MAMMALIAN *SPOROTHRIX* INFECTIONS IN SOUTHERN AFRICA - RESEARCHES ON THEIR DEVELOPMENT, DYNAMICS AND CONTROL

by

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A TRIBUTE TO MY MENTOR -
THE LATE
PROFESSOR GEORGE HUDSON FINDLAY

"What is all knowledge too but recorded experience,
and a product of history; of which therefore,
reasoning and belief, no less than action and passion,
are essential materials"

Thomas Carlyle (1795 - 1881)

CONTENTS

CHAPTER I

HISTORICAL INTRODUCTION

The only causative organism in human and animal sporotrichosis is the dimorphic fungus, *Sporothrix schenckii*[1] Hektoen and Perkins (1900). *Sporothrix schenckii* is the type species of the form-genus *Sporothrix*, and is provisionally classified as Deuteromycotina (*Fungi Imperfecti*), Hyphomycetes, Sympodulosporae, Amerosporae (De Hoog, 1974). Some twelve other anamorphic *Sporothrix* species are known to be classified in this genus.[2]

The generic name *Sporotrichum* for the agent of sporotrichosis is inappropriate (Carmichael, 1962). The lectotype species, namely *Sporotrichum areum* Link, of the genus *Sporotrichum*, does not resemble *Sporothrix schenckii* at all. When Schenck in 1898 first described sporotrichosis from the John's Hopkins Hospital in Baltimore, USA, the consultant mycologist of the United States Department of Agriculture in Washington, Dr. E. F. Smith, tentatively assigned the organism isolated to the genus *Sporotrichum* (Schenck, 1898; Carmichael, 1962). Smith's conclusions on the isolate, as quoted by Schenck (1898), were: "It is conidial fructification only, and on this account it is impossible to give more than a guess as to its position in the natural system of classification. To determine this the perfect spore form would have to be obtained. It can only be put in form genus, in other words, into some artificial system of classification, until the natural one is known. It seems to me that it might be classified in either of these genera, according to the system given in Saccardo's *Sylloge Fungorum*,

[1] See list of synonyms - page 207

[2] See list of species - page 205

which is the commonly accepted standard of the artificial classification. (1) It is not unlike the *Botrytis bassiana*, the muscardine, or calicino disease of silk-worms, and might therefore be regarded as a *Botrytis*. Against this classification, however, it is the fact that the spore-bearing branches are not erect, which is a rather trifling distinction. It might also be classed as (2) *Sporotrichum*, but inasmuch as it becomes dusky when it is old it might also be classed as (3) *Trichosporium*. Saccardo separated out the dusky forms of Sporotrichum into a separate genus under this name. The more difference in the colour, as we know from cultures of many fungi, it is often a very trifling matter, the early stages of the fungus often being white and the later stages dusky or even brown. On this ground I think that his distinction is of no value, and I think that we may throw out the genus *Trichosporium* altogether. As regards the other two, my own judgement would be that it fits best into *Sporotrichum*. In his *Sylloge Fungorum* Saccardo describes more than one hundred species of *Sporotrichum*, but most of them are described very imperfectly, and I cannot identify this fungus as belonging to any of them."

Even before Schenck's (1898) first case description, Link in 1809 (Castleton and Rees, 1952) and Lutz and Splendore in 1907, described cases well comparable to sporotrichosis, but no fungus was isolated. Hektoen and Perkins (1900) reported the second proven case of sporotrichosis, from the United States (Chicago) and named the fungus isolated *Sporothrix schenckii*. Their report was entitled "Refractory subcutaneous abscesses caused by *Sporothrix schenckii*: A new pathogenic fungus". Since they mention Smith's identification in the genus *Sporotrichum*, it is clear they intended a new genus. In 1903, sporotrichosis was described for the first time in France by De Beurmann and Ramond. Matruchot and Ramond (1905) named this isolate *Sporotrichum beurmanni*. They examined Schenck's original isolate and regarded it distinct from the organism isolated by De Beurmann and Ramond due to the lack of pigment. *Sporotrichum beurmanni* produced brown to black colonies. Matruchot re-described Schenck's organism as "*Sporotrichum schencki*" in 1910, erroneously dropping the final "i". Until recently this spelling was commonly used.

In an excellent review on sporotrichosis, which is considered a classic in the medical literature, De Beurmann and Gougerot (1912) described ten more cases of the disease between 1906 and 1911, and by 1912 they tabulated 200 additional cases. Their detailed mycological descriptions made it clear that *Sporothrix schenckii* is synonymous with *Sporotrichum schenckii*, and is regarded as the same species as *Sporotrichum beurmanni* (Matruchot and Ramond, 1905). Decades following the naming of *Sporothrix schenckii* as such, this generic name has been ignored, and *Sporotrichum* preferred. A possible reason for this confusion is that both words Sporotrichum and Sporothrix, when translated literally, have the same obscure Latin meaning of "spore hair". The binomial is sometimes attributed to Matruchot (Conant *et al.*, 1971) and sometimes to Hektoen and Perkins (Rippon, 1988). Only in 1973 did Nicot and Mariat validate the genus *Sporothrix* and the type species *S. schenckii*. The South African variety, *Sporothrix schenckii* var. *luriei* differs from the type species only in forming unusual fungal cell forms *in vivo* (Ajello and Kaplan, 1969). Recent examination of this species indicate that it may warrant species or subspecies status (Staib and Blisse, 1974).

The relation of certain *Ceratocystis* species in the class ascomycetes to *Sporothrix schenckii* is well documented. Many *Ceratocystis* species synthesize rhamnomannans (Gorin *et al.*, 1977; Mendonça-Previato *et al.*, 1980; Travassos *et al.*, 1973; Travassos and Lloyd, 1980; Weijman and de Hoog, 1985), which are also the main surface antigens of *S. schenckii* but which are not found in the most other pathogenic fungi. The coexistence of some *Ceratocystis* species and *S. schenckii* in the same habitat as well as the close resemblance of some conidial forms of *Ceratocystis* species to that of the *Sporothrix* type, validates such a relationship (Mariat, 1975; Travassos and Lloyd, 1980). A number of *Sporothrix* species are known to have a teleomorphic state[3]. However, the true teleomorph of *S. schenckii* has not been established beyond any doubt. *Ceratocystis stenoceras* (Robak) C. Moreau has a conidial state distinguishable from that of *S. schenckii*. Hunt (1956) wrote a concise taxonomic

[3] See list of species - page 205

history of the genus *Ceratocystis*. Weijman and de Hoog suggested a subdivision of the genus *Ceratocystis* in 1985. Those species with an endogenous conidiogenesis or with *Chalara* states were considered *Ceratocystis* sensu strictu, whereas those species with exogenous conidiogenesis or with *Graphium*-like states were classified as *Ophiostoma*. Teleomorphic species related to *S. schenckii* belong to the genus *Ophiostoma*. Shadomy and Davis (1988) described *Chaetonium sp.* as another possible teleomorph for this fungus, but this has still to be validated.

Sporothrix schenckii gains entrance into the body by traumatic implantation of the conidia of this organism into the skin. The fungus is present in soil and on decaying vegetation. Handling sphagnum (Adam *et al.*, 1982; D'Alessio *et al.*, 1965; Grotte and Younger, 1981; McDonought *et al.*, 1970; Powell *et al.*, 1978) and peat moss (Figure 1), potting soil (Kenyon *et al.*, 1984), reeds, grasses and a variety of plants, for example, roses (Morgan, 1972), carnations, the barberry bush (Kenyon *et al.*, 1984), thorn trees, eucalyptus and wattle trees, and many others, have been associated with the development of sporotrichosis.

FIGURE 1. Prepared peat moss, pre-packed and stacked outside - Bucks County, Pensylvania, USA. Peat moss is often the source of Sporothrix schenckii infections.

Minor injuries most often mentioned include cuts or scratches from plants (Auld and Beardmore, 1979), brushing against tree bark or timber and injuries from thorn pricks or splinters. Even insect stings (Mathieu-Serra and Collins 1978), cat scratches (Dunstan *et al.*, 1986a, 1986b; Read and Sperling, 1982; Schippacasse *et al.*, 1985), rat bites (Fischman *et al.*, 1973), handling fish (Mayorga *et al.*, 1978), dogs (Goad and Goad, 1986; Londero *et al.*, 1964; Scott and Horn, 1987; Woodard, 1980) and parrot bites, hammer blows or injuries from metal tools (Gonzàlez-Ochoa, 1968; Lane and Garrison, 1970), building bricks (Sanders, 1971), etc., have been blamed for the onset of sporotrichosis. Sporotrichosis is therefore considered as much an occupational hazard in greenhouse workers, plantation nurseries, builders, farmers and miners (Brown *et al.*, 1947; Cox and Reller, 1979; England *et al.*, 1988a, 1988b, 1989; Ewing *et al.*, 1980; Foerster, 1926; James, 1965; Pijper and Pullinger, 1927; Powell *et al.*, 1978; Richard, 1979; Sanders, 1971), and even in orchid growers (Rippon, 1988) and rose gardeners (Keddes, 1964; Morgan, 1972), as it is a hazard of outdoor work or recreation (Harris, 1985; Vaisrub, 1971). *S. schenckii* seems to thrive in soil and moist, rich loam and compost which is often used in the aforementioned occupations (Feuerman *et al.*, 1976; Lavell and Mariat, 1983). Some insects, for example, mosquitoes, mites, ants, flies and even cockroaches, may also be vectors in contracting the disease as conidia have been isolated from them (Brühl and Fuchs, 1973; Kenyon *et al.*, 1984; Mayorga *et al.*, 1978; Rippon, 1988). Laboratory personnel handling infected material or experimental animals and veterinary surgeons handling infected animals, may also be at risk although transfer by these means have only seldom been described (Disalvo, 1987; Nusbaum *et al.*, 1983; Read and Sperling, 1982; Samorodin and Sina, 1984; Thompson and Kaplan, 1977). Auto-inoculation through contaminated dressings from suppurative lesions or direct transfer, for example, from a mother to her child, has also been reported (Smith, 1945).

Localized cutaneous-subcutaneous sporotrichosis is the most common form of infection and is characterized by ulcerative lesions, associated with involvement of regional lymphatics and lymphadenopathy, in which hard,

spherical nodules form along the lymphatic vessels. Less classic and less easily recognizable sporotrichotic disease includes disseminated cutaneous, subcutaneous and extracutaneous forms. Here primary skin lesions are often absent, but subcutaneous nodules may occur at random any where on the body. Disseminated, pulmonary, osseous and mucous involvement was first described by de Beurmann and Gougerot (1912). Over the last two to three decades, cases of sporotrichosis involving the lungs, joints, bones, muscles, the conjunctiva and cornea (Sun and Chu, 1966), the nose, mouth, pharynx, the brain and meninges (Parker, 1972; Scott *et al.*, 1987; Swartz, 1987), the liver and other internal organs, especially in a primary capacity, have increasing been reported (Agger *et al.*, 1978, 1985; Alvarez and Lòpez-Villegas, 1966; Altner and Turner, 1970; Anees *et al.*, 1986; Arango *et al.*, 1981; Atdjian *et al.*, 1980; Berson and Brandt, 1977; Brook *et al.*, 1977; Castro *et al.*, 1981; Chang *et al.*, 1984; Churchill, 1982; Dewan *et al.*, 1986; England and Hochholzer, 1985, 1987; Fields *et al.*, 1989; Goveia *et al.*, 1981; Govender *et al.*, 1989; Gullberg *et al.*, 1987; Haponik *et al.*, 1989; Klein *et al.*, 1966; Kumar *et al.*, 1984; Michelson, 1977; Mohr *et al.*, 1972; Naimark and Tiu, 1979; Schwartz, 1989; Velji *et al.*, 1988; Watts and Chandler, 1987; Winn, 1988). Disseminated disease is usually associated with patients with an underlying disease or compromised immunologic status, including alcoholism (Khan, 1975; Khan *et al.*, 1983; Rippon, 1988) and acquired immunodeficiency syndrome (AIDS) - (Shaw *et al.*, 1989).

Healthy patients in endemic areas may be resistant to sporotrichosis or may develop a localized cutaneous form. Exposure over long periods of time to small numbers of conidia, may gradually confer immunity (González-Ochoa, 1968; González-Ochoa and Ricoy, 1970). Self-limited sporotrichosis with spontaneous cure can also occur (Bargman, 1981, 1983; Iwatsu *et al.*, 1985; Pueringer *et al.*, 1986; Rippon, 1988).

Histopathologically the fungus is difficult to demonstrate. The organism is scarce and when present is seen as an asteroid body in the centre of a microabscess. Serial sections are usually needed (Simson *et al.*, 1947). By using a fluorescent-antibody technique (Kaplan and

Ivens, 1960; Kaplan and Gonzàlez-Ochoa, 1963) or the diastase method described by Sarosi *et al.* (1985), some greater success has been achieved in finding organisms in sections.

Since the earliest discoveries of sporotrichosis in America and France, it has been reported from all the continents, with the exception of the Arctic regions, i. e. the regions north of the fiftieth latitude above the equator and south of the fiftieth latitude below the equator. In Central and South America the disease has the highest incidence in Mexico, Brazil and Uruguay (Aceves Ortega, 1974; Conti-Díaz, 1989; Crevasse and Ellner, 1960; Gelders *et al.*, 1973; González-Ochoa, 1970; González-Ochoa, 1974; Kaplan *et al.*, 1982; Mackinnon *et al.*, 1969; Mayorga, 1970; Mayorga *et al.*, 1978; Sanders, 1971; Velasco Castegon and González-Ochoa, 1971). In the United States of America, the disease is most common in the Missouri valley and Mississippi basin. Here, as well as in France and Canada and other temperate countries, gardening and contact with soil is regarded as the main source of the infection (Kenyon *et al.*, 1984; Mathieu-Serra and Collins, 1978). Japan currently also ranks as a country with high incidence (Fukushiro, 1984; Ito *et al.*, 1970; Itoh *et al.*, 1986). The largest number of cases from any one area ever described, occurred in the South African Gold Mines between 1941 to 1945, where approximately 3000 cases of sporotrichosis were reported (Helm and Bernman, 1947; James, 1965; Simson *et al.*, 1947).

Reports on natural sporotrichotic infections in animals appeared in the early years of the twentieth century shortly after the first human cases came to note, the rat being the first animal in which such an infection was described from Brazil (Lutz and Splendore, 1907, 1908). Pigouet and Vigne (1912) found several rats with sporotrichosis during a survey of the plague in France. The animals suffered mainly from the gummatous or lymphatic skin forms of sporotrichosis. Lesions on the buccal mucosa of these rats were also present and were attributed to the animals feeding on contaminated vegetable material in nature. In France, Gougerot (1908) first described the disease in dogs. Subcutaneous tissue infections and dissemination to bones, perineum or the liver were

prevalent. Carougeau (1909) reported sporotrichosis in Madagascar in the horse and mule and named the organism *Sporothrix equi*, a species now regarded synonymous with *S. schenckii* (Carmicheal, 1962). Page *et al.* (1910) reported on the first cases in horses from the United States (as quoted by Watson, 1920). Thereafter several publications involving mainly domestic and farm animals have appeared (Blackford, 1984; Blood and Henderson, 1968; Davis and Worthington, 1964; Dion and Speckman, 1978; Fishburn and Kelley, 1967; Humphreys and Helmer, 1943; Jubb and Kennedy, 1970; Londero *et al.*, 1964; Morgan *et al.*, 1984; Read and Sperling, 1982; Saunders, 1948, 1955; Scott *et al.*, 1974; Smith *et al.*, 1972; Thorold, 1951). Wild boars (Smith, 1968), foxes, camels, chimpanzees (Saliba, *et al.*, 1968), armadillos (Kaplan *et al.*, 1982) and even dolphins can also be added to the list (Medway, 1980; Migaki *et al.*, 1978).

It is suspected that sporotrichosis in animals occurs worldwide, but it has only been reported from Brazil, Canada, Colombia, France, Greece, Indo-China, Italy, Madagascar, Syria, the United States and South Africa (Ainsworth and Austwick, 1973; Benham and Kesten, 1932; Jungerman and Schwartzman, 1972; Rippon, 1988; Theron, 1987). Moreover, animal models in experimental sporotrichosis for the better understanding of immunological responses to *S. schenckii* are now available (Barbee *et al.*, 1977; Hachisuka and Sasai, 1981; Lehmann, 1985).

S. schenckii is a dimorphic fungus, i. e. it grows in a mycelial phase at 25°C and a yeast phase at 37°C. Biological studies on *S. schenckii* indicate that apart from temperature dependent morphological changes, there are also several other factors which contribute to cellular changes in this organism in its dimorphism. At 25°C conidia arise directly from vegetative hyphae or are formed on conidiophores, while at 37°C abundant yeasts are formed. The carbohydrate composition of the cell walls of the conidia (mycelial phase), yeasts, yeast primordia (on the vegetative hyphae during transition from hyphal to yeasts phase), consist of mainly monorhamnosyl-rhamnomannans, while the hyphal cell walls contain dirhamnosyl-rhamnomannans. Human sera reacts primarily with monorhamnomannans (Lloyd and Travassos, 1975).

Additional to the biological studies, there have been reports on electron microscopy as well as scanning electron microscopy of *S. schenckii* (Boehm *et al.*, 1982; Font and Jakobiec, 1976; Garrison *et al.*, 1975, 1976, 1977, 1979, 1982; Garrison and Mirikitani, 1983; Lane *et al.*, 1969; Lane and Garrison, 1970; Lurie and Still, 1969; Maeda *et al.*, 1987, 1988).

De Beurmann and Ramond (1903) first demonstrated the efficacy of potassium iodide in the treatment of sporotrichosis. However, the mechanism of its action remains obscure. Several hypotheses and solutions have been proposed, but without a satisfactory explanation (Davis, 1919; Hiruma and Kagawa, 1987; Honbo *et al.*, 1985; Rex and Bennett, 1990; Stone, 1969; Urabe and Nagashima, 1969; Wada, 1966). Application of heat to sporotrichotic lesions as a curative measure has been used with varying success (Hiruma and Kagawa, 1983; Hiruma *et al.*, 1987). New antifungal drugs, including itraconazole and terbinafine, are under investigation and according to the results obtained so far seems to be promising (Baker *et al.*, 1989; Borelli, 1987; Lavalle *et al.*, 1987; Restrepto *et al.*, 1986; Viviani *et al.*, 1987, 1990; current study).

The history of sporotrichosis in South Africa dates back to 1914 when the first cases of the disease were discovered in the Transvaal gold mines (Pijper and Pullinger, 1927). It has since been established that the mines provided ideal conditions for the proliferation of *S. schenckii* underground, viz. a temperature of 25-27°C and a humidity of 92-100%. The sporotrichosis epidemics which followed can be attributed to the use of untreated timber, mainly eucalyptus and wattle props, which sustain fungal growth under the special mining conditions mentioned above (Figure 2). *S. schenckii* was found to be present on the outermost layers of the timber and mine labourers are constantly exposed to skin injuries allowing the fungus to enter the body. Much of the epidemiology of sporotrichosis became clear between 1927 and 1947 which explained why sporotrichosis was occurring in the mines (Brown *et al.*, 1947; Du Toit, 1942; James, 1965; Simson *et al.*, 1947).

FIGURE 2. Stacked mine timber props (here eucalyptus), used underground in South African gold mines. S. schenckii proliferates under underground conditions (25-27°C, 92-100% humidity), to become a high risk of infection for mine workers.

Dr. Davidine Pullinger who was involved in the original investigations into the epidemic of sporotrichosis on the mines was visited by Prof. G. H. Findlay and the author in Johannesburg. She disclosed how the original discovery of sporotrichosis in the Transvaal in 1914 came about and was subsequently published (Pijper and Pullinger, 1927). Findlay (1985) described her story as follows. Dr. Adrianus Pijper who was in private practice, had a microbiology laboratory at his home at that time and it was here where the isolation and identification of the first strain of *S. schenckii* took place. Mrs Kettle (neé Pam), an assistant editor of the Lancet at that time, had a brother, Mr Edgar Pam, who was the manager of the Modder East Gold Mine. His wife, Dr. Millicent Pam, was a fellow medical student of Dr. Davidine Pullinger at St. Mary's in London. The mine doctor, Dr. Orenstein, asked Dr. Pam to have a look at some troublesome skin cases on the mine. Dr. Pam showed the cases to Dr. Pullinger on a weekend visit who made the diagnosis of sporotrichosis. Dr. Pam sent specimens to Dr. Pijper to convince him that these were indeed cases of sporotrichosis. Drs. Pullinger and

Pijper cultured *Sporotrichum beurmanni*, as they named the isolate, from the specimens (Figure 3). The "Pam" in Figure 3 is thus explained. The personal contact of Mrs. Kettle at the Lancet ensured that the paper appeared in the 1927 issue (Figure 4).

Concerning the cases outside the mines, Pijper described sporotrichosis in a white man in the Transvaal in 1931 and in the same year with Goldberg, a case from Botswana area (Bechuanaland as it was known then) (Goldberg and Pijper, 1931). Pijper claimed that in the two decades that followed no other cases were seen (Findlay, 1970). Four reports on sporotrichosis appeared in the 1950's and 1960's (Gluckman, 1965; Lowenthal, 1959; Lurie, 1963b; Van Dijk and Der Kinderen, 1958).

Sporotrichosis is regularly seen in private practices in Pretoria (Findlay, 1970), cases principally coming from the Transvaal Highveld[4], while in the Orange Free State[5] they come mainly from the eastern part. Sporotrichosis is also known to occur in Natal and the Cape province, but geographical and epidemiological information is still lacking in these two provinces of South Africa. Likewise, in Zimbabwe, several cases have been reported (Ross and Gelfand, 1978), but their geographical origin as well as the epidemiology of the disease has not yet been delineated[6].

[4] Private dermatologists in Pretoria (1985-1991) - personal communication; see also Chapter III

[5] Prof F Scott (1984) - personal communication

[6] Dr V Robertson (1988) - personal communication

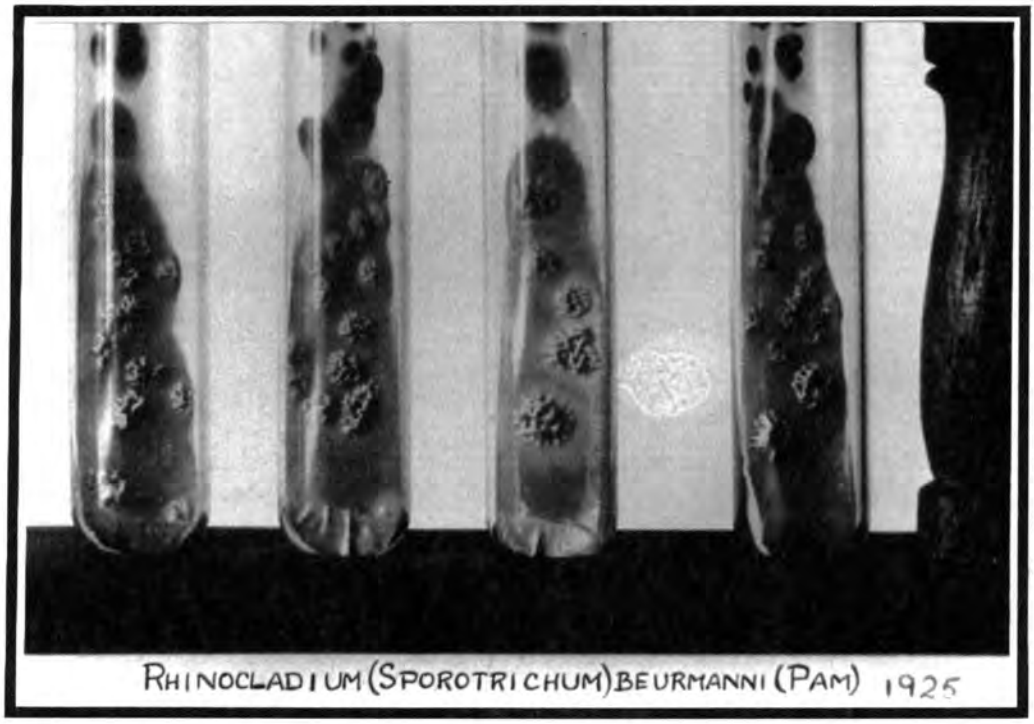
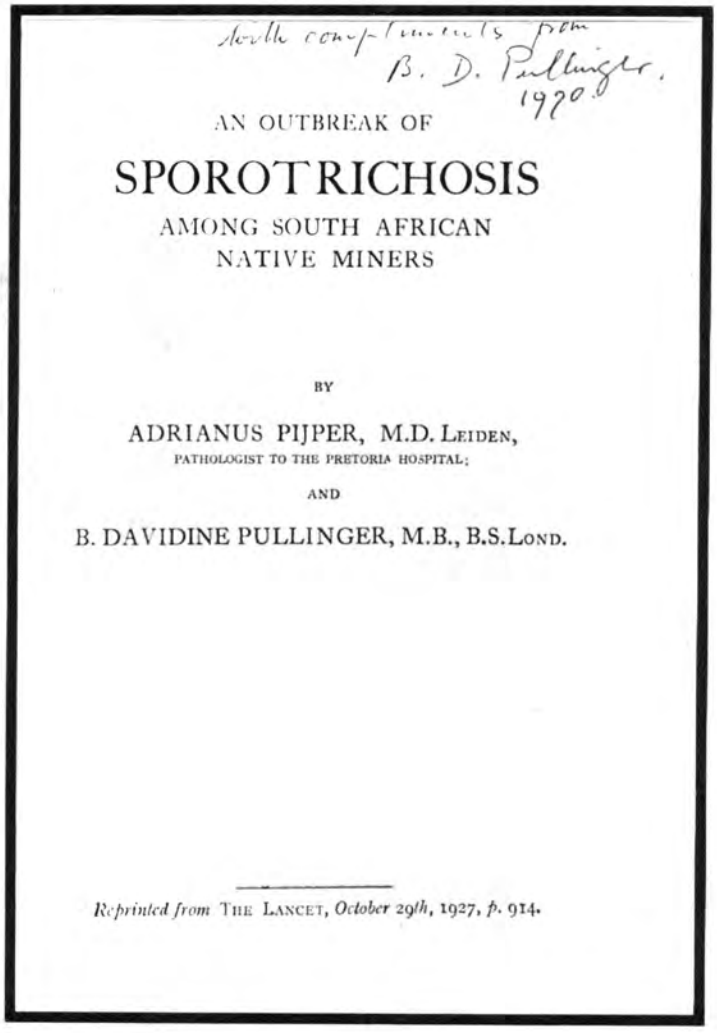


FIGURE 3. The first culture of Sporothrix schenckii isolated in the Transvaal in 1914 by Dr A Pijper and Dr Davidine Pullinger.



With compliments from
B. D. Pullinger.
1970.

AN OUTBREAK OF

SPOROTRICHOSIS

AMONG SOUTH AFRICAN
NATIVE MINERS

BY

ADRIANUS PIJPER, M.D. LEIDEN,
PATHOLOGIST TO THE PRETORIA HOSPITAL;

AND

B. DAVIDINE PULLINGER, M.B., B.S. LOND.

Reprinted from THE LANCET, *October 29th*, 1927, *p.* 914.

FIGURE 4.

The front page of the publication by Pijper and Pullinger, appearing in the Lancet in 1927. This publication was inscribed by Dr Davidine Pullinger in 1970 while visiting at her home.

CHAPTER II

MATERIALS AND METHODS

1 SOURCES OF MYCOLOGICAL MATERIAL AND PATIENTS

1.1 Human sporotrichosis

Patients who had attended the skin clinics at the H. F. Verwoerd Hospital (whites, coloureds and Asian) and the Kalafong Hospital (blacks) in Pretoria, contributed some material for the present study. Whites and a few black patients seen by dermatologists in private practice in Pretoria, most of whom were being considered for antimycotic drug trials, added to the total number of cases of sporotrichosis examined in the present study. Most patients were resident in the Transvaal, and represented all age groups. It must be emphasized that due to the nature of the clinical symptoms of sporotrichosis, all patients with the infection seek professional services. Cases are also regularly referred to dermatologists by general practitioners. Data from a large number of cases of sporotrichosis in South African gold mines, supplied by the South African Institute for Medical Research (SAIMR), Johannesburg, were analyzed for the purpose of this study.

Four groups of patients are therefore distinguished:

Group I H F Verwoerd and Kalafong hospitals - 88 cases, 1966 - 1990, Department of Dermatology, University of Pretoria - From mycological, histological and photographic records.

Group II Sporanox® (itraconazole)[7] trials - 61 cases, 1986 - 1990, Private Dermatologists, Pretoria.

Group III Lamisil® (terbinafine)[8] trials - five cases, 1989 - 1990, Private Dermatologists, Pretoria.

Group IV South African gold mines - 1580 cases, 1967 - 1989, SAIMR records, Johannesburg.

The specimens for mycological examination were collected at the discretion of the medical officers or dermatologists concerned, who confirmed the diagnosis of sporotrichosis and excluded other subcutaneous or deep mycoses. In most cases the material was collected by the dermatologist.

1.2 Animal sporotrichosis

Mycological specimens from animals were occasionally examined. Consultations came from the Onderstepoort Research Institute, where horses were the main concern. Small domestic animals, especially cats, seen either by private dermatologists or private veterinary surgeons, also contributed to a total number of eight cases of sporotrichosis over some 15 years. It must be emphasized that these were isolated cases of animal sporotrichosis examined in mycological consultations, and do not represent the true incidence of the disease in animals. In most cases, the dermatologist or veterinary surgeon collected the material himself.

[7] Janssen Pharmaceutica Pty (Ltd)

[8] Sandoz Products Pty (Ltd)

2 COLLECTION AND PROCESSING OF THE MATERIAL

2.1 Human sporotrichosis

For mycological examination, crusts, skin scrapings and curetted material from active skin lesions of sporotrichosis were placed onto a clean microscope slide by means of a sterile scalpel blade (No. 15 or 21) or forceps. This slide was covered with a second one and the sample was wrapped in paper for transport purposes. Larger crusts or curettings were placed in dry sterile containers for transport. In some cases skin biopsies were taken by dermatologists following standard clinical procedures. For mycology, such specimens were also placed aseptically in dry sterile containers for transport. Biopsy material for histopathological examination was fixed in 10% formaldehyde in appropriate containers, and sent to be processed[9]. Pus swabs were taken from suppurating lesions. Specimens were processed for mycological examination, within hours or one to two days after being taken. All the material was processed in a Type II laminar flow cabinet, following the standard procedures.

2.1.1 Direct microscopy

Azo black clearing and staining solution was prepared according the method of Burke and Jones (1984) by first dissolving 100mg Azo black (Chorazol black E[10]) in 10cm^3 dimethyl sulfoxide (DMSO) in a small glass beaker. A second solution containing 5g of potassium hydroxide in 65cm^3 of distilled water was prepared and mixed with the first solution. Several rinses of the glass beaker with the dye solution were necessary to remove all the dye effectively. The original recipe of Burke and Jones (1984) was modified by adding

[9] Biopsy material from patient Group I - H F Verwoerd and Kalafong hospitals was processed by the Department of Anatomical Pathology of the Institute for Pathology, University of Pretoria. Biopsy material from Groups II & III was processed by private pathologists.

[10] Gurr

25cm^3 glycerol to 65cm^3 distilled water instead of using 90cm^3 of water to dissolve the potassium hydroxide. The stain could be used directly without filtration. It was kept in a brown glass bottle in a dark cupboard due to its sensitivity to light. With these precautions it remained stable for approximately three to four months.

Azo black stains selective for chitin, whereby fungal elements are detected (Burke and Jones, 1984). Due to this selectivity, very few artifacts were noted and they could easily be distinguished from fungal material.

Biopsies, crusts or curetted material were macerated in a sterile petri dish. The larger quantity of the material was reserved for culture. One to two drops of the Azo black stain was placed on a microscope slide, to which the material was transferred by means of a pre-sterilized needle or loop and mixed well to separate the pieces and to spread them in the thinnest possible layer. A coverslip was placed on the preparation, which was gently heated over a flame for 30 seconds, and then carefully pressed out. Overheating or boiling was avoided to prevent damage or destruction of the tissue cells and the fungus. It is very difficult to detect the yeasts of sporotrichosis by this method, which had however to be employed to exclude the presence of other subcutaneous or deep mycoses in some cases.

The Azo black mixture acted upon the tissue as follows: Proteinaceous material was digested, clarified and pigments were bleached and the thick tissue layers separated and dispersed into single cell layers by both KOH and DMSO. The fungal elements and especially the chitinacious fungal cell walls, were selectively stained a light green. Modifying this stain to contain 25% glycerol had a dual purpose. It not only prevented the preparation from drying out, where it was necessary to digest thickly layered material overnight, but it also prevented complete digestion of tissue and fungal material by the DMSO. The preparation could also

be kept for a few days for further examination or micro-photography when necessary, by placing it in a sterile petri dish containing a damp sponge and rested upon a U-shaped glass rod.

Pus swabs were inoculated onto appropriate media (see paragraph 2.1.2 below), after which approximately 2cm^3 of 0,85% sterile saline was added to the tube, enough to cover the cotton wool bud containing the specimen, and incubated at 37°C overnight. Any material sticking to the side of the tube, was first washed off by rotating the swab. As a rule it was difficult to demonstrate *S. schenckii* in direct preparations, but this method ensured proliferation of the yeast phase and a larger number of organisms could be demonstrated by the conventional microbiological Gram stain.

2.1.2 Cultural techniques and media used

Sabouraud-dextrose (4%) agar (SDA)[11], pH 6,5 - 6,8, containing 0,05g/cm^3 chloramphenicol[12] and 0,4g/cm^3 actidione[13], in 9cm diameter disposable petri dishes, was routinely used for the primary isolation of *S. schenckii*. SDA slopes without antibiotics in MacCartney bottles, either in 7cm^3 or 30cm^3 volume sizes, were used for the maintenance of stock cultures. Although *S. schenckii* grows relatively fast, i. e. within four to six days, and is able to survive in the presence of both actidione and chloramphenicol, these additives assist considerably in the successful isolation of this fungus. Chloramphenicol controls bacterial growth, while the actidione, first used by Georg *et al.* (1954) for this purpose, prevents the growth of most contaminant saprophytic fungi.

[11] Difco, Detroit Michigan

[12] Park Davis

[13] Cycloheximide - BDH Chemicals and Upjohn

Pus swabs were streaked on SDA plates or slopes following conventional microbiological methods. Biopsies, curetted material or crusts from sporotrichotic lesions were inoculated in the same manner, after being aseptically cut into small pieces.

When the presence of a fungus other than *S. schenckii* needed to be excluded or confirmed, for example, chromoblastomycosis or blastomycosis, it was necessary to use SDA containing only chloramphenicol, because some of these fungi are sensitive to actidione. SDA containing both drugs and SDA containing only chloramphenicol were therefore used routinely.

2.2 Material from animal cases of sporotrichosis

Material for mycological examination, namely biopsies, curetted tissue, crusts from skin lesions, skin scrapings or pus swabs from suppurating lesions, was collected, transported and prepared in the same manner as for the human cases.

3 CULTURAL CHARACTERISTICS OF *S. SCHENCKII* AT 25°C

3.1 Techniques and media used

The media described in paragraph 2.1.2 (p. 17), were routinely used for the isolation of *S. schenckii* at 25°C and to determine its macroscopic cultural morphology. Cultures were examined every two days for the presence of growth. Microscopic preparations were attempted when the colonies reached a diameter of 0,5mm or greater.

Once *S. schenckii* was isolated, cultures were grown on SDA without antibiotics or actidione, at 25°C for four weeks (two sets per strain), morphologically verified, and stored at 4°C with the original isolate. Satisfactory isolates were also inoculated into

sterilized distilled water in $10cm^3$ Bijou bottles, incubated at 25°C until ample growth was present, and stored at 4°C. These cultures were checked and subcultured every 6 to 12 months. Desiccated cultures will be kept at the Medical Research Council for reference purposes.

3.2 Microscopic cultural morphology of *S. schenckii* at 25°C

Lactophenol Cotton Blue (LCB)[14] mounting medium was used to make microscopic preparations of fungus material from positive cultures. Small pieces of the colony were removed using a sterilized hook-shaped needle, placed in a drop of 90% ethanol on a microscope slide, and teased out. A drop of LCB was added, covered with a coverslip, pressed out gently and examined microscopically. The preparation could be kept indefinitely by sealing the edges of the coverslip with nail varnish.

Conventional fungal block cultures were made to identify the isolates when necessary (see also paragraph 5.1, p. 20).

4 MYCELIAL TO YEAST CONVERSION OF *S. SCHENCKII* AT 37°C

4.1 Macroscopic cultural morphology of *S. schenckii* at 37°C

Conversion from the mycelium phase of *S. schenckii* grown at 25°C, to the yeast phase, was achieved by spreading small pieces of the mycelial thallus and conidia on standard microbiological blood agar and incubating the cultures at 37°C for 7 to 14 days. Brain Heart Infusion (BHI)[15] broth to which 2% agar-agar was added, could be used equally well to achieve conversion to the yeast phase at 37°C. In

[14] Gurr

[15] Merck Chemicals

some cases a second transfer, on either of the media mentioned, was necessary to achieve full conversion to the yeast phase.

4.2 Microscopic cultural morphology of *S. schenckii* at 37°C

Microscopic preparations from both blood agar and BHI broth cultures were prepared in the same manner. Colonies were scraped from the agar by using a sterilized loop, placed in a drop of 90% ethanol on a microscope slide, and spread out. A drop of LCB was added, covered with a coverslip, pressed out gently and examined microscopically. The preparation could be kept indefinitely by sealing the edges of the coverslip with nail varnish.

Gram stains from cultures grown at 37°C were made following conventional microbiological methods.

5 CONIDIUM STUDIES IN *S. SCHENCKII*

5.1 Conidium ontogeny and colony formation

Two sets of block cultures were made by cutting 1cm agar blocks from malt agar plates and placing them on sterilized microscope slides. These agar blocks were inoculated at the eight corners with a sterile needle carrying conidia from pigmented areas of human isolates of *S. schenckii*. A sterile coverslip was placed on each block. The cultures were kept moist with sterile water in a sterile chamber, incubated at 25°C and examined microscopically on days one to nine and on day 21. One set was employed for light microscopy with phase contrast and the other set for the study of the fungal nuclei by using a fluorescent DNA stain (see paragraph 8, p. 26).

5.2 Conidial pigment

5.2.1 Histochemical staining

Pigmented colonies of *S. schenckii* ranging 2-3cm in diameter on SDA plates were sliced into 2-3mm strips, fixed in 10% formalin, sectioned and treated histochemically for melanin with Masson Fontana and Schmorl's stains, with and without bleaching with H_2O_2, following standard methods. Simultaneous controls were processed by using unpigmented colonies of the same organism.

5.2.2 Conidial pigment production

To induce or to initiate pigment production in the conidia of unpigmented human and soil isolates of *S. schenckii*, the following media were used. The number of strains inoculated upon them are indicated in brackets. SDA (50); malt agar (50); water agar (30); neopeptone agar (20); neopeptone broth (20); distilled water (50); wattle agar (20); eucalyptus agar (20); wattle "broth" and agar (10 each); eucalyptus "broth" and agar (10 each); Fries' minimal medium ± sucrose ± agar (10 for each combination). Large rectangular wedge-shaped slopes were made in 250cm^3 flat medicine bottles, containing 60-80cm^3 of the various media. These were inoculated with standardized conidium suspensions in sterile water containing a trace of Tween 80 (2-3drops per 500cm^3); 2cm^3 for a 250cm^3 flask and 0,5cm^3 for a 30cm^3 MacCartney bottle. Cultures were incubated at 25°C and observed for five to six months.

The wattle and eucalyptus broths were prepared by cutting dry twigs into 2-4cm pieces, weighing out 40g quantities and placing them into 100cm^3 distilled water in flat medicine bottles with large mouths. They were then autoclaved for 30 minutes at 120°C. In order to prepare the wattle and eucalyptus agars, 2% agar-agar was added to the decanted extracts, steamed to dissolve, and quantities of 20cm^3

distributed into MacCartney bottles. The bottles were autoclaved for 15 minutes at 120°C and sloped.

5.3 Conidial volume

For this study Archer's method of using the conidia of *Absidia corymbifera* to calibrate the Coulter ZBI counter channelyser for volume measurement of human platelets (Archer *et al.*, 1977), was applied to measure the size by volume of the conidia of human and soil strains of *S. schenckii*. As standards two strains of *Absidia corymbifera* were obtained - one from the Commonwealth Mycological Institute (CMI 123261) and the other from the Centraalbureau voor Schimmelculturen (CBS 429.75), the Netherlands. The freeze-dried cultures were suspended according to instruction and grown on SDA for 14 days. The conidial volumes were measured directly on the Coulter "S plus" counter, calibrated with a standard "4C plus" control suspension. Calculations could be made directly from the histograms produced on a coupled plotter. Thereafter Archer's method was applied to various strains of *S. schenckii* except that conidial suspensions were not made up for later use as calibration standards. For easier referral, *S. schenckii* cultures were grouped according to the colour of the colony, namely unpigmented, buff, black/unpigmented and black. Seven unpigmented strains and one each of the other three colours were selected. Conidia were harvested between 14 and 21 days, and after 54 days in case of the buff-coloured culture. One-litre Roux flasks containing 250cm^3 SDA, and 250cm^3 sized flat medicine bottles, containing 80cm^3 SDA, were found to have large agar surfaces when sloped to allow for ample fungus growth, thus yielding high enough concentrations of *S. schenckii* conidia for volume determinations. In some instances, as indicated by the plotter results, it was necessary to dilute the suspensions of conidia.

5.4 Pigmentation in single-conidium cultures

Two stock cultures, one on SDA producing hyaline conidia, and the other on malt agar, producing pigmented conidia, were used to initiate single-conidium cultures on SDA. The germinating conidia were individually isolated under the light microscope from streaked inocula on SDA slide cultures and incubated at 25°C. These were carefully excised and inoculated into petri dishes containing SDA. Successful transfer took place best on the second or third day. These subcultures were observed for four to eight weeks.

5.5 Conidium viability

The conidium viability of *S. schenckii* was tested by storing 60 stock cultures of *S. schenckii* without subculturing for four years in 30cm^3 MacCartney bottles containing SDA without antibiotics or actidione. Seventeen strains were unpigmented, of which seven showed spotted grey to brown pigmentation and the other strains produced predominantly buff to brown or black pigment. Conidium viability was tested at random by subculturing various coloured parts of these cultures into SDA with and without actidione and chloramphenicol. Brain heart infusion broth was employed to hydrate any naturally dried out cultures. All the subcultures were incubated at 25°C for at least four weeks.

6 GROWTH OF *S. SCHENCKII* ON WOOD

6.1 Growth on wattle and eucalyptus

Eight unpigmented strains of *S. schenckii* from SDA, six human and two soil isolates, were inoculated on wood shavings, leaves, bark, green twigs and dead sticks of wattle and eucalyptus. Before

inoculation the plant material had been autoclaved at 120°C for 30 minutes, and placed in sterile 500cm^3 Erlenmeyer flasks or in large petri dishes lined with moist blotting paper. The inoculated cultures were incubated at 25°C for five to six months.

6.2 Growth on indigenous and other exotic woods

Standardized unpigmented conidial suspensions from five human isolates of *S. schenckii* were used to inoculate 20 indigenous and 17 exotic wood species. Wood blocks of 5mm X 5mm X 10mm were cut from sapwood specimens obtained from the National Institute for Timber Research (CSIR, Pretoria) and sterilized in the same manner as described for wood flours (see paragraph 7 below). Four sterilized wood blocks of each wood type were placed on 50cm^3 unsloped agar-agar cultures prepared in 100cm^3 Schott bottles. The inoculated cultures were incubated at 25°C, and observed for five to six months.

7 GROWTH OF *S. SCHENCKII* ON VARIOUS WOOD MEALS, WOOD FLOURS AND CELLULOSE

For the purpose of this study, wood meals are defined as coarsely or finely grated wood, while wood flours are powdered wood substances.

A 1000cm^3 phosphate buffer solution, pH 5,7, was made up by mixing 80cm^3 of Na_2HPO_4 (28,39g/1000cm^3) 920cm^3 of NaH_2PO_4 (29,6g/1000cm^3), to which 20g of agar-agar was added. After steaming to dissolve the agar, 20cm^3 agar quantities were dispersed in MacCartney bottles and autoclaved for 15 minutes at 120°C.

Six different wood meals and wood flours were obtained from the National Timber Research Institute (CSIR, Pretoria). Wood meals

included course and fine *Pinus pinaster* and *Eucalyptus saligna* and the wood flours, purified extracted Bagasse lignin (Ref. E7/1-1 (a)) and Bagasse hemi-cellulose (Ref. G6/91). Cellulose sheets were coarsely grated. One gram quantities were weighed out into 7cm diameter disposable petri dishes or $30cm^3$ MacCartney bottles. The petri dishes were placed in surgical theatre paper bags, closed and taped, and exposed to ethylene oxide without heat. The ethylene oxide was withdrawn by opening the bags and exposing them to UV light for 24 hours in an extraction fume cupboard. The openings of the paper bags were closed aseptically and the samples were kept until needed. All samples were used within 2-3 weeks of preparation. Six samples of each were prepared. Wood meals were not autoclaved, as according to Pizzi (1987)[16], this may break down the composition of their larger carbohydrate molecules. Due to the stability of cellulose, hemi-cellulose and lignin, these substances were autoclaved in $30cm^3$ MacCartney bottles for 20 minutes at 120°C.

Two methods for making up wood flour agars were employed. First, the sterilized wood flours were sprinkled aseptically onto the set of buffer agar plates which had been poured from the already made-up MacCartney bottles. Secondly, the $20cm^3$ quantities of sterilized buffered agar were melted, cooled and poured evenly over the wood flours prepared in the petri dishes, stirred with a sterile glass-rod to disperse the wood flours evenly and allowed to set. The 2% agar concentration proved to be of an ideal thickness to keep the wood flours in suspension until the agars had set.

Six pathogenic strains of *S. schenckii* were grown on SDA for four to six weeks, after which 0,5cm diameter punches were taken approximately 1cm from the outer edge of the colony. After the SDA agar was removed from the punches, they were inoculated onto the wood flours. Due the strong matting ability of *S. schenckii* colonies the agar portion of the punch could easily be removed. All

[16] Personal communication

plates were incubated at 25°C and observed every two to four days for eight weeks.

8 FLUORESCENT DNA-STAINING STUDIES OF *S. SCHENCKII*

This was performed on block cultures made in both SDA and malt agar (see paragraph 5.1, p. 20). The fungal growths, on either the microscope slides or coverslips, were fixed in absolute alcohol for five minutes, and stained for ten minutes in the dark with a solution 1$\mu g/cm^3$ bisbenzimide (H33258, Riedel de Haen) in 0,05 M phosphate buffer (pH 7,4) and 2M sodium chloride (Latt and Stetten, 1976; Lemke *et al.*, 1978). This fluorescent substance binds quantitatively to DNA (Labarca and Paugen, 1980), and allows ready examination by fluorescent microscopy. Background staining is slight, but sufficient to be topographically useful. Fluorescent intensity was graded subjectively, in comparison to nuclear size.

9 SCANNING ELECTRON MICROSCOPY OF *S. SCHENCKII*

The S450 Hitachi scanning electron microscope was used to study the morphology of cultures of *S.schenckii* grown on plant material and in block cultures.

9.1 Growth of *S. schenckii* on wattle pods, twigs and leaves

Wattle pods, twigs and leaves were sterilized with ethylene oxide as described for the wood meals and wood flours in paragraph 7 (p. 24), inoculated with ten pathogenic and two soil isolates of *S. schenckii* and kept moist at 25°C for five months. Fragments of plant material overgrown with fungus were fixed in cacodylate-buffered glutaraldehyde for two hours, rinsed in pure buffer and

post-fixed in 2% osmium tetroxide. After further buffer rinsing, dehydration in ascending alcohol concentrations, critical point drying (Hitachi HCP-2) and attachment to brass stubs, the samples were treated with carbon and gold evaporation before viewing.

9.2 Growth of *S. schenckii* on eucalyptus wood chips

Selected eucalyptus wood chips overgrown with fungus were placed in 12cm diameter glass petri dishes and prepared by the same method as described in paragraph 9.1 (see p. 26).

9.3 Block cultures of *S. schenckii* on malt agar

Malt agar blocks, punched out with a 0,5cm diameter sterile glass tube, were mounted between 12mm diameter sterile coverslips, inoculated with conidial suspensions of *S. schenckii* and kept moist at 25°C for three to ten days. The coverslips with fungal growth were prepared in the same manner as described for the plant material in paragraph 9.1 (see p. 26).

10 ELECTRON MICROSCOPY OF *S. SCHENCKII*

10.1 Conidium ultrastructure

The structure of the classical ovate conidia of *S. schenckii* compared to its triangular conidia was of particular interest in this study.

A case of disseminated sporotrichosis of skin and bone provided the strain of *S. schenckii* used in the electron microscopic study of the conidia of this fungus. An early isolate from subcutaneous abscesses yielded a filamentous phase on SDA at 25°C, with the

classical small oval conidia, while nine months later an isolate from a granulomatous cyst in the patella from the same patient was intensely pigmented with large oval and triangular conidia (see Chapter III, paragraph 1.7.3, p. 52).

The original isolate as well as the pigmented strain, were grown for 30 days on SDA containing actidione and chloramphenicol, after which the conidia were harvested, suspended and fixed in 5% phosphate-buffered glutaraldehyde at pH 7,3 for eight hours and spun down. The pellet that formed was consolidated with a drop of agar, washed overnight in phosphate buffer, and post fixed in 1% phosphate-buffered osmic acid. Following ethyl alcohol dehydration, with propylene oxide transition to imbedding into Spurr's epoxy resin, sections were cut on a L.K.B. microtome with glass knives, stained with uranyl and lead citrate and examined under a Phillips 301 electron microscope.

10.2 Ultrastructure of *S. schenckii* yeasts in tissue

Conventional methods for electron microscopy study of tissue sections were employed. Biopsy material from a patient described in Chapter III, paragraph 1.7.3 (p. 52), was obtained for the study.

11 *S. SCHENCKII* IN SOIL, COMPOST OR VEGETATION SAMPLES

Samples of suspect soil, compost and vegetation were collected as soon as possible after the diagnosis of sporotrichosis in a particular patient. Collections were made from the location or site where injuries were sustained. However, only where patients remembered an injury associated with soil or plant matter, could sampling be attempted. In the case of patients living in remote areas, the method of collection was explained, and requested to bring the samples with on a follow-up visit to the dermatologist.

In one case the patient collected soil from his wrecked car after he developed sporotrichosis due to an injury sustained during the accident.

It was found preferable to collect dry soil as too much moisture in an enclosed container could instigate growth, proliferation or conidium production of soil fungi other than *Sporothrix schenckii*. The overgrowth by fast growing fungi, often producing large numbers of conidia, could greatly minimize the chances of recovering the slower growing *S. schenckii*. Only the top one to two centimeters of dry soil was collected and placed in a $100cm^3$ or $200cm^3$ sized container with a screw cap. The method of collecting soil was explained to the patients who placed the soil samples into clean plastic bags where it had not been possible to supply them with containers. In a few cases, instructions were not complied with, and large bags of soil or plants from 10 to 20 different locations were collected. Only selected samples were tested in these cases.

Dry plant material, including dead branches, cut into small easily transportable pieces, as well as suspicious pieces of bark or other plant material, i. e. pieces with grey or black marks on them, were placed in plastic bags of a size suitable for transport. These marks were excised to a depth of 2 - 3mm from large pieces of wood such as fencing poles or branches. Care was taken to ensure that as little as possible moisture developed in the bags by only partially closing them. In cases where compost was collected the same precautions were taken.

11.1 Direct microscopy

Direct microscopy was not performed on soil samples. Vegetation samples, pieces of bark and excised pieces of wood, were examined under a stereo-microscope to determine whether the marks, which often had irregular borders, were fungal growth. Direct

preparations of the material were also attempted by scraping off the marks and preparing mounts in Lactophenol colourless stain for microscopy.

11.2 Cultural techniques and media used

Samples were treated by a method similar, to those described by Feuerman *et al*. (1976) and Kenyon *et al*. (1984). Two quantities of 25g soil for each sample were placed in sterile 50cm^3 test tubes. Four times the volume of 0,85% saline containing 2μg/cm^3 streptomycin and 5μg/cm^3 penicillin was added to the samples, after which they were shaken vigorously for five to ten minutes. The suspensions were left to settle for one hour. Allocates of 0,5cm^3 of the supernatant were spread evenly onto Malt and SDA agars, containing chloramphenicol and actidione in the same quantities described before. Four plates of each agar type per sample were set up. Plates were incubated at 25°C and examined daily for ten days.

11.3 Pathogenicity of soil isolates

Comparative pathogenicity was assessed by intratesticular injection in rats of standardized conidial suspension from five soil isolates. As "controls" the pathogenic isolates from the corresponding cases were also inoculated into rat testes. Histological examination of rat testes were made from the parallel inocula at 5, 15 and 27 days. From 40 sacrificed rats, two per isolate, some 100 sections were prepared and stained with the conventional Haematoxylin and Eosin (H&E) and Periodic Acid Schiff (PAS) stains by the department of Anatomical Pathology, Institute for Pathology, University of Pretoria.

12 TREATMENT OF SPOROTRICHOSIS

Mycological consultations for patients in treatment trials were done in close collaboration the specialist concerned, and formed an integral part of the trials. Experience with potassium iodide, itraconazole (Sporanox) and terbinafine (Lamisil) will be described and discussed fully in Chapters III and IV, respectively. For numbers of the respective patients see paragraph 1.1 (pp. 13-14) of this Chapter.

CHAPTER III

RESULTS

1 HUMAN SPOROTRICHOSIS IN THE TRANSVAAL

1.1 Frequency and clinical incidence

The four groups of patients with sporotrichosis reported in this study include: Group I - H F Verwoerd and Kalafong hospitals (88 cases, 1966 - 1990); Group II - itraconazole trials (61 cases, 1986 - 1990); Group III - terbinafine trials (five cases, 1989 - 1990); Group IV - South African gold mines (1580 cases, 1967 - 1989, SAIMR records, Johannesburg). A total of 154 cases of sporotrichosis in the general population can therefore be considered for analyses in this study by adding the patients included in Groups I, II & III together.

Group IV represents by far the largest group and are all patients who contracted sporotrichosis in South African gold mines, mainly in the Witwatersrand area. During the 22 year period under consideration, the mines with the highest incidences are: Crown Mines (350 cases); Leslie Gold Mines (280 cases); Gold Fields West (200 cases); Durban Deep (178 cases); West Driefontein (30 cases); Winkelhaak (25 cases). The remaining mines contributed 20 cases or less and include, Bracken Mines, Discoverers, Carltonville, Luipaardsvlei, Springbok Collieries, Westonaria Gold Fields, Venterspost, Coronation, Duiwelskloof and several other smaller mines.

1.2 Age, sex and race distribution

Groups I to III (Hospital and trial consultations):

In the total number of patients in groups I, II and III, males predominate in a ratio of 3-4:1. Age is fairly evenly distributed between patients from less than 10 years of age to patients over 90 years old. The age distribution is shown for the H F Verwoerd and Kalafong patients (group I) in figure 5 and for the trial patients (groups II and III) in figure 6. The age and sex distribution in all three groups are combined in figure 7 which indicate that the incidence of sporotrichosis is lower in some age groups, i. e. 41-50 and 71-91 years.

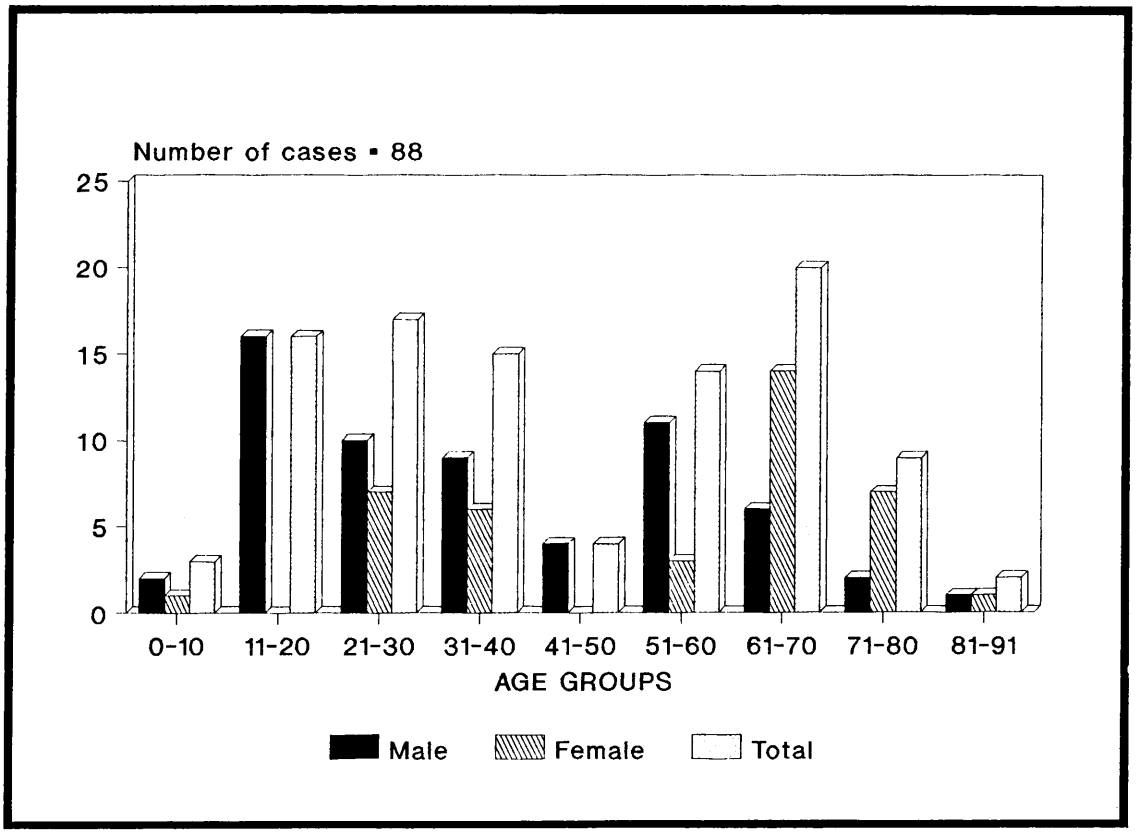


FIGURE 5. Group I - Sporotrichosis. H F Verwoerd & Kalafong hospitals - Age and sex distribution of patients

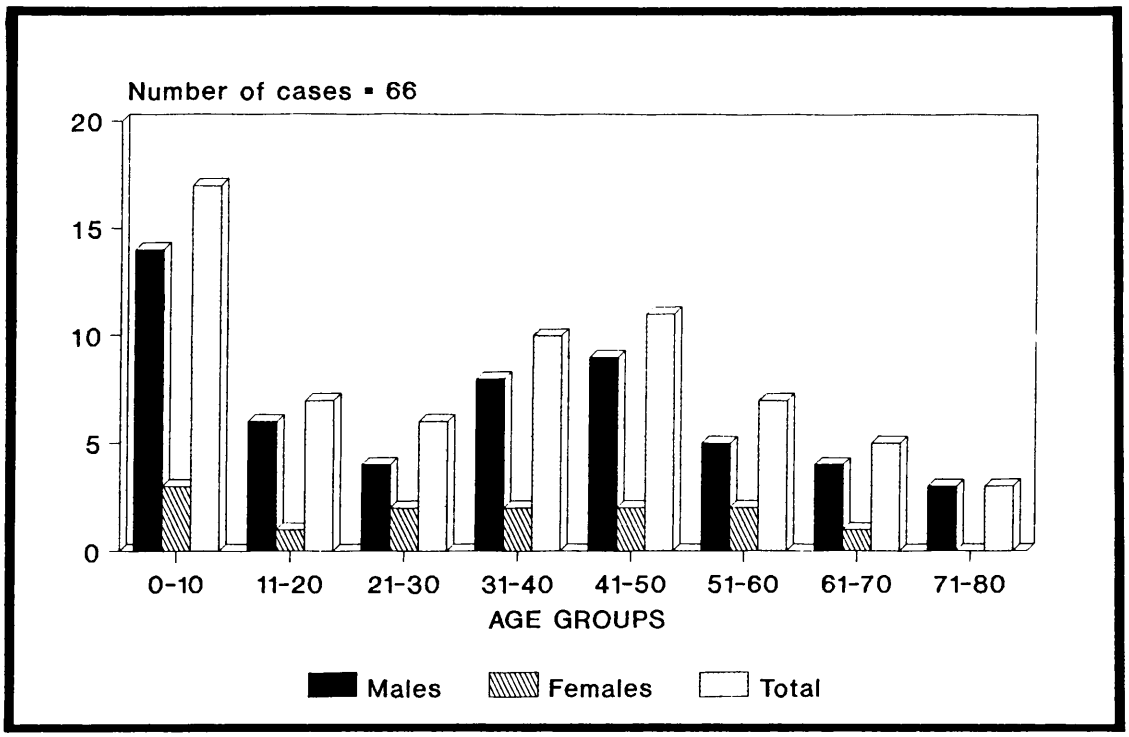


FIGURE 6. Groups II & III - Sporotrichosis. Itraconazole and terbinafine trials - Age and sex distribution of patients.

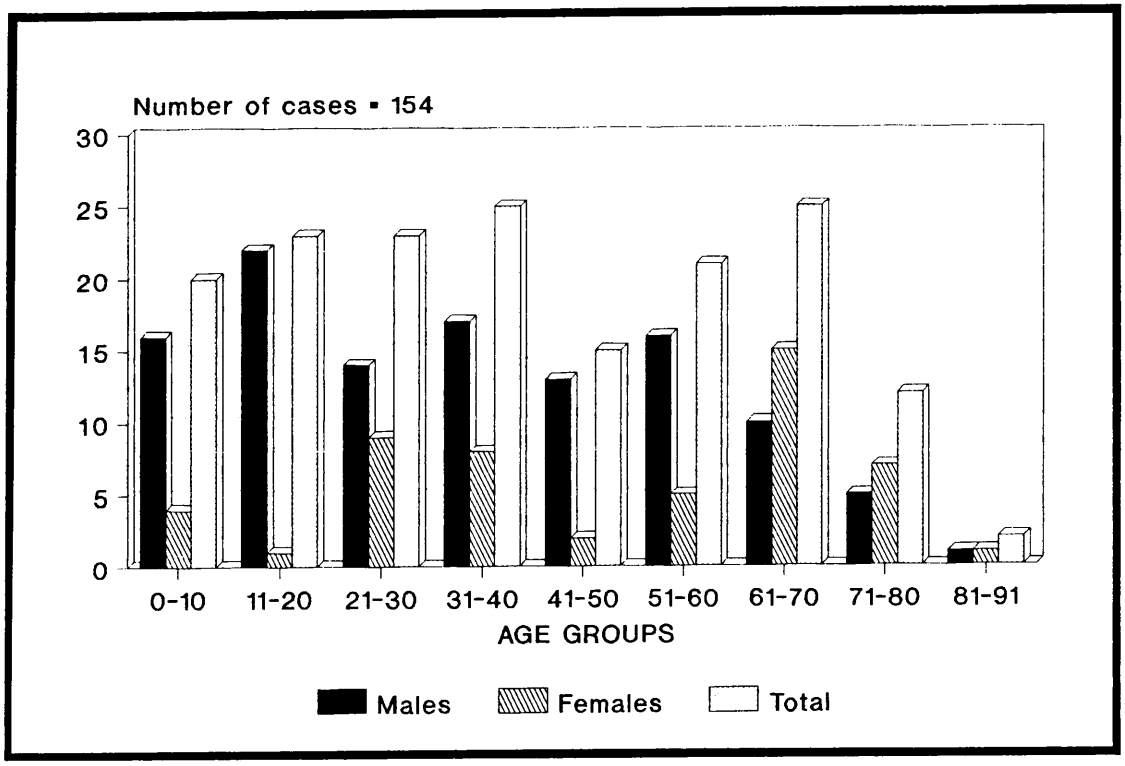


FIGURE 7. Age and sex distribution of total number of cases of sporotrichosis in groups I, II & III.

As far as race and sex are concerned, it is clear from Table 1 that approximately the same number of whites and blacks, but more males than females were seen from the H F Verwoerd and Kalafong hospitals. Table 2 indicates that only two blacks and one Asian patient, all males, were included in the itraconazole trial. Only five patients were on terbinafine treatment. They were all white males and are included in Table 2.

The preponderance of whites in the drug trials is due to the fact that more whites seek private specialist services while other races report to provincial hospitals.

TABLE 1. Sporotrichosis - Group I. H F Verwoerd & Kalafong hospitals - Race and sex distribution of patients

SEX	WHITE	BLACK	TOTAL
M	30	33	63
F	11	14	25
TOTAL	41	47	88

TABLE 2. Sporotrichosis - Groups II & III. Itraconazole & terbinafine trials - Race and sex distribution of patients.

SEX	WHITE	BLACK	ASIAN	TOTAL
M	50	2	1	53
F	13	-	-	13
TOTAL	64	1	1	66

Group IV (Gold mine cases):

Cases from the gold mines were all males and due to their employment in the mining industry they were all healthy adults. With the exception of a few whites, all were black.

1.3 Sources of the infection

Groups I to III (Hospital and trial consultations):

Each age group which contracts sporotrichosis has a different range of activities and associated injuries which act as a port of entry for the infection. For example, under 10 years, insect bites, falls and abrasions acquired outdoors; from 11 to 20 years - the above and sporting activities, such as rugby and cricket; 21 to 30 years - outdoor sports and occupations such as the building trade, brickworkers, farmers, farm labourers, etc.; the 31 to 40 year age group follows the same pattern but also includes gardeners; from 41 to 50 years preoccupation with gardening, other out of doors activities and insect bites. In the 51 year age group and above similar injuries or associations as for the 31 to 51 age group have been documented.

An association with soil or plant material was evident in most patients. Thorn pricks from roses and contact with untreated eucalyptus and wattle poles could be regarded as sources of the infection in some patients.

Group IV (Gold mine cases):

In gold miners abrasions and other slight or major injuries to the skin as well as the favourable underground conditions allowing for the proliferation of *Sporothrix schenckii*, enhance the risk of acquiring sporotrichosis underground. Although some of the mining staff working above ground also contracted sporotrichosis, the number of cases is negligible when compared with those contracting the disease underground.[17]

[17] Dr C Thomas, Dr A Calver, Dr B Cowey, Dr D Davis and Dr Gose - personal communication

1.4 Seasonal incidence

Groups I to III (Hospital and trial consultations):

No definite seasonal pattern in the occurrence of sporotrichosis could be established. Although on average patients were seen four to eight weeks after the infection, as much as 12 months might have elapsed before the diagnosis was made. This may be due to the fact that in some cases the disease is initially misdiagnosed and treated as a bacteriological infection, and several months elapse before a correct diagnoses is made, by which time many patients have forgotten the initial injury. Figure 8 shows the months in which the patient reported to the dermatologist and summarizes the seasonal distribution of the time of diagnosis of sporotrichosis in groups I, II and III. In most cases it was not possible to accurately establish the date of onset of the disease.

