Table 13. Average Rf values x 10 of general soluble protein bands of thirty strains of pathogens and saprobes in the electrophoretic pattern of protein extracts in liquid CPYG medium at 25°C for 14 days

Rf values X10	*P. verr* (ok)	*P. verr* (mac)	*P. verr* (tex)	*P. verr* (269)	*P. verr* (264)	*P. jean* (msu)	*P. jean* (bon)	*F. der* (msu)
	0.25	0.25	0.35	0.25				0.36
	0.42			0.41				0.45
	0.51			0.66	0.50	0.50		
1			0.88					0.80
	1.33	1.31	1.32	1.15	1.17	1.08		1.16
			1.67	1.31	1.42	1.50	1.50	1.58
			1.64					
2					1.83	1.92	1.92	
			2.11					
	2.58	2.54	2.28	2.31	2.33			
						2.42	2.42	
			2.81	2.89	2.97			2.68
3	3.17	3.12	3.25	3.28		3.08	3.08	3.21
		3.44	3.39					
	3.50			3.61	3.67	3.50	3.50	
	3.75	3.69						
4			3.95					
			4.30	4.02	4.16	4.08		
	4.58	4.59			4.67		4.33	4.20
	4.75		4.74					4.55
				4.92				
5						5.00	5.00	5.09
			5.26	5.25				5.27
				5.57	5.58			
		5.74						
6	5.83		5.88		6.00			

Table 13 (Cont'd.)

Rf values X10	*P. verr* (ok)	*P. verr* (mac)	*P. verr* (tex)	*P. verr* (269)	*P. verr* (264)	*P. jean* (msu)	*P. jean* (bon)	*F. der* (msu)
				6.07		6.08		6.16
		6.39	6.49					
	6.50				6.57			6.70
7			6.93			6.92		
		7.13						
	7.35				7.25		7.25	
		7.54						
	7.67		7.63					
8			7.90		7.91			
	8.42	8.42			8.42			
		8.61				8.67		
			8.77				8.75	
9					9.08			
				9.18		9.17		
								9.20

	F. pedro (belo)	*F. pedro* (msu)	*F. pedro* (tex)	*F. pedro* (259)	*F. pedro* (261)	*F. pedro* (262)	*F. comp* (msu)	*F. comp* (bon)
	0.50	0.50	0.16		0.57	0.29	0.44	0.44
			0.32	0.29		0.44		
	0.70		0.48	0.44		0.58	0.52	
				0.58		0.87		
1-	1.10	1.10	1.12	1.15	1.13	1.16	1.03	1.03
	1.14	1.33		1.30		1.30	1.32	
	1.18	1.83	1.30	1.72	1.64	1.72		
			1.72					
					1.91			
2-	2.32	2.00	2.32			2.01	2.21	
		2.17					2.35	2.27

Table 13 (Cont'd.)

Rf values X10	*F. pedro* (belo)	*F. pedro* (msu)	*F. pedro* (tex)	*F. pedro* (259)	*F. pedro* (261)	*F. pedro* (262)	*F. comp* (msu)	*F. comp* (bon)
				2.45	2.50	2.45	2.50	
			2.82	2.76		2.75		2.74
	2.95				2.96			
3-		3.00	3.23	3.04		3.20	3.02	3.36
	3.57	3.42	3.40	3.20		3.48	3.24	
	3.84	3.58	3.71	3.72	3.79		3.82	
						3.91		3.91
4-	4.02	4.28	4.00	4.04	4.27	4.20	4.12	4.18
			4.20					
	4.64	4.58	4.67		4.76	4.78	4.71	
5-		4.92		4.93				4.91
		5.42		5.36	5.16	5.00	5.15	
	5.54		5.75	5.51	5.40	5.51	5.64	5.64
	5.89					5.73		
6-		5.92	5.97	5.94	5.95			6.00
	6.07		6.29	6.24	6.29	6.09		
	6.43		6.53	6.52		6.38		6.27
	6.61	6.60	6.94			6.67		6.64
					6.94			
7-			7.10	7.10		7.10		
		7.33			7.42			
	7.68	7.67				7.67		
			7.74	7.83			7.82	7.82
8-			8.14		8.07			8.36
	8.30	8.30	8.47			8.48		
		8.67			8.71			
	8.75							
9-		8.92						
					9.03			9.09
			9.44				9.46	9.46
		9.50		9.58				

Table 13 (Cont'd.)

Rf values X10	*C. carri* (g8)	*C. carri* (g9)	*C. carri* (tex)	*C. tric* (msu)	*C. tric* (g10)	*C. resi* (9257)	*C. resi* (7898)
	0.24		0.24				
				0.40	0.40		
	0.48	0.48	0.48				
	0.65		0.65			0.70	0.79
1-				0.97	0.97	0.90	0.97
	1.21	1.21	1.21			1.23	
							1.58
	1.69	1.69	1.69	1.61	1.61		
				1.86	1.86		1.93
2-	2.18	2.18	2.18	2.17	2.17	2.11	2.19
				2.26	2.26		
				2.66	2.66	2.54	
		2.74		2.79			
3-					2.98	2.90	2.90
	3.07						
	3.47	3.47	3.47	3.47	3.47	3.33	
		3.71		3.87	3.87	3.68	3.86
4-	3.95	3.95	3.95				
				4.27	4.27	4.04	4.04
	4.52	4.52	4.52			4.56	4.56
				4.84	4.84		
						4.91	
5-	5.08	5.08	5.08				
				5.16	5.06		5.44
	5.40		5.57				
				5.73	5.73		
6-	5.97	5.97	5.97				5.97
	6.13	6.13		6.21	6.21		
						6.49	6.40
		6.53	6.53	6.77	6.77		

Table 13 (Cont'd.)

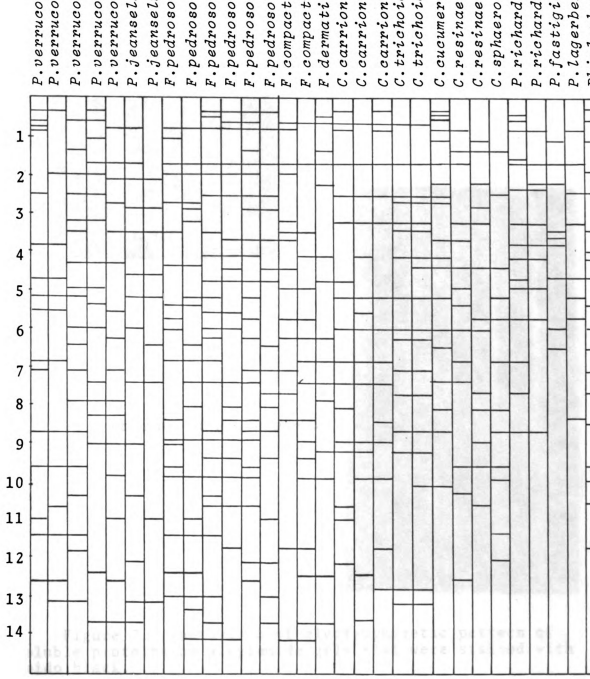
Rf values X10	*C. carri* (g8(	*C. carri* (g9)	*C. carri* (tex)	*C. tric* (msu)	*C. tric* (g10)	*C. resi* (9257)	*C. resi* (7898)
7-	7.12					7.02	7.28
	7.34						
			7.74				
8-	8.23						
		8.31					
			8.47	8.47	8.47	8.42	
				8.79	8.79		
9-							
		9.11					

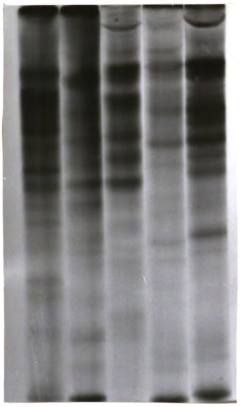
	C. cucum (F-26)	*C. sphae* (9494)	*C. richa* (263)	*C. richa* (6808)	*P. fast* (8008)	*P. lage* (267)	*P. unk* (msu)
	0.25			0.31			0.25
	0.33			0.39			0.42
	0.49			0.62		0.62	
	0.57	0.97	0.62	0.77	0.73		0.75
1-	0.82						
	1.23		1.23	1.23	1.16	1.23	1.30
	1.48		1.54		1.52		
	1.72	1.77	1.62	1.62		1.62	1.67
	1.86						
2-							
			2.15	2.15	2.03	2.15	2.25
	2.46		2.54	2.54	2.39		
	2.62			2.77	2.61		2.67
	2.95	2.90	2.82				2.83

Table 13 (Cont'd.)

