A Survey and Study of the Aquatic Fungi at Ton Nga Chang Wildlife-Sanctuary, Songkhla Province

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ชื่อวิทยานิพนธ์ การสำรวจและศึกษาเชื้อราในแหล่งน้ำบริเวณเขตรักษาพันธุ์สัตว์ป่าโตนงาช้าง จังหวัดสงขลา

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บทคัดย่อ

การสำรวจราน้ำในเขตป่าร้อนชื้นทางภาคใต้ของประเทศไทย ได้ทำการศึกษาจากแหล่งน้ำในเขตรักษาพันธุ์สัตว์ป่าโตนงาช้าง จังหวัดสงขลา โดยมีวัตถุประสงค์เพื่อศึกษาความอยู่รอดของโคนิเดียราน้ำที่อยู่ในฟองธรรมชาติและภายใต้สภาวะในห้องปฏิบัติการ รวมทั้งศึกษาความหลากหลายทางชีวภาพของราน้ำในแหล่งน้ำธรรมชาติ จากการตรวจนับเชื้อที่มีชีวิตทั้งหมดในฟองโดยวิธี total viable plate count พบปริมาณเชื้อในฟองใหม่อยู่ในช่วง 2.3×10^3 ถึง 3.4×10^5 CFU/ml และปริมาณเชื้อในฟองเก่าอยู่ในช่วง 5×10^3 ถึง 3.2×10^5 CFU/ml ซึ่งไม่มีความแตกต่างอย่างมีนัยสำคัญ ($P>0.05$) ส่วนการตรวจนับเชื้อที่มีชีวิตโดยวิธีการย้อมสี 3 ชนิดได้แก่ tetrazolium bromide (MTT), acridine orange และ DAPI พบว่าภายหลังการย้อมด้วย acridine orange และ DAPI เปอร์เซนต์ของโคนิเดียที่อยู่รอดในฟองมีค่าสูงกว่าการย้อมด้วย MTT แต่ไม่มีความแตกต่างอย่างมีนัยสำคัญ ($P>0.05$) อย่างไรก็ตามการย้อมด้วย MTT ให้ความสะดวกในการศึกษานอกห้องปฏิบัติการและเห็นความแตกต่างระหว่างเซลล์ที่มีชีวิตและเซลล์ตายชัดเจนกว่า acridine orange และ DAPI โดยโคนิเดียที่มีชีวิตมีเซลล์อย่างน้อยหนึ่งเซลล์ที่มีการสะสมของ formazan ซึ่งมีสีม่วงแดง ส่วนเซลล์ตายไม่มีสี จากการใช้ MTT ย้อมตัวอย่างฟองพบว่า 44-77% ของโคนิเดียทั้งหมดในฟองใหม่มีชีวิตรอด ส่วนในฟองเก่าโคนิเดียอยู่รอดได้ 42-69%

การศึกษาความอยู่รอดของราน้ำ 4 สายพันธุ์ได้แก่ *Anguillospora* sp., *Helicomyces* sp., *Thozetella* sp. และ *Volutella* sp. ภายใต้สภาวะในห้องปฏิบัติการ พบว่าหลังจากปล่อยให้โคนิเดียแห้งเป็นเวลา 10 ชั่วโมง ราน้ำทั้ง 4 สายพันธุ์อยู่รอดได้ 3-45% ในขณะที่หลังจากให้อากาศเป็นเวลา 7 วัน ราน้ำทั้ง 4 สายพันธุ์อยู่รอดได้ 83-88% ดังนั้นการอยู่รอดภายใต้สภาวะให้อากาศในห้องปฏิบัติการอาจเป็นตัวทำนายความอยู่รอดของโคนิเดียราน้ำที่ถูกดักอยู่ในฟองในแหล่งน้ำธรรมชาติได้

การศึกษาความหลากหลายของราน้ำในฟอง สามารถจัดจำแนกถึงระดับสกุลได้ทั้งสิ้น 35 สกุล 48 ชนิด โดยส่วนใหญ่เป็นกลุ่ม hyphomycetes รองลงมาคือกลุ่ม coelomycetes พบ 3 สกุลและ basidiomycetes พบเพียง 1 สกุล ราน้ำชนิดที่พบมากและตรวจพบอย่างสม่ำเสมอทุกบริเวณที่เก็บตัวอย่างในระหว่างเดือนมิถุนายนถึงกันยายน 2541 ได้แก่ *Anguillospora* sp. และ *Triscelophorus* sp. เมื่อเปรียบเทียบกับชนิดของเชื้อที่อยู่ในน้ำบริเวณใกล้กับแหล่งเกิดฟองพบเชื้อส่วนใหญ่ได้แก่ *Anguillospora* sp. และราในกลุ่ม dematiaceous hyphomycetes ซึ่งส่วนใหญ่มีรูปร่างกลมรีและมีสีน้ำตาลเข้ม

ทำการแยกเชื้อบริสุทธิ์ได้ 65 ไอโซเลท โดยเชื้อส่วนใหญ่ร้อยละ 63.1 ไม่สร้างสปอร์บนอาหารเลี้ยงเชื้อ สามารถจำแนกเชื้อบริสุทธิ์ได้ 9 ชนิด จำนวน 15 สายพันธุ์ได้แก่ *Volutella* sp. 5 สายพันธุ์, *Pestalotia* sp. 3 สายพันธุ์ รวมทั้ง *Anguillospora* sp., *Beltrania rhombica*, *Helicomyces* sp., *Robillarda* sp., *Thozetella* sp., *Varicosporium* sp. และ *Wiesneriomyces* sp. อย่างละ 1สายพันธุ์

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Abstract

A survey of freshwater fungi was conducted in a stream at Ton Nga Chang Wildlife-Sanctuary, Songkhla Province. The objectives of the study were to estimate the viability of conidia trapped in foam and under laboratory conditions, to study the biodiversity of fungi in freshwater habitats.

The viability of conidia estimated by total viable plate count in fresh foam was 2.3 x 10^3 to 3.4 x 10^5 CFU/ml, and these values were 5 x 10^3 to 3.2 x 10^5 CFU/ml in old foam. These values in fresh and old foam were not significantly different at $P>0.05$. Three vital stains; tetrazolium bromide (MTT), acridine orange and DAPI, were used to estimate the conidial viability in foam samples. Acridine orange and DAPI gave higher percentages of viability than MTT staining, but were not significantly different ($P>0.05$). However, MTT staining is more practical than those two vital stains. The viable and non-viable cells can easily be distinguished. The conidia were considered to be viable when at least one cell formed formazan complex, whereas non-viable cells were colourless. The viability of conidia in foam estimated by MTT staining showed 44-77% viable in fresh foam, and 42-69% in old foam.

Four species; *Anguillospora* sp., *Helicomyces* sp., *Thozetella* sp. and *Volutella* sp. were tested for their viability under laboratory conditions. The conidial viability after drying for 10 hours was 3-45%. However, after aeration for seven days, the conidia were viable for 83-88%. This indicated that survival of laboratory-produced conidia under aerated conditions may be a predictor of fungal viability in natural foam.

Thirty-five genera, 48 species of fungi were identified in the foam samples. The dominant genera were hyphomycetes. Three genera of coelomycetes and only one genus of basidiomycetes were found. The predominant species in foam throughout period of study (June- September, 1998) were *Anguillospora* sp. and *Triscelophorus* sp. The species found in stream water samples were mostly *Anguillospora* sp. and dematiaceous hyphomycetes with round, ovoid and brown in colour.

Sixty-five pure cultures were isolated from foam samples, 63.1% of them did not sporulate on agar media. The following 9 genera, 15 isolates have been identified; five isolates of *Volutella* sp., three isolates of *Pestalotia* sp., only one isolate of *Anguillospora* sp., *Beltrania rhombica, Helicomyces* sp., *Robillarda* sp., *Thozetella* sp., *Varicosporium* sp. and *Wiesneriomyces* sp.

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List of Abbreviations

cm	=	centimetre
CFU/ ml	=	Colony Forming Unit per millilitre
°C	=	degree Celcius
DAPI	=	4′-6, diamidino-2-phenylindole
Fig.	=	Figure
g	=	gram
km	=	kilometre
l	=	litre
m	=	metre
µg	=	microgram
µm	=	micrometre
mg	=	milligram
ml	=	millilitre
mm	=	millimetre
nm	=	nanometre
pers. comm.	=	personal communication
unpubl. obs.	=	unpublished observation

Chapter 1

Introduction

1.1 Background problem to this research

Biodiversity, as widely used with animals, plants, microorganisms, and humans, has various meanings. Hawksworth (1991) suggested the level of biodiversity can be in terms of species diversity, population diversity and ecosystem diversity. Species diversity, a commonly used parameter, is an important ecological characteristic of variation in species of biotic communities (Miller, 1995; Ogawa *et al.*,1996).

Fungi are one group of microorganisms which play an important role in natural ecosystems. Examples of fungal functions are listed in Table 1. They have been estimated to be 1.5 million species on earth based on the ratio of numbers of vascular plants species to the numbers of described fungal species in the British Isles (ca. 1:6). It means only 5% (69,000 species) of total species have been identified on earth (Hawksworth, 1991).

Tropical forests are expected to have more species diversity of microorganisms than other regions of the world (Lodge *et al.*, 1996).

Table 1. Functions of fungi in natural ecosystems.

Physiological and metabolic
- Decomposition of organic matter: volatilization of C, H, and O - Elemental release and mineralization of N, P, K, S and other ions - Accumulation of toxic materials - Synthesis of humic materials
Ecological
- Facilitation of energy exchange between above ground and below ground systems - Promotion and alteration of niche development
Mediative and integrative
- Facilitation of transport of essential elements and water from soil to plant root - Regulation of water and ion movement through plants - Regulation of C allocation below ground - Participation in saprotrophic food chains - Production of environmental biochemicals (antibiotics, enzymes and immunosuppresants)

(After: Miller, 1995)

According to the functions of fungi in ecosystem, it can be concluded that fungi control many vital processes for the maintenance and survival of tropical forests (Hawksworth & Cowell, 1992).

Little is known about the biology, genetics, ecology, host range, biochemistry and distributions of fungi (Hawksworth *et al.*, 1995), including the study of aquatic hyphomycetes. Aquatic hyphomycetes is one group of mitosporic fungi, growing on leaf litter in streams, producing enormous numbers of conidia (Bärlocher, 1982). The typically branched and sigmoid shaped conidia can be trapped by air bubbles and accumulate in persistent foam below waterfalls or rapids (Iqbal & Webster, 1973a; Ingold, 1975). There are some taxonomic studies of aquatic hyphomycetes accumulating in foam in Thailand (Tubaki *et al.,* 1983; Hywel-Jones, unpubl. obs.). Most of the studies were carried out in the South and central part of Thailand. Moreover, the estimation of conidial viability in foam has been determined only in the temperate region (Sridhar & Bärlocher, 1994). Little information is known about the taxonomy, ecology and viability estimation of aquatic hyphomycetes in the South of Thailand.

Ton Nga Chang Wildlife-Sanctuary represents one of the tropical rain forests in Southern Thailand, which still remain in good condition, with less destruction and pollution. It contains a diversity of plants, animals and microorganisms, including fungi. The taxonomic study and viability

estimation of aquatic hyphomycetes were conducted in this study area in order to collect more information for a database of the fungal biodiversity of Southern Thailand.

1.2 Outline of the research

This study was conducted at Ton Nga Chang Wildlife-Sanctuary, Songkhla Province. The foam samples were collected from 4 sites in the same stream. The total number of viable conidia in fresh and old foam was determined by total viable plate counts. The viability of fungal conidia in two types of foam was estimated by using 3 different stains; tetrazolium bromide (MTT), acridine orange and 4′-6 diamidino-2-phenylindole (DAPI). The fungi in foam were examined, identified under a light microscope, and isolated into pure culture. The culture collections were kept at the Department of Microbiology, Prince of Songkla University, Songkhla and the Biotec Culture Collection, National Center for Genetic Engineering and Biotechnology, Bangkok.

1.3 Benefits of the research

The investigation of species diversity and the viability of fungal conidia can contribute to a database of the freshwater fungal biodiversity of Southern Thailand. The cultures isolated will be the base for further development such as the specimens for taxonomic study in the university, searching for new antibiotics, bioactive compound screening, and genetical studies in the future.

Chapter 2

Literature Review

2.1 General introduction to the fungi

Fungi are a diverse group of microorganisms and referred to the Kingdom Fungi (Hawksworth *et al.*, 1995). Fungi are eucaryote, lack chlorophyll, cell walls composed of polysaccharides, cellulose and/or chitin, mannan or glucan, and produce hyphae as the vegetative stage of their life cycle (Moore-Landecker, 1990; Hugo & Russell, 1992; Rees & Dickinson, 1992; Carlile & Watkinson, 1994).

For reproduction, fungi produce specialised structures and may have sexual (teleomorph state) or asexual (anamorph state) spores. In the higher fungi (ascomycetes, basidiomycetes) sexual spores are produced by special reproductive structures called ascomata and basidiomata, respectively. Mitosporic fungi (Deuteromycetes or Fungi Imperfecti), asexual spores are produced by various means on vegetative hyphae, sporodochia, acervuli, synnemata or pycnidia (Alexopoulos *et al.,* 1996).

Fungi are heterotrophic organisms that consume organic matter as an energy source. Some are saprotrophs, obtain their nutrients from dead organisms such as plants or animals. These groups play an important role in

the decomposition of organic matter in the natural ecosystem (Carlile & Watkinson, 1994; Alexopoulos *et al.*, 1996), because they are able to produce extracellular enzymes such as amylases, cellulase, chitinases, cutinase, hemicellulase, ligninase, pectinase, proteinase, ribonuclease and xylanase (Carlile & Watkinson, 1994; Dix & Webster, 1995).

Fungi are found in many different habitats such as soil, associated with plants, ruminant guts, associated with other fungi, associated with vertebrates and invertebrates, and in water. Hawksworth *et al.* (1996) listed the fungal habitats in tropical forests and this is reproduced in Table 2.

Additionally, fungi may be parasites of plants, insects, animals, and humans. Some fungi produce harmful compounds to animals and man such as mushroom toxins and aflatoxins. Fungal activities may also be beneficial to humans, for example in the production of bread, cheese, fermented milk, wine and beer, whisky, soy bean products and other asian and african fermented foods (Moore-Landecker, 1990; Rees & Dickinson, 1992; Carlile & Watkinson, 1994). Fungal products used commercially include organic acids, alcohols, antibiotics, pigments, vitamins and variety of enzymes. Some fungal metabolites are used in the processing of foods to change their flavor and composition. Examples of important commercial fungal metabolites are listed in Table 3.

Table 2. Fungal habitats in tropical forests.

Living vascular plant-associated fungi
- Biotrophs and necrotrophs of leaves, stems, fruits, seeds, etc. - Endophytes of leaves, stem, bark and roots - Secondary colonizers of dead attached tissue - Arbuscular mycorrhizal and ectomycorrhizal species
Fungi associated with plant exudates
- Fungi on leaves and fruit surfaces - Yeasts and other fungi associated with nectar, resin etc.
Dead plant-associated fungi
- Saprobes of wood, bark and litter - Fungi on soil surfaces - Fungi isolated from soil core - Fungi associated with burnt plant tissue - Fungi of submerged and inundated vegetation - Chytrids associated with pollen in water samples
Fungi associated with non-vascular plants
- Lichenized fungi on leaves, bark, rock and soil surfaces - Algal parasites on leaves and trunks, in water etc. - Fungi associated with bryophytes - Fungi associated with aquatic algae

Table 2. (continued)

Fungi associated with other fungi and fungal analogues
- Biotrophs, necrotrophs and saprobes of other fungi - Lichenicolous species - Myxomyceticolous species
Fungi associated with vertebrates
- Mammal and bird skins, feathers, hair, bone etc. - Fungi from dung, pellets etc. - Fungi associated with nests, lairs etc. - Ruminant guts - Fungi on fish scales, in fish guts etc.
Fungi associated with invertebrates
- Biotrophs and necrotrophs of insects - Fungi from invertebrate guts (arthropods, annelids etc.) - Fungi associated with insect nests (bees, termites etc.) - Fungi on arthropod scales - Nematode-trapping fungi, fungi associated with rotifers etc.
Fungi in water
- Water and foam isolations from streams, permanent and temporary ponds etc. - Water retained in plants

(After: Hyde & Hawksworth, 1997)

Table 3. Examples of important commercial fungal metabolites and enzymes.