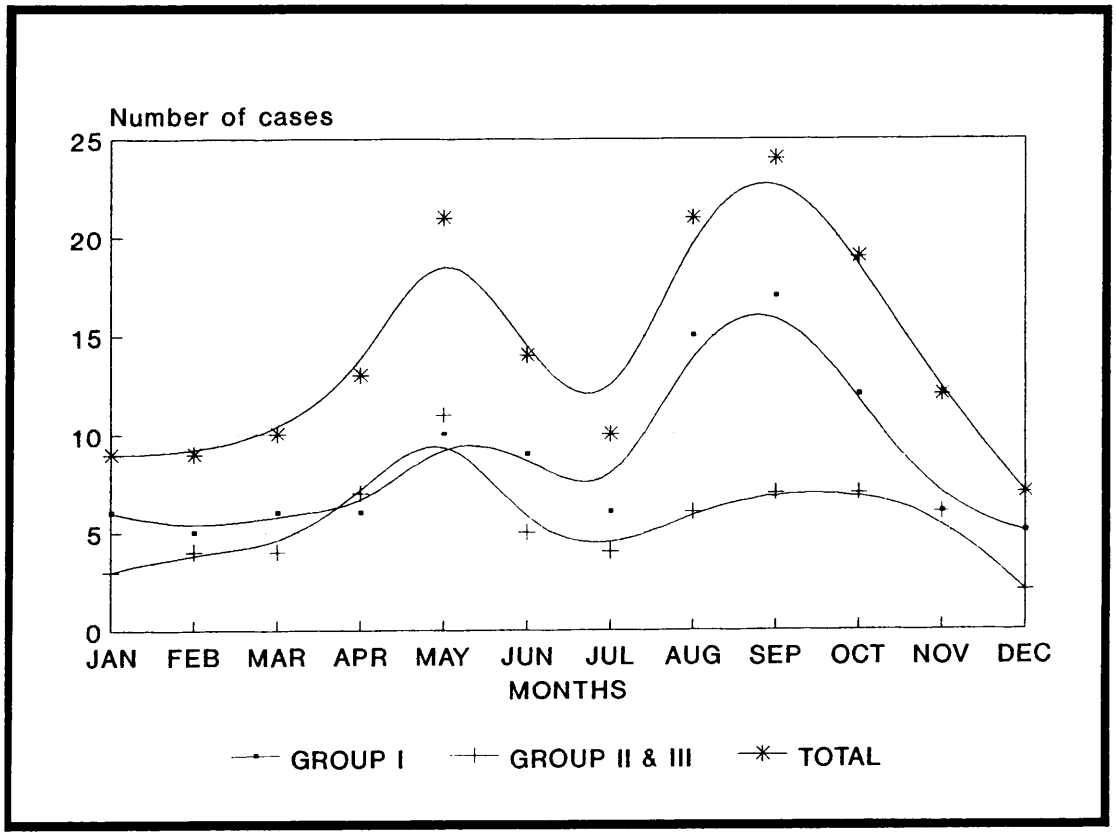


FIGURE 8. Seasonal distribution of sporotrichosis according to date of diagnosis. Groups I to III

An accurate assessment of a seasonal incidence of sporotrichosis was not possible as minor injuries which are the onset the infection, heal spontaneously and are not associated with sporotrichosis which manifests itself only later. There was a greater tendency for cases to be diagnosed during May, August, September and October, with a slight decline in June, July and the other months of the year (Figure 8, smoothed curve), but this is not of statistical significance. Although the results do not necessarily indicate the time of onset of the disease it still seems warranted to conclude that sporotrichotic infections occur more readily in the cooler and dryer months of the year in the Transvaal as an average incubation period of four to eight weeks was experienced in most cases.

Group IV (Gold mine cases):

Taken over the total period of 22 years, 1967-1989, the seasonal incidence in the cases of sporotrichosis in the gold mines portrays a similar picture as illustrated for groups I to III (see Figure 9).

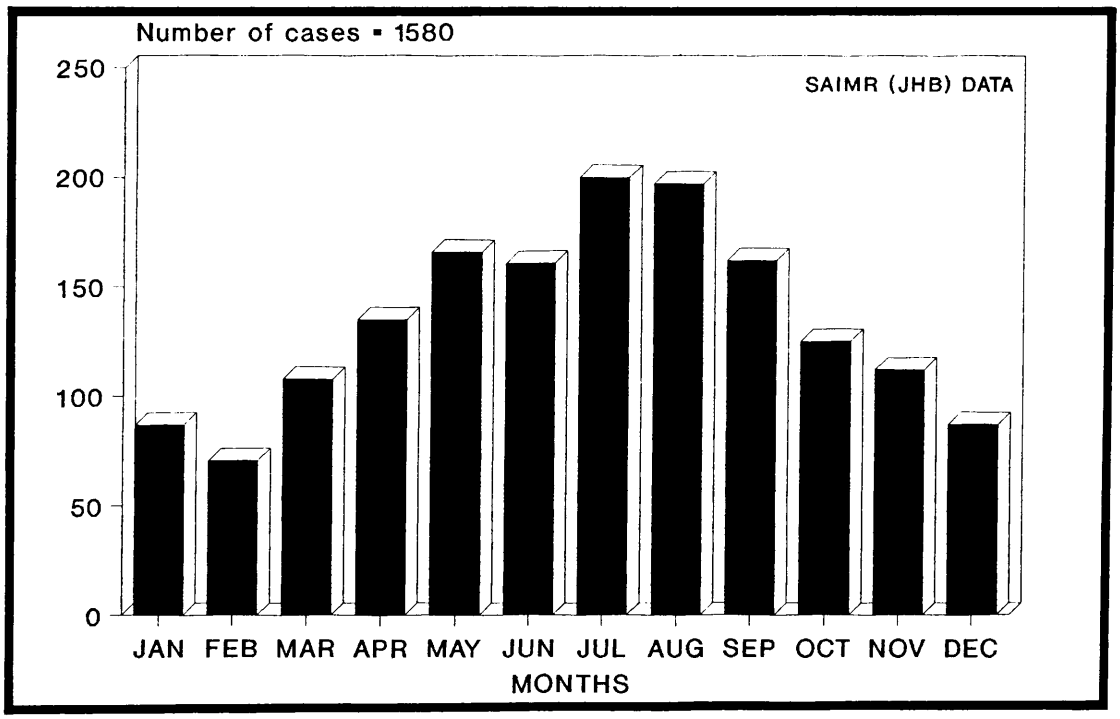


FIGURE 9. Seasonal distribution of cases of sporotrichosis in South African gold mines (1967 - 1989)

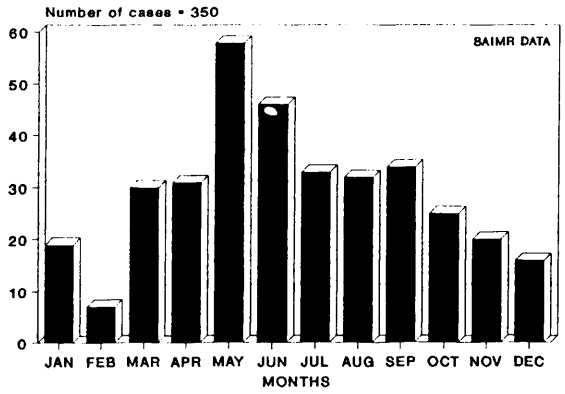


FIGURE 10. Crown Mines

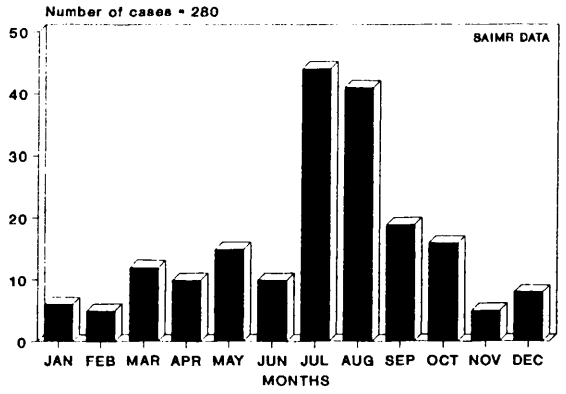


FIGURE 11. Leslie Gold Mine

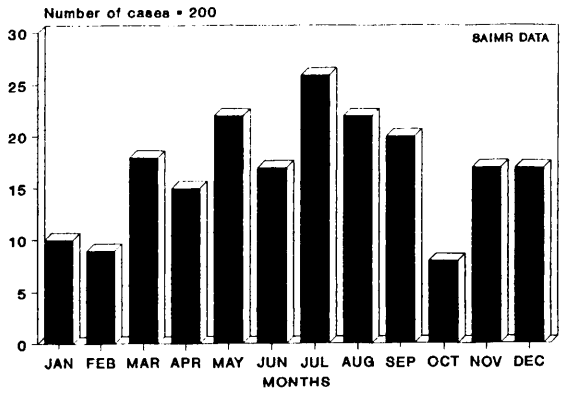


FIGURE 12. Gold Fields West

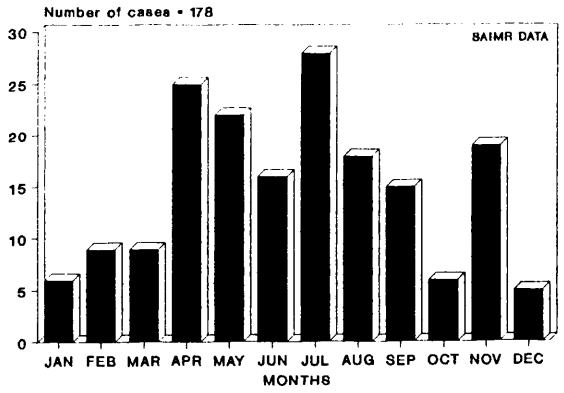


FIGURE 13. Durban Deep Mine

FIGURES 10 - 13. Seasonal distribution of cases of sporotrichosis in four South African gold mines (1967 - 1989).

It was impossible to establish the onset of the disease in the miners as the SAIMR records only indicate the date on which the specimens were taken from the respective patients. When the seasonal distribution of cases in the mines with the highest incidence of sporotrichosis are compared, the distribution is quite varied over the 22 year period under discussion (Figures 10 - 13). There was a clustering of cases in some months which can be attributed to small outbreaks experienced at a specific mine. For example, 28 cases were diagnosed at Crown Mines in May 1968, contributing half of the total number of cases for May for the 22 years (Figure 10). At Leslie Gold Mine, peaks of cases were experienced during July and August of which 40 and 38 cases for the respective months were seen in 1968, thus contributing more than 90% of all cases during those months for the 22 years (Figure 11). At Gold Fields West and Durban Deep mines the cases were more evenly distributed, i. e. one to four cases per month per year. When these are added together over the 22 year period, the months as indicated in figures 12 and 13, showed the higher incidences. It is interesting to note that the months in which the small outbreaks occurred, fall into the cooler months of the year, even when the onset of the disease is taken at an average of three to four weeks before the patients seek medical attention[18]. Therefore as in the cases of sporotrichosis in the general population, the cases in the mines tended to occur in the cooler months of the year.

1.5 Geographic distribution

When the known areas from which the 154 cases of sporotrichosis in groups I to III came, are plotted, it is evident that most cases came from within 250 kilometers of Pretoria (Figure 14). This may be due to the fact that all cases in this study were diagnosed by dermatologists in Pretoria.

In Pretoria itself there seems to be no tendency for sporotrichosis to occur in any particular suburb. The following suburbs in Pretoria

[18] Dr A Calver - personal communication 1989

contributed cases of sporotrichosis (in alphabetic order and the number of cases in brackets): Annlin (1), Arcadia (3), Brooklyn (1), Colbyn (1), Constantia Park (1), Daspoort (1), Dorandia (1), Doornkloof (1), East Lynne (1), Elarduspark (1), Eldoraine (1), Erasmia (1), Groenkloof (1), Gezina (1), Hatfield (1), Irene (2), Kilnerpark (1), Lynnwood (1), Lynnwood Ridge (2), Nieuw Muckleneuk (1), Murrayfield (2), Menlo Park (1), Meyerspark (1), Pretoria Gardens (1), Queenswood (1), The Reeds (1), Rietfontein (2), Rooihuiskraal (1), Silverton (1), Sinoville (2), Suiderberg (Mountain View) (1), Sunnyside (3), Valhalla (1), Villieria (1), Voortrekkerhoogte (1), Wapadrand (2), Waterkloof (1), Waterkloof Heights (1), Waverley (1), Wierda Park (1), Wingate Park (1) and The Willows (1). It is seen that most cases come from the northern, eastern and southern parts of Pretoria (Figure 15).

Towns and areas outside Pretoria, but still limited to the Transvaal which also contributed cases of sporotrichosis, include Amersfoort (1), Brits (3), Boksburg (1), Bronkhorstspruit (7), Carolina (1), Cullinan (4), Delmas (1), Dennilton (2), Devon (1), Ermelo (1), Hartebeespoortdam (2), Heidelberg (1), Hennopsmeer (1), Johannesburg (2), Krugersdorp (2), Koster (1), Middelburg (Transvaal) (2), Rayton (4), Rustenburg (3), Schagen (Nelspruit) (1), Standerton (1), Tierpoort (1), Volksrust (2), Vorna Valley (Half Way House) (1) and Zonderwater Prisons (Cullinan (2) - see figure 14.

1.6 Impressions of the epidemiological dimorphism seen in sporotrichosis.

Several factors are of importance when the epidemiology of sporotrichosis in the general population are compared to those in gold miners[19]

[19] Prof G H Findlay - personal communication

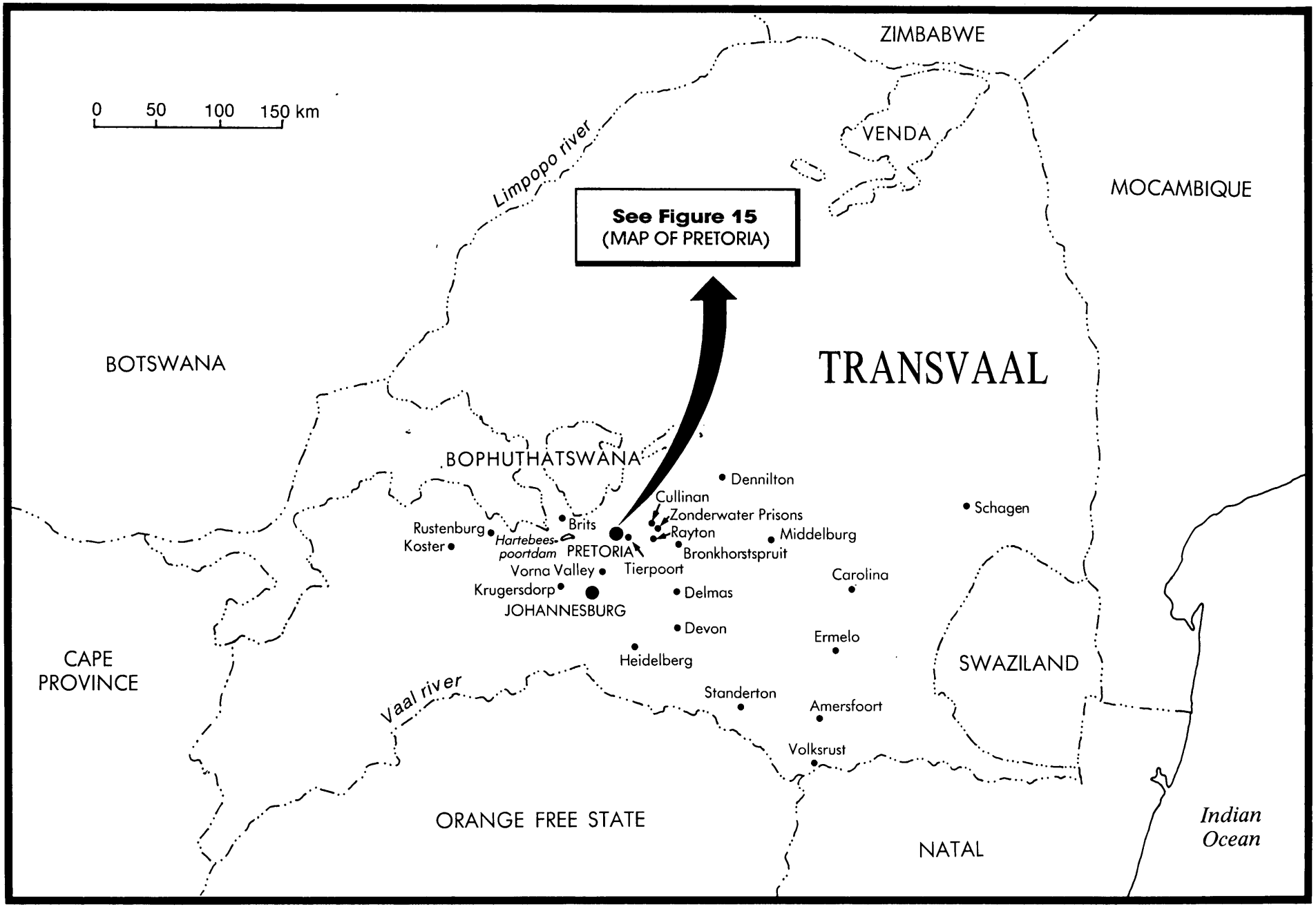


FIGURE 14. Geographic distribution of sporotrichosis cases resident in the Transvaal

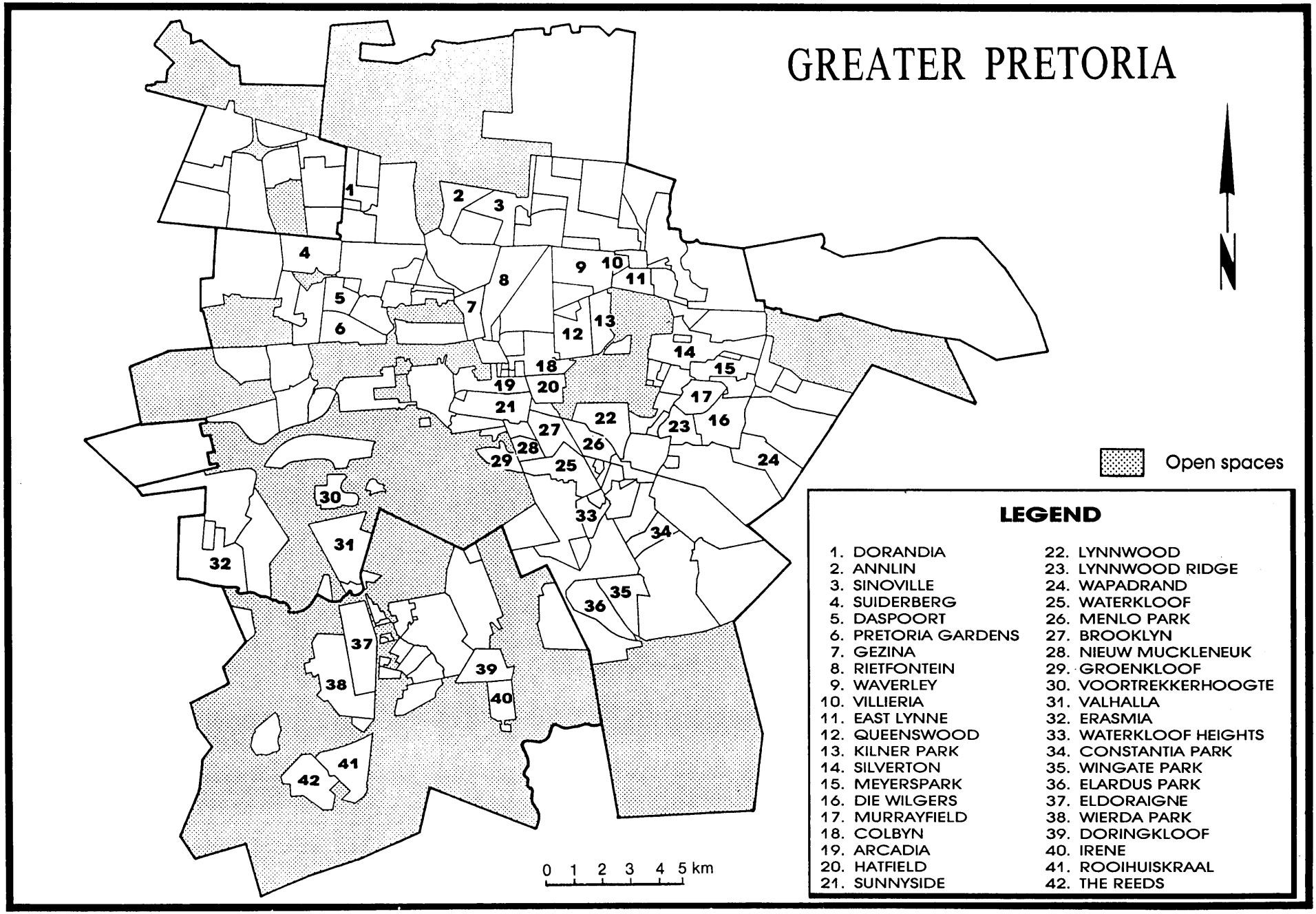


FIGURE 15. Geographic distribution of suburbs in Pretoria from which cases of sporotrichosis came

- The clinical types seen

Three cases of which two were disseminated sporotrichosis and a third occurring in an immunocompromised patient, were documented in groups I, II and III, i. e. three out of the total number of 154 patients. The other cases were of the localized or lymphocutaneous type. As far as could be established the mine cases were also of the localized or lymphocutaneous type.[20] Systemic or extracutaneous cases, for example, cases with bone or lung involvement, were not mentioned in the data obtained from the SAIMR. See paragraph 1.7 (p. 45) for a detailed description of the clinical types of sporotrichosis occurring in this study.

- The fitness of the subject

The cases in the general population are sometimes unfit, debilitated or immunocompromised while, the miners were always healthy young adults.

- The influence of season

The cases in the general population and the gold mines tend to cluster in the cooler months of the year, as summarized in Figures 8 - 13.

- The response to treatment

Response to conventional treatment with potassium iodide is satisfactory in the fit subject but often less so in the compromised individuals. However, new treatments, such as itraconazole and terbinafine, are constantly being investigated. This will be discussed fully in paragraph 1.9 (p. 64).

[20] Dr C Thomas, Dr B Cowey, Dr A Calver, Dr Davis and Dr Gose - personal communication

- The risk of infection

  The risk of infection is lower in the general public when compared to the higher risk of trauma underground and the abundant presence of the fungus.

1.7 Clinical types of sporotrichosis

For the purpose of this study, the clinical types of sporotrichosis[21] will only be discussed in general in order to correlate some clinical findings with the mycological research.

1.7.1 Lymphocutaneous sporotrichosis

Lymphocutaneous sporotrichosis accounted for 78% of the cases in groups I to III. *S. schenckii* gains entrance into the body through traumatic implantation. After an average incubation period of three to four weeks, a small hard mobile subcutaneous nodule or superficial ulcer develops at the site of inoculation. Nodule formation usually indicates a deeper subcutaneous inoculation of the organism into the skin, while in the case of ulcerative lesions the fungus is usually present in the dermis or epidermis. This initial lesion is not tender and is pink to purple, or black in colour. It becomes attached to the underlying skin, accompanied by local or regional lymphadenopathy. Nodular lesion may become necrotic and break through the skin. Left untreated, these lesions become chronic and develop into the classic form of the disease, producing multiple subcutaneous nodules along the lymphatic vessels. Any of these nodules can break down and ulcerate. The connecting lymphvessels become hard and cord-like. Depending upon the initial site of the inoculation, sporotrichosis of this kind may be present on any of the extremities, the neck, the face or even the scalp.

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[21] Prof G H Findlay and consultants, Dr P R Hull and Dr J S van Heerden - personal communication. They saw most of the sporotrichosis cases included in patient groups I, II & III.

To illustrate some of these clinically pathognomic lesions, Figure 16 indicates a primary lesion on the finger, i. e. site of inoculum, with spread of the disease on the dorsum of the hand and up the arm. A lymphocutaneous form with lesions spreading up the inner arm is shown in Figure 17. Similar lesions on the arm of a black patient, with the primary lesion in the soft tissue between the thumb and the forefinger can be seen in Figure 18.

A rare case of sporotrichosis is shown in Figures 19 and 20. The primary lesion started as a pimple on the tip of the nose, developed into cerebriform lesions and spread along the regional lymphvessels of the face, forming sporotrichotic nodules. Due to the location of the lesion, and possibility of spread to the orbit, the eyes and the brain, standard treatment with potassium iodide was not attempted. The patient was hospitalized and amphotericin B was administered intravenously. She reacted favourably with complete healing of the sporotrichotic lesions.

1.7.2 Localized cutaneous sporotrichosis

In localized cutaneous sporotrichosis the lesion is restricted to the site of inoculation, without lymphatic involvement, and is thus "fixed". Lesions may form ulcerative, granulomatous, verrucous, infiltrated erythematous plaques which can become crusted and weeping (Figures 21 and 22); or the lesions may present as a scaling, macular or papular rash. Satellite lesions in localized cutaneous sporotrichosis are not uncommon (Figure 21). An unusual, localized cutaneous sporotrichotic lesion under the eye of an avid gardener is illustrated in Figure 23. This patient probably initiated the infection by rubbing the skin under his eye with soiled hands. The mild trauma was enough to infect this superficial skin abrasion with the *Sporothrix* organism from contaminated garden soil. A similar case is illustrated in Figure 26, where a single lesion developed on the foot after a car accident. The wrecked car containing soil and plant material contaminated with *S. schenckii* is shown in figure 25.

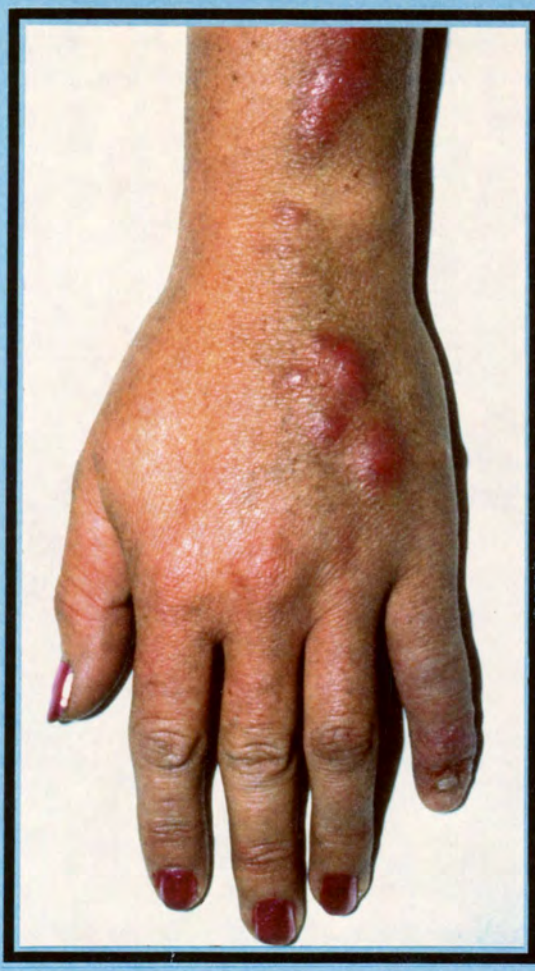

FIGURE 16. Lymphocutaneous sporotrichosis Primary lesion on finger with spread on hand dorsum and arm

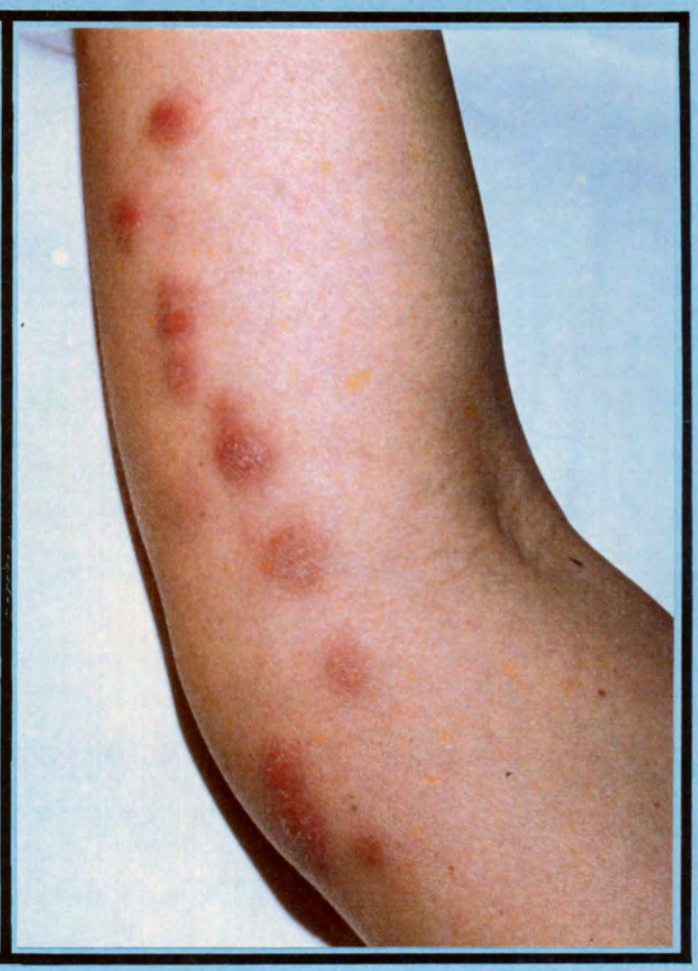

FIGURE 17. Classical lymphocutaneous sporotrichosis on inner arm

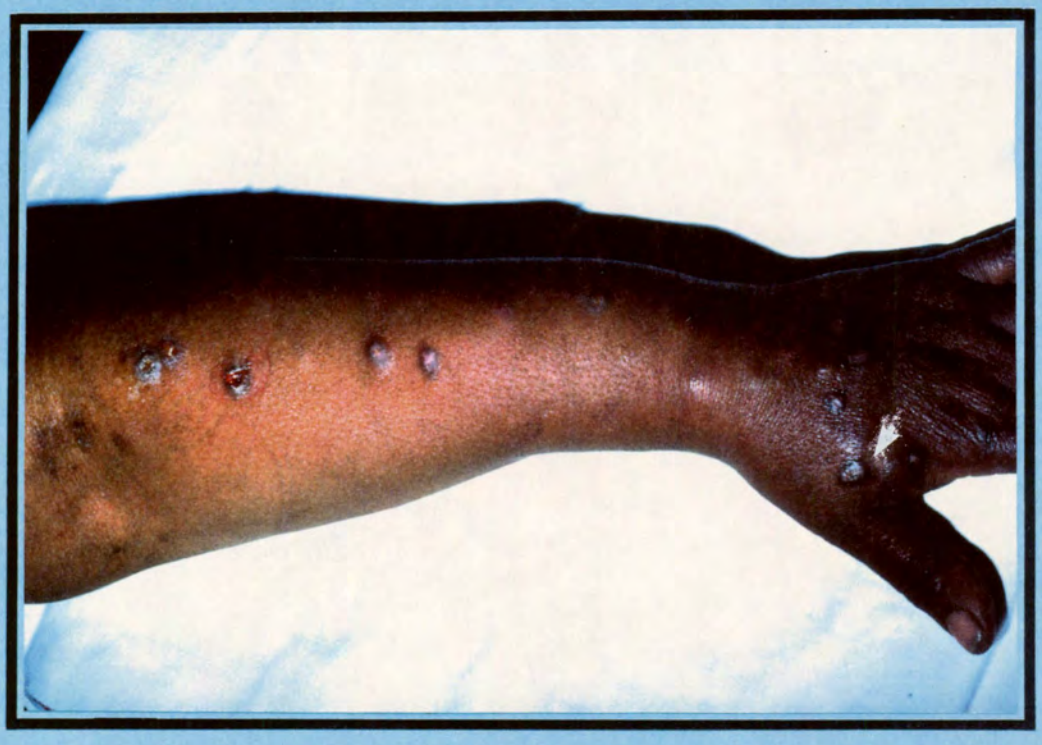

FIGURE 18. Primary soft tissue inoculation (arrow) and lymphocutaneous spread along the arm

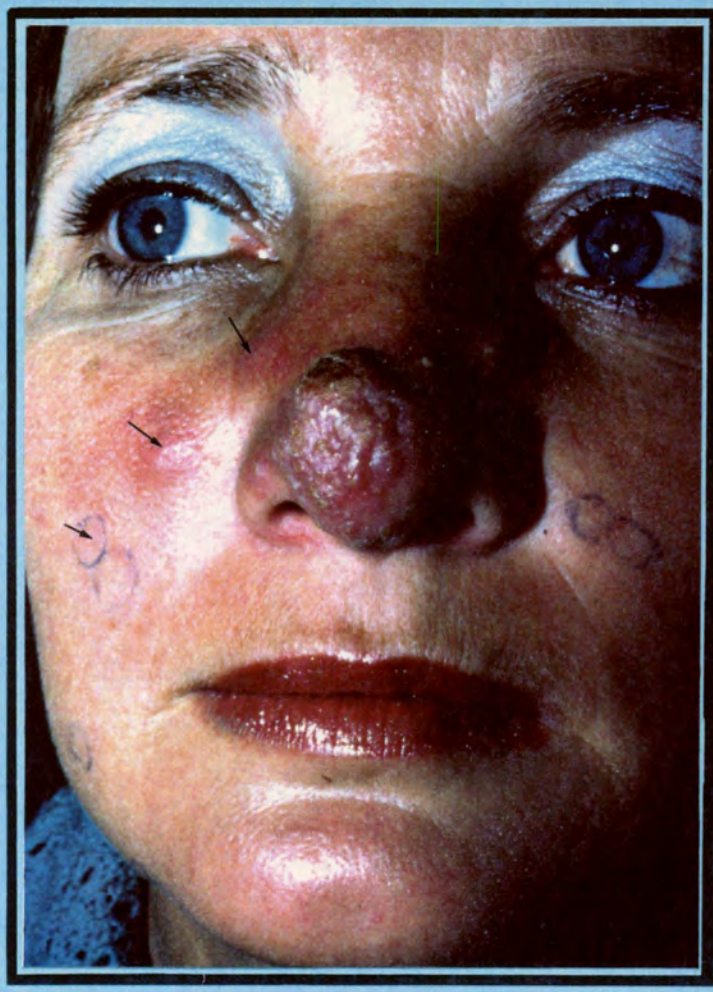

FIGURE 19. Lymphocutaneous sporotrichosis. Cerebriform lesions on the nose with spread along the lymphatics of the face (arrows)

FIGURE 20. Same case as in Figure 19. Side view showing the extent the lesion

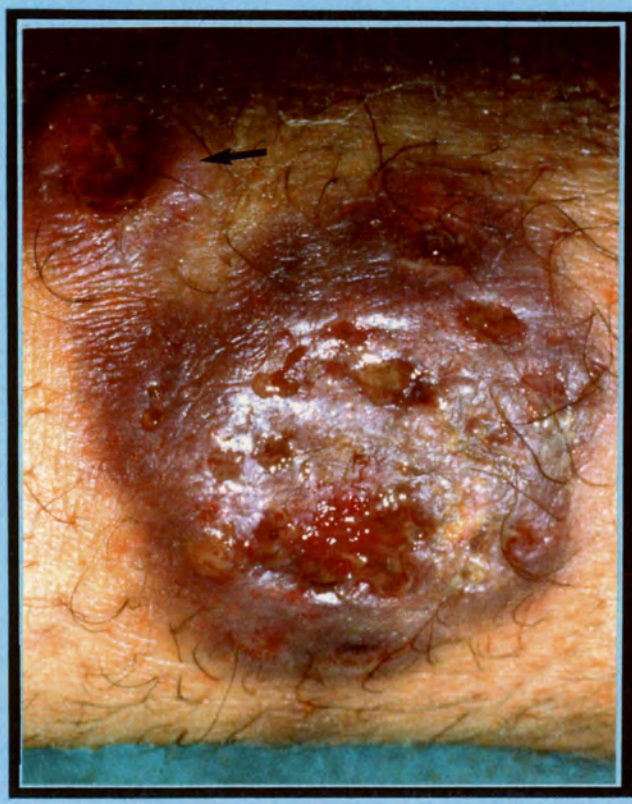

FIGURE 21. Ulcerative fixed sporotrichosis. Note the satellite lesion (arrow)

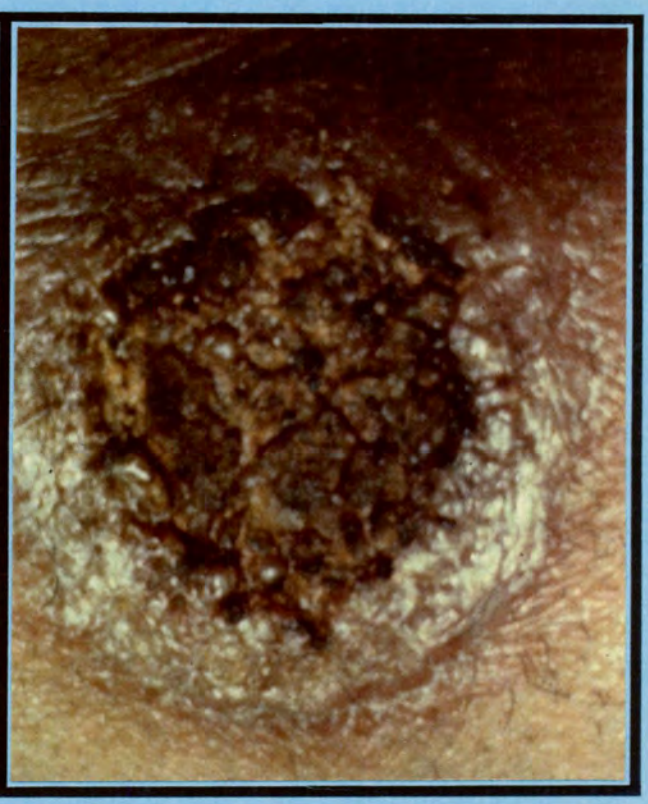

FIGURE 22. Localized sporotrichosis. Note the scaling and black colour of the lesion

Clinical differentiation from many other cutaneous diseases, including verrucous tuberculosis, atypical mycobacterial infections, late syphilitic lesions, cutaneous leishmaniasis, rosacea and even cancerous lesions (Figure 24), is necessary. Other fungal diseases from which this form of sporotrichosis must be differentiated, include mainly chromoblastomycosis, blastomycosis and more rarely histoplasmosis. The last two diseases normally show additional systemic symptoms.

Multiple fixed cutaneous lesions can occur in the same patient where several sites on the body are inoculated from the same source. A four year old girl, living on a small holding near Half Way House (Vorna Valley), between Pretoria and Johannesburg contracted multiple fixed cutaneous lesions of sporotrichosis. The source of the infection proved to be the sand pit she played in. From close examination of the plot and discussion with the child's parents, it became clear that unwashed building sand was used to fill the sand pit. A *Sporothrix*-like organism was cultured from specimens collected from this sand.

In debilitated or immunocompromised patients sporotrichosis can take on clinical forms not easily recognizable as such. In the present study localized sporotrichosis was seen in a 77-year-old white female who had received corticosteroids for eight years for bullous pemphigoid, a skin disease unrelated to sporotrichosis. There was no history of trauma to the skin. She had however visited the countryside some four to five weeks before the onset of the sporotrichosis. The lesion started as a small pimple on her left arm, developing pustules over her upper arm within three weeks (Figure 27). It deteriorated rapidly in the following three weeks to form a large spreading lesion over the upper and lower arm (Figure 28).

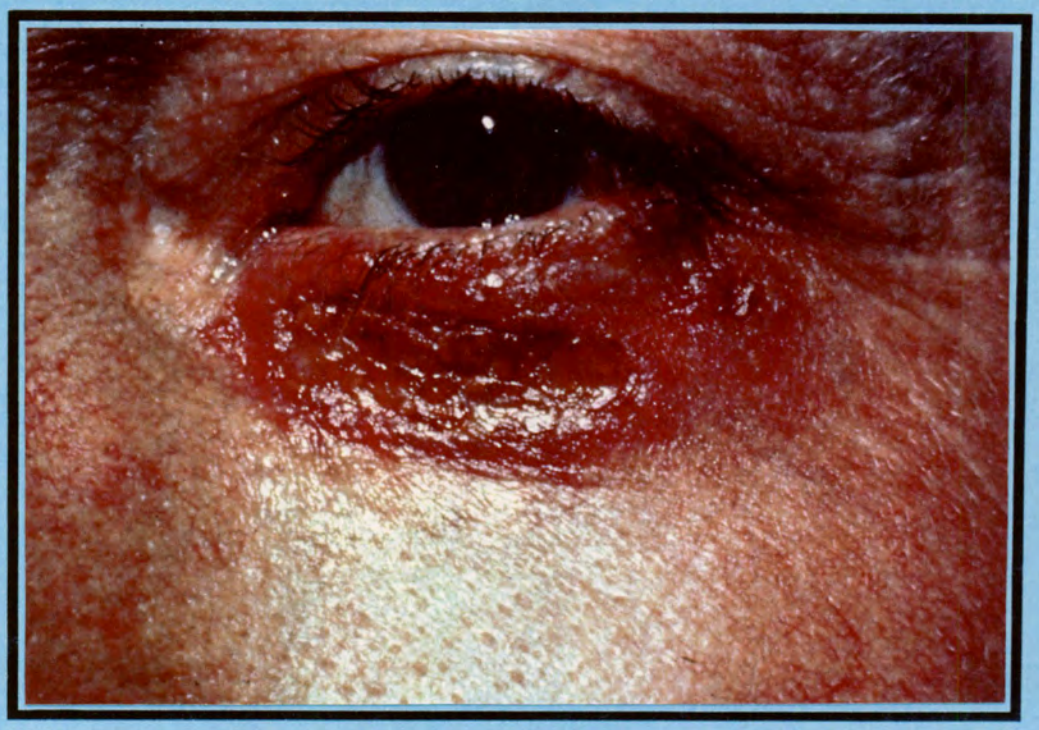

FIGURE 23. Fixed cutaneous sporotrichosis under the eye

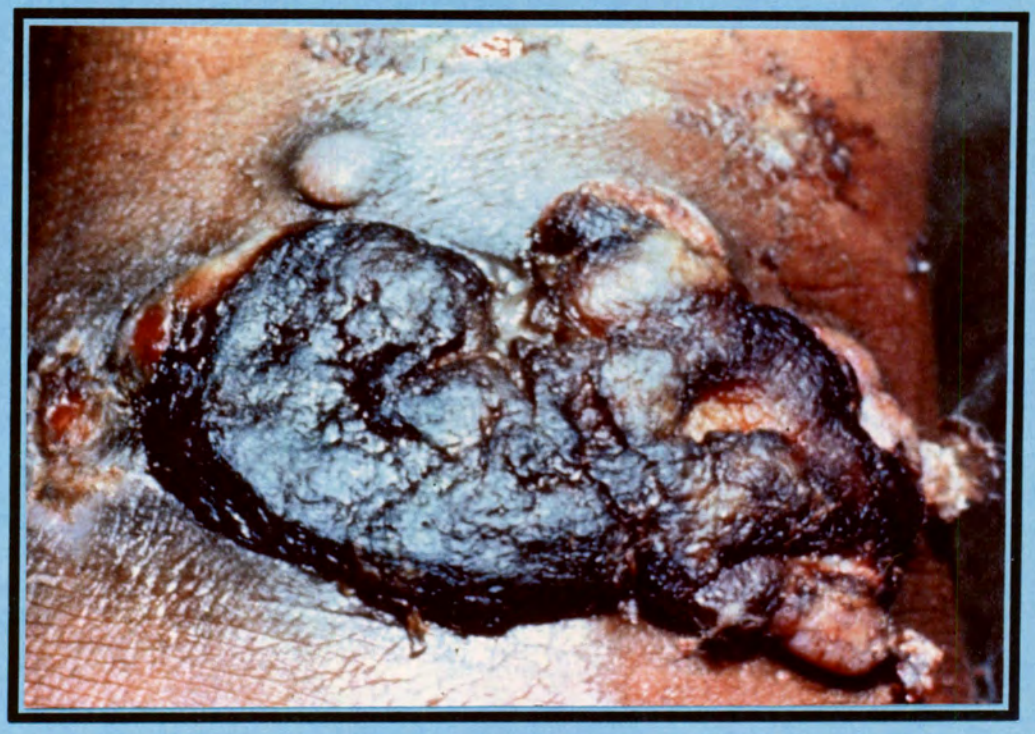

FIGURE 24. Sporotrichosis mimicking a cancerous lesion on the ankle

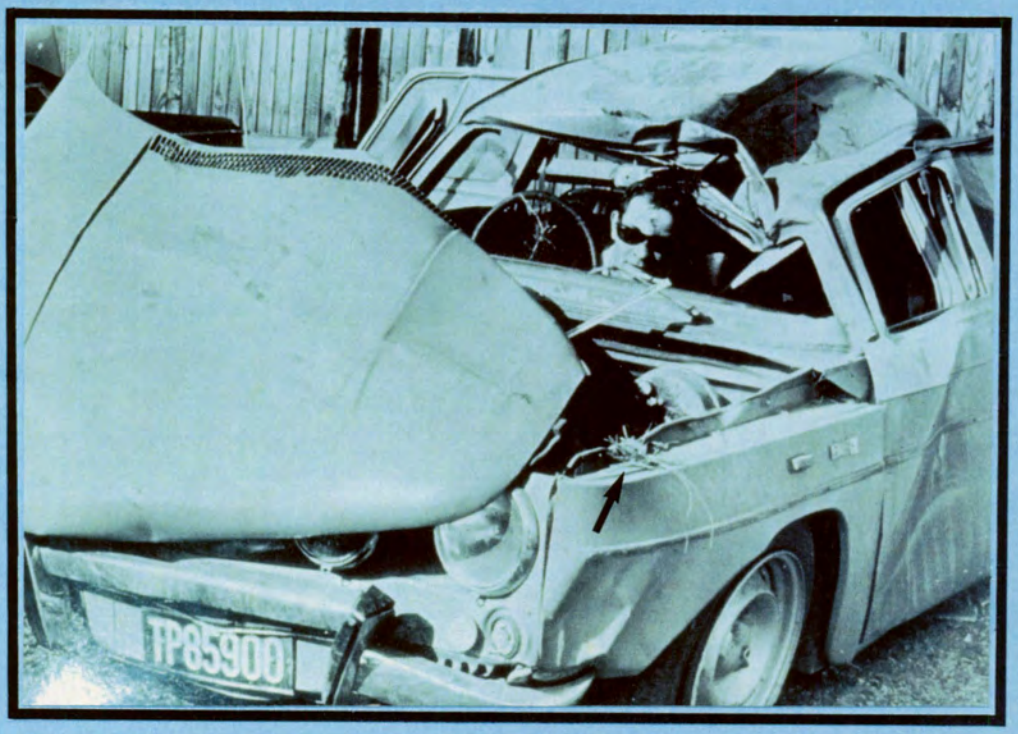

FIGURE 25. Wrecked car containing soil and plant material (arrow) contaminated with S. schenckii - see Figure 26

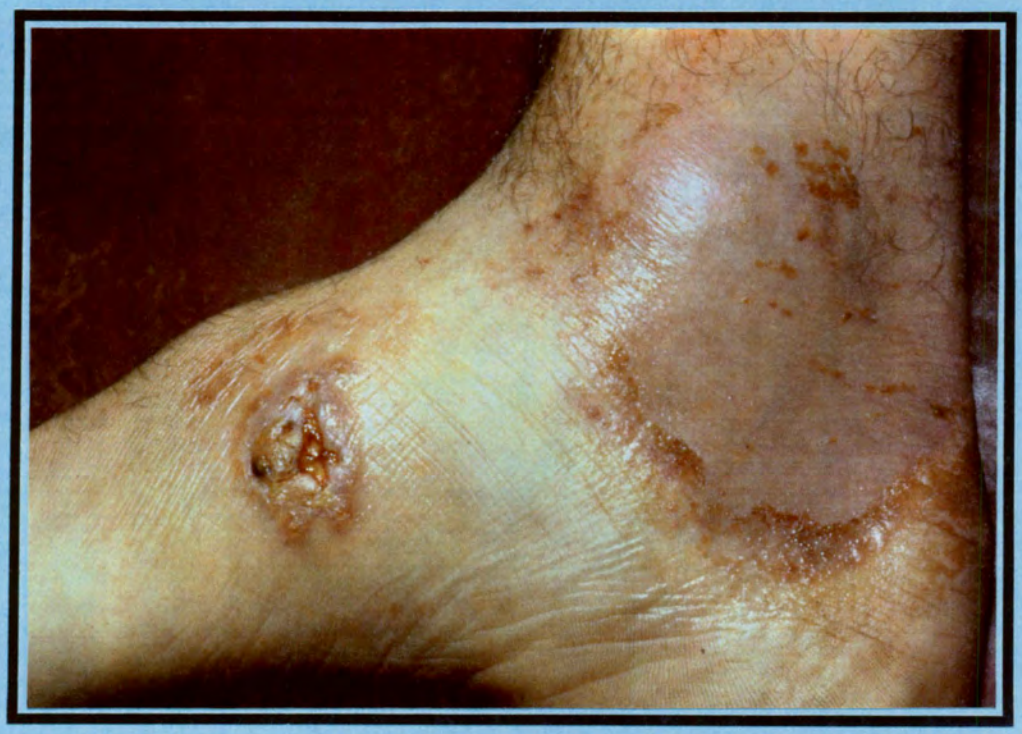

FIGURE 26. Localized sporotrichosis on the foot after a car accident.

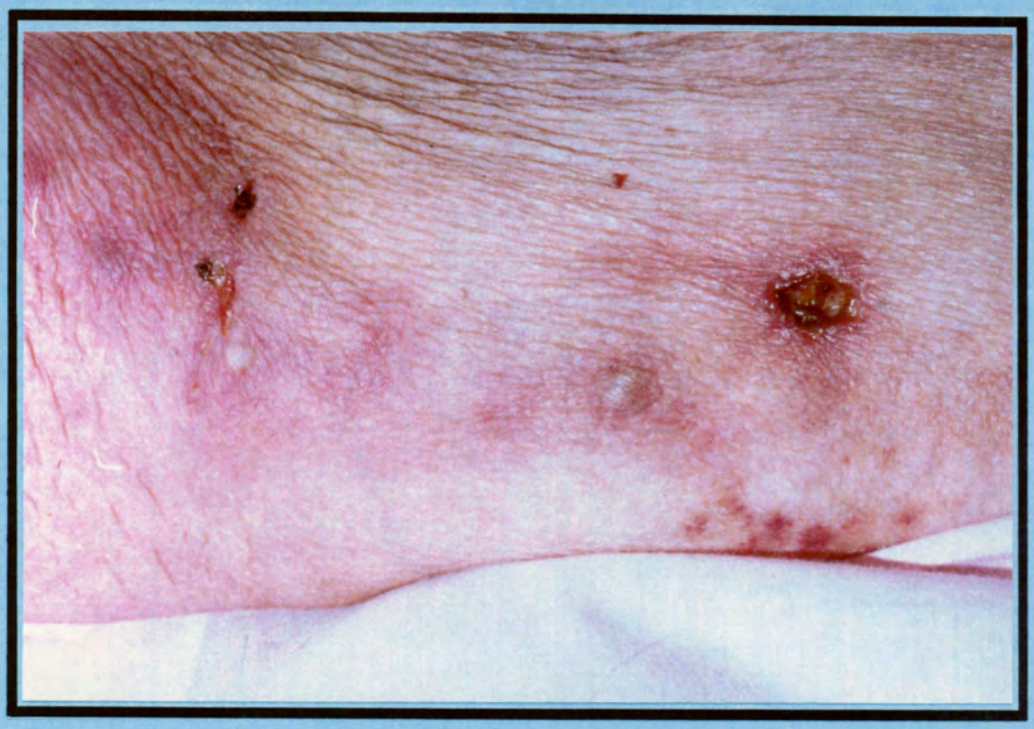

FIGURE 27. Pustular lesions of the upper arm of a patient treated with corticosteroids for an unrelated skin disease for eight years

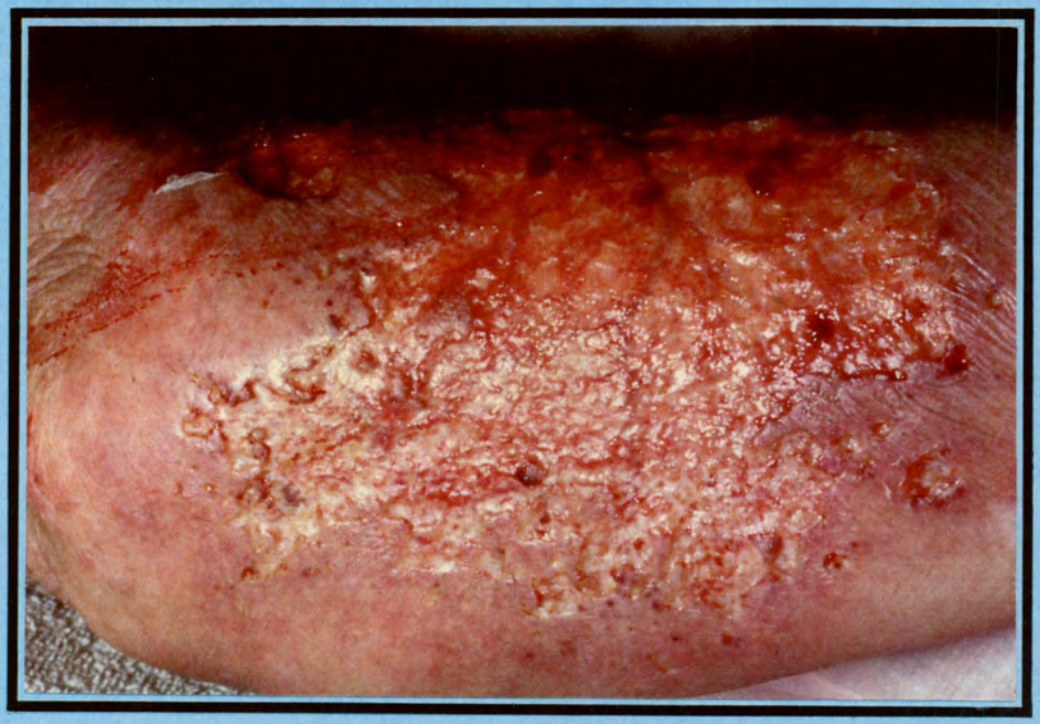

FIGURE 28. Same patient as illustrated in Figure 27 - three weeks later showing deterioration of the lesion

1.7.3 Extracutaneous and systemic sporotrichosis

Extracutaneous and disseminated sporotrichosis are general terms used to describe several forms of sporotrichosis involving organs other than subcutaneous tissues. Of these osseous involvement is most common. Skin lesions invariably precede or accompany sporotrichosis involving bones and joints. Only two examples of these forms of sporotrichosis were encountered in the current study.[22]

A 55-year-old white man presented with a systemic history unrelated to sporotrichosis, namely pernicious anaemia and a protein losing enteropathy for six years. He was occupied as a store keeper in a rural area. He had suffered an injury to the fourth finger and the dorsum of one hand and an ankle which marked the onset of the sporotrichosis (Figure 29). Multiple ulcerating subcutaneous abscesses of eight months duration were present on all the extremities and trunk. No lymphatic involvement could be established (Figures 30 and 32). Antibiotic treatment had no healing effect but the lesions remained static and no new ones appeared. An ulcerating skin lesion on a shoulder was surgically removed (Figure 30) and revealed a non-specific histological picture. Macular and papular skin lesions on the face subsequently appeared (Figure 31). The patient also complained of several episodes of a painful swollen knee and ankle joint in the left leg during the following eight months (Figure 32) and experienced progressive limitation of movement in the leg. He was hospitalized and during routine examinations, *S. schenckii* was cultured from all the skin lesions including the face. X-rays examination of the left knee showed a large osteolytic lesion in the patella (Figure 33). Surgical exploration confirmed the extent of this bony lesion (Figure 34), and curetted tissue obtained during the operation grew pure cultures of *S. schenckii*. The diagnosis of primary subcutaneous sporotrichosis with dissemination to the bone and joints, i. e. osseous and arthritic sporotrichosis, thus confirmed.

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[22] Both patients were under the care of Prof G H Findlay from whom permission was obtained to publish the abridged clinical findings

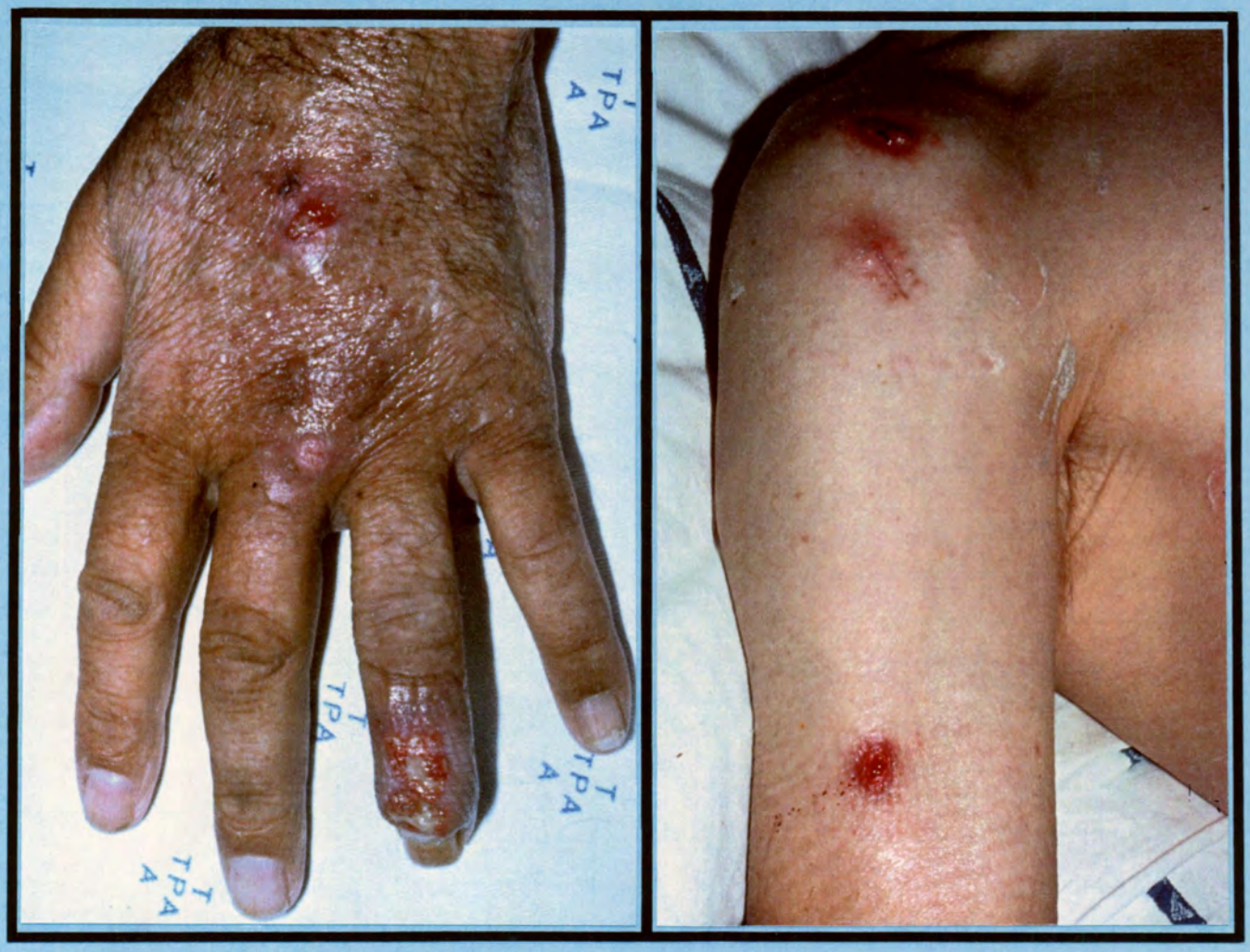


FIGURE 29. Extracutaneous sporotrichosis. Primary lesions on the finger and hand dorsum

FIGURE 30. Extracutaneous sporotrichosis. Several ulcerating lesions on the arm

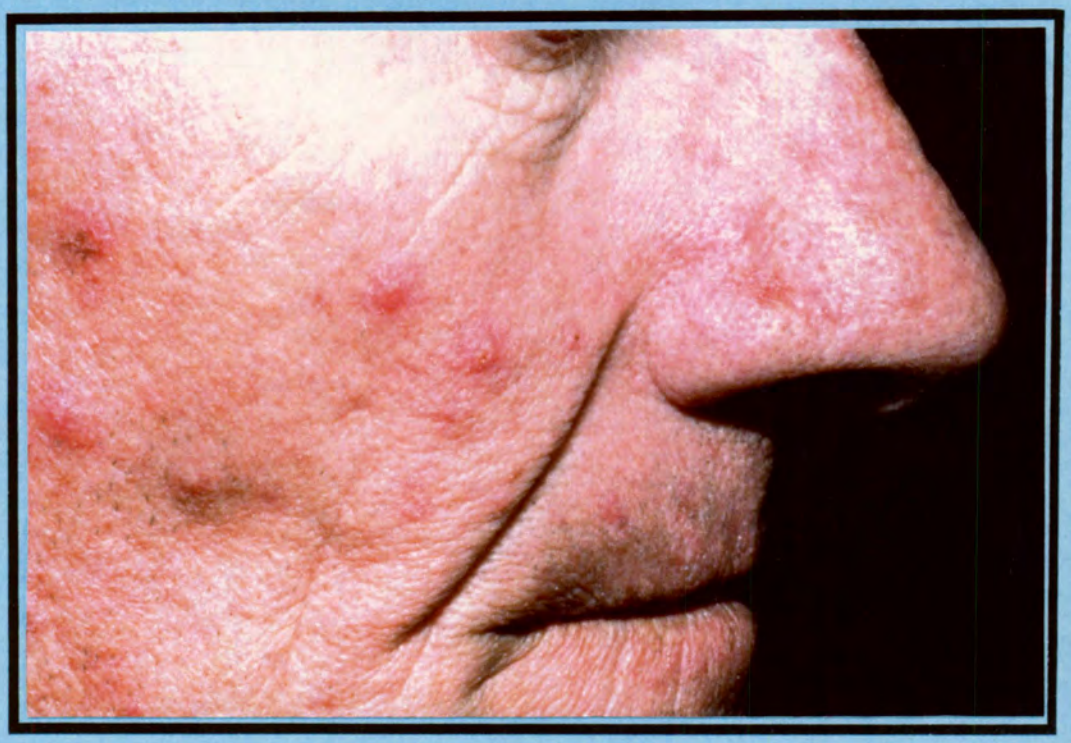


FIGURE 31. Macular and papular lesions of extracutaneous sporotrichosis of the face

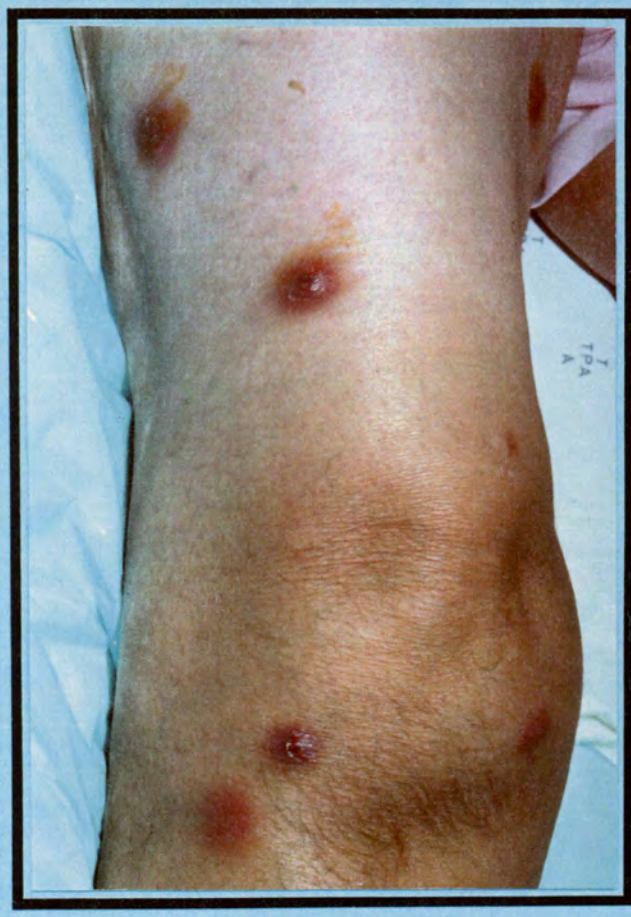


FIGURE 32. Extracutaneous sporotrichosis. Ulcerating lesions on leg and swelling of knee joint.

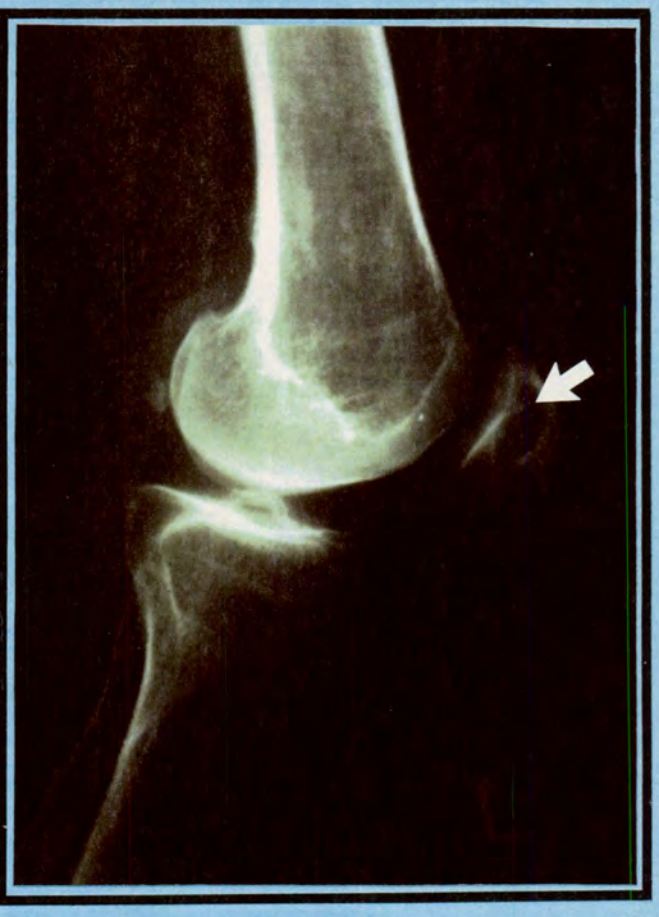


FIGURE 33. X-ray of the knee showing a large osteolytic lesion in the patella (arrow).