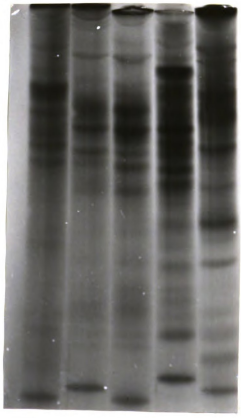
Rf values X10	*C. cucum* (F-26)	*C. sphae* (9494)	*C. richa* (263)	*C. richa* (6808)	*P. fast* (8008)	*P. lage* (267)	*P. unk* (msu)
3-				3.15	3.19	3.15	3.16
	3.44		3.39	3.39		3.39	
		3.55					3.67
		3.87	3.85			3.85	
4-	3.93				3.91		4.00
	4.42						4.42
		4.52		4.62			4.83
	4.92						
5-				5.15			5.25
	5.33	5.48				5.45	
				5.69	5.65		5.67
			5.92				
6-				6.00			
		6.45					6.33
		6.69					6.67
7-					6.93		
							7.17
		7.58					
					7.73		7.75
8-		8.07					
					8.54		

Figure 6. Electrophoretic pattern in acrylamide gels of protein extracts stained with amido black for thirty pathogens and saprobes of species of *Phialophora*, *Fonsecaea* and *Cladosporium*.





3 strains of *C. carrionii*
2 strains of *C. trichoides*
(left to right)



5 strains of *P. verrucosa*

Figure 7. Photograph of electrophoretic pattern of soluble proteins on acrylamide gels that were stained with amido black.

A number of matching bands can be seen in the genera, although not all species may have the matching bands. In some cases the matching bands extend through a portion of the species of one genus to another genus.

Average Rf values of each protein band in the electrophoretic patterns were compared in all possible paired combinations to determine the number of bands which had equal Rf value for different organisms and strains. The bands which were within one Rf value of each other were considered matching. The number of matching bands are shown in Table 14. There are a greater number of matching bands among intraspecies than the number of bands among interpsecies. Three strains of *P. verrucosa*, including *P. verrucosa* (oklahoma), *P. verrucosa* (mac) and *P. verrucosa* (tex) show a greater number of matching bands than two other strains, *P. verrucosa* (269) and *P. verrucosa* (264).

The list of thirty isolates used in disc gel electrophoresis.

No.	1	*Phialophora verrucosa*	(Oklahoma strain, ok)
	2	*P. verrucosa*	(McCurdy strain, mac)
	3	*P. verrucosa*	(Texas Univ., tex)
	4	*P. verrucosa*	(Bonny, Natick, bon 269)
	5	*P. verrucosa*	(Bonny, Natick, bon 264)
	6	*P. jeanselmei*	(Michigan State Univ., msu)
	7	*P. jeanselmei*	(Bonny, Natick, bon 270)
	8	*F. pedrosoi*	(Belo Horizonte, belo)

9	*F. pedrosoi*	(Michigan State Univ., msu)
10	*F. pedrosoi*	(Texas Univ., tex)
11	*F. pedrosoi*	(Bonny, Natick, bon 259)
12	*F. pedrosoi*	(Bonny, Natick, bon 261)
13	*F. pedrosoi*	(Bonny, Natick, bon 262)
14	*F. compactum*	(Michigan State Univ., msu)
15	*F. compactum*	(Bonny, Natick, bon)
16	*Fonsecaea dermatitidis*	(Michigan State Univ., msu)
17	*Cladosporium carrionii*	(George, CDC, g8)
18	*C. carrionii*	(George, CDC, g9)
19	*C. carrionii*	(Texas Univ., tex)
20	*C. trichoides*	(Michigan State Univ., msu)
21	*C. trichoides*	(George, CDC, g10)
22	*C. cucumerinum*	(DeZeeuw, F-26)
23	*C. resinae f. avellanium*	(Bonny, Natick, 9257)
24	*C. resinae f. avellanium*	(Bonny, Natick, 7898)
25	*C. sphaerospermum*	(Bonny, Natick, 9494)
26	*P. richardsiae*	(Bonny, Natick, 6808)
27	*P. richardsiae*	(Bonny, Natick, 263)
28	*P. fastigiata*	(Bonny, Natick, 8008)
29	*P. lagerburgii*	(Bonny, Natick, 267)
30	*Phialophora* sp.	(Univ. of Mich., E-286)

In Table 14, the number of matching bands of *P. verrucosa* intraspecies appear to be greater than when compared with the number of the bands of one strain to the next strain. At any rate, the number of matching bands of *P. verrucosa* and *F. pedrosoi* appear to be less than that of *Fonsecaea* intraspecies per se.

Table 14. The number of matching soluble protein bands of thirty human pathogenic, plant pathogenic and saprobic strains in the gel electrophoretic gels stained with amido black

	P.ver ok	P.ver mac	P.ver tex	P.ver 269	P.ver 264	P.jea msu	P.jea bon	P.ped belo	P.ped msu	P.ped tex	P.ped 259	P.ped 262	P.ped 261	P.com msu	P.com bon	P.der msu	C.car g8	C.car g9	C.car tex	C.tri msu	C.tri g10	C.cuc F-26	C.res 9257	C.res 7898	C.sph 9494	P.ric 6808	P.ric 263	P.fas 8705	P.lag 8707	P.unk msu
P.ver ok	15																													
P.ver mac	10	13																												
P.ver tex	8	7	19																											
P.ver 269	5	4	8	17																										
P.ver 264	7	4	4	6	16																									
P.jea msu	3	3	3	5	6	14																								
P.jea bon	3	3	4	4	3	7	9																							
P.ped belo	4	5	7	7	8	4	3	19																						
P.ped msu	5	5	7	8	6	4	5	12	21																					
P.ped tex	6	8	8	7	7	4	3	10	11	25																				
P.ped 259	6	6	5	8	6	4	3	7	9	15	21																			
P.ped 262	6	8	7	5	6	5	3	8	8	14	14	24																		
P.ped 261	5	4	6	4	5	6	4	5	8	9	9	10	18																	
P.com msu	2	2	4	5	5	4	2	7	6	11	10	10	3	17																
P.com bon	3	1	6	4	5	3	1	6	6	6	9	9	6	9	16															
P.der msu	4	2	3	5	5	3	2	4	5	6	6	7	5	3	3	16														
C.car g8	7	5	4	6	3	6	3	5	9	11	11	11	8	4	5	5	17													
C.car g9	6	3	5	6	4	5	3	6	8	11	9	8	5	3	9	6	10	15												
C.car tex	6	3	4	6	3	5	2	7	8	10	11	10	5	6	8	4	11	10	15											
C.tri msu	5	2	6	4	5	5	2	4	4	8	5	8	5	7	5	5	6	8	7	17										
C.tri g10	5	2	7	5	4	4	2	5	5	8	6	8	6	8	4	5	7	7	7	15	18									
C.cuc F-26	5	2	5	6	3	5	3	5	9	10	11	10	9	5	4	5	8	7	7	8	9	18								
C.res 9257	4	3	5	5	2	3	1	6	6	6	6	7	4	5	5	5	6	6	6	3	4	6	14							
C.res 7898	1	2	3	2	0	2	1	5	5	2	5	6	4	4	2	2	5	3	3	3	4	5	7	13						
C.sph 9494	5	3	5	2	2	2	1	6	6	6	4	7	5	2	3	3	5	4	5	3	4	5	4	5	12					
P.ric 6808	5	3	4	5	4	1	6	3	3	3	4	5	3	4	5	6	5	5	4	6	5	4	4	3	3	15				
P.ric 263	3	2	3	3	2	1	0	2	2	3	3	3	4	3	4	3	3	3	3	4	4	5	4	3	2	9	10			
P.fas 8705	2	2	3	3	3	5	3	3	2	3	2	1	2	3	2	3	3	2	2	3	3	6	3	3	0	3	3	9		
P.lag 8707	3	2	2	5	2	2	1	2	3	4	4	3	3	3	2	2	6	4	5	4	4	4	3	2	3	5	4	2	8	
P.unk msu	6	5	5	6	2	1	1	4	3	6	9	8	3	4	3	2	4	2	3	6	5	4	2	4	1	3	0	4	2	19

The number of common bands of *F. compactum* and *P. verrucosa* (see Table 14) are fewer than the number of common bands seen between *F. compactum* and *F. pedrosoi*. There is little difference in the number of matching bands of two strains of *P. verrucosa* with *P. jeanselmei* and of *P. jeanselmei* with *F. pedrosoi*.

Fonsecaea dermatitidis also shows a similar number of matching bands with every strain of *P. verrucosa*, *P. jeanselmei*, *F. pedrosoi*, *F. compactum*, *C. carrionii* and *C. trichoides*.