Metabolites	Produced by	Use as
Organic acids		
Citric acid	*Aspergillus* spp. *Penicillium* spp.	Flavoring ingredients
Gluconic acid	*A. niger*	Manufacture of tooth paste
Itaconic acid	*A. terreus*	Manufacture of plastics
Alcohol		
Ethyl alcohol	Yeasts	Commercially important chemicals
Glycerol	*Saccharomyces cerevisiae*	Explosives
Fats	*Penicillium* spp. Yeasts	Soaps, manufacture of foods
Antibiotics		
Griseofulvin	*P. griseofulvum*	Oral and topical antibiotics
Penicillin	*P. chrysogenum*	Oral and parental antibiotics
Cephalosporins	*Cephalosporium acremonium*	Oral antibiotics

Table 3. (continued)

Metabolites	Produced by	Use as
Vitamins, Pigments and Growth factors		
Vitamin B	Yeasts	Nutritional supplement and medical therapy
Riboflavin	*Ashbya gossypii, Eremothecium ashbyii*	Nutritional supplement and medical therapy
Gibberellin	*Fusarium moniliformis*	Growth-promoting substances
Enzymes		
Amylases	*Aspergillus* spp.	Starch removal agent in food preparation
Invertase	*S. cerevisiae*	Production of artificial honey and invert sugar
Pectic enzymes	*Penicillium* spp.	Removal of pectins before concentrate juice
Protease	*Aspergillus* spp., *P. roqueforti, Agaricus brunnescens*	Food processing

(After: Ainsworth & Sussman, 1965; Moore-Landecker, 1990; Rees & Dickinson, 1992; Carlile & Watkinson, 1994)

Additionally, because of their unique characteristics, fungi offer many advantages as model systems in research. They can be grown easily in simple culture media, do not require much space and have high growth rates. Most of their life cycle is haploid, they have both sexual and asexual reproduction, and mutants can readily produced. Some are heterokaryotic or have a parasexual life cycle, which are convenient for studying the relationship between different nuclei and in gene mapping. Fungi can be widely used for biological research in biochemistry, molecular biology, genetic sequencing and recombinant DNA technology (Moore-Landecker, 1990; Bridge *et al.*, 1998).

Many different groups of fungi occur in freshwater habitats, including the lower fungi: Mastigomycotina, Zygomycota and the higher fungi: Ascomycota, Mitosporic fungi and some Basidiomycota (Dix & Webster, 1995).

Freshwater environments can be divided into:

1. Lotic habitats comprise running or flowing water such as streams, rivers, creeks and brooks (Frederick & Sparrow, 1965).

2. Lentic habitats comprise aquatic environments which have stagnant or lack continuous flowing water, such as lakes, temporary ponds, man-made pools, swamps and dams (Frederick & Sparrow, 1965; Wong *et al.*, 1998).

Goh & Hyde (1996) reviewed and divided the fungi in freshwater habitats into 3 main groups: (i) Chytrids and oomycetes (ii) Aquatic ascomycetes on wood, and (iii) Freshwater hyphomycetes occurring on decaying leaves.

(i) Lower fungi: Chytrids and oomycetes

Chytrids and oomycetes are lower fungi that are very common and widespread in freshwater environments and play an important role in the freshwater ecosystem. Some are biotrophic parasites of algae and aquatic macrophytes, or cause diseases of aquatic animals. These fungi generally lack the capability of degrading cellulose, but are able to degrade insect skeletons, keratin and pollen grains (Laidlaw, 1985; Bruning, 1991; Powell, 1993).

(ii) Aquatic ascomycetes on wood

Freshwater ascomycetes can occur as parasites of aquatic plants or as saprotrophs of decaying plant parts and submerged wood (Shearer, 1993). Shearer (1992) suggested that wood can be a better substratum for freshwater ascomycetes than the other parts of the plant because of its persistence and nutritive value.

(iii) Freshwater hyphomycetes on decaying leaves

Freshwater hyphomycetes (often referred to as Ingoldian fungi) are anamorphs or asexual states of ascomycetes and basidiomycetes. Their whole life cycle, dispersal, vegetative growth and sporulation, occur under water or in predominantly aquatic or semi-aquatic habitats (Thomas, 1996; Marvanová, 1997).

2.2. The specialised features of Ingoldian fungi

The special feature of this group of fungi is their conidial shape. They produce large conidia 50-100 μm or more in size which are thin walled and hyaline (Ingold, 1975). Conidial morphology is distinctive with branched conidia, often with four arms (tetraradiate), they may also be helicoid or sigmoid in shape (Fig. 1).

Conidial morphology is believed to be an adaptation for survival in aquatic environments (Ingold, 1966; 1975). Ingold (1966; 1975) also noted that branched conidia may be the result of convergent evolution. Many Ingoldian fungi which have the tetraradiate form have the advantage for increasing the probability of contact with the substratum in water, especially in fast-flowing streams (Ingold, 1966; Read *et al.,* 1992a).

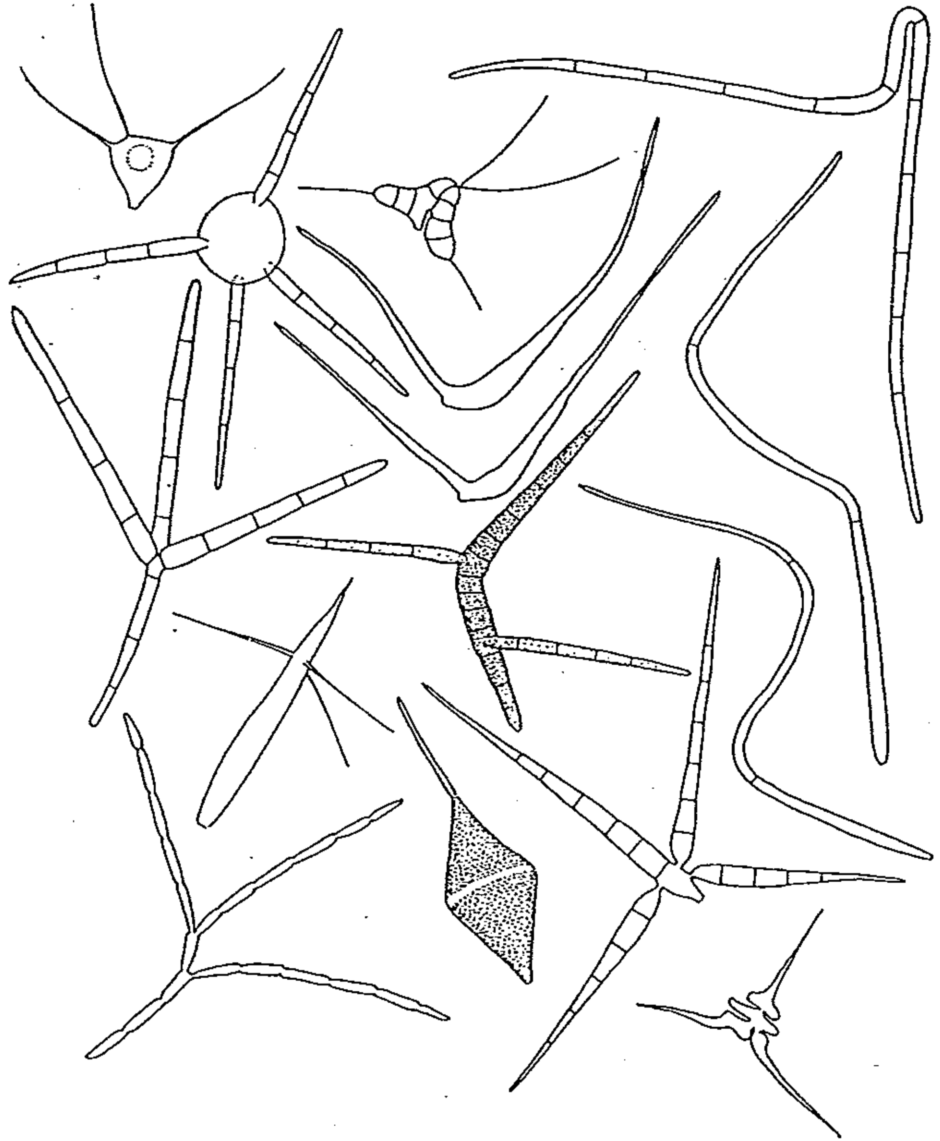

Fig. 1. Conidial morphology of some Ingoldian fungi.

(After: Goh, 1997)

Ingold (1966, 1973) and Bandoni (1975) proposed that the tetraradiate conidial form was advantageous to the fungi: the tetraradiate conidia are denser than water and settle slower than spherical or oval conidia with the same volume. Therefore, it allows more time and facilitates their liberation and dispersal by water movement, before sinking into the bottom of the streams. The other advantage is that since the tetraradiate conidia have four long arms, which are in more than one plane, thus, when they settle, they act as a tripod which have three points of contact with the surface of the substratum in the flowing water.

Examples of genera with branched or tetraradiate conidia are: *Alatospora, Actinospora, Articulospora, Campylospora, Clavariopsis, Jaculispora, Lemonniera, Tetrachaetum, Tetracladium, Tricladium* and *Triscelophorus* (Ingold, 1975; Webster & Descals, 1981; Marvanová, 1997). Other Ingoldian fungi are sigmoid or helicoid in shape, e.g. *Anguillospora, Angulospora, Filosporella, Flagellospora, Helicomyces, Helicosporium,* and *Lunulospora* (Ingold, 1975; Webster & Descals, 1981; Marvanová, 1997). The possible significance of the sigmoid form seems to be to enable attachment with substrata at two points of contact with the substratum. However, because of these attachment points, the tetraradiate conidia represent a more stable adhesion than some sigmoid or ovoid shaped spores (Ingold, 1966; Bandoni, 1975; Read, 1990).

Generally, Ingoldian fungi quickly germinate, within 2-6 hours (Ingold, 1975; Webster & Descals, 1979). The strength of adhesion resulting from the formation of appressoria and mucilage production in their attachment to substrata is important in an aquatic habitat (Webster, 1959; Ingold, 1975; Read *et al.*, 1992b, c). High germination rates of aquatic hyphomycete conidia confer a competitive advantage compared with other groups of fungi such as terrestrial fungi and other aquatic organisms (Read *et al.*, 1992a).

While the conidia of most aquatic hyphomycetes rapidly germinate, some species germinate slowly: *Anguillospora crassa, Heliscus lugdunensis*, and *Tumularia aquatica*. It is noted that the conidia of these species have large reserves of glycogen (Webster & Descals, 1979). These reserves enable the conidia to remain viable for longer periods before utilizing extracellular nutrients (Read *et al.*, 1992a).

Read *et al.* (1992b, c) and Au *et al.* (1996) studied the germination and development of the attachment structures of some aquatic hyphomycetes by using light microscopy and electron microscopy. They noted that:

(i) Most of the conidia of aquatic hyphomycetes germinated within 2 hours after contact with the substratum.

(ii) Mucilage secretion occurred around the conidia, germ tubes and appressoria, while the strength of attachment increased with settlement time.

Adhesive mucilage comprised polysaccharides with neutral or carboxyl functional groups (Read *et al.,* 1992a,b). However, the mucilage of the sigmoid conidia of *Mycocentrospora filiformis*, did not appear to stain with any stains (Read *et al.*, 1992b). In tetraradiate conidia, mucilage was produced at the poles of ungerminated arms before settlement, while for sigmoid conidia, the mucilage was secreted at the curvature points of contact with substrata.

(iii) Appressorial formation is an adaptation to increase fungal adhesion and may be dependent on the substrata. Appressoria are formed by many tetraradiate and branched conidia at the conidial arm poles immediately after germination. Read *et al.*(1992b) indicated that sigmoid conidia of *M. filiformis* produced few appressoria compared with conidia of *Lemonniera aquatica*. However, the appressorium formation results from chemical, nutrient or physical stimulation.

Read *et al.* (1992b) also suggested other roles for the appressoria in aquatic hyphomycetes:

1. To aid fungal adhesion with the substratum by increasing the surface area of contact and secretion of mucilage.
2. Appressoria may act as assimilation organs, and possess high metabolic activity, and,

3. To produce an infection structure (penetration pegs) to aid penetration into the host.

This indicates that aquatic hyphomycetes are a group of fungi that are successful in the colonization of submerged substrata in freshwater environments, especially in fast-flowing streams.

2.3. Habitats and substrata of Ingoldian fungi

2.3 A: Foam

Lotic habitats comprise rapidly-flowing streams or rivers. Foam is the result of accumulated air bubbles, formed on the surface below rapids or waterfalls and particularly after heavy rainfall (Ingold, 1975). Two types of foam are distinguished:

- Fresh foam: colour and consistency similar to whipped egg white and
- Old foam: yellow-brown, and partly dried up (Sridhar & Bärlocher, 1994).

The origin of foam is not known, but it is thought to result from the initial decay of leaf leachates (Ingold, 1975). Ingold (1975) has reported that foam not only forms after heavy rain or under fast-flowing waterfalls, but can occur above a little barrier of twigs and leaves in streams.

Numerous species of Ingoldian fungi have been reported in both fresh and old foam, with conidia easily trapped by air bubbles in the foam

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(Shearer, 1993; Thomas, 1996; Hyde *et al.,* 1997). Therefore foam acts as a trap or reservoir for fungal spores.

Ingoldian fungi produce variously shaped conidia: sigmoid, branched, tetraradiate, or may be helicoid. Tetraradiate and branched conidia are trapped more efficiently than the regular shaped spores, such as, sigmoid or ovoid conidia (Iqbal & Webster, 1973a). The reason why the tetraradiate or branched conidia can be trapped in foam more easily than other types is that they are denser than water. Therefore they have more chance to be brought to the surface of water by air bubbles, they are caught up in the foam and do not sink into the water. The other reason is that because surface tension at the air-water interface may cause the conidia to be caught up by the air bubbles more efficiently (Iqbal & Webster, 1973a).

Iqbal & Webster (1973a) investigated how tetraradiate and sigmoid conidia accumulated at the air bubbles surface in foam. Tetraradiate conidia (e.g. *Articulospora tetracladia*) were arranged so that three arms were tangential to the bubble surface, and one arm pointing outwards. Sigmoid conidia (e.g. *Anguillospora crassa*) adapted their curvature to the air bubble surface so that the body of the spore lies tangential to the bubble surface. Whenever the air bubbles are increased into a larger surface, the conidia are removed and easily ejected upwards, then the conidia are accumulated again into the streams.

Iqbal & Webster (1973a) studied the number and percentage of conidia of various types of aquatic hyphomycetes in foam and compared them with the conidia in river water. They found that the percentages of tetraradiate conidia or multi-branched conidia found in foam were higher than those in river water. For example, 17.6% of *Articulospora tetracladia* conidia occurred in a foam samples compared with only 0.9% in river water. *Tetracladium marchalianum*, with branched conidia, 50% were found in foam compared with only 3.9% in river water. For the sigmoid conidia of *Lunulospora curvula*, 5.9% occurred in foam samples, while there were 61.8% in river water.