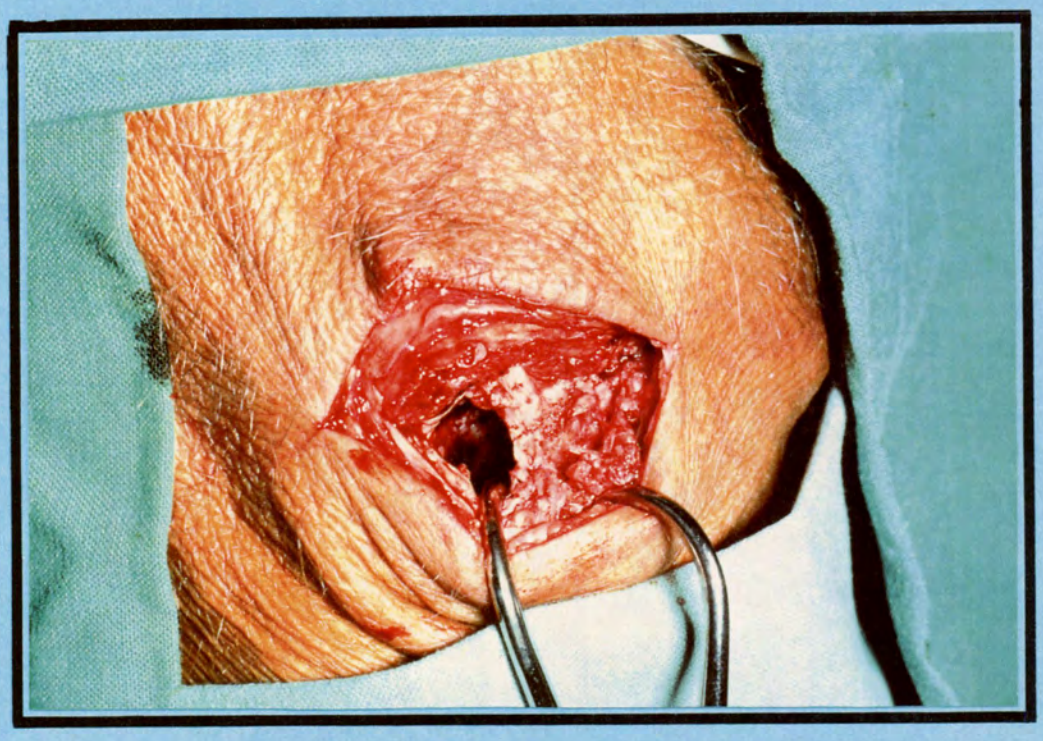


FIGURE 34. Surgical exploration indicating the extent of the patellar lesion shown in Figure 33.

This patient did not respond to the conventional treatment for sporotrichosis, namely oral potassium iodide, probably due to the systemic complications mentioned before. Intravenous amphotericin B was necessary to effect a cure.

The second case that falls into this category of sporotrichosis, was a 67-year old white female clinical history of idiopathic suppressed cellular immunity. She had fully recovered from pulmonary aspergillosis some years before. She developed primary pulmonary sporotrichosis which remained undiagnosed until she was admitted to hospital where a septic ulcer on the left medial malleolus of four months duration was diagnosed as sporotrichosis (Figure 35). Ulcers similar to the ones described in the previous patient were present on the limbs. Cultures of sputum as well as skin scrapings were positive for *S. schenckii*. Chest X-rays revealed a mottled picture throughout both lung fields (Figure 36). Blood cultures for fungi were negative. A diagnosis of primary pulmonary sporotrichosis with skin dissemination was made.

These two patients were the only cases of extracutaneous and systemic sporotrichosis included in the current study. None of the other types of extracutaneous sporotrichosis, involving for example the sinuses and orbit, internal organs (other than skeletal) and the central nervous system can therefore be illustrated here.

As far as the mine cases are concerned, lymphocutaneous sporotrichosis occurred in the majority of patients, while fixed cutaneous sporotrichosis was seen in a much smaller number.[23] Exact percentages could however not be determined from the SAIMR records.

[23] Dr C Thomas, Dr B Cowey, Dr A Calver, Dr Davis and Dr Gose - personal communication

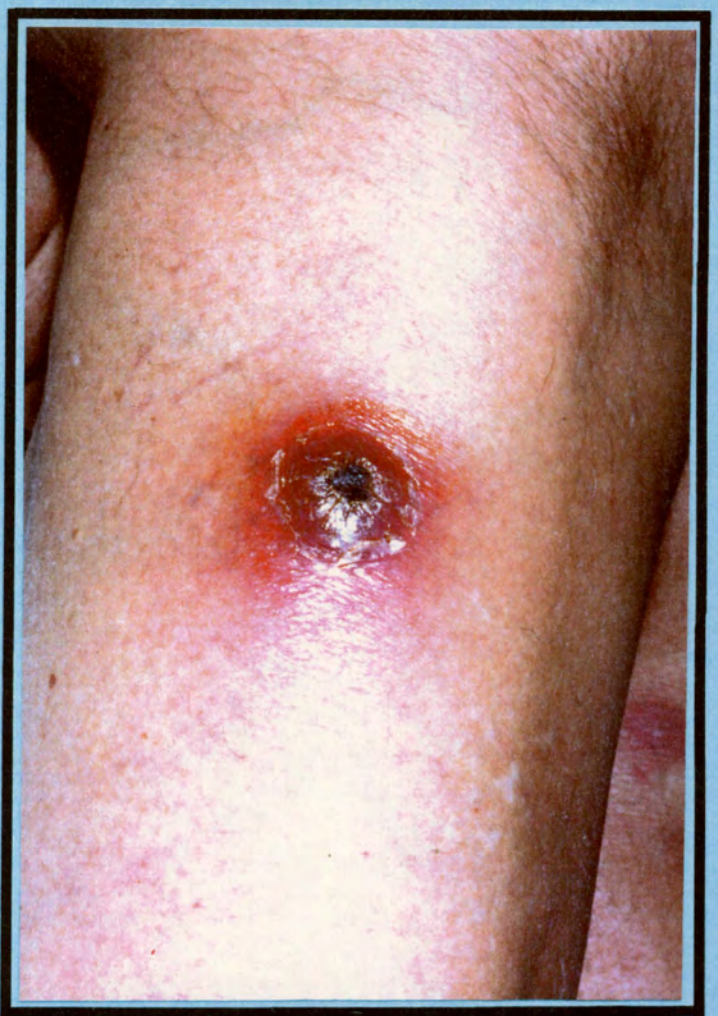


FIGURE 35. Disseminated sporotrichosis. Ulcerative subcutaneous lesion on leg

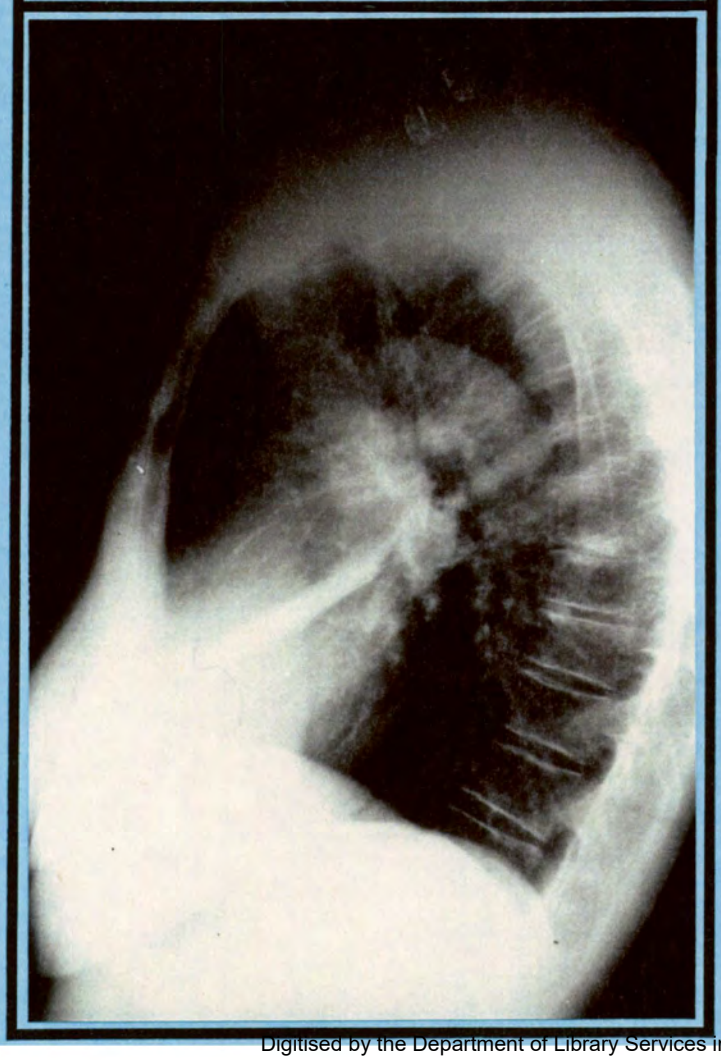


FIGURE 36. X-ray of the chest showing mottled infiltrates in primary pulmonary sporotrichosis.

1.8 HISTOPATHOLOGY OF *S. SCHENCKII*

1.8.1 General

Histologically lesions of cutaneous and subcutaneous sporotrichosis consist of a combination of granulomatous and pyogenic reactions. Three main granulomatous patterns may be found alone or in combination, viz. a sporotrichotic, a tuberculoid or a foreign body reaction. Basically a granulomatous reaction consists of masses of epithelioid histiocytes, which have the tendency to form concentric rings. The central area consists of neutrophils or necrotic material surrounded by an infiltrate of neutrophils, some plasma cells and lymphocytes. In the case of a tuberculoid reaction, this central area merges into an outer zone of epithelioid cells mixed with fibroblasts, lymphocytes and Langerhans giant cells. A prominent outer layer of plasma cells suggests a syphilid. Microabscesses are frequently seen, but in some cases without the epithelioid histiocyte component. Pseudoepitheliomatous hyperplasia is a feature most often seen in chronic sporotrichotic lesions (Rippon, 1988; Lurie, 1963a).

Although the classical histopathological picture of sporotrichosis could not be demonstrated in biopsy material from all cases included in this study, most of these features could be illustrated (Figures 37, 38, 39a, 39b and 43). The fungus is difficult to demonstrate in tissue sections, but when present, fungal elements appear as short oblong rods or "cigar" bodies, measuring 2 - 5 x 1 - 3μm (Figures 39a and 39b). They represent the yeast phase of the fungus. They are granular and basophilic initially becoming oxyphilic in the degenerated stage and are surrounded by an unstained areola. The last feature is best illustrated by means of electron microscopy of the organisms in tissue (see paragraph 10, p.130). Cigar bodies may lie free in the tissue or are present in phagocytes. Larger globose, double contoured yeast-like organisms of varied size may also occur (Figures 39a and 39b).

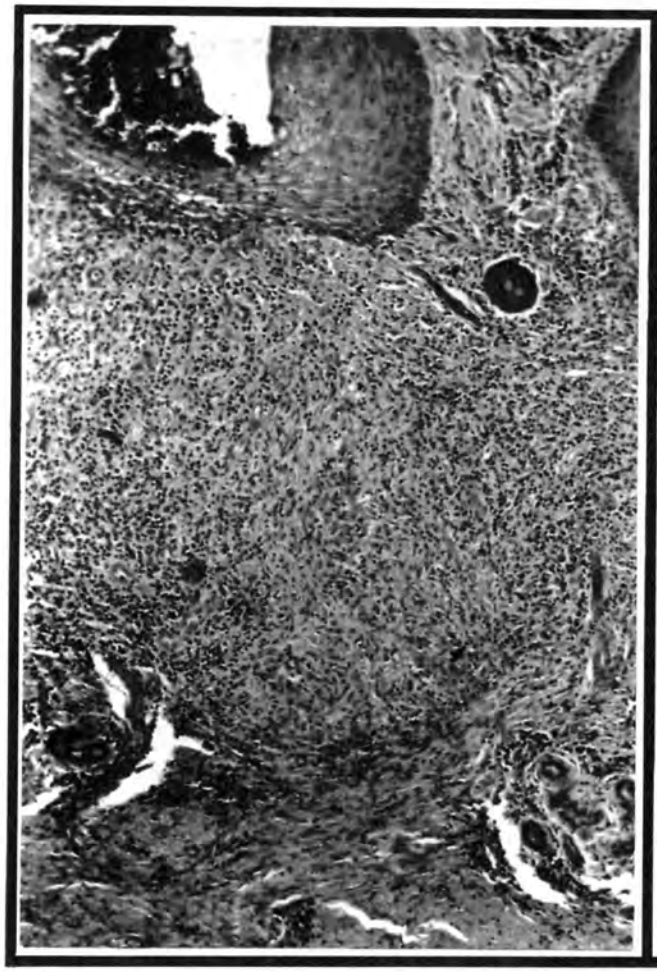


FIGURE 37. Sporotrichotic granuloma with central necrosis and polymorph infiltration. (H & E, 4x lens)

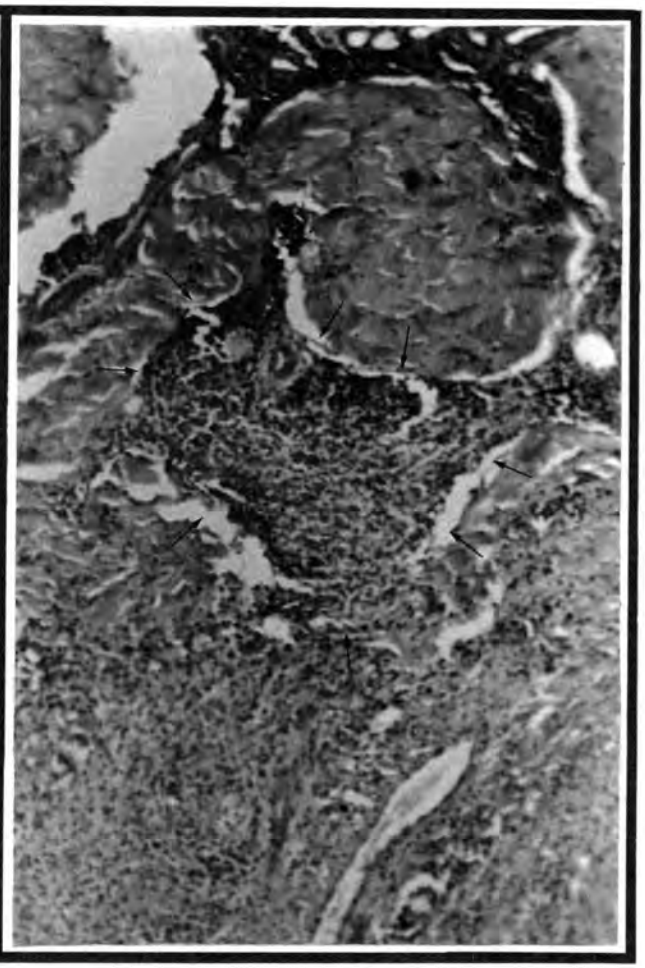


FIGURE 38. Sporotrichotic microabscess (arrows). PAS, 4x lens

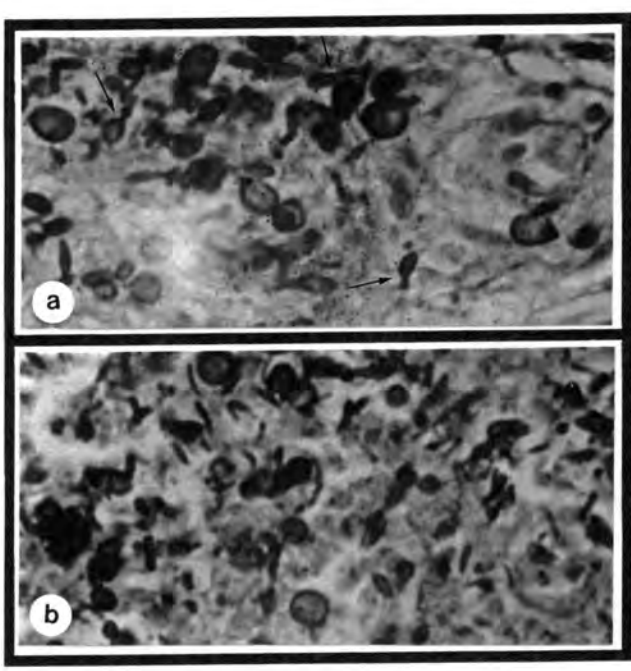


FIGURE 39a&b. Cigar bodies, budding yeasts, (arrows) and large globose yeast-like organisms in tissue (PAS, 40x lens)

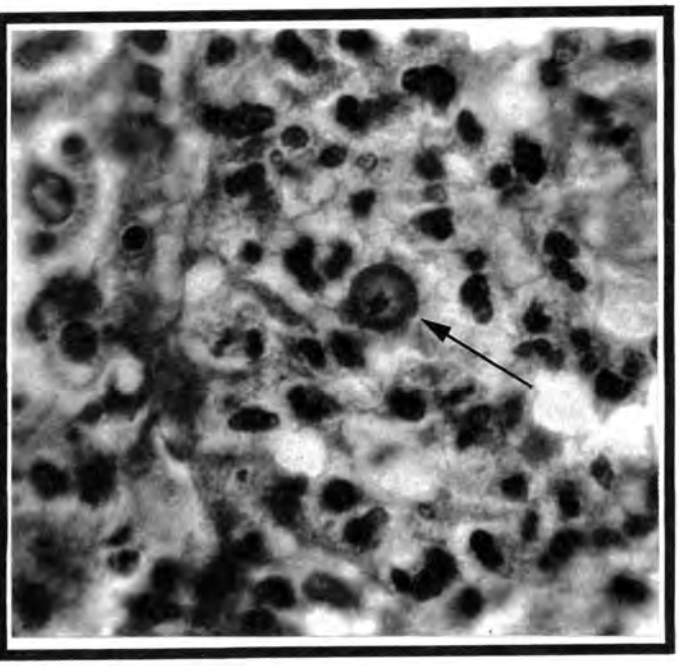


FIGURE 40. Pre-asteroid in sporotrichotic skin lesion (arrow). PAS, oil immersion lens

Although asteroids are diagnostic in sporotrichosis they are not a unique feature as other fungal diseases also produce these in tissue (see Chapter IV for discussion).

Asteroid bodies represent an antigen-antibody reaction between the yeasts cells of *S. schenckii* and the host tissues, and their formation can be described as follows. Conidia and hyphae from contaminated plant material and soil, i. e. the filamentous phase of *S. schenckii*, are inoculated into the skin. Due to the higher body temperature and the physiology of the fungus, they transform into a yeast phase and produce budding yeast-like forms and cigar bodies in the tissue. In turn some of these yeasts and cigar bodies round off to form pre-asteroids. Pre-asteroids are larger rounded or globose yeast cells with a double contoured cell wall (Figure 40) which may go over into asteroid formation once an antigen-antibody reaction precipitates out around them (Figure 41). Generally, pre-asteroids are more often seen than asteroids. The central cell of an asteroid is viable, as can be shown in Figure 42, where it is seen to germinate.[24] This unique *in vivo* demonstration of a germinating asteroid body was seen in lung tissue infected with *S. schenckii* which was processed three days after the death of a patient with primary pulmonary sporotrichosis (Berson and Brandt, 1977). The cooler body temperature after death had allowed the yeast cells to germinate and to transform back into the filamentous form of the fungus.

Although it was not possible to established in how many cases included in patient groups I-IV, asteroid bodies were present on histology, it is estimated that they occur in approximately 30% of cases.[25]

[24] With courtesy of Dr Berson (Berson and Brandt, 1977)

[25] Prof I W Simson - personal communication.

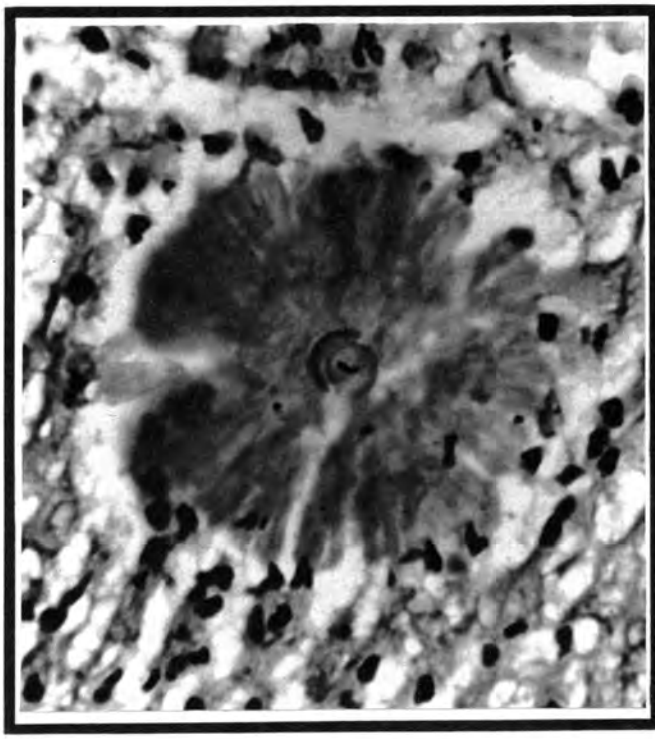


FIGURE 41. Classical asteroid body in tissue. Localized sporotrichosis. (PAS stain; Oil immersion lens).

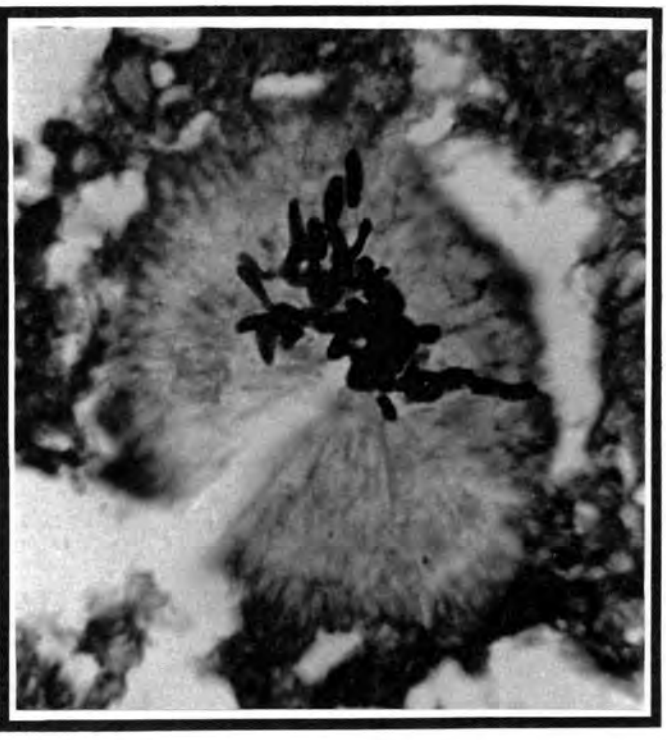


FIGURE 42. Germinating asteroid body in lung tissue. (PAS stain; Oil immersion lens).

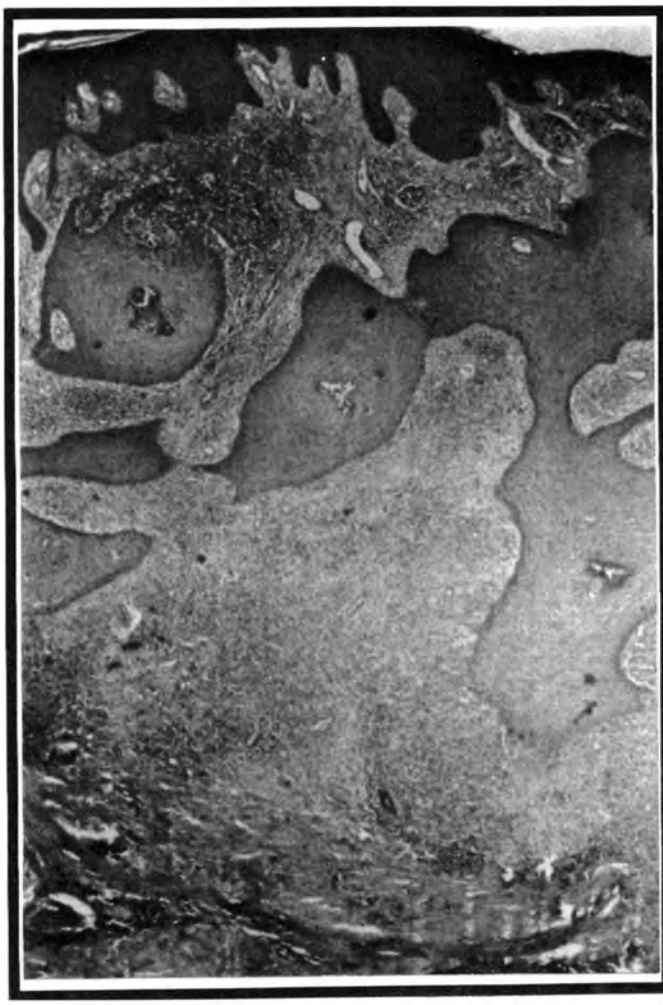


FIGURE 43. Microabscess formation and pseudoepitheliomatous hyperplasia. (H&E stain; 4x lens).

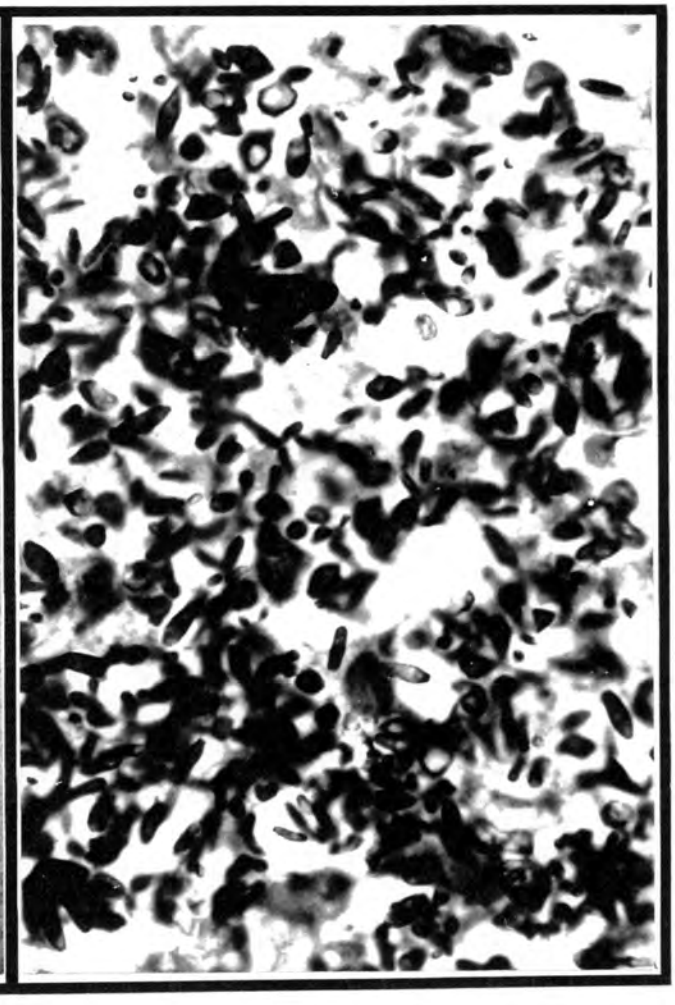


FIGURE 44. Intracellular and extracellular masses of yeasts and cigar bodies. (PAS stain; Oil immersion lens).

1.8.2 Tissue reaction and forms of *S. schenckii* in a immunocompromised host

The 77-year-old patient described in paragraph 1.7.2 (p. 46) is of relevance here. Histologically this unique case of localized sporotrichosis showed that -

- There was full thickness skin necrosis in the lesions. No surface vegetation or spread in the papillary layer of the skin was present. Pseudoeptheliomatous hyperplasia was noted at the periphery of the lesion. An example of pseudoeptheliomatous hyperplasia is illustrated in figure 43;

- Intracellular and extracellular masses of yeast-like and cigar body forms, outnumbering the host's inflammatory cells, could be demonstrated. Multiple budding, aggregation and conglomeration of the organisms was noted (Figure 44);

- Asteroid body formation was absent.

With the conventional Gram stain, direct microscopy done on pus swabs obtained from the lesions of this patient showed numerous Gram positive budding yeast cells and cigar bodies (Figures 45a, 45b, 46 and 47). Thick pus smears from the same patient, kept overnight at room temperature and then stained with PAS and Gram stain, showed germinating cigar bodies, partial yeast elongation as well as hyphal formation. With the Gram stain, the yeast cells and yeast elongation stained gram positive while the hyphal parts stained gram negative (Figures 45a, 45b and 46). All organisms and hyphal filaments were PAS positive (Figure 47).

These features are unusual in the direct microscopy of pus from sporotrichotic lesions. Even after incubation and using the methods described in Chapter II, paragraph 2.1.1 (p. 15), yeasts and cigar bodies are seldom detected on direct microscopy from patients with either lymphocutaneous or localized sporotrichosis.

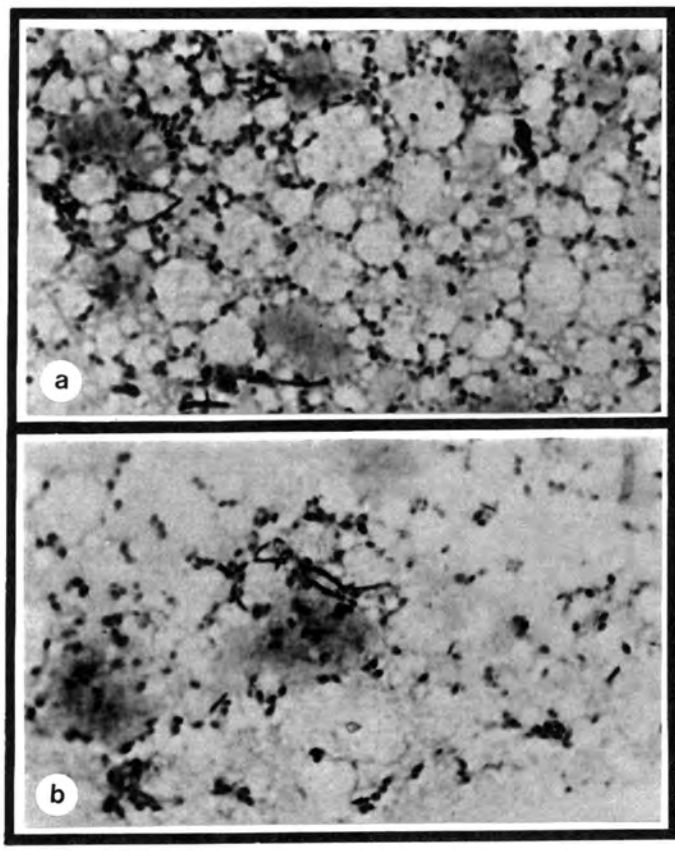


FIGURE 45a&b. Gram positive cigar bodies from pus. (Oil immersion lens)

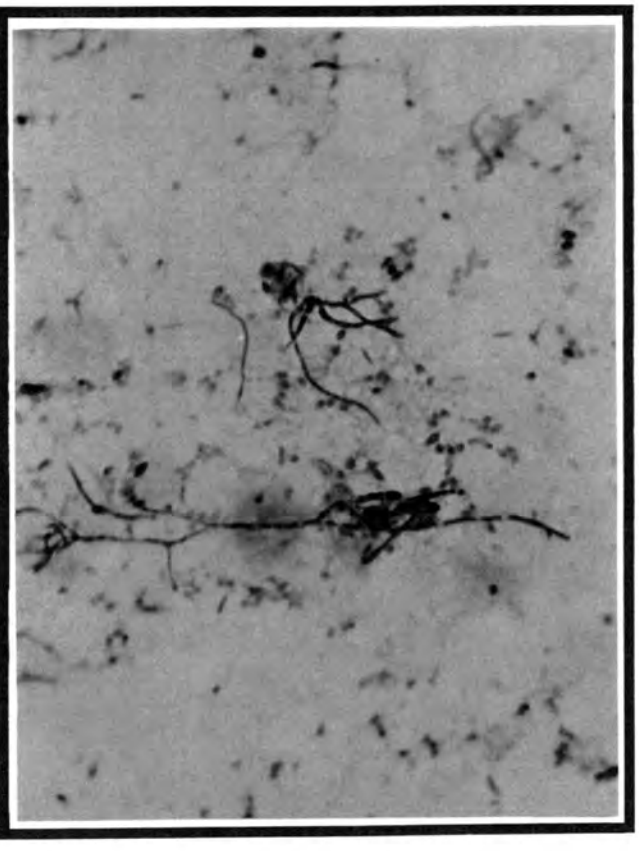


FIGURE 46. Gram stain of pus. Yeast to mycelial formation. (Oil immersion lens)

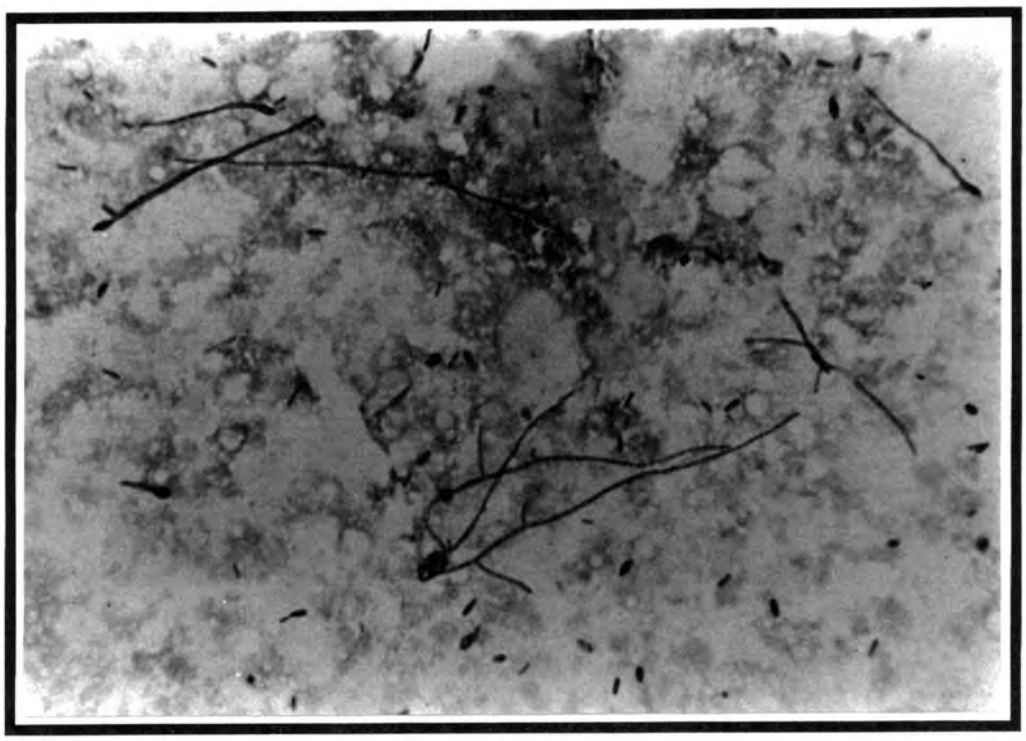


FIGURE 47. Several germinating yeasts cells and cigar bodies in pus smears incubated overnight at room temperature (PAS stain; Oil immersion lens)

1.9 TREATMENT OF SPOROTRICHOSIS

The methods used to collect and process material and to culture *S. schenckii* from clinical specimens and to prepare such cultures for microscopy, for all treatment regimens, are described in Chapter II, paragraphs 2 (p. 15) and 3 (p. 18), respectively.

Since the early 1970's mycological research consultations were done in close collaboration with Prof G H Findlay, dermatology consultants at the H F Verwoerd and Kalafong Hospitals, as well as private dermatologists in Pretoria. During this time it was found that good cultural results could be obtained by removing crusts or taking superficial scrapings from sporotrichotic lesions. A primary positive culture rate of 97% could be obtained. This method was particularly valuable in the case of small children and babies, making the invasive procedure of taking biopsies unnecessary. Cultures were therefore a reliable means to confirm the clinical diagnosis of sporotrichosis. Because of the reliability of this method it was used throughout the treatment trials to determine the viability of the fungus. The protocols of both the itraconazole and terbinafine trials called for the confirmation of the clinical diagnosis of sporotrichosis by either culture or histopathology. In cases where the first culture was negative, a second attempt to culture the fungus was often positive to confirm the diagnosis of sporotrichosis.

For the purpose of this study the efficacy of the antimycotic drugs, itraconazole and terbinafine in sporotrichosis with emphasis on the mycological findings, and less so on the clinical details, will be entered into. The same strategy will be followed for the more conventional treatments for sporotrichosis, namely potassium iodide and amphotericin B.

1.9.1 Potassium iodide and amphotericin B

Potassium iodide has been used in the treatment of sporotrichosis since the turn of the century when its value as a sporotrichotic drug was discovered (De Beurmann and Ramond, 1903). Most patients with cutaneous and lymphocutaneous cases of sporotrichosis respond well to potassium iodide. In extracutaneous and systemic sporotrichosis the reaction is not as favourable and amphotericin B, which has to be administered intravenously and has severe side-effects , must be used. For this reason alternative drugs have been investigated.

Potassium iodide was used in the patients in Group I (patients from H F Verwoerd and Kalafong Hospitals) with localized and lymphocutaneous sporotrichosis. A total of 88 cases could be traced (1966-1990) and were retrospectively evaluated by means of mycological, photographical and histopathological records. Their age, sex and race is recorded in paragraph 1.2 (p. 33), while the seasonal and geographic distributions for these patients are summarized in paragraphs 1.4 (p. 37) and 1.5 (p. 40) respectively. Infection sites were mainly on exposed parts of the body and extremities, as illustrated in Figures 16 to 26.

From the records of these patients, documented side-effects caused by potassium iodide included malaise, gastrointestinal upsets, headache, fever and coryza-like symptoms as well as acneform rashes and iododermas. As far as could be deduced, decrease in the dosage of the potassium iodide generally overcame these symptoms. Treatment periods varied from 4 weeks to 5 or 6 months. On average four to eight weeks were necessary to see a marked improvement in the clinical picture and clearing by three to four months treatment.

By using the methods described in Chapter II, paragraphs 3 (p. 18) and 4 (p. 19), mycology cultures had been obtained in all cases of sporotrichosis to confirm the diagnosis. Where clinical progress was not satisfactory, additional culture material was taken on return visits. Repeat cultures were also done during routine follow-up visits. The results showed that in spite of good clinical improvement,

and in some cases only minor scaling of the lesions being present, *S. schenckii* could still be cultured from the lesions. The fungus could also be recultured in cases where minor flare-ups or relapses of the sporotrichosis occurred, due to other systemic disorders or side-effects of the drug.

The unusual case of localized sporotrichosis described in paragraph 1.7.1 of this Chapter (p. 45), cleared completely on potassium iodide after initial hospitalization and controlled medication for both unrelated skin diseases. Mycological examinations done on several occasions during treatment also remained positive.

The second patient in this group that did not react favourably to potassium iodide was a case of cutaneous plus arthritic sporotrichosis (see paragraph 1.7.2, p. 46). Potassium iodide was not absorbed systemically due to enteropathy. Amphotericin B was given intravenously to effect cure in this patient. Mycological cultures became negative within the first three weeks of treatment and remained so for the full course of the treatment. The skin lesions cleared completely, but the arthritic sporotrichosis recurred. An additional course of amphotericin B resulted in cure. Cultures were not obtained at this time.

In one patient the use of potassium iodide was not considered because the lesions were dangerously near the eye. See paragraph 1.7.1 (p. 45) and figures 19 and 20. Hospitalization and treatment with amphotericin B, effected clinical and mycological cure without any relapse.

1.9.2 Itraconazole (Sporanox)

Potassium iodide and amphotericin B have been the only effective drugs for the treatment of sporotrichosis until recently when imidazole, triazolic and allylamine compounds were developed as possible alternative drugs for mycoses including sporotrichosis.

Itraconazole is a very lipophilic triazole derivative developed by Janssen Pharmaceutica (Pty) Ltd. Its mechanism of action involves the inhibition of ergosterol biosynthesis in the fungal cell membranes. The resultant impaired cell development and/or irreversible cell degeneration cause fungal death.

Sixty one patients with sporotrichosis completed this treatment trial (patient Group II). Their ages, sex and race are given in paragraph 1.2 (p. 33) , while the seasonal and geographic distributions are given in paragraphs 1.4 (p. 37) and 1.5 (p. 40) respectively. It may be seen that patients of all ages and both sexes were therefore treated with this drug. All suffered from cutaneous sporotrichosis, were otherwise healthy and had no systemic illnesses. Itraconazole was given in a dose of 100mg daily. Only patients with a positive culture or histology compatible with a diagnosis of sporotrichosis were entered into the trial. Two patients had negative cultures, but the histological examination confirmed sporotrichosis. The cultures remained negative for the full period of the treatment and the treatment was curative in both. The patients were assessed clinically by the dermatologist at monthly intervals when cultures were repeated. The required special laboratory investigations, according to the clinical protocol were done at these visits.

In 56 patients the disease had been present for at least 20 weeks before the specialist was consulted. Three patients gave histories of a year while two had the disease for more than a year.

An average treatment period of 12 to 16 weeks was necessary to establish a clinical and mycological cure. Clinical improvement was seen within the first four to eight weeks of treatment and the cultures became negative on average eight weeks after the treatment was started. The additional four to eight weeks treatment was necessary to ensure complete clinical resolution of all lesions. Fifty nine of the 61 patients were cured within 16 weeks. In one of the other two patients cultures were negative at eight weeks, but again became positive at 12 weeks. From 16 weeks onward cultures remained negative. The second

case relapsed with positive cultures at 16 weeks after treatment had been started and then twice again in the following year before a cure was effected.

In summary therefore, all 61 patients with cutaneous sporotrichosis were cured with itraconazole. No adverse clinical side-effects was documented by the dermatologist.

1.9.3 Terbinafine (Lamisil)

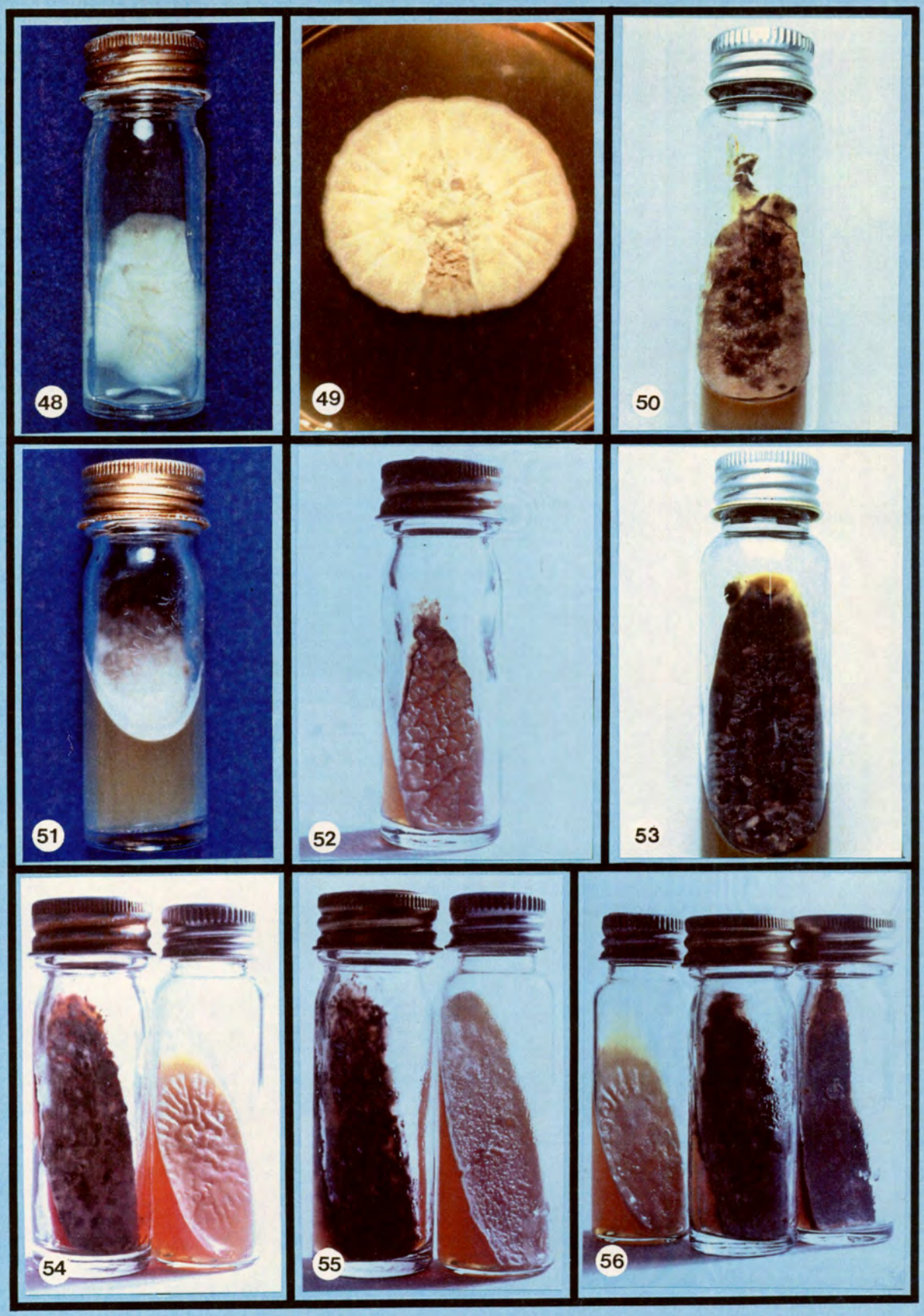
This is the first trial in which terbinafine has been used to treat cutaneous sporotrichosis. For patients to be entered into this trial, they had to be older than 18 years, not pregnant or known to be suffering from any systemic or haematological diseases. Five patients, all white males between the ages of 20 and 73 years old qualified. They are included in patient Group III and their statistics are recorded in Table 2 and paragraphs 1.2 (p. 33) and 1.5 (p. 40). All five patients had cutaneous sporotrichosis, four with the lymphocutaneous type and one with localized sporotrichosis. In four cases the duration of the disease was 8, 10, 11 and 13 weeks respectively, while sporotrichosis had been present for 40 weeks in the fifth case. Terbinafine was given in a dose of 500mg daily in two divided doses. Duration of the treatment was 8, 12, 14, 19 and 20 weeks, given until a clinical and mycological cure was achieved. Cultures were done at monthly intervals using the same methods described previously. Cultures became negative within eight weeks in three cases, while in the remaining two patients it took 12 and 30 weeks respectively to achieve negative cultures. Treatment was continued for two weeks (one case), four weeks (three cases) and 15 weeks (one case) after the cultures became negative. No correlation could be established between the number of weeks treatment, the number of weeks to a negative culture and the clinical type of the disease. No adverse side-effects to terbinafine were recorded in any patients and all laboratory parameters remained within normal limits.

2 CULTURAL MORPHOLOGY OF *S. SCHENCKII* AT 25°C

2.1 Macroscopic cultural morphology of *S. schenckii* at 25°C

The methods used to collect and process material and to culture original isolates of *S. schenckii* from clinical specimens, are described in paragraphs 2 (p. 15) and 3.1 (p. 18) of Chapter II.

S. schenckii is resistant to actidione, which was used to advantage to selectively culture this organism. The cultural morphology of *S. schenckii* varied greatly, even under similar conditions. Initial colonies on SDA at 25°C grew within three to five days after inoculation. They were star-like, glabrous and yeast-like in appearance, and became tough, wrinkled or folded in time. The colour of the colonies were either white-buff, to dark-buff, to brown or black (Figures 48 to 52). Cerebriform wedges were occasionally seen (Figure 49). The entire colony could have this appearance. More often however, both in the original and subculture, colonies formed radiating folds from the central point of the inoculum (Figures 48 and 49). Fine spicules, commonly centered around the inoculum, especially in young cultures, were present in any of these colony types (Figures 48 and 49). These disappeared with age. A variegated combination of all the colours mentioned, was common (Figure 50). Some cultures gradually transformed from the lighter to the darker colours with age, while others remained light or turned black after a short incubation period at 25°C. Pigmentation started in the thinner part of the agar, thus at the top end of the slope, where colonies spread over the entire SDA slope (Figure 53). Pigmentation also occurred along the margin of the agar, where it came in contact with the glass of the medium bottle (Figure 51). Pigment remained limited to the mycelial thallus (Figure 52), and did not diffuse into the agar. A brown diffusible pigment was however occasionally present in aged cultures. Sharp contrasts in the colour of cultures were evident where different media were used. For example, on 30cm³ SDA slopes, in 7 to 14 day old cultures, the colour remained classically buff, while on malt agar the



FIGURES 48 - 56. Cultural morphology of the mycelial phase of S. schenckii at 25°C: 48 - white to buff colony; 49 - darker buff colony with a cerebriform wedge; 50 - variegated culture form; 51 & 52 - white and buff colonies respectively, turning black in the thinner parts of the agar; 53 - older culture with more advanced pigmentation; 54 - malt agar, mottled black and brown colony (left), SDA - unpigmented colony (right); 55 - older culture on malt agar (left), SDA - unpigmented colony (right); 56 - cultures on SDA (left), malt agar - younger culture (middle), malt agar - older culture with a powdery appearance and completely black (right).

colonies were dark brown to black after three to four days (Figures 53 - 56).

Of special interest is the fact that there seems to be a correlation between pigment production in primary isolates and their clinical source. Strains of *S. schenckii* isolated from lymphocutaneous lesions produced pigment more readily then those isolated from localized lesions. *S. schenckii* grown from the osteolytic lesion in the patella, of the previously described case of extracutaneous sporotrichosis, was a black pigmented strain. These cultures remained pigmented on subculture into SDA. In contrast, the strain isolated from the case with pulmonary sporotrichosis, proved to be unpigmented and remained so in subculture (for these case descriptions see paragraph 1.7.3, p. 53).

When *S. schenckii* was subcultured, single point inoculations were difficult to perform as several additional colonies grew in the line of transferal. Stereo-microscopy, at 40 times magnification, showed detached conidia on the surface of the agar, germinating at the same time as the larger inoculum. Thus, conidia easily "dropped" from the inoculum onto the agar. Special care had therefore to be taken, not to contaminate the surroundings or to inoculate the hands or eyes while working with the fungus.

In addition, once the colony thallus was fully developed, it became difficult to remove small pieces of the colony, as the entire mycelial mat tended to be lifted free from the agar surface with a needle, especially in cultures with a lighter colour, due to the structural formation of the mycelial mat (see descriptions paragraph 9.2, p. 123). Subculturing was done by cutting pieces out of the mycelial mat by means of a sterilized scalpel, in order to obtain inocula of required size.

In summary, under the same cultural conditions, it was found that strains of *S. schenckii* do not behave alike. There is considerable variation in the time when the pigment appears, the intensity of the

pigment, the shade of the pigment as well as the distribution of the pigment in a colony. In general, the lighter colonies were more velvety and had a tough matted thallus, while the black cultures were more wrinkled and corrugated, but less tough and matted.

2.2 Microscopic cultural morphology of *S. schenckii* at 25°C

Microscopic preparations made from cultures using either lactophenol colourless or lactophenol cotton blue as mounting and staining media, were found to be successful. The method described in Chapter II, paragraph 3.2 (p. 19), was followed.

For the purpose of this study a synnema will be referred to as a group of parallel running hyphae, producing conidia laterally, and synnemata as groups of these structures. Coremia are considered synonymous with synnemata.

S. schenckii is a hyphomycete belonging to the Deuteromycotina (Fungi Imperfecti). It is a dimorphic fungus and grows in the mycelial phase at 25°C. Microscopically this phase of the fungus consisted of thin, septate branching hyphae, 1 - 2μm in diameter. Undifferentiated hyphae had the tendency to run parallel to one another, forming synnemata consisting of several mycelial strands each. An actively growing hypha could "turn" to run parallel to another already existing hypha (Figures 57a, 57b, 58a and 58b). Anastomoses (cross-connections) were regularly formed between mycelia in a synnema (Figures 58a and 58b). Conidiophores arose at right angles on any of the undifferentiated hyphae, therefore erect, and they tapered towards the tip (Figure 59). Some could be recumbent. Conidiophores had the same diameter as hyphae at their base, 1 - 2μm, and measure 0,5 - 1μm at the tip. They could also develop at any hyphal tip, thus terminating hyphal growth and forming conidia (Figures 60 and 61). Conidiophores varied greatly in length (Figure 59), averaging 15 - 20μm, in most isolates.

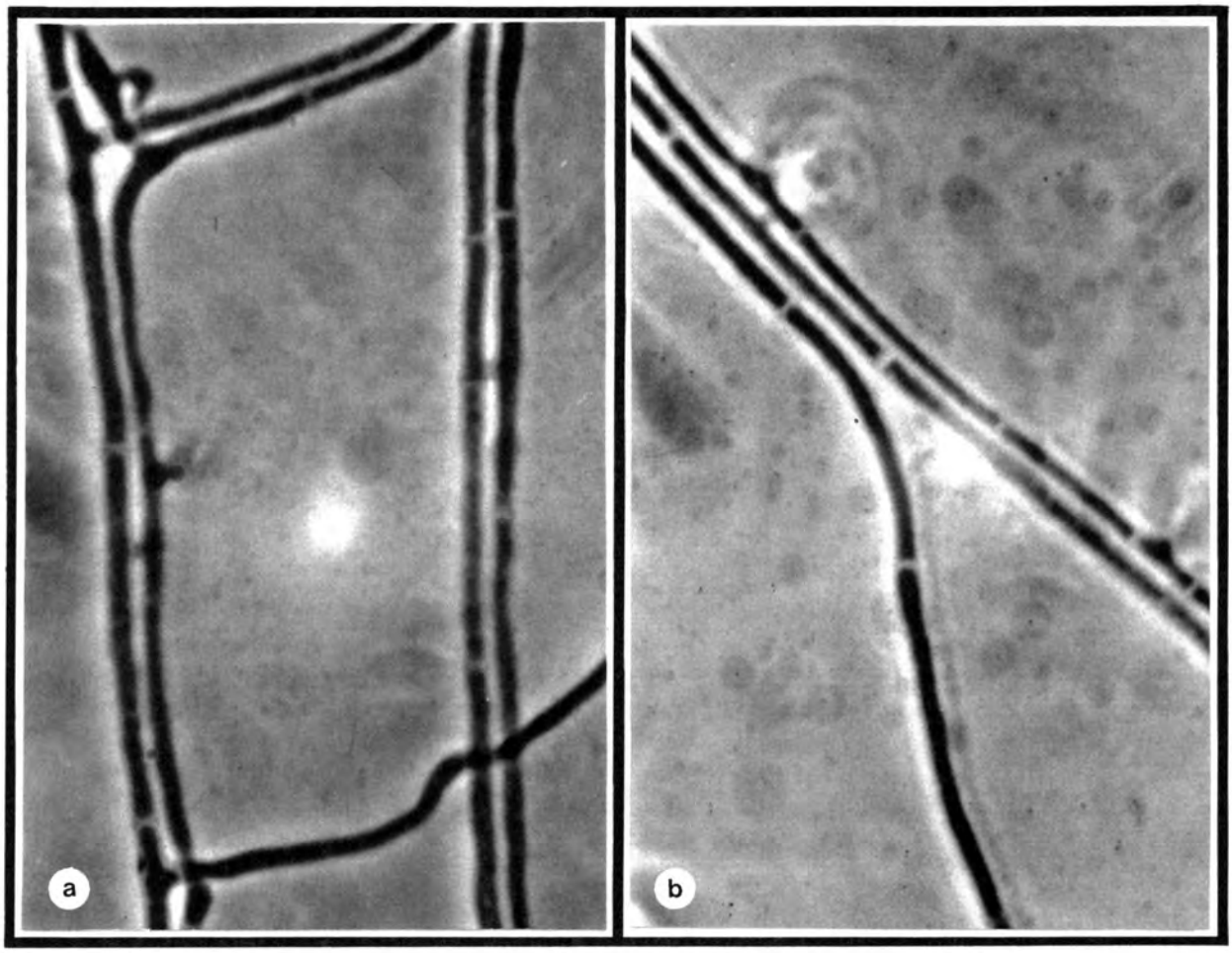


FIGURE 57a&b. Synnemata formation - undifferentiated hyphae running parallel. (40x lens)

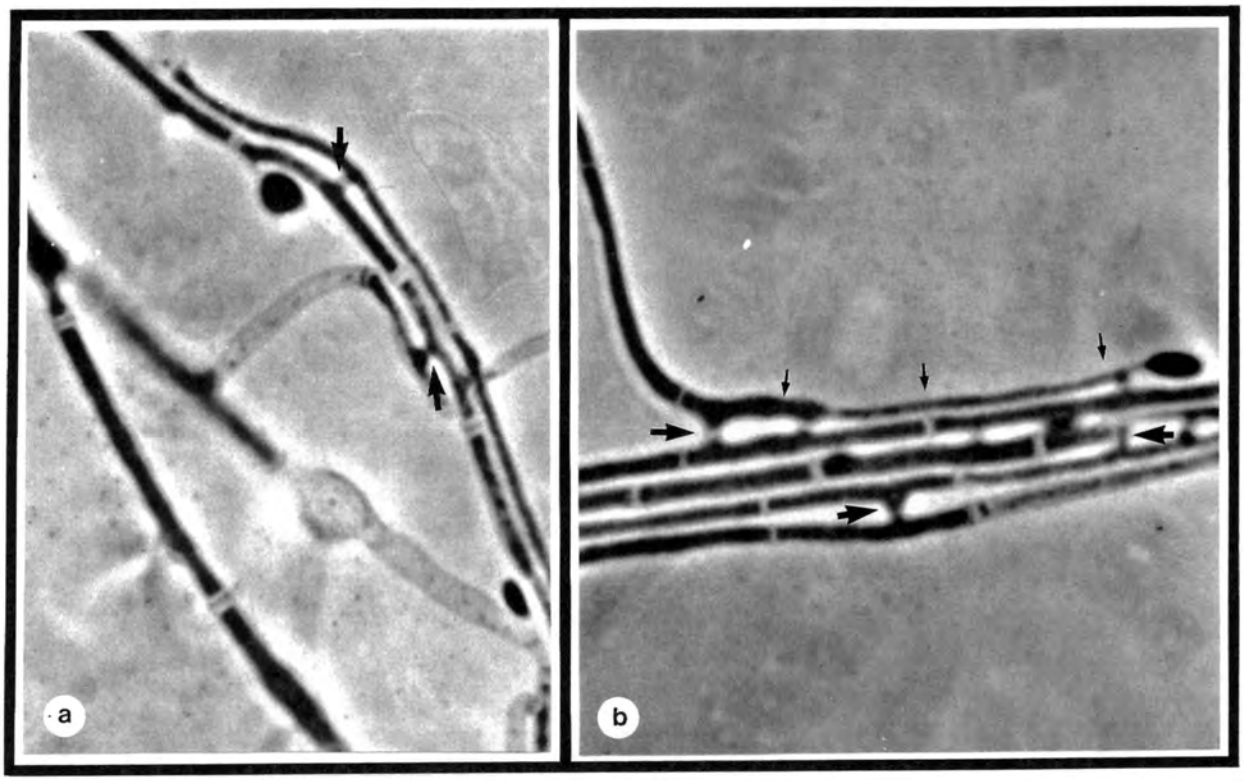


FIGURE 58a&b. Synnemata formation. Conidiophore producing a terminal conidium which forms part of the synnema (small arrows). Anastomoses between the hyphae are evident (large arrows). 40x lens

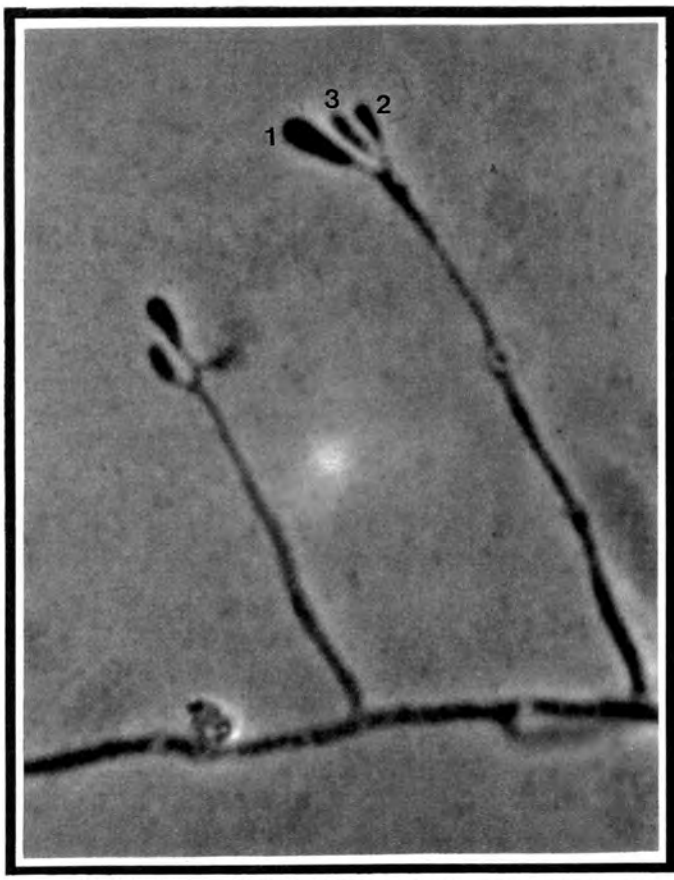


FIGURE 59. Conidiophores arising at right angles to the hyphae. 1, 2 & 3 = older to younger conidia on denticles. (40x lens)

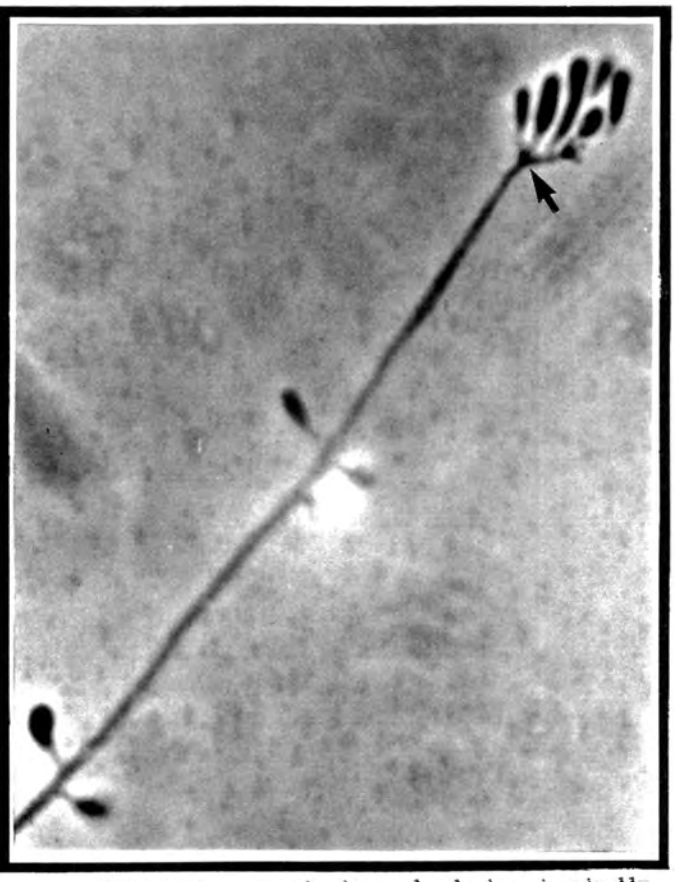


FIGURE 60. Conidiophore developing terminally. Conidia on denticulate vesicle (arrow). (40x lens)

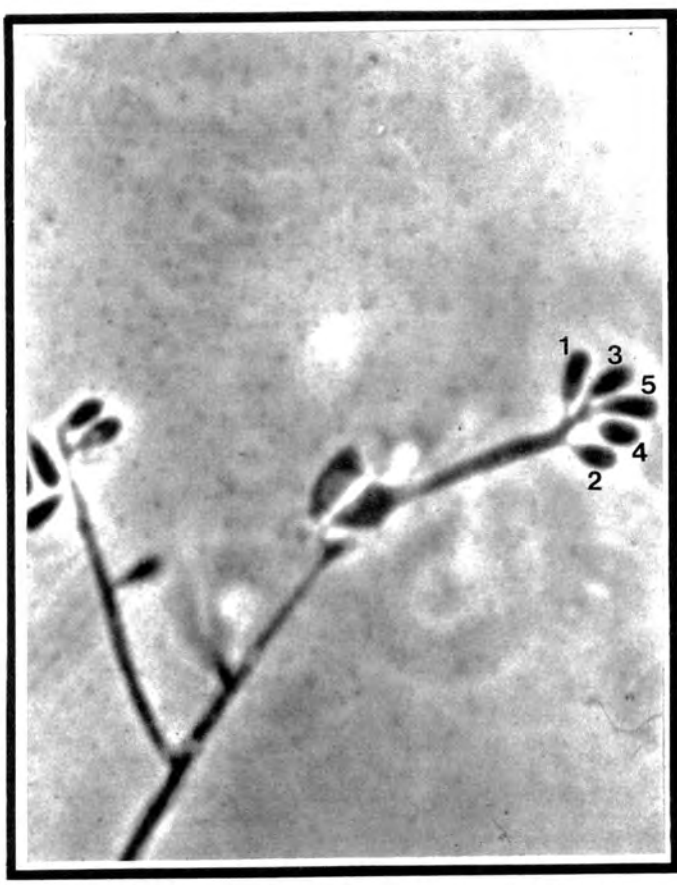


FIGURE 61. Conidiation advancing. Rosette of 5 conidia. 1 = older conidium, 5 = younger conidium. (40x lens)

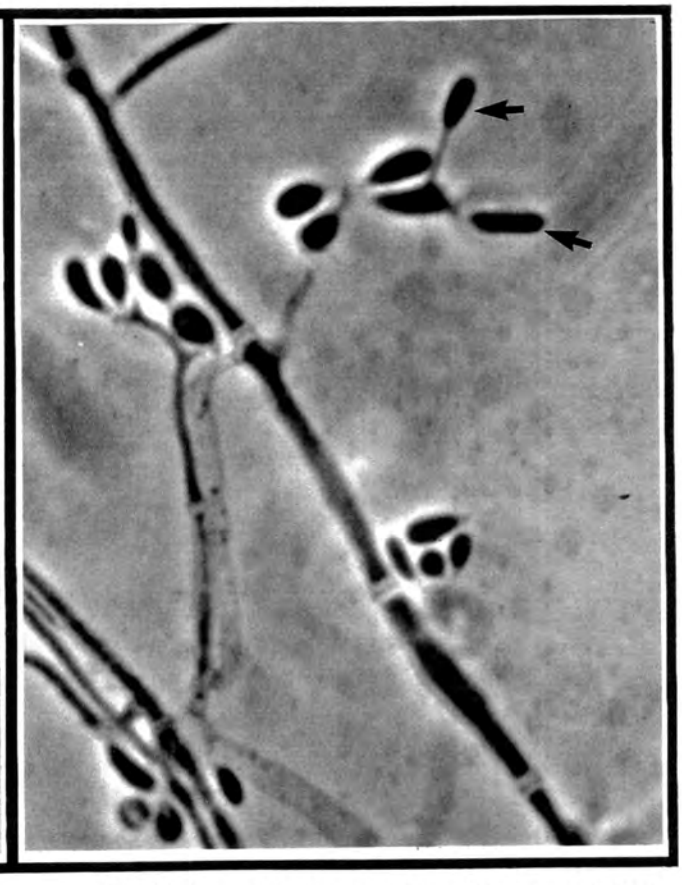


FIGURE 62. Primary conidium with two secondary conidia on denticles (arrows). (40x lens)

Conidium formation was holoblastic. Initial conidiation took place at the apex of the conidiophore, after the formation of a denticulate vesicle which bore conidia sympodially (Figure 60). At first, one or three to five conidia were present per vesicle, the older conidium being at the proximal end and the youngest at the distal end (marked 1, to 3 or 5, in Figures 59 to 61, respectively). A rosette of conidia was formed (Figures 59 - 61). These young conidia were simple, thin-walled, ovate, hyaline, 2 - 3μm broad and 3 - 6μm long, and were attached by means of a thin denticle. A distinct detachment septum left the conidia with a straight detachment base. Part of the denticle therefore remained on the vesicle. These features are best illustrated by means of scanning electron microscopy (see paragraph 9.2, p. 125). Secondary conidia could form at the unattached end of any of the conidia, thus acropetalously forming a cluster in some cases (Figure 62). Conidiation progressed beyond this, forming sleeves of conidia along the conidiophores (Figures 63 and 64). Any cell of the undifferentiated hyphae could also produce conidia laterally (Figure 65a), forming thick sleeves of conidia along the hyphae (Figure 65b). These laterally produced conidia could not be correlated with sessile conidia, but they were formed in a similar manner as on the conidiophore, i. e. on a denticle, therefore by orthotopic branching (Figures 63 - 65). Secondary conidia could also be present here. All conidia were of fairly uniform or constant shape at this stage of development, namely ovate and slightly tapering at the tip, and measuring the same as before. As the colony aged, the sizes of the conidia could vary quite considerably. This variation could be related to the stage of development of the conidium and its volume, i. e. younger hyaline conidia (4 to 14 days) were smaller (Figure 65a), while the older conidia (14 to 21 days) with pigment starting to appear, were larger. As soon as pigmentation became evident in the conidia, their size increased to 6 - 8μm long and 4 - 6μm broad (Figure 65b), and their measured volume was larger (see paragraph 5.3, p. 92).