The number of matching bands of *F. compactum* and *F. pedrosoi* is greater than with *F. compactum* and *C. carrionii*. However, the number of matching bands appear to be fewer when *F. compactum* is compared with *F. dermatitidis*, *P. verrucosa* or with *P. jeanselmei*.

Cladosporium cucumerinum (F-26), a plant pathogen, shows a greater number of matching bands when compared with *F. pedrosoi*, *C. trichoides* and with *C. carrionii*, but the number of matching bands is less with the human pathogenic *Phialophora* species (Table 14).

The matching bands between pairs of saprobes are less in numbers than between pairs of plant pathogenic species. The matching bands between saprobes and human pathogenic strains are also less in numbers.

2. Specific protein staining for intracellular enzyme and isoenzyme locations

The developed bands of enzymes and isoenzymes with specific substrates in gel electrophoresis showed more

varying number of bands among saprobes than among human pathogenic strains. Uniform pattern of enzymatic activity and of isoenzymes were seen in human pathogenic strains.

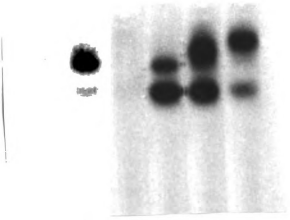
Peroxidase

Peroxidase activity was shown as a brown-colored band in gel electrophoresis when catechol was used as an electron donor (see Figure 8). The Rf values of peroxidase activity are shown in Table 15. All five strains of *P. verrucosa* showed a single band of peroxidase activity at Rf 2.5. Each of the two strains of *P. jeanselmei* had double bands for peroxidase activity, one at Rf 2.2 and the other at Rf 2.5. *Fonsecaea pedrosoi*, *F. compactum*, *F. dermatitidis* and *C. carrionii* showed a similar peroxidase activity which was composed of two different bands: one was at Rf 1.7 and the other at Rf 2.5. Three different bands of peroxidase activity were observed in gel electrophoresis of *C. trichoides*. *Phialophora* and *Cladosporium* saprobes showed a variation in the enzymatic activity as illustrated by the varied location of representative bands in Figure 9.

Alpha-and beta-D-glucosidases

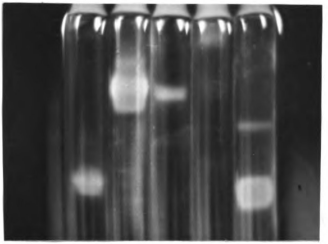
Human and animal pathogenic strains showed no intracellular alpha-D-glucosidase activity. A rather low activity was detected in *Cladosporium resinae* f. *avellaneum* (7998).

The yellow colored band was developed for beta-D-glucosidase activity, with *P. verrucosa*, *P. jeanselmei*,



Peroxidase

Acid phosphatase



Amylase

Figure 8. Photographs of electrophoretic bands of enzymes and isoenzymes with their specific substrates for species of *Phialophora*, *Fonsecaea*, and *Cladosporium*.

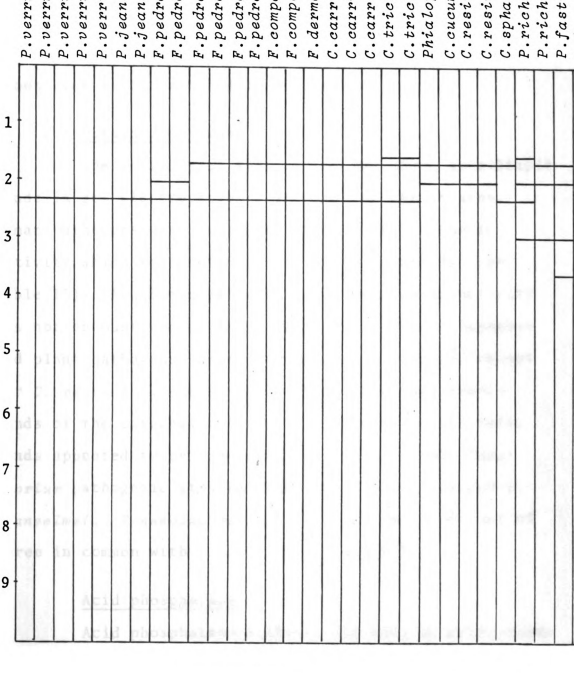
Table 15. Migration distances of peroxidase, beta-D-glucosidase and catechol oxidase isoenzyme bands developed and stained in acrylamide gels for 30 pathogens and saprobes

Organism	Migration distances		
	peroxidase	beta-D-glucosidase	catechol oxidase
P. verrucosa (ok)	2.5	1.8	1.3 1.9
P. verrucosa (mac)	2.5	1.8	1.3 1.9
P. verrucosa (tex)	2.5	1.8	1.9 2.5
P. verrucosa (264)	2.5	1.8	2.8
P. verrucosa (269)	2.5	1.8	2.5 3.0
P. jeanselmei (msu)	2.2 2.5	1.8	2.1 3.5
P. jeanselmei (270)	2.2 2.5	1.8	2.1 3.5
F. pedrosoi (belo)	1.7	1.8	0.8 1.9 2.5
	2.5		3.8
F. pedrosoi (msu)	1.7	1.8	1.9 2.1 2.8
	2.5		3.0
F. pedrosoi (259)	1.7	1.8	1.9 2.5 2.8
	2.5		3.0
F. pedrosoi (tex)	1.7	1.8	1.9 2.5 2.8
	2.5		
F. pedrosoi (262)	1.7	1.8	0.7 1.9 2.5
	2.5		3.0 3.4 2.8
F. pedrosoi (261)	1.7	1.8	0.8 1.9 2.5
	2.5		3.0
F. compactum (msu)	1.7	1.8	0.8 1.9 2.8
	2.5		
F. compactum (bon)	1.7	1.8	0.8 1.9 2.8
	2.5		

Table 15 (Cont'd.)

Organism	Migration distances				
	peroxidase		beta-D-glucosidase		catechol oxidase
F. dermatitidis (msu)	1.7		1.8		0.8 2.8
C. carrionii (g8)	1.7		2.3		1.3 1.9 2.8
	2.5				3.4
C. carrionii (g9)	1.7		2.3		2.8 3.0 4.4
	2.5				4.8
C. carrionii (tex)	1.7		2.3		1.3 1.9 2.8
	2.5				4.4
C. trichoides (msu)	1.6	1.7	1.8		21. 2.8 3.0
	2.5				4.4
C. trichoides (g10)	1.6	1.7	1.8		1.3 1.9 2.8
	2.5				3.4 3.6
Phialophora sp. (E-286)	1.7	2.2	1.8		2.1
P. lagerbergii (267)	1.7	2.2	1.8		0
P. richardsiae (263)	1.7	2.2	1.8	2.3	0
P. richardsiae (6808)	1.7	2.2	1.8	2.3	0
P. fastigiata (265)	1.7	2.5	1.5		0
C. resinae f. *avellaneum* (7998)	1.6 2.5	2.2 3.0	2.3		0
C. resinae f. *avellaneum* (9257)	1.7 2.5	2.2 3.0	2.3		0.8 2.1
C. sphaerospermum (9494)	1.7 2.5	2.2 3.0 3.7	1.5		0
C. cucumerinum (F-286)	1.9 3.0 3.7	2.2	1.5	1.8	0

Figure 9. Electrophoretic pattern in acrylamide gels of peroxidase of soluble protein extracts from thirty pathogens and saprobes.



F. pedrosoi, *F. compactum*, *H. dermatitidis*, *C. trichoides* and *Phialophora* species (E286) showing a single band of beta-D-glucosidase at Rf 1.8 (see Table 15). *Cladosporium carrionii* also showed a single band of the enzymatic activity at Rf 2.3. Two isolates of *P. richardsiae* showed two different bands of isoenzymes, one at Rf 2.3 and one at Rf 1.8. A single band of the enzymatic activity at Rf 1.5 was detected in *Cladosporium* saprobes and in *P. fastigiata*. The pattern of beta-D-glucosidase and its isoenzymes is illustrated in Figure 10.

Catechol oxidase

The activity of this enzyme was shown as multiple bands in gel electrophoresis for most of the strains. Human pathogens gave multiple bands of the enzymatic activity which were noted in different locations (see Table 15). A uniform pattern of this enzymatic activity was not encountered in the pathogenic strains. Saprobes and plant pathogens showed no enzymatic activity, except for *C. resinae* f. *avellaneum* (7998) which gave double bands of the catechol oxidase. The number of enzymatic bands appeared to be greater in *F. pedrosoi* and *Cladosporium* pathogenic strains than for *P. verrucosa* and *P. jeanselmei*. *Fonsecaea dermatitidis* had two bands out of three in common with *F. compactum* (Figure 11).