Although Ingoldian fungal conidia are trapped in foam or in water, germination does not occur. Whenever the conidia make contact with any solid substratum, germination will occur within an hour (Ingold, 1975). The strength of attachment will increase as the result of mucilage secretion and appressorium formation (Ingold, 1975; Webster & Descals, 1979).

Descals *et al.* (1995) listed the advantages of studying foam:

1. Identification of unknown species, and estimation of their component in stream ecology,
2. They can be applied for the biotechnology or pharmacology,
3. Check whether *in vitro* sporulation conditions are adequate,

4. Detect the following natural phenomena such as;

    - species interaction (such as mycoparasitism of hyphomycetes on the conidia of some Ingoldian fungi)
    - microcycle conidiation (production of phialides directly on conidia of macroconidia)
    - synanamorph (microconidial developing on macroconidia)
    - repetition (production of daughter conidia of the same morph)
    - conidial lysis or disarticulation (release of part-conidia)
    - adaptation for anchorage (apical mucilage production)
    - characteristic modes of germination (production of appressoria) and

5. Preserved foam collections in accessible herbaria may prove invaluable, in future taxonomic or ecological study, especially with molecular identification techniques.

There were many reports of the common aquatic hyphomycete genera found in foam samples and these are listed in Table 4.

Several researchers in temperate and tropical regions have studied the ecology of fungal conidia trapped in foam. The conidial shape, concentration, distribution, new species, longitudinal changes in conidial diversity in the rivers have been studied (Ingold, 1974; Descals, Nawawi & Webster, 1976; Marvanová & Muller-Haeckel, 1980; Descals & Webster, 1982; Nawawi & Webster, 1982; Iqbal, 1995).

Table 4. Common genera of aquatic hyphomycetes found in foam samples with various spore shapes.

I. Genera with unbranched, filiform or sigmoid conidia
Anguillospora *Angulospora* *Calcarispora* *Condylospora* *Flagellospora* *Lunulospora* *Mycocentrospora*
II. Genera with helicoid conidia
Helicomyces *Helicosporium*
III. Genera with branched, tetraradiate conidia
Actinospora *Alatospora* *Articulospora* *Brachiosphaera* *Campylospora* *Clavariopsis* *Dendrospora* *Diplocladiella* *Flabellospora* *Isthmotricladia*

Table 4. (continued)

III. Genera with branched, tetraradiate conidia
Laridospora *Lemonniera* *Tetracladium* *Tricladium* *Triscelophorus* *Varicosporium*

(After: Ingold, 1975; Marvanová, 1997)

In 1942, Ingold originally described aquatic hyphomycetes which had many different shaped conidia from submerged alder leaves in fast-flowing streams. He reported that this special group of fungi were thin-walled, had septate mycelium, and most of their life cycle occurred in water. The vegetative growth, conidiophores and conidial production, liberation and local dispersal occurred below water (Ingold, 1966; Bandoni, 1975).

Subsequently several other works have studied aquatic hyphomycetes in foam samples and some of these are summarized in Table 5.

Table 5. The selected examples of aquatic hyphomycetes observed from river foam.

Author/ date of studies	Location	Total species reported	Most common species	Parameters affecting spores density	Other features of interest
Ingold, 1968	Britain	10	*Culicidospora aquatica*, *C. gravida*, *Dendrospora* sp., and *Tricladium eccentricum*		Many species were unknown and new records to Britain and Europe
Alasoadura, 1968	Nigeria	9	*Actinospora megalospora*, *Anguillospora longissima*, and *Campylospora chaetocladia*		Found new specie *Pyramidospora densa*
Ingold, 1974	North-western Scotland	17	*Campylospora parvula*, *Dactylella appendiculata*, *Gyoerffyella* spp., *Tetracladium* spp., and *Tricladium* sp.		Many species were undescribed

Table 5. (continued)

Author/ date of studies	Location	Total species reported	Most common species	Parameters affecting spores density	Other features of interest
Marvanová & Muller-Haeckel, 1980	Njakajokk stream, Sweden	41	*Alatospora acuminata, Anguillospora longissima, Articulospora tetracladia,* and *Lemoniera aquatica*	Seasonal variation	Twenty species were known, 21 were unknown species, these can be expected to be involved in angiosperm leaf degradation
Tubaki, *et al.*, 1983	Northern and western Thailand	20	*Campylospora chaetocladia, Campylospora* sp., *Clavariopsis aquatica, Scorpiosporium* sp., and *Triscelophorus* sp.	Rainfall	
Sridhar & Kaveriappa, 1984	Konaje stream, India	19	*Lunulospora curvula, Triscelophorus monosporus, Triscelophorus* sp., and *Flagellospora penincillioides*	Rainfall and leaf decomposition	*L. curvula, T. monosporus,* and *Triscelophorus* sp. were found throughout the year

Table 5. (continued)

Author/ date of studies	Location	Total species reported	Most common species	Parameters affecting spores density	Other features of interest
Swart, 1986	Victoria, Australia	28	*Tricladium* spp. and *Triscelophorus* spp.		This report was a preliminary survey of aquatic hyphomycete genera in foam and leaf samples
Hywel-Jones, 1990; 1991 (unpubl. obs.)	Central part of Thailand	11	*Brachiosphaera tropicalis*, *Clavariopsis tentaculata*, *Erynia conica*, *E. rhizospora*, *Flagellospora pennicillioides*, *Isthmotricladia laevis*, *Lunulospora curvula*, *L. cymbiformis*, *Phalangispora constricta*, *P. nawawi* and *Triscelophorus acuminatus*		For isolation and bioactive compound screening

Table 5. (continued)

Author/ date of studies	Location	Total species reported	Most common species	Parameters affecting spores density	Other features of interest
Chauvet, 1991	South-western France	54	1. *Heliscus tentaculus, Campylospora chaetocladia, Lunulospora curvula,* and *Triscelophorus monosporus* were common in high pH waters and at high temperature. 2. *Tetrachaetum elegans* and *Clavatospora longibrachiata* were abundant in acidic water	Water temperature, pH, altitude, seasonal variation, quality of foam (varies in time and space), efficiency of foam in trapping spores varies with fungal species	The species assemblage is determined by substrata quality varying with riparian vegetation.
Czeczuga & Oreowska, 1993	River Supras'l, Poland	56	*Anguillospora longissima, Lemoniera aquatica, Heliscus lugdunensis,* and *Tetracladium marchalianum*	Seasonal variation, concentration of various chemical components in water	More hyphomycete species found in foam than in river water
Sridhar & Kaveriappa, 1993	Western Ghats, India	9			The conidial shape correlated with those found in Thailand, Malaysia and South Africa.

Table 5. (continued)

Author/ date of studies	Location	Total species reported	Most common species	Parameters affecting spores density	Other features of interest
Webster *et al.*, 1994	Transvaal and Natal, South Africa	More than 100			Twenty six species were new to South Africa, some of them should be confirmed in culture.
Descals *et al.*, 1995	Gredos and Be'jar mountians, Central Spain	66	*Alatospora acuminata* and *Tetracladium setigerum*	Efficiency of foam in trapping spores varies with fungal morphology, foam age, seasonal variation, water chemistry	For poorly known reasons, foam formation can be strongly seasonal in temperate climates, often being frequent in wetter months, but rare in summer.
Tan & Koh, 1995	A lowland tropical forest , Singapore	18	*Beltrania rhombica*, *Triscelophorus acuminatus*, *Trichocladium cylindroclavatum*, *Ingoldiella hamata*, and *Trichocladium singaporense*	With heavy rainfall, the stream flowing, and with thick foam formation.	The abundance of *B rhombica* indicated that it can be adapted to an aquatic form of life, and constituted a dominant resident species in the stream.

Table 5. (continued)

Author/ date of studies	Location	Total species reported	Most common species	Parameters affecting spores density	Other features of interest
Raviraja *et al.*, 1998	Western Ghats region, India	47	*Lunulospora curvula*, *L. cymbiformis*, *Flabellospora verticillata*, and *Triscelophorus acuminatus*	Water chemistry	
Rajashekhar & Kaveriappa, 1998	River Cauvery at Srirangapatna, India	33	*Lunulospora curvula* and *Triscelophorus monosporus*	Rainfall	The range of pH and dissolved oxygen may affect number of species between the two collecting sites

Additionally, many investigations of new species in foam have been carried out in Malaysia. Examples of new species of aquatic hyphomycetes from Malaysia include: *Campylospora filicladia, Flabellocladia gigantea, Flabellocladia tetracladia, Flabellospora multiradiata, Flabellospora verticillata, Isthmotricladia laeensis, I. gombakiensis, Laridospora appendiculata, Tricladium aciculum, Tricladium fuscum, Triscelophorus acuminatus, Varicosporium helicosporum* and *V. macrosporum* (Nawawi, 1974a; b; 1975a; b; 1976a; b; 1985a; b).

2.3 B Spores in river water

Table 6 lists selected examples of fungi found in water samples from streams and rivers.

Table 6. Selected observations of aquatic hyphomycetes in river water.

Author / Date of studies	Location	Most common species reported	Other features of interest
Iqbal & Webster, 1973b	River Exe and its tributaries, Britain	*Clavariopsis aquatica* and *Flagellospora curvula*	The spore concentration was higher during August-September than May-July. The peak period of spore concentration depends on substratum preference and availability, and the physiology of growth.
Sridhar & Kaveriappa, 1984	Konaje stream, Mangalore, India	*Lunulospora curvula, Triscelophorus monosporus* and *Triscelophorus* sp.	Rainfall and leaf decomposition may affect spore density.
Thomas *et al.*, 1991	Lees creek, Australia	*Clavariopsis aquatica* and *Lunulospora cymbiformis*	The variation in spore density with time and longitudinal change was studied. It is possible that spore production of some species of aquatic hyphomycetes may be enhanced by light.
Rajashekhar & Kaveriappa, 1996	Western Ghats, India	*Triscelophorus monosporus*	Water was collected from a sulphur spring

Table 6. (continued)

Author / Date of studies	Location	Most common species reported	Other features of interest
Rajashekhar & Kaveriappa, 1996	Western Ghats, India	*Triscelophorus monosporus*	Water was collected from a sulphur spring
Fabre, 1998a; b; c	South-western, France	*Alatospora acuminata* and *Clavariopsis aquatica*	Has studied the same river as Chauvet's (1991) for many years. He noted that: 1. The conidial concentration of fungi found in the river water was higher during the autumn than in summer and winter. 2. Species richness was maximal in the autumn and winter. 3. Three groups of aquatic hyphomycetes were recognized and related to the seasonal pattern in conidial concentration: A. Species appeared with a consistent seasonal pattern of conidial concentration. They peaked simultaneously at all three rivers. For examples, *Alatospora acuminata* and *Lemonniera aquatica* peaked highest in late autumn, *Articulospora tetracladia* in winter, *Heliscella stellata* in

Table 6. (continued)

Author / Date of studies	Location	Most common species reported	Other features of interest
			summer and *Lunulospora curvula* in early autumn. B. Species appeared with a different seasonal pattern of conidial concentration. For example, *Pyricularia submersa* peaked in early autumn in the Adour river, and 71 and 92% occurred in spring in the Nive and Tech rivers, respectively. C. Species appeared with no seasonal pattern of conidia concentration, for examples, *Clavariopsis aquatica,* and *Clavatospora longibrachiata* and were more or less amount throughout the year.
Gönczöl *et al*., 1999; Gönczöl & Révay, 1999	Morgó stream, Hungary	*Alatospora acuminata* , *Flagellospora curvula* and *Heliscus lugdunensis*	Sixty-nine species were identified, mainly aquatic hyphomycetes.The factors affecting the variation of conidial concentration were the spatial heterogeneity of environmental factors and seasonal changes during the period of study.

2.3 C. Spores on leaves

Freshwater hyphomycetes are commonly collected on leaves. Many leaf species have been examined for freshwater hyphomycetes and the most common are listed in Table 7.

Since the pioneer studies of Ingold on aquatic hyphomycetes associated with alder leaves, many others have sampled a variety of leaves for freshwater fungi. Bärlocher (1982) studied four streams in the Black forest and Swiss Jura, when oak, larch leaves and spruce needles were placed in litter bags and exposed in the streams. Periodically, conidium production was determined. The highest spore production was on oak leaves, followed by larch and spruce needles. *Heliscus lugdunensis* was the dominant species and the initial colonizer of spruce needles, but was later replaced by *Alatospora acuminata.* He also studied the fungal colonization of dried and fresh leaves in the river Teign, England (Bärlocher, 1991). Alder leaves (*Alnus glutinosa*) were used in determining the weight loss during colonization by aquatic hyphomycetes. Dried leaves lost weight more quickly than fresh leaves during the first few weeks. Colonization of fresh leaves was delayed because leaching had not occurred prior to exposure. In this study, *Anguillospora filiformis* was the dominant species found in the alder leaves.

Table 7. The most common leaf species which are colonized by freshwater hyphomycetes.

Common name	**Scientific name**
Alder	*Alnus glutinosa*
Aquatic macrophytes in salt marshes and estuaries with brackish water	*Spartina alterniflora, Juncus* sp., *Phragmites australis, Typha latifolia*
Ash	*Fraxinus pennsylvanica*
Aspen	*Populus tremuloides*
Banyan	*Ficus bengalensis*
Beech	*Fagus sylvatica*
Birch	*Betula* sp.
Cashew	*Anacardium occidentale*
Coffee	*Coffea arabica*
Conifer	*Pinus roxburghii*
Elm	*Ulmus americana*
Eucalypt	*Eucalyptus globulus*
Hornbeam	*Carpinus betulus*
Jack fruit tree	*Artocarpus heterophyllus*
Larch	*Larix decidua*
Mango	*Mangifera indica*
Maple	*Acer saccharum*
Mat grass	*Nardus stricta*
Oak	*Quercus velutina*
Oak	*Q. serrata*
White oak	*Q. alba*
Pignut hickory	*Carya glabra*

Table 7. (continued)

Common name	Scientific name
Rubber	*Hevea brasiliensis*
Sycamore	*Acer pseudoplatanus*
Sycamore	*Platanus occidentalis*
Tapioca	*Manihot utilissima*
Teak	*Tectona grandis*
Yellow poplar	*Liriodendron tulipifera*

(After: Ingold, 1966; Bärlocher & Oertli, 1978a, b; Bärlocher, 1982; Shearer & Lane, 1983; Sridhar & Kaveriappa, 1989; Bärlocher, 1991; Polishook *et al.,* 1996; Abdel-raheem, 1997; Maamri *et al.,* 1998; Raviraja *et al.,* 1998a, b; Canhoto & Graca, 1999)

Sridhar & Kaveriappa (1989) examined the colonization of aquatic hyphomycetes in a Konaje streams, India. Sterilized leaves of banyan (*Ficus bengalensis*), cashew (*Anacardium occidentale*), coffee (*Cofea arabica*), mango (*Mangifera indica*) and rubber (*Hevea brasiliensis*) were submerged in the stream. Sixteen species from 13 genera of hyphomycetes colonized the leaves. All colonized the banyan leaves. More species were found during the monsoon and after the monsoon season. *Ingoldiella hamata, Lunulospora curvula, Triscelophorus acuminatus, T. monosporus* and *T. konajensis* were found on all leaf species. Colonization of leaf litter by these aquatic hyphomycetes may be controlled by many factors, such as, growth promoting or inhibiting substances, preference, competition, differences in growth rate and time required for sporulation (Willoughby & Archer, 1973; Suberkropp & Klug, 1976).