An interesting feature regularly found in cultures was the enclosure of laterally produced conidia on a hypha by other parallel running

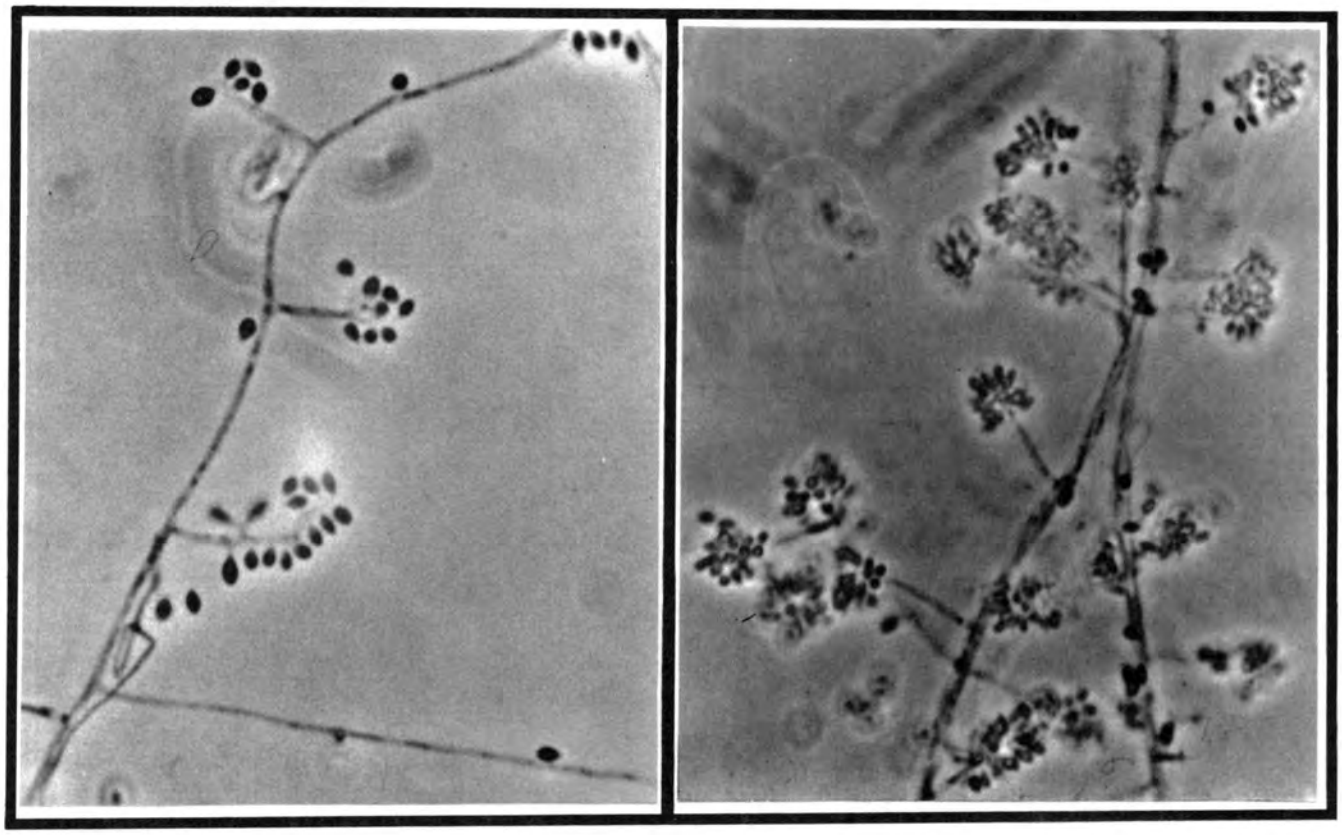


FIGURE 63. Sleeves of conidia present along the conidiophores. (20x lens)

FIGURE 64. Conidiation in a more advanced phase than illustrated in Figure 63. (20x lens)

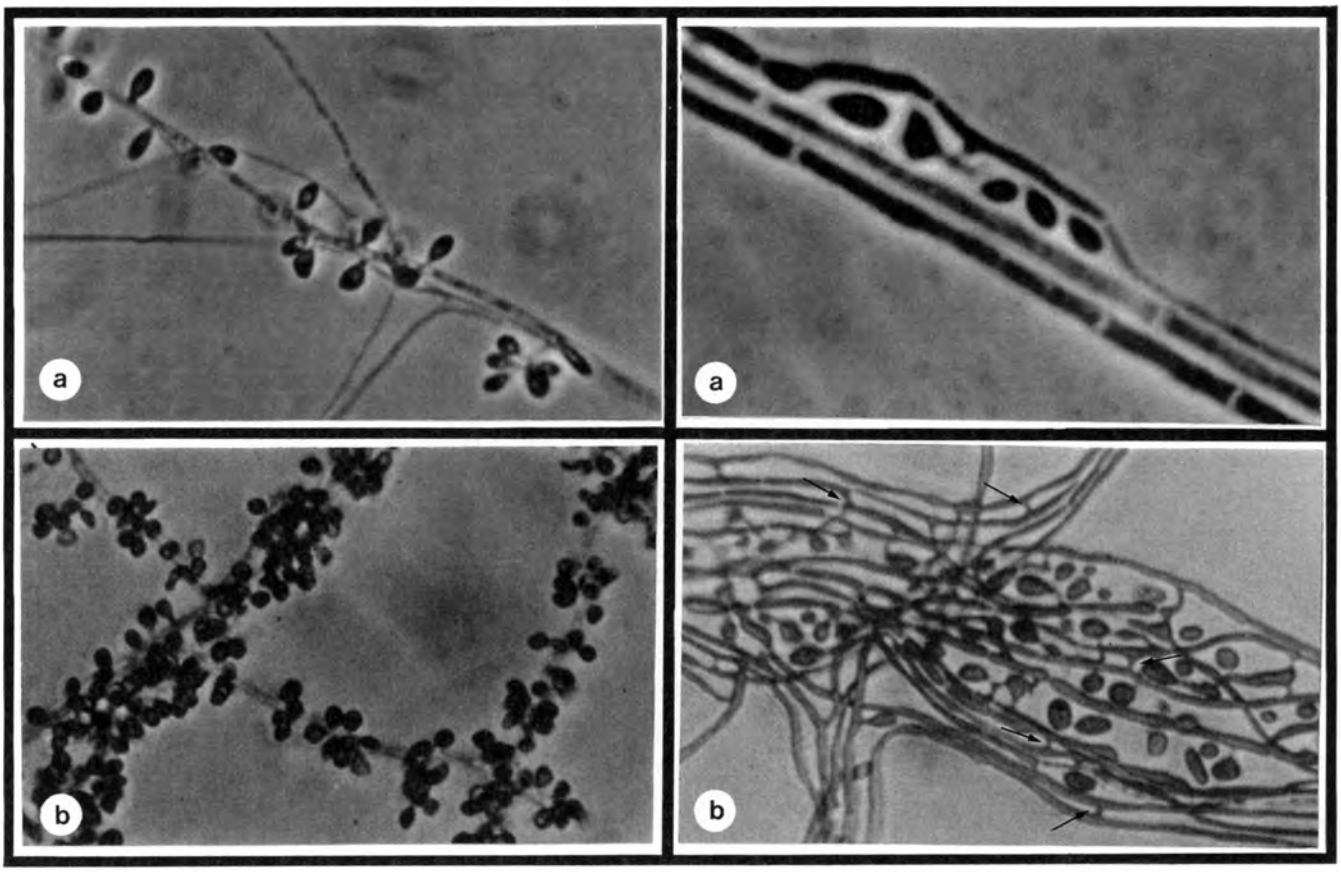


FIGURE 65. a) Single conidia forming laterally on undifferentiated hyphae (20x lens). b) Thick sleeves of conidia along the hyphae. (20X lens)

FIGURE 66a&b. Conidia enclosed by parallel running hyphae. Anastomoses are present (arrows). (40x lens)

hypha. Although these conidia seemed to be endoconidia, they could not be confirmed as such (Figures 66a and 66b).

Triangular conidia were only found in pigmented strains of *S. schenckii*. They normally appeared on SDA after two to six months incubation at 25°C. On malt agar they became evident after three weeks in most strains.

3 MYCELIAL TO YEAST CONVERSION OF *S. SCHENCKII* AT 37°C

3.1 Macroscopic cultural morphology of *S. schenckii* at 37°C

Dimorphism in *S. schenckii* could be confirmed by applying the methods described in paragraph 4.1 of Chapter II (p. 19).

All pathogenic isolates of *S. schenckii* converted readily from the mycelium phase grown at 25°C to the yeast phase grown on blood and BHI agar at 37°C. On blood agar the colonies were buff coloured, smooth to slightly roughened and waxy in texture. They were submerged into the agar and reached a maximum size of approximately 1-2mm in diameter after 7 to 14 days incubation (Figure 67). Once these cultures were removed from the higher temperature and kept either at room temperature or at a constant temperature of 25°C, they converted back to the mycelial phase on both blood and BHI agars, as also happened when they were re-inoculated into SDA. The yeast to mycelial conversion took place within three to six days.

3.2 Microscopic cultural morphology of *S. schenckii* at 37°C

Cultures of *S. schenckii* were monitored for mycelial to yeast conversion by using the methods described in Chapter II, paragraph 4.2 (p. 20). Either lactophenol cotton blue preparations or gram stains, viewed under oil immersion, were used to monitor the extent of

conversion. In fully converted colonies, yeast cells were uniformly oval in shape with or without buds, and were gram positive. Some larger globose yeast cells were also present. Hyphal remnants and conidia from the mycelial phase as well as non-viable yeast cells, stained gram negative (Figure 68). At the higher temperature, conidia from the mycelial phase germinated. The germ tubes did not extend, but directly gave rise to blastoconidia (yeasts cells). Hyphae from the mycelial phase tended to loose their rigid structure and form "oidia" within the hyphae, which fragmented. Oidia in turn formed blastoconidia which formed yeasts by single or multiple budding. Some club shaped structures developed either at the hyphal tips or laterally on the hyphae, directly giving rise to blastoconidia.

4 PRESENCE OF *S. SCHENCKII* IN SOIL, COMPOST OR ON VEGETATION SAMPLES

The methods of Feuerman *et al.* (1976) and Kenyon *et al.* (1984) were used to isolate *S. schenckii* form soil (see Chapter II, paragraph 11.2, p. 30). Such isolations were only attempted where patients had become infected from their surroundings, and soil and/or plant material could be collected.

Once a strain was isolated from soil and it showed macroscopic and microscopic morphological characteristics similar to *S. schenckii*, confirmation that it was indeed *S. schenckii* was difficult. There are no physiological tests that can be applied to identify *S. schenckii*. It has a requirement for thiamine like many other fungi. One way is to test the ability of these strains to convert from the mycelium phase at 25°C to the yeast phase at 37°C. Pathogenicity to animals further confirms soil isolates as being *S. schenckii*. Therefore, all soil isolates were tested for conversion at 37°C, and some selected soil strains were injected into rats.

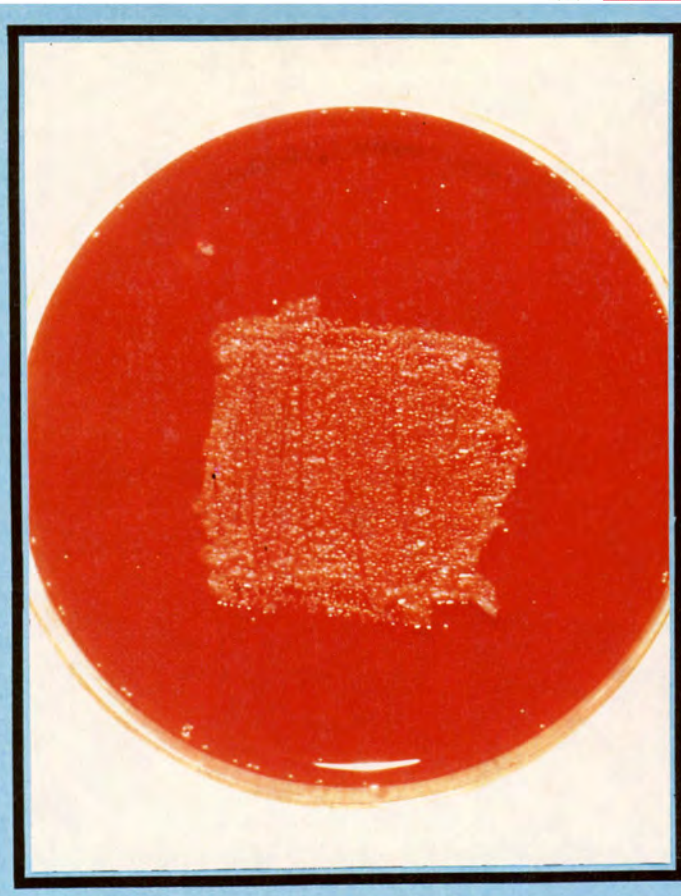


FIGURE 67. Yeast phase colonies of S. schenckii grown on blood agar at 37°C.

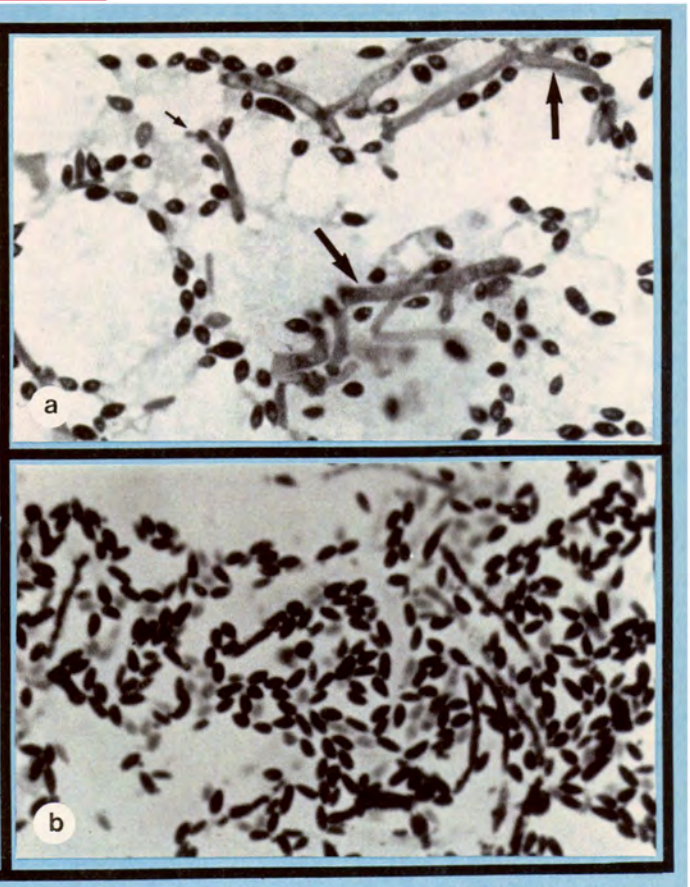


FIGURE 68. Yeast phase of S. schenckii at 37°C. a) Hyphal remnants - large arrows. Hyphae forming yeasts - small arrow. PAS stain. b) Gram stain.

FIGURE 69. S. schenckii isolated from soil. Arrows indicate smooth wax-like colonies with spicules.

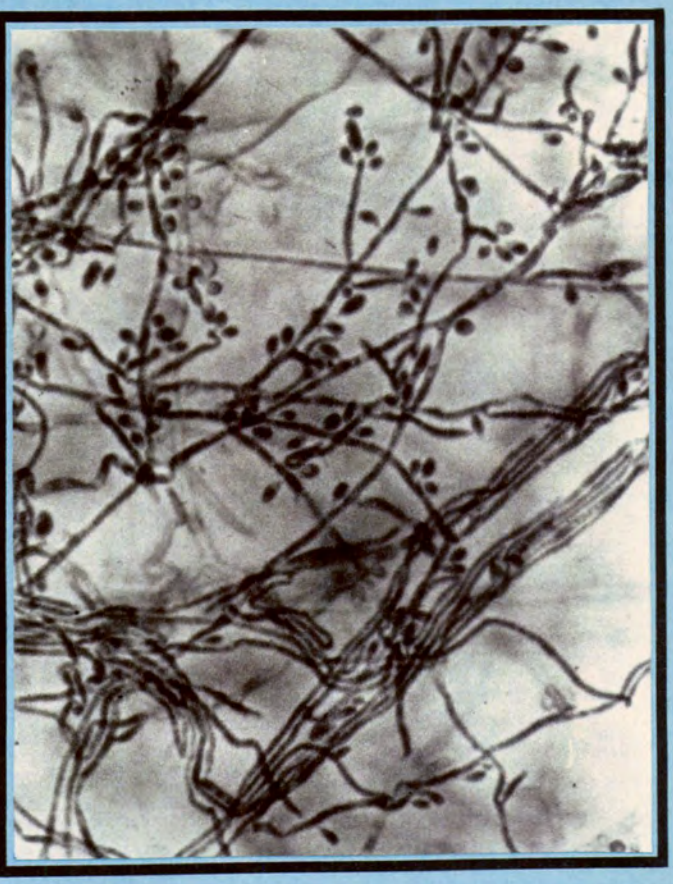


FIGURE 70. General microscopic picture of S. schenckii isolated from soil. (40x lens)

4.1 Direct microscopy

Direct microscopy was done where visible mycelial growth was evident on dead plant material. The objective here was to find triangular conidia or other conidia of the *S. schenckii* type. This method proved to be of little value in the case of plant material gathered from nature as the microscopic characteristics of *S. schenckii* are not distinctive enough to allow definite identification of the fungus. This method could however be applied with success where wood was inoculated with strains of *S. schenckii* in order to stimulate the formation of triangular conidia.

4.2 Macroscopic cultural morphology of soil isolates of *S. schenckii*

The ability of *S. schenckii* to grow in the presence of actidione makes it possible to isolate this fungus from soil as many of the other saprophytic organisms present in soil are suppressed. However, several saprophytic soil fungi are resistant to actidione. Cultures on SDA at 25°C were therefore examined for growth on a daily basis once the soil supernatant had been inoculated onto several SDA agar plates following the methods described (see Chapter II, paragraph 11.2, p. 30). Any small wax-like colonies appearing within the first three to four days of incubation were subcultured onto additional SDA plates and further incubated at 25°C. This was necessary in order to select positive *S. schenckii* colonies before the sporulation or overgrowth of saprophytes. Obviously saprophytic fungi were discarded and colonies with a macroscopic cultural morphology similar to *S. schenckii* were then examined microscopically. Such colonies were inoculated onto blood agar or BHI agar and incubated at 37°C for possible conversion to the yeast phase. Soil isolates were also inoculated into malt agar, but none produced the characteristic black pigmentation on this medium to the same extent as found with the pathogenic isolates. Only spotted areas of the colonies turned brown with an occasional black spot among them.

In general, soil isolates of *S.schenckii* can be described as follows. Initial isolates on SDA at 25°C, were wax-like, smooth, white to buff-coloured, with spicules present in the centre of the colonies (Figure 69). Some colonies became a darker buff colour with age, but most remained unpigmented. Variegated pigmentation was however also seen. Soil strains did not readily have wrinkled thalli as seen in pathogenic strains, but they had the same tough, well matted thallus that could be lifted free from the agar. Colonies were smooth with only a few shallow radiating folds. Pigmentation was slight and was not formed to the same degree on malt agar as described for the pathogenic isolates.

4.3 Microscopic cultural morphology of soil isolates of *S. schenckii*

Soil isolates of *S. schenckii* had a microscopic cultural morphology very similar to the pathogenic ones. The same microscopic methods were used to examine them.

Apart from the fact that the hyphae were slightly thicker, i. e. 1 - 3μm in diameter, the conidia formed in some of the soil strains slightly longer (7μm - 9μm) and more slender, all the other microscopic characteristics were similar to those seen in the pathogenic isolates (Figure 70). Pigmented conidia, with sizes similar to the pathogenic strains, were encountered only from the pigmented areas of the colonies. Triangular conidia were seen to form only in some soil strains, and only after six to eight months incubation at 25°C.

4.4 Pathogenicity of soil isolates

In order to test the pathogenicity of some selected soil isolates, rat testes were inoculated by the methods described in Chapter II, paragraph 11.3 (p. 31). One hundred histopathological sections were microscopically viewed. The findings are summarized in Table 3.

By comparing the pathogenicity of the soil strains and their corresponding pathogenic strains, the histopathology could be interpreted as follows[26]. In the soil isolates the periorchitis was diffuse, with small areas of initial necrotizing granuloma formation, progressing to histiocyte infiltration, giant cell formation, and massive caseation necrosis (gumma production). The orchitis was slower to develop. Organisms were rare, and seen only as phagocytosed yeast-like cells (Figures 71 and 72). There was no sign of the larger globose double contoured free-lying elements characteristic of the virulent human strains. In the pathogenic "controls", the changes were of the typical kind, as given in Table 3 (Figures 73, 74a and 74b).

[26] Prof G H Findlay - personal communication and combined publication; Findlay and Vismer (1986)

TABLE 3. Comparative histopathology of the pathogenicity of S. schenckii isolates in rat testis.

SOIL ISOLATES (Figures 71 and 72)	HUMAN ISOLATES (Figures 73, 74a and 74b)
Periorchitis	**Periorchitis**
5 Days:	5 Days:
▪ Slight diffuse non-specific periorchitis. ▪ Foamy spaces were present. ▪ No organisms could be detected.	▪ "Abscess" in periorchitic tissue from the inoculation. ▪ Marked degenerative pyknosis of polymorphs and other poorly identifiable cells.
15 Days:	15 Days:
▪ Histiocytic minigranulomas were formed. ▪ Small foci of necrosis noticed. ▪ Organisms doubtfully identifiable.	▪ No organisms or PAS positivity. ▪ Periorchitis consists of a pannus of granulation tissue, and follicle-like arrangement of giant cell granulomas.
27 Days:	27 Days:
▪ Large area of caseation necrosis. ▪ A dermal nodule shows necrotic-hyaline granuloma (gumma). ▪ Small numbers of phagocytosed fungal cells seen in histiocytic giant-cell masses.	▪ Ghost-like traces of fungus in giant-cells. ▪ PAS positivity of cytoplasm of fungal cells. ▪ Miliary abscesses turn into tubercles. ▪ Lymphatic spaces filled with masses of cigar bodies.
Orchitis	**Orchitis**
5 Days:	5 Days:
▪ Reactionless injection trauma. ▪ Tubule dissociation and loss of interstitial colloid.	▪ Reactionless injection trauma. ▪ Intertubular spaces broadened with oedema.
15 - 27 Days:	15 - 27 Days:
▪ Diffuse interstitial histiocytic orchitis, packed solid with cells. ▪ Some giant cells present. ▪ Occasional phagocytosed yeasts, often present in clusters. ▪ Organisms never lying loose. ▪ No cigar bodies or large globose yeasts seen. ▪ Testis tubules severely damage by inflammation.	▪ Intertubular spaces filled with masses of necrotic cells. ▪ Globose cryptococcoid bodies in clusters. ▪ Masses of cigar bodies present. ▪ Inflammatory cells with clumping and disrupted nuclear material. ▪ Organisms surviving well in midst of necrosis - extracellular. ▪ Testis tubules lytic but not invaded

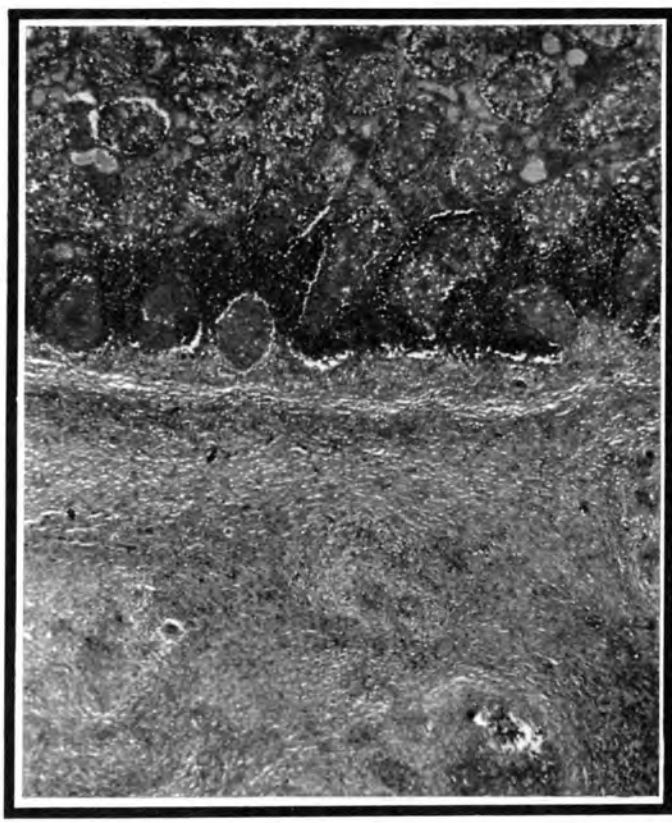


FIGURE 71. General histology of rat testis infected with soil isolates of S. schenckii. (4x lens)

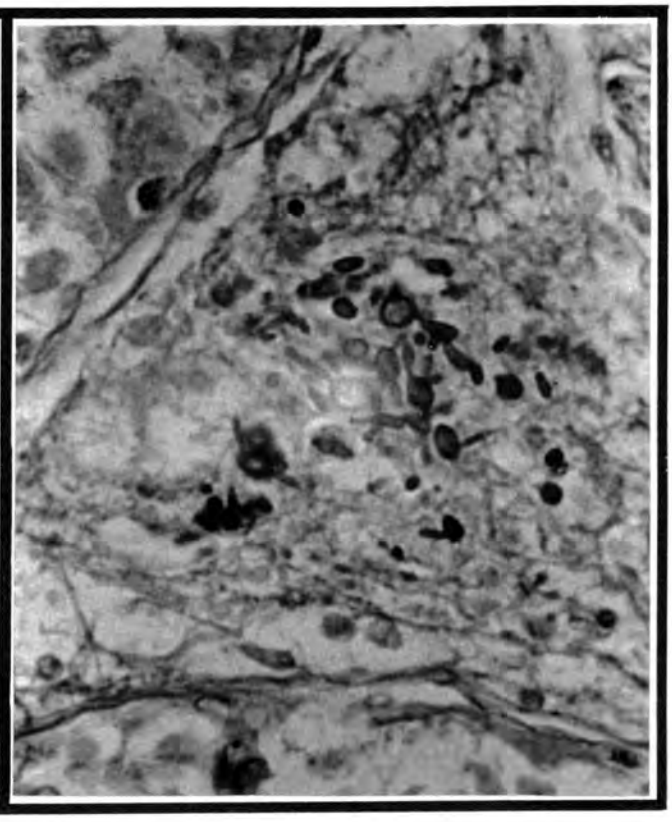


FIGURE 72. Organisms of S.schenckii in rat testis - soil isolates. PAS stain. (40x lens)

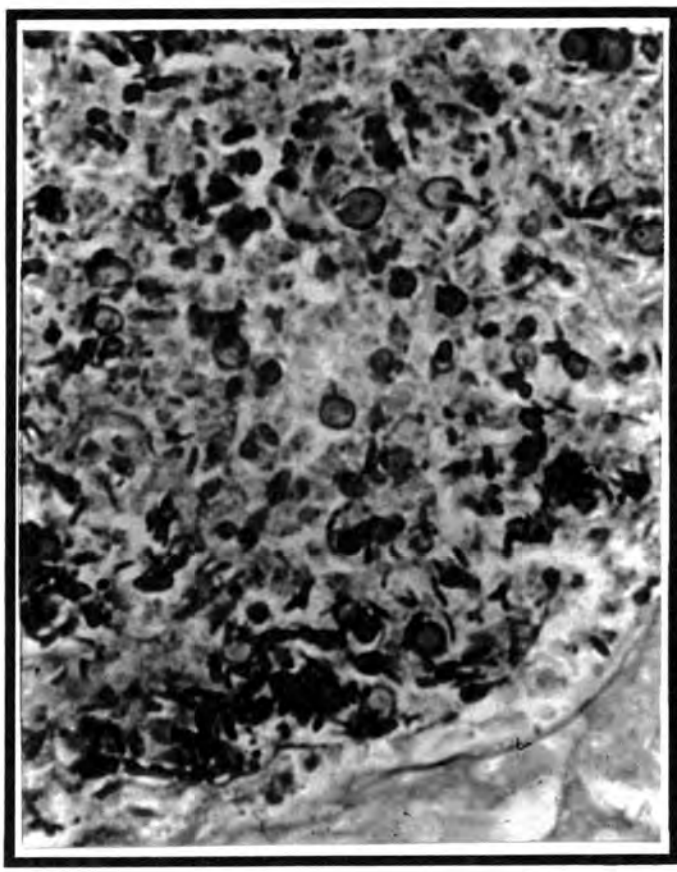


FIGURE 73. S. schenckii infection in rat testis showing numerous organisms - human isolate. PAS stain. (40x lens)

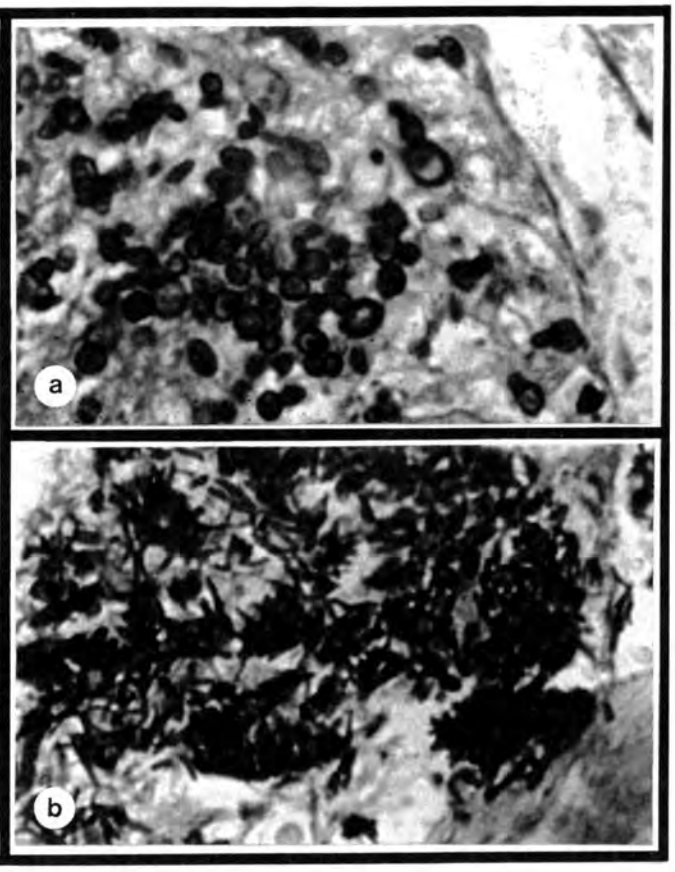


FIGURE 74. a) Masses of yeast-like organisms and b) cigar bodies in rat testis infected with S. schenckii - human isolate. PAS stain. (40x lens)

5 CONIDIUM STUDIES IN *S .SCHENCKII*

The process of conidium enlargement, which is associated with maturation and changes in pigmentation, shape and viability, was investigated quantitatively.

5.1 Conidium ontogeny and colony formation

The method described in Chapter II, 5.1 (p. ..), was used to study the conidium ontogeny and colony formation in *S. schenckii*. Block cultures viewed under the light microscope with phase contrast, after being stained with lactophenol cotton blue, gave satisfactory results.

The studies of the macroscopic and microscopic morphology of *S. schenckii* at 25°C already discussed, mainly gave only general impressions (see paragraphs 2.1, p. 69 and 2.2, p. 72). The following controlled study in block cultures portrays the morphogenetic sequences in *S. schenckii* over a three week period. Conidiation was satisfactory on both SDA and malt agar. As the latter allowed for the formation of pigmented as well as triangular conidia in a shorter incubation period, it was used for this study. Table 4 summarizes the findings.

Colony formation started with a rhomboidal lattice-work of hyphae branching at wide angles, spreading from the point of inoculation on the block culture on days 1 to 3 (Figure 75). Hyphae closest to the original inoculation appeared turgid, then extended in straight pathways. Where these hyphae met (at any angle) they ran in parallel lines, thus massing together to form synnemata - synnema formation was already pronounced from day 3 onward (Figures 76 and 77). The star-like appearance of young colonies could be correlated to groups of synnemata radiating from the central inoculum (Figures 75, 76 and 76). Thinner, more curving hyphae filled up the lattice, and the structure was secured by anastomoses (cross-connections) between them from day

TABLE 4. Morphogenetic sequences in colonies of S. schenckii

DAYS	1	2	3	4	5	6	7	8	9	21
• Germination, original inoculum	+++	++	±	-	-	-	-	-	-	-
• Hyphae broad & granular	++	+	-	-	-	-	-	-	-	-
• Hyphae straight with synnemata production	+	++	+++	+++	Hyphal degeneration →→					
• Hyphae curving between synnemata	-	+	+	++	++	Obscured →→				
• Synnemata - radiating from center inoculum	-	-	+	++	++	----------→→				
• Hyphal inter-connections	-	+	++	++	++	++	+++	----------→→		
• Conidiophore formation with rosettes, unpigmented conidia	-	-	+	+	+	Production zone displaces outward				
• Conidium production, pigmented conidia	-	±	++	+++	+++	Detaching ----------→ Triangular				
• Secondary germination unpigmented conidia	-	-	-	+	+	+	+	+	+	++
• Zone differentiation: outer/inner	-	-	-	++	++	Outward displacement →→				

→→	=	continued
-	=	no development
±	=	slight development
+	=	developed
++	=	well developed
+++	=	very well developed

two onward (Figures 75 - 82). As the central hyphae degenerated, the growing zone moved outward (Figures 77 and 81). Conidium production on this hyphal network passed through a sequence, starting from small, hyaline, ovate, laterally borne conidia on the young undifferentiated vegetative hyphae (from day 3), to larger pigmented conidia where the hyphae were degenerating and disappeared - this sequence was fully developed by day 3 (Figures 78, 79, 80 and 81). With time, as the older unpigmented conidia became progressively assimilated in the pigmented group, the junction zone between the two types of conidia moved outward - this was particularly noticeable from day 7 onwards (Figure 81). Conidium formation on conidiophores was observed

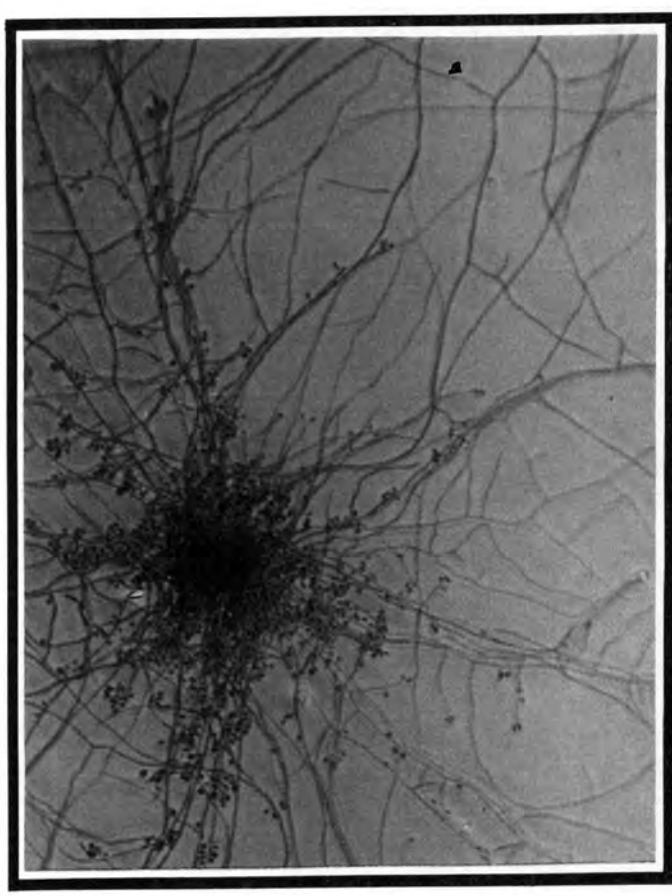


FIGURE 75. Lattice work of hyphae branching at wide angles. (4x lens)

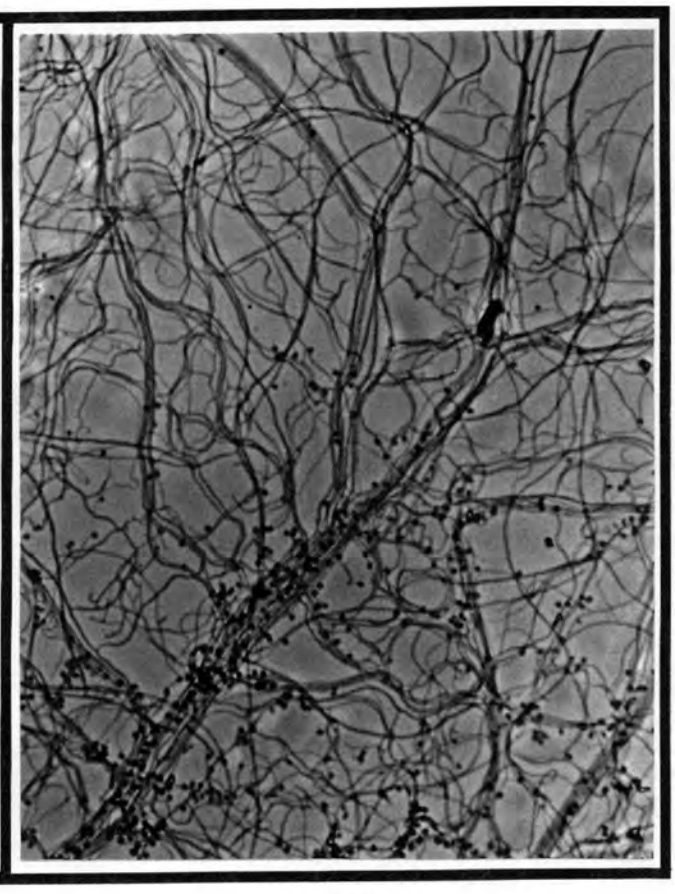


FIGURE 76. Rhomboidal lattice work of hyphae in block cultures. (4x lens)

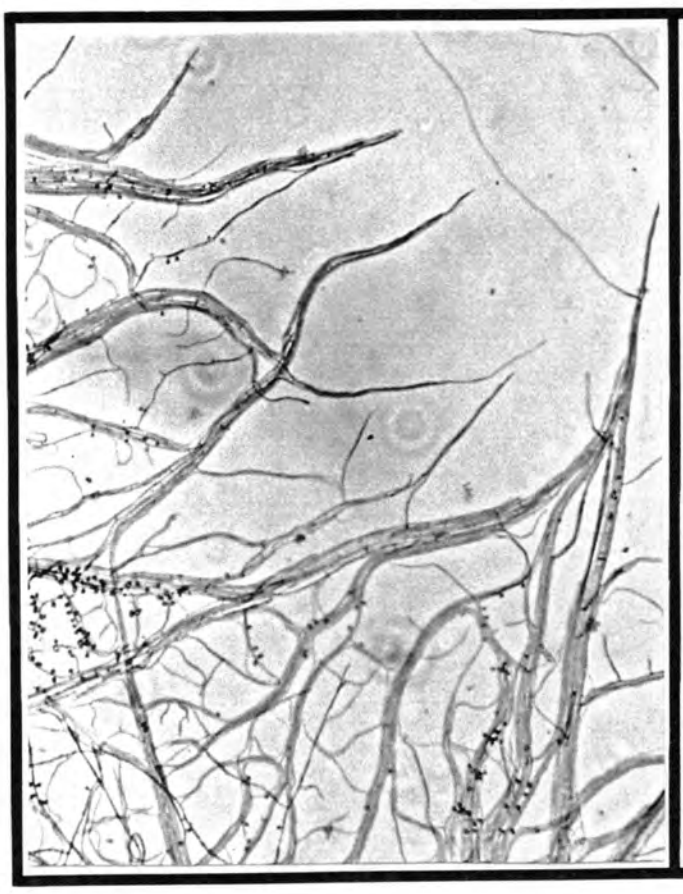


FIGURE 77. Growing zone moving outward. (4x lens)

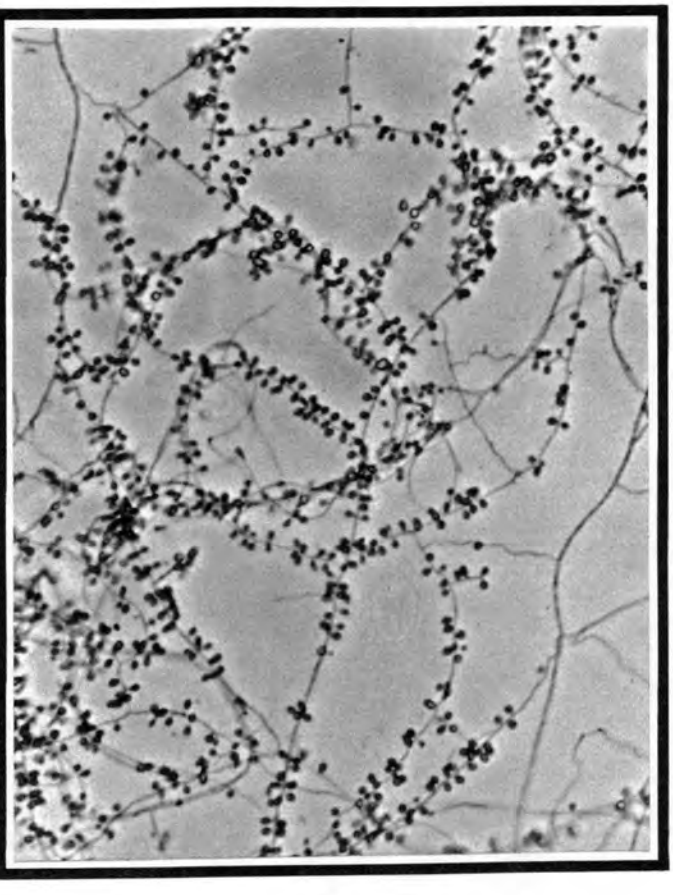


FIGURE 78. Conidia born on laterally undifferentiated hyphae. (20x lens)

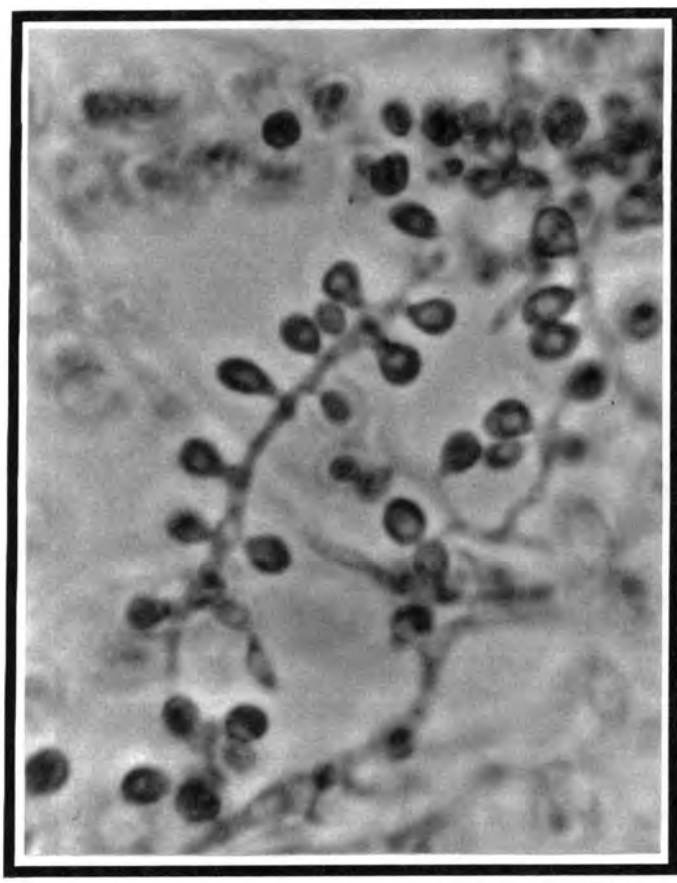


FIGURE 79. Larger pigmented conidia present with hyphal degeneration. (Oil immersion lens)

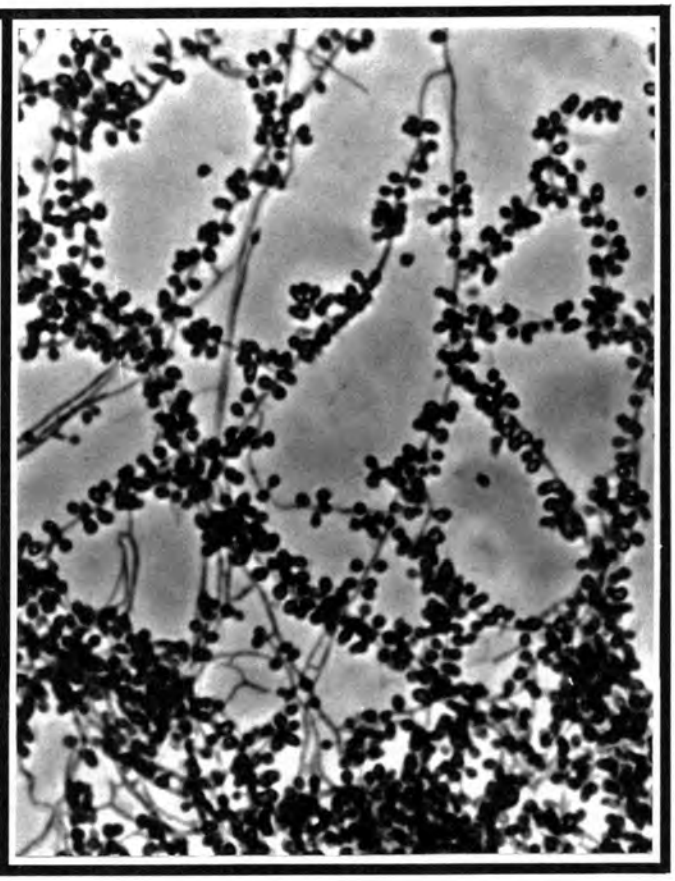


FIGURE 80. Masses of pigmented conidia born laterally on the hyphae (20x lens - enlarged)

FIGURE 81. Assimilation of zones between unpigmented and pigmented conidia. (10x lens)

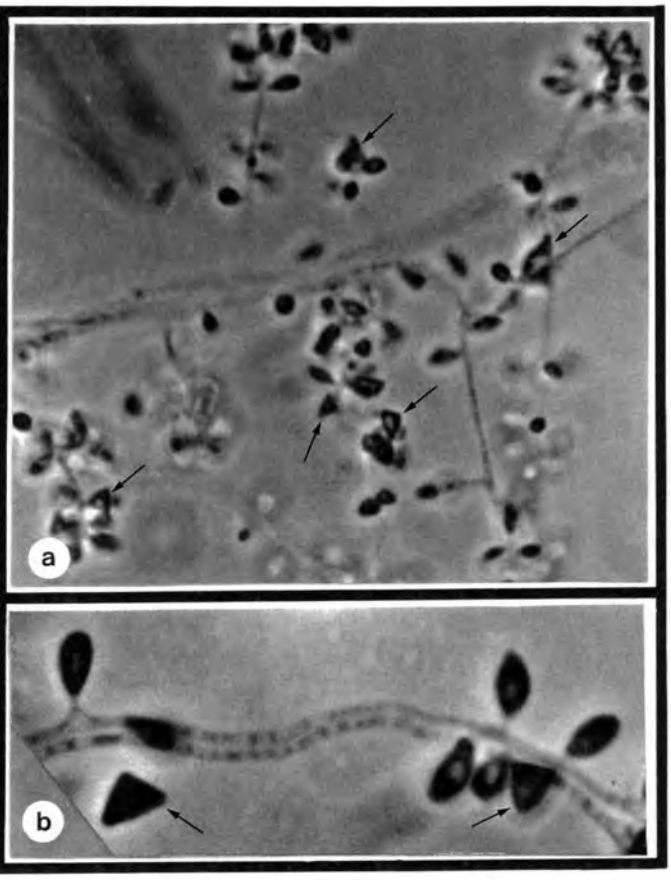


FIGURE 82. Triangular conidium formation - arrows: a) 20x lens b) Oil immersion lens, enlarged.

particularly on the "young" side of this junction from day 3, and becoming obscured by day 6 to 7 and onward (Figures 75 and 81).

The sizes of the hyphae, conidiophores, hyaline and pigmented conidia were the same as those measured in the previous study (see paragraph 2.2, p. 72).

The previous description of the mycelial mat of the colonies as being tough and matted can now be explained by the ability of the hyphae to form anastomoses interconnecting the entire mycelial thallus. In the unpigmented colonies there seems to be less hyphal degeneration, with the formation of hyaline conidia which are present on conidiophores and to a lesser extent laterally on undifferentiated hyphae. Once conidial pigmentation sets in, hyphal degeneration commences due to advanced lateral conidiation, conidia being formed by orthotopic branching on each septated segment on a hypha. Each segment of the hyphae contains one nucleus which ascends into the conidium structure (see paragraph 8.2, p. 116). Thus the less tough thallus of the pigmented colonies consists mainly of detached conidia and hyphal remnants left after degeneration of the hyphal structure.

Triangular conidia appeared after 21 days of incubation. They were present on conidiophores as well as laterally on the hyphae. They are not considered to be a separate entity, but a mature form of the pigmented conidia (Figures 82a and 82b).

Malt agar promoted not only pigmentation in originally unpigmented strains of *S. schenckii*, but also the formation of triangular conidia.

5.2 Conidial pigment

5.2.1 Histochemical staining

Pigmented and unpigmented colonies of *S. schenckii* (the latter as controls) were fixed, sectioned and treated histochemically for melanin with and without bleaching. The methods used are described in paragraph 5.2.1 (p. 21) of Chapter II.

Results indicated that pigment was present in the cell walls of the conidia only, and that the cell walls were thin enough to appear translucent and brown in colour. Histochemically the pigment behaved like a melanin. No pigment could be detected in the unpigmented conidia or in the hyphal cell walls from either type of colony. Similarly, diffusible pigment could also not be found in the surrounding agar.

5.2.2 Conidial pigment production

The nutritional requirements for good growth and pigment production by conidia at 25°C were investigated. The methods used were those described in paragraph 5.2.2 (p. 21) of Chapter II. See also previously illustrated figures 48 to 56.

- **Sabouraud dextrose agar** (see paragraph 2.1, p. 69)

  Pigmentation set in after 2-3 weeks in the 30cm³ slopes and after 2-3 months in the 250cm³ slopes. Pigmentation usually started at the thinner parts of the medium - at the thinner end of the wedge and along the margin of the medium - becoming general in a few months. Not all strains produced pigment equally well; some turned buff in colour with time, while others assumed variegated appearance. Most of the soil strains remained unpigmented on this medium, some forming single spots of pigmentation with time.

- **Malt agar** (see paragraph 2.1, p. 69)

  All unpigmented pathogenic strains of *S. schenckii* transferred onto malt agar produced a black pigment within 7 to 14 days. Some soil strains remained unpigmented, while others showed limited pigmentation, mostly of the variegated type. None develop the full black pigmentation seen in the pathogenic strains.

- **Neopeptone agar and broth**

  Neopeptone agar or broth without dextrose produced fairly good growth but no pigmentation. The growth in the broth was in the form an unpigmented superficial pellet, with some submerged flakes being present. Once dextrose was added to these media, pigmentation follow the same pattern as for SDA.

- **Fries' minimal salt medium**

  This medium containing 4% sucrose and no yeast extract, allowed growth but no pigmentation. Without sucrose this medium supported limited growth and very mild pigmentation restricted to conidia.

- **Minimal media**

  Distilled water and water agar yielded restricted growth and mild pigmentation. On water agar the growth was invariably submerged, forming clustered mycelia and the pigmentation remained restricted to the few pigmented conidia produced.

- **Wattle and eucalyptus 'broth' and agar**

  Crude watery extracts of dead dry wattle and eucalyptus twigs produced growth similar to that in distilled water. A "tanned" effect, i. e. reddish-brown colour, of the growing fungus was

evident and could not be related to pigment production either in the hyphae or in the conidia of the fungus itself. Fairly good growth appeared on the agars, which also had a diffuse reddish-brown "stained" appearance. It was deduced that this was due to the soluble tannins present in both types of wood.

These results indicated that media which were rich in peptone as a nitrogen source, and dextrose as a source of carbohydrate resulted in good growth but poor pigmentation. Media rich in either nitrogen or carbohydrate resulted in no pigmentation, but fairly good growth. Media with declining levels of both nitrogen and carbohydrate, as judged by duration of growth in layers of differing thickness in the medium, stimulated pigmentation. Poor nutritional media resulted in poor growth and poor pigmentation. The rapid growth and rapid pigmentation on malt agar in the case of the pathogenic strains was noteworthy. It was clear that *S. schenckii* has the ability to grow in the presence of wattle and eucalyptus extracts, and that these extracts "tan" or stain the fungus but do not enhance growth or pigment production.

5.3 Conidial volume

Archer's (1977) methods described in Chapter II, paragraph 5.3 (p. 22), were applied to ascertain the conidial volumes in unpigmented, buff coloured, variegated and black cultures of *S. schenckii*. Large culture flasks were used in order to obtain large enough numbers of conidia. *Absidia corymbifera* conidia from both CMI and CBS were used as controls of mean conidial volume, and it was found that the conidia from these strains were about 1μm^3 (*fl*) larger than those reported by Archer *et al*. (1977). In other respects our results were the same as those described by Archer *et al*. (1977). The results are given in Tables 5 and 6.

Seven unpigmented strains were used, six pathogenic (strains 1-6, Table 6) and one soil isolate (strain 7, Table 6). The mean volumes ranged between 4.06 - 5.65μm and the volumes of the six pathogenic strains

averaging 4.91μm. The unpigmented soil isolate's volume was virtually the same, being 4.94μm. Larger culture flasks (1000cm^3) seemed to produce smaller conidia even with an extended incubation period of seven days in the case of strain 2 (Table 5, strains 1 and 2). Conidial volumes were the same whether conidia were harvested using Tween 80 or Nonidet p40, but the latter gave larger conidium yields. Furthermore, the mean conidium volumes for the buff, the variegated and black strains increased steadily with increasing pigmentation, although not with a constant factor (strains 8, 9 and 10, Table 6). A volume increase of 1.36μm occurred from the buff to the variegated strains, whereas from the unpigmented to the buff, and the variegated to the black it was 0.7μm and 0.34μm, respectively. As expected, the largest difference in volume was found between the unpigmented and the black conidia, i. e. 2.4μm. Figure 83 graphically illustrates these findings.

TABLE 5. Conidial volumes of Absidia corymbifera as determined in the Coulter S-Counter

Absidia corymbifera Source	Modal volume μm^3; *fl*	Mean volume μm^3; *fl*
Archer *et al.* (1977)	8.17[27]	9.0
CMI (123261)	10.12	10.82
CBS (429.75)	10.12	10.76

[27] From the graph published by Archer et al. (1977).

TABLE 6. Conidial volumes of S. schenckii as determined in the Coulter S-Counter

S.schenckii Number	Modal volume μm^3; *fl*	Mean volume μm^3; *fl*	Colony type	Culture flask size	Conidium harvesting day
1 (P)	3.12	4.06	Unpigmented	1000cm³	21
2 (P)	2.56	4.50	Unpigmented	1000cm³	14
3 (P)	3.68	5.01	Unpigmented	250cm³	21
4 (P)	3.68	5.07	Unpigmented	250cm³	14
5 (P)	3.68	5.17	Unpigmented	250cm³	14
6 (P)	2.56	5.65	Unpigmented	250cm³	14
7 (S)	3.96	4.94	Unpigmented	250cm³	14
8 (P)	4.24	5.62	Buff	1000cm³	54
9 (P)	7.32	6.98	Variegated	250cm³	21
10 (P)	7.32	7.32	Black	250cm³	21

P = Pathogenic strains; S = Soil strain;

5.4 Pigmentation in single-conidium cultures

The methods described in Chapter II, paragraph 5.5 (p. 23), were used to produce single-conidium cultures in SDA.

Pigment production in the colonies as well as the colony type which developed from these single-conidium cultures did not correspond to the conidium source. At two months colonies from the unpigmented conidium source were grey and produced slight central pigmentation in some cultures. One colony produced pigmentation in a wedge of 110°. Colonies developing from the pigmented conidia were more variable, showing several lighter and darker concentric rings. Some cultures were variegated while others developed central pigmentation.

In the stock culture from which the unpigmented conidia were removed, the surface of the colony had been disturbed when a sterilized loop was run over it in order to pick up conidia to streak out for single conidia. It was interesting to note that at the scraped site, speckled

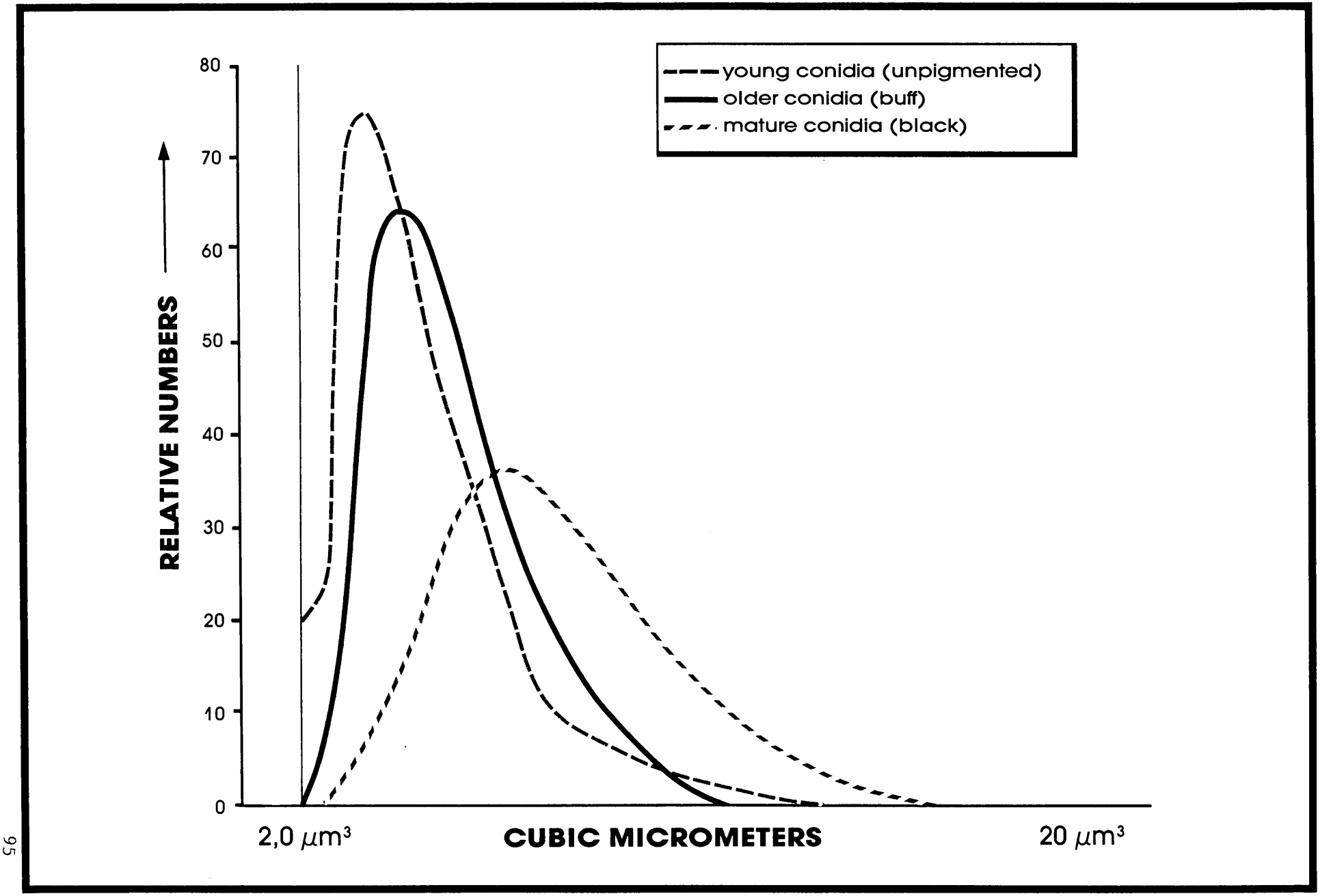


FIGURE 83. Conidium volumes of pathogenic and soil isolates of S. schenckii as determined in the Coulter S-Counter

black areas developed, showing pigmented conidia on microscopy. The rest of the colony remained unpigmented.

Pigment production is not consistently transmitted in the conidium lines, as judged by single conidium isolates. Regardless of the original conidium characteristics, pigmentation develops either in the older parts of the colonies, in segments, in concentric rings or in a variegated pattern. It appears therefore that pigment production is potentially present in all conidium types, but their development is affected by circumstances in the growth phase of the colony.

5.5 Conidium viability

The methods described in Chapter II, paragraph 5.5 (p. 23), were used to test the viability of the conidia in *S. schenckii*.

Ten of the originally unpigmented strains had shown slight pigment production within the first three months of incubation while the others remained unpigmented or turned a light buff colour. After hydration of the naturally dried out cultures in BHI broth, all pigmented or slightly pigmented cultures were viable. Of the other 40 unpigmented strains only two regrew. Any nutritional circumstance promoting pigmentation therefore promotes viability under adverse conditions.

6 GROWTH OF *S. SCHENCKII* ON WOOD

6.1 Growth on wattle and eucalyptus

Using the methods described in paragraph 6.1 (p. 23) of Chapter II, dead wattle leaves and twigs, inoculated with *S. schenckii*, yielded abundant growth (Figures 84 - 86). On wattle bark and dried out pods (Figure 84), growth was slight and very superficial. No visible growth occurred on green twigs. The colonies first appeared as delicate,

spider web-like lattice of mycelia, which became encrusted with massive sheaths of conidia. The hyphae were seen to mat around the epidermal hairs present on the wattle twigs and leaves (Figures 85 and 86). These conidial masses, together with the plant hairs, were detachable, and readily disintegrated into a fine "ash". One could therefore appreciate how even the slightest contact with such a fungal web would deliver a heavy inoculum of infective conidia at any given site on the body. Scanning electron microscopy of *S. schenckii* growing on wood, illustrates these masses of conidia present on small pieces of wood (see paragraph 9, p. 122).

Eucalyptus wood shavings and particles also produced abundant growth (Figure 87), while limited growth appeared on bark. Again a closely knit web-like mycelial lattice formed (Figures 87, 88a and 88b). It is clear from these figures that the fungus grew superficially and that the structure of the wood was not affected in any way.

Both on wattle and eucalyptus, visibly thicker mycelial strands were made up of several hyphae representing synnemata (Figures 88a and 88b).

Microscopic examination of the growth on wood showed that the previously described anastomoses between hyphae were also present, and all the other microscopic characteristics of *S. schenckii* could be confirmed. Although present, the classical conidiophore and conidium rosettes were obscured by the formation of lateral conidia on undifferentiated hyphae at an early stage. Unpigmented conidia appeared within one to two weeks, ovate to oval pigmented conidia in three weeks, larger club-shaped to globose pigmented conidia in two months, and triangular conidia appeared with increasing frequency up to six months.

The findings were similar for pathogenic and soil strains, except that some soil strains produced a white woolly growth in contrast to the flat darker growth of the pathogenic isolates.