Acid phosphatase

Acid phosphatase activity was seen as white bands in acrylamide gels after the staining procedure (Figure 8).

Figure 10. Electrophoretic pattern in gels of beta-D-glucosidase of soluble protein extracts from the thirty pathogens and saprobes.

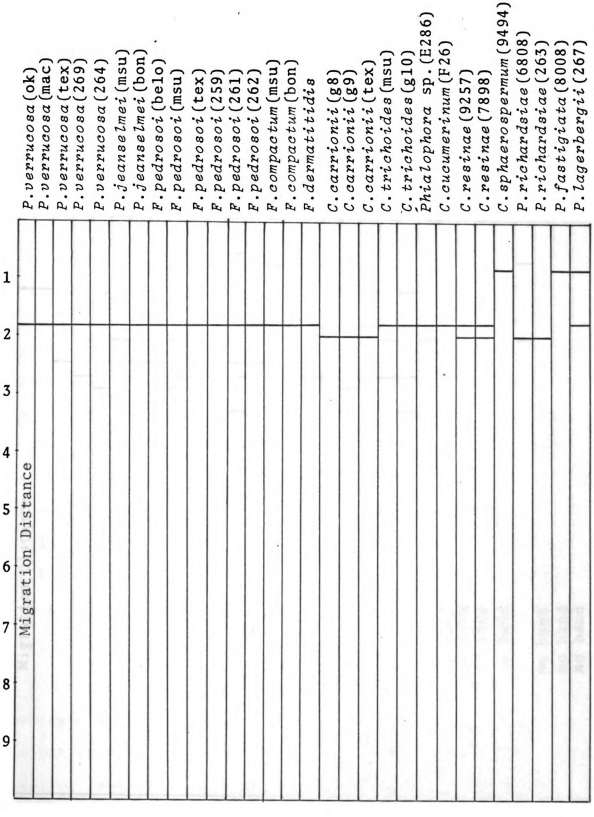
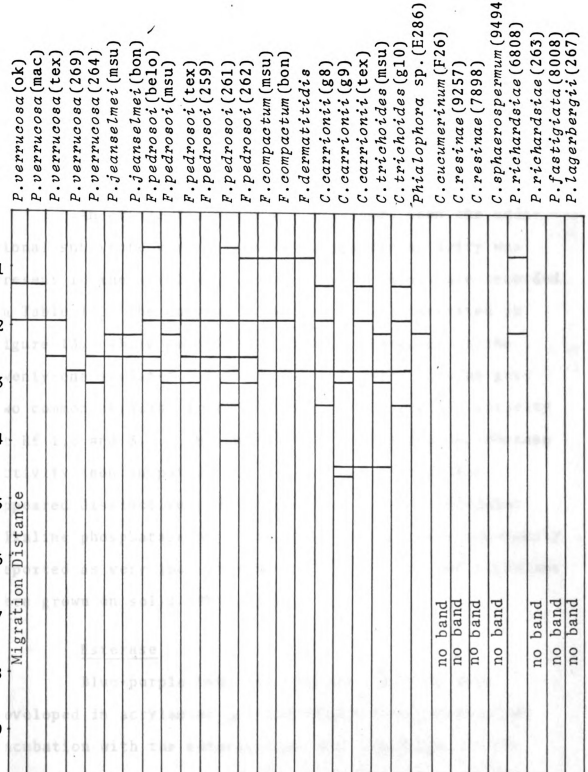


Figure 11. Electrophoretic pattern in acrylamide gels of catechol oxidase soluble protein extracts from thirty pathogens and saprobes.



Twenty-two pathogenic strains showed a common straight band at Rf 3.5 and multiple bands of isoenzymes among species of pathogens (Table 16). A rather non-uniform enzymatic pattern was found among saprobes. Multiple bands were seen at different Rf values and this is illustrated in Figure 12. Five isoenzymes were encountered for acid phosphatase in *P. verrucosa* (tex).

Alkaline phosphatase

An orange-red color was developed upon the additional substrate when alkaline phosphatase activity was present in the acrylamide gels. The Rf values are recorded in Table 16. The enzymatic patterns are illustrated in Figure 13. There were four isoenzymes detected in the twenty-one isolates. Twenty-one pathogenic strains gave two common straight lines of alkaline phosphatase activity at Rf 1.5 and 3.3. The intracellular alkaline phosphatase activity seen in pathogenic species and in saprobes appeared distinctively different from the extracellular alkaline phosphatase activity of human pathogens previously reported as very low, with none detected when the organisms were grown on solid CPYG medium.

Esterase

Blue-purple bands of esterase activity were developed in acrylamide gel electrophoretic column after incubation with the esterase specific substrate. Five bands of isoenzymes of esterase were detected as shown in Figure 14 and Table 16. No esterase activity was found in

Table 16. Migration distance of acid phosphatase, alkaline phosphatase, esterase, and amylase isoenzyme bands developed with their specific substrates within acrylamide gels

Organism	Migration distances			
	acid phosphatase	alkaline phosphatase	esterase	amylase
P. verrucosa (ok)	1.4 3.5	1.5 3.3	1.3 1.6 3.2	0
P. verrucosa (mac)	1.4 3.5	1.5 3.3	1.3 2.0 3.2 3.8	0
P. verrucosa (tex)	1.4 1.6 2.0 3.5 5.0	1.5 3.3	1.3 3.2 3.8	1.1 3.0
P. verrucosa (264)	3.5	1.5 3.3	3.8	0
P. verrucosa (269)	1.4 3.5	1.5 3.3	1.3 1.6 2.0 2.3 3.8	0
P. jeanselmei (msu)	1.4 1.6 2.0	1.5 3.3	0	1.1 1.3 3.0
P. jeanselmei (270)	1.4 2.0 3.5	1.5 3.3	0	1.1
F. pedrosoi (belo)	1.4 3.5	1.5 3.3	3.8	0
F. pedrosoi (msu)	3.5	1.5 3.3	1.3 3.8	0

Table 16 (Cont'd.)

Organism	Migration distances			
	phosphatase	alkaline phosphatase	esterase	amylase
F. pedrosoi (259)	1.4 3.5	1.5 3.3	3.2 3.8	0
F. pedrosoi (tex)	1.4 3.5	1.5 3.3	3.2 3.8	0
F. pedrosoi (262)	1.4 3.5	1.5 3.3	3.2 3.8	0
F. pedrosoi (261)	1.4 3.5	1.5 3.3	1.3 3.2 1.6 3.8	0
F. compactum (msu)	3.5	1.5 3.3	0	0
F. compactum (bon)	3.5	1.5 3.3	0	0
F. dermatitidis (msu)	1.4 3.5	1.5 3.3	3.2 3.8	0
C. carrionii (g8)	3.5	1.5 3.3	3.2 3.8	0
C. carrionii (g9)	3.5	1.5 3.3	3.2 3.8	0
C. carrionii (tex)	1.4 3.5	1.5 3.3	1.3 1.6 2.0 3.8	1.1 1.7

Table 16 (Cont'd.)

Organism	Migration distances			
	phosphatase	alkaline phosphatase	esterase	amylase
C. trichoides (msu)	3.5	1.5 3.3	3.8	0
C. trichoides (g10)	3.5	1.5 3.3	1.3 1.6 2.0 3.2	0
Phialophora sp. (E-286)	1.4	2.2	1.3	1.1
P. lagerbergii (267)	2.8 3.9	3.3	1.3	2.5
P. richardsiae (263)	2.2	1.8 2.3	0	1.1 1.3 2.5 3.0
P. richardsiae (6808)	2.2	1.8 2.3	0	1.1 1.5 2.5 3.0
P. fastigiata (265)	2.2	1.5	0	1.1
C. resinae (7998)	1.5	2.3	3.8	1.5 4.7
C. resinae (9257)	3.3	2.3	1.6	1.1
C. sphaerospermum (9494)	3.7	1.5	0	1.1 2.5
C. cucumerinum (F-26)	2.2	1.8	0	0

Figure 12. Electrophoretic pattern in acrylamide gels of acid phosphatase of soluble protein extracts from thirty pathogens and saprobes.