Maamri *et al.* (1998) studied the aquatic hyphomycetes and terrestrial fungi developing on decaying leaves in a temporary Moroccan river. Aquatic hyphomycetes processed the leaf litter rapidly when water flowed in the spring season. *Anguillospora longissima* was the dominant species in this survey.

Although most types of leaves can be easily decomposed by aquatic hyphomycetes, some need more time for colonization to occur (Canhoto & Graca, 1999). The colonization of eucalypt (*Eucalyptus globulus*) leaves by aquatic hyphomycetes was delayed because of the chemical components in the leaves. Waxy leaf cuticles, polyphenolic compounds, tannins, and eucalypt oil may inhibit or delay the colonization by fungi (Bärlocher *et al.*, 1995; Raviraja *et al.*, 1998).

Canhoto & Graca (1999) reported that eucalypt oil or tannic acid completely inhibited or depressed the growth of some aquatic hyphomycetes. However, the removal of tannins or phenolic compound by solvents accelerated fungal colonization (Bärlocher *et al.*, 1995). Bärlocher & Oertli (1978a; b) showed that powdered conifer (*Pinus leucodermis* and *Sequoia gigantea*) depressed the growth of some aquatic hyphomycetes. They indicated that phenolic compounds are the plant's chemical defense against herbivores and pathogenic organisms.

2.4 Wood

Woody debris serves as a habitat, reservoir, nutrition source, and site of sexual and asexual reproduction (Shearer, 1992). The principal components of wood are lignocellulose, celluloses, starch, hemicellulose, lignin, but a low nitrogen content (Wong *et al.*, 1998). Many workers have reported that aquatic hyphomycetes have the capability to degrade submerged wood as shown by the examples listed in Table 8.

Besides their ability in degrading cellulose, starch and hemicellulose, they can also utilize lignin (Dix & Webster, 1995), which is an insoluble complex aromatic polymer. Soft and hard woody plant tissue comprise 30% and 20% of lignin, providing rigidity and resistance to biological attack. It is found in a complex with cellulose and hemicellulose (Reid, 1995). The lignin-degrading fungi are able to utilize lignin by a variety of extracellular enzymes such as lignin peroxidase, manganese peroxidase, laccase, and cellobiose: quinone oxidoreductase (Reid, 1995). The assimilation of lignin by fungi can occur by three mechanisms:

i. Depolymerization by cleavage of bonds within the polymer,

ii. Removal and modification of side-chains with substitution on the benzene ring, and,

Table 8. Fungi reported colonizing submerged wood.

Anguillospora longissima
A. crassa
A. gigantea
Actinospora megalospora
Alatospora acuminata
A. flagellata
Brachiosphaera jamaicensis
Campylospora chaetocladia
Casaresia sphagnorum
Clavariopsis aquatica
Clavatospora longibrachiata
Condylospora gigantea
Dendrospora erecta
Dimorphospora foliicola
Diplocladiella foliicola
Filosporella annelidica
Flagellospora curvula
Heliscus lugdunensis
Isthmotricladia britannica
Lemonniera aquatica
Lunulospora curvula
Mycocentrospora acerina
Mycocentrospora angulata
Tetracladium marchalianum
Tricladium splendens
T. giganteum
T. gracile
Tumularia aquatica
Varicosporium elodeae
Yinmingella mitriformis

(After: Willoughby & Archer, 1973; Sander & Anderson, 1979; Jones, 1981; Re′vay & Gönczöl, 1990; Shearer & Webster, 1991; Shearer, 1992; Goh *et al.*, 1998; Wong *et al.*, 1998)

iii. Fission by ring-splitting enzymes to convert aromatic nuclei into respirable aliphatic compounds (Dix & Webster, 1995).

Aquatic hyphomycetes, which are able to grow on submerged decaying wood branches of plants, can still survive when substrata are no longer submerged in water. This group is called lignicolous aquatic hyphomycetes (Shearer, 1992). They have been reported to have the ability to degrade lignin and other substrates, such as, cellulose, pectin, and starch (Thornton, 1963; Fisher & Likens, 1972; Charmier & Dixon, 1982).

Several experiments monitoring the production of lignin degrading enzymes have been undertaken and a few examples will be reviewed here.

Zare-Maivan & Shearer (1988) tested the ability of freshwater hyphomycetes to produce extracellular enzymes, such as, amylases, xylanase, cellulases and polyphenol oxidase. Laccase activity was also detected in the cultures of some freshwater hyphomycetes. The presence of laccase was detected by the appearance of a red to purple colour zone around a fungal colony after adding a solution of 0.1 % syringaldazine in 95 % ethanol. They reported that *Heliscus lugdunensis, Pyramidospora* sp., and *Trichocladium lignincola* showed a positive result.

Abdel-Raheem (1997) tested for laccase activity in some lignicolous aquatic hyphomycetes. The oxidation of the diammonium salt of 2, 2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) in laccase plate assay and the spectrophotometric assay method were used. Most of the fungi tested in this study produced varying levels of laccase activity. This result indicated that many aquatic hyphomycetes play a role in lignin degradation in the freshwater environment.

2.5 The ecology and role of aquatic hyphomycetes.

Inputs of allochthonous organic matter, such as autumn shed leaf litter in temperate climates, provide an important sources of energy for food webs in woodland streams (Bird & Kaushik, 1981). The main factors that control the rate of leaf decomposition are temperature, quality of water and the initial chemical composition of the leaves (Kaushik & Hynes, 1971). Decomposition has been subdivided into three phases by Cummins (1974): A. Leaching, B. Microbial colonization, and C. Invertebrate palatability.

A. Leaching

Leaching is the process that occurs immediately after immersion of leaves into water. Dissolved organic matter, such as amino acids, soluble carbohydrates, and polyphenols are removed from the leaves (Willoughby &

Archer, 1973; Suberkropp & Klug, 1976) with the maximum leaching of carbohydrates from twigs within 3 weeks of submersion, however; some leaching continued up to 12 weeks. Webster & Benfield (1986) concluded that most soluble substances can be leached from leaves within the first 24-48 hours after submersion in a stream and result in a loss of up to 30% of the original weight depending on the leaf species.

B. Microbial colonization

Microorganisms are important mediators in leaf breakdown in streams (Suberkropp, 1997). In the first few days to weeks of decomposition, the leaves have been changed chemically and physically. It has been called a conditioning process associated with an increase in the food quality of the detritus (Suberkropp & Klug, 1976; Anderson & Sedell, 1979; Findlay & Arsuffi, 1989).

During the early stage of leaf decomposition, fungi are the dominant group of microflora rather than bacteria. Aquatic hyphomycetes have been reported to produce many types of extracellular enzymes that are able to degrade the major plant polysaccharides (Suberkropp & Klug, 1981) e.g. cellulases, hemicellulases, pectinases, laccase, polygalacturonases, pectin lyase, lignin-degrading enzymes. These enzymes are able to degrade simple

polysaccharides, cellulose, hemicellulose, pectin, lignin and other cell wall polymers of plants (Charmier & Dixon, 1982; Chamier, 1985).

Suberkropp & Klug (1976) examined the interactions between fungi and bacteria involved in leaf processing in streams. It was concluded that bacterial biomass, estimated by ATP levels, during the early phase of the first 12 weeks of the decomposition were lower than fungal biomass. The fungi have a greater capability in degrading leaf litter because of the variety of extracellular enzymes they produce, and their ability to penetrate deep into the substratum.

Few bacteria have the ability to degrade structural polymers and then only slowly. However, bacterial biomass can increase during the final stage of leaf processing by fungi, since the leaf matrix increases in surface area and also with increased temperature of the water. An increase in surface area resulted in a competitive advantage for bacteria in colonization the leaf remains and they are capable of using the soluble compounds released by fungal activities.

Generally, leaf detritus (Coarse Particulate Organic Matter, CPOM) is transferred into Dissolved Organic Matter (DOM), Fine Particulate Organic Matter (FPOM) and CO_2. The major end-products of the detritus inputs are CO_2 and FPOM, and fungal spores can be part of FPOM released from leaf detritus and represented an important food source for filter-feeder organisms.

However, the influence of stream water temperature during diurnal and seasonal fluctuations, dissolved nitrate and phosphate, forest clear cutting can affect microbial activities and production in stream ecosystems (Peters *et al.*, 1987; Findlay & Arsuffi, 1989).

C. Invertebrates palatability

Leaf litter serves as a food source for invertebrates or shredders living in streams. Examples of shredders found in freshwater streams are amphipods (*Gammarus fossarum, G. minus* and *G. pulex*) and insects (*Tipula abdominalis, T. lateralis, Peltoperla* sp. and *Clistoronia magnifera*). These shredders utilize the softened leaf detritus as a food source during microbial colonization and transformation. However, the fungi colonizing the leaf detritus can change the food quality of leaf detritus affecting the growth rate of shredders (Suberkropp & Klug, 1981; Bärlocher, 1985; Charmier *et al.*, 1989; Canhoto & Graca, 1999).

2.6 Interaction of aquatic hyphomycetes with other organisms

1. Mycoparasitism

Some aquatic fungi are associated with freshwater hyphomycetes by growing on each other. For example *Janetia curviapicis* grows on other hyphomycetes on submerged wood in a stream (Goh & Hyde, 1996), while *Crucella subtilis* (anamorph of *Camptobasidium hydrophilum*) is a mycoparasite of several aquatic hyphomycetes. *Nectria* species have also been observed to develop on the fruiting bodies of ascomycetes on submerged wood (Wong *et al.*, 1998).

2. Pathogens

Some aquatic hyphomycetes act as pathogens of plant and animals living in freshwater habitats. They cause some diseases in plant and animals (Goh & Hyde, 1996; Wong *et al.*, 1998).

3. Role of aquatic hyphomycetes to the shredders in freshwater habitats.

Aquatic hyphomycetes can change the food quality of leaf detritus during the conditioning process, and may affect the growth rate of some shredders. Some shredders prefer to feed on leaves that are colonized by fungi (Graca *et al.,* 1993). Moreover the palatability of leaf detritus is

affected by a number of factors, including leaf softness, nitrogen content and fungal biomass (Suberkropp, 1992).

2.7 Viability testing

Vital stains

2.7A. Tetrazolium salts

Tetrazolium salts are heterocyclic organic compounds used for detecting redox potential of cells in chemistry and industrial technology and different branches of biological sciences e.g. medicine, pharmacology, immunology and botany, but especially in biochemistry and histochemistry (Seidler, 1991).

Tetrazolium salts form uncharged, brightly coloured formazans which are a special redox system acting as proton acceptor or as an oxidant. Several of the formazans precipitate out of solution and are useful for histochemical localization of the sites of reduction or after solubilization in organic solvents, for standard spectrophotometric techniques. The tetrazolium salts which are widely used in biological studies are listed in Table 9.

Table 9. Tetrazolium salts used for detecting redox potential in living cells and tissues.

Name	Tetrazolium salts	Colour of formazan	Water solubility of formazan	Applications
I-6496 INT	4-Iodonitrotetrazolium violet	Orange	No	Cytochrome b-ubiquinolone complex activity, succinic dehydrogenase histochemistry, heavy metal toxicity in yeast
M-6494 MTT	3-(4,5-Dimethylthiazol-2-yl) 2,5diphenyltetra zolium bromide	Purple	No	Superoxide generation by fumarate reductase and nitric oxide synthase, mitochondrial dehydrogenase activity, cell viability and proliferation, neuronal cell death, *In vitro* toxicity testing
N-6495 NBT	Nitro blue tetrazolium chloride	Deep blue	No	Superoxide generation by xanthine oxidase, superoxide release from single

Table 9. (continued)

Name	Tetrazolium salts	Colour of formazan	Water solubility of formazan	Applications
				pulmonary alveolar macrophages, neutrophil oxidative metabolism, succinic dehydrogenase histochemistry
N-6498 NTV	4-Nitrotetrazolium violet chloride	Purple	No	Catalytic properties of xanthine dehydrogenases and oxidoreductases in organelles
T-6490	Tetrazolium blue chloride	Blue	No	Detection of impaired electron transport in *Chlamydomonas*, succinic dehydrogenase histochemistry
X-6493 XTT	2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl) 2H-tetrazolium-5-carboxanilide	Orange	Yes	Antifungal susceptibility , drug sensitivity of cells, parasitic nematode viability, tumor cell cytotoxicity

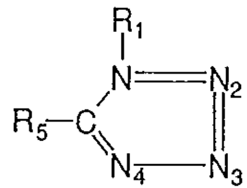
(After: Humason, 1972; Prescott, 1978; Weiss, 1988)

a) Structure of tetrazolium salts

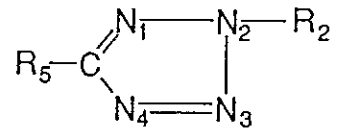
Tetrazolium salts are organic compounds that can be derived formally from the tetrazoles which exist in two isomeric forms (Fig. 2A and 2B). Their five-membered unsaturated ring contains two double bonds, one carbon and four nitrogen atoms.

b) The formation of tetrazolium formazan

Tetrazolium salts accept electrons from oxidized substrates or appropriate enzymes, such as NADH and NADPH. In particular, MTT is reduced at the ubiquinone and cytochrome b and c sites of the mitochondrial electron transport system and is the result of succinate dehydrogenase activity. This reaction converts the yellow salts to blue-coloured formazan crystals (Fig.3) that can be dissolved in an organic solvent whose concentration can be spectrophotometrically determined (Weiss, 1988).



A 1-tetrazol



B 2-tetrazol

Fig. 2. Chemical structures of tetrazolium salts, R_n = phenolic radicals

$$R_5-C(=N^{+}(R_1)-N_2(R_2)-N=N) \xrightarrow{x^-} R_1-N_1=N_2-C_3(R_3)=N_4-N_5<^{H}_{R_5}$$

Tetrazolium salt

Formazan complex

Fig. 3. The reduction reaction of tetrazolium bromide.

c) Fungal viability testing by tetrazolium salts

An & Hendrix (1988) reported using a variety of vital stains in the estimation of the viability of fungal spores. Trypan blue, triphenyl tetrazolium chloride, and rose bengal were used in viability testing, but it was difficult to differentiate living from non-living spores. MTT, a tetrazolium salt, has been used in order to determine the viable and non-viable conidia of *Glomus* sp. (An & Hendrix, 1988). Living cells were stained by MTT and can be seen by the formation of a red-coloured formazan complex. With longer incubation, the conidia become dark blue or purple.

Miller *et al.,* (1993) used MTT to estimate the viability of the spores of the basidiomycetes: *Rhizopogon rubescens* and *Suillus tomentosus.* Positive MTT staining was indicated when reddish compounds were formed in the mitochondria. It was concluded that the succinate dehydrogenase enzymes in the mitochondria reduce the MTT and formed the coloured formazan.

Sridhar & Bärlocher (1994) estimated the viability of conidia in fresh and old foam in a temperate stream. They used MTT to test the conidial viability and found that the percentage viability of conidia found in fresh foam was 76-91%, and in old foam only 20-43%.

2.7 B Acridine orange and DAPI

a) Acridine orange

Acridine orange (3,6-bisDimethylaminoacridine hydrochloride) (Fig. 4) is a fluorescent vital stain which is commonly used to estimate cell viability and has many other applications (Albert, 1966; Mason, 1993; Korber *et al.*, 1994).