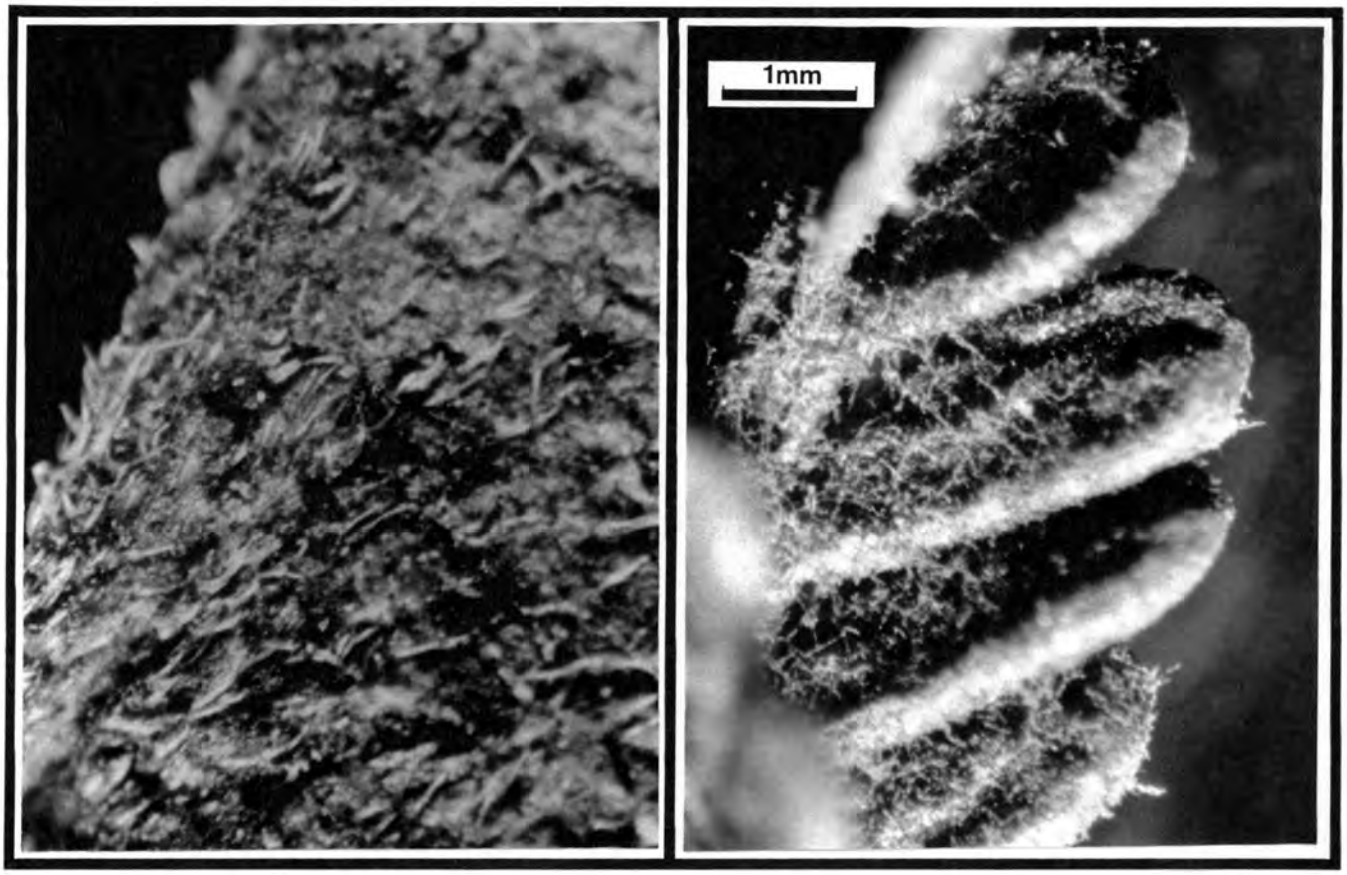


FIGURE 84. Superficial and slight growth on wattle pods.

FIGURE 85. Abundant growth of on wattle leaves.

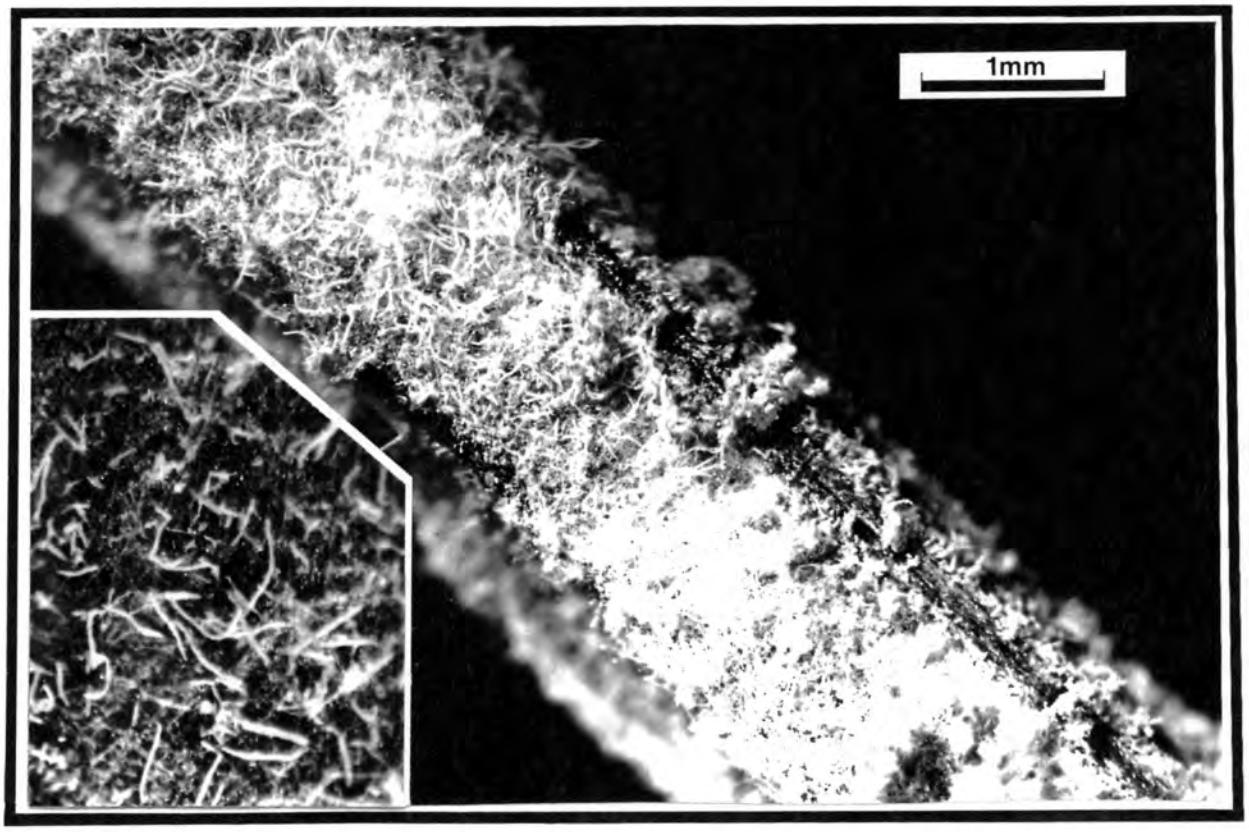


FIGURE 86. Mycelial lattice among epidermal hairs on a dead wattle twig.
Inset: Enlargement.

FIGURES 84 - 86. Stereo-microscopy of S. schenckii growing on wattle plant material.

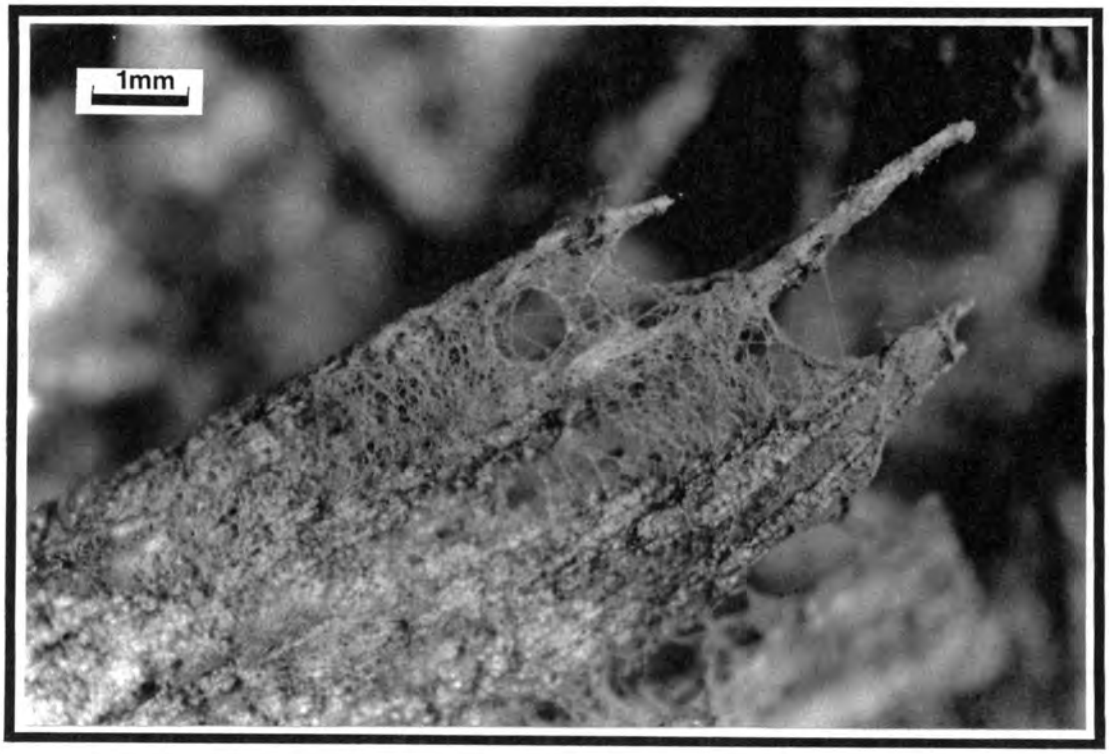
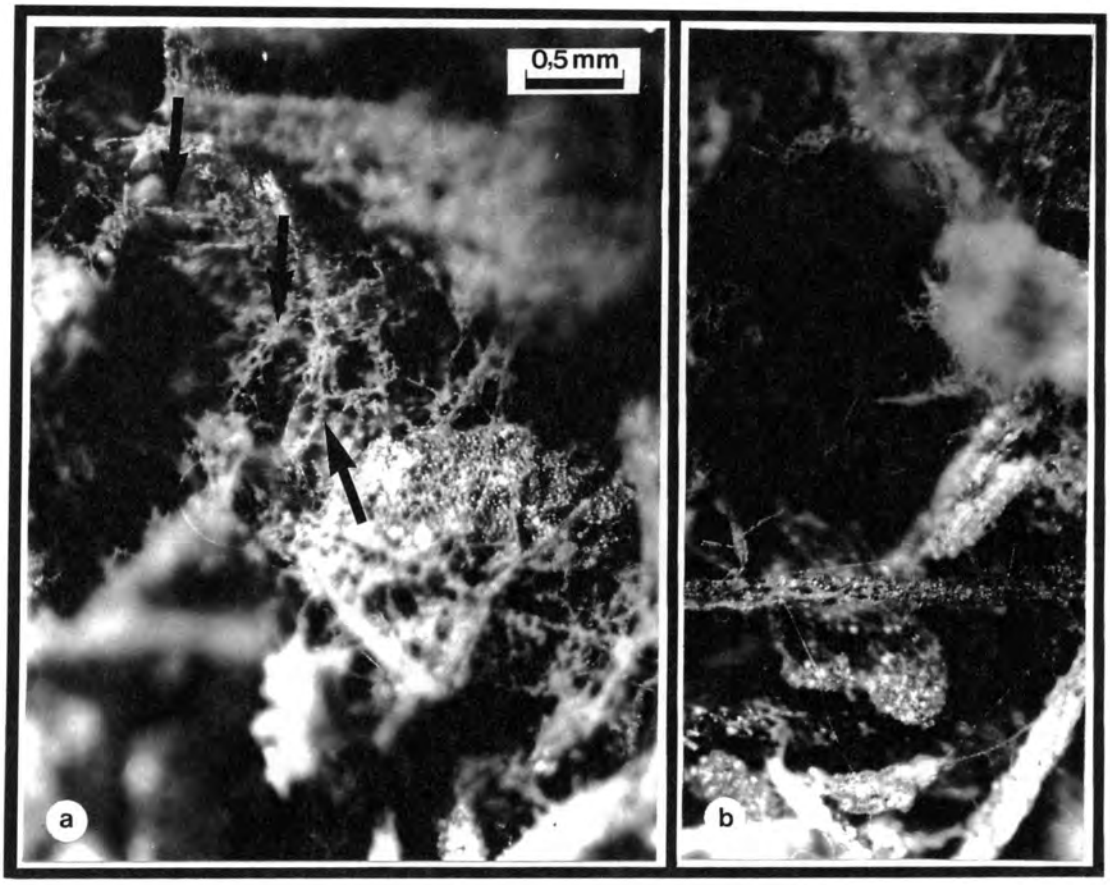


FIGURE 87. Euclyptus shavings overspun with S. schenckii growth. Stereo-microscopy.



FIGURES 88a&b. Spider web-like growth of S. schenckii on eucalyptus shavings. Thicker mycelial strands (arrows) may represent synnemata. Stereo-microscopy.

6.2 Growth on indigenous and other exotic woods

The methods described in Chapter II, paragraph 6.2 (p. 24), were used to monitor the viability and abundance of growth and conidium production of *S. schenckii* on wood. Wood samples were chosen to be as varied as possible and included 20 indigenous and 17 exotic species. Their botanical names, as supplied by the National Institute for Timber Research (NITR - CSIR, Pretoria), were used for the purpose of this study and are summarized in Table 7 together with some of the findings (see also List of Species).

In order to compare the growth of *S. schenckii* on wood with growth on agar, controls grown on agars without wood blocks were set up, incubated and examined at the same time. All of the five *S. schenckii* isolates used in this experiment, grew fairly well on the agar-agar surfaces, the growth spreading evenly over the surface within the first six days. After 14 days the growth became submerged to a depth of up to 0,5cm and mildly pigmented areas became evident. Up to 30 days, the growth submerged to a maximal depth of 2cm and remained so for the rest of the three months. An uninoculated control agar bottle was incubated for three months, in order to compare its colour to the colour of the pigment diffusing from the wood blocks into the agar. The control agar remained a dull white colour during incubation throughout the three month period.

In assessing the growth on the indigenous wood blocks on agar, *S. schenckii* grew with increasing abundance on *Brachylaena discolor* (Coast Silver Oak), the growth being restricted to the bottom of the wood blocks. Starting at day six, light brown pigment leached out from the wood into the agar, and at 30 days the pigment was distinctly lighter to a depth of approximately 1cm. Submerged growth, present in the lighter area, produced a pigmented line of growth where the darker pigment in the agar started (Figure 89). On *Schotia brachypetala* (Weeping Boer-Bean), *S. schenckii* grew in white woolly form, covering the wood blocks entirely. A light yellowish pigment diffused from this

TABLE 7. Growth of S. schenckii on indigenous and exotic woods

WOOD SPECIES

INDIGENOUS WOODS:	6 DAYS	14 DAYS	30 DAYS	3 MONTHS
Brachylaena discolor (Coast Silver Oak)	+[1]	+[1]	++[1]	++[1]
Schotia brachypetala (Weeping Boer-Bean)	-	+[2]	++[2]	++[2]
Calodendrum capense (Cape Chestnut)	-	-	+	+
Erythrina lysistemon (Common Coral Tree)	±	±	±	±
Acacia xantophloea (Fever Tree)	±	±	±	±
Syzygium cordatum (Water Berry)	-	±	±	±
Faurea saligna (Transvaal Beech)	-	-	±[3]	±[3]
Ficus capensis (Broom Cluster Fig)	-	-	-	±
Breonadia salicina (Matumi)	-	-	-	±
Ekebergia capensis (Cape Ash)	-	-	-[4]	-[4]
Albizia adianthifolia (Flat-Crown)	-	-	-	-
Parinari curatellifolia (Mobola Plum)	-	-	-	-
Anthocleista grandiflora (Big-Leaf Tree)	-	-	-	-
Podocarpus latifolius (Real Yellowwood)	-	-	-	-

INDIGENOUS WOODS contd:	6 DAYS	14 DAYS	30 DAYS	3 MONTHS
Ocotea bullata (Stinkwood)	-	-	-	-
Olea capensis (Ironwood)	-	-	-	-
Trichilia emetica (Natal Mahogany)	-	-	-	-
Widdringtonia nodiflora (Mountain Cypress)	-	-	-	-
Gonioma kamassi (Kamassi)	-	-	-	-
Rapanea melanophloeos (Cape Beech)	-	-	-	-

EXOTIC WOODS:

Cinnamomum camphora (Camphor Tree)	++	+++	+++	+++
Eucalyptus grandis (Rose Gum)	$++^{3}$	$+++^{3}$	$+++^{3}$	$+++^{3}$
Eucalyptus sideroxylon (Black Ironbark)	+	$++^{3}$	$++^{5}$	$++^{5}$
Ginkgo biloba (Maidenhair Tree)	++	$++^{3}$	$++^{5}$	$++^{5}$
Acacia melanoxylon (Australian blackwood)	-	+	$++^{3}$	$++^{5}$
Sequoia sempervirens (Californian Redwood)	±	±	+	+
Grevillea robusta (Australian Silky Oak)	±	±	±	±
Jacaranda mimosaefolia (Jacaranda)	±	±	±	±
Khaya nyasica (East African Mahogany)	±	±	±	±

EXOTIC WOODS contd:	6 DAYS	14 DAYS	30 DAYS	3 MONTHS
Melia azedarach (Seringa)	±	±	±	±
Pinus elliottii (Slash Pine)	±	±	±	±
Pinus pinaster (Cluster Pine)	±	±	±	±
Populus deltoides (Match Poplar)	±	±	±	±
Pinus radiata (Radiata Pine)	-	-	-	-
Pinus roxburghii (Chir Pine)	-	-	-	-
Platanus acerifolia (London Plane)	-	-	-	-
Populus alba (White Poplar)	-	-	-	-

-	=	No growth
±	=	Mild growth
+	=	Growth
++	=	Good growth
+++	=	Very good growth

1	=	Growth at bottom of wood block
2	=	White woolly growth
3	=	Grey to black growth
4	=	Growth form agar to wood
5	=	Brown to black growth

wood (Figure 90). A similar growth pattern was seen in the case of soil strains being inoculated on eucalyptus and wattle (see paragraph 6.1, p. 96). From 30 days onward growth was also present on *Calodendrum capense* (Cape Chestnut); it was being pigmented, mainly restricted around the wood blocks, and submerged 0,8 - 1cm into the agar. Again a light yellow pigment diffused from the wood (Figure 91). Mild growth appeared after six days on *Erythrina lysistemon* (Common Coral Tree), and *Acacia xantophloea* (Fever Tree); after a month the growth was submerged to a depth of 1,5cm and 2cm respectively. In the latter a light brown pigment diffused into the agar (Figures 92 and 93). At 14 days mild growth was detected on *Syzygium cordatum* (Water Berry), producing a brown diffusible pigment. Only superficial growth was noted on the agar (Figure 94). After 30 days on *Faurea saligna* (Transvaal Beech), mild grey to black growth became noticeable; it remained restricted around the wood blocks in the agar, in the presence of a light brown diffusible pigment. Growth remained static on the last four woods after three months. Mild growth with no further development was seen on *Ficus capensis* (Broom Cluster) and on *Breonadia salicina* (Matumi; Figure 95) after three months. Only mild growth was detected on both these agars, appearing only after 30 days. From six days onward a red-brown diffusible pigment was present in the case of *Breonadia salicina*. With *Ekebergia capensis* (Cape Ash) and *Albizia adianthifolia* (Flat Crown), no growth was noted on the wood blocks. However, on the agars prolific black growth became apparent around the wood blocks at 30 days and later, and a light brown pigment diffused into the agar. The growth was submerged to a depth of 1,5cm (Figures 96 and 97). At three months the black growth was more abundant and crept up from the agar onto the wood blocks in the case of *Albizia adianthifolia* (Figure 97). Similar, but less prolific growth was present on *Parinari curatellifolia* (Mobola Plum). No submerged growth but a diffusible brown pigment was evident (Figure 98). No growth on the wood, but growth similar to that detected in the control agars, with submerged growth, was noted for *Anthocleista grandiflora* (Big-Leaf Tree) (Figure 99). *Podocarpus latifolius* (Real Yellowwood) also did not allow growth, but black star-like colonies developed on the surface of the agar as well as black submerged growth (Figure 100).

FIGURE 89. Brachylaena discolor

FIGURE 90. Schotia brachypetala

FIGURE 91. Caledendrum capense

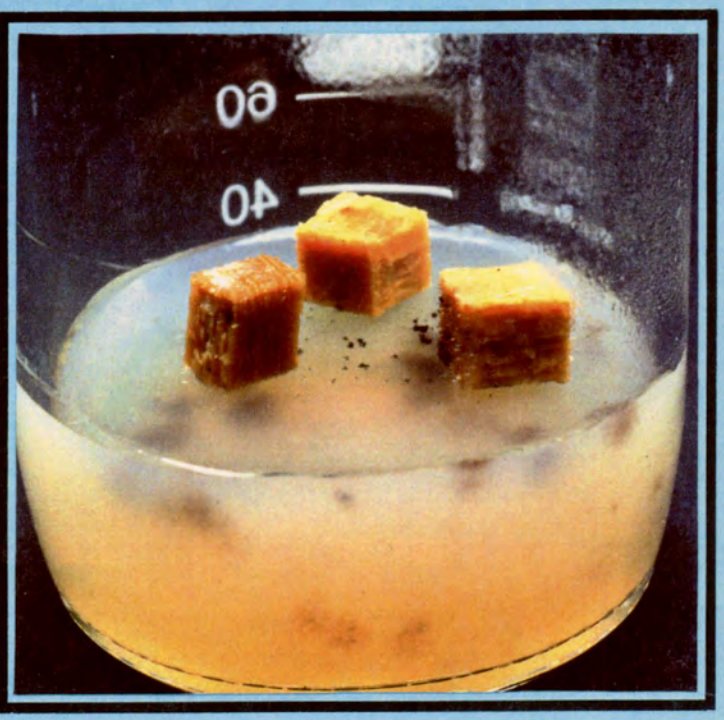


FIGURE 92. Erythrina lysistemon

FIGURES 89 - 92. Growth of S. schenckii on wood - Stereo-microscopy.

FIGURE 93. Acacia xanthophloea

FIGURE 94. Syzygium cordatum

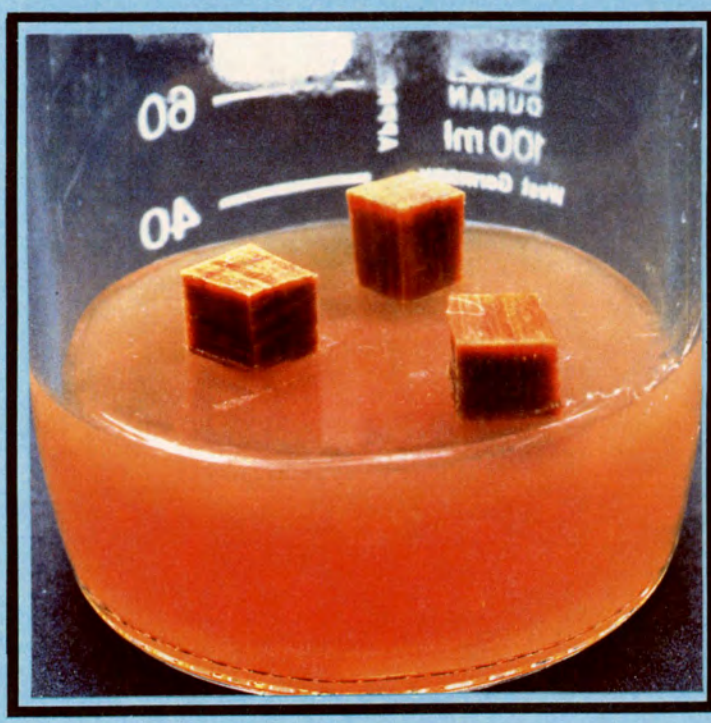


FIGURE 95. Breonadia salicina

FIGURE 96. Ekebergia capensis

FIGURES 93 - 96. Growth of S. schenckii on wood - Stereo-microscopy.

No growth was detected on the other six indigenous wood species over the three month period (see Table 7). Their agars produced very mild growth, invariably starting after 30 days with limited submerged growth.

Growth on the exotic wood species proved to be much more prolific. On *Cinnamomum camphora* (Camphor Tree), a woolly layer of grey growth covered the wood from day six, later turning black. Submerged pigmented growth was equally prolific throughout the three month period. On *Eucalyptus grandis* (Rose Gum) growth appeared grey and spiky at first, developing into a flat grey growth at 30 days and three months. Submerged growth was present as pigmented "fungal balls" directly below the wood blocks. Brown pigment diffused from the wood blocks into the agar (Figure 101). Similar results were seen on *E. sideroxylon* (Black Iron Bark) and *Ginkgo biloba* (Maiden Hair Tree), except that the growth turned brown to black after 30 days and the submerged growth was less pronounced and less pigmented, and no pigment diffused from these woods (Figure 102). *Acacia melanoxylon* (Australian Blackwood) also produced grey growth at 30 days and black growth by three months. A dark diffusible pigment was evident in the agar, and the growth on the agar was superficial (Figure 103). Eight other exotic wood species propagated mild growth from day six (indicated as (±) in Table 7); this did not increase over the three month period, except for *Sequoia sempervirens* (Californian Redwood). No diffusible pigment could be seen in all the last eight woods, and submerged growth was restricted to around the wood blocks. Four out of 17 (23,5%) exotic woods failed to show any growth, i. e. *Pinus radiata* (Radiata Pine), *Pinus roxburghii* (Pitch Pine), *Platanus acerfolia* (London Plane) and *Populus alba* (White Popular), while a larger percentage of the indigenous species failed to show any growth, viz. 11 out of 20 (55%) (see Table 7). As in the case of the indigenous wood, *Albizia adianthifolia*, black growth crept up from the agar onto the bottom of the wood blocks of *Populus alba* (Figure 108). No pigment leached from these woods and submerged growth was also restricted to around the wood blocks. Examples are illustrated in figures 104 - 108.

FIGURE 97. Albizia adianthifolia

FIGURE 98. Parinari curatellifolia

FIGURE 99. Anthocleista grandiflora

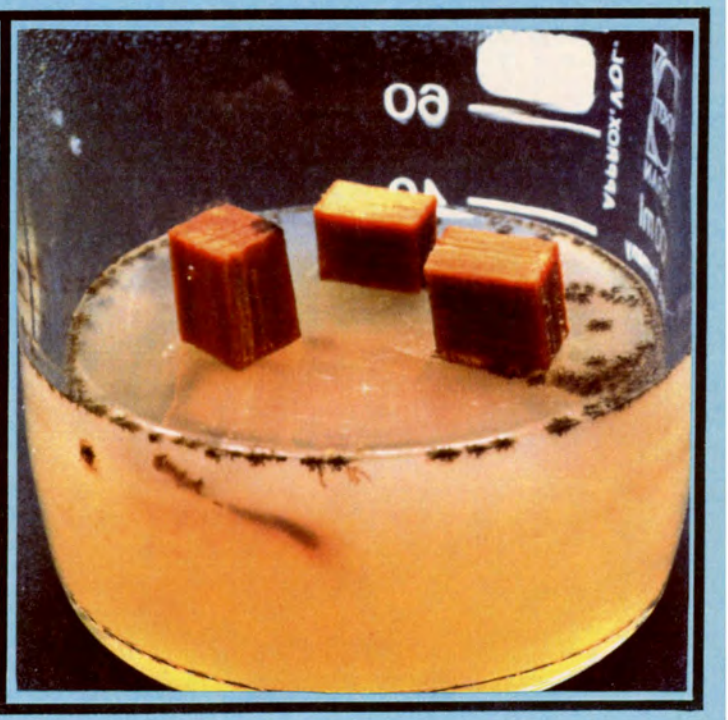


FIGURE 100. Podocarpus latifolius

FIGURES 97 - 100. Growth of S. schenckii on wood - Stereo-microscopy.

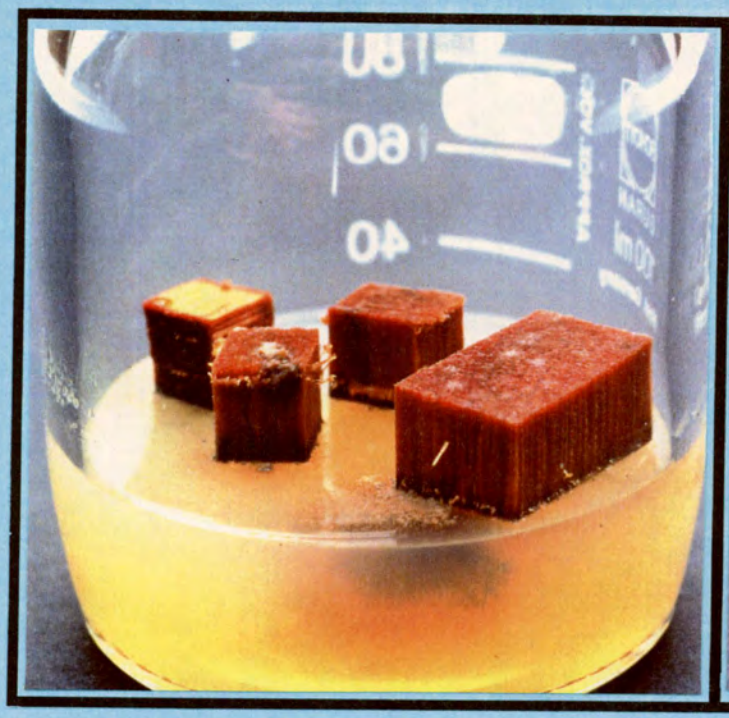


FIGURE 101. Eucalyptus grandis

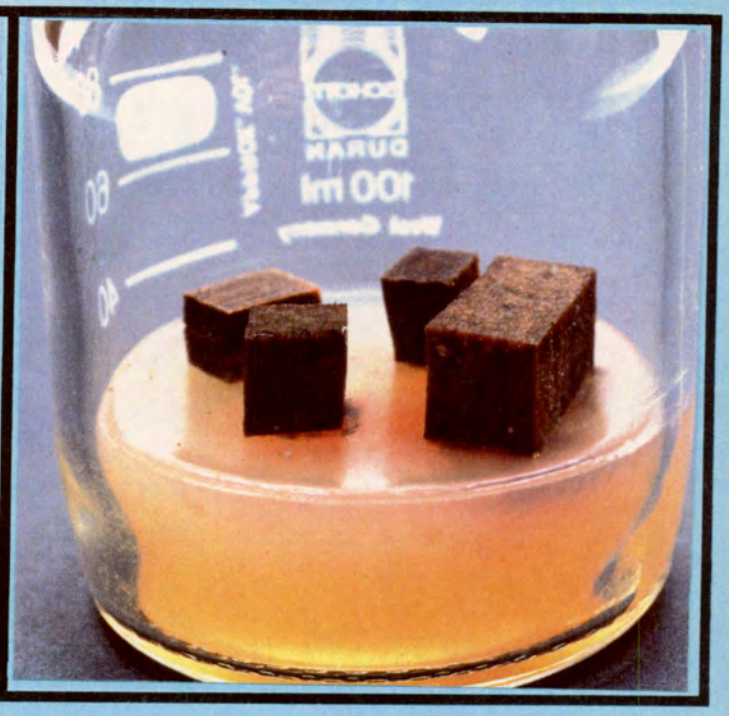


FIGURE 102. Eucalyptus sideroxylon

FIGURE 103. Acacia melanoxylon

FIGURE 104. Grevillea robusta

FIGURES 101 - 104. Growth of S. schenckii on wood - Stereo-microscopy.

FIGURE 105. Jacaranda mimosaefolia

FIGURE 106. Pinus radiata

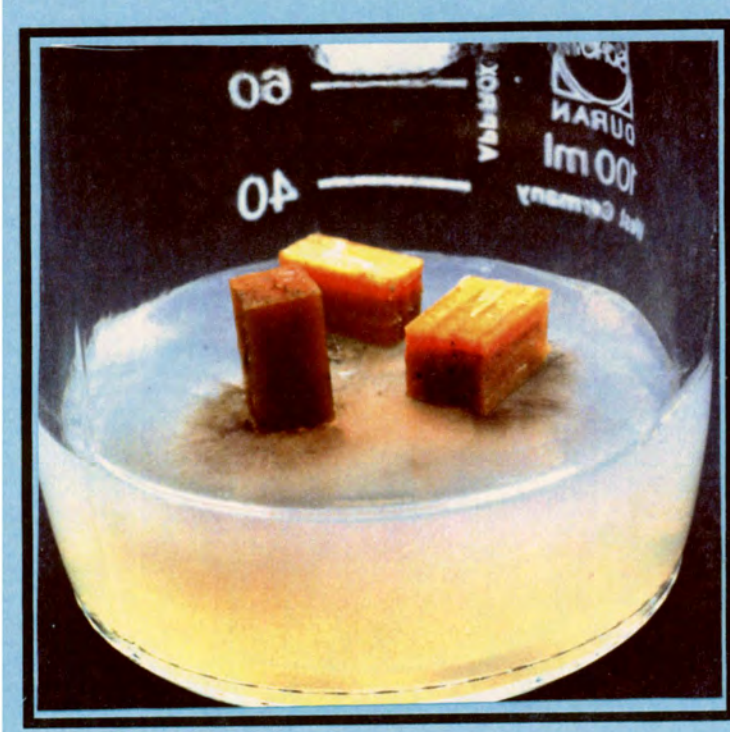


FIGURE 107. Pinus roxburghii

FIGURE 108. Populus alba

FIGURES 105 - 108. Growth of S. schenckii on wood - Stereo-microscopy.

As far as the microscopy of the growth on wood and on agar is concerned, black growth invariable produced pigmented ovate to globose conidia and pigmented triangular conidia. Pigment remained restricted to conidium formation. Where black sclerotic bodies were present on the agar surfaces of cultures with the indigenous wood species *Calodendrum capense* (Figure 91), *Erythrina lysistemon* (Figure 92), *Ekebergia capensis* (Figure 96) and *Anthocleista grandiflora* (Figure 99) pigment was seen in these sterile hyphal structures. The white and grey growth on *Schotia brachypetala* and *Cinnamomum camphora* respectively, proved to have microscopic characteristics of *S. schenckii*, but only a limited number of hyaline ovate conidia were present.

7 GROWTH OF *S. SCHENCKII* ON WOOD MEALS, WOOD FLOURS AND CELLULOSE

The methods described in Chapter II paragraph 7 (p. 24), were used to grow *S. schenckii* on purified bagasse lignin, various coarsely ground wood meals, fine and ultra-fine purified extracted wood flours and grated purified cellulose. Bagasse is the dry pulp of sugar cane after the juices have been extracted.

The results were the same for the six pathogenic strains of *S. schenckii* used. After 21 days incubation at 25°C, stereo-microscopy revealed that the buffered agar-agar controls gave similar growth patterns as seen with the agar-agar controls for the previous experiment. Growth appeared to be slightly more prolific, radiating from the 0,5cm punch inoculum; it was less submerged, but pigmented like before (Figures 109 - 111).

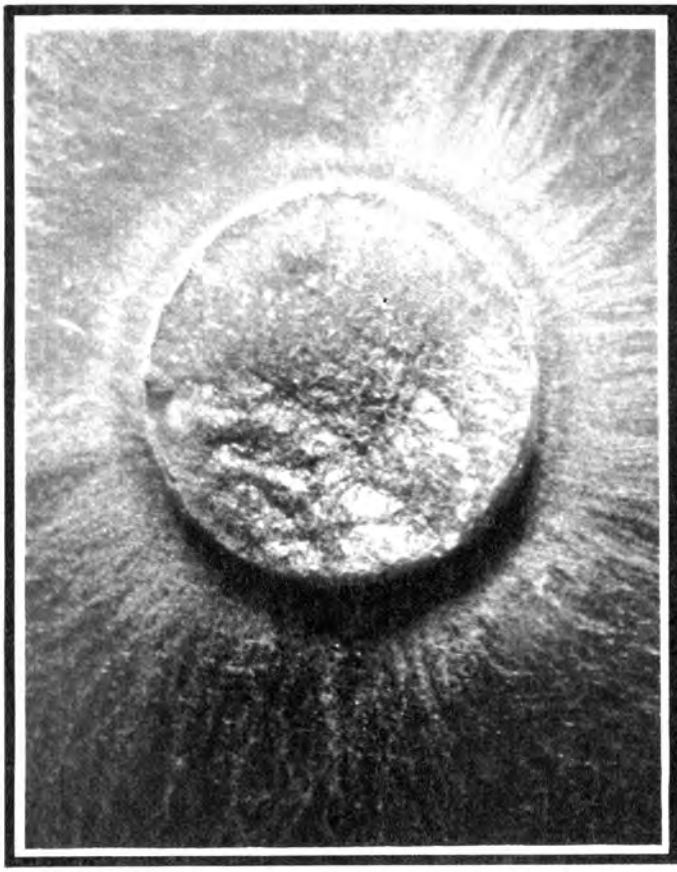


FIGURE 109. Radiating growth of S. schenckii on bufferred agar-agar (14 days). Stereo-microscopy, 1,5x lens.

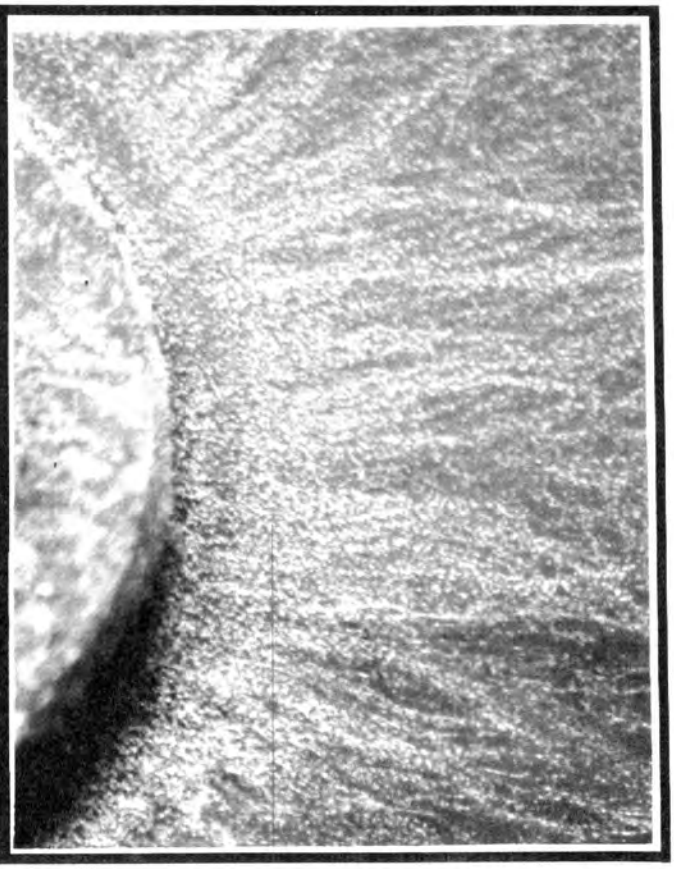


FIGURE 110. Enlargement of Figure 109.

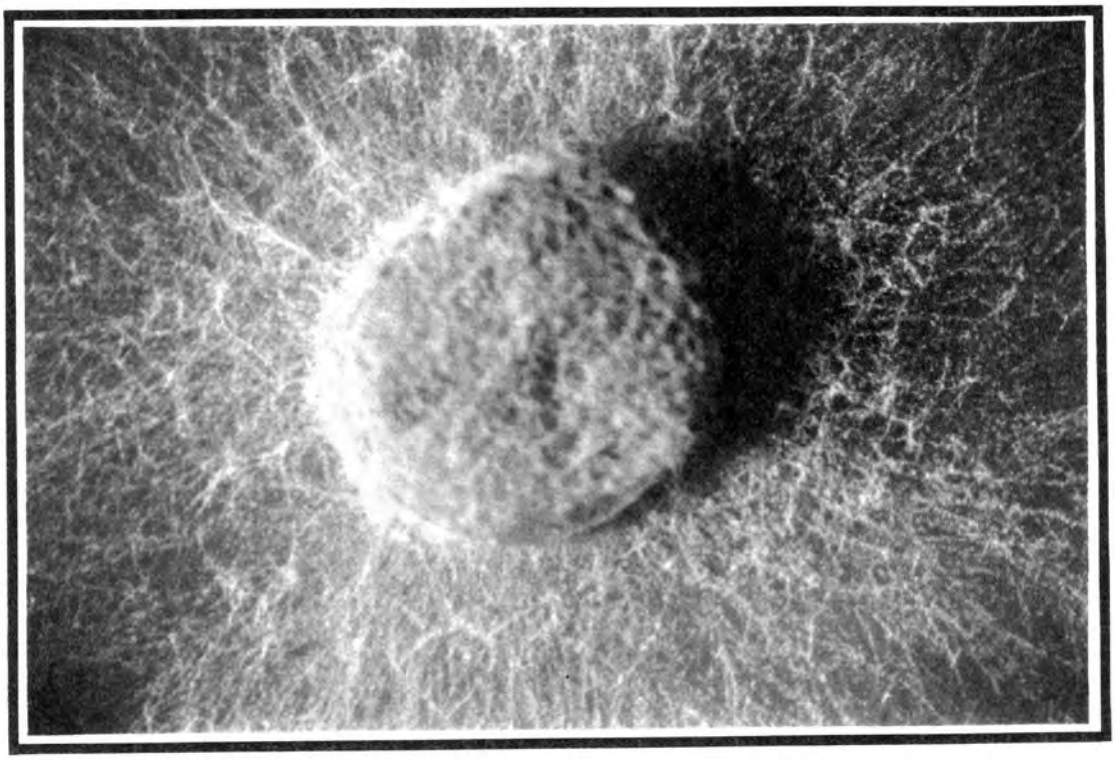


FIGURE 111. S. schenckii growing on agar-agar (21 days). Stereo-microscopy, 1,5x lens.

- **On coarse and fine *Pinus pinaster* wood meal:**

  Growth was restricted to the area around the inoculum and tended to form a few "aerial synnemata" after 21 days. No submerged growth developed after a month, and spider web-like growth was present on the surface of the agar. No pigment was noted (Figure 112).

- **On *Eucalyptus saligna* wood meal (fine):**

  Here the growth remained restricted around the inoculum site. Sparse mycelial growth did however spread over the agar, with no submerged growth. Although the "aerial synnemata" formation was more pronounced, the growth was not pigmented (Figure 113).

- **On bagasse lignin wood flour:**

  S. schenckii grew well on this wood-flour, and "aerial synnemata" were produced in abundance. No pigment could be detected in the colony, but radiating and slightly submerged growth was seen (Figure 114).

- **On bagasse hemicellulose wood flour (fine and ultra-fine):**

  The inoculum took up the colour of the hemicellulose within the first two weeks of incubation, but no growth at all could be seen either on the fine or ultra-fine hemicellulose agars (Figure 115).

- **On cellulose:**

  Growth was similar to that seen on the control agars, but no pigment was produced. No changes in the cellulose pieces could be detected.

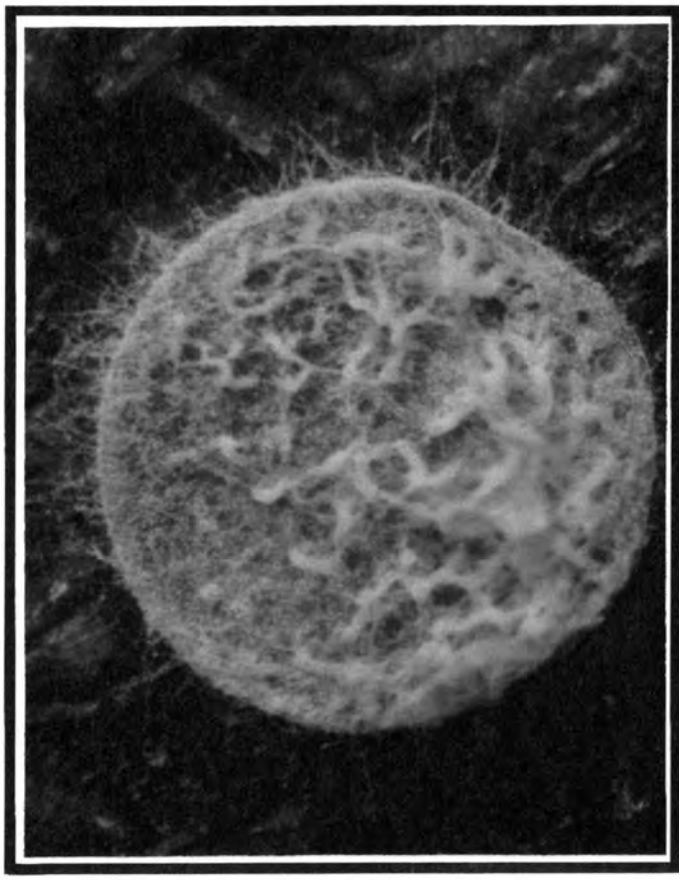


FIGURE 112. Superficial spider web-like growth of S. schenckii on Pinus pinaster wood meal.

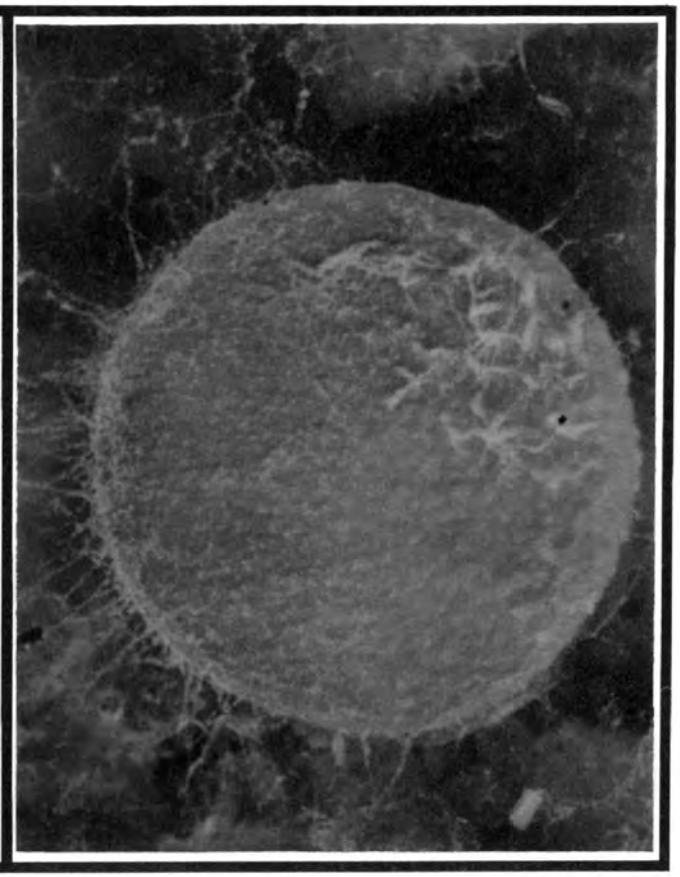


FIGURE 113. "Aerial synnemata" forming on Eucalyptus siligna wood meal.

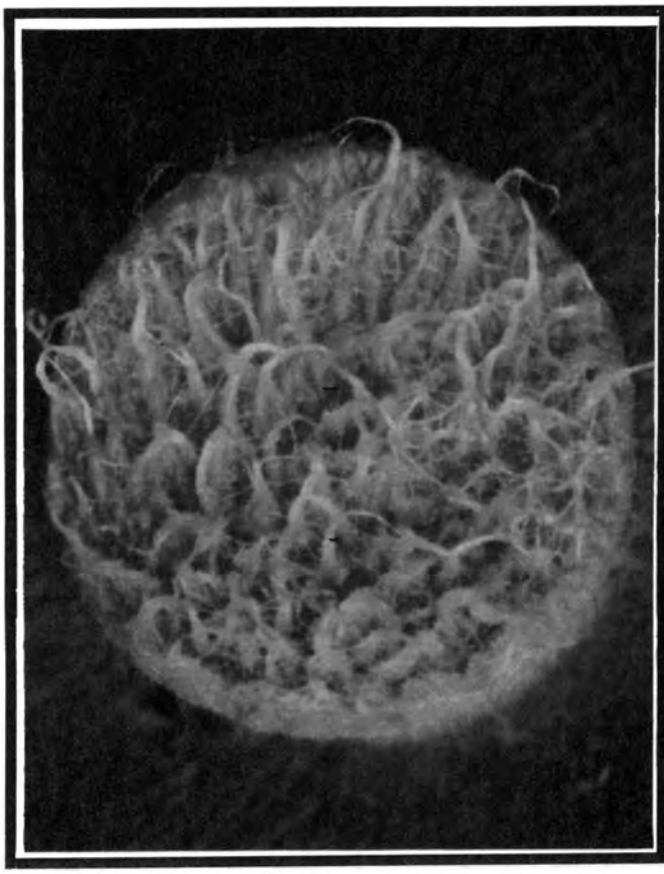


FIGURE 114. Pronounced "aerial synnemata" production on bagasse lignin.

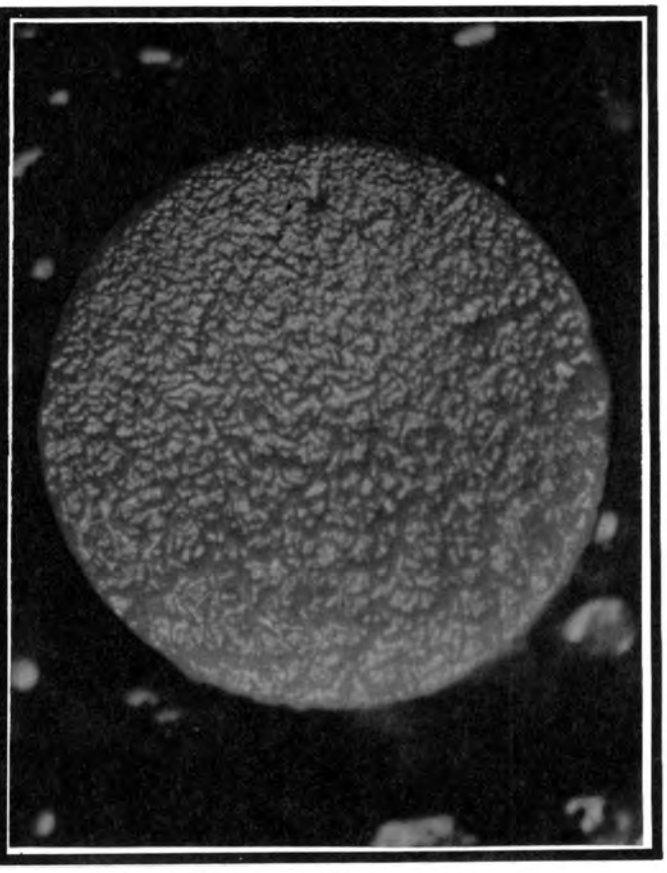


FIGURE 115. No growth on bagasse hemicellulose.

FIGURES 112 - 115. Growth of S. schenckii on wood flours and wood meals. Stereo-microscopy. 1,5x lens.

The extraction process for hemicellulose is done at a very alkaline pH (pH 9 - pH 13), this being the main reason for use of buffered agar suspensions. It was found however that when the pH of the hemicellulose agar was measured at a temperature of 40°C, the pH was 9,3. In subsequent experiments on buffered agars, with a pH between 3,0 and 9,5, *S. schenckii* grew at a maximum from pH 5,0 to 6,6, and at an optimum at pH 5,6, when incubated at 25°C. The fungus produced very mild and restricted growth from pH 8,0 to pH 8,5, and very poor growth at higher pH values. Mild growth could still be detected at pH 4,5, but not at lower pH values. An hypothesis that *S. schenckii* prefers an acidic pH range, can therefore be put forward. Furthermore, it was found that hemicellulose acts as a strong buffer, because the pH of the hemicellulose agar, kept in a water bath at 40°C, drifted from pH 6,0 to 9,3, increasing and decreasing when stirred. This transient increase in hemicellulose alkalinity seems a reasonable explanation why *S. schenckii* could not grow in the presence of hemicelluloses.

These experiments indicate that while *S. schenckii* grew fairly well on agar-agar it does not have the ability to utilize purified cellulose or lignin. Because *S. schenckii* could not be grown successfully on hemicellulose, such a deduction could not be made for hemicellulose. No changes in the wood structure could be detected in the case of the course or fine wood meals.

As far as the microscopy of the "aerial synnemata" is concerned, their structure was similar to that seen on block cultures of *S. schenckii* (see paragraphs 2.2 (p. 72) and 5.1 (p. 85). They also consisted of closely knit mycelial strands, running parallel, with classical conidiation of *S. schenckii*.

8 FLUORESCENT DNA-STAINING STUDIES OF *S. SCHENCKII*

By use of the methods described in Chapter II, paragraphs 5.1 (p. 20) and 8 (p. 26), fluorescent DNA-staining could be applied successfully to the duplicate sets of prepared block cultures of *S. schenckii*. The results for the colony development and conidium ontogeny are applicable here (see paragraph 5.1, p. 85).

The aim of the study was to determine whether there were any qualitative fluorescent differences between the nuclei in the classical hyaline ovate conidia first produced by *S. schenckii* and the later developing larger pigmented conidia and triangular conidia. Nuclear division and migration into vegetative hyphae, conidiophores and conidia could also be detected by this method.

Background staining with bisbenzimide was slight, showing up the hyphal cell walls and the placement of septa. As the hyphae degenerated, the staining of the walls, septa and nuclei diminished.

Microscopic examination was done on the day the block cultures were fixed and stained as the fluorescence deteriorated within 8 to 12 hours after preparation.

8.1 Conidium germination

Conidium germination commenced on day one with incubation at 25°C. Only one germ tube per conidium developed at the unattached or distal end of the conidium (Figures 116a and 116b).

8.2 Hyphal growth and hyphal nuclei

Hyphal growth took place at the tips, and septa formed once nuclear division was complete and the daughter nucleus had migrated into the newly formed hyphal segment (Figures 116c and 117). Thus, vegetative

hyphae with one nucleus per hyphal segment were produced (Figures 117 and 120). In the active hyphal growing phase of *S. schenckii*, i. e. days one to nine in the block cultures, hyphal nuclei were most commonly elongated, like rice-grains, with a dull fluorescence, capable of curving, bending and forming knuckles into hyphal side branches (Figures 118 - 122). Except directly after division, the nuclei were evenly and regularly spaced in the centre of each hyphal cell throughout the length of the hyphae (Figure 120). In certain hyphal regions the nuclei were compact and spherical and more brightly fluorescent than the "rice-grain" shaped nucleus, and their containing cells were often smaller (Figure 120). The nuclear size was seemingly equal to that of the conidia as was the degree of fluorescence (Figures 120 and 121). No quantitative estimations of fluorescence were made, but it was seen that increased fluorescence paralleled reduced nuclear volume. Hyphal nuclei also migrated between parallel running or angulating hyphal strands via anastomoses. More than one nucleus per H-shaped or Y-shaped hyphal segment was therefore present (Figures 119 and 121). No nuclear fusion was detected.

Conidiophore formation progressed by receiving nuclei from the vegetative hyphae (Figure 119). Multiple nuclei in a conidiophore were each separated by a septum, as seen in the case of the nuclei in hyphal segments.

8.3 Conidium formation and conidial nuclei

As previously described, conidium formation commenced on days two or three at 25°C in block culture (see Table 4). Large nuclei were seen in the hyphal segments near the sites of lateral conidium production and in conidiophores producing rosettes of conidia. At these sites of conidium production the hyphal cells were smaller. Nuclear fluorescence was uneven and fragmented, evidently preceding division. Increased DNA content seemed likely. These nuclei remained in the rice-grain shape, but had a more drawn appearance, dividing to supply nuclei to the forming conidia, with the one daughter nucleus moving

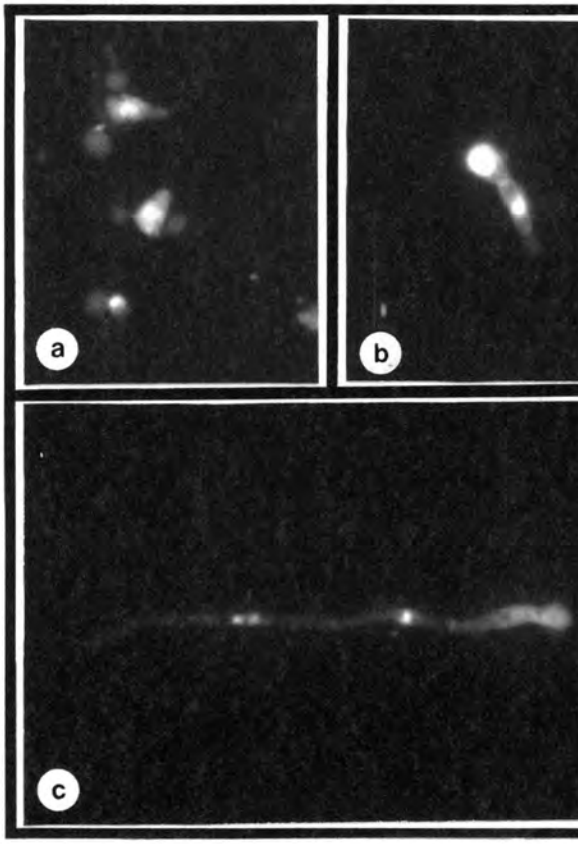


FIGURE 116a-c. Germinating conidium, nuclear division and germ-tube formation. (Oil immersion lens)

FIGURE 117. Nuclear division (arrow), hyphal growth and septal formation. (Oil immersion lens)

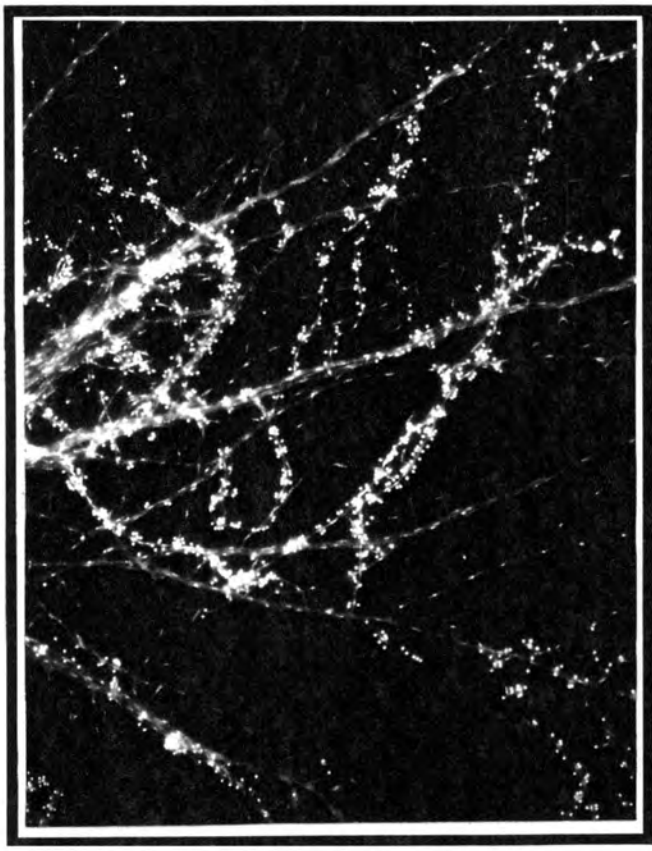


FIGURE 118. Actively growing hyphal lattice, dull fluorescing "rice-grain" nuclei and synnemata formation. (4x lens)

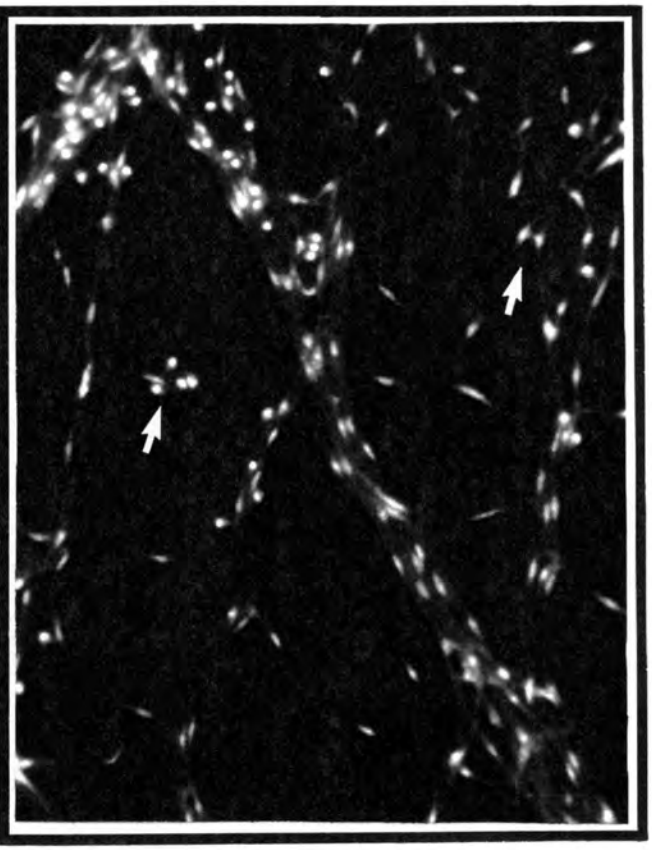


FIGURE 119. Hyphal nuclear migration via anastomoses; Conidiophore with conidium rosette (arrows). (20x lens)

FIGURES 116 - 119. DNA-fluorescence with bisbenzimide in S. schenckii block cultures.