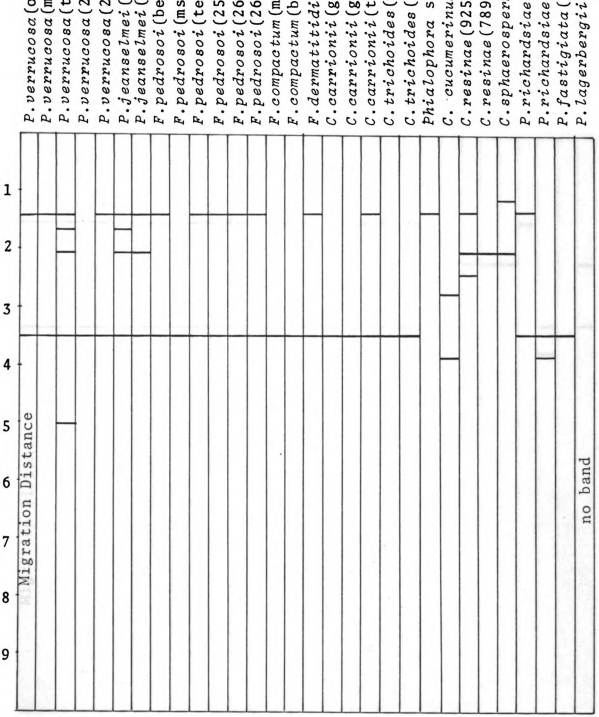


Figure 13. Electrophoretic pattern in acrylamide gels of alkaline phosphatase of soluble protein extracts from thirty pathogens and saprobes.

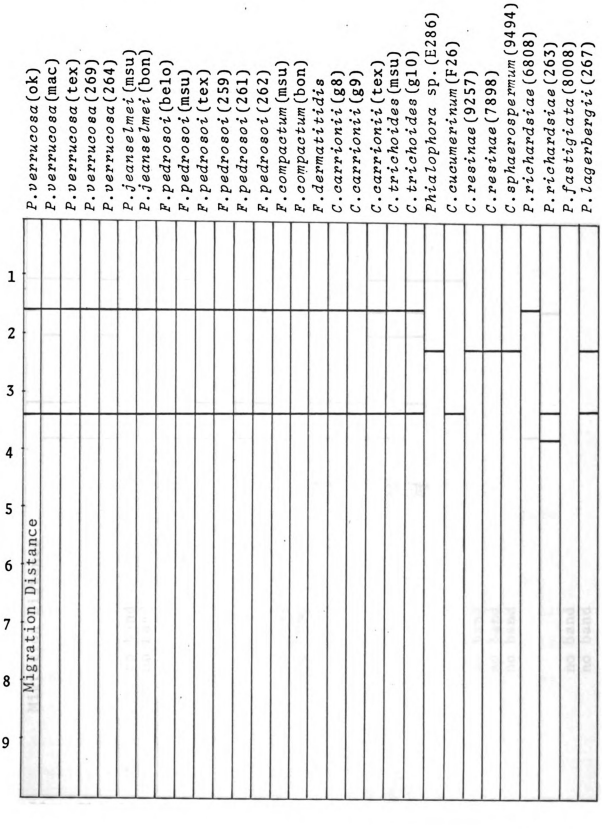
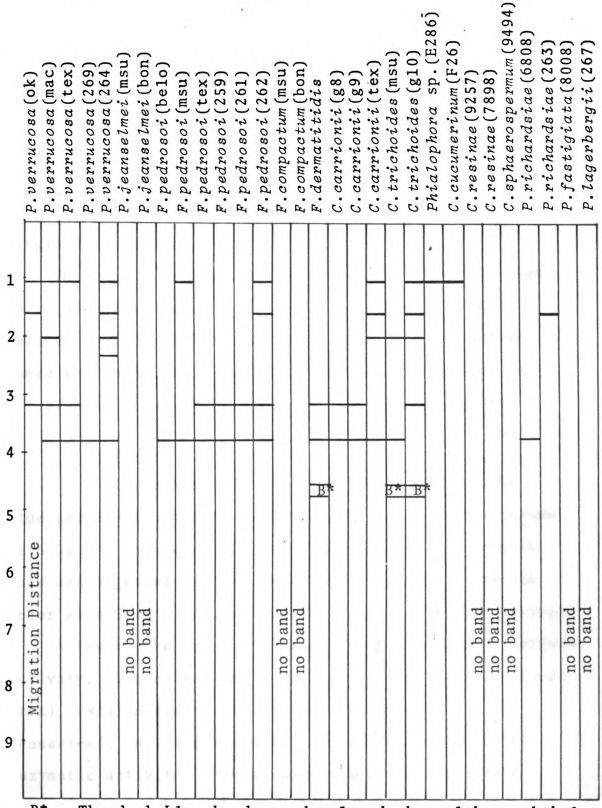


Figure 14. Electrophoretic pattern in acrylamide gels of esterase of mycelial soluble protein extracts from thirty pathogens and saprobes.



B* - The dark blue band was developed when alpha-naphthyl acetate was used for esterase specific substrate.

F. jeanselmei and *F. compactum*. Enzymatic bands with the same Rf values were observed for *P. verrucosa* isolates. *Phialophora verrucosa* isolated from wood pulp showed five bands for esterase while *P. verrucosa* isolated from human infection had one to five bands for esterase. The enzymatic band seen at Rf 3.8 was a common band among most pathogenic strains. Six out of eight strains of saprobes showed no esterase activity. The remaining two strains, *C. resinae* f. *avellaneum* No. 7998 and No. 9275, had a single band each.

The same Rf values for the enzymatic bands are seen in *F. dermatitidis*, *C. carrionii*, *C. trichoides* and *Phialophora* sp. (E-286), which was isolated from a frog. There was an unidentified dark blue band recognized in *C. trichoides* and in *F. dermatitidis* (see Figure 14).

Amylase

Colorless bands of amylase were noted in the blue gel electrophoretic column after staining the column with Gram's iodide as a control. The large starch molecule was digested with amylase and the products gave no color with Gram's iodide staining (Figure 8, lower picture).

A few of the human and animal pathogens showed amylase activity. These included *P. verrucosa* (tex), *C. carrionii* (tex), *Phialophora* sp. (286), and two isolates of *P. jeanselmei*. All eight isolates of saprobes showed this enzymatic activity. The maximum number of isoenzymes detected among these thirty isolates was four bands. The

Rf values and diagrams of the enzymatic bands are shown in Table 16 and in Figure 15, respectively. Determination of amylase activity in the mycelial extracts of these fungi was carried out by using liquid CPYG medium in the previous experiment but failed to show any promising results.

Comparison of Enzymatic Common Bands Between Pairs of Pathogens and Saprobes

Comparison of enzymatic bands or enzymatic activity of all tested enzymes, including acid phosphatase, alkaline phosphatase, beta-D-glucosidase, peroxidase, catechol oxidase, esterase and amylase, of the thirty isolates are shown in Table 17. Sequential combination of this table would give a complete chart of pair comparative gel electrophoresis of the seven enzymes. Several pathogens, including *F. pedrosoi* (261) and *C. trichoides* (g10) had an especially high number of common bands for all strains. A greater number of enzymatic matching bands were found among human pathogenic strains. A lesser number of the matching bands was seen among saprobes, animal pathogens and among plant pathogens. The results of the enzyme patterns here show some correlation to the previous results of soluble protein patterns seen in Table 14. The number of enzymatic matching bands were greater in intraspecies organisms than for interspecies organisms, including: *P. verrucosa, P. jeanselmei, F. pedrosoi* and *F. compactum*. *Cladosporium carrionii, C. trichoides* and *F. dermatitidis* showed closer relationship to *F. pedrosoi* when the matching

Figure 15. Electrophoretic pattern in acrylamide gels of amylase of mycelial soluble protein extracts from thirty pathogens and saprobes.

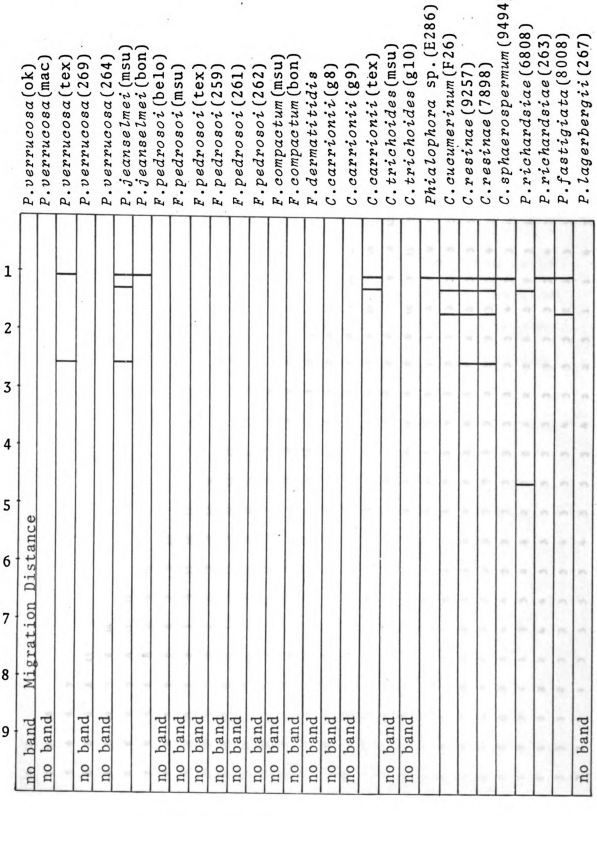


Table 17. The number of common isoenzyme bands developed with seven specific substrates with gel electrophoresis of thirty human pathogenic, plant pathogenic and some saprobic strains