The colour of acridine orange fluorescence has been used to estimate cellular viability, with viable cells emitting a green fluorescence while non-viable cells emitted a red fluorescence (Korber *et al.*, 1994).

b) DAPI

DAPI (4′-6 diamidino-2-phenylindole) is a fluorescent dye which has been reported to be more stable and specific for DNA. DAPI is generally believed to bind with DNA at the A-T rich regions within the minor groove of B-DNA in solution. DAPI also binds to poly [d(G-C)$_2$], presumably in the major groove of the polynucleotide. Matsuzawa & Yoshikawa (1994) suggested that DAPI molecules interact with DNA via the phosphate groups along the DNA strand. The DAPI bound to these sites seems to be governed by the pH of the medium. DAPI exists as a protonated form with a

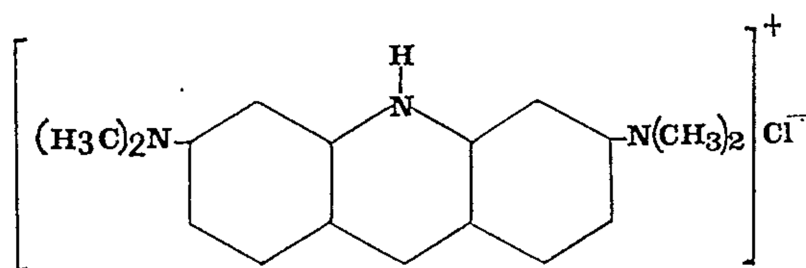


Fig. 4. The chemical structure of acridine orange.

fluorescent emission maximum near 490 nm and as an unprotonated form with a fluorescent emission maximum at 440 nm. Hence, the maximum fluorescence of the DAPI is between 440 nm. (blue) and 490 nm. (blue-green), depending on whether the DAPI is bound inside or outside the minor DNA groove. (Saby *et al.*, 1997).

c) Fungal viability testing by acridine orange and DAPI

Acridine orange and DAPI, have been used to test for fungal spore viability: stained cells or spores revealed the precence of DNA material. *Rhinosporidium seeberi* was stained by acridine orange and examined under the fluorescent microscope, this indicated the occurrence of DNA in spores and sporangia (Moses *et al.*, 1991).

DNA content, localization, variations in content, the nuclear distribution during spore germination, the numbers of nuclei in spores, basidiospore viability and germination were estimated by using the DAPI stain in many species of fungi: *Phytophthora infestans*, ectomycorrhizal, saprotrophic basidiomycetes, *Neurospora crassa* and *Colletotrichum* sp. (Moses *et al.*, 1991; Whittaker *et al.*, 1991a, b; Barja *et al.*, 1992; Miller *et al.*, 1993).

Objectives

1. To estimate the viability of conidia trapped in foam samples
2. To estimate the viability of laboratory-produced conidia under different conditions
3. To study the biodiversity of fungal conidia in foam
4. To isolate the fungi and collect the culture collections

Chapter 3

Materials and Methods

3.1 Materials and equipment

3.1.1 Chemicals

Media (Appendix A)

- Corn meal agar (Difco)
- Potato dextrose agar (Difco)

Antibiotics

Kanamycin, Streptomycin, Tetracycline (Sigma)

Other chemicals

- Acridine orange (Sigma)
- Agar granulated (BBL)
- DAPI (sigma)
- Ethanol 95%
- Formalin
- Glacial acetic acid (Merck)
- MTT (Sigma)
- NaCl (Merck)

3.1.2 Equipment

Equipments	company
Autoclave machine	Tomy SS- 320
Compound microscope	Olympus
Fluorescent microscope	Leitz Wetzlar
Hot air oven	MMM Medcenter
Hot plate stirrer	IKAMAG REC-G
Incubator 25 °C	Sanyo
Laminar air flow cabinet	ISSCO
Refrigerators	Mitsubishi, Sanyo
Rotary shaker	Forma Scientific
Stereo microscope	Nikon
Vacuum pump	Gelman Science
Water bath	Memmert
Balance	Mettler, Sartorius

3.1.3 Other apparatus

- air pump
- fine insect pin
- glass slides and cover slips
- ice box

- plastic bottles
- plastic jars
- translucent millipore membrane (Nuclepore)
- haemacytometer slide

3.2 Study sites

Ton Nga Chang Wildlife-Sanctuary was chosen for the study, located in Songkhla and Sathun province, Southern Thailand (6 °5′ to 7°3′ North and 100°8′ to 100°16′ East). The northern part joins with Rathaphum District, Songkhla province, eastern part joins to Hat Yai district, Songkhla province. The western part joins Muang District and the southern part joins Thaleban National Park, Sathun province.

Four collecting sites were located along the Ton Nga Chang waterfall at approximately 50-m intervals and ca. 5-10 m wide. The samplings sites were located in the direction from upstream to downstream (Fig. 5).

3.3 Sampling procedures

The studies were conducted every two weeks at each site from June to September, 1998. Foam samples were collected during June to September, stream water samples were collected from August to September.

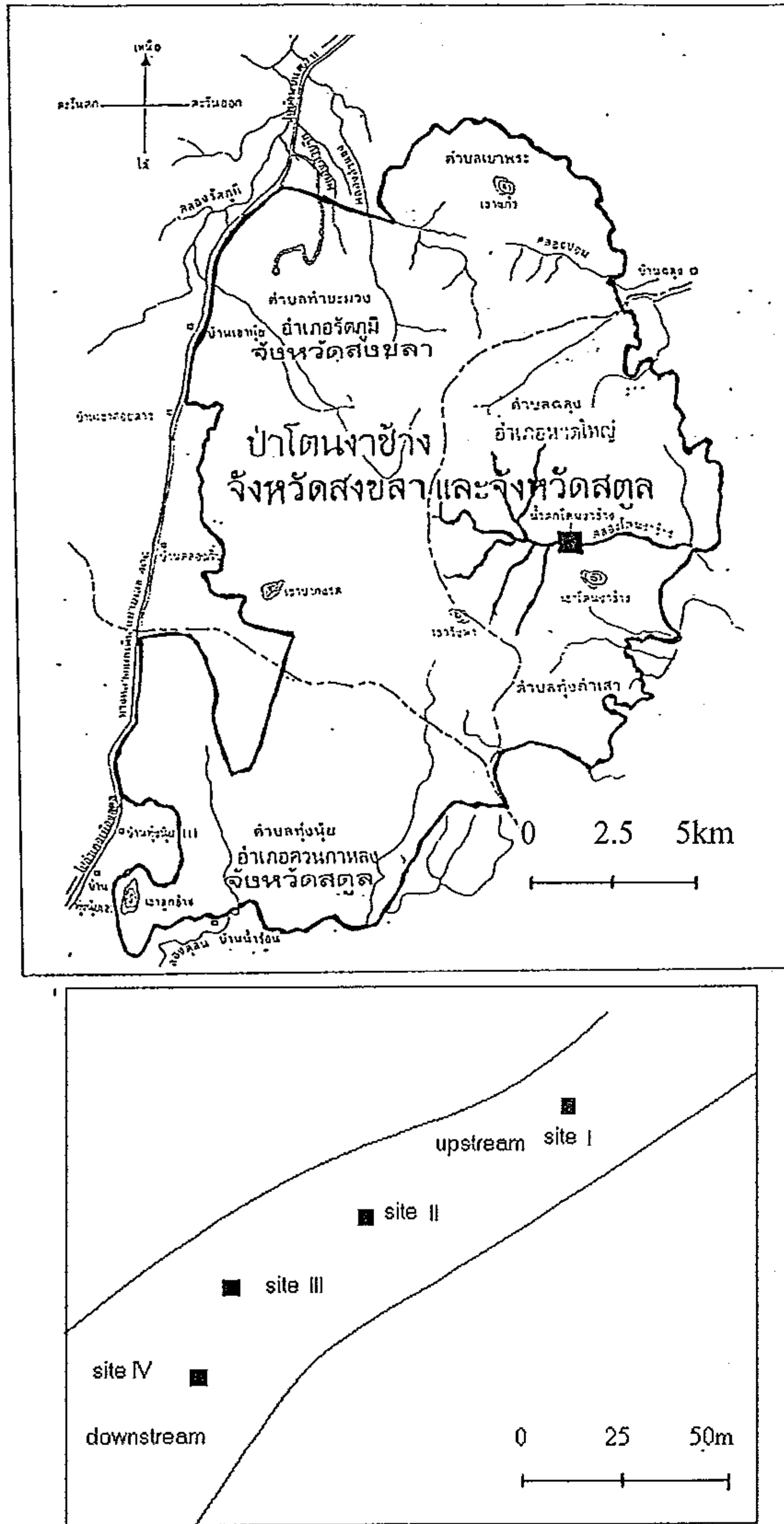


Fig. 5. Map showing the location of sampling sites along Ton Nga Chang waterfall (■).

3.3A Foam collection

Screw-capped plastic jars were labelled, and the number and the appearance of foam samples noted. Fresh and old foam below the waterfall, along the rapids, behind twigs and fallen leaves were collected (Fig. 6). The appearance of the foam are shown in Fig. 7. Foam samples were scooped with a spoon or jar lid, and transferred into the jars. Excess water was immediately decanted. Sometimes foam was scarce and not thick enough, subsamples were collected and pooled in order to have enough material for study. Two to three replicates of fresh and old foam were collected at each study sites.

Collected foam were divided into 2 sets, the first set was kept on ice in an ice box in order to continue their study in the laboratory, at the Department of Microbiology, Prince of Songkla University. The other set was fixed by adding a few drops of Formalin-Acetic Alcohol (FAA) into the foam jars immediately after sampling in order to examine the fungal conidia under a microscope.

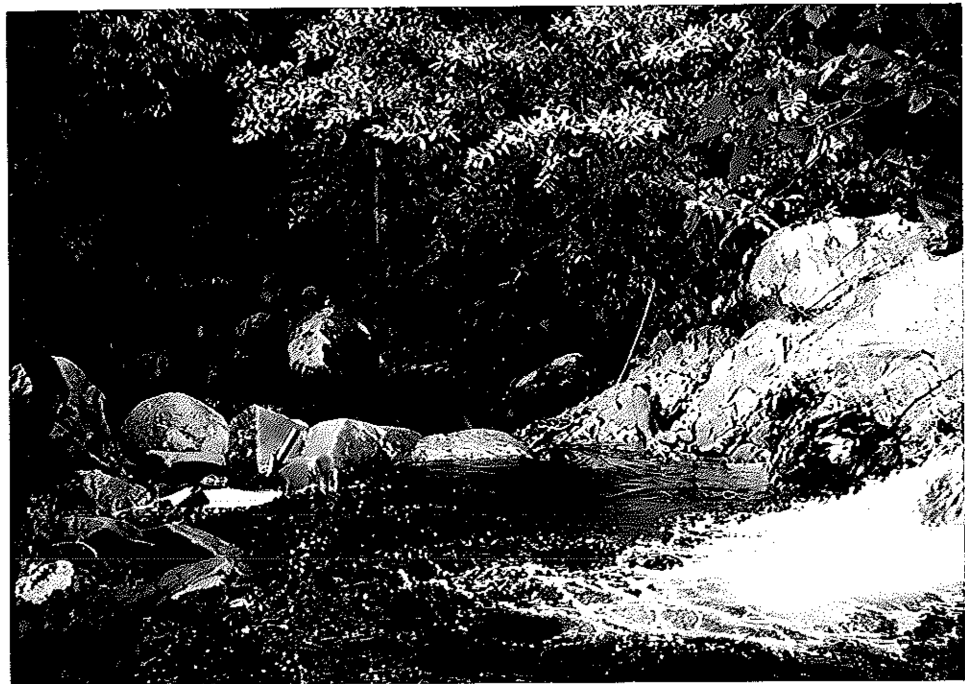


Fig. 6. The accumulation of foam (arrow) below the waterfall.

A

B

Fig. 7. The appearance of foam samples, A: Fresh foam B: Old foam

3.3B. Stream water collection

One-litre of water was taken with a plastic bottle in the fast-flowing stream below the waterfall. Bottles were placed on ice in an ice box, and returned to the laboratory.

3.4 Estimation of fungal concentration and species diversity in stream water samples

Fungal conidia were counted under a microscope to estimate the numbers of conidia in the stream water. One-litre of water was collected and filtered through a translucent millipore membrane filter (diameter 5 cm, pore size 8 μm). Filters were air dried, and the fungal conidia stained with lactophenol cotton blue. Stained filters were cut into small pieces, placed on clean, glass slides, and covered with a cover slip. These were scanned for fungi under a microscope and the species identified. The different conidial types were recorded for each stream and study site.

3.5 Estimation of the viability of conidia in foam samples

3.5.1 Total viable plate count method

Total viable plate counts were made to enumerate the numbers of viable fungal conidia in fresh and old foam samples. Foam samples were returned to the laboratory within an hour. The foam was immediately liquefied by shaking the collecting jars a few times. One ml of foam sample was pipetted into a clean test tube and 1 ml of sterile distilled water added. The suspension was vigorously shaken, then 1 ml of the suspension was mixed with 10 ml of sterile distilled water. Serial 10-fold dilutions of foam samples were made. The desired dilutions were selected, usually 1/100, 1/1,000 and 1/10,000. 100 μl aliquots of the selected dilutions were plated to an isolation medium (corn meal agar, containing antibiotics) and a bent glass rod was used to spread the sample over the surface of the plate. Plates were incubated in 25 °C incubator for 3-5 days, and the fungal colony forming units counted and expressed as number of units per ml of foam sample.

3.5.2 Vital staining method

The viabiltity of conidial cells was tested by using 3 different stains: tetrazolium bromide (MTT, Sigma 2128), acridine orange (Sigma) and DAPI (Sigma).

1) Tetrazolium bromide staining

A few drops of diluted fresh and old foam samples were mounted on a clean, glass slide, and an equal volume of MTT solution concentration 0.5 mg/ml was added (Sridhar & Bärlocher, 1994). These were mixed with a Pasteur pipette, the slides placed in a moistured petri dish and incubated at room temperature in the dark for 1-2 hours. Slides were scanned for fungi under a compound microscope and the viable and non-viable cells enumerated. A total of 200 conidia per sample were scanned. Conidia were determined to be viable when at least one cell had reduced the stain and formed the red-coloured formazan product. Non-living conidia were colourless after staining. Both fresh and old foam samples were counted and the percentage conidial viability trapped in fresh and old foam was calculated.

2) Acridine orange staining

A few drops of diluted fresh and old foam samples were transferred onto a clean, glass slide and acridine orange solution (concentration 0.2 mg/ml (Saby *et al.,* 1997)), added and mixed with a Pasteur pipette, and covered with a cover slip. Slides were scanned immediately for conidia staining under a fluorescent microscope. A total of 200 conidia per sample were examined. Conidia were determined to be viable when at least one cell

became green, while non-viable conidia were red. Percentage viability conidia trapped in fresh and old foam was calculated.

3) DAPI staining

A few drops of diluted fresh and old foam samples were transferred onto a clean glass slide and DAPI solution added (concentration 0.5 μg/ml (Miller *et al.*, 1993)), mixed gently with a Pasteur pipette added, covered with a cover slip, and incubated at room temperature for 5 minutes in the dark. After incubation, slides were scanned immediately for conidia staining under a fluorescent microscope. A total of 200 conidia per sample were examined. Conidia were determined to be viable when at least one cell fluoresced blue under a microscope, while non-viable conidia were colourless. Percentage viability conidia trapped in fresh and old foam was then calculated.

3.6 Morphological examination

The semi-permanent slides from foam samples were prepared. A few drops of a foam sample were transferred to a clean-glass slide and air-dried or quickly heated over a flame. A few drops of the mountant, lacto phenol cotton blue, was added. Slides were sealed with fingernail polish and allowed

to dry. Slides were examined for fungal conidia which were measured under a microscope and photographs were taken.