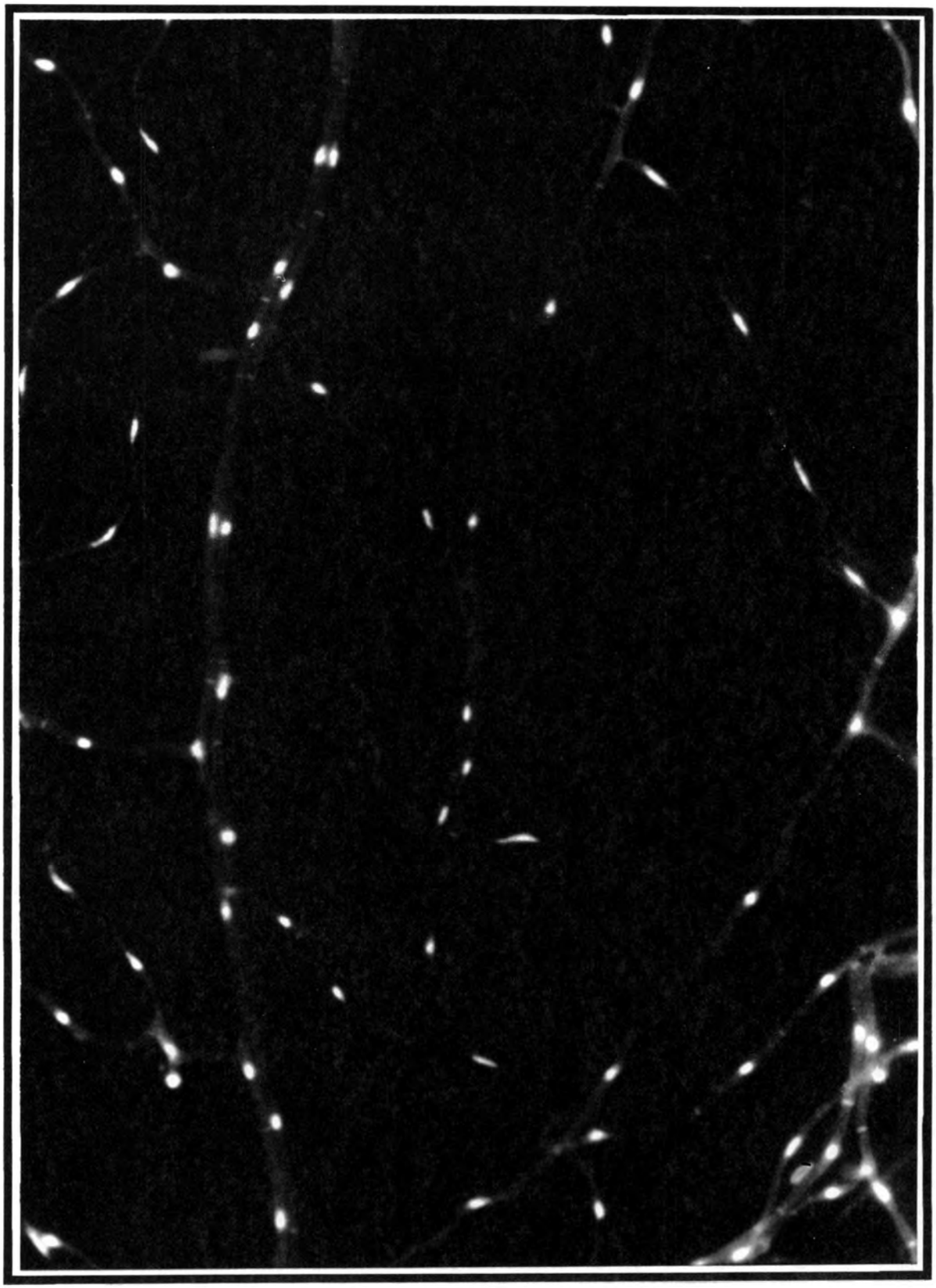


FIGURE 120. DNA-fluorescence with bisbenzimide in S. schenckii block cultures. One nucleus per hyphal segment, evenly spaced. "Rice-grain" and spherical nuclei present - the latter contained in smaller hyphal cells. (Oil immersion lens)

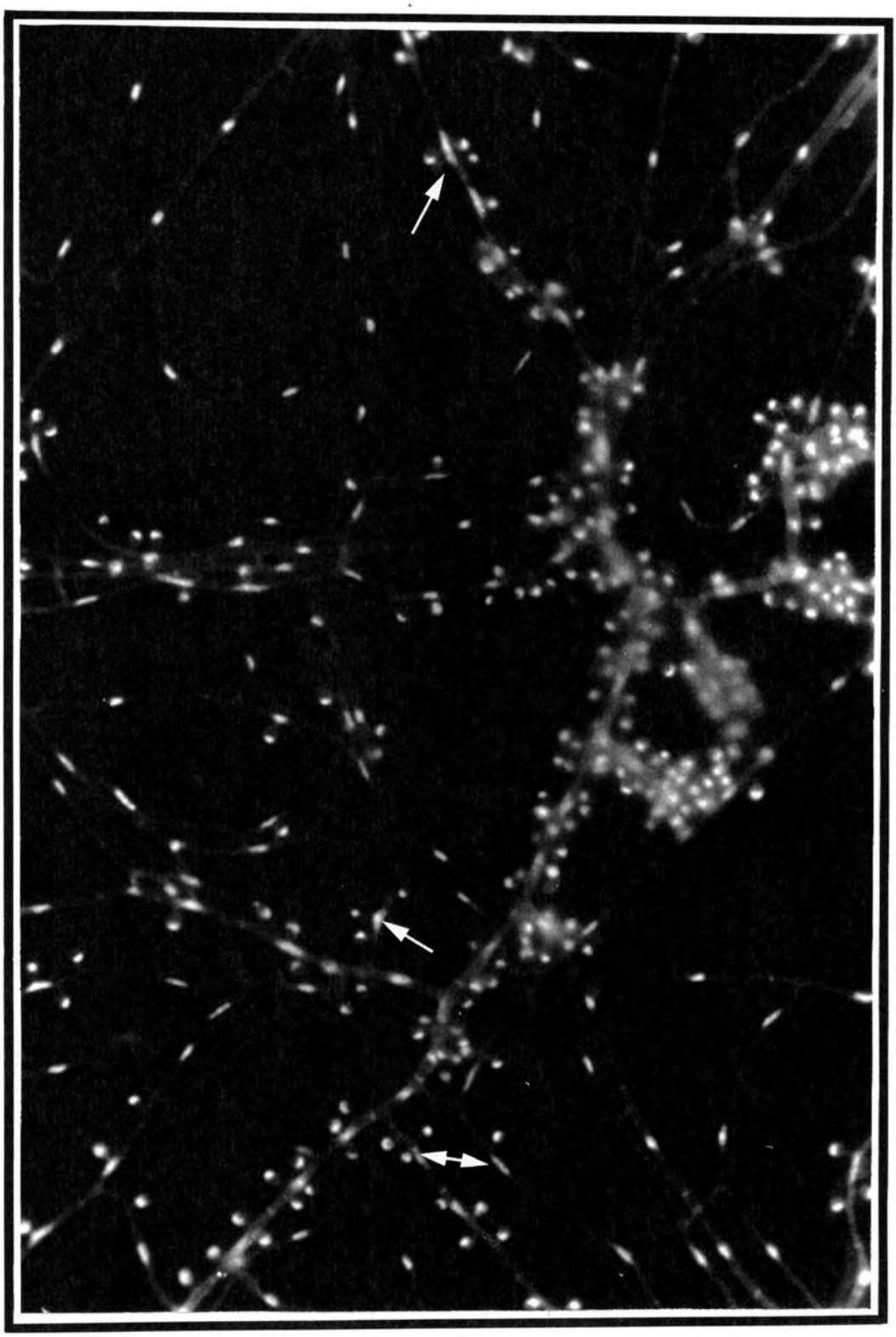


FIGURE 121. DNA-fluorescence with bisbenzimide in S. schenckii block cultures. H- and Y-shaped hyphal segments. Dividing hyphal nuclei supplying lateral conidia with nuclei (arrows). Mature conidia with compressed crescentic shaped nuclei - mainly distally, but also laterally or basally. (40x lens)

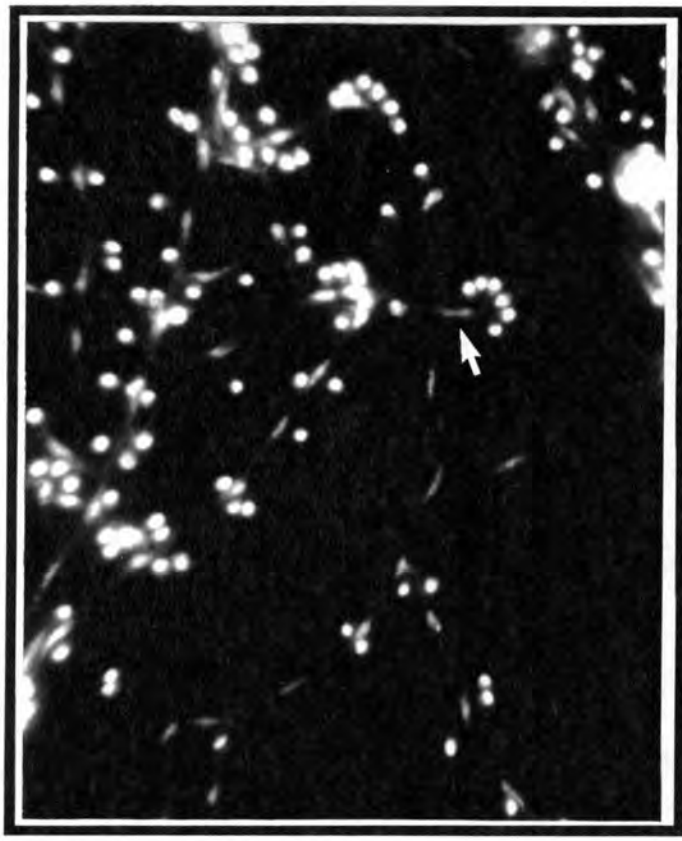


FIGURE 122. Rosette of six conidia formed from one dividing nucleus in conidiophore (arrow). (40x lens)

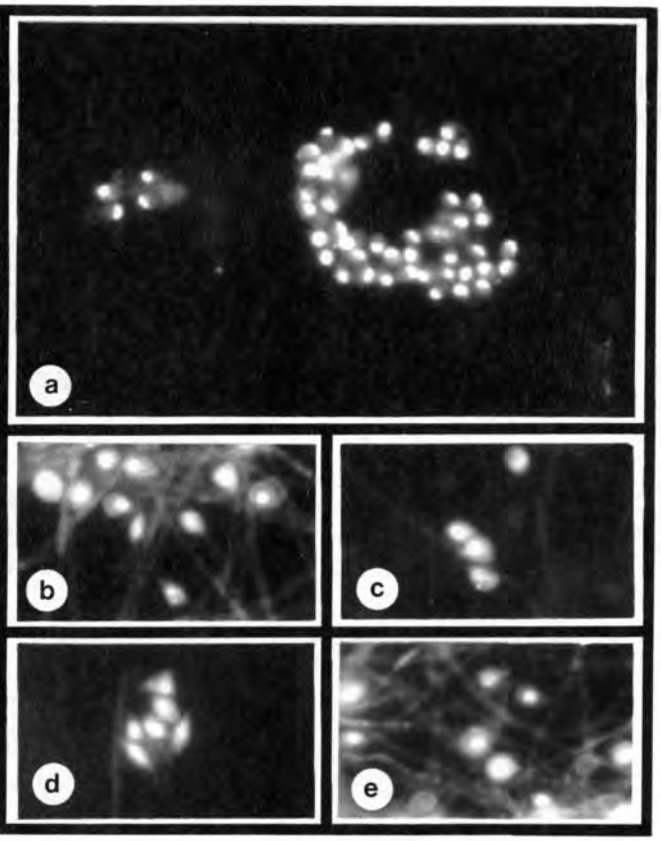


FIGURE 123. a) Detached mature conidia with distal crescentic nuclei. (40x lens) b-e) Triangular conidia with one nucleus each. (Oil immersion lens)

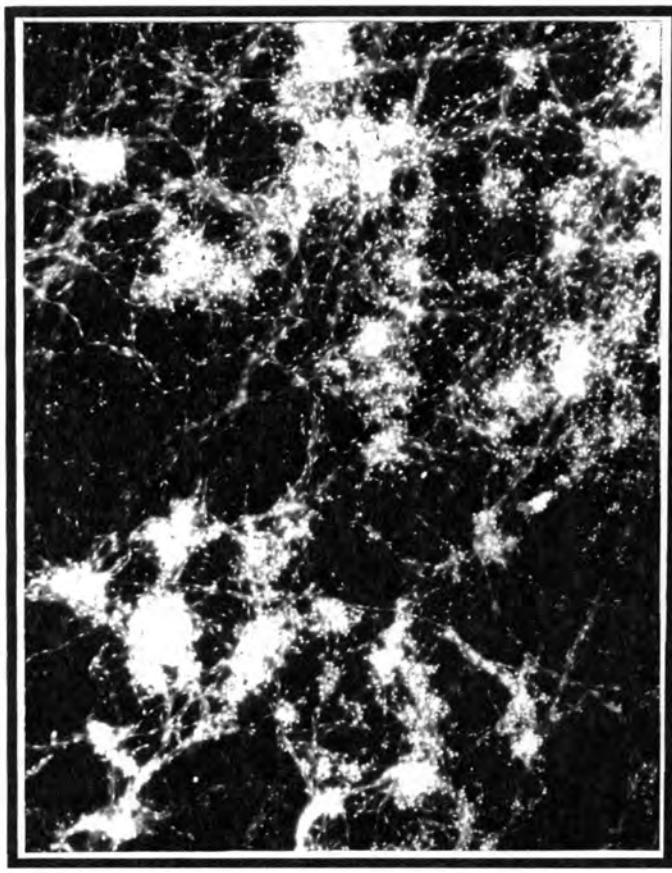


FIGURE 124. Mass conidial and hyphal nuclear fluorescence - Nine day old block culture. (10x lens)

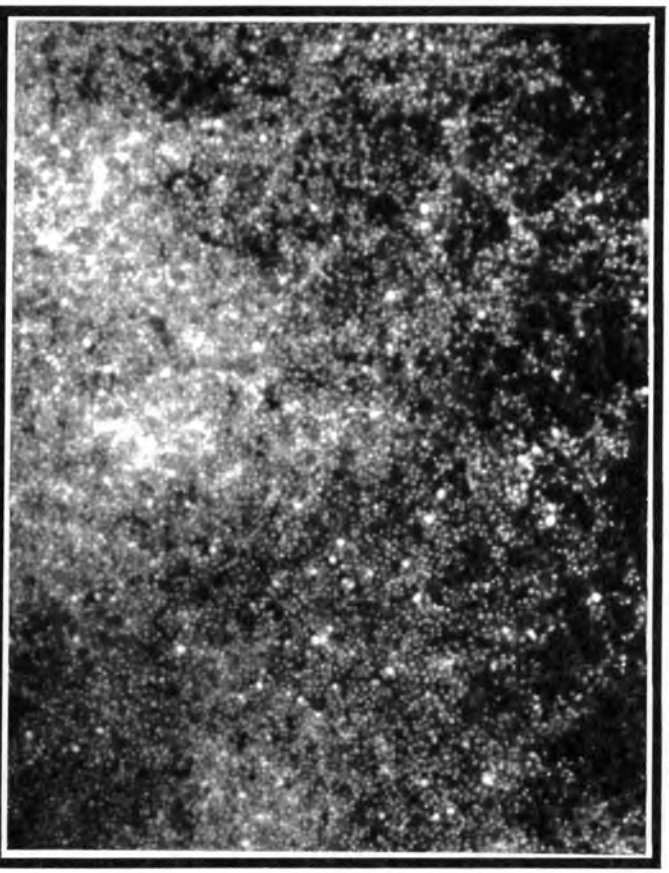


FIGURE 125. Mass conidium and hyphal nuclear fluorescence - Twenty one day old old block culture. (10x lens)

FIGURES 122 - 125. DNA-fluorescence with bisbenzimide in S. schenckii block cultures.

into the new conidium and the other remaining in the hypha or conidiophore. It was usual for such cells to yield five to six conidia initially, later forming cuffs of conidia around the hyphae and conidiophores (Figures 119, 121 and 122). Nuclear fluorescence in the newly formed conidium filled the entire structure (Figures 121 and 122). As the conidia matured, the nuclei were usually compressed into the distal part of the conidium, giving them a hemispherical or indented, crescentic shape (Figures 121 and 123a). Conidia were occasionally seen to have basally or laterally compressed nuclei (Figure 121). The intensity of fluorescence in triangular conidia was the same as for the ovate to globose conidia. Only one nucleus per triangular conidium could be detected (Figures 123b-e). As the block culture aged, the fluorescence of the dense masses of conidia increased, obscuring all other microscopic features (Figures 124 and 125).

9 SCANNING ELECTRON MICROSCOPY OF *S. SCHENCKII*

9.1 Growth of *S. schenckii* on eucalyptus chips, wattle pods, twigs and leaves

In situ scanning electron microscopic (SEM) examination of the fungal growth on wood seemed necessary to better assess the occurrence of *S. schenckii* in nature on plant material. Of special interest was the formation of triangular conidia in either pathogenic or soil isolates on dead plant material. Original pathogenic and soil isolates which had not been seen on microscopic examination to produce triangular conidia in SDA cultures, were therefore inoculated onto the plant material under discussion. The pathogenic strains, isolated from localized, lymphocutaneous and extracutaneous sporotrichosis, included unpigmented, buff, variegated and brown to black cultures. The soil strains were unpigmented. The methods described in paragraphs 9.1 (p. 26) and 9.2 (p. 27) of Chapter II, were employed, and the

macroscopic findings described in paragraph 6.1 (p. 96) of the current Chapter are applicable.

Visible mycelial growths, from both pathogenic and soil isolates of *S. schenckii*, were abundant and could easily be detected by means of SEM on eucalyptus wood chips and on dead wattle twigs and leaves. On wattle pods the growth was more scanty and superficial (Figures 126a, 126b, 126c, 131a and 131b). Fungal penetration into the wood was very superficial, i. e. to a maximum of 1 to 2mm, and assumed the appearance of hyphal weaving through the outer layers of the wood rather than true penetration (Figures 126a, 126b, 127, 129 and 130).

By monitoring the growth with the light microscope, conidiation in these strains was seen to follow the same pattern described before, and all other microscopic features of *S. schenckii* could be confirmed. Irrespective of the original clinical source of the ten pathogenic strains used, and whether they were pigmented or not, when inoculated onto both eucalyptus and wattle wood materials, triangular conidia developed within four to six months (Figures 126a, 126b, 126c, 130, 131a and 131b). The type of plant material, i. e. leaves, twigs, pods or shavings, did not seem to enhance or inhibit triangularity in any way. However, very few hyphal strands without dense sleeves of conidia could be seen after the five months' incubation, and both ovate and triangular conidia were present on the same hyphae (Figures 126a, 126b, 126c, 131a and 131b). This resulted in a large number of conidia per square millimeter or centimeter of plant material. The results were the same for the soil strains used, except that they did not produce triangular conidia within the initial five month or an extended five month incubation period, thus over ten months incubation (Figures 132a, 132b and 132c). Microscopic morphology of some soil isolates producing longer and more slender conidia (see paragraph 4.3, p. 81), could effectively be illustrated by SEM (Figure 132d). Other microscopic features, identical to those seen in the pathogenic strains of *S. schenckii*, can be seen in detail in figures 132a and 132d.

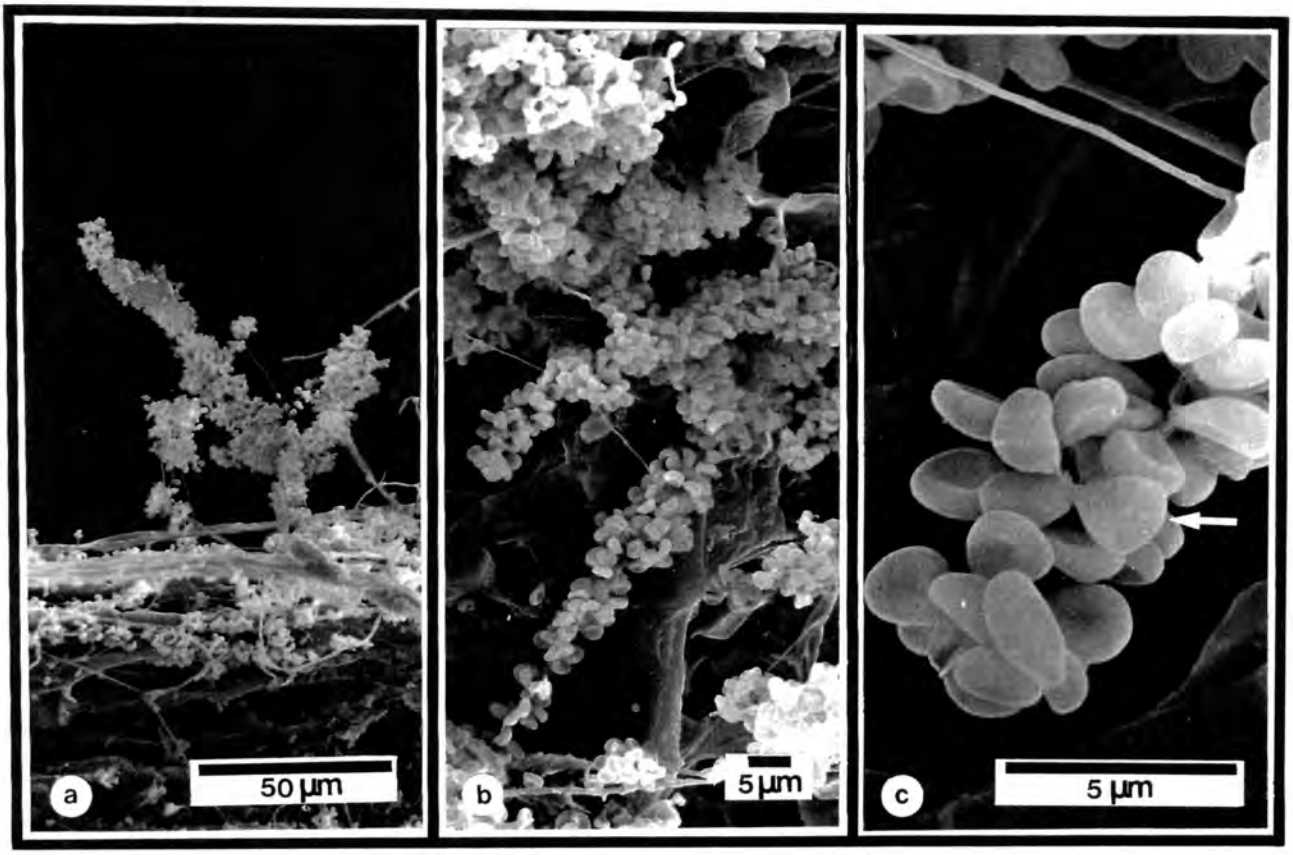


FIGURE 126. a) Superficial growth showing massed conidial sleeves on hyphae, projecting away from wood.
b) Higher magnification showing woven pattern of S. schenckii on structurally sound wood.
c) Masses of ovate and triangular conidia (arrow) on the same hypha.

FIGURE 127. Hyphal lattice of S. schenckii on a wattle leaf.

FIGURE 128. Full transition from ovate to triangular conidia on wattle leaves.

FIGURES 126 - 128. Scanning electron microscopy of S. schenckii growth on eucalyptus wood chips and wattle leaves

The pathogenic strains showed a complete transition from ovate to triangular conidia during the longer incubation period of ten months (Figure 127). Natural dehydration of some wood cultures over the same period did not promote the formation of triangular conidia. Although some ovate conidia showed indented sides, probably due to natural dehydration or processing of the material, the distal end of the ovate conidium was always rounded (Figures 126a, 126b, 126c, 128, 131a and 131b). Triangular conidia could therefore not be considered as artifactual changes in the ovate conidia due to dehydration. "Ears" on either end of the flat distal plane, i. e. unattached end, were detected in some of these triangular conidia. Triangular conidia with one "ear" more prominent than the other were also evident (Figures 128, 131a and 131b).

9.2 Block cultures of *S. schenckii* in malt agar

The microscopic features of the mycelial phase of *S. schenckii* at 25°C and the process of conidium ontogeny in block culture (see Chapter III, 2.2, p. 71), could be illustrated in more detail by means of scanning electron microscopy. The methods described in Chapter II, 9.3, p. 27, were applied.

Conidia germinated in a asymmetrical fashion from their distal unattached end to form one germ-tube per conidium (Figure 133). Parallel running hyphae became prominent from day three onward, and in some places enclosed non-germinating conidia (Figure 134). Bright bulges corresponding to hyphal and conidial nuclei were also evident (Figure 134). Conidiation was confirmed to be holoblastic and commenced at the apex of the conidiophore as shown in figure 135a, where the two younger conidia are still attached and the older conidium has come free. The cell wall sculpture of the younger conidia was markedly more textured than that of the older ones (Figures 135a and 135b). As conidiation progressed, the apex of the conidiophore became vesiculate, forming conidia on denticles in a sympodial manner (Figures 136a and 136b). Conidial cell wall ornamentations appeared citrus

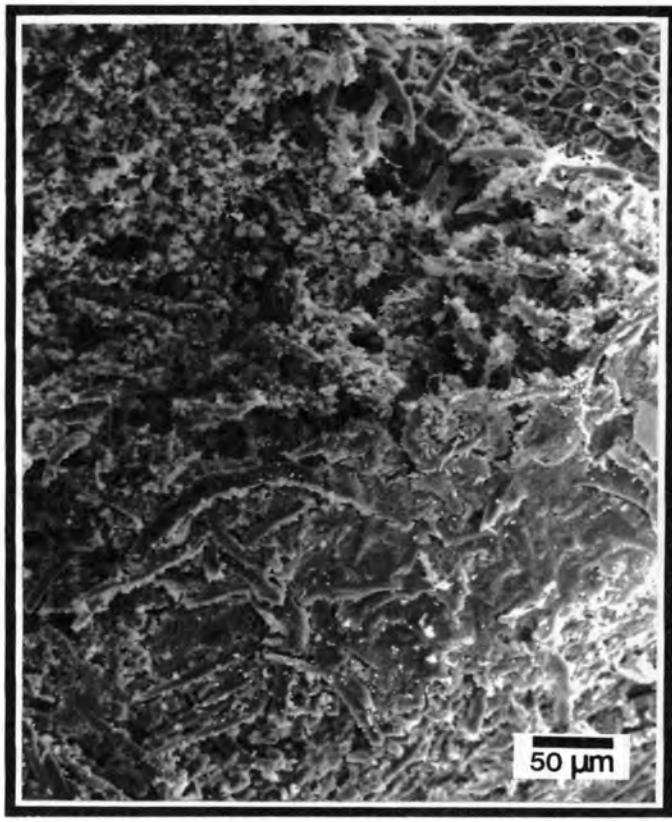


FIGURE 129. Hairy rough wood surface covered by dense masses of conidia.

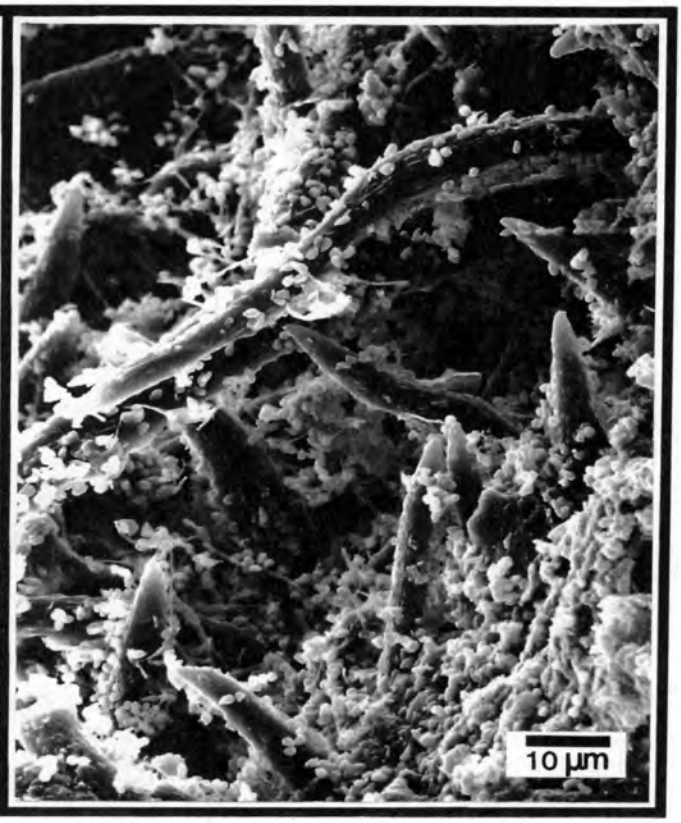
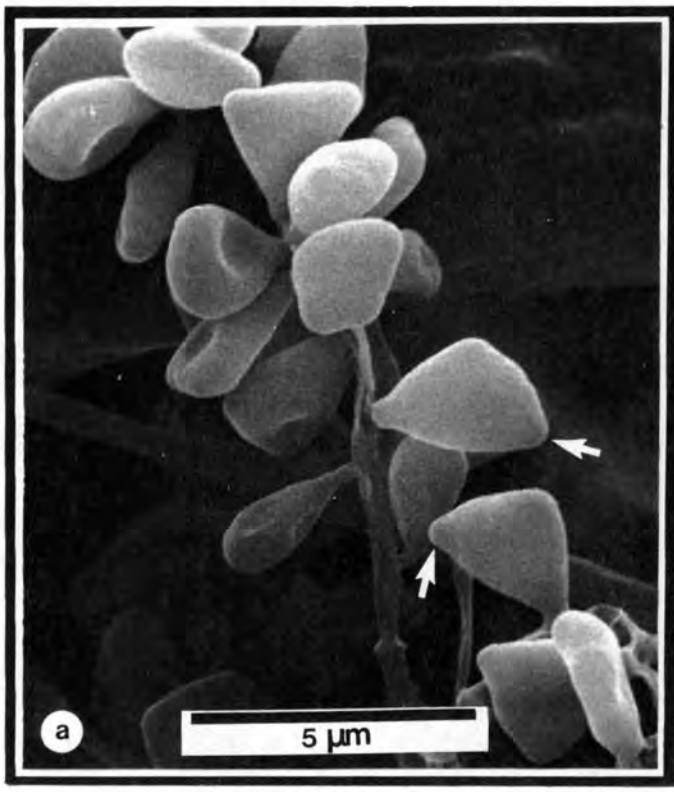
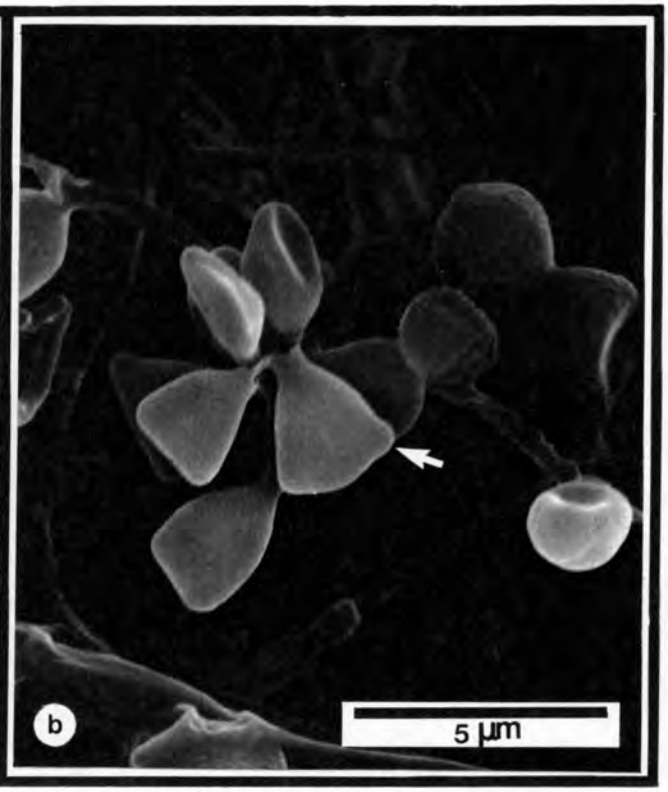


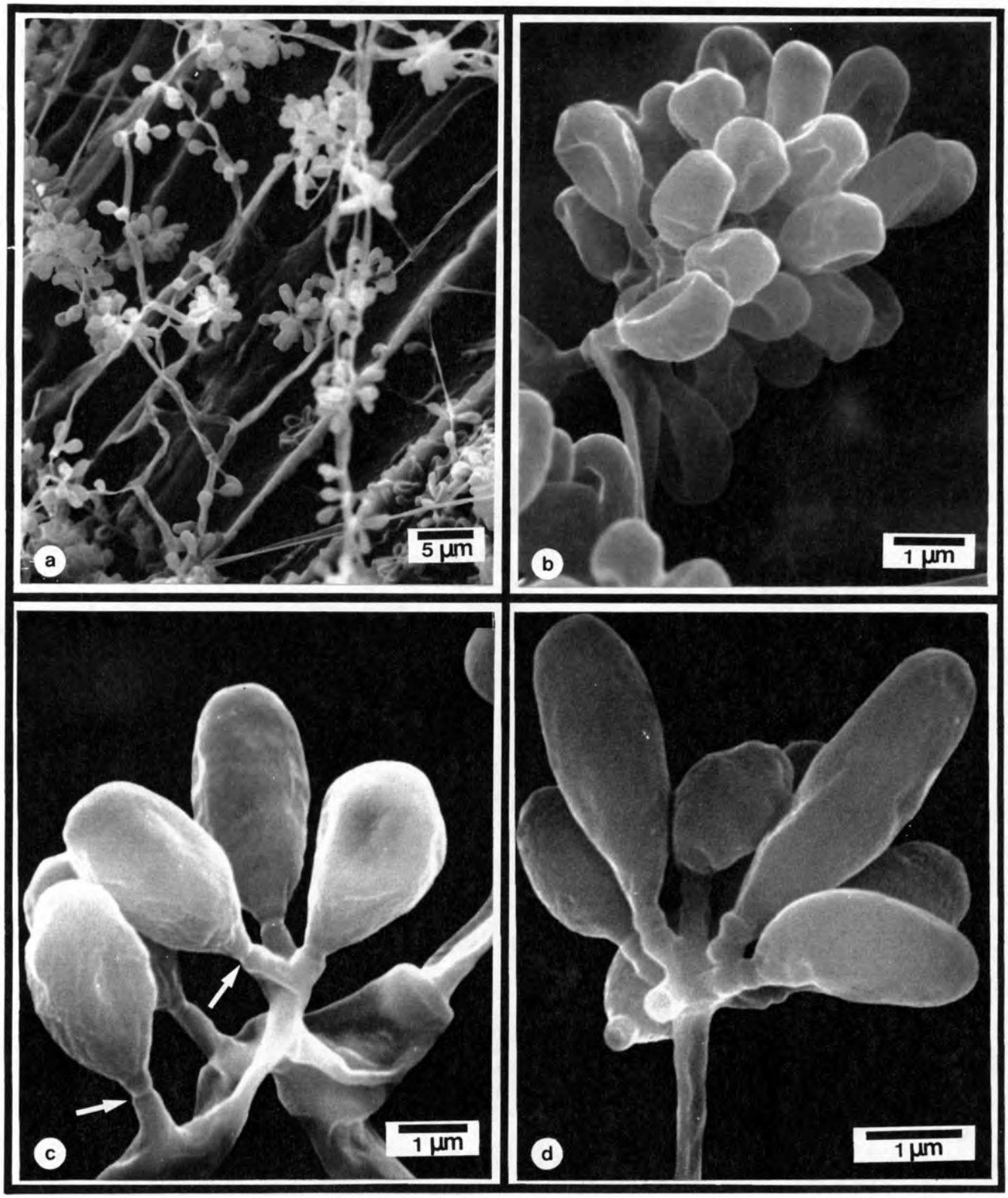
FIGURE 130. Higher magnification of hairy area in Figure 129.





FIGURES 131a&b. Triangular and ovate conidia present on the same hypha. The arrows indicate the "ears" evident in triangular conidia.

FIGURES 130 - 131. Scanning electron microscopy of S. schenckii growth on wattle twigs.



FIGURES 132a-d. Scanning electron microscopy of soil isolates of S. schenckii grown on wattle: a) Growth after fifteen months incubation. b) Production of only ovate conidia. c) Conidium rosette - arrows indicating detachment septa. d) Longer and more slender conidia seen in some soil isolates - conidium rosette on vesiculate conidiophore.

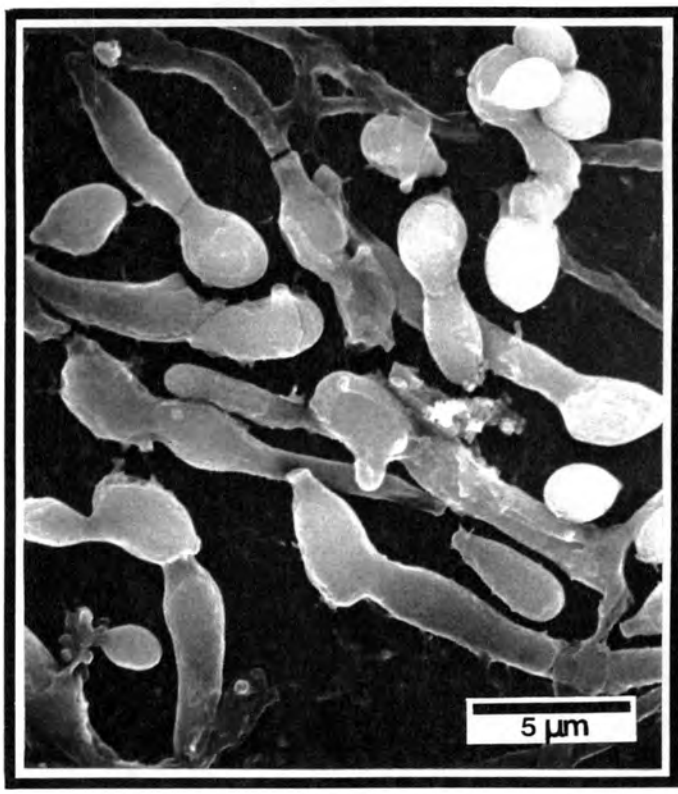


FIGURE 133. Germinating conidia - one distal germ-tube per ovate conidium.

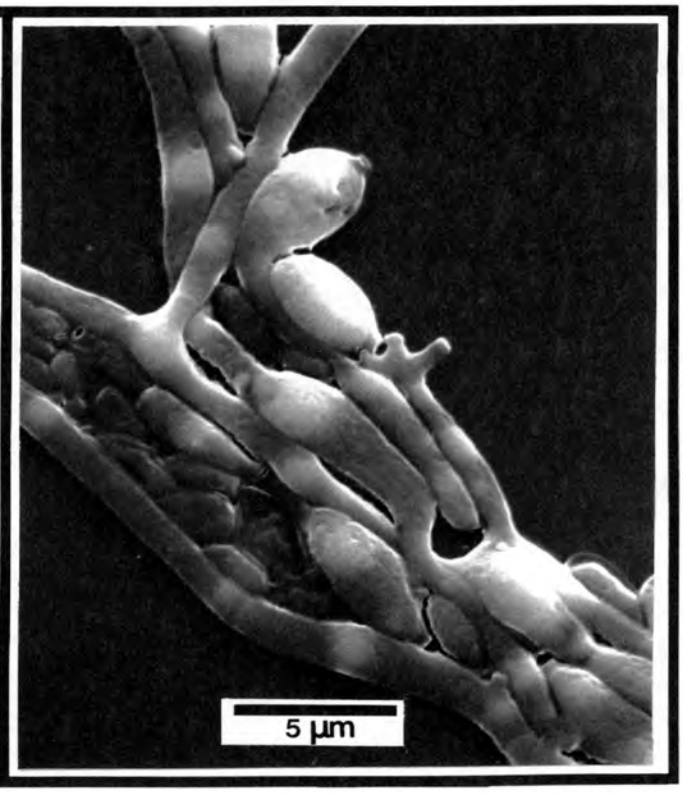
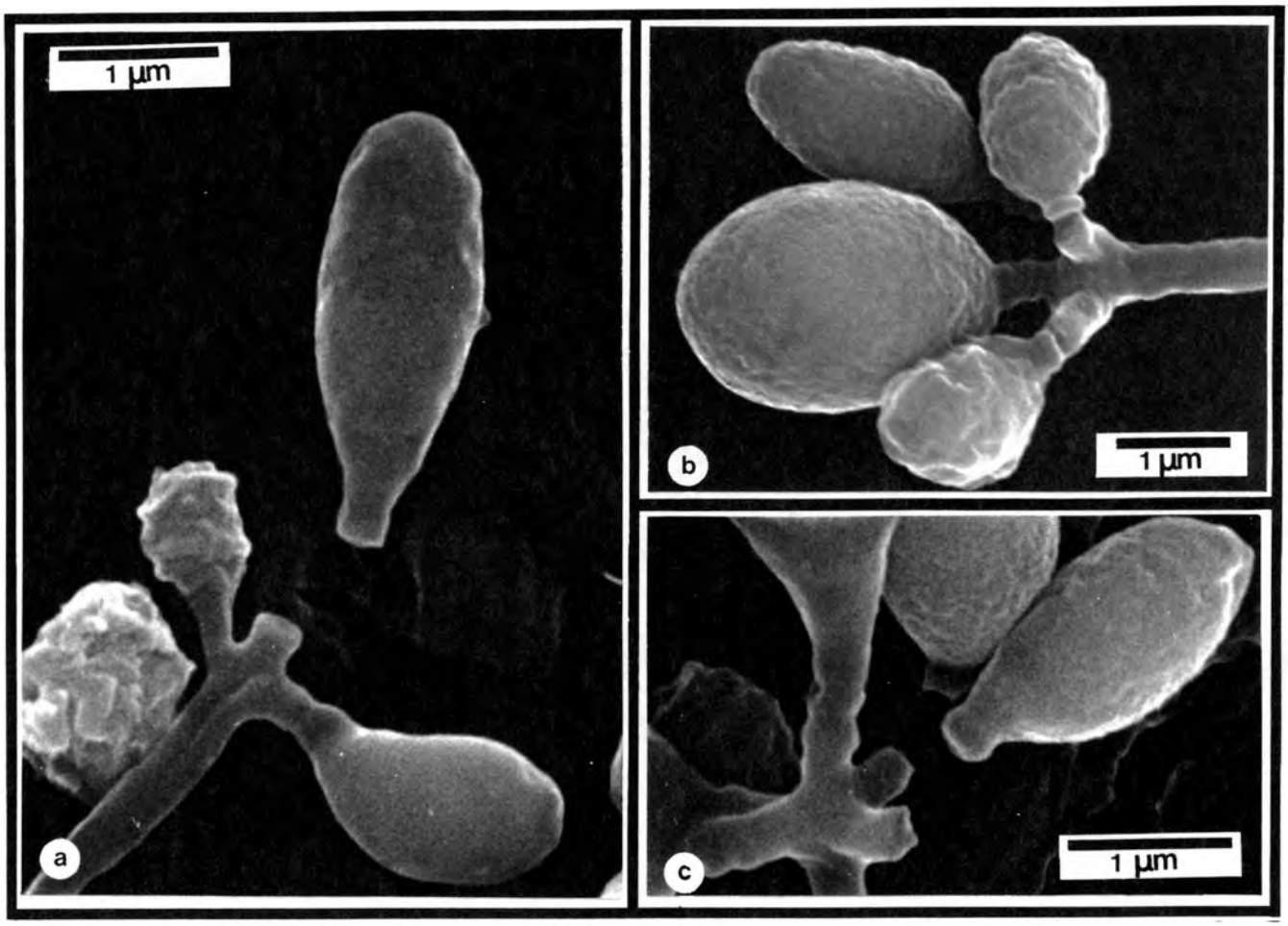
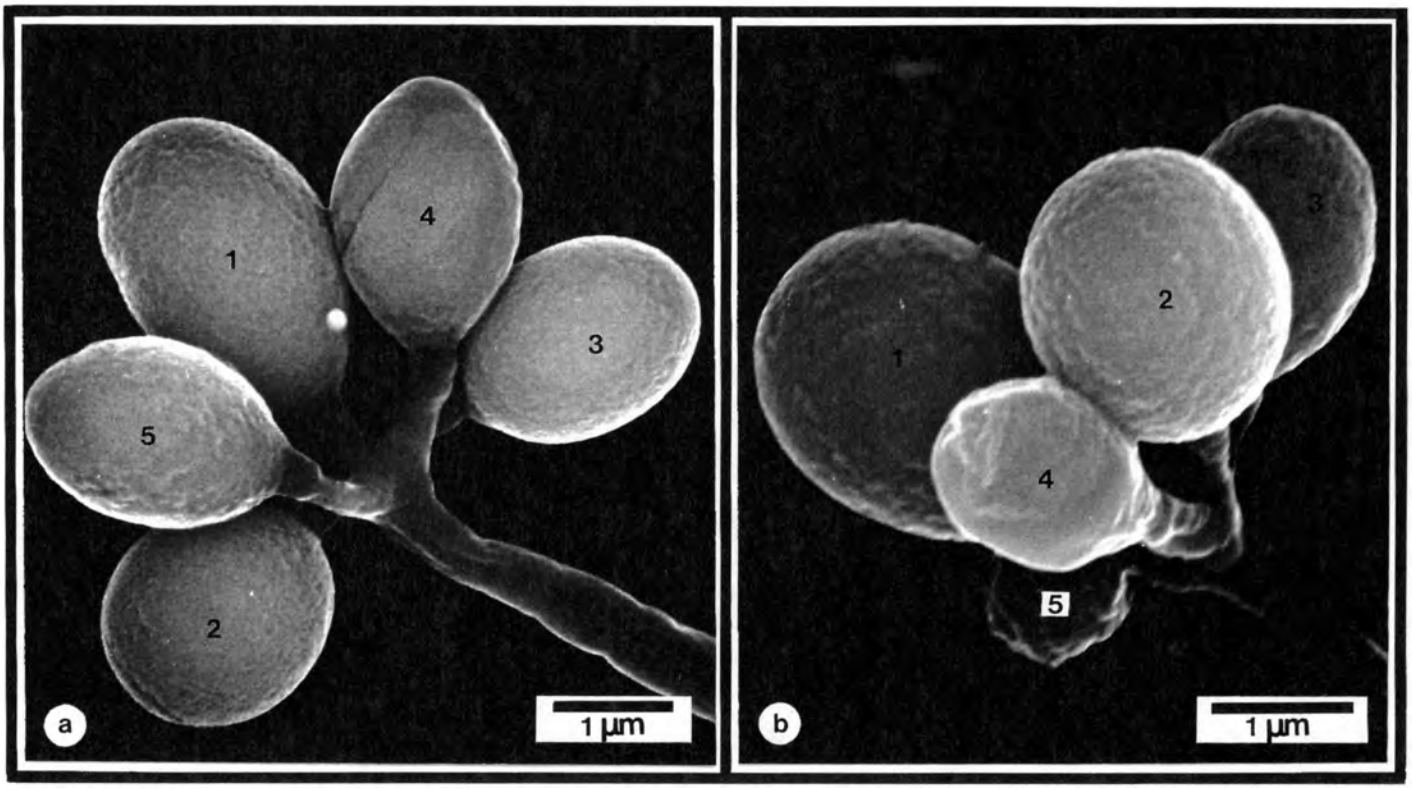


FIGURE 134. Parallel running hyphae enclosing non-germinating conidia. Bright bulges correspond to nuclei.



FIGURES 135abc. a) Conidiophore with three conidia, 1 = oldest & 3 = youngest. b) Four stage conidiation on vesiculate conidiophore. c) Conidia detaching. Note remaining denticles on the conidium and conidiophore vesicle.

FIGURES 133 - 135. Scanning electron microscopy of S. schenckii grown in block culture.



FIGURES 136a-b. Five stage conidiation. 1 = oldest sympodially produced conidium, 5 = youngest. Note the distinct detachment septum line with double ringed collar.

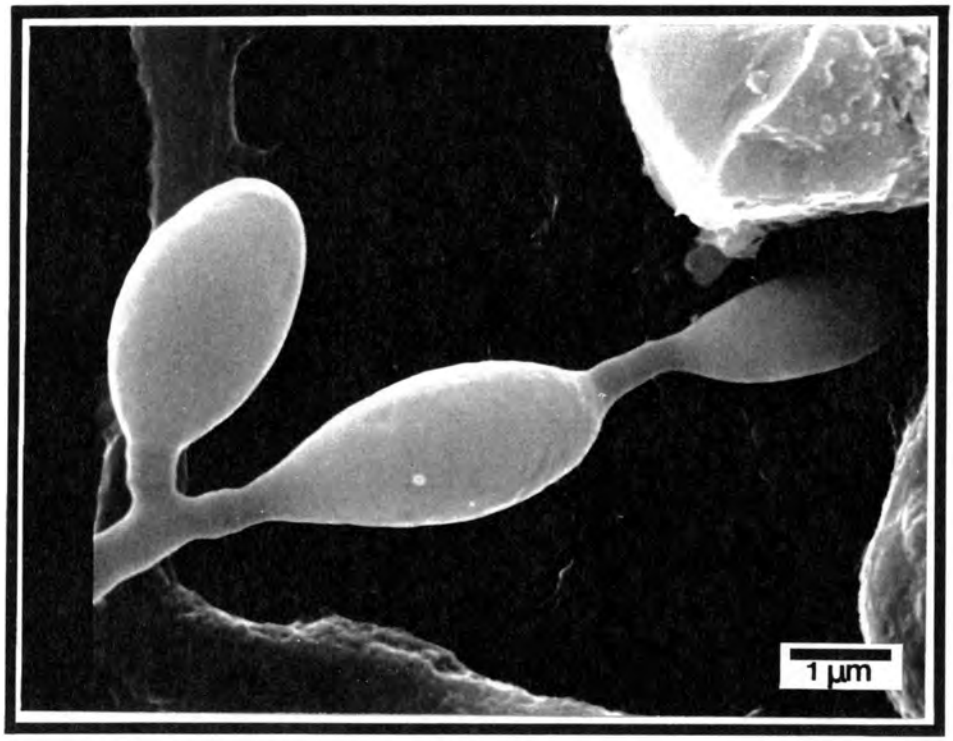


FIGURE 137. Secondary conidium formation from the distal end of a primary conidium

FIGURES 136 - 137. Scanning electron microscopy of S. schenckii grown in block culture

peel-like once the conidium was matured (Figures 135a, 135b, 135c, 136a and 136b). A distinct line, representing a detachment septum, developed in the centre of the denticle, forming a double ringed collar on the denticle (Figures 135a, 135c, 136a, and 136c). Once conidium separation had taken place, remains of the denticle were visible on the vesicle of the conidiophore as well as on the conidium (Figures 135a and 135c). Secondary conidia were occasionally produced from the distal ends of the primary conidia (Figure 137).

10 ELECTRON MICROSCOPY OF *S. SCHENCKII*

The ultrastructure of the classical ovate conidia and the triangular conidia produced by *S. schenckii* were compared according to the methods described in Chapter II, 10.1 (p. 27). Some hyphal strands were present in each type of conidial suspension used as no attempt was made to filter them out of the material used to prepare the suspensions.

10.1 Conidium ultrastructure

It can be well appreciated that in the ultrastructural examination of triangular conidia, the planes of sectioning do not always demonstrate triangularity. Numerous sections were necessary in order to see triangular conidia as such.

10.1.1 Conidial cell wall

The cell wall of the young, recently produced, hyaline ovate conidium (OV), consisted of an electron dense outer cell wall (ocw), which apart from a little roughness, had no other prominent ornamentations. (Figure 138). This correlated well with the scanning electron microscopy described for these structures (see paragraphs 9.1 (p. 122) and 9.2 (p. 125)). The outer cell wall layer ran continuously over either the

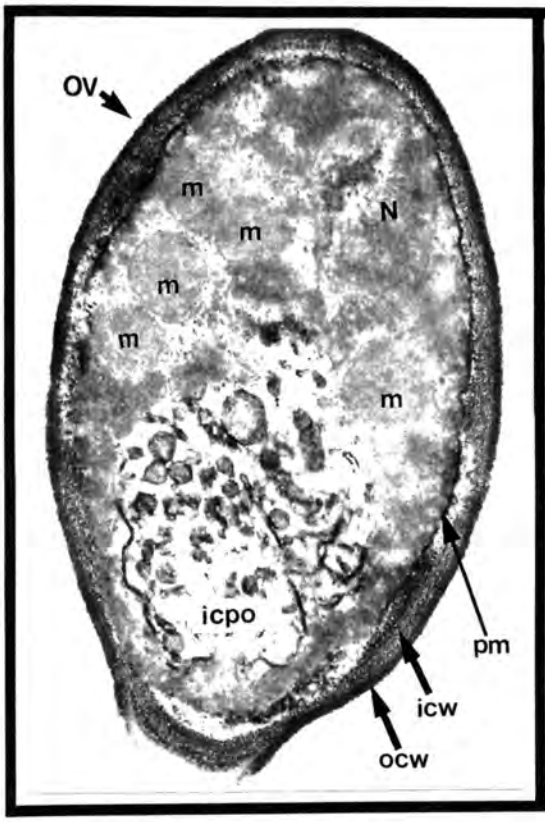


FIGURE 138. x 37 500

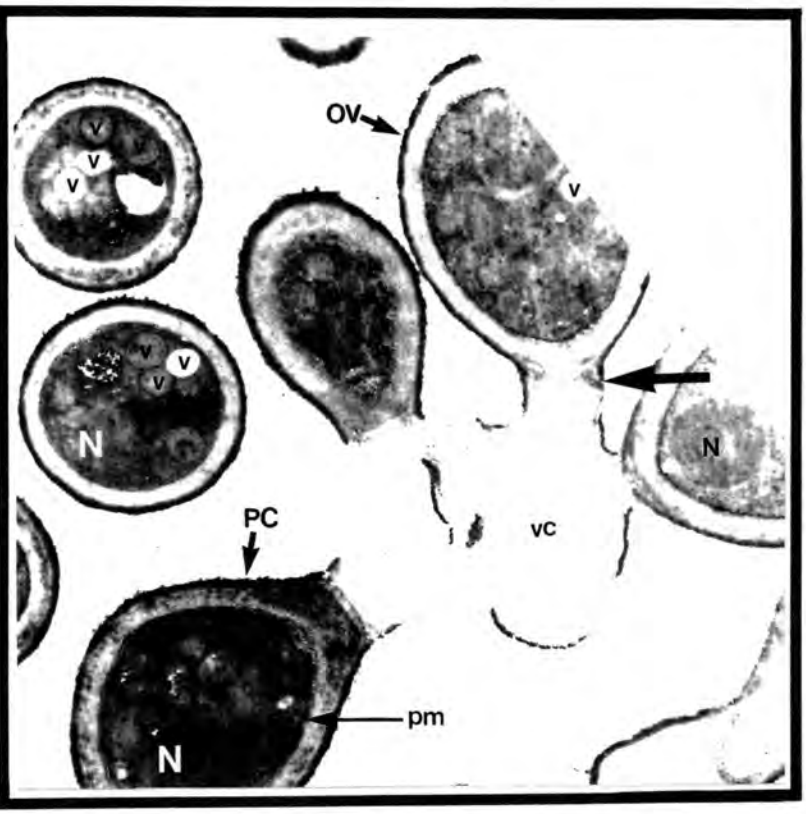


FIGURE 139. x 16 500.

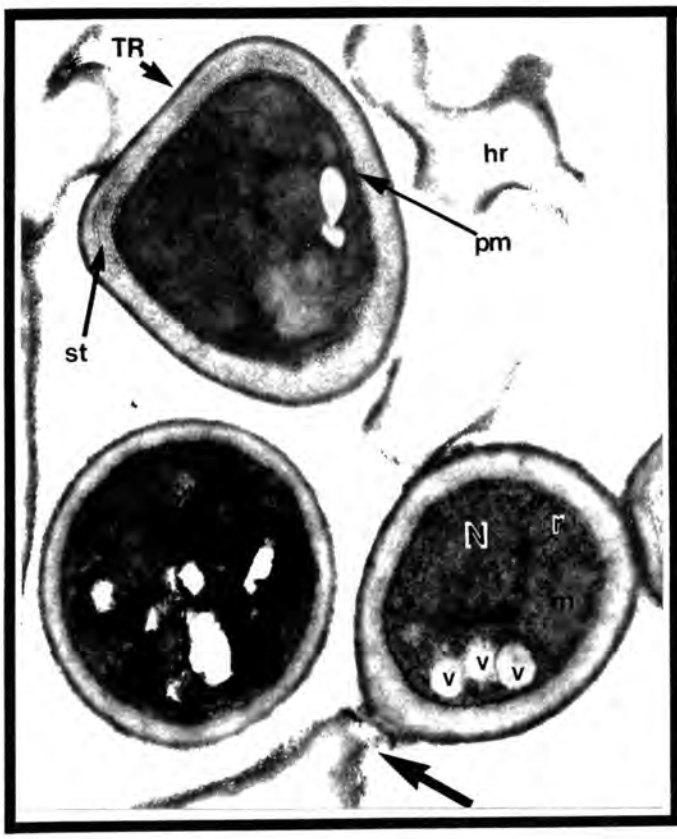


FIGURE 140. x 22 500

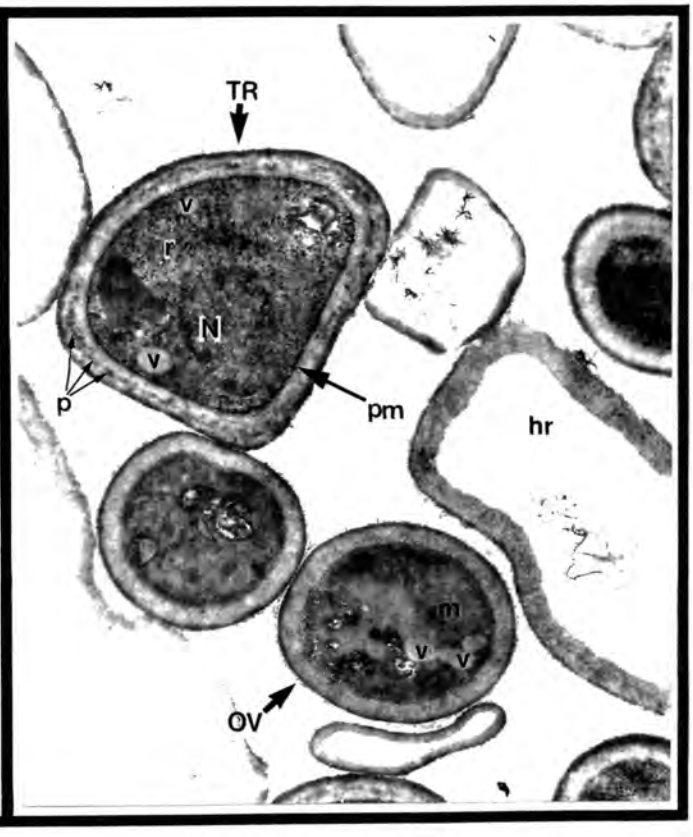


FIGURE 141. x 16 500.

FIGURES 138 - 141. Electron microscopy of S. schenckii conidia: OV = ovate conidium; TR = triangular conidium; PC = pigmented conidium; ocw = outer cell wall; icw = inner cell wall; pm = plasma membrane; m = mitochondria; v = vacuole; N = nucleus; r = ribosomes; icpo = intracytoplasmic organelles; vc = vesiculate conidiophore; hr = hyphal remnants; p = possible pigment deposits in icw; st = shoulder-like thickenings in conidium cell wall. Largest arrow = conidium detachment.

conidiogenous hyphal cell or the vesiculate conidiophore (vc) from which the conidia were formed (Figures 139 and 142). This layer appeared broken at the attachment end of the conidium after it had separated from its producing cell (Figures 139, 140, 145 and 146). Remains of the vesiculate conidiophore (vc) and detachment septum are shown in figures 139 and 146. Electron microscopy thus confirms holoblastic conidiation. To the inside of the outer cell wall layer there was an inner cell wall layer (icw). The latter was generally more translucent, about three times as thick as the outer layer, showed a diffuse granularity and projected against the plasmalemma (pl; Figures 138 - 142). The inner cell wall extended over the full conidium, including its point of attachment (Figure 138). The conidium denticle showed in addition to this inner cell wall material, a translucent layer of material, corresponding to one of the collars on either side of the detachment septum of the conidium (Figures 138 and 139). This was in sharp contrast to the denticle of the conidiogenous cell, which only showed a pale convex bulge of the hyphal or conidiophore inner cell wall (Figures 139, 142 and 146).

Pigmented (PC) and triangular (TR) conidia had similar cell wall structures. A thinner, more electron dense outer layer (ocw), was followed by a thicker layer consisting of granular, flocculent, and laminated deposits with intervening translucent zones (Figures 141 and 142). Oval structures without a basal attachment and with thickening of the cell wall at one or both ends, were considered to be triangular conidia, cut across at a level just below their unattached ends. In several sections it was seen that the deposits over the shoulder-like thickenings at the unattached ends of the triangular conidia formed part of the inner cell wall; these deposits were electron dense, flocculent or more laminated compared to the rest of the cell wall (Figures 140 - 146). Electron dense granular material in the centre of the inner cell wall may represent pigment observed in pigmented and triangular conidia (Figures 142, 143a, 143b, 144 and 145). Figure 142 illustrates a four staged conidium production of pigmented and triangular conidia on a vesiculate conidiophore.

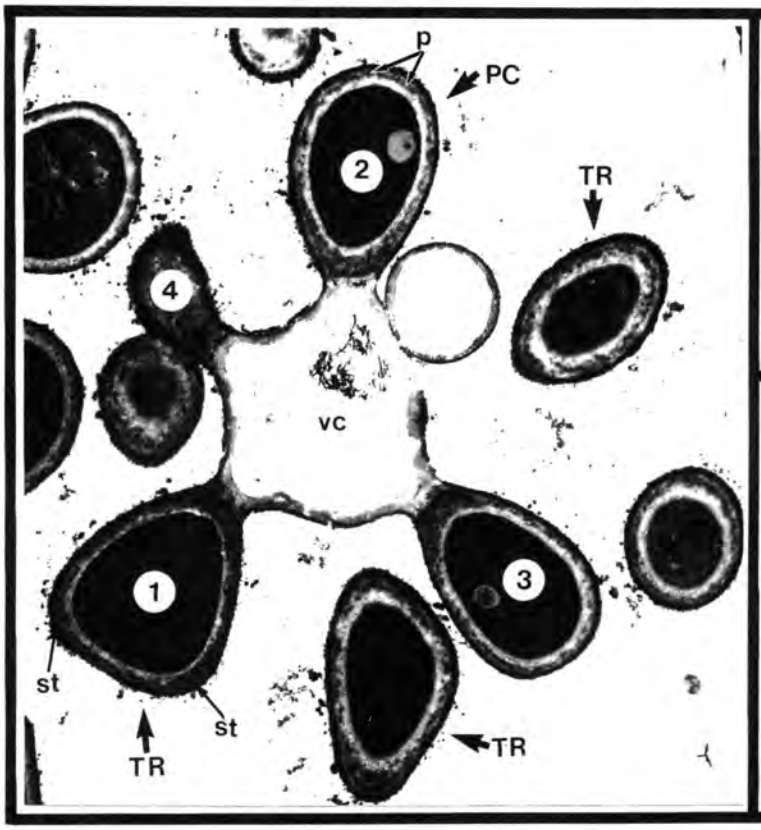


FIGURE 142. x 10 600.

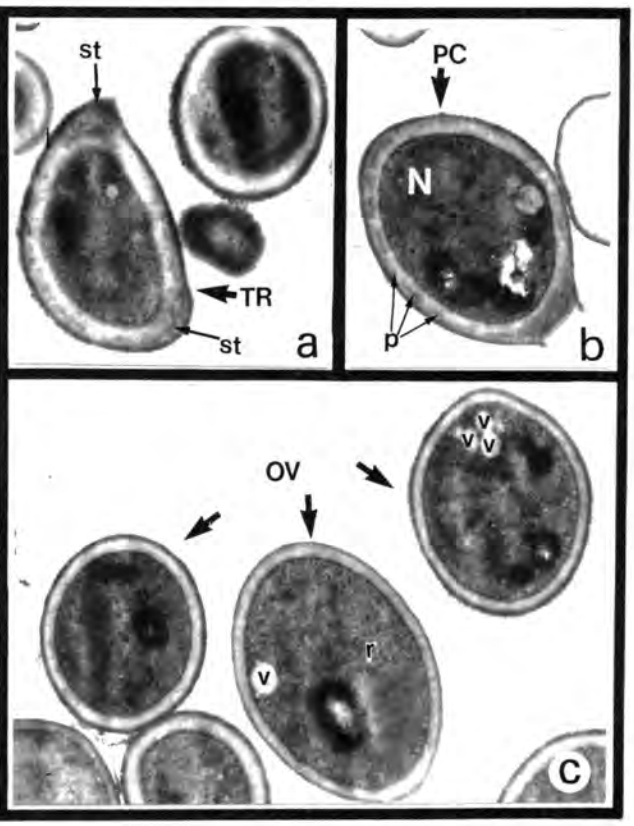


FIGURE 143a-c. x 13 600.

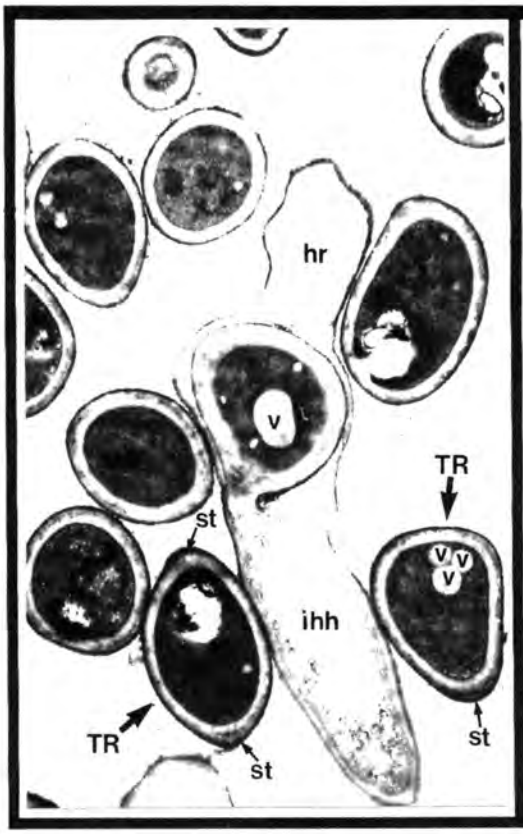


FIGURE 144. x 8 500.

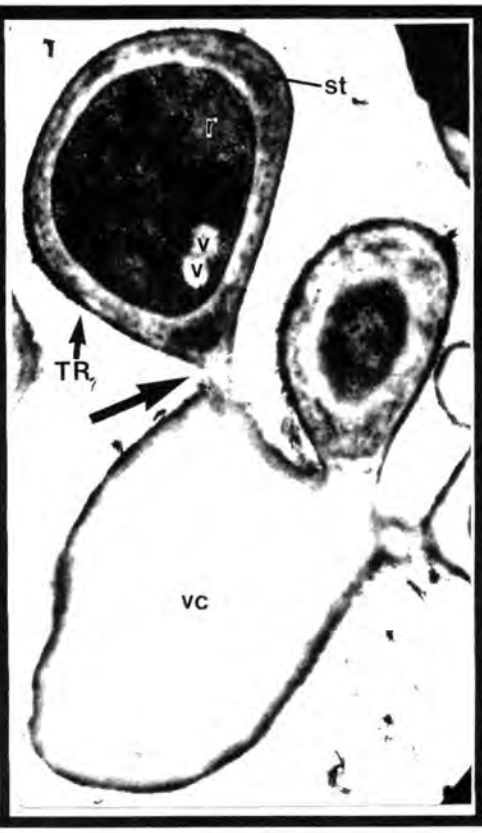


FIGURE 145. x 16 500.

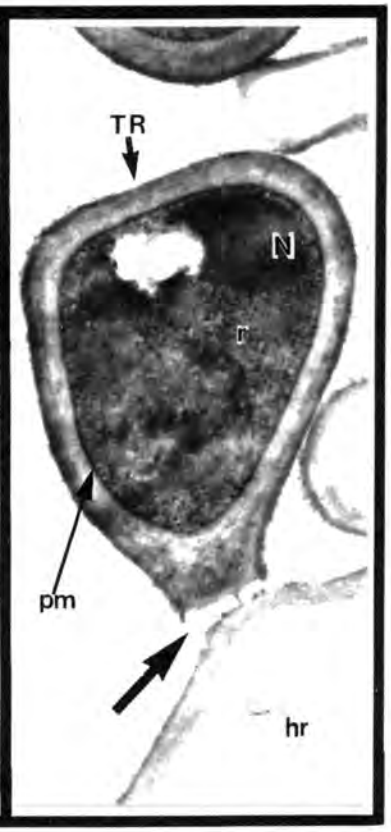


FIGURE 146. x 22 500.

FIGURES 142 - 146. Electron microscopy of S. schenckii conidia: OV = ovate conidium; TR = triangular conidium; PC = pigmented conidium; ocw = outer cell wall; icw = inner cell wall; pm = plasma membrane; m = mitochondria; v = vacuole; N = nucleus; r = ribosomes; icpo = intracytoplasmic organelles; vc = vesiculate conidiophore; hr = hyphal remnants; p = possible pigment deposits in icw; st = shoulder-like thickenings in conidium cell wall; ihh = intrahyphal hyphae. Largest arrows = conidium detachment.

10.1.2 Conidial cytoplasm

The cytoplasm of the younger conidia was clearly structured but later became densely obscured (Figure 138, 139, 143a, 147 and 148). The cytoplasmic content was enclosed in the plasma membrane:

- **Plasma membrane (pl)**

  The plasma membrane was the site of some particular features. In the younger ovate conidia it was closely interlocked with the inner cell wall and in places it was difficult to distinguish it from the cytoplasm. It mostly appeared as a smooth or wavy double-lined tubular layer, enclosing the entire cytoplasm in all types of conidia (Figures 138, 140 and 146). Depending upon the plane of sectioning the folds of the plasma membrane produce a variety of images - such as a woven, zig-zag or toothed pattern with lines and rods, resembling a tyre-thread or a rush-mat pattern (Figures 138, 143c, 145 and 146). The plasma membrane was seen to retract in most ovate conidia with some folding and reduplication and the formation of a space filled with hairy, thready and granular connections to the adjoining inner cell wall (Figures 138, 147 - 149). Where the plasma membrane was intact, laminated inner cell wall layers were produced to the outside of the in the ovate conidium and especially in triangular conidia (Figures 140, 143a and 146). From this it can be deduced that the cell wall layers originate from the plasma membrane.

- **Nuclei (N)**

  Nuclei were occasionally clearly visible and tended to occur at the distal end of conidia, but were also noted basally. Only one nucleus per conidium was present (Figures 140, 141, 143b and 146).

- **Mitochondria (m)**

  Several mitochondria per conidium were seen. In cases where the cytoplasmic structures became obscured, only ghost-like relics remained (Figure 138).

- **Vacuoles (v)**

  Vacuoles occurred regularly in conidia. Some conidia had up to three or four vacuoles, clearly surrounded by tonoplasts (Figures 139 - 146).

- **Ribosomes (r)**

  Ribosomes occurred freely in the cytoplasm, unassociated with endoplasmatic reticulum (Figures 140, 141, 146). The last was very scarce in all the sections viewed.

- **Other intracytoplasmic structures**

  Some intracytoplasmic structures were of uncertain nature. They included annular spaces with peripheral radiations, various droplets and tubular structures. They had no special or structural features and were indistinguishable from the normal intracytoplasmic structures (Figures 138, 147 and 148).

10.2 Enclosed conidia and hyphal degeneration

Conidium-like structures lying within membranous enclosures were common in our material (Figures 147, 149 and 150). Such enclosures comprised the inner part of an empty or degenerated hyphal structure. The outer and inner cell walls and septa were still present in places, but the cytoplasmic content was lost, except for a few crystals and perhaps a ring of retracted plasma membrane (Figures 147, 149 and 150).

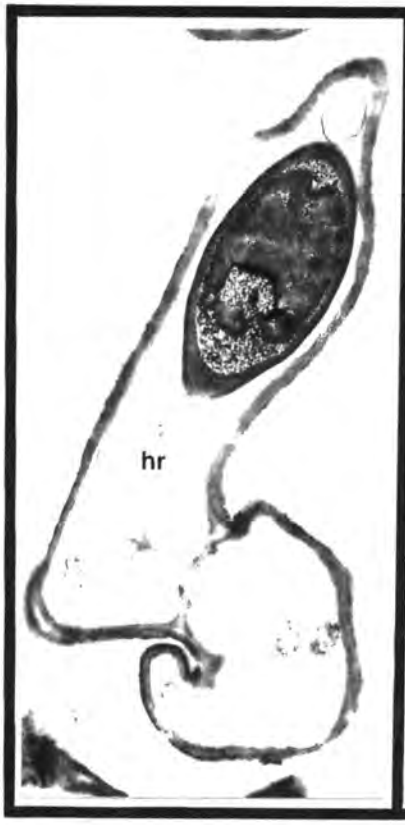


FIGURE 147. x 13 600.

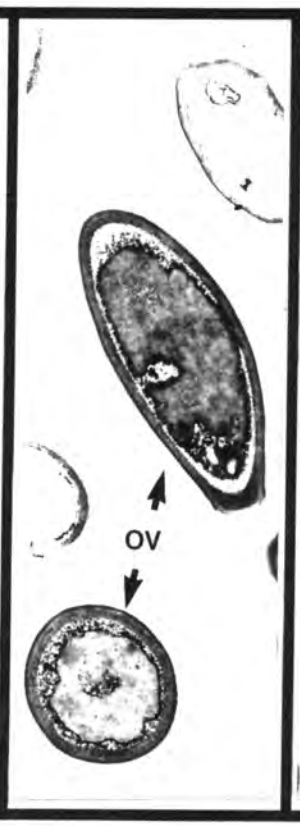


FIGURE 148. x 10 600.