	P.ver ok	P.ver mic	P.ver tex	P.ver 269	P.ver 264	P.jea asu	P.jea bon	F.ped belo	F.ped asu	F.ped tex	F.ped 259	F.ped 262	F.ped 261	F.com asu	F.com bon	F.der asu	C.car g8	C.car g9	C.car tex	C.tri asu	C.tri g10	C.cuc F-26	C.res 9257	C.res 7898	C.sph 9494	P.ric 6808	P.ric 263	P.fas 8008	P.lag 8707	P.sp 8286
P.ver ok	11																													
P.ver mic	10	12																												
P.ver tex	9	10	16																											
P.ver 269	5	6	6	7																										
P.ver 264	8	8	8	6	13																									
P.jea asu	6	6	10	5	6	14																								
P.jea bon	6	6	8	5	6	11	11																							
F.ped belo	7	8	9	6	8	6	6	12																						
F.ped asu	7	8	8	7	8	6	6	8	12																					
F.ped tex	8	9	10	7	9	6	6	10	10	13																				
F.ped 259	7	8	9	7	8	6	6	10	9	12	12																			
F.ped 262	9	9	8	8	7	6	6	11	9	13	13	15																		
F.ped 261	9	10	11	6	10	6	6	10	10	12	11	12	15																	
F.com asu	6	6	6	6	5	5	5	7	8	8	8	8	8	9																
F.com bon	6	6	6	6	5	5	5	7	8	8	8	8	8	9	9															
F.der asu	7	8	8	7	7	6	6	8	8	10	10	10	10	8	8	11														
C.car g8	7	8	6	6	5	4	4	7	8	9	9	10	8	7	7	8	12													
C.car g9	5	6	6	6	6	4	4	6	8	9	8	7	7	6	6	8	9	12												
C.car tex	9	8	10	6	8	6	6	8	9	9	9	9	10	7	7	8	10	9	17											
C.tri asu	5	6	6	7	7	6	6	7	9	9	8	9	8	7	7	8	7	9	8	12										
C.tri g10	10	10	8	6	8	5	5	7	9	9	9	10	10	8	8	8	10	7	11	8	16									
C.cuc F-26	1	1	1	1	1	2	2	1	1	1	1	1	1	1	1	1	0	0	0	1	1	8								
C.res 9257	2	1	2	1	2	4	4	2	3	2	2	2	4	3	3	3	3	3	5	3	3	2	11							
C.res 7898	1	2	2	2	2	2	2	2	2	2	2	2	2	1	1	2	3	3	3	3	2	1	5	10						
C.sph 9494	2	2	3	2	2	3	3	3	3	3	3	3	3	3	3	3	3	3	4	3	3	4	5	3	10					
P.ric 6808	2	2	3	2	2	4	4	3	3	3	3	3	3	3	3	3	4	3	3	4	3	3	4	2	4	11				
P.ric 263	1	1	3	1	1	5	3	2	2	2	2	2	2	2	2	2	2	2	3	2	2	4	5	2	4	6	11			
P.fas 8008	2	2	3	2	2	4	4	3	3	3	3	3	3	3	3	3	3	3	4	3	3	2	3	1	5	3	3	6		
P.lag 8707	3	3	3	2	3	3	3	3	4	3	3	3	4	3	3	3	2	2	3	3	4	2	2	1	3	3	3	1	8	
P.sp 8286	3	3	4	1	3	5	5	3	4	3	3	3	4	2	2	3	1	1	4	3	3	4	4	1	3	4	4	2	4	9

bands of the enzymes were compared on the bases of intra-species and interspecies.

Table 17 shows that six pairs of matching enzymatic bands occurred between *P. jeanselmei* and each strain of *F. pedrosoi*. The matching bands of all seven enzymes of *F. pedrosoi* and *P. verrucosa* were similar to that of *P. verrucosa* and *Cladosporium* pathogenic strains.

Phialophora sp. (286) showed a great number of matching bands with *P. jeanselmei*, *C. cucumerinum* (F-26), *C. resinae* (9257) and with *P. richardsiae* No. 6808 and No. 236. The plant pathogen of cucumber, *C. cucumerinum* (F-26), also showed a great number of matching bands with those of human and animal pathogens. The intraspecies of pathogenic strains showed an equal number of matching bands with the plant pathogen as indicated in Table 17.

DISCUSSION

Extracellular Enzymatic Studies

The dematiaceous pathogenic fungi, *P. verrucosa, F. pedrosoi, F. compactum, F. dermatitidis, C. carrionii, C. trichoides* and *P. jeanselmei*, showed low extracellular enzymatic activity in most strains with twelve paranitrophenol derivatives, including the alpha-D-glucoside, beta-D-glucoside, alpha-D-galactoside, beta-D-galactoside, N-acetyl-beta-glucosaminide, caprylate, stearate, laurate, palmitate and butyrate. Low acid phosphatase and alkaline phosphatase were also observed when the organisms were grown for 15 and 25 days at 25°C on solid CPYG medium, which was considered to be a good synthetic medium for production of extracellular enzymes of *Blastomyces dermatitidis* (Beneke *et al.*, 1969). The extracellular enzymatic activities of forty-five dematiaceous fungi that are saprobes and plant pathogens, including *Phialophora* species and *Cladosporium* species, were high in most cases at 15 days of mycelial growth in the medium and usually increased through 25 days on the same medium.

The lack of detectable or low extracellular enzyme activities for the human and animal pathogenic dematiaceous fungi in contrast to the higher extracellular enzyme activities for the saprobes and plant pathogens is similar in pattern

to other reports in the literature. Montemayer (1949) found from the studies of biochemical activities of the fungi that none of these, including *F. compactum*, *F. pedrosoi*, *P. jeanselmei* and *P. verrucosa*, could liquify gelatin, Loeffler's serum or coagulated milk, in contrast to the positive activity for two saprophytic *Cladosporium* isolates. In 1952 De Vries also reported that *C. carrionii* and *C. trichoides* did not exert proteolytic activity on Loeffler's serum while on the other hand extensive activity occurred with the saprophytic species of this genus. In addition, he found that twenty-four saprophytic species of *Cladosporium* produced tributyrine hydrolyzing lipases in varying degree whereas the pathogenic strains showed no activity.

The extracellular fatty acid esterase activities varied in the organisms investigated. Activity toward PNP caprylate was found in most of the pathogenic and saprophytic species while only an occasional strain showed activity toward PNP lauric acid. None of the strains tested showed any extracellular activity toward PNP derivatives of butyrate, palmitate or stearate. It is possible that these enzymes may be bound to the cell in some of the strains of *Cladosporium*, *Fonsecaea* and *Phialophora*, and do not escape into the medium unless cell disintegration occurs or those which are liberated to a significant extent into the medium are from intact cells under varied physiological conditions of the organism. There may be good reasons to believe that several cell bound enzymes are situated outside the relatively impermeable barrier provided

by the cytoplasmic membrane, so only certain enzymes may diffuse through pores of the cell wall as proposed by Pollock (1962), who called these kinds of enzymes "partially cell-bound enzymes."

Mitchell and Moyle (1959) state that it is not surprising that a small amount of the potentially extracellular enzymes should be detected on the outer layers of the cell. The problem of the enzymatic liberation would be devolved upon the cell wall, thus partially or completely preventing enzyme released from the cell. Thus, as Mitchell and Moyle speculated, it would be plausible to say that the extent of enzymes produced would presumably depend upon the properties both of the cell wall and of the enzyme per se.

Szaniszlo *et al.* (1972) analyzed the chemical compositions of hyphal walls of three chromomycotic agents, including *P. verrucosa*, *F. pedrosoi* and *C. carrionii*, and found that the hyphal walls were composed of glucose, mannose, glucosamine, and protein components which presented the similar amount of the chemical compositions in these three genera. No records of analyses of the chemical compositions of the hyphal wall of *Cladosporium* and *Phialophora* saprobes exist, to my knowledge. The mycelial mats of plant pathogens and saprobes were more difficult to homogenize than those of human and animal pathogens. It seems reasonable to conjecture the chemical compositions of the cell walls of plant pathogens and saprobes may be different from those of the human pathogens. This may be a factor in the variation that could account for the marked

differences in certain extracellular activities between the human pathogens, and the plant pathogens and the saprobes.