3.7 Fungal isolation

3.7A Single spore isolation

Foam samples were brought back to the laboratory and conidia immediately isolated. The foam was liquefied by shaking, and aliquots of foam pipetted onto the isolation medium containing antibiotics. Foam was diluted with sterile distilled water when it was rich in fungal spores. A bent glass rod, or wide loop, was used to spread the foam sample all over the surface of the isolation medium. Plates were incubated at room temperature for 1 hour and then scanned for fungal conidia with a low power of a compound microscope. Conidia were transferred to fresh medium with a fine insect pin. These were incubated at 25 °C incubator and examined for hyphal growth everyday. Subcultures of the identified geminating conidia were made until pure cultures were obtained. The appearances of the pure colony cultures were recorded: e.g. colour, sporulation.

3.7B Hyphal tip isolation

After the total viable plate count from foam samples was counted, the germinated fungi were isolated by cutting hyphal tips of germinating conidia and transfering to new growth plates. These were subcultured onto a fresh

media until pure cultures were obtained. The appearances of the pure colony were recorded: e.g. colour, sporulation.

3.8 Pure culture preservation

Pure cultures were maintained on corn meal agar and potato dextrose agar slants under sterile paraffin oil, and maintained at the Mycology Laboratory, Department of Microbiology, Prince of Songkla University, Songkhla, and at the Mycology Laboratory, National Center for Genetic Engineering and Biotechnology, Bangkok.

3.9 Fungal identification

Fungi in foam were identified on the basis of detached conidia collected from the foam or stream water. Identification of the pure cultures was based on the laboratory-produced spores and the characteristics of the colony appearance. Identification keys used were by: Nawawi (1973; 1974a, b; 1975a, b; 1976a, b), Ingold (1975), Webster & Descals (1981), Marvanová (1997).

3.10 Estimation of laboratory-produced conidia viability under different conditions.

3.10A. Estimation of laboratory-produced conidia viability under aeration condition.

Four cultures were selected, and conidial production induced. Several thin slices of the culture were cut from the margin of the 2 week old colony, placed into the sterile conical flask containing sterile distilled water. Flasks were connected with an air pump and air flowing into the flasks equilibrated. Flasks were aerated at room temperature for 24-48 hours, and checked regularly for sporulation by decanting a few drops of the suspension onto a haemacytometer slide. The number of conidia per ml of suspension was counted. A spore suspension approximately 10^3 conidia per ml was used. Viability of conidia was tested with MTT (see section 3.5.2). The experiment was run for 3, 5 and 7 days, and spore viability determined.

3.10B Estimation of laboratory-produced conidia viability under drying condition.

Four species producing conidia in culture were chosen. A conidial suspension was prepared, counted and equilibrated to a concentration of ca. 10^3 conidia per ml of suspension. The conidial suspension was transferred to a clean glass slide, left in a laminar-flow cabinet for 1, 5, 15, 30 minutes, 1, 5

and 10 hours respectively and conidial viability determined using MTT. Conidial viability under the different drying time was calculated.

3.11 Statistical analysis

Analysis of variance and non-linear regression analysis were used in the data analysis (Genstat and SPSS Program).

Chapter 4

Results and Discussions

4.1 Estimation of the viability of conidia in foam samples

4.1.1 Total viable plate count

The total numbers of fungi trapped in foam varied from 2.3 x 10^3 to 3.4 x 10^5 cfu/ml in fresh foam and from 5 x 10^3 to 3.2 x 10^5 cfu/ml in old foam throughout the period of study (Fig. 8). These values in fresh and old foam were not significantly different at $P>0.05$ (Table A1). The number of fungi in both fresh and old foam started to increase from week 4 (early July) until maximum in week 10 (late August). The highest number of total fungi was in week 10 (late August) and the lowest in the first collection (mid June). After week 10, the numbers decreased again until the end of the study.

In Ton Nga Chang Wildlife-Sanctuary, the water temperature, water pH, air temperature, relative humidity and dissolved oxygen content were consistent for the whole period of study (Fig. 9). However, rainfall appeared to be the main factor affecting the total numbers of fungi trapped in the foam. Leaf fall or leaf litter submerged in the stream in the study area were the

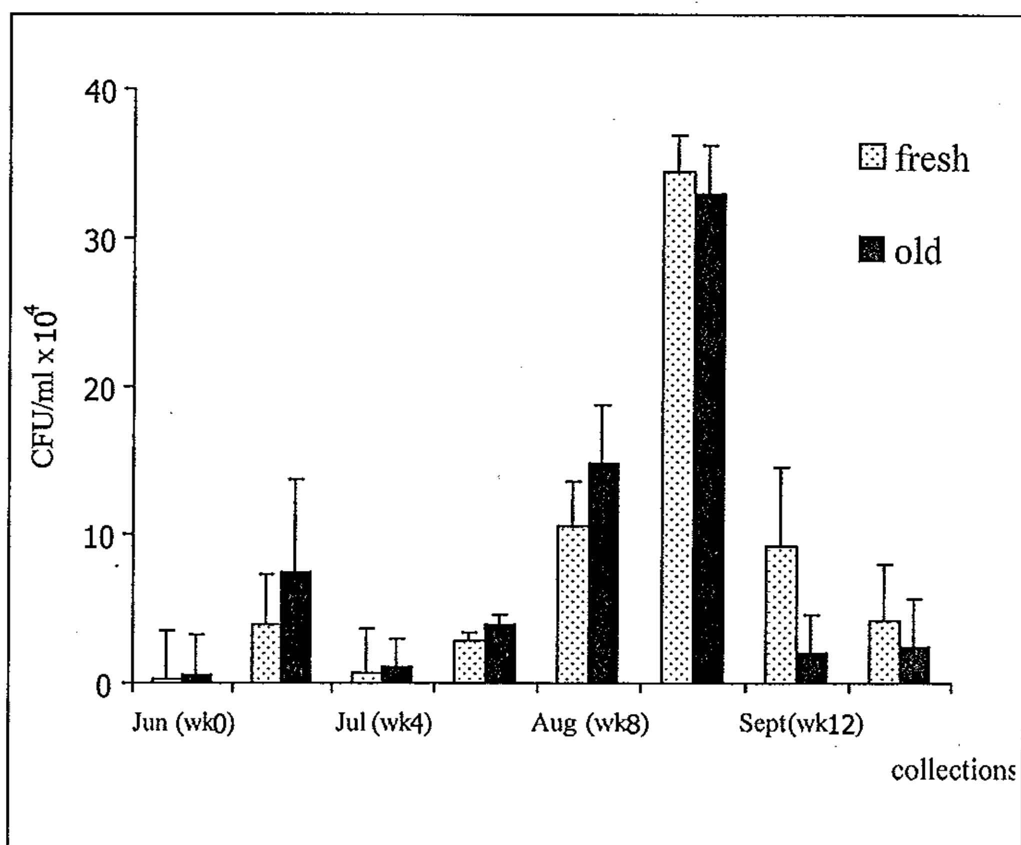


Fig. 8. Total viable plate count in foam (CFU/ml).

(Error bars represent standard error)

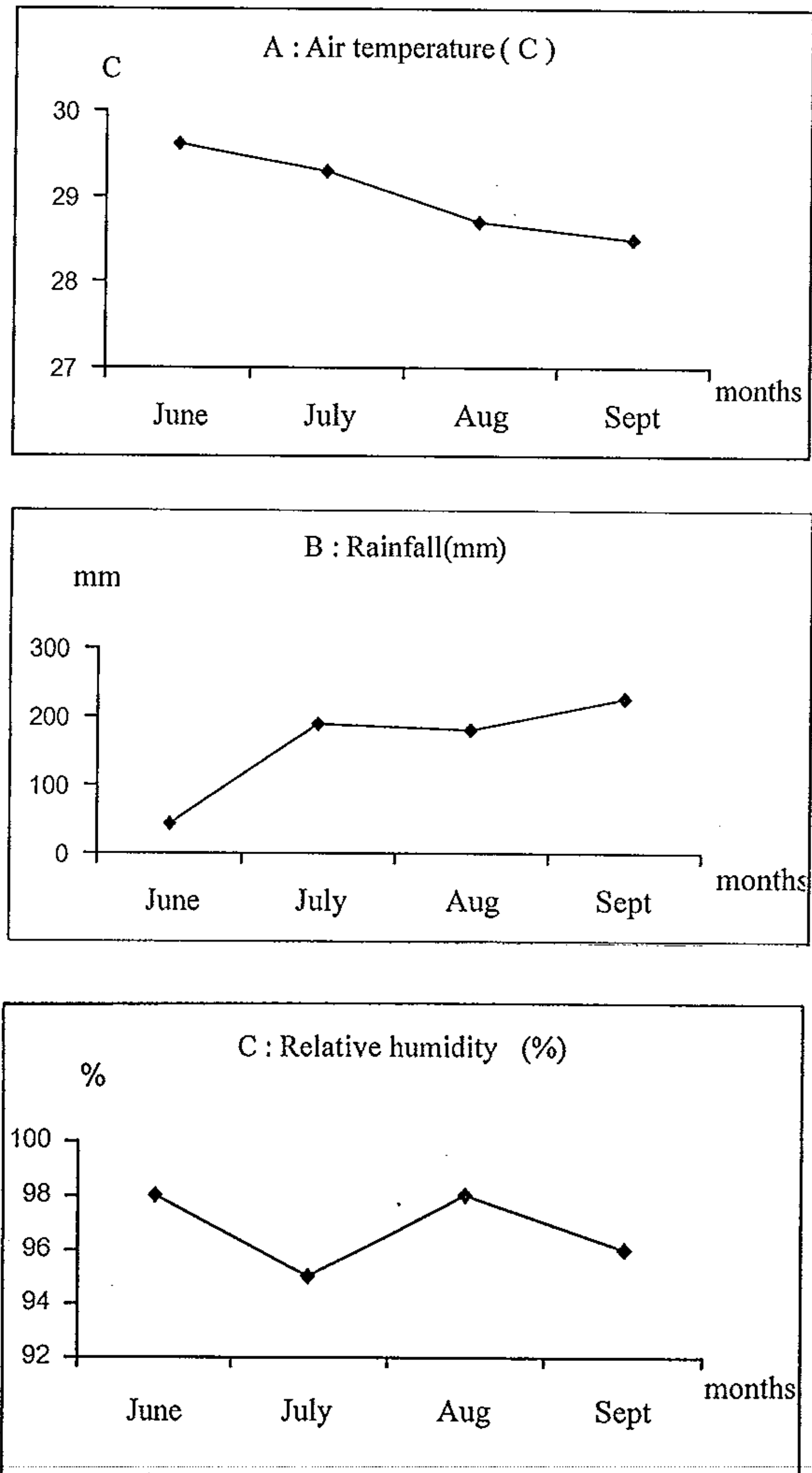


Fig. 9. Agroclimatological data in Songkhla

A. Air temperature B. Rainfall C.Relative humidity

important habitats of fungi. After heavy rainfall, it may increase the water current in the stream which may cause the release of the conidia from the leaf litter into the stream and establish thicker air bubbles in the foam. The water flowing system can enhance sporulation and spore released of some species in the laboratory (Sanders & Webster, 1980). Additionally, the rainfall probably washed the conidia from terrestrial habitats, such as, leaves and soil near the bank into the stream. This may lead to higher numbers of fungi in foam samples.

4.1.2 Vital staining

4.1.2 A comparison of different vital stains

The viability of conidial cells was tested using three different vital stains; MTT, acridine orange and DAPI. MTT was used to determine the viability by the presence of a purple-coloured formazan (Weiss, 1988). MTT was reduced by dehydrogenase enzymes in living cells to form coloured formazan products, whereas non-living cells were colourless. This oxidation-reduction reaction occurs in the mitochondrial electron transport chain (Weiss, 1988).

Acridine orange and DAPI are fluorescent vital stains which interact with nucleic acid (DNA) in living cells. The maximum adsorption

wavelength for acridine orange is 530 nm. Viable cells emitted a green fluorescence while non-viable cells emitted a red fluorescence (Korber *et al.*, 1994). The maximum adsorption wavelength for DAPI is 440-490 nm. Living cells emitted a blue-green fluorescence while non-living cells remain colourless (Saby *et al.*, 1997).

After staining with MTT, acridine orange and DAPI, the conidia were considered to be viable when at least one cell had formed coloured formazan, emitted a green fluorescence or emitted a blue-green fluorescence, respectively. In this experiment, started from week 8 to week 14, different vital stains were used to estimate the viability of conidia in fresh and old foam (Fig. 10). Acridine orange and DAPI staining gave higher estimates of percentages of viable conidia than MTT staining, but were not significantly different at $P>0.05$ (Table A2, A3). These fluorescent vital stains were more stable and specific to nucleic acid in living cells leading to higher percentages of viability in fresh and old foam.

However, MTT staining is routinely more practical for the estimation of fungal viability since there is no need to use a fluorescence microscope. This is especially important when conducting such work in the field away from the laboratory. The viable and non-viable cells can easily be distinguished using an ordinary compound microscope where living cells stained red or purple, while non-living cells remain colourless (Fig. 11).

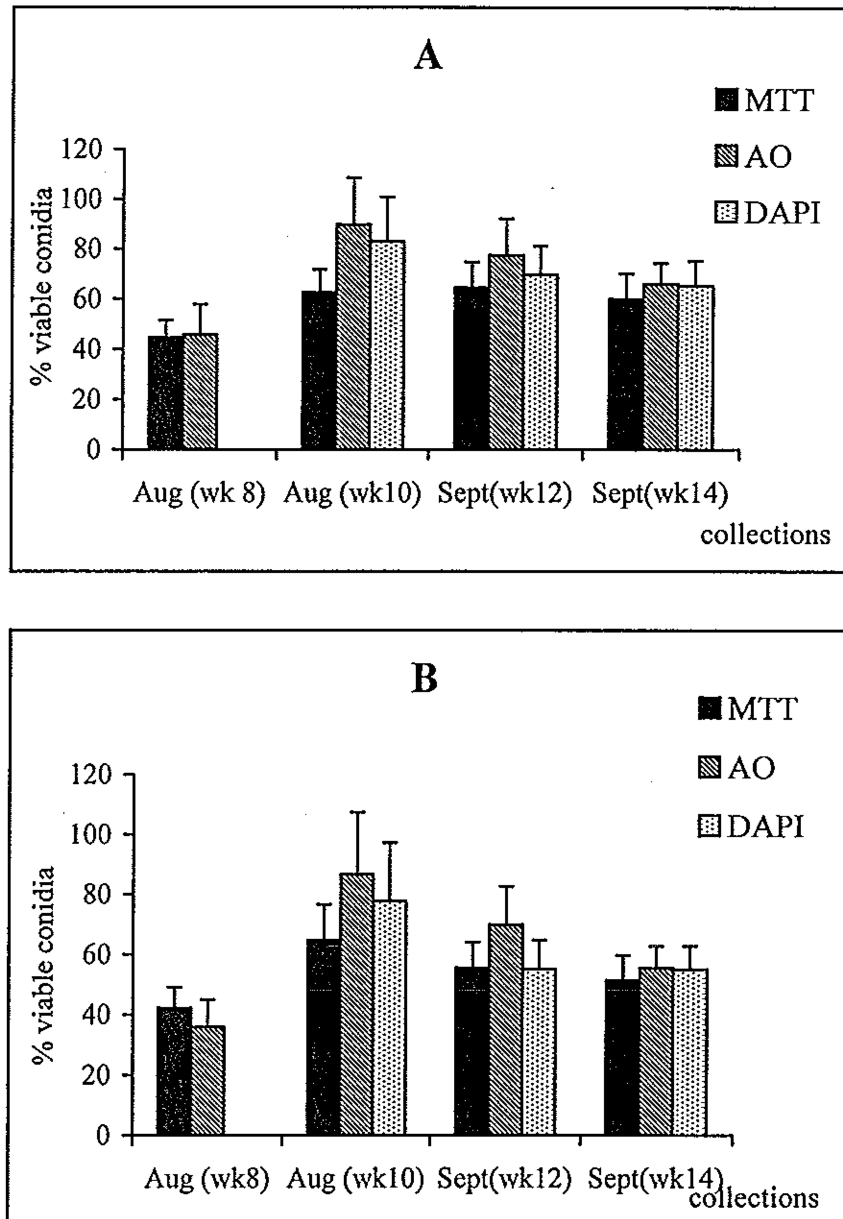


Fig. 10. Percentage viability of conidia using three different stains; MTT, acridine orange (AO) and DAPI.