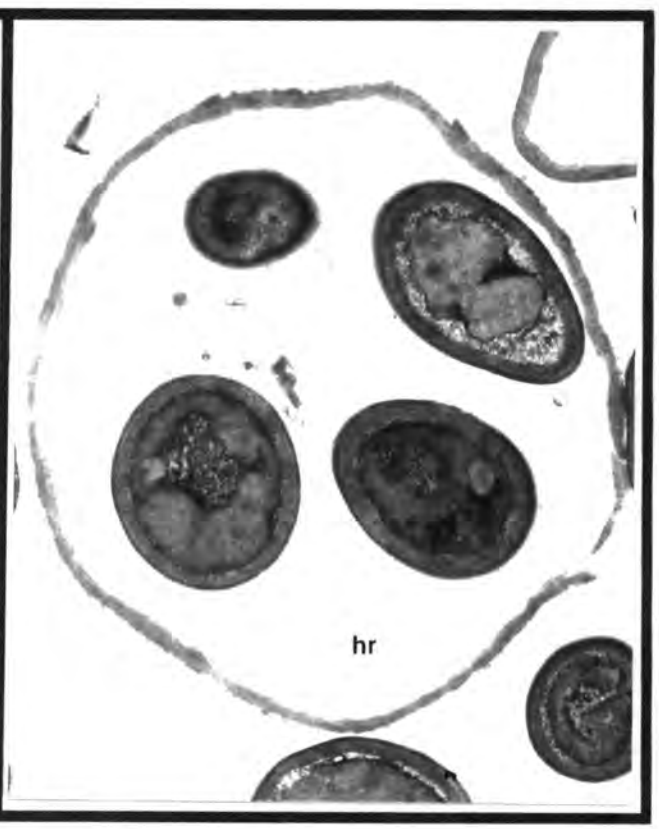


FIGURE 149. x 16 500.

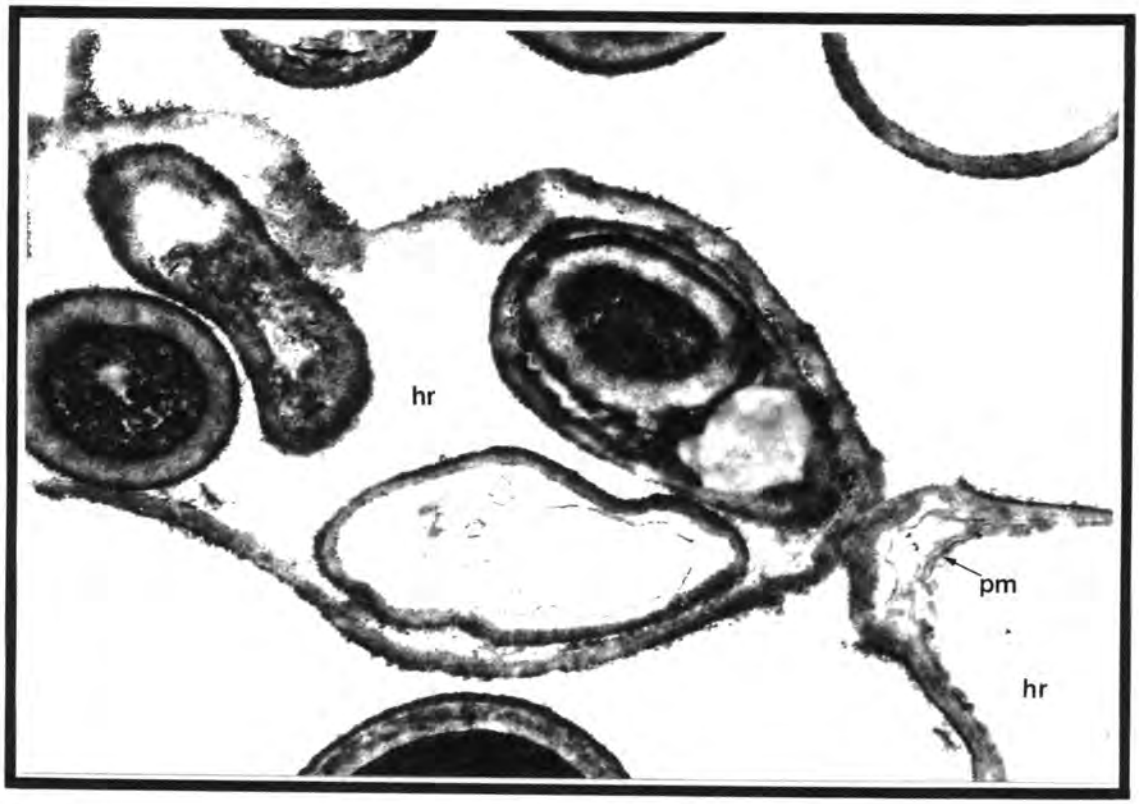


FIGURE 150. x 20 500.

FIGURES 147 - 150. Electron microscopy of S. schenckii conidia: Examples of intrahyphal conidium production and intrahyphal conidium inclusions. OV = ovate conidium; pm = plasma membrane; hr = hyphal remnants.

The occurrence of conidium-like structures within membranous enclosures was due to conidium production on intra-hyphal hyphae or conidia being enclosed by parallel running hyphae as seen in the light microscope and SEM studies (see paragraphs 2.2, p. 72 and 9.2, p. 125, respectively). Intra-hyphal hyphae developed where hyphal degeneration had occurred and new hyphal growth took place within the old hyphal shell. Conidia forming on the new actively growing hyphae could therefore not be considered true endoconidia, but rather enclosed conidia. This was mostly a feature of hyaline ovate conidia (Figures 147, 149 and 150). "Endoconidia" lying in the vacant and bulging intrahyphal space showed the constriction and detachment collar or rim round the conidium exactly as seen in conidia borne externally (Figures 147, 149 and 150).

10.3 Ultrastructure of *S. schenckii* yeasts in tissue

Biopsy material known to contain numerous organisms were employed to study the yeast phase of *S. schenckii in vivo* (see Chapter II, paragraph 10.2 (p. 28) and paragraph 1.7.3, p. 53 of this Chapter).

Both yeast-like organisms and classical cigar bodies were present in the material. Characteristically, one or more organisms were found in phagocytes (PH) (Figure 151), and appeared to be surrounded by a broad, but often irregular "space" filled with scanty microfibrillar material. Although this space created the impression of a capsule, it could not be considered as such due to its irregular shape (Figures 151, 152 and 154). The radiating microfibrillar outer cell wall layer (ocw) of the organism appeared to be the origin of the material present in this space. However, it seemed more likely that the organism had shrunk away from the double membrane (dm) which outlined the space, and that the microfibrils developed in the process (Figures 152 and 154). Towards the inside of the organism, the microfibrillar outer cell wall layer was followed by an inner cell wall (icw) consisting of two distinct layers, viz. a thinner electron dense layer and a more translucent, thicker, laminar layer, which lay against the plasma membrane (pm). The cytoplasm was enclosed by the plasma membrane,

which appeared to be a double unit membrane (trilaminar) and was smooth, slightly wavy or serrated in most organisms (Figures 151 - 154). The cytoplasm contained the following clearly identifiable structures:

- **Mitochondria (m):** Mitochondria were spherical, oval or sausage-shaped, with parallel running cristae. The mitochondria were included in a double-unit membrane, the mitochondrial membrane (Figure 151).

- **Ribosomes (r):** Structures which corresponded to ribosomes were mostly unattached and occurred diffusely throughout the cytoplasm (Figures 152 - 154).

- **Endoplasmic reticulum (er):** Tubuli of endoplasmic reticulum were scarce, were only occasionally seen in sections and were unassociated with the ribosomes.

- **Osmiophilic bodies (ob):** These structures probably represent storage granules. Those with a homogenous content and lacking detectable internal structure, were regarded as lipid granules. They were found in all organisms. Some of the osmiophilic bodies appeared to be connected with the plasma membrane (Figures 152 and 153).

- **Nuclei (N):** In most organisms the nucleus occupied approximately one third of the cytoplasm. It was encapsulated by a porous nucleomembrane. The nucleoplasm was not homogeneous. Occasionally it contained thread-like structures or tubuli of uncertain nature, as well as electron dense globules (Figure 151).

- **Vacuoles (v):** Vacuoles were enclosed by a single membrane, the tonoplast, and occurred frequently in all organisms. They varied in number and size in any one yeast-like organism (Figures 152 and 153).

- **Glycogen (g):** Characteristic glycogen granules were present in two forms, either as conglomerates in one area of the yeast cell (Figure 154), or as star-like electron dense bodies in close association with the plasma membrane (Figures 153 and 154).

Other material more difficult to identify was also seen in the cytoplasm in some sections (Figures 151 - 153). Many yeasts cells were degenerated, with retraction of the cytoplasm, creating a "space" between the plasma membrane and the cytoplasm (Figures 151 and 154). In these areas the plasma membrane was also clearly in a degenerated form, with only electron dense granules of the structure remaining (Figures 151 -inset and 154).

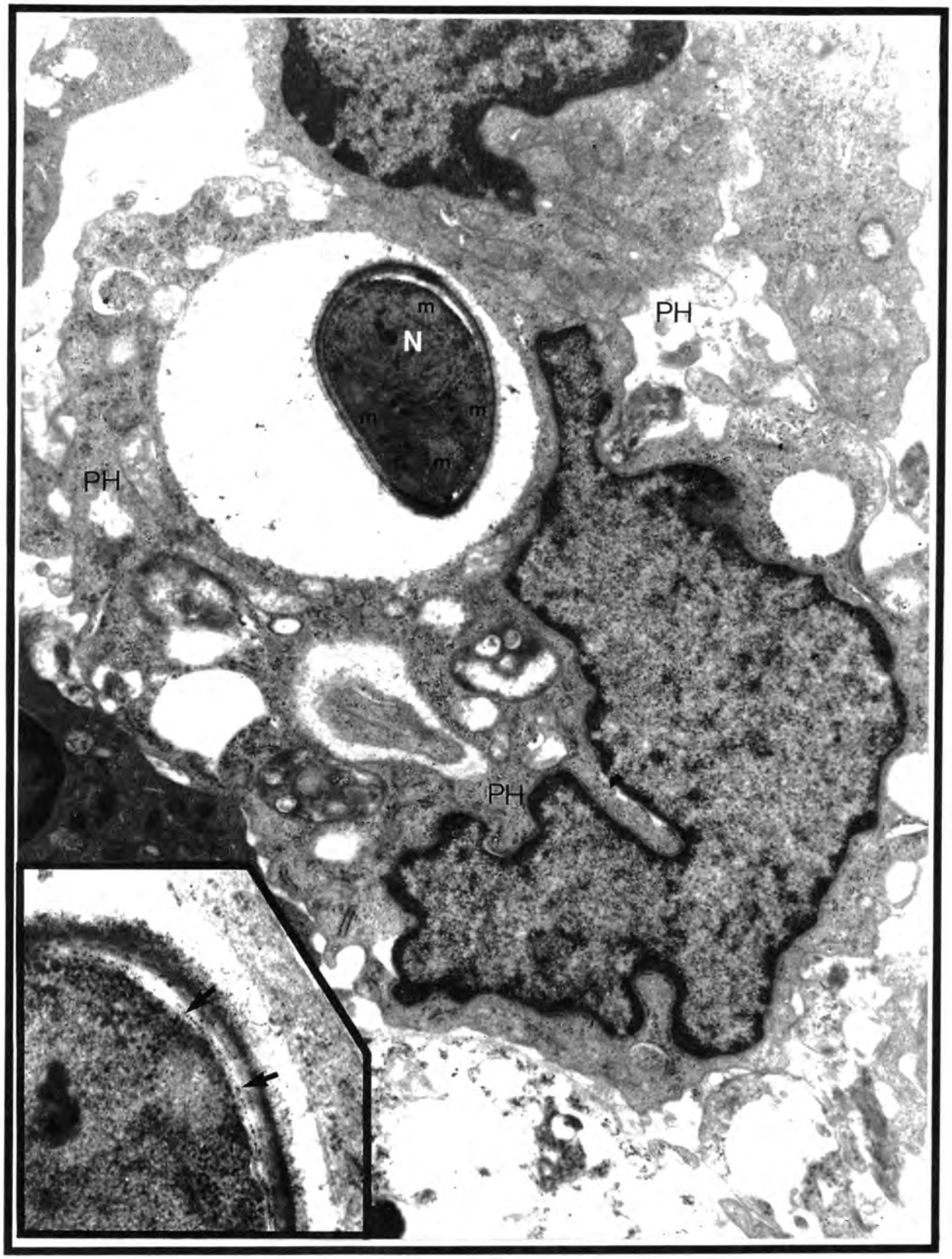


FIGURE 151. Electron microscopy of S. schenckii yeasts in tissue: PH = phagocyte; m = mitochondria; N = nucleus. x 15 000. Inset: Arrows indicate the granular remains of the plasma membrane found when the yeast cells degenerate in tissue. x 46 500.

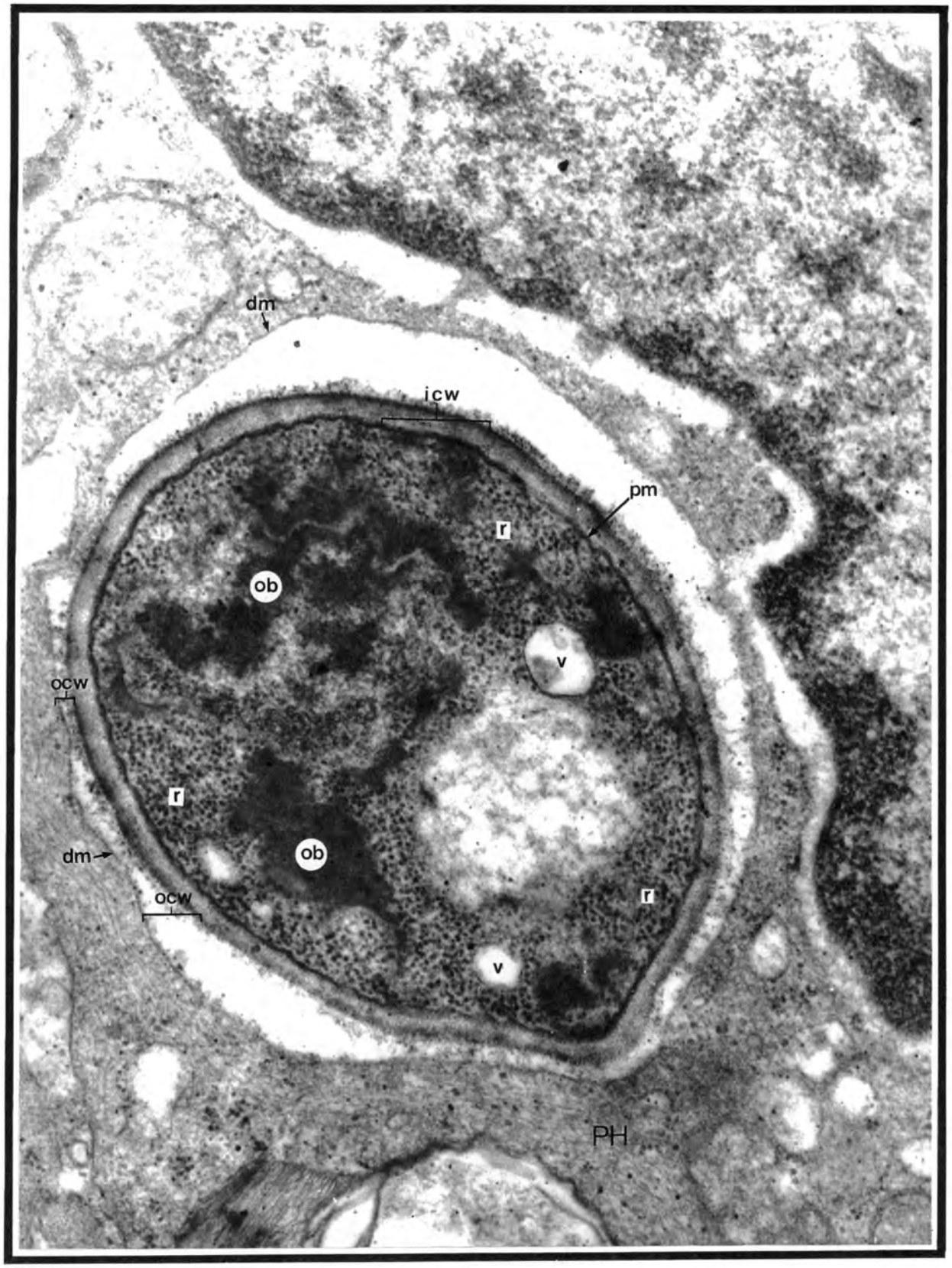


FIGURE 152. Electron microscopy of S. schenckii yeasts in tissue: PH = phagocyte; ocw = outer cell wall; icw = inner cell wall; dm = double membrane; pm = plasma membrane; r = ribosomes; v = vacuole; ob = osmiophilic body. x 46 200

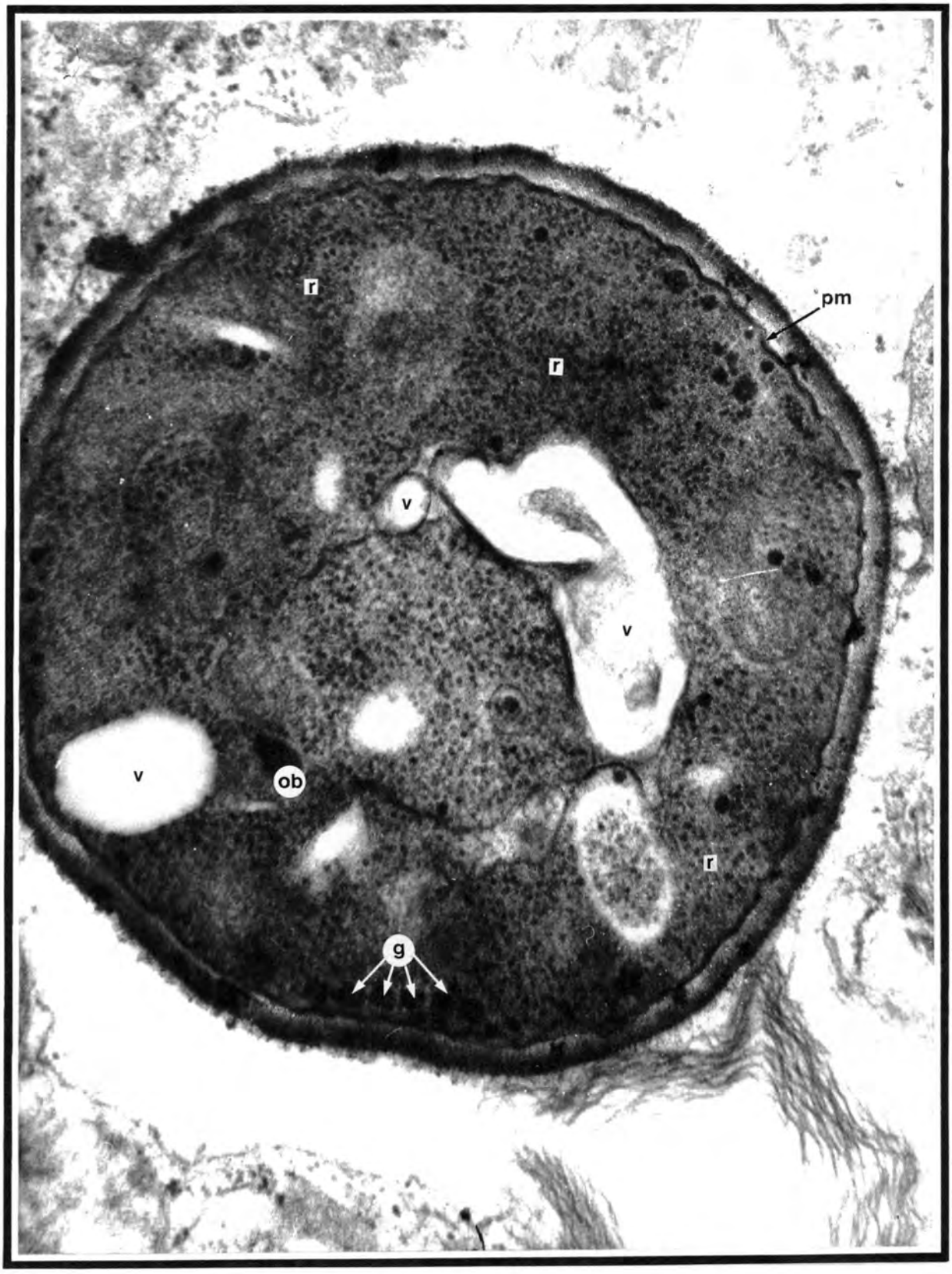


FIGURE 153. Electron microscopy of S. schenckii yeasts in tissue: pm = plasma membrane; r = ribosomes; v = vacuole; g = glycogen; ob = osmiophilic body. x 65 100.

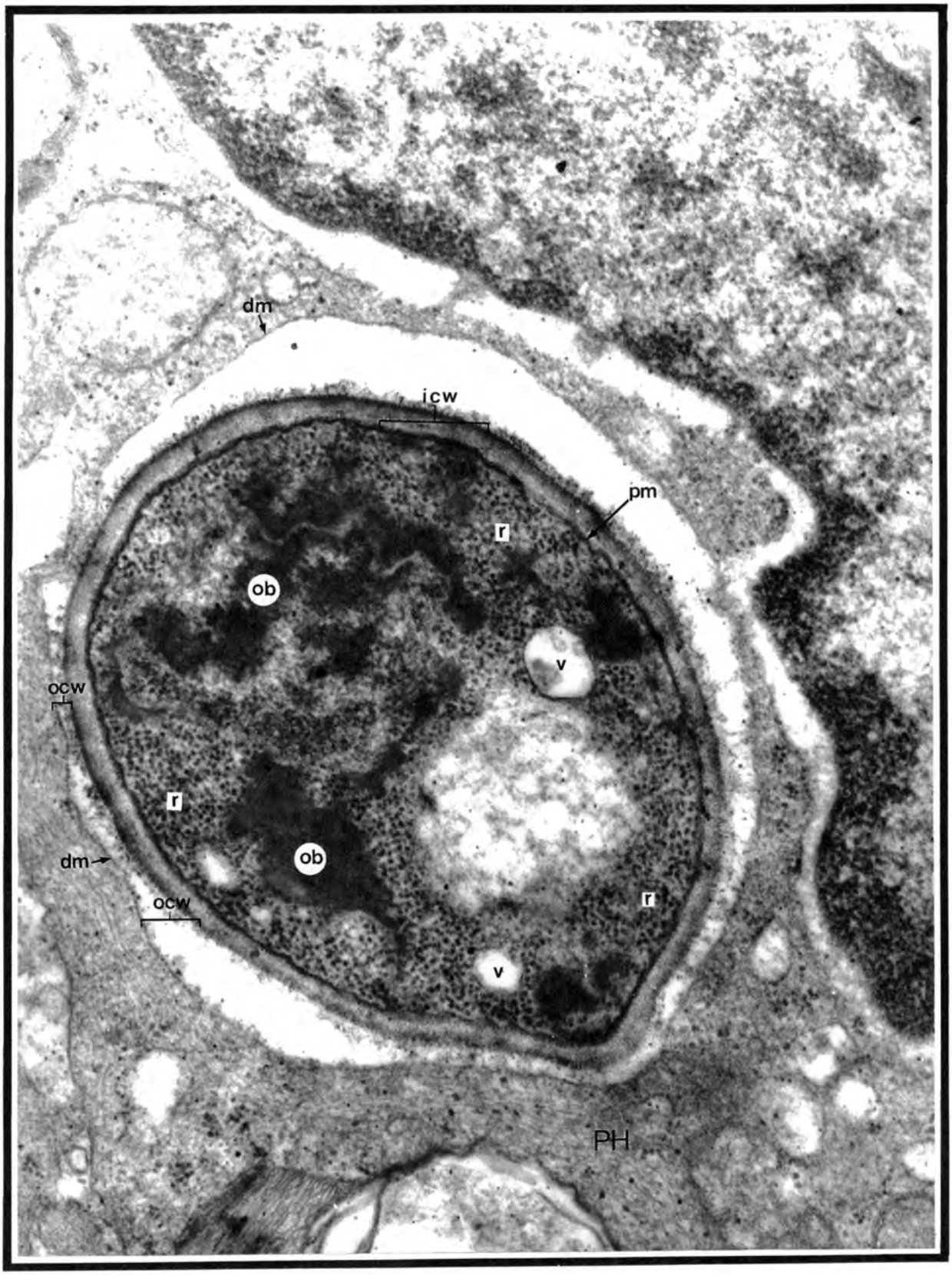


FIGURE 154. Electron microscopy of S. schenckii yeasts in tissue: PH = phagocyte; dm = double membrane; pm = plasma membrane; r = ribosomes; g = conglomerate of glycogen. The arrows indicate the start of yeast cell degeneration. Note the granular plasma membrane remaining in these areas. x 28 600.

12 ANIMAL SPOROTRICHOSIS

As indicated in Chapter II, paragraph 1.2 (p. 14), the number of cases of sporotrichosis seen was not sufficient to draw conclusions about the geographic distribution of the disease in animals in the Transvaal. Individual case studies will therefore be discussed. Of a total of eight cases of sporotrichosis seen in animals, six were in cats and two were in horses. These were all ad hoc consultations from Onderstepoort, private veterinarians and dermatologists.

The clinical findings in the cats were very similar. In the four cats for who a history was available, the lesions were of the localized type and were crusted and suppurative. In four of these cases, sporotrichosis had not been considered and a suppurative dermatophytosis or combined fungal and bacterial infection suspected. The lesions were mainly present in the facial area, with two animals showing lesions on the forepaws and neck in addition. These were considered to be multiple inoculations, rather than spread of the disease. It is speculated that the infections could have resulted from the cats fighting or scuffling, sustaining minor injuries or scratches and inoculating themselves with the fungus from soil. Although it was not possible to follow up the treatment in these cats, potassium iodide, 40mg per kilogram body weight (reduced to 20mg per kilogram body weight), was suggested. In one animal however, it was confirmed that the lesions healed spontaneously.

Two cases of sporotrichosis in horses were encountered, the one a stallion and the other a mare. Both animals were two to three years old. The two horses had shared the same camp continuously for some months when the lesions appeared. They showed no obvious external injuries. The lesions were similar in each case, starting as subcutaneous nodules in the neck behind the jaw bone, and spreading along the lymphatics of the neck. Several nodules also developed over the flanks and the rump, and in one animal down the leg, following the regional lymphatics (Figures 155, and 156). None of the lesions in either animal broke down to ulcerate. No satisfactory explanation for

the presence and distribution of the nodules, other than lymphatic spread, could be put forward (Dr M Henton - personal communication). The source of the infection was not clear either. It could have resulted from the habit of horses to chew on wooden poles or rub and scratch their bodies on wooden poles. Investigation of the camps where the animals were kept, revealed that only the fencing poles planted vertically were treated with creosote, while raw untreated fencing poles were used horizontally. Several of these untreated horizontal poles were found to have been chewed or gnawed at, or were used as scratching poles by the infected horses. It is possible that contaminated wood splinters could have got stuck in the buccal mucosa of the animals or that contaminated soil or wood splinters could have infected their hides through minor injuries.

Several attempts to culture the organism from collected soil, plant debris and samples from the chewed cross-poles of the fences, yielded *S. schenckii*-like organisms from the soil samples. These strains did not convert to the yeast phase at 37°C and did not produce pigment or triangular conidia when grown on malt agar. Their pathogenicity to laboratory animals was not tested. To identify these strains as *S. schenckii* was therefore not possible.

Both animals were left untreated. The mare was in foal and it was feared that intravenous sodium iodide or potassium idodide per mouth might effect the foetus. Because of their size, horses would require large amounts of drugs during the long treatment period, and the newer drugs would be costly. It is not known whether the lesions healed spontaneously.

The *S. schenckii* strains isolated from the horses and cats did not differ in any way from the human isolates when examined macro- and microscopically at 25°C. They converted easily to the yeast phase when incubated at 37°C on blood agar, and also showed macro- and microscopic morphology identical to the yeast phase isolates of humans.

FIGURE 155. Sporotrichosis in a horse. Nodular cord-like lesions following the lymphatics of the neck.

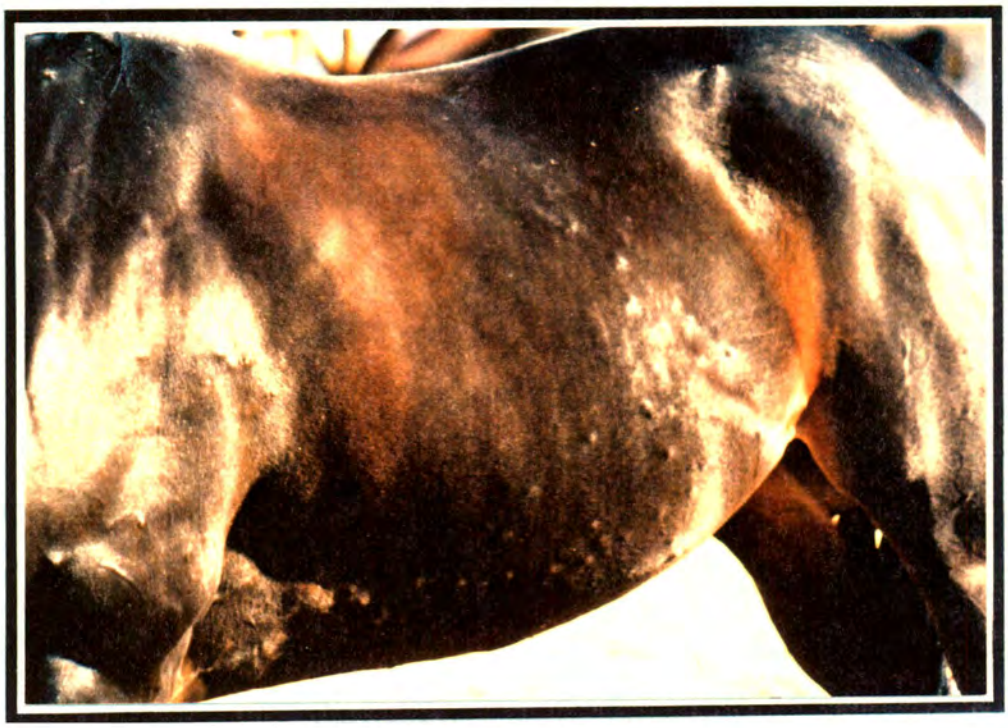


FIGURE 156. Similar nodular cord-like sporotrichotic lesions on the flank and the rump of the animal as illustrated in figure 155.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

1 EPIDEMIOLOGY OF SPOROTRICHOSIS

The discovery of sporotrichosis in South Africa dates back to 1914, when this disease was first diagnosed in the Witwatersrand gold mines in the Transvaal (Pijper and Pullinger, 1927). Two publications on sporotrichosis occurring outside the mines appeared in 1931 (Goldberg and Pijper, 1931; Pijper, 1931). Pijper claimed that in the more than 20 years that followed, the organism was not isolated again (Findlay, 1970). Pijper (1931) named his isolate *Sporotrichum carougeaui* Langeron 1922, but currently this organism is regarded as synonymous with *Sporothrix schenckii* (Aram, 1986). Thereafter, until 1965 only six reports appeared on sporotrichosis, dealing mainly with clinical aspects of the disease, viz.

- a superinfection of sporotrichosis on sarcoidosis (an unrelated dermatological condition), in a patient from the Boksburg area (Van Dijk and Der Kinderen, 1958);
- treatment of sporotrichosis with griseofulvin, an antimycotic normally used for dermatophytoses (Lowenthal, 1959);
- the description of unusual clinical manifestations in osseous sporotrichosis (Lurie, 1963b);
- sporotrichotic infections in children (Gluckman, 1965) and
- histopathological studies on asteroid bodies in sporotrichosis (Lurie, 1963a; Lurie and Still, 1969).

No satisfactory explanation can be given for the lack of reports on sporotrichosis between 1931 and 1957. In 1970 Findlay however, concluded that sporotrichosis occurred regularly in the Transvaal, according to his experience in private practice since 1958. The current study follows on Findlay's work and portrays the situation since 1966 at the provincial hospitals in Pretoria, as well as other private practices in the city. It does not include the patients previously described by Findlay nor the patients seen by him in private practice since 1966. The current study confirms Findlay's finding that sporotrichosis is endemic in the Transvaal, and that it remains the most prevalent subcutaneous mycosis in the area, averaging about 15 to 20 cases per year. This refers only to cases seen outside the mines. The number is obviously higher, as cases seen in other centres in the Transvaal have not been included.

Pretoria lies in the central highveld plateau of the Transvaal, which has a summer rainfall. The most rain falls between November and February (500-750mm), with an average of 65% relative humidity. The highest temperatures are experienced during December, January and February (25°C-32°C), while the coldest months are June and July with temperatures of 5°C-15°C. Our study indicates that the seasonal variation in cases of sporotrichosis in Pretoria and the Transvaal is not very pronounced. But the onset of the disease seems to be mainly in the cooler and dryer months of the year (May to September), coinciding well with what has previously been reported, namely that no definite seasonal pattern can be established, and that in some years more cases are seen in the first half of the summer (August to December) (Findlay, 1970).

Males predominated in the current study, accounting for 75% to 80% of the cases. This can be related to the occupation of the male patients, especially as farmers and builders. Females seemed to be at far lesser risk in our particular population, mainly becoming infected by gardening injuries, insect bites or other minor injuries due to outdoor activities. These played a role in both sexes. *S. schenckii* has been

found to occur saprophytically on wasps, ants and flies; although its presence is not common enough for these insects to be considered as a natural habitat of the fungus, it incriminates insects as a possible source of sporotrichosis (Benham and Kesten, 1932). Insect bites could be contaminated by soil and inoculation of the fungus into the skin could occur through scratching. Cockroaches may also act as vectors of pathogenic and toxin producing fungi (Brühl and Fuchs, 1973). However, a large number of patients did not remember any trauma preceding the onset of their sporotrichosis, while small pimples were mentioned by others as the start of the infection. Chung *et al*. (1990) described a case very similar to ours with the lesion on the nose. No trauma could be established, and the method of inoculation remains obscure. Our patient had however been in contact with untreated eucalyptus poles, but without obvious injury or trauma at the spot on the nose where the disease started.

There is general consensus in the literature that sporotrichosis is a disease occurring at all ages. Similarly in this study, patients of under ten years of age to over 90 years of age were seen. No particular predisposing factors are known to be present in any particular race. Exposure to possible sources of the fungus either from recreational or occupational activities is one of the main determining factors in acquiring the disease.

2 GEOGRAPHIC DISTRIBUTION OF SPOROTRICHOSIS

Patients with sporotrichosis came from 42 suburbs in the Pretoria municipal area. Cases were seen more than once from some of these suburbs, but none showed a particularly higher incidence. The western suburbs of Pretoria were less well represented, but this can be attributed to their smaller population, due to many undeveloped areas and open fields in that part of the city. Most cases came from the eastern suburbs, which are the most densely populated parts of Pretoria.

Homes in Pretoria have lush gardens, with a large variety of indigenous and exotic plants growing in them. Apart from roses, no other plants could be particularly associated with the onset of sporotrichosis. In some cases garden soils were confirmed to be the source of the fungus while in others special circumstances related to the source of the fungus, as in the case of a positive soil isolate from soil samples taken from a crashed car. A fairly regular association was found with wattle and eucalyptus in the case of farmers and builders.

Twenty three towns and areas outside the Pretoria municipal boundary also produced cases of sporotrichosis. Again no particular area could be shown to have a higher incidence of sporotrichosis. However most cases came from the Transvaal central highveld plateau. Previously Findlay (1970) had found that the subtropical Transvaal Lowveld (Nelspruit), the central Bushveld (Naboomspruit), Pietersburg plateau, Western Ridge Basin (Rustenburg) and Eastern Ridge Basin (Lydenburg) yielded cases of sporotrichosis, findings very similar to the results obtained in this study. The geographic distribution of sporotrichosis in the Transvaal appears to have remained constant for the past three decades, with a steady flow of cases from especially the highveld plateau, and sporadic cases from the rest of the province.

The major source of sporotrichotic infection in the Transvaal appears to be garden soil, in which compost, mosses, and potting soil are regularly used. Although several smaller and larger epidemics have been documented worldwide (Carrada-Bravo, 1975; Crevasse and Ellner, 1960; Dahl *et al.*, 1971; D'Allessio *et al.*, 1965; Frumkin and Tisserand, 1989; Kohl and Rosen, 1980; Laur *et al.*, 1979), the South Africa gold mine epidemics remain unparalleled. Epidemics other than in mines have been recorded and are often traced to one source of the organism. In South Africa a small outbreak occurred among workers at a Pretoria brick field between 1959 and 1961. Padding grass from the south-east of Pretoria where sporotrichosis is known to occur, was presumably responsible for the infection (Findlay, 1970). In North Texas, where a familial epidemic occurred, the source could be traced to "prairie hay" used as a mulch for potato plants (Laur *et al.*, 1979). In

Wisconsin, Pennsylvania, a large number of forest workers packaging pine seedlings in sphagnum moss, contracted sporotrichosis. The initial report resulted in further investigations and cases of sporotrichosis were confirmed from 14 States, all traceable to two nurseries in Pennsylvania, who supplied pine seedlings packed in sphagnum moss from Wisconsin (Coté *et al.*, 1988; England *et al.*, 1988a, 1988b, 1989). Sphagnum moss is a well known source of *S. schenckii* and several other authors have described the moss as an occupational hazard in forest workers and nursery staff (Crevasse and Ellner, 1960; Schwartz and Kaufman, 1977). In areas where sporotrichosis is a common occupational disease in forest workers, for example in the Mississippi valley, warnings have been published in the Tree Planter's Notes (no 41, April 1960), explaining the hazards of sporotrichosis (Taylor, 1977). The wood of the trees in the forests has never been implicated as a source of sporotrichosis among these workers. Sphagnum moss has not been associated with sporotrichosis in the current study. Several reports implicate sphagnum moss as a source of the disease in America (Coté *et al.*, 1988); Grotte and Younger, 1981; Powell *et al.*, 1978; Tan *et al.*, 1988). Dixon *et al.* (1991) pointed out that even though epidemics may be traced to one or more sources of sphagnum moss at nurseries, it is not clear when the moss becomes infected with *S. schenckii*; the fungus could not be recovered from the bogs where the moss is grown (Crevasse and Ellner, 1960; Dixon *et al.*, 1991). It is possible that the moss becomes infected after it has been harvested and transported to distribution sites, or that the concentration of the fungus is too low for it to be detected in the large bogs. Adam *et al.* (1982) pointed out that the plastic bags used for transport of sphagnum moss present an ideal environment for the fungus to grow; this was later confirmed by Tan *et al.* (1988). Further research is necessary to fully understand the role played by commercial compost, mosses and potting soil in the occurrence of sporotrichosis in this country.

With regard to the other three provinces in South Africa, there is little published information available. In Natal, it is believed that sporotrichosis had a particularly high incidence in forest workers in the 1940's. It was during that time that eucalyptus was introduced into

Kwazulu (previously known as Zululand) in order to dry out large swamp areas and to combat mosquito populations and malaria. This was achieved, but the incidence of sporotrichosis increased among the forest workers. Later, as the disease became known, it showed a decline for reasons that are not clear (Leeming, 1989). Bayles (1992) regards Natal as an endemic area for sporotrichosis, and claims that the disease is regularly encountered in this province, especially in the black male population, among farm labourers and builders.

In the Orange Free State, cases are seen in the eastern parts which forms a prolongation of the Southern Transvaal Highveld and include Frankfurt, Warden and Harrismith (Findlay, 1970). In the lower lying areas, cases are rare but not unknown and have been reported from Boshoff, Winburg and Bultfontein (Findlay, 1970). Sporadic cases are still seen in the Orange Free State (Venter, 1989).

In the Cape Province one or two cases of sporotrichosis per year are encountered in the large centres such as Grootte Schuur (Strauss, 1991).

An important epidemiological aspect of sporotrichosis in South Africa is its occurrence in the Witwatersrand gold mines. Pijper and Pullinger discovered sporotrichosis among 14 miners at the Modder East gold mine in 1914 (Pijper and Pullinger, 1927). Only in 1941 did Dangerfield and Gear report on the second, much larger outbreak of the disease in which 74 miners were affected. In 1942 Du Toit described an outbreak of 650 cases at the Venterspost gold mine. He observed that this particular mine had a cooler temperature and was particularly "wet", and attributed much of the outbreak to these two factors. James (1965) summarized the prevalence of sporotrichosis between 1942 and 1943, stating that more than 1900 cases were recorded during that time, while between 1960 and 1964 a further 617 cases occurred in several gold mines. The research that followed clarified much of the epidemiology of sporotrichosis in the mines in this country, confirming that untreated contaminated wood props were the source of the disease (Brown *et al.*, 1947; James, 1965).

A fact seldom mentioned in the literature is that *S. schenckii* has the ability to grow on fabrics such as flannel, hessian, coarse felt and white canvas (Du Toit, 1942). No growth has however been noted on dyed material. The undyed fabrics may play a role in spreading and contracting the fungus, as they are often used in packaging, but plant material and soil seem to predominate as possible sources of the disease. *S. schenckii* has been isolated from old mattresses, but it is uncertain whether this source could cause infection (Cooke and Foter, 1958). Du Toit (1942) found that the conidia of *S. schenckii* may remain viable for more than two years. Further research is needed to establish the possible role of fabrics and similar materials in the spread of the disease.

Contaminated wood props seem to be the only external sources of sporotrichosis in the gold mines. The proliferation of *S. schenckii* on untreated wood props poses a high risk of infection to miners. Contrary to statements in the world literature that sporotrichosis has been eradicated in the South African gold mines (Rippon, 1988), due to the treatment of the timber props, we have found from analyzed SAIMR data, that minor epidemics as well as a constant flow of cases have regularly been recorded over the past two decades. James (1965) stated that efforts to keep records of sporotrichosis in the mines were abandoned after 1944; mines no longer wished to report on these cases because of economical implications as far as labour was concerned because mine workers feared that they may contract the disease and seeked employment elsewhere. It must be borne in mind that wood rotting fungi and fire, are of far greater concern to the mining industry than sporotrichosis. Knowledge of the disease gained during the early epidemics, which had far reaching implications as some mines had to be closed down due to the treat of sporotrichosis, has made medical officers and mine workers alike, realize that they are at a high risk of contracting the disease. Personal interviews with mining staff revealed that certain shafts in mines are known "to have the disease" and special care is taken not to come into contact with the wood in those shafts. Mine workers are also informed about the presence of

S. schenckii in certain areas. Replacement of the infected wood is not always practicable and treatment of the wood against fungal decay to expensive.

Wood still plays an important role in the mining industry. Mine timbers that were used and are still being used are mainly *Eucalyptus grandis (saligna)* and *Acacia mearnsii (millissima)* - the black wattle (Barnard and Lubbe, 1987; Brown *et al.*, 1947). According to recent estimations of the demand for wood in the mining industry, a growth rate of 2% per year in wood production, mainly *Eucalyptus grandis*, is necessary to keep abreast of mining demands (Barnard and Lubbe, 1987). Barnard and Lubbe (1987) also stated that some 2,4 million tons of wood per year are needed in the mining industry for support timbers, while 90 000 tons and 2000 tons are used for planks and sleepers, respectively. Sleepers are usually imported from other African countries. Of these demands, 85% is from gold mines, 3% from coal mines and 12% from platinum mines. In spite of the required 2% growth rate, a gradual decline in timber usage has been experienced in the mining industry and the nature of the timber supports have undergone major adaptations over the last ten years (Barnard and Lubbe, 1987). With the development of mechanized mining, the socalled "pack design", i. e. the way in which the wood props are stacked, supporting mining roofs has changed and less solid timber is currently needed. Indirectly these changes have resulted in a decline in the number of cases of sporotrichosis in the gold mines. Genetic cloning of *Eucalyptus grandis* for faster growers and the replanting of seedlings have resulted in the production of 35% more wood rather than with self regenerating crops. *S. schenckii* may be less able to proliferate on these new, genetically different wood (Hall, 1965; Riemann and Faure, 1987). Another interesting fact about mining timbers is that the yield force (i. e. bending strength) of the wood is not influenced by the props standing in water or by a high humidity. Wood props perform well under these circumstances, but at these high humidities, up to 17% of wood props are lost per year, due to fungal deterioration (Hall, 1965; Riemann and Faure, 1987). Fungal infection greatly decreases the yield strength of the wood and constitutes the main reason for props being changed on a more regular basis in order to

prevent total fungal decay. It was found to be more economical to use these timbers as sleepers, than to treat them against fungal decay. The presence of *S. schenckii* is coincidental as it proliferates underground. It disappears when from the wood props when the latter is attacked by wood rotting fungi (for example *Polyporus rugulosus*, *Poria* sp. and *Fomes* sp.). *S. schenckii*'s destruction seems to be due to enzymatic action of these Basidiomycetes, as the conidia of *S. schenckii* appeared mutilated, their cell walls pitted or corroded and in some instances only fragments of the conidia remained (Brown *et al.*, 1947). *S. schenckii* was isolated only from sound timber underground by these authors. It is clear that the organism does not use the broken down metabolites of these lignin destroying fungi for survival.

Analysis of data available for cases of sporotrichosis in the gold mines showed that most cases were recorded in the cooler months of the year (April to September). It is difficult to find a suitable explanation for this phenomenon as the conditions underground, i. e. temperature, humidity as well as the pH of the water remain constant throughout the year. No correlation could be found with the introduction of new wood props underground. New poles are introduced throughout the year, depending on the phase of development of the specific shafts. Perhaps the seasonal distribution of cases in the mines may partially be explained by the fact that some mines experienced a small epidemic around one particular month, which contributed the largest percentage of cases. Other factors must also be considered when analyzing the mine results, for example, the time the wood was introduced into a specific mine, whether it was contaminated with the fungus, the working traffic in the area, and the presence of other fungi, which may effect the survival of *S. schenckii*. Underground conditions are also of great importance for the proliferation of the fungus.

An important question is why sporotrichosis has never occurred in the coal mines of South Africa. Wooden props similar to those used in the gold mines are also used in the coal mines, and the sources of the wood are probably the same. A limited investigation into coal mining methods indicated that the pH of the water seems to be a major problem.

Socalled "acid in mining" is cause for great concern in coal mines, and involves the leaching of sulfides and sulfates from the coal ore. These substances dissolve in the water, making it acidic (pH range 2.0 - 3.0). To stop metal instruments from rusting specialized neutralization plants are installed in the mines (Lindhout, 1990). A similar situation prevails in the gold mines. An unique feature of gold-bearing ore is its almost exclusive association with pyrite. When water and air come into contact with the broken pyrite reef, the pyrite oxidase dissolves in the water to form a weak sulfuric acid solution (Lindhout, 1990). It is therefore necessary for neutralization (liming) of water to be an ongoing and constant process. In both types of mine, sodium chloride and calcium sulphate salts also dissolve in the water, creating buffer solutions. In the coal mines the process of neutralizing the water seems to be more complicated and the pH of the water normally remains low. In the gold mines on the other hand, the pH of the water is more easily maintained between pH 5,5 and 6,5. In summary therefore, coal mines use less wood and water, the pH of the water is very acidic, and the temperature in general is much cooler than in the gold mines (Brown, 1990). These factors may explain why sporotrichosis has not been seen in the coal mines of South Africa.

Very little published information regarding sporotrichosis is available from the countries bordering on South Africa. Ross and Gelfand (1978) felt that the clinical presentation and perhaps also the histopathological features of sporotrichosis are not well appreciated in Zimbabwe. They could find eight cases of the disease over a ten year period by examining histological sections. Most of the cases were black patients from the northern parts of the country.

Sporotrichosis is also not well documented in the rest of Africa. In North Africa, by 1978, only three cases of sporotrichosis had been encountered in the Sudan (Gumaa, 1978; Mahgoub, 1990), and two in Nigeria (Jacyk *et al.*, 1981). It is suggested that the soil in North Africa differs from that in countries with endemic sporotrichosis, and that the plants normally associated with the disease are not found in

that part of Africa (Mahgoub, 1991). On the other hand, unawareness of the disease may be a major factor and many cases may go undiagnosed and untreated (Mahgoub, 1991).

Some intriguing and puzzling shifts in the incidence and geographic distribution of sporotrichosis can be noted from Europe, Asia and the Americas. In Europe and especially France, several hundred cases had been described by 1912 (De Beurmann and Gougerot, 1903, 1908a, 1908b, 1912; De Beurmann, 1912), but after the first two decades of this century sporotrichosis became rare in Europe, and is still uncommon today. The classical French descriptions of De Beurmann and Gougerot (1912) were so explicit that a lack of knowledge of the disease cannot be regarded as one of the contributing factors for a further lack of records of sporotrichosis. Recent reviews confirm that only 44 cases of sporotrichosis were described between 1976 and 1988 from seven of the 35 European countries (Alberici *et al.*, 1989). Spain (23 cases) and Italy (14 cases) headed the list, with three cases from France and only one case each from the United Kingdom, The Netherlands, Czechoslovakia and Romania. Even though Spain and Italy report a relatively high number of cases, the incidence of sporotrichosis is considered low in both these countries (Grasa *et al.*, 1980; Ventin *et al.*, 1987). Puncture wounds from pine needles (*Pinus pinea*), and from the thorns of *Rubus fruticosus*, were regarded as the source of the fungus in some Spanish cases (Grasa *et al.*, 1980). Although cases were previously reported from Portugal and Germany, currently sporotrichosis seems to be non-existent in these countries (Cabrita, 1974; Goncalves, 1973).

With regard to Asia, literature in English from Russia (USSR) and countries of Asia is scarce. In a review, Stepanishtcheva *et al.* (1972) reported that sporotrichosis is the second most prevalent subcutaneous or deep mycosis in Moscow. He mentions unusual clinical forms of the disease, presenting as acne conglolata and folliculitis in the nape of the neck, which were confirmed to be sporotrichosis, and occurred in most of the patients described.

India is also a country with little sporotrichosis. The first report dates back to 1932, and since then only about 25 cases are known to have occurred there (Khan, 1975; Kini *et al.*, 1986; Mishra and Sandhu, 1972; Singh *et al.*, 1983; Sur Roy Chowdhury and Mondal, 1979). It is interesting to note that 95% of the population are rural cultivators and tea garden workers and that large numbers work in forestry based industries, yet the incidence of sporotrichosis remains low (Baruah *et al.*, 1976). Similar findings appear in the latest publications from India, and show a female preponderance, which can be related to their involvement in the above mentioned occupations (Kini *et al.*, 1986). Singh *et al.* (1983), pointed out that heavy rainfall, high humidity and lower temperatures, contributed to the prevalence of *S. schenckii* in the northern parts of India.

Unlike the Asian countries mentioned so far, Japan has a high incidence of sporotrichosis. It is the most common subcutaneous mycosis in that country. Sporotrichosis was first documented in 1920 and more than 2000 cases has been reported from Japan by 1981 (Fukushiro, 1984). The female to male ratio is 2:1. The fact that twice as many women contract the disease, can be related to the agricultural work done by women in Japan. In these women the hands and face are most often affected (Fukushiro, 1984; Itoh *et al.*, 1986; Kariya and Iwatsu, 1979; Kusuhara *et al.*, 1988; Tamachi *et al.*, 1980). Tamachi *et al.* (1980) reported that their cases came mainly from districts in the Chikugo plains, representing an agricultural and horticultural area, where the male to female ratio was almost equal. Here both sexes work in the same type of agricultural occupations. A steady increase of cases over the past two decades has been noted in Japan. It is due to the awareness of the disease, the active collection of cases, improved medical facilities and better diagnostic techniques. Additional factors are a nationwide health insurance system, improved roads and traffic networks with hospitals being more accessible and an increase in the population (Kariya and Iwatsu, 1979; Tamachi *et al.*, 1980). Kariya and Iwatsu (1979) and Tamachi *et al.* (1980) also showed that the distribution of cases is closely related to climate, and maintained that a monthly average temperature of 0°C - 4°C, and a rainfall of less

than 50mm in the cold season, favours the onset of sporotrichosis in Japan.

Sporotrichosis has been infrequently recorded in the Australasian literature. Although the eucalyptus and wattle were originally introduced into South Africa from Australia (Sherry, 1971; Poynton, 1979), there does not seem to be an association between these plants and the occurrence of sporotrichosis in Australia as there is South Africa. In fact, a very low incidence of sporotrichosis is noted in Australia. The first case was described only in 1951 (Robinson and Orban, 1951). Most cases come from Queensland and are associated with pine seedlings packed in sphagnum moss and forest workers contracting the disease. Sphagnum moss is a well known source of *S. schenckii* in Australia (Auld and Breadmore, 1979; Barrack and Powell, 1952; Breadmore, 1979; Mead and Ridley, 1957; Minty *et al.*, 1956; O'Donnell, 1962). Sporadic cases of sporotrichosis have also been encountered in several other states in Australia, namely Brisbane, Maryland and Sydney (Bullpitt and Weedon, 1978; Durie, *et al.* 1961; Muir and Pritchard, 1984; Robertson, 1967). Males predominate in all these reports, but seasonal predilections are not mentioned. The latter may be due to the small number of patients seen, and the specific association with sphagnum moss as a source of the infection.

Numerous reports from the Americas and Canada indicate that sporotrichosis is well known and occurs regularly in these parts of the world. A distinct rise in the number of cases in the United States since the 1930's has been experienced, and especially an increase in "opportunistic" sporotrichosis and disseminated disease (Remington *et al.*, 1983; Rippon, 1988). Recently a large epidemic occurred among forest workers, traceable to sphagnum moss as the source of the infection (Dixon *et al.*, 1991). Sinski *et al.* (1976) reported the first case of sporotrichosis in Arizona (USA), acquired from rotted shavings of an ash tree.

In Latin America sporotrichosis frequently occurs in temperate and tropical zones including Brazil, Peru, Puerto Rico, São Paulo, Colombia,

Paraguay and Uruguay (Caudros *et al.*, 1990; Conti-Díaz, 1989; González-Ochoa, 1970; Itoh, *et al.*, 1986; Restrepo, *et al.*, 1986; Sampaio and Lacaz, 1959). In contrast to these countries with moist, humid climates, Mexico is semi-arid with a low humidity, but also has a high incidence of the disease. Grass seems to be the main source of the organism (González-Ochoa and Ricoy, 1970; Velasco Castegan and González-Ochoa, 1971). Mexicans come into contact with grass, when gathering it, weaving baskets or using it as packing material. It is interesting to note that half of the cases of sporotrichosis reported from Uruguay are related to armadillo hunting. The source of the infection is not the animals themselves but the dry pangola grass (*Digiparia decubens*) which they use for nesting. The rough manner in which the animals are pulled from their burrows during the hunt, poses a high risk for injury and inoculation of the fungus (Mackinnon and Conti-Díaz, 1969; Mackinnon *et al.*, 1969). Mackinnon *et al.* (1969) could not find *S. schenckii* in 15 seven-banded armadillos, *Dasupus septemcinctus*, examined for the fungus suggesting that these animals are not carriers of the disease. Two cases of spontaneous disseminated systemic sporotrichosis were described in nine-banded armadillos (*Dasupus novemcinctus*) by Kaplan *et al.* (1982). In Uruguay sporotrichosis seems to be contracted after short periods of abundant rain or fog, when the relative humidity is high. A constant temperature of 16-20°C further favours the occurrence of sporotrichosis in that country (Mackinnon and Conti-Díaz, 1962). Mackinnon (1947-1949) had previously found that sporotrichosis occurred during April, May, June and July, months during which the temperature averages 16°C - 19°C, and the rainfall and relative humidity is highest. In Brazil, straw is the common source of the organism and epidemics occur among brick yard workers who use it for padding bricks. *S. schenckii* gains entrance through minor skin injuries and skin abrasions sustained during work (Mayorga *et al.*, 1970, 1978; Sanders, 1971). In Colombia, sporotrichosis has the highest incidence of subcutaneous mycoses, with females slightly more often affected than males. Extracutaneous disease is rarely encountered (Restrepo, *et al.*, 1986).

In summary of the epidemiology, the geographical distribution and seasonal incidence of sporotrichosis, it can be said that it has a worldwide distribution. Patients of all ages, races and of both sexes are affected. There is however no general pattern emerging from the description of the climates in which sporotrichosis occurs, for example, in Uruguay and Rio Grando do Sul, the majority of cases occur in the winter and autumn (temperatures 14°C-15°C), which is the rainy season; in Mexico and South Africa (current study), it also occurs in the autumn and winter months, which are during the dry season (González-Ochoa, 1968, 1974; Londero *et al.*, 1964; Mackinnon, 1947-1949; Mackinnon *et al.*, 1964, 1969; Mackinnon and Conti-Díaz, 1962, 1969). Thus, there is no relationship with climatic conditions. Plant and soil types may influence the proliferation of *S. schenckii* and in turn its ecology and epidemiology, but much research is needed to determine the predilections of this organism in its natural habitat.

3 CLINICAL ASPECTS OF SPOROTRICHOSIS

The lymphangitic form of the disease was the most common type encountered in the current study. In most reports from other countries lymphangitic sporotrichosis accounts for 70%-80% of the total number of cases (Altner and Turner, 1970; Restrepo *et al.*, 1986). Even though the organism spreads along the lymph vessels, the lymph nodes are very rarely involved (Benham and Kesten, 1932; Rippon, 1988). Localized sporotrichosis accounts for almost all the remaining cases, with the disseminated and extracutaneous forms of the disease being rare.

In both lymphatic and localized sporotrichosis, the time that elapse between the inoculation and diagnosis of the disease, varied from three weeks to more than 12 months, averaging two to three months in our study. Itoh *et al.* (1986) reported periods varying from as short as ten days, to as long as nine years. Restrepo *et al.* (1986) found that in

Colombia, an average period of almost ten months elapses before a diagnosis of sporotrichosis is made.

The very early descriptions of sporotrichosis indicated that the clinical pictures from France and America differed. American cases appeared to be more of the lymphangitic type while in France syphiloid and tuberculoid lesions seemed more common (Adamson, 1911). It may be postulated that the French population had a higher immunity against sporotrichosis, which had developed over the years; this could also explain why the disease is currently so rare in that country. Lavell and Mariat (1983) regard localized sporotrichosis as a type of reinfection in a patient who has developed immunity against the fungus. Several authors have shown that the localized form (i. e. more arrested form) of the disease occurs in patients who have previously been in contact with *S. schenckii*, while the lymphangitic form occurs in patients who have not been in contact with the organism before. The size of the inoculum is likely however also to play an important role. Small inocula implanted through minor injuries involving contact with soil, plants and decaying vegetation, may induce immunity, enabling the body to suppress the infection. Larger and perhaps deeper inocula would result in infections of the localized or lymphangitic type, depending on whether there had been previous contact with the fungus (Goncalves, 1973; Lavell and Mariat, 1983; Rippon, 1988; Travassos and Lloyd, 1980). This development of immunity during infection of the skin, the usual site of primary inoculation, would also explain the rarity of disseminated and extracutaneous cases in the healthy population.

The incidence of sporotrichosis in Australia is low, and the localized form predominates. No systemic cases have been documented in Australia (Auld and Beardmore, 1979; Muir and Pritchard, 1984). The reasons put forward by Auld and Beardmore (1979) include possible lower pathogenicity of the fungus and the occurrence of subclinical infections. They also postulate that severe solar damage common to white Australians may hamper spread of organisms in the skin. In addition, the incidence of sporotrichosis in Australia may be underestimated due to the high incidence of skin cancers in that

country. Excised skin lesions revealing a non-specific histological picture, and where no mycology is done, may be misdiagnosed as skin cancers or solar keratoses (Auld and Beardmore, 1979; Barrack and Powell, 1952; Black and McAleer, 1975; Bullpitt and Weedon, 1978; Mead and Ridley, 1957; Minty *et al.*, 1956).

Localized forms of sporotrichosis can mimic several other fungous and bacterial infections especially those of long duration, for example nocardiosis, chromoblastomycosis, blastomycosis and paracoccidioidomycosis (Dolezal, 1981; Gluckman, 1965; Laur *et al.*, 1979; Rippon, 1988; Wlodaver *et al.*, 1988). Other skin diseases which need to be differentiated from sporotrichosis include sarcoidosis, tuberculosis, leishmaniasis, psoriasis, gummatous syphilis, pyogenic granuloma (Boehm *et al.*, 1982; Dolezal, 1981; Everett and Terry, 1978; Rippon, 1988; Spiers *et al.*, 1986).

Osteolytic and osseous lesions caused by *S. schenckii* have been increasingly reported over the last two decades. Ajello and Kaplan (1969a) described an osteolytic skull lesion caused by *S. schenckii* var. *luriei*. Lurie (1963b) first reported this variant of *S. schenckii*, isolated from five gold miners with bone involvement in South Africa. The organism differs from *S. schenckii* only in its appearance *in vivo*. Var. *luriei* produces large yeast-like organisms in tissues including bony lesions, averaging 15μm - 20μm in diameter, which multiply by budding or by fusion. These tissue forms are not seen in *S. schenckii*. It is interesting to note that in histoplasmosis caused by *Histoplasma capsulatum*, a similar histological variant, i. e. *Histoplasma capsulatum* var. *duboisii*, has also been described from South Africa. In other countries where both sporotrichosis and histoplasmosis are endemic, these variants have not yet been seen.

Predisposing factors for osseous and arthritic infections reported in the literature include alcoholism, cirrhosis, diabetes, tuberculosis and syphilis. Sporotrichotic arthritis often simulates tuberculous arthritis. Confirmation of sporotrichotic arthritis depends on culture, and for this reason the diagnosis is often delayed. The rarity of the

disease and the low index of suspicion are additional delaying factors (Bayer *et al.*, 1979; Birnbaum and Walter, 1987; Khan *et al.*, 1983; Levinsky, 1972; Mackenzie *et al.*, 1988; Molstad and Strom, 1978; Weitzner, *et al.* 1977; Wilson *et al.*, 1988). An early diagnosis in sporotrichotic arthritis or synovitis is necessary to prevent irreversible destruction to the joints (Marrocco *et al.*, 1975). Sporotrichotic arthritis may simulates rheumatoid arthritis, and treatment with systemic steroids for a mistaken diagnosis of rheumatic disease may enhance the growth of *S. schenckii* (Ehrlich, 1978). Although there is very little supporting evidence, the lung seems to be the portal of entry in cases of extracutaneous and arthritic sporotrichosis where no skin or lung lesions are present (Bayer *et al.*, 1979; Brook *et al.*, 1977; Ortiz and Lefkovitz, 1991).

The incidence of both arthritic sporotrichosis and bone involvement is rare in South Africa. Only five among thousands of mine cases of sporotrichosis were recorded by Lurie (1963b). The only other South African report of sporotrichotic arthritis comes from the Cape (Grootte Schuur Hospital) (Kreft and Amihood, 1972). In this case a deep puncture wound into the knee joint caused by a thorn was responsible for the disease. Our case of sporotrichotic arthritis did not sustain any injury to the knee before developing the arthritis. His protein losing enteropathy is considered to be the predisposing factor for the development of multiple skin lesions and haematogenous spread to the knee joint.

The knee joint is the most common site for arthritic sporotrichosis, and repeated cultures are often necessary to make a final diagnosis. The average time form the onset to the positive diagnosis of arthritic sporotrichosis is about two years (Bayer *et al.*, 1979; Yacobucci and Santilli, 1986; Yoa *et al.*, 1986). In our case this period was shorter perhaps due to presence of the multiple skin lesions which made the diagnosis of sporotrichotic arthritis easier. There is no set regimen as far as the treatment of patients with arthritic sporotrichosis is concerned. Individual evaluation is necessary but amphotericin B seems to be the only reliable drug for the treatment of osseous sporotrichosis

and a cure rate of 70-80% is reported (Bayer *et al.*, 1979; Chang *et al.*, 1984; Gladstone and Littman, 1971; Gullberg *et al.*, 1987; Khan *et al.*, 1983; Lurie, 1963b; Wilson *et al.*, 1988). Surgical debridement has been shown to be valuable in combination with systemic medication (Govender *et al.*, 1989). Although the disease is curable, permanent damage to the joint results in all cases (Altner and Turner, 1970; Bayer *et al.*, 1979; Birnbaum and Walter, 1987; Chang *et. al.*, 1984; Chowdhury *et al.*, 1991; Crout *et al.*, 1977; Dehaven *et al.*, 1972; Ehrlich, 1978; Friedman and Doyle, 1983; Halverson *et al.*, 1985; Janes and Mann, 1987; Khan *et al.*, 1983; Lesperance *et al.*, 1988; Levinski, 1972; Lipstein-Kresch *et al.*, 1985; Mackenzie *et al.*, 1988; Yoa *et al.*, 1986). In our case of sporotrichotic arthritis a combination of amphotericin B and surgical intervention gave good therapeutic results, but the patient was left with restricted movement of the knee joint.

As far as pulmonary sporotrichosis is concerned, it is of interest that the conidia of the mycelial phase of *S. schenckii* measure 2 - 3μm in diameter, which would allow these conidia to lodge in the alveoli of the lung. However, primary pulmonary disease is rare. Conti-Díaz and Civila (1969) showed experimentally that it is difficult to infect mice via a broncho-pulmonary route; contact with large numbers of conidia in a arid state is necessary for the animals to contract lung disease. Low host resistance plays an important role in the development of primary lung disease (Velji *et al.*, 1988; Haponik *et al.*, 1989).

Only 30 documented cases of primary pulmonary sporotrichosis had been reported by 1972, and a few single cases have been described since (Fields *et al.*, 1989; Kinas and Smulewicz, 1976; Mohr *et al.*, 1972; Pluss and Opal, 1986). All the reports confirm that primary pulmonary sporotrichosis is difficult to diagnose, and that abnormal cell-mediated immunity is almost always present (Velji *et al.*, 1988; Haponik *et. al.*, 1989). Based on radiographical and clinical features, sporotrichosis often presents with a picture similar to other pulmonary diseases, such as tuberculosis. It is usually only when the disease becomes systemic that the possibility of a fungal disease is considered. Some authors

feel that primary pulmonary sporotrichosis may be more common than indicated by reports in the literature (Fields *et al.*, 1989; Rohatgi, 1980; Velji *et. al.*, 1988). The reasons for this are: the organisms are scarce and not easily detected by ordinary stains in direct smears; the yeast form of the fungus on bacteriological media may be mistaken for other commonly found yeasts, such as *Candida albicans*; the culture may fail because of inappropriate handling of the specimen. As in the case of bone and joint disease, in patients with underlying disease or alcohol abuse in cases of undiagnosed lung disease, a differential diagnosis of sporotrichosis should always be considered and the correct laboratory methods applied in order to detect the organism (Arango *et al.*, 1981; Fields *et al.*, 1989; Hay *et al.*, 1986).