Extracellular enzymatic activity of intraspecies of plant pathogens and saprobes, *Phialophora* and *Cladosporium* species, showed a variation in relationship to one another at 25 days of growth in culture. *Phialophora* saprobe strains showed high activities for beta-D-glucosidase and N-acetyl-β-glucosaminidase, while no activity was detected for the pathogenic strains. *Cladosporium* saprobe also showed a high activity for beta-D-glucosidase, and N-acetyl-D-glucosaminidase, whereas these activities were not detected for the human pathogens. Both genera showed similar extracellular enzyme patterns, except alpha-D-galactosidase activity was high. This could possibly be used for differentiation of *Phialophora* and *Cladosporium* saprobes.

Since N-acetyl-beta-glucosaminidase was detected in a rather high activity in *Cladosporium* and *Phialophora* saprobes and none in human pathogens, it might be speculated that the growth rate of the saprobes would be faster than that of the pathogens. The speculation is based on the fact that N-acetyl-β-glucosaminidase will hydrolyze PNP-N-acetyl-β-glucosaminide and a free group of N-acetyl-β-glucosaminide will be released and directly absorbed for chitin synthesis. It is reasonable to conclude that the rate of cell wall formation of the saprobes is faster than that of the pathogens, and reflect in the faster rate of growth of the saprobes. The observations

on the rate of growth seem to correlate with the observations in the work of Silva (1960), who found that rate of growth of *Cladosporium* saprobes was faster than that of the pathogens.

From the results of the twelve enzymes studied, it was noted that there were differences of extracellular enzymatic activity among *Cladosporium* interspecies. For instance, five isolates of *C. oxysporum* showed extracellular alkaline phosphatase while four strains of *C. resinae* and two strains of *C. cladosporioides* had no enzymatic activity. The combination of morphologic appearance of these fungi and the differences in the enzymatic activity may be good criteria for taxonomic classification of *C. oxysporum*.

The relationship of increase in extracellular enzymatic activity and growth rate in culture of all human and animal pathogens, and the plant pathogens and saprobes except *Fonsecaea* pathogens were compared (Figures 2, 3, 4 and 5).

Most of the increased enzymatic activity, including beta-D-glucosidase, N-acetyl-beta-glucosaminidase, caprylase, acid phosphatase, alkaline phosphatase and alpha-D-galactosidase of some of these fungi appeared to increase during the logarithmic phase of growth at 15 days. This evidence would indicate that extracellular enzymes were liberated from the series of individual cells during the normal growth and metabolism of the cells and not released after cell autolysis (Pollock, 1962).

It is interesting to note that *P. jeanselmei*, a human pathogen, showed surprisingly low enzymatic activity through the first 15 days of growth and rapidly increased enzymatic activity right after the logarithmic phase of growth. The increased enzymatic activities might have been due to released enzymes from autolytic cells, which seems to be less convincing because it is unlikely to assume that there was an autolysis of the fungus right at the beginning of the stationary phase. There may be a correlation between the rapid rate of enzyme activity at about the 15-day growth period and the change that occurs in the yeast type to mycelial type colony after the first 10 days of colony growth.

Peroxidase activity

There was no significant difference of peroxidase activity seen among dematiaceous pathogenic and saprophytic isolates.

Adenosine triphosphatase activity

According to a report by West (1967) the sclerotic cells of *P. verrucosa* (A-126) were formed when L-proline was utilized as a nitrogen source. Basically about 60-70% of total nitrogen of the fungal wall is incorporated into protein and a small amount into nucleic acids and chitin according to Moore and Landecker (1972).

By using L-proline as a nitrogen source, it was found that all five strains of *P. verrucosa* did form the sclerotic cells. There was no significant relationship between

formation of the sclerotic cells and the release of adenosine triphosphatase as three of the five strains of *P. verrucosa* had some enzyme activity.

Fonsecaea and *Cladosporium* species failed to form sclerotic cells in West's liquid medium. However, sclerotic cells were formed when species of both genera were cultured on six synthetic media by Silva (1967).

Again, West (1967) found there was no significant difference in adenosine triphosphatase activity among the fungi when DL-isoleucine was utilized as a nitrogen source in experiments designed to get more mycelial mat weight without formation of the sclerotic cells.

In conclusion, it would be reasonable to propose that there was no significant difference in adenosine triphosphatase activity between human pathogens and saprobes when L-proline or DL-isoleucine was used as nitrogen source. On the other hand, the ability to produce extracellular ATPase in the fungi not only depends on the nutrient utilized but also is dependent on the releasing capability of the organism.

Comparative Gel Electrophoresis of Intracellular Soluble Proteins

Intracellular extracts of chromomycotic pathogens and saprobes

1. General soluble proteins stained with amido black

The results showed that low concentrations of soluble protein were extracted from mycelium of saprobes harvested

at the same period of time on identical medium when compared with those from pathogens. It seems possible to expect a difference in the type of proteins obtained, which appear to depend on the physiological activity of the cell at the time of harvesting; since the soluble proteins reflect the physiological state of the cell rather than morphological strucutre, it is reasonable to anticipate that the saprobes not only have a variation in composition of the cell wall but also have a physiological difference from pathogenic species.

It is possible to separate a mixture of globular proteins in solution on the basis of their different rates of migration in the electric field at a given pH. The migratory distance depended on charges and sizes of the protein molecule. The matching bands between a pair of gel electrophoresis could be considered as similar kinds of proteins in the different isolates.

Gel electrophoresis permits high resolution analysis of extremely small samples of complex mixtures of proteins. It is also used for detecting mutant forms of hemoglobin and other proteins. Lehninger (1970) stated that difference of a single charged group per molecule is sufficient to distinguish the mutant from the normal form of protein. Gel electrophoresis was an accurate method to use for the study of interspecies relationship in the protein bands of the dematiaceous fungi.

Schechter (1972) devoted a great deal of effort to making interesting studies on the difference in soluble

proteins of *Candida* species by using gel electrophoresis, although his diagrammatic interpretation seems to create more complications than those of Shannon (1972), who utilized starch gel electrophoresis for studying of intracellular enzymes of *Polyporus*. The later diagrammatic interpretation was, therefore, adopted here for the human and animal pathogenic species of *Phialophora*, *Fonsecaea* and *Cladosporium* with the saprobic and plant pathogenic species of *Phialophora* and *Cladosporium*.

The number of matching bands by gel electrophoresis was low between pairs of five strains of *P. verrucosa* and six strains of *F. pedrosoi* (Table 14). The findings indicate a distinctive difference of interspecies relationship between *F. pedrosoi* and *P. verrucosa*.

The results of comparison of matching bands of soluble proteins by gel electrophoresis showed a greater relationship between *F. pedrosoi* and *F. compactum* than that of *F. compactum* and *P. verrucosa*. The number of matching bands was low between pairs of five strains of *F. pedrosoi* and six strains of *F. pedrosoi*. The findings indicate a distinctive difference of interspecies relationship between *F. pedrosoi* and *P. verrucosa*. This finding was comparable to the serological studies of Conant and Martin (1937). They found that the sera of rabbits immunized with *Fonsecaea* (*Hormodendrum*) *pedrosoi* and *F. compactum* not only had a high titer of complement fixing antibodies for their respective antigens but also had a high titer for each other, and reacted to appreciable degree only with the

homologous fungus. However, Martin *et al.* (1936) demonstrated a cross-antigenic relationship between strains of *P. verrucosa* and *Fonsecaea* (*Hormodendrum*) *pedrosoi*. Promising results on relationships were demonstrated by Buckley and Murray (1966), who utilized the agar gel-diffusion test to determine that there was more cross-reactivity between *F. pedrosoi* and *F. compactum* and very little with *P. verrucosa*. Their findings by the agar gel diffusion method support the results obtained by gel electrophoresis that there are similar kinds of protein molecules in the matching bands between *P. verrucosa* and *F. pedrosoi* which can serve as an antigenic protein for inducing antibodies. If the matching bands of soluble proteins contained antigen-determinant groups which can induce antibodies, they could show antigenic relationship between them.

In another study Gordon and Al-Doory (1965) utilized fluorescent antibody technique to compare relationships of the dematiaceous fungi. They noted that there was very little serological relationship between *P. verrucosa* and *F. compactum* or *Cladosporium* sp. The conjugates of two strains of *P. verrucosa* did not react with any species of *Fonsecaea*. They also found that *F. dermatitidis* conjugate did not react with any strain of *P. jeanselmei*, when using the fluorescent antibody technique. However, by using disc gel electrophoresis, there were three matching bands in one strain and two matching bands in another strain of *P. jeanselmei* with *F. dermatitidis*. One might conclude that

either the molecular size of these similar soluble proteins are not big enough to serve as antigens, or they lack capable antigenicity.