A Fresh foam B. Old foam

(Error bars represent standard error)

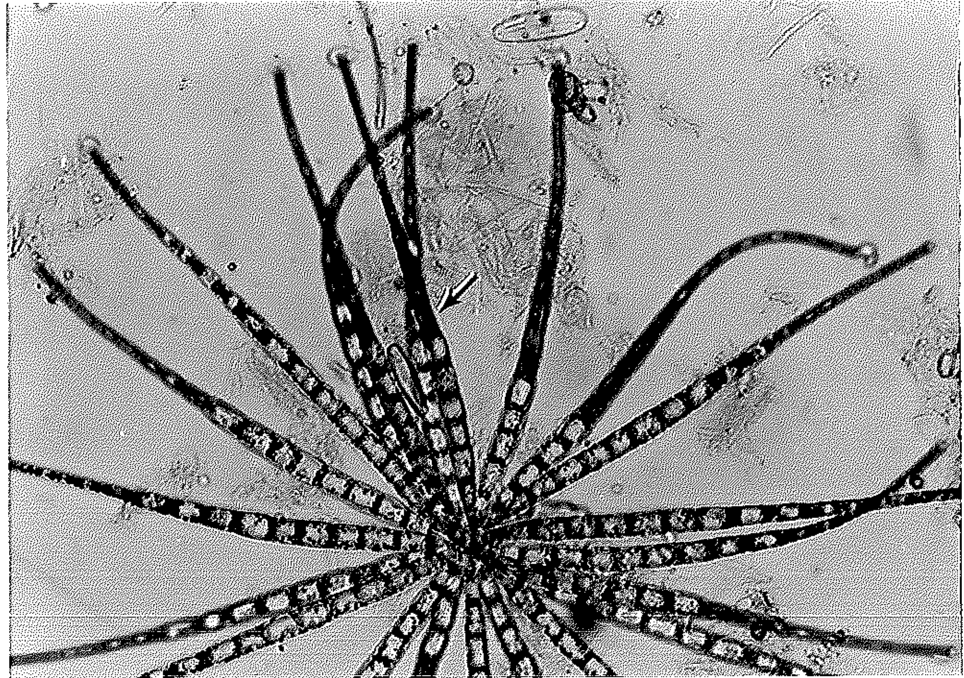


Fig. 11. The MTT staining conidia, living cells stained red-purple (arrow), non-living cells were colourless.

4.1.2 B. Viability of conidia in foam samples (MTT staining)

In my study, the conidial viability in fresh and old foam was in the range of 44-77% and 42-69%, respectively (Fig. 12). There was variation in the viability of conidia in fresh and old foam for every collecting time, but not significantly different at $P>0.05$ (Table A4). It was observed that the viability of conidia in fresh foam was not much higher than in old foam. Percentage viability in fresh foam decreased from the first collection to week 8 (early August), then increased again from week 10 to the end of the study. However, the viability of conidia in old foam fluctuated throughout the period of study.

In this study, the viability in fresh and old foam was similar. The reason is probably that the physicochemical parameters such as water temperature, water pH and dissolved oxygen content in tropical streams were consistent (Tan & Koh, 1995). Another possibility is that because there was rainfall during the period of my study, this will have increased the stream water current. The conversion from fresh to old foam might, therefore, be within a week. Thus the conidia were probably continuously being trapped in old foam resulting in the viability of conidia in fresh and old foam being in a similar range.

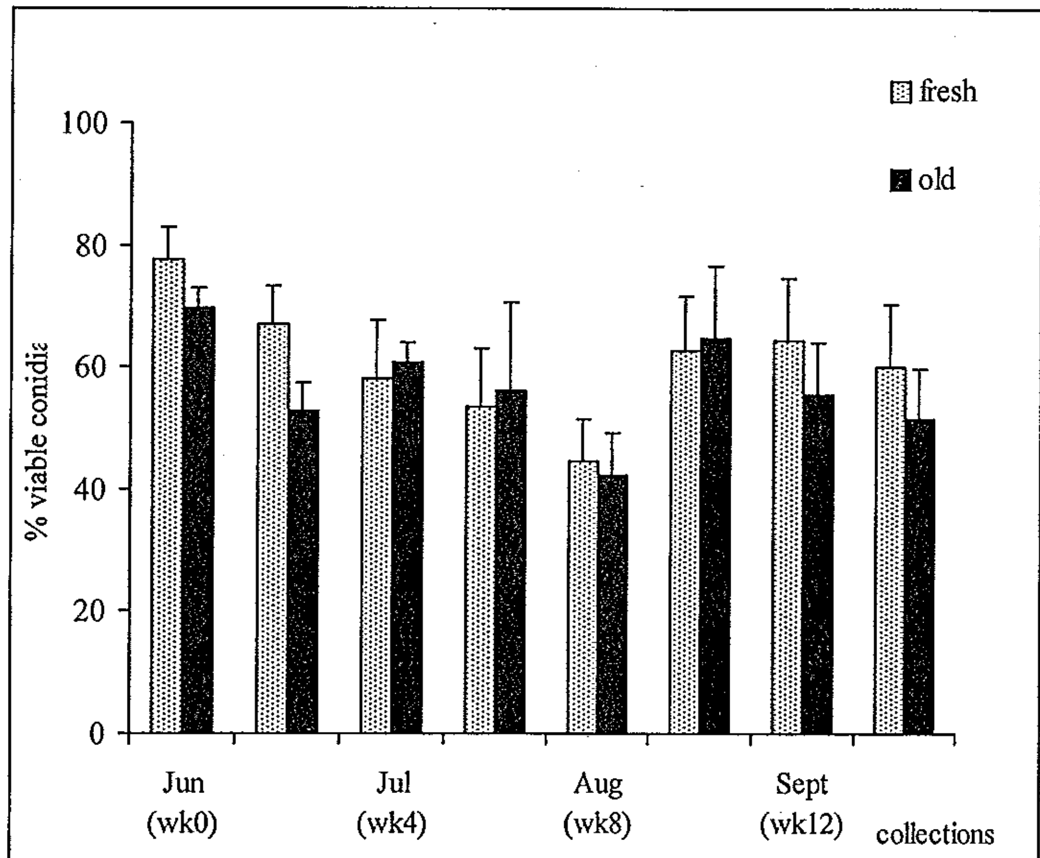


Fig. 12. Percentage viability of conidia in fresh and old foam (MTT staining). (Error bars represent standard error)

Fresh foam occurs because of the accumulation of newly-produced air bubbles (Ingold, 1975). The conidia trapped in fresh foam probably obtain their oxygen from these air bubbles. Some conidia may not survive, however, because the fast-flowing water current may break the conidial cells. The conversion from fresh to old foam depends on the rainfall and season but can be as little as one week in a temperate summer (Sridhar & Bärlocher, 1994). In Thailand, therefore, this conversion could be in less than one week. Foam was considered to be old when the colour had changed from white to yellow-brown and partly dried up. In temperate regions, the transition from fresh to old foam was delayed from 2-4 weeks because of low temperatures which caused ice formation resulting in less water flowing in the stream (Sridhar & Bärlocher, 1994). Additionally, in temperate regions, the conidia could survive in fresh foam in higher percentages (79-91%) possibly because they could preserve their metabolic activities under low temperature conditions. However, the conidia trapped in old foam have got older and some have died, only 20-43% survived (Sridhar & Bärlocher, 1994).

4.2 Estimation of the viability of laboratory-produced conidia under different conditions

4.2.1 Fungal viability under aerated conditions

In natural environments, little is known about the fate of the conidia after they are released from the conidiophores and suspended in the stream before being trapped in foam. Some spores may lose their viability while floating along the stream. The aerated conditions in the laboratory demonstrated the fast-flowing stream conditions. The viability of four species of laboratory-produced conidia was estimated in order to compare this with the viability of conidia in natural foam.

Anguillospora sp., *Helicomyces* sp., *Volutella* sp. and *Thozetella* sp. were tested in this experiment. *Anguillospora* sp. and *Helicomyces* sp. are muticellular, septate, sigmoid and helicoid in shape, respectively. Whereas *Thozetella* sp. and *Volutella* sp., are single-celled, ovoid and sickle-shaped, respectively.

Results were analysed using Genstat to fit the data to the most appropriate model. The numbers of viable conidia of the four species and aeration time were programmed using non-linear regression analysis. Four types of curves

were tested on the same data set; Exponential decay, Logistic, Generalised logistic and Gompertz curve. These different curves have different regression equations with different constant parameters showing the relationship between the viable conidia for all four species with aeration time. A single curve with common parameters was the first model which was fitted. In this model, the constant parameters for viable conidia for all four species were not separated and this resulted in less percentage variance being explained by the model compared with those which separated the parameters between each species. For examples, a single curve of Exponential decay gave 86.4% of variance explained and individual curve explained more of the variation with 88%. Also the single curve of Gompertz explained 86.7% variance while the individual curve gave 88% (Appendix B).

These results demonstrate that in biological systems it is not practical to fix the same parameters for all species since different species will not all behave the same way. The four species have different conidial shapes, sizes and other physiological characteristics. By analogy we can take the example of longevity of humans, dogs and rats. They each have different general morphology, size and other physiological characteristics even though they all are mammals. Therefore a constant parameter for their life span can not be adopted. Thus, a non-linear regression curve should be fitted to biological species using separated parameters.

In my study the Exponential decay curve was chosen as the most appropriate curve for the interpretation of the viability of all four fungal species under aeration and the most suitable for biological prediction.

The viabilities of the four types of conidia after aeration for seven days are given in Fig. 13. All four species showed a steady decrease in viability over time. The viability, estimated by MTT staining, started in the range of 95-98% after three days. From day 5 to day 7, the viability decreased to 83-88% for all four species. It had been expected that *Anguillospora* sp. and *Helicomyces* sp., which have a similar shape and size, should have the same pattern of viability under aerated conditions. In my experiment, however it was observed that the viability pattern of *Anguillospora* sp. was similar to *Volutella* sp. while *Thozetella* sp. was similar to *Helicomyces* sp. It may be because these two groups of fungi had a similar way of adaptation under the aerated conditions. However, the viability for all four species showed little variation, the values were still in the same range from 83-88% over the aeration time.

Most fungi are strictly aerobic (Emerson & Natwig, 1981) and need oxygen for their mycelial growth, sporulation and respiration (Griffin, 1994). The air bubbles under laboratory conditions provide a good source of oxygen.

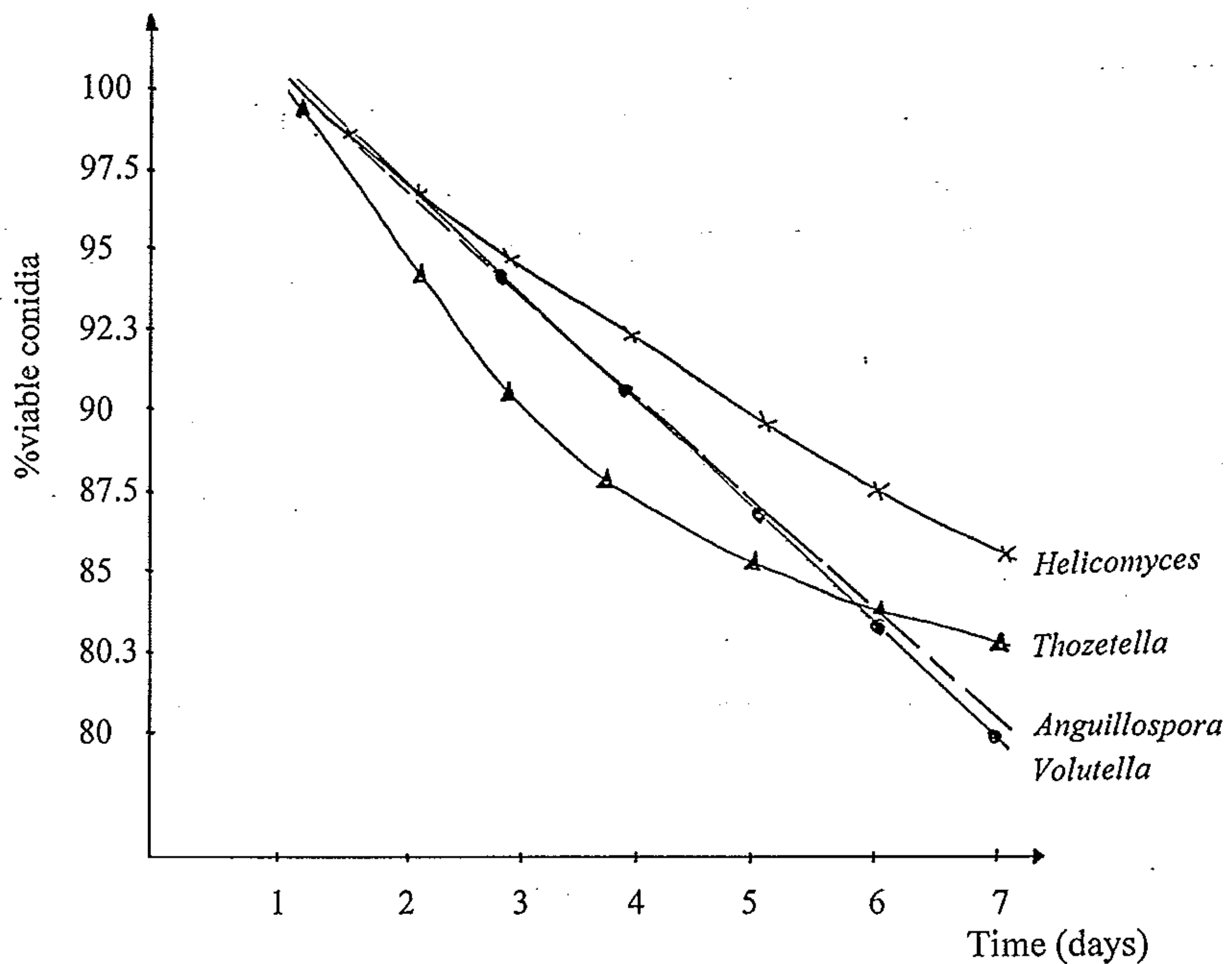


Fig. 13. The Exponential decay curve fitted for the viability of four species after aeration for seven days.

Therefore, the survival of the laboratory-produced conidia for all four species was still high (83-88%) after aeration for seven days.

The level of viability after seven days was comparable to that of conidia in natural foam which varied from 42-77 % in fresh and old foam. Therefore it may be possible that the survival of laboratory-produced conidia under aerated conditions could be a predictor of fungal viability in foam.

For example, the regression equation of this Exponential decay curve;

$$Y = A + BR^{x},$$

where Y = numbers of viable conidia

X = aeration time (days)

A,B and R = constant parameters for each fungal species

can be used. It is interesting to continue the experiment by estimating the viability for all four species after an extended period of aeration. For example, if the aeration time is 14, 21 and 28 days, the predicted numbers of viable conidia can be estimated and are given in Table 10.

Table 10. The predicted percentages of viable conidia for four species under aerated conditions.

Species \ Aeration time	14 days	21 days	28 days
Anguillospora sp.	56.5	27.5	4.5
*Helicomyces sp.**	78	71.5	70
*Thozetella sp.**	83	82.5	82
Volutella sp.	65	50.5	38.5

*Although the Exponential decay curve was used for all four species this may not be the most appropriate model for *Helicomyces* and *Thozetella* over a longer period of time. Since in a biological system, it is impossible that any living cells are still alive forever with longer aeration time.

4.2.2 The effect of drying on spore viability

The same four species of laboratory-produced conidia were used in this experiment. Non-linear regression analysis was used in order to analyse and fit the data to the most appropriate model. In this experiment three types of curve; Exponential curve, Logistic curve and Hyperbolic curve were fitted with the same data set. The Hyperbolic curve with separated constant parameters was chosen as the most appropriate curve. Fig. 14 shows the viability of the four laboratory-produced conidia when dried for 10 hours. All four species had viability of 3-45% after 10 hours drying. The viability of conidia started to decrease after five minutes drying and decreased rapidly after 15 minutes drying. From 30 minutes to 10 hours drying, it was observed that two groups of conidia were distinguished; *Anguillospora* sp. and *Helicomyces* sp. had viability of 37-45%, whereas *Thozetella* sp. and *Volutella* sp. had viability of 3-7%. Fungi, like all organisms, require water as an essential medium for substrate and enzymatic activities (Griffin, 1993). Drying or lack of water is one of the environmental factors affecting the viability and physiology of fungal conidia. When cells are dried, water moves out by osmotic-diffusion and enzymatic reactions can not occur leading to denaturation of the nucleic acids (Griffin, 1993).

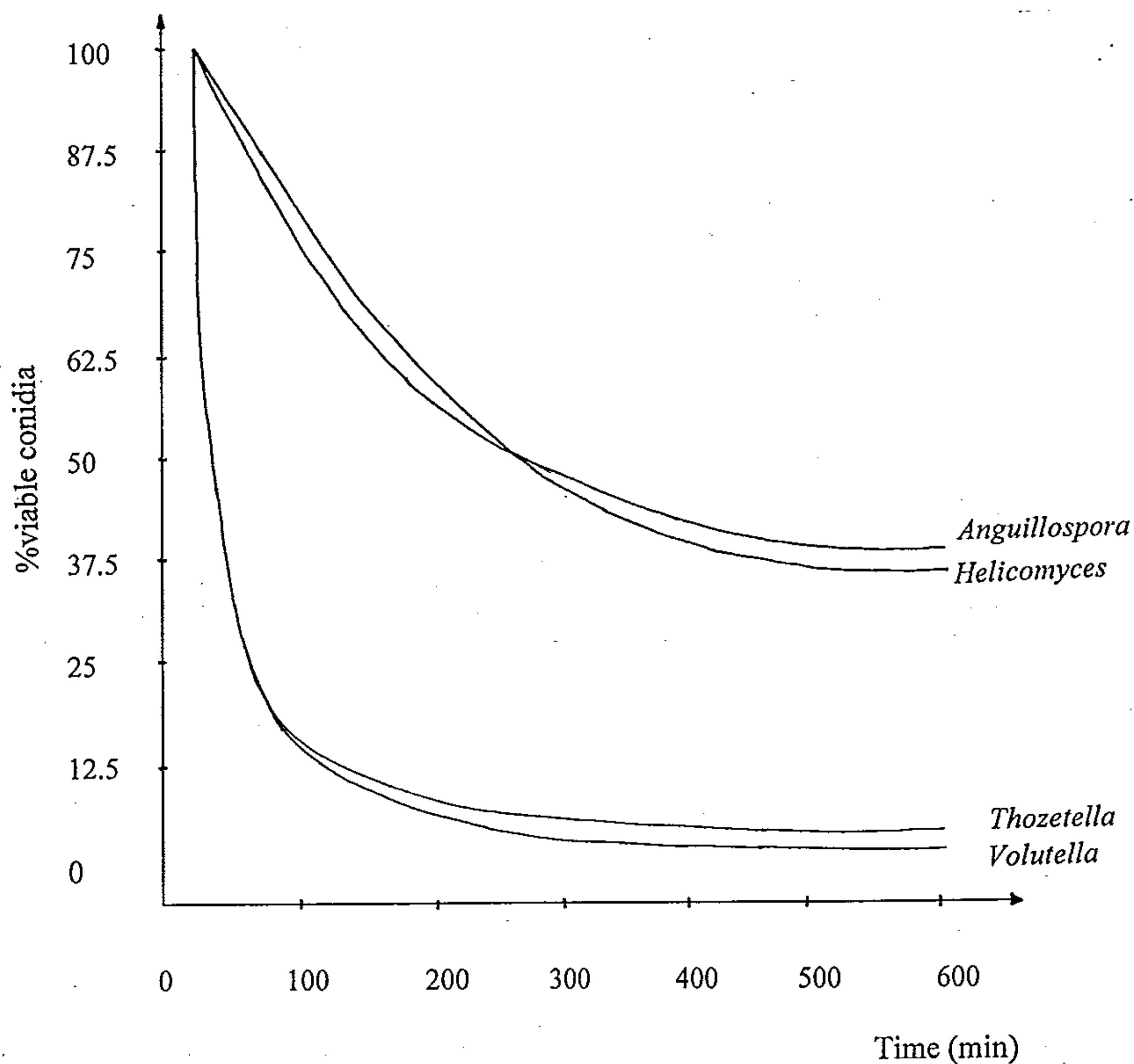


Fig. 14. The Hyperbolic curve fitted for the viability of four species after drying for 10 hours.

The viability of *Anguillospora* sp. and *Helicomyces* sp. was clearly higher than those of *Thozetella* sp. and *Volutella* sp. (Fig. 14). The reasons may be because of their conidial morphology. *Anguillospora* sp. and *Helicomyces* sp. are sigmoid and helicoid, respectively, and are multicellular, septate and bigger than the other two species. Additionally, it may be possible that these two species contain more glycogen as a food reserve. Whereas *Thozetella* sp. and *Volutella* sp., which are ovoid and sickle-shaped, respectively, are single-celled and smaller in size and probably have less food reserve. Thus, the possibility of survival under drying conditions with at least one cell had formed formazan complexes of *Anguillospora* sp. and *Helicomyces* sp. is much higher than the other two species. Thus, the viability of *Thozetella* sp. and *Volutella* sp. were much lower than those of *Helicomyces* sp. and *Anguillospora* sp.

The regression equation of the Hyperbolic curve is;

$$Y = A + B\,(1 + D^{x}),$$

where Y = number of viable conidia

X = drying time (minutes)

A, B and D = constant parameters for each fungal species.

As before, it is interesting to continue the experiment by testing the viability for all four species after a longer drying time, as shown in Table 11.

Table 11. The predicted percentages of viable conidia for four species under drying conditions.

Species \ Aeration time	15 hours	20 hours	24 hours
Anguillospora sp.	36	32	30
Helicomyces sp.	27	21	19
*Thozetella sp.**	ND	ND	ND
*Volutella sp.**	ND	ND	ND

ND = Not determined

*Using the Hyperbolic curve zero viability occurred between 10 and 15 hours for *Thozetella* sp. and *Volutella* sp.

4.3 Diversity of freshwater fungi in foam samples

4.3.1 Foam samples

Foam samples were examined for fungal conidia using a light microscope and identification based on conidial shape (Ingold, 1975). A total of 65 different conidial shapes of mitosporic fungi were observed throughout the period of study (June-September, 1998). Thirty-five genera, 48 species were identified as listed in Table 12. The identified fungi were dominated by hyphomycetes with three genera of coelomycetes and only one genus of basidiomycetes.

For the hyphomycetes their conidia were produced directly from conidiophores (Hawksworth *et al.*, 1995). Various general conidial shapes of hyphomycetes were distinguished in foam samples, such as, sigmoid (Fig. 15.1), tetraradiate (Fig. 15.2), branched (Fig. 15.3), ovoid (Fig. 15.4) and helicoid (Fig. 15.5). A further division of the hyphomycetes could be made based on colour. While many were hyaline there were also dematiaceous hyphomycetes (Fig. 15.6).

Three genera of coelomycetes were identified in this study (Fig. 15.7). Their conidia were produced within the cavity of fungal tissue (Hawksworth *et al.*, 1995). However only one genus of basidiomycetes was identified based on its unique morphology; the presence of clamp connections (Fig. 15.7).

The occurrence of fungi in foam samples for each month from June to September (1998) is listed in Table 12. The number of fungi increased steadily from June to August. After August there was a slight decrease in species numbers. There are several factors affecting spore density and species richness in foam, such as, seasonal leaf fall, water temperature, water pH, dissolved O_2 and rainfall (Tubaki *et al.*, 1983; Sridhar & Kaveriappa, 1984; Chauvet, 1991; Tan & Koh, 1995; Rajashekhar & Kaveriappa, 1998). The availability of substrata and rainfall may be the important parameters affecting the species diversity in the tropics (Tan & Koh, 1995). In my study, a higher number of species correlated with the rainfall data (Fig. 9). Heavy rainfall increased from July to September and this may establish thicker foam samples and enhance sporulation and spore release of some species (Sanders & Webster, 1980).

Table 12. List of identified fungal species found in collected samples.

Genus, species	June	July	August	Sept.	Pure culture
Hyphomycetes					
1. Long, sigmoid shape					
- *Anguillospora* sp. **	F	F	F	F	/
- *Anguillospora*-like	-	-	F	F	
- *Condylospora spumigena*	-	F	F	-	
- *Lunulospora cymbiformis*	F	F	F	F	
- *Wiesneriomyces* sp. 1	-	-	F	-	/
- *Wiesneriomyces* sp. 2	-	-	F	-	
2. Tetraradiate shape					
- *Brachiosphaera tropicalis*	F	-	F	F	
- *Clavariopsis* sp.	F	F	-	-	
- *Quadricladium*-like	F	F	-	F	
- *Tetrachaetum*-like	-	F	F	F	
- *Triscelophorus acuminatus* **	F	F	F	F	
3. Branched shape					
- *Campylospora chaetocladia*	F	F	-	-	
- *C. filiformis*	F	F	-	F	
- *Dendrospora* sp.	-	-	F	F	

Table 12. (continued)

Genus, species	June	July	August	Sept.	Pure culture
- *Dwayaangam* sp. 1	-	F	F	-	
- *Dwayaangam* sp. 2	-	F	F	-	
- *Flabellospora crassa*	F	F	F	F	
- *F. multiradiata*	F	F	F	F	
- *F. verticillata*	F	F	F	F	
- *Isthmotricladia gombakiensis*	-	-	F	-	
- *I. laeensis*	-	F	F	F	
- *Laridospora appendiculata*	F	-	F	-	
- *Nawawia* sp. 1	-	F	F	F	
- *Nawawia* sp. 2	-	F	F	F	
- *Nawawia* sp. 3	-	F	F	F	
- *Phalangispora constricta*	F	F	F	F	
- *Tricladium aciculum*	F	F	-	F	
- *Trinacrium -like*	-	-	F	-	
- *Varicosporium giganteum*	-	-	F	F	
- *V. macrosporum*	-	-	F	F	/
4. Ovoid shape					
- *Thozetella* sp.	F	-	-	F,W	/
- *Volutella* spp.	-	-	-	W	/

Table 12. (continued)

Genus, species	June	July	August	Sept.	Pure culture
5. Helicoid shape					
- *Helicomyces* sp.	F	F	F	F	/
- *Helicosporium* sp.1	F	F	F	F	
- *Helicosporium* sp.2	F	F	F	F	
6.Dematiaceous hyphomycetes					
- *Beltrania rhombica*	F	F	F	F	/
-*Camposporium*-like sp.1	-	-	F	F	
-*Camposporium*-like sp.2	-	-	F	F	
- *Diplocladiella appendiculata*	F	F	F	F	
- *D. scalaroides*	F	F	F	F	
- *Pseudobeltrania* sp.	-	-	F,W	F,W	
- *Scutispora* sp.	F	F	W	F	
-*Sporidesmium tropicalis*	-	-	F	F	
- Other dematiaceous hyphomycetes			W	W	

Table 12. (continued)

Basidiomycetes - *Ingoldiella hamata*	-	-	F	F	
Coelomycetes - *Chaetospermum camelliae*	-	-	F	F	
- *Chaetospermum*-like	-	F	F	W	
- *Pestalotia* sp.	F	F	F	F,W	/
- *Robillarda* sp.	-	-	F	F	/
Total species found in foam	**23**	**30**	**40**	**34**	
Total species found in stream water	**ND**	**ND**	**4**	**7**	

F = foam samples

W = water samples

ND = non detected

\- = not found

** = common species

/ = obtained in pure culture

Fig. 15. The conidia trapped in foam samples

Fig. 15.1. Long, sigmoid shape

A *Anguillospora* sp.

B,C *Anguillospora*-like

D *Condylospora spumigena*

E *Lunulospora cymbiformis*

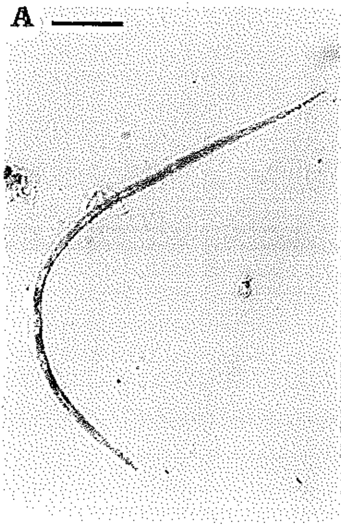
F *Wiesneriomyces* sp. 1

G *Wiesneriomyces* sp. 2

H unidentified JL 004

I unidentified JL 006

Scale bars = 20 μm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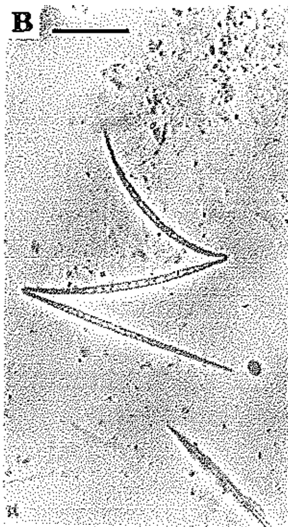
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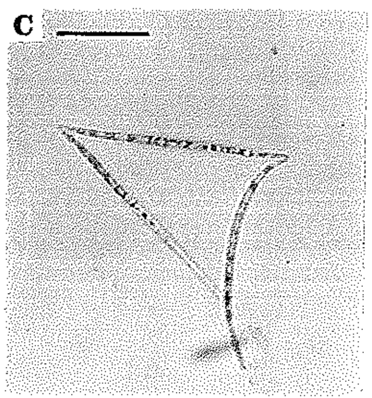
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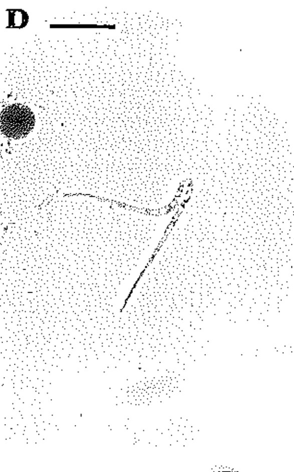
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