Lung involvement in sporotrichosis is very rare in South Africa. In addition to our undocumented patient with primary pulmonary sporotrichosis, Matthews *et al.* (1982) described a case with skin involvement and systemic spread to the lungs, while Berson and Brandt (1977) reported a sporotrichotic lung infection diagnosed post mortem in a seemingly uncompromised host.

Several unusual cases of sporotrichosis have been noted in the literature. The most unusual and severe localized and disseminated forms of sporotrichosis seem to occur in Mexico (Dominguez-Cherit, 1986; Lavalle *et al.*, 1987). Good therapeutic results with potassium iodide and amphotericin B, have been achieved in these patients.

Chronic sporotrichotic meningitis associated with alcoholism (Freeman and Ziegler, 1977; Schwartz, 1987) is another rare form of sporotrichosis. It is not clear why only alcoholism and not other debilitating diseases predispose to the disease. Occupation and type of exposure to the fungus may play an important role. A very unusual case was reported in an underground "timber boy" from the Western Deep Levels gold mine on the Witwatersrand, who developed meningo-encephalitis due to *S. schenckii*. He had no visible skin lesions, and came to post mortem ten days after admission to hospital (Parker, 1972). Cerebrospinal fluid taken before death was negative on

fungal culture. The diagnosis was made histologically on brain tissue. This is the only documented case of sporotrichosis causing death in a mine worker in South Africa. The immunological status of this patient is not known.

Intraocular sporotrichosis is an equally rare disease. Gordon (1947) in reviewing the literature, could find 48 reports, two of which came from South Africa. Since then no other cases have been reported in this country, but several other cases have occurred elsewhere (Cassady and Foerster, 1971; Castro *et al.*, 1981). Sporotrichotic infections of the conjunctiva have not been reported from this country, and are rarely described elsewhere (Adamson, 1911; Alvarez and Lòpez-Villegas, 1966; Levy, 1971; Witherspoon *et al.*, 1990).

Little is known about the occurrence of subclinical infections and the incidence of sporotrichosis in endemic areas. The latter is expected to be higher than generally believed. Reports of sporotrichosis occurring for the second time in the same patient are extremely rare (Grekin, 1984). It is not known whether such cases are not reported or whether they seldom occur. The fairly high percentage of positive hypersensitivity reactions found in large populations not known to have had the disease before, indicates the former to be more likely. In South Africa no attempt has been made to study the epidemiology of sporotrichosis in the population at large as well as in the gold mines by skin testing with sporotrichin. A preliminary study in which 30 hospital patients were tested at random with a French antigen gave negative results. The occupations of the patients were of such nature that their contact with *S. schenckii* seemed likely (Findlay and Vismer, 1978 - unpublished data). Twenty five years experience of sporotrichin testing in Mexico has shown it to give nearly 100% accurate results in cases of sporotrichosis. It is considered to be of value not only as a diagnostic tool, but also for studying the epidemiology of the disease (Dominguez-Soto and Hojyo-Tomoka, 1983).

In summary it can be said that *S. schenckii* attacks the skin and subcutaneous tissue, bones and joints, the lungs, the brain and meninges and the eye, but that other internal organs are not affected.

4 HISTOPATHOLOGY OF *S. SCHENCKII*

Organisms of *S. schenckii* are rarely seen in tissue. The organism is however readily cultured (Khan, 1975; Rippon, 1988; Sarosi *et al.*, 1985; current study). The importance of serial sections in order to demonstrate asteroid bodies was first emphasized by Simson *et al.* (1947). Although asteroid bodies are regarded as one of the characteristic features in sporotrichosis they also occur in other diseases. Moore (1946) and Moore and Ackerman (1946) described asteroid bodies in aspergillosis. They consist of a radiate formation around a double contoured large yeast cell of *S. schenckii*, which is thought to be due to a reaction between a product of the fungus and a substance produced by the host (Kinbara and Fukushiro, 1982). Kinbara and Fukushiro (1982) found that immunofluorescence is negative on the radiate formation when tested by the fluorescent antibody technique, and consider it to be of unknown origin. After an extensive histopathological examination of cases of sporotrichosis they concluded that there is no significant difference between the fungal elements found in cutaneous lymphatic and localized lesions of sporotrichosis. Lurie and Still (1969) incubated *S. schenckii* yeasts with anti-sporotrichotic serum, and found an amorphous precipitate around these cells, which they regard as an immune reaction. If biopsies are taken at an early stage of the disease, i. e. before engulfment by phagocytes takes place, organisms are more plentiful in the tissues (Hiruma and Kagawa, 1985).

Cigar bodies can either lie free in tissue or be present in phagocytes (Adamson, 1911). They may bud at more than one point and are thin walled. No radiating phenomenon is present around cigar bodies or

yeasts (Brandt and Van Niekerk, 1969; Rippon, 1988). Once these organisms round off and become double contoured to form pre-asteroids, a fungal and host tissue reaction is established and asteroid bodies are formed.

Sarosi *et al*. (1985) shed some light on the long standing question of why *S. schenckii* is easily cultured, yet difficult to see the organism in tissue sections or in direct pus smears. He maintains that loosely bound non-fungal polysaccharides precipitate on the cell walls of yeasts of *S. schenckii* formed in the tissues, acting as a barrier which prevents the uptake of conventional stains used in histopathology and microbiology. He recommends diastase digestion of the loosely bound polysaccharides before the staining procedure, in order to expose the cell wall of *S. schenckii* to the stains. Some authors have used this procedure with success (Dellatore *et al*., 1982), while others are testing this method for validity (Friedman and Doyle, 1983). We incubate pus swabs overnight in saline at 37°C. This removes the outer polysaccharide layer which Garrison *et al*. (1983) and Waldbaum *et al*. (1978) have found necessary to remove in order to stain the organism. On the other hand, yeasts do proliferate overnight, become more numerous and easier to detect. This method proved to be valuable in direct examinations of material from pus swabs.

The application of the fluorescent antibody technique as a means of a rapid and direct microscopic identification of sporotrichosis, has been a major breakthrough in detecting organisms on smears from lesions (González and Kaplan, 1964; Kaplan and Ivens, 1960; Kaplan and González-Ochoa, 1963). Criticism regarding the method is the difficulty of preparation of the labelled immuno-globulins and the maintaining a supply in smaller laboratories. Heed should also be taken of possible cross-reactivity with members of the genus *Ceratocystis* and other related fungi (Ishizaki *et al*., 1978, 1979, 1981). This method was not used in the current study.

The presence of hyphae in histological sections in sporotrichosis is rather unique. Collins (1947) found hyphae in post mortem tissue 24

hours after death in a case of disseminated sporotrichosis. Berson and Brandt (1977) detected germinating asteroid bodies and yeasts as well as hyphae in lung tissue at post mortem. This germination indicates that the double contoured yeast in the centre of an asteroid body is viable. The lower temperature of the body and the relative lack of serum components in the tissues after death, could explain the germination and hyphal growth. Maberry *et al*. (1966) in reviewing the literature, found five reports describing hyphae in tissue sections of sporotrichosis in live subjects. These included Lurie's (1963a) report in the South African literature. No satisfactory explanation could be found for this phenomenon. The localization and depth of the lesion, the size of the inoculum, host resistance and fungal virulence could play a role.

In the compromised host, unusual tissue reactions and forms of *S. schenckii* have often been encountered. Patients with cutaneous disease appeared to have normal cell-mediated immunity (Nakamura *et al*., 1977; Plouffe *et al*., 1979). Infectious diseases are however influenced by both humoral and cellular immune responses (Hachisuka and Sasai, 1980a, 1980b, 1981; Hachisuka *et al*., 1982). By inducing experimental sporotrichosis in mice, Hachisuka and Sasai (1980a, 1980b, 1981) and Hachisuka *et al*. (1982) found that cell-mediated immunity play an important role in the defense mechanism of sporotrichosis, and that humoral immunity may be needed for opsonization of organisms. This however does not explain the susceptibility of the host to sporotrichosis. Several reports confirm our finding that patients who contract sporotrichosis, while being treated with systemic corticosteroids for other ailments, may develop large numbers of organisms in tissue (Bickley *et al*., 1985; Fukushiro, 1984). In Japan, where the incidence of sporotrichosis is high, cases with abundant organisms in tissues are increasingly detected (Hiruma and Kagawa, 1985; Mohri *et al*., 1987). Most of these Japanese patients are not compromised in any way, although corticosteroids and underlying disease, such as diabetes mellitus, play a role in some. Underlying defects in immunity are thought to be responsible for the extracutaneous manifestations of sporotrichosis. Systemic disease occurs in patients

with defects in cell-mediated immune response as measured by delayed hypersensitivity skin tests or lymphocyte transformation studies (Nakamura *et al.*, 1977; Plouffe *et al.*, 1979).

5 TREATMENT OF SPOROTRICHOSIS

At present effective treatment of sporotrichosis depends upon two medications, namely potassium iodide, which is effective and cheap, for the lymphangitic and cutaneous forms of the disease, and amphotericin B which is administered intravenously for the extracutaneous and systemic disease. Both potassium iodide and amphotericin B have side-effects and the availability of the new orally active drugs, such as itraconazole and terbinafine, opens new avenues for therapeutic intervention. Amphotericin B is always considered first in severe cases, as experience with itraconazole and terbinafine is still too limited to be sure of their efficacy.

De Beurmann and Ramond (1903) first demonstrated the efficacy of iodides in the treatment of sporotrichosis. It is still considered the drug of choice in this disease (Itoh *et al.*, 1986; Fukushiro, 1984). There seems to be no uniform approach to the treatment of sporotrichosis with potassium iodide, especially in children. Dosage and duration of treatment should be individualized (Chandler *et al.*, 1968; Prose *et al.*, 1986).

The mechanism of action of potassium iodide remains uncertain. Two hypotheses have been formulated, namely that potassium iodide is absorbed in the body and changed to iodine, which has a direct fungicidal effect (Urabe and Nagashima, 1969), or that potassium iodide enhances the cellular immune reactions in the host (Davis, 1919). Hiruma and Kagawa (1987), Honbo et al. (1985) as well as Wada (1966), could find no experimental evidence that potassium iodide has any fungicidal effect *in vitro*, and assumed that it is the conversion of

potassium iodide to iodine, that is responsible for the antifungal effect *in vivo*. Davis (1919) on the other hand found that potassium iodide will not prevent experimental sporotrichosis in animals, but will cure the disease.

For the treatment trials conducted in this study, the criteria for the protocol set up by the respective companies as well as those put forward by Dismukes *et al*. (1980) were met, in order to make published reports comparable to those from different parts of the world. Restrepo *et al*. (1986) reported on the treatment of 17 cases of sporotrichosis with itraconazole (Sporanox). Results equally favourable to ours were obtained by her with this drug. All patients were cured, most within 120 days, and less than 10% of her patients needed 150 days of treatment for cure. In some cases however, the dosage needs to be increased, i. e. from 100mg per day to 200mg per day to effect cure (Borelli, 1987). Duration of treatment of more than one year have been needed in some cases. Experience in Mexico in the treatment of sporotrichosis is extensive because sporotrichosis is the second most prevalent mycosis in that country, mycetoma being the most common (Lavalle *et al*., 1987). Lavalle *et al*. (1987) found that lymphocutaneous and localized sporotrichosis took three to four months (90-120 days) to be cured with 150mg per day and 100mg per day of itraconazole respectively. The higher dose seems to shorten the period of treatment by about four weeks, without notable side-effects.

From the results of our study and also from the results of others, itraconazole appears to be an effective drug against *S. schenckii*, and may provide a means of avoiding the side-effects of the other available drugs and decreasing the length of therapy and the relapse rate.

Apart from our study on terbinafine (Lamisil), no other reports regarding this drug as a treatment for sporotrichosis could be traced (Hull and Vismer, 1992). Our five patients reacted very favorably to treatment without showing notable side-effects. Further investigations with regards to sporotrichosis are needed to determine its efficacy and possible side-effects in the long term.

Although thermotherapy was not used in this study, it is an additional method of treatment used in sporotrichosis (Galiana and Conti-Díaz, 1963; Hiruma and Kagawa, 1986; Hiruma *et al.*, 1987; Mackinnon and Conti-Díaz, 1962; Mayorga *et al.*, 1978). The mechanism of action of hyperthermia on *S. schenckii* in tissue is not well understood. Hiruma and Kagawa (1983, 1986) found that yeasts ingested by polymorphonuclear leukocytes become more sensitive to high temperatures which also damage *S. schenckii* yeasts *in vitro*. Others have found that sustained temperatures between 36°C and 38°C retard the multiplication of the yeasts in tissue, high temperatures may hamper the metabolism of the organism and favour the defence mechanisms of the host (Galiana and Conti-Díaz, 1963).

Spontaneous cure in sporotrichosis has seldom been reported, but may be more common that realized (Bargman, 1981, 1983; Itwasu *et al.*, 1985; Rippon, 1988). Pueringer *et al.* (1986) reported spontaneous remission in extensive pulmonary sporotrichosis, and self-limited asymptomatic pulmonary infection is possible. Once sporotrichosis is diagnosed, it is usually treated and not left to heal spontaneously but minor cases may not seek medical attention and remain unrecorded. It should be emphasized that sporotrichosis should not be left untreated because it may become chronic, flare up or become disseminated, especially if the patient is immunosuppressed by drugs or other infections (Pepper and Rippon, 1980).

In summation of our findings with regards to treatment of sporotrichosis, it is firstly not necessary to take biopsies in order to follow the mycology of the infection during treatment trials. Crusts or pus swabs from the lesions are quite sufficient for culture of the organism. This is of special importance in babies and small children. A positive culture is unequivocal proof of sporotrichosis (Laur *et al.*, 1979; Rippon, 1988). In patients treated with potassium iodide, cultures for *S. schenckii* remained positive until the clinical lesions had virtually disappeared, while in the case of itraconazole and terbinafine it was found that the cultures became negative at an average

of eight weeks. These drugs are expensive, especially when patients do not react favourably, and the dosage has to be increased or the treatment period prolonged. On the other hand side-effects from potassium iodide occur regularly, in which case itraconazole and terbinafine can be useful alternatives.

6 MYCOLOGY OF *S. SCHENCKII*

The importance of using a class II laminar flow cabinet when examining pathogenic fungi is stressed by DiSilvo (1987). Some 4000 laboratory related infections have been documented, of which 353 were due to fungi. Five deaths from laboratory contracted fungal disease have occurred. Non-medical mycologists in particular, who are at special risk, because they come into contact with fungi on a regular basis are alerted by this report to be aware of the dangers of predisposing factors for fungal disease, such as antibiotics, chemotherapy and steroid treatment. In research laboratories experiments are often conducted with very large concentrations of organisms, and inhalation of fungal conidia can lead to invasive disease (DiSilvo, 1987). Lymphocutaneous sporotrichosis developing pricks with contaminated laboratory instruments, bites while inoculating laboratory animals, splattering of cultures into eyes, have been reported (Thompson and Kaplan, 1977). Dupont (1991) of the Pasteur Institute in Paris warned all HIV positive laboratory assistants to take special care when they work with fungi.

It has been emphasized in the literature that *S. schenckii* is not a common laboratory or environmental contaminant and positive cultures should always be repeated and correlated with the clinical data. After inhalation *S. schenckii* may exist as a lung saprophyte, without causing disease, but its presence in visceral specimens should always be considered pathogenic (Ewing *et al.*, 1980; Lowenstein *et al.*, 1978). Brandt and Van Niekerk (1968) isolated strains of *S. schenckii* of low virulence from the foot soles of miners working above ground indicating

the presence of organisms in these surroundings where they do not cause any particular problem. *S. schenckii* has occasionally been isolated from skin lesions other than sporotrichosis, where it was not considered of any clinical importance (Iwatsu, 1980). Staib and Grosse (1983) found *S. schenckii* on the floor of an indoor swimming pool, showing that the organism can be present in obscure places without causing disease. *S. schenckii* has also been isolated from cold-stored frankfurters but contaminated food is unlikely to pose an epidemiological treat (Ahearn and Kaplan, 1969).

The classification of *S. schenckii* is purely phenetic (De Hoog, 1974; De Hoog *et al.*, 1985). Most of the strains encountered in this study fit the classical descriptions of the organism. (Ajello *et al.* 1962; Barron, 1972; Carmichael, 1962; Davis, 1914, 1915; De Beurmann and Gougerot, 1912a, 1912b; Rippon, 1988). Confirmation could be found in the literature regarding the sympodial, holoblastic conidiation in *S. schenckii*. The high degree of polymorphism associated with conidia formation has also been experienced by others, and the sympodial proliferation of conidia at the apex of a conidiophore in the initial stages of conidium formation, is considered classic (Cole, 1976; De Hoog, 1974; Rippon, 1988).

Identification of the conidial state of *S. schenckii* rests with the conidial formation of the *Sporothrix*-type; the ability of the fungus to grow in the presence of cycloheximide; conversion to the yeast phase at 37°C; and its requirement for thiamine. However, as Mariat (1971, 1975) and Mariat and Diez (1971b) pointed out, these characteristics also apply to *Ceratocystis* spp., and that the identification of the latter species depends on the formation of perithecia in a balanced medium with wood fragments (Mariat *et al.*, 1963; Mariat, 1971; Mariat and Diez, 1971a, 1971b). In the current study, in spite of several inoculations onto wood, wood chips, wood meal and wood flours, no *Ceratocystis*-like perithecia were noted. Only some sclerotic bodies were found in association with certain wood samples. In spite of the similarities in these genera regarding their cultural and morphological characteristics (Lloyd and Travassos, 1975; Mariat, 1971; Mariat and Diez, 1971b;

Nicot and Mariat, 1973; Taylor, 1970a), their serological (Ishizaki *et al.*, 1978, 1979, 1981; Lloyd and Travassos, 1975; Nakamura *et al.*, 1976, 1977), and chemical properties (De Bièvre and Mariat, 1981a; Mendonça-Hagler *et al.*, 1974; Mendonça *et al.*, 1976; San-Blas, 1982; Travassos *et al.*, 1973, 1974, 1978; Toriello and Mariat, 1974), they are not identical. Thus, the teleomorph of *S. schenckii* has not yet been elucidated. Mitochondrial-DNA restriction profiles (Mt-DNA) also proved inconclusive as neither *C. ceratocystis* nor *C. minor* were identical to *S. schenckii* (Suzuki *et al.*, 1988). Suzuki *et al.* (1988) interestingly observed that *S. schenckii* var. *luriei* also did not produce the same Mt-DNA restriction profiles as *S. schenckii* and should therefore be regarded as a separate species of *Sporothrix*. Staib and Blisse (1974) found that *S. schenckii* var. *luriei*, does not have the ability to assimilate creatine, creatinine and guanido-acetic acid, contrary to all other strains of *S. schenckii*; they agree that this variant should be reconsidered for species status. *S. curviconia* and *S. inflata* are closely related to *S. schenckii*, due to similar polysaccharides present in their cell walls, and Suzuki *et al.* (1988) could confirm similarity in one Mt-DNA band of these organisms with that of *S. schenckii*, but they are still considered as species separate to *S. schenckii*. The twelve anamorphic *Sporothrix* species very closely related to *S. schenckii* include *S. curviconia*, *S. inflata* and *S. schenckii* var. *luriei*. These species contain rhamnose, an antigen not widely present in fungi, as determined by gas liquid chromatography and immuno-diffusion (Ishizaki *et al.*, 1979; Gorin and Haskins, 1977). The source of some of these *Sporothrix* species is quite unique. The rare species, *S. fungorum*, was isolated from a 500 year old mummy found in the tomb of Umanak in Greenland (Bodenhoff *et al.*, 1979); on a few other occasions it was isolated from Europe, and once in South Africa by J A Stalpers in 1972 at Savelbos (De Hoog and De Vries, 1973).

Secondary conidia produced from primary conidia, either on conidiogenous hyphae or conidiophores, were frequently seen in our cultures and have also been described by others (Garrison *et al.*, 1982; Taylor, 1970b). Higher temperatures and humidity as well as sufficient nutrition seem to enhance their formation. Although not described in the results of

study, unusual irregularly shaped hyphal and conidial forms were seen to develop in the pathogenic and soil isolates alike, when temperature instability in incubators occurred as a result of unusually high ambient temperatures (28°C - 33°C). Conjugating hyphae with ascus formation were described by Ansel and Thibaut (1970), Thibaut (1972) as well as Thibaut and Ansel (1973) who created the genus *Dolichoascus* for teleomorphic phase of *S. schenckii*. This is in opposition to the view held by Mariat that a species of *Ceratocystis* is the teleomorph of *S. schenckii* (Mariat, 1971; Mariat *et al.*, 1978). It is also proposed that the findings of "asci" were really those of senescence and comprised endoconidia and not ascospores as stated by Ansel and Thibaut (1970) (Mariat and Diez, 1971b; Nicot and Mariat, 1973). As confirmed by electron microscopy, our material showed similar structures as described by the above mentioned authors, but these evidently result from hyphal death, intrahyphal regrowth, and conidium formation within the bulging dead cell which may be attached at either end to empty cell wall relics. These cannot be regarded as a teleomorphic phase of the fungus.

The conidia in *S. schenckii* are not well adapted for dispersal, neither by the manner by which they are borne on the conidiophores or conidiogenous hyphae, nor by the conidial size and shape. Although they detach easily, dispersal in air will probably only take place once the conidia are dried out. Their dispersal seem more likely in water, as conidia from dried out cultures readily go into suspension and do not seem to have a high surface tension. Damp skin would therefore be ideal for the conidia to adhere to, and then be inoculated by abrasion or minor trauma.

Both Garrison *et al.* (1982) and Rippon (1988) described triangular conidia as macroconidia. The developmental features of the conidia followed throughout this study made it clear that these triangular conidia are not a separate entity, but that they are fully matured conidia. Nutritional and other external factors promote their formation. The findings of this study confirming this statement are as follows:

- Any originally unpigmented pathogenic strains of *S. schenckii*, producing only hyaline conidia and no triangular conidia, could be made to do so by either culturing it on dead eucalyptus or wattle plant material or by subculturing the strain onto malt agar.

- On wood a total conversion from the hyaline ovate conidia to pigmented triangular conidia could be achieved over a period of five to six months.

- Scanning electron microscopy demonstrated that ovate and triangular conidia occurred on the same hypha.

- DNA fluorescent staining of the conidial nuclei indicated that ovate and triangular conidia have a common nuclear source.

- Triangular conidia showed no DNA increase with fluorescent nuclear staining.

- Electron microscopy showed that pigmented and triangular conidia had additional cell wall deposits developing as the conidia matured.

Our attempt to alter the conidial shape by adding crude wattle and eucalyptus extracts to cultures of *S. schenckii* on Sabouraud's medium failed to stimulate the formation of triangular conidia or to induce pigmentation in conidia. It remains unclear why *S. schenckii* has a predilection to grow on these wood species.

When Mariat (1971) studied the triangular conidium, he regarded it at first as a growth-form arising by a mutation which at the same time cancelled the power of the wild strain to form an ascocarp and thick pigmented perithecial hyphae. The possible plurality of conidium colour and form of pathogenic strains was not elucidated. In other studies, Mariat *et al*. (1978) correlated maximal pigmentation induced by certain media with specific appearance of electron dense masses attached to the external surface of the conidial cell wall. In our pigmented conidia which were not "force-fed" to produce pigment, the differences between

the triangular pigmented conidia and the ovoid hyaline ones lay entirely in the dense granularity or otherwise of the cell wall, the outer surface being at most slightly fuzzy and not covered with adherent precipitate.

As far as the viability of conidia is concerned, it is generally accepted that pigmented conidia are more resistant to the environment and remain viable for longer periods. In our experiment, where cultures were left to dry out naturally, we found that the pigmented strains survived better. In controlled experiments, fungi with melanized conidia have been found to be more resistant to killing by ultra violet light or by solar radiation (Durrell, 1964). The degree of protection is proportional to the melanin concentration in the conidial walls (Wheeler and Bell, 1988). The exact role of melanin in the virulence of an organism has not been adequately studied and is poorly understood (Wheeler and Bell, 1988). For example, in the case of *Wangiella dermatitidis*, a pathogenic dematiaceous fungus, causing especially central nervous system and brain infections in humans, melanin depleted strains were less virulent than melanin enhanced strains (Polak and Dixon, 1991). On the other hand, Davis (1915) found no difference in the pathogenicity or virulence when inoculating either pigmented or unpigmented strains of *S. schenckii* into rats.

Single-conidium cultures produced distinct culture lines, and the type of the conidium, i. e. either black or white, developed speckled black, grey or white colonies as well as colonies with a ring or wedge of pigment from the original black and white cultures. We therefore regard pigment potentially present in all conidial types of *S. schenckii*, i. e. white, buff, brown, grey and black. We consider that the colour depends on external influences in the artificial substrates in the laboratory or natural sources of food in nature, such as compost and plant material. Substrates which provide the necessary nutrients in excess, stimulate the formation of hyaline ovate conidia. A decline in nutrients instigates pigment production in the cell wall and makes the conidium more durable and resistant. In our patient with bone and joint infection, the later cultures showed

increased pigmentation and the production of triangular conidia. In addition, from this particular isolate, single conidium cultures could be segregated into two or three types. After making this last observation we also found that it had been described by Davis in 1915. This negates the theory that pigmentation is purely a consequence of nutrition.

In their famous monograph De Beurmann and Gougerot (1912) concluded that pleomorphism in *S. schenckii* was not due to fixed variation. Mutations and the development of stable strains were not considered responsible. They implied that it was useless to relate morphology to either nutrition or development in this species. While confirming the variability in growth style, we have done what these earlier investigators did not attempt namely, study the circumstances initiating the morphological changes. The pigmented conidium was found to develop from the unpigmented one as the food supply declines, while no gross changes in the nuclei were detected. Such a conidium has a long survival time. High infectivity in an environmental site is related to growth circumstances and conidium density rather than to clinical virulence. From our experience clinical virulence of *S. schenckii* infection is related to lowered host resistance which promotes a more intense infection. The size of the inoculum also contributes to infectivity.

7 DIMORPHISM IN *S. SCHENCKII*

S. schenckii is a dimorphic fungus, falling into the unique group of fungi which have the ability to invade tissue and cause disease associated with the changing to yeast-like forms in tissue (Howard, 1961; Rippon, 1980). Dimorphism in pathogenic fungi was previously believed to be only temperature related. Temperature is the main factor promoting morphogenesis, but Romano (1965) divided dimorphic pathogenic fungi into three groups: those in which temperature alone is sufficient

to cause transformation (*Blastomyces dermatitides* and *Paracoccidioides brasiliensis*; those in which temperature and supplementary nutrition are necessary (*Histoplasma capsulatum*, *H. farcinimosum* and *S. schenckii*), and a third controversial group where temperature, nutrition and other environmental factors may play a role (*Candida* sp. and Mucorales).

The importance of the ability of *S. schenckii* to convert to the yeast phase has long been recognized and has been used over the years as a test for the authenticity of cultures of *S. schenckii* isolated from humans and animals. Virulence has often been correlated with the ease with which a particular strain converts to the yeast phase at 37°C.

Soil isolates identified as *S. schenckii* strains, that are not able to convert to the yeast phase at 37°C, are suspect. Species that are very closely related to *Sporothrix* include some *Ceratocystis* spp., *Graphium* spp. and *Europhium* spp. When these strains were compared by means of serological tests, it was found that *S. schenckii* has a multiple antigenic system, and that it is similar but not identical to very closely related *Ceratocystis* species, namely *C. stenoceras* (a phytopathogen), *C. minor* and *C. pilifera* (Ishizaki *et al.*, 1978; Garrison *et al.*, 1977; Harada *et al.*, 1976). Harada *et al.* (1976) concluded from serological testing that *S. schenckii* is probably an anamorphic exoconidial form of a species of one of the closely related ascigerous phytopathogenic genera of *Ceratocystis* or *Europhium*, according to cross-reactivity among the species in the above mentioned genera. Importantly they also noted that some of these species grew well at 34°C and produced yeast-like colonies, while others grew poorly and some not at all. The inability to grow at 37°C is another reason why these *Ceratocystis* species cannot be considered as true *S. schenckii* teleomorphs.

In the morphogenic process according to Garrison *et al.* (1975, 1982), yeasts are formed directly from hyphae; this could be confirmed in the current study and is an unique feature of *S. schenckii* (Rippon, 1980). Contrary to the findings of Garrison *et al.* (1983), we could not find

any evidence in this study that conidia directly gave rise to yeast blastoconidia at 37°C.

In addition to temperature, disulfide reductase is another factor playing a role in the mycelial to yeast morphogenesis; this enzyme softens the rigid disulfide linkages in the hyphal cell wall of *S. schenckii*, allowing for yeast-like growth (Betancourt *et al.*, 1985). Thus, yeast transformation will occur when free sulphydryl groups are incorporated into the medium, for example as fresh blood or cystine (Cambell, 1945; Rippon, 1980). Other factors that influence the transformation from yeast to mycelium and *vice versa*, include the presence of divalent cations, especially Zn^{2+}, Mg^{2+} and Ca^{2+}. Zinc and magnesium ions have an inhibitory action of the regulation in yeast to mycelium transformation, while calcium ions play a stimulatory role (Alsina and Rodríguez-del Valle, 1984; Rodríguez-del Valle, 1989).

Tsuboi *et al.* (1987, 1988) found that the pH of the medium in which *S. schenckii* grows is important for the production of extracellular proteinases I and II. These enzymes have been isolated in media with different pH's, imply that their production is pH dependent, and that they are key enzymes for fungal growth. Either proteinase is expressed, depending upon the pH of the medium, allowing for the corresponding growth form. These authors also showed that no proteinases are produced in Brian Heart Infusion and Sabouraud agars; probably because proteinase production is unnecessary in media which already contain enough aminopeptides. Inhibitory profiles suggest that extracellular proteinases are essential for fungal growth in nitrogen-restricted media. Virulence of *S. schenckii* may also be related to the proteinases it produces (Tsuboi *et al.* 1987, 1988). Investigations into the influence of the pH, temperature, aeration and carbon source on the development of the yeast and mycelium phase from conidia of *S. schenckii*, have no specific parameter as an exclusive determinant of morphology. The specific forms that develop are dependent upon the interrelationship between the available nutrients and culture conditions, for example, yeasts were obtained at 25°C, and the mycelial phase at 30°C by varying the pH and source of carbon (glucose) and

nitrogen (neopeptone) of the medium (Arnold, *et al.*, 1987a; Catchings and Guidry, 1973; Rodríguez-Del Valle *et al.*, 1983).

Differences in the cultural characteristics of strains of *S. schenckii* isolated from sporotrichotic lesions have been subject to much debate. Kamalam and Thambiah (1982) and Kwon-Chung (1979) found that strains isolated from fixed cutaneous disease could grow at a maximal temperature of 35°C, while the organisms isolated from lymphangitic and disseminated disease could grow at a higher temperature, i. e. 37°C. No immunological differences could be established between the groups of patients. De Albornoz *et al.* (1986) found that their strains could all grow at the higher temperature and even at 38°C. However, *S. schenckii* cannot grow above 38,5°C (Urabe and Honbo, 1986). Certainly in the current study no difference could be noted in the temperature requirements for yeast conversion in different strains. Different temperature requirements of different strains of *S. schenckii* do not explain differences in clinical forms.

It is generally accepted that the cell-mediated immunity of the host determines the degree of invasion by pathogenic fungi. This is true of both cutaneous and extracutaneous sporotrichosis, particularly the latter (De Albornoz *et al.*, 1984, 1989; Plouffe *et al.*, 1979). However, our studies of the growth of *S. schenckii* on wood, demonstrated that very large numbers of conidia may be present on very small areas of plant material. As in the case of several other pathogenic dimorphic fungi, the size of the inoculum *in vivo* plays an important role (Rippon, 1988).

In conclusion, as far as dimorphism in *S. schenckii* is concerned, it is becoming clear that yeast formation is initiated not only by higher body temperature of the host, but that external factors such as pH, carbon and nitrogen sources and enzyme production also determine fungal growth forms. The size of the inoculum and the immune response of the host may play additional roles. Even the melanin pigmentation of the strain may be of importance in some cases. It is much like "being in the

wrong place at the right time" - the fungus does not depend upon infection to complete its life cycle.

8 FLUORESCENT MICROSCOPY OF *S. SCHENCKII*

Fluorescent DNA-staining and electron microscopic studies of *S. schenckii* confirmed that conidia contain only one nucleus, as previously reported by us (Findlay *et al.*, 1979; Findlay and Vismer, 1986). Extensive DNA and RNA determinations indicated that germ tube formation precedes nuclear division in the conidia of *S. schenckii*, and that the first septum in the germ tube may be present before nuclear division was complete. Septum formation was not complete still allowing for nuclear migration. Germination has been found in an average of 80% of conidia within the first 9 - 12 hours of incubation (Ayala and Rodríguez-Del Valle, 1988; Resto and Rodríguez-Del Valle, 1988). In yeasts cells the situation seems to be different, as these authors found two nuclei present in the yeast cell before germ tube formation commenced.

9 SCANNING ELECTRON MICROSCOPY OF *S. SCHENCKII*

Scanning electron microscopy of the growth on wood confirm the finding that *S. schenckii* does not have the ability to use the wood structure for nutritional purposes. However, the large number of conidia that are produced on 1 - 2mm of plant material, ensures or poses a high risk of infection to anyone coming in contact with the contaminated plant material.

10 ELECTRON MICROSCOPY OF *S. SCHENCKII*

On the electron microscopy of *S. schenckii*, osmiophilic bodies, other than lipid bodies, as seen by Garrison *et al.* (1976, 1977), could not be found in the conidia in our material. Their finding that matured lipid bodies were enclosed in a unit membrane could also not be confirmed. But we confirmed their finding that lipid bodies were present in young and matured conidia. Extensive biochemical testing has indicated that the osmiophilic bodies have lipase activity, are partially solubilized with lipid solvents and are closely associated with lipid bodies (Garrison *et al.*, 1977). No lomasomes, as described by Ansel *et al.* (1969), could be detected in our material.

An interesting study of the plasma membrane in aging cells was reported by Maeda *et al.* (1987, 1988). The degeneration of the plasma membrane, with only granules remaining, was elegantly portrayed by means of freeze fracture by these authors. They concluded that cells in the degenerated stages are more susceptible to antifungal treatment. Degeneration of the yeasts in tissue may be a natural phenomenon or may be due to host reaction towards these cells (Svoboda and Trujillo-González, 1990).

Polysaccharides and peptido-polysaccharides are frequently isolated from the outer cell wall layers of fungi (Gorin *et al.*, 1977; Previato *et al.*, 1979). The cell wall surface polysaccharides synthesized in *S. schenckii* determine the antigenic specificity of the organism (Gorin *et al.*, 1977). Some *Ceratocystis* species are fungi very closely related to *S. schenckii* but only the latter produces exocellular mannans in the early growth stages (Gorin *et al.*, 1977). The original multi-species concept of sporotrichosis was invalidated by the work of Lurie (1948), when he showed that *S. schenckii*, *S. beurmanni*, *S. asteroides* and *S. equi*, all had the same antigenic properties. This furthered a one species concept of the disease.

The cell wall components of *S. schenckii* are qualitatively and quantitatively similar, in both the hyphae and the conidia. Slightly higher levels of protein and carbohydrates, and slightly lower levels

of lipids and phosphate have been detected in conidia, as compared to hyphae. In the yeast phase, a higher content of carbohydrate was found, and the amino acid composition of proteins differed in the hyphae and conidia of *S. schenckii* (Previato *et al.*, 1979).

Pigmentation in cultures was found to be very variable in this study as no two cultures, even of the same strain, behaved alike under similar conditions. As mentioned, the time when the pigment appeared and the intensity, shade and distribution of the pigment varied considerably in the strains isolated. The conidia are the chief seat of pigment formation. Davis (1915) found that the pigment in *S. schenckii* conidia was insoluble in aqueous solutions and undiffusible in fat solvents, weak acids and weak alkalis, thus behaving like melanin. Strong acids and alkalis dissolve the entire organism and destroy the pigment. Our finding that the pigment reacted histochemically like a melanin concurs well with these findings. Nickerson (1947) reported similar findings, but the natural substrate for the pigment in *S. schenckii* has not yet been identified. Black cultures contained mainly black conidia, each having a brownish tint when examined microscopically. Because the cytoplasm of the conidia is not pigmented in a granular fashion but homogeneously or diffusely brown, Davis (1915) concluded that the pigment is uniformly distributed in the conidium. Examination of pigmented conidia by means of electron microscopy reveals that the pigment in the cell wall of the conidia constitutes a granular opaque line deposited in the centre of the inner cell wall. Strong evidence of this could be found in the triangular conidia. It is debatable whether the pigment is also present in the cytoplasm, as other organelles clustered and filled it. The view that the electron dense granules present in the cell walls of the pigmented ovoid and triangular conidia is melanin has been confirmed by the extensive work done on melanins in pathogenic fungi by Wheeler and Bell (1988).

Davis's (1915) observation that hyphae were unpigmented, but that mycelial masses occasionally contained small amounts of pigment, could not be confirmed in our strains. He described a yellow diffusible pigment in old cultures. In our collection old cultures produced a

light to dark brown diffusible pigment, and was regarded as pigment leaching from degenerating or broken down conidia, rather than from the hyphae.

On electron microscopy features similar to those described by others were demonstrable in our material of *S. schenckii* yeasts in tissue (Agar and Douglas, 1957; Avram *et al.*, 1975; Lane *et al.*, 1969; Lane and Garrison, 1970; Garrison *et al.*, 1975, 1977, 1979, 1982; Maeda *et al.*, 1987, 1988; Rippon, 1980, 1988). The ultrastructure of asteroids was described for the first time by Hiruma *et al.* (1991), who concluded that the radiate formation around the asteroid might be composed of disintegrated host cells deposited in a crystalline manner around a fungal cell. We did not encounter any asteroid bodies in our material.

The cell walls of the conidia in culture and yeasts in tissue were found to be similar, except for the presence of microfibrils and an additional double membrane to the outside of yeasts. Garrison *et al.* (1982) found these microfibrils to be peptido-rhamnomannans, but their function remains obscure. Later Garrison *et al.* (1983) and Arnold *et al.* (1986, 1987a, 1987b) posed the question whether the increased number of isoenzymes in the pathogenic phase of *S. schenckii* play a role in the pathogenesis of the disease. The inclusion of isoenzymes into the microfibrillar layer would allow for interaction with macrophages and other host cells. Lurie (1963a) stated that the yeasts of *S. schenckii* in tissue do not have a surrounding capsule, but that these organisms shrink away from the outer cell wall structure. This phenomenon is described in the current study and other investigators have reached similar conclusions (Streeton *et al.*, 1974). So far there is no cytochemical evidence that this is a true capsule (Garrison and Mirikitani, 1983). The limiting membrane, interpreted by us as to be part of the outer cell wall membrane in the yeasts cells seen in tissue, is similar to that described by Garrison *et al.* (1979) in a case of feline sporotrichosis. They concluded that there is no evidence that the fibrillar space between this membrane and the rest of the fungal cell forms part of a capsule.

11 ECOLOGY OF *S. SCHENCKII*

The general statement frequently made in the literature that *S. schenckii* "has a wide distribution in nature", is seldom substantiated by soil and plant surveys undertaken to detect the presence of the organism. Often only a few samples are found positive (Rogers and Beneke, 1964; Mackinnon and Conti-Díaz, 1969). Ventin *et al.* (1987) examined several samples of soil, plants and decomposing vegetable material in a geographic area related to the occurrence of cases of sporotrichosis and found *S. schenckii* only in decomposing vegetable material. In similar ecological searches for pathogenic fungi done in Virginia and Baltimore indicated that a *Sporothrix* sp. was isolated on one occasion only, and this isolate was found to have a low virulence in hamsters. The potential hazard of potting soil as a source of infection is real; in routine testing of the national brands of potting soil in USA 17% proved to be positive for *S. schenckii* (Kenyon *et al.*, 1984).

S. schenckii is not considered to be phytopathogenic, but is able to grow on injured or debilitated plant tissue, as shown experimentally by Benham and Kesten (1932). They injected conidia into scarified carnation buds, which subsequently became diseased. *S. schenckii* is commonly found on decaying vegetation and in soil, but the conditions which enhance its growth in soil remain obscure (McDonough *et al.*, 1970; Mackinnon *et al.*, 1969; Howard and Orr, 1963). *S. schenckii* is usually found in topsoil. In most cases the fungus can be isolated from specimens taken from a depth of 2 - 3cm. It is interesting to note that in our study where the fungus was grown on wood blocks, the growth penetrated into the water agar also up to a maximum depth of 2cm, indicating *S. schenckii*'s aerobic nature.

Problems exist in validating the authenticity of the anamorph when soil strains of *S. schenckii* are associated with *Ceratocystis* species (Goncalves, 1973). No studies have been done to determine whether the quality of the soil is a determining factor for the occurrence of *S. schenckii*. Poor quality of soil may explain why sporotrichosis does

not occur in certain countries or why the organism has disappeared from a specific environment (Mahgoub, 1991). The identification of *S. schenckii* isolated from nature is complicated by the simultaneous occurrence in the same habitats of morphologically related species of *Ceratocystis* and of *Graphium* species. Ajello and Kaplan (1969) described perithecium-like structures in *S. schenckii* var. *luriei*, when this variant of *S. schenckii* was cultured on oatmeal agar. These structures present as spherical bodies, or dematiaceous pseudoparenchymous mycelia, 125μm in diameter and may represent abortive attempts at sexual reproduction. Similar sclerotic structures were seen in water agar cultures of *S. schenckii*, but consisted only of clumped mycelial masses. Mackinnon *et al.* (1969) used tongue depressors made from willow wood (*Salix alba*) to grow *S. schenckii*, and the fungus produced pigment on them. The production of pigment was used to distinguish *S. schenckii* from *Ceratocystis* spp. and *Graphium* spp. as according to these authors, the last two species do not produce pigment on this wood.

The growth of *S. schenckii* on wattle and eucalyptus wood in our study was very similar to that described by James (1965) on the wood props in the gold mines. James (1965) found that the colour of the growth varied from white to grey, brown or black, and that the colour and the texture of the colonies were affected by age, moisture and the substrate. *S. schenckii* was also readily overgrown by other fungi, and was destroyed by wood-rotting fungi. Importantly, triangular conidia only appeared after five to six months on the wood, and most infections seemed to be correlated with their presence (James, 1965; Brown *et al.*, 1947). The findings of James (1965) relevant to this study are as follows:

- *S. schenckii* grew well on the sapwood of eucalyptus and wattle timber, but not on the heartwood of these wood species.
- The fungus grew poorly on the sapwood of the pitch pine (*Pinus rigida*), and very poorly on the heartwood, no growth occurred on oregon fir.

- All the mine recordings of *S. schenckii* were on wattle and eucalyptus.
- *S. schenckii* grew very superficially on the wood, but in the cut ends (cross-section) of the wood, penetration was measured up to a maximum of 2cm.
- *S. schenckii* grew poorly on mine mud, in mine water, and on wet rock, but the fungus grew in the water around the wattle and eucalyptus poles standing in water.
- Optimal fungal growth was seen at temperatures between 27,8°C - 29,4°C (82°F - 85°F). No growth was recorded at 36°C.
- Free dripping water and the moisture in the air seemed to be a critical factor in the growth of *S. schenckii* underground, as the relative humidity underground determined the growth of the fungus. Growth was significant at a relative humidity of 92,5%, and rapid good growth was seen at a relative humidity of 95%. These findings also correlated well to the growth on eucalyptus and wattle wood, as the growth was good at 100%, very slight at 92,5% and no growth was found at 90% relative humidity. Colony development seemed to be much slower with the lower humidity, but a thin film of water on the wood was sufficient to sustain growth.

Brown *et al.* (1947) confirmed that the conidia of *S. schenckii* are not easily dispersed into the air currents of the mine shafts, and that contamination from one shaft to the other, by means of ventilation seemed very unlikely (Brown *et al.*, 1947). Under experimental conditions, an air current with a speed of 200 FPM (feet per minute) can liberate conidia from wood artificially infected with *S. schenckii*. The number of liberated conidia is inversely proportional to the degree of humidity of the substrate, findings similar to those of Conti-Díaz and Civila (1969). This may explain why no cases of primary pulmonary sporotrichosis were included in the large epidemics experienced in the mines. The fact that the mine workers were healthy probably played an additional role.

Growth of *S.schenckii* on indigenous and exotic wood indicated that it grew better on some wood species exotic to South Africa, including

Eucalyptus grandis, *E. sideroxylon*, *Cinnamomum camphora*, *Ginkgo biloba* and *Acacia melanoxylon*. No obvious common factor could be found on familial or genus level nor in the chemical characteristics of these wood species. It is possible that pigmented and possible unpigmented compounds initially leached out and restrict fungal growth. Later the fungus grew on the agar and crept up onto the wood, producing heavily pigmented growth, for example in the case of *Albizia adianthifolia*. Growth occurred only after 30 days and three months on some wood species, for example, *Calodendrum capense*. Where growth on the wood was apparent, pigmented and triangular conidia were detected on microscopy. There were two exceptions, viz. *Schotia brachypetala* and *Cinnamomum camphora*, simulating pleomorphic growth in culture. Volz and Pan (1976) did an extensive study of *S. schenckii*'s growth on various plants in Taiwan, and also found that the fungus seems to have a preference for certain plants. In results similar to ours, abundant growth occurred on one plant species, but not on other species of the same genus. They also could not find a common factor among an extensive list of plants species collected from all over the island. It is clear that *S. schenckii* shows a predilection for certain plants, but does not grow on others.

Growth of *S. schenckii* in various wood flours and wood meals indicated that lignin was definitely not digested by *S. schenckii*. This fungus probably proliferates on sugars diffusing from dead woods and leaves, and other nitrogen sources from nature. As shown it can grow in the presence of wattle and eucalyptus extracts, which do not however enhance the growth in any way. Depletion of limited food sources leaching from the wood, promoted the formation of pigmented and triangular conidia.

When the pH of the wood species used in our experiment was determined, it was found to be quite varied (range pH 3,2 - 6,3), and mainly between 4,0 and 4,8. The pH of *Eucalyptus grandis* was one of the lowest, with a pH of 3,3 taken at three hours, six hours, and after wood shavings were soaked overnight in distilled water. In most instances where growth was detected, the pH was below 5,0. Of particular interest is that sphagnum mosses, peat and grasses also grow in acidic environments.

The ecological niche of *S. schenckii*, in conjunction with its enzymatic system and other nutritional requirements, may be closely linked to the pH values of the environment. Further research is necessary to determine these factors, and to perhaps correlate them with the symbiotic existence of insects as vectors of the fungus when considering its association with disease.

A further explanation as to why certain soil isolates are not pathogenic may lie in the findings that strains of *S. schenckii* are serologically different, and that they do not all possess the socalled "unique" *S. schenckii* antigen irrespective if their source was from the soil or from clinically different sporotrichotic diseases (Nishikawa *et al.*, 1975).

Soil samples are very often collected after the disease has taken its course and periods of delay before collection can vary considerably - from three months to a year or more in our series. The natural cycle of the fungus is still unknown. It may be present in a complete different form in nature as to when it is collected after it has caused disease. Furthermore, the human host may prove to be more susceptible than other animals.

The striking morphological similarities of soil isolates to human pathogens has been widely reported but the ability of the soil strains to grow and convert to a yeast phase at 37°C, as well as the virulence of these strains are quite variable (Howard and Orr, 1963). Consistency in the ability of pathogenic strains to produce black pigment in malt (current study) or carrot agar (Davis, 1915), and characteristics present in the conidia of the fungus have been found. Only some soil strains are able to produce pigment in the media mentioned, and to show mild virulence. It may speculated that only true strains of *S. schenckii* are able to display these characteristics and that all other strains originally unpigmented, are not *S. schenckii*.

Some soil isolates showed more slender conidia, measuring 1-2μm longer than the characteristic conidia. This does not seem to be unusual as

variation in the length of conidia has been reported (Travassos and Lloyd, 1980; Shadomy and Wang, 1988). The sympodial manner in which these conidia were formed on the conidiophores and the denticles remaining after conidium detachment were identical to those of normal length. Results very similar to ours, namely that soil isolates constantly produce slender, elongated conidia, were published by Shadomy and Wang (1988) for soil isolates in America. Their strains also converted to the yeast phase at 37°C, but their soil isolates seemed more virulent to mice, producing lesions typical of sporotrichosis.

The guanine-cytosine (G-C) content in *S. schenckii* is closest to that of *C. minor*, so that the latter is considered the strongest possible teleomorph for *S. schenckii* (Polonelli and Morace, 1982); Mariat, 1971; De Bièvre and Mariat, 1978a, 1978b, 1981b). De Bièvre and Mariat (1978a, 1978b, 1981b) summarized the ecology and epidemiology of the fungal complex *Sporothrix - Ceratocystis* and showed that these fungi occurred on the same fragments of plants, for example pine and eucalyptus. De Bièvre and Mariat (1978a, 1978b, 1981b) found that *S. schenckii* could not be isolated from the same ecological area at different times of the year. We had similar negative results in some of our cases when some months had elapsed between the time of the injury and the taking of the soil sample. The presence *S. schenckii* in nature, even abundance, is not enough to explain the development of the disease after inoculation into the host. The immunological reaction of the host is very important, and immunological defects must be suspected particularly in unusual cutaneous cases and systemic and extracutaneous sporotrichosis (De Bièvre and Mariat, 1978b; Hachisuka *et al.*, 1980a). In AIDS patients several cases of sporotrichosis have been described, as an infection additional to others (Bibler *et al.*, 1986; Fitzpatrick and Eubanks, 1988; Keiser and Whittle, 1991; Kurosawa *et al.*, 1988).

An interesting observation of Happ *et al.* (1975) is that a variety of *Ceratocystis minor*, in a *S. schenckii* "imperfect state", exists as an ectosymbiont of the southern pine beetle, *Dendroctonus frontalis* Zimm. In the mycangium (fungal transport pocket) of the beetle, *S. schenckii* grew as a yeast-like form, but on culture or in the tree, growth was

mycelial. Their electron microscopic work showed that the yeast-like structures in the mycangia were similar to the yeast phase in human tissues. *C. minor*, a species phylogenetically related to *S. schenckii* (Mendonça-Hagler *et al.*, 1974), and considered by some the teleomorph closest to this fungus, is often associated with bark beetles in coniferous hosts (Hunt, 1956). However, the species of bark beetles implicated here are not parasites on either wattle or eucalyptus so that they may form part of the life-cycle of *S. schenckii* (Poynton, 1979; Sherry, 1971). It has long been known that the ambrosia fungi live in symbiosis with beetles and are disseminated by them (Batra, 1963). A similar relationship can therefore not be considered in the case of the southern bark beetle and *S. schenckii* as yet. It remains an interesting fact that the mycangium of the beetle carries a selective medium for the fungus it is transporting, and also that *S. schenckii* is in a yeast form in the insect's fungal transport pocket; this may be a way in which *S. schenckii* is selected to become more virulent.

We conclude provisionally that *S. schenckii* is polymorphic, and that some selection of strains takes place in nature, due to environmental factors, or in the patient; so far without evidence of mutation. An hypothesis can be formulated to explain the epidemiology of sporotrichosis occurring in the population at large. It has been found that unpigmented pathogenic strains which produce only hyaline conidia, convert to pigmented strains, producing larger ovate to triangular conidia. In the spring and during the rainy season (September through to February) *S. schenckii* proliferates and exists as hyaline, non-infective or mildly infective type of conidia. Thereafter as nutrition begins to decline, the conidia start pigmenting. By the winter season they are triangular in form, perhaps not more virulent, but being strongly pigmented, they can cause inflammatory responses when inoculated into the skin. This latent phase of the conidia lasts until the rainy season commences again. Other factors to be considered are the risk of injury and infection in adults and children alike.

12 SPOROTRICHOSIS IN ANIMALS

Saunders (1948, 1955) reviewed the occurrence of sporotrichosis in animals, and listed the species in which the disease has been documented. Mainly domestic animals, including cats, dogs, horses, mules, donkeys, cattle, fowl, and even camels are affected. Sporotrichosis occurs sporadically in horses and does not have the tendency to spread in a herd, nor from one animal to another. Chimpanzees, mice and swine can also be added to the list (Jungerman and Schwartzman, 1972; Kier, 1979; Scott and Horn, 1987). Sporotrichosis has even been described in a dolphin (Medway, 1980; Migaki *et al.*, 1978). Smith *et al.* (1972) confirmed the importance of a positive culture in sporotrichosis especially when this disease is included in the differential diagnosis of cutaneous glanders, epizootic lymphangitis and ulcerative lymphangitis. Although sporotrichosis disseminates in dogs most often (Woodard, 1980), dissemination in cats has also been seen (Kier *et al.*, 1979). Our experience of sporotrichosis in animals apart from two horses is limited to six domestic cats, in which the face and forelegs were most affected.

Robinson and Parkin (1929) described the first cases in horses in South Africa from the Pretoria North area. In 1951 Thorold reported a further two cases, and since then only one other report of sporotrichosis in animals, also in horses, in South Africa appeared. The author, Theron (1987), entitled this publication "Don't mix horses and roses"; he described sporotrichosis as "a disease that may kill a horse if not diagnosed in time - and an unlikely host is the rose bush". At a recent annual congress of the South African Veterinary Society (Marshall, 1990a), examples of sporotrichosis in horses were presented in which there were widespread lesions, often with sparing the forelegs, but with extensive lesions down the back legs and the fetlock above the hoof (Blackford, 1984; Marshall, 1990a, 1991; Muller *et al.*, 1983). In the early reports of sporotrichosis, *S. schenckii* was described on the skin and the buccal mucosa of healthy horses after they had been in contact with infected animals (Adamson, 1911; De Beurmann and Geogerot,

were no obvious injuries or areas of chaffed skin to account for the sporotrichotic infection. It seems most likely that the source of the infection was infected buccal mucosa. Carougeau (1909) reported similar findings in mules and horses from Madagascar.

The histological examination of sporotrichosis in animals was found to be very similar to that seen in humans (Smith, Jones and Hunt, 1972). Intravenous sodium iodide, and oral potassium iodide are both effective in horses, and the former treatment is preferred (Blackford, 1984; Blood, 1986; Davis and Warrington, 1964). Dissemination in cats is best treated with amphotericin B. Ketaconazole is ineffective in the treatment of sporotrichosis in cats (Scott *et al.*, 1987).

Sporotrichosis is a health hazard for veterinary surgeons, veterinary students and assistants as well as pet owners. Several cases in humans have been described where infected animals were the source of the infection (Caravalho *et al.*, 1991; Nusbaum *et al.*, 1983; Read and Sperling, 1982; Samorodin and Sina, 1984; Schiappacasse *et al.*, 1985; Scott and Horn, 1987; Zamri-Saad *et al.*, 1990).

There is general agreement that not sufficient data is available regarding sporotrichotic infections in animals, because not all cases are reported, and epidemioligcal studies have not yet been undertaken. It is unlikely that the incidence of sporotrichosis in animals is as low as the available figures suggest. A countrywide survey needs to be done to clarify the situation.

"Fungi are a mutable and
treacherous tribe"

This is an old adage describing the only
constant in the study of mycology:

VARIATION

John Willard Rippon
1988

SUMMARY

THESIS TITLE: MAMMALIAN *SPOROTHRIX* INFECTIONS IN SOUTHERN AFRICA - RESEARCHES ON THEIR DEVELOPMENT, DYNAMICS AND CONTROL.

CANDIDATE: Hester Francis Vismer

PROMOTER: Professor A Eicker

ASSOCIATE PROMOTER: Professor E J Schulz

DEPARTMENT: Faculty of Science, Department of Botany
University of Pretoria. Pretoria.

DEGREE: Philosophiae doctor

In the historical introduction of this manuscript, a worldwide literature review, regarding aspects of mammalian *Sporothrix* infections, is given.

A research programme concentrating on the epidemiology, morphology and ecology of *Sporothrix schenckii* in the Transvaal, is presented.

Two epidemiological forms of human sporotrichosis exist in the aforementioned province. One type is due to an infection acquired from a source in nature (soil, plants and decaying vegetation), and the other type was found in the Witwatersrand gold mines due to fungal proliferation on the timber props underground, where the disease took on epidemiologic proportions in the early part of the century. Changes in the mining methods have almost eliminated the problem in the latter environment. Sporadic cases and small outbreaks do however still occur.

Pathogenic *S. schenckii* strains derived from human and animal disease could be converted on moist wood to produce triangular and

pigmented conidia, similar to those seen in the gold mine isolates. Pigmented conidia proved to be more viable than unpigmented ones, and pigment production was found to be potentially present in all conidium types. The larger numbers of conidia produced in pigmented strains are indicative of higher infectivity rather than increased pathogenicity or virulence.

Larger conidium volumes were unrelated to genetic material but were due to an increased cytoplasmic content and cell wall thickening in the older conidia, as confirmed by fluorescent nuclear DNA staining and electron microscopy.

Results of this study suggest that *S. schenckii* possesses an intrinsic polymorphism, as the two types of pathogenic strains could be interconverted solely by altering the nutritional conditions.

This study also describes the occurrence of *S. schenckii* infections in animals and reports on the successful treatment of the disease with two new drugs.

OPSOMMING

TESIS TITEL: SOOGDIER *SPOROTHRIX* INFEKSIES IN SUIDELIKE AFRIKA - NAVORSING OOR DIE ONTWIKKELING, DINAMIKA EN BEHEER DAARVAN

KANDIDAAT: Hester Francis Vismer

PROMOTOR: Professor A Eicker

MEDE PROMOTOR: Professor E J Schulz

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GRAAD: Philosophiae Doctor

'n Wêreldwye literatuuroorsig wat handel oor die aspekte van soogdier *Sporothrix* infeksies, word in die historiese inleiding van hierdie proefskrif uiteengesit.

'n Navorsingsprogram wat op die morfologie, ekologie, epidemiologie en behandeling van *Sporothrix schenckii* infeksies in die Transvaal konsentreer, word aangebied.

Twee epidemiologiese vorms van menslike *S. schenckii* infeksies kom in die Transvaal voor. Die een uiterste van die siekte het sy oorsprong uit 'n omgewingsbron, naamlik besmette grond, plantmateriaal en kompos, terwyl die ander vorm in die Witwatersrandse goudmyne opgedoen word waar die organisme weelderig op ondergrondse houtstutte groei. Die laasgenoemde vorm het epidemiese afmetings in die eerste helfte van dié eeu aangeneem. Tans is die probleem feitlik uitgeskakel weens veranderde ontginingsmetodes in die mynbedryf asook 'n meer deeglike kennis van die siektetoestand. Sporadiese gevalle en klein epidemies kom egter nog voor.

Patogeniese *S.schenckii* stamme kan deur hulle op klam hout te laat groei, omgeskakel word na gepigmenteerde kolonies wat driehoekige kodidiums vorm. Laasgenoemde is soortgelyk aan die konidiums wat in die Witwatersrandse goudmyne gevind is. Gepigmenteerde konidiums bly langer lewensvatbaar as die ongepigmenteerde konidiums, alhoewel pigmentproduksie potensieel in alle konidiumtipes teenwoordig is. Die groter aantal konidiums wat deur die gepigmenteerde isolate geproduseer word, dui op 'n hoër infeksie risiko eerder as 'n verhoogde patogenisiteit of virulensie van 'n betrokke stam.

Groter konidium volumes by gepigmenteerde konidiums, kon nie met genetiese materiaal gekorreleer word nie maar wel met 'n verhoogde sitoplasmiese inhoud en verdikking van die selwand by ouer en volwasse konidiums. Dit kon met fluoresente kern-DNA-kleurings en elektronmikroskopie bevestig word.

Navorsing suggereer dus 'n intrinsieke polimorfisme in *S.schenckii*, aangesien die twee tipes patogeniese stamme interkonversie kan ondergaan deur slegs die voedingsbronne aan te pas.

Enkele gevalle van sporotrigose by diere word ook bespreek.

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It is with pleasure and much gratitude that I acknowledge those who assisted me during this study. Professor Albert Eicker accepted the task of being my promoter in preparing for this degree. His generous time, advice, valuable criticism, and constant support at all stages of the preparation of this manuscript deserve special mention.

A tribute to the late Professor George Findlay for letting me share his special interest in sporotrichosis and for allowing me to use the clinical data of his patients. His constructive advice and assistance in interpreting the results throughout the larger part of this investigation, until shortly before he died, were invaluable.

Professor Joy Schulz who agreed to being associate promoter, and who assisted with the clinical interpretations, and also generously gave of her time to read and edit this manuscript in its entirety - therefore rendering the passages more readable than they would otherwise have been.

I thank Dr Peter Hull sincerely for the mycological consultations, the clinical and other patient data on the sporotrichosis treatment trials, which he entrusted to me. His collaboration and assistance in collecting soil and vegetation samples from the dwellings of this group of sporotrichosis patients, is greatly appreciated. Our discussions throughout the trials were invaluable.

My thanks are also extended to Prof W Jacyk, head of the Department of Dermatology, University of Pretoria, for putting at my disposal

original clinical colour slides from their unique collection as well as the clinical records of the respective patients. The clinical photographs used for this document comprise the patients in which the author confirmed the clinical diagnosis of sporotrichosis by mycological investigation, and that are housed in this department.

The following people are also sincerely thanked:

- The mining directors and medical officers of several gold mines, for their assistance in obtaining information regarding the mine cases.

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CURRICULUM VITAE

The author was born and grew up in the Transvaal, RSA. After she completed matric, employment at the Institute for Pathology, Department of Microbiology, University of Pretoria followed. In 1970 a student post was taken up at this Institute and the first two years of a BSc degree completed at the same University. Special interest in Medical Mycology developed during this time, after an introduction to Mrs V Davis (Medical Mycologist) and the late Professor George Findlay, dermatologist and director of the Photobiology Research Unit (Medical Research Council) at the time. She joined this Unit as part time research assistant in 1972, acquiring a BSc degree in the same year, majoring in Botany and Genetics.

After getting married and a two and a half year stay in Zimbabwe, where teaching and laboratory work filled the time, she returned to South Africa, acquiring a BSc Hons Status and an MSc (Cum Laude) in Botany (Mycology) at the University of Pretoria in 1978 and 1986 respectively.

Research was continued at the Photobiology Research Unit and later at the Research Institute for Environmental Diseases (RIED) when it was included under RIED when Professor George Findlay retired in 1988. He sadly passed away in November 1989. Currently the post as Senior Medical Researcher is held at RIED. Some twenty two scientific papers of mycological interest have been published over the years and several medical mycology papers have been delivered at national and international conferences and scientific meetings. Guest lectures have also been delivered throughtout South Africa. Professional memberships include the International Society of Human and Animal Mycology, The South African Society for Microbiology, The South African Association for Botanists, Allergy Society of South Africa. The author has been registered as a Medical Scientist with the South African Medical and Dental Council since 1978.

LIST OF SPECIES

FUNGAL SPECIES

From Barron (1972), De Hoog (1973, 1974) and Olchowecki & Ried (1974)

Sporothrix Hektoen & Perkins

Sporothrix schenckii Hektoen & Perkins (1900)

S. schenckii var. *luriei* Ajello & Kaplan, var. nov.

S. alba (Petch) de Hoog, comb. nov.

S. curviconia de Hoog, sp. nov.

S. cyanescens de Hoog & de Vries, comb. nov.

S. foliorum J. J. Taylor

S. fungorum de Hoog & de Vries

S. ghanensis de Hoog & Evans, sp. nov.

S. inflata de Hoog, sp. nov.

S. insectorum de Hoog & Evans, sp. nov.

S. isarioides (Petch) de Hoog, comb. nov.

S. luteoalba de Hoog, sp. nov.

S. ramosissima Aruad ex de Hoog, sp. nov.

S. setiphila (Deighton & Pirozynski) de Hoog, comb. nov.

Ceratocystis Ellis & Halsted emend.

Ceratocystis stenoceras (Robak) C. Moreau

C. minor (Hedgc.) Hunt

C. pilifera (Fries) C. Moreau

Ophiostoma H. & P. Syd.

Ophiostoma stenoceras (Robak) Melin & Nannf.

PLANT SPECIES

Botanical names as supplied by the National Institute for Timber Research (CSIR, Pretoria)

Indigenous woods

Acacia xantophloea Benth.

Albizia adanthifolia (Schumach.) W F Wight

Anthocleista grandiflora Gilg

Brachylaena discolor DC subsp.*discolor*

Breonadia microcephala (Del.) Ridsd.

Calodendrum capense L. F. Thunb.

Ekebergia capensis Sparrm.

Erythrina lysistemon Hutch.

Faurea saligna Harv.

Ficus capensis Thunb.

Gonioma kamassi E. Mey.

Ocotea bullata (Burch.)

Olea capensis L. subsp. *marocarpa* (C. H. Wr.) Verdoorn

Parinari curatellifolia Planch. ex Benth.

Podocarpus latifolius (Thunb.) R. Br. ex Mirb.

Rapanea melanophloeos (L.) Mez

Schotia brachypetala Sond.

Syzygium cordatum Hochst.

Trichilia emetica Vahl.

Widdringtonia nodiflora (L.) Powrie

Exotic woods

Acacia melanoxylon R.Br.

Cinnamomum camphora (L.) J. Presl.

Eucalyptus grandis Hill. ex Maid.

Eucalyptus sideroxylon A. Cunn. ex Woolls

Ginkgo biloba L.

Grevillea robusta A. Cunn.

Jacaranda mimosaefolia D. Don.

Khaya nyasica Stapf ex Bak. f.

Melia azedarach L.

Pinus elliottii Engelm.

Pinus pinaster Ait.

Pinus radiata D. Don.

Pinus roxburghii Sarg.

Platanus acerifolia (Ait.) Willd.

Populus alba L.

Populus deltoides Bart. ex Marsh

Sequoia sempervirens Endl.

LIST OF SYNONYMS

Sporotrhix schenckii Hektoen and Perkins 1900
= *Sporotrichum* sp. Smith 1898
= *Sporotrichum schencki* Matruchot 1910
= *Sporotrichum beurmanni* Matruchot et Ramond 1905
= *Sporotrichum asteroides* Splendore 1909
= *Sporotrichum equi* Carougeau 1909[1]
= *Sporotrichum jeanselmei* Brumpt et Langeron 1910[1]
= *Sporotrichum carougeaui* Langeron 1913[1]
= *Sporotrichum councilmani* Wobach 1917[1]
= *Sporotrichum grigsby* Dodge 1935
= *Sporotrichum fonsecai* Filho 1930[2]
= *Sporotrichum oculare*
= *Sporotrichum lipsiense* Benedek 1926[1]
= *Sporotrichum epigoeum* Castellani 1929[1]
= *Sporotrichum anglicum* (1937)[1]
= *Sporotrichum biparasiticus*
= *Sporotrichum tropicale* Panja, Dey & Ghosh 1947[1]
= *Rhinocladium beurmanni*
= *Rhinocladium schencki* Verdun et Mandoul 1924[2]
= *Rhinocladium schenckii* Ota 1928[2]

Ceratocystis stenoceras (Robak) C. Moreau[3]
= *Ophiostoma stenoceras* (Robak) Melin & Nannf.

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[1]Aram (1986)
[2]Rippon (1988)
[3]Hunt (1956)

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