By using immunodiffusion (ID) and immunoelectrophoresis (IEP), Cooper *et al.* (1970) and Biguet *et al.* (1965) found that there were more common antigens between *P. verrucosa* and *C. carrionii* than between either these two species and *F. pedrosoi*. They concluded that *F. pedrosoi* should be retained in the genus *Fonsecaea*. They found a low number of different lines of identification between pairs of *C. carrionii* - *P. verrucosa* and *C. carrionii* - *F. pedrosoi*. On the other hand, *C. carrionii* showed a closer relationship to *P. verrucosa* and *F. pedrosoi* than to *P. verrucosa* and *F. pedrosoi*. These results were comparable to the results obtained by acrylamide gel electrophoresis. Moreover, there was a greater number of matching bands for *Cladosporium* human pathogens, *Cladosporium* saprobes and *Cladosporium* plant pathogen with *Fonsecaea* pathogenic species. These results might suggest a closer relationship between *Cladosporium* pathogens, *Cladosporium* saprobes and *Fonsecaea* sp. There are a considerable number of matching bands between *Cladosporium* saprobes and two species of *Fonsecaea*, including *F. pedrosoi* and *F. compactum* as well as *F. dermatitidis*. It would be appropriate to say that there was a close relationship between *Cladosporium* saprobes and the two species of *Fonsecaea* and *F. dermatitidis*. These results appear to be comparable to those reported by Gordon and

Al-Doory (1965), who applied fluorescent antibody technique to the study and comparison of three *Fonsecaea* sp., including *F. pedrosoi*, *F. compactum* and *F. dermatitidis* and *Cladosporium* saprobes. They found that all three *Fonsecaea* sp. showed a considerable reaction with the saprophytic *Cladosporium* sp. and *F. dermatitidis* was found to be most closely reactive with saprobe *Cladosporium*.

Again, a lower number of matching bands was seen when *Fonsecaea* pathogens were compared with *Phialophora* saprobes and pathogens. The findings indicate a closer relationship of *Fonsecaea* sp. with *Cladosporium* sp. than to *Fonsecaea* and *Phialophora* species.

It appears that comparable results are obtained from both techniques, namely, fluorescent antibody test and disc electrophoresis. Moreover, it appears that more detailed findings can be obtained from the latter technique than the former one.

Vaughan *et al.* (1965) compared protein bands of several *Brassica* species obtained by gel immunoelectrophoresis against those obtained by the relatively simple acrylamide gel electrophoresis. They concluded that the latter method was at least equal to the former in resolution of protein bands. In addition, Lester *et al.* (1963) found unique protein bands were in electrophoresis patterns from protein of *Baptisia* while no such difference could be detected by the serological techniques. It would be worthwhile to compare the results obtained from disc electrophoresis with the aforementioned methods.

In a comparison of the various methods utilized for the study of the relationships of the dematiaceous fungi, there is similar evidence from agar gel-diffusion, immunodiffusion (ID), immunoelectrophoresis (IEP), fluorescent antibody technique and by disc gel electrophoresis to indicate that *F. pedrosoi* and *F. verrucosa* are less closely related than *F. compactum* and *F. pedrosoi*.

2. Staining of enzymes and isoenzymes with their specific substrates

The diagrammatic interpretation of Shannon *et al.* (1973) was also adopted for comparison of enzymatic and isoenzymatic bands by adding the total number of similar bands together for pairs of organisms.

There was a distinctive difference of enzymatic bands noted between pathogenic isolates and saprobes. A high number of matching bands of enzymes and isoenzymes were shown among pathogens. This may reflect a closer relationship of physiological activity of these human pathogenic fungi.

Furthermore, it was found that there are a greater number of common bands in pathogenic intraspecies than in pathogenic interspecies. These results are comparable to those of the general soluble protein pattern, which offers some basis of support for the taxonomic classification of these species of fungi.

In order to find the relationship between *Cladosporium* sp., *Phialophora* sp. and *Fonsecaea* sp., the number of matching bands of specific enzyme and isoenzyme of these

species were then compared (Table 17), and it was found that there was similarly close relationship between the human and animal pathogenic *Cladosporium* sp. and *Fonsecaea* sp. and between *Cladosporium* sp. and *Phialophora* sp. However, there were less common matching bands between the plant pathogenic and saprobic *Cladosporium* sp. and *Phialophora* sp. The results are somewhat different from those obtained by general soluble protein staining, which shows close relationship between *Cladosporium* sp. and *Fonsecaea* sp. but less close between *Cladosporium* sp. and *Phialophora* sp. These findings with a higher number of matching bands for interspecies of *Fonsecaea* and a lower number between *Cladosporium* species seems to correspond with the work by Cooper (1970), who tried to show that *Fonsecaea* was a distinctive genus and was included neither in *Phialophora* nor in *Cladosporium*.

The results obtained from this study also show a close relationship between *F. dermatitidis* and *F. pedrosoi* when their matching enzymatic bands are compared. This would support the current classification as *Fonsecaea dermatitidis*.

However, Frank and Berry (1972) have indicated that disc acrylamide gel electrophoresis is applicable to species identification, although it seemed to be complicated within and even between genera. The patterns were never so close by this method as to contradict the traditional taxonomy; when based on morphology according to

Martin and Alexopoulos (1969), this is a statement that is also applicable to the dematiaceous fungi.

In conclusion, the determination of soluble proteins, enzymes and isoenzymes by disc electrophoresis is considered a sensitive technique for the study of the relationship of dematiaceous fungi. The difference of banding patterns of soluble proteins could be obtained from the same organism if they were grown under the different condition and harvested at different times according to Shannon (1973). Therefore, it is essential that standardized parameters be used to provide valid results with disc acrylamide gel electrophoresis.

The results from the use of disc acrylamide gel electrophoretic banding patterns of general soluble protein, enzymes and isoenzymes have contributed to support of the current concept of speciation of the dematiaceous fungi. The differentiation of pathogens from saprobes by determination of the presence or absence of beta-D-glucosidase and N-acetyl-beta-glucosaminidase may be a valuable method to include in the clinical laboratory procedures. More strains of the pathogens and saprophytes need to be examined for these extracellular enzyme activities to see if the difference continues to exist between the saprophytic species and the pathogenic species.

SUMMARY

1. All the saprophytic species of *Phialophora* showed extracellular N-acetyl-beta-glucosaminidase activity. No activity was detected in the human pathogenic species.

2. All of the saprophytic species of *Cladosporium* had extracellular N-acetyl-beta-glucosaminidase activity. No activity was detected in the human pathogenic species of *Cladosporium* and *Fonsecaea*.

3. All saprophytic species of *Phialophora* had extracellular beta-D-glucosidase activity, while the pathogenic species had none when assayed at 15 days.

4. All saprophytic species of *Cladosporium* except *C. carpophilum* have extracellular alpha-D-galactosidase, while no activity was detected in the pathogenic species of *Cladosporium*, *Fonsecaea*, and *Phialophora* except for one strain of *P. jeanselmei*.

5. All twenty-two pathogenic species and forty-five saprobes were capable of releasing extracellular peroxidase, when grown in liquid CPYG medium at 25°C, at 5-, 10- and 15-day intervals.

6. A few of the human pathogens and saprobe isolates showed extracellular adenosine triphosphatase activity. Some increase in ATPase activity in strains occurred by changing the nitrogen source from DL-isoleucine to L-proline.

7. No relationship could be found between adenosine triphosphatase and sclerotic cells in cultural studies as the sclerotic cells found in the tissue phase did not develop.

8. Comparative disc gel electrophoretic patterns of soluble proteins showed little significant relationship between human pathogens and saprobes.

9. There was significantly greater number of matching bands of soluble proteins of pathogenic intraspecies than for the interspecies.

10. Comparative gel electrophoretic patterns of soluble proteins showed closer relationship between *Fonsecaea* sp. and *Cladosporium* sp. than between *Fonsecaea* sp. and *Phialophora* sp.

11. *Fonsecaea compactum* showed the closest relationship with *F. pedrosoi* on the basis of soluble protein and enzyme gel electrophoretic patterns.

12. Enzyme and isoenzyme gel electrophoretic patterns showed correlative relationship with the general soluble protein patterns.

13. The evidences from the correlative relationships of matching bands indicated that *Fonsecaea dermatitidis* is closer to other species of *Fonsecaea* than to the former genus *Hormodendrum* (*Cladosporium*).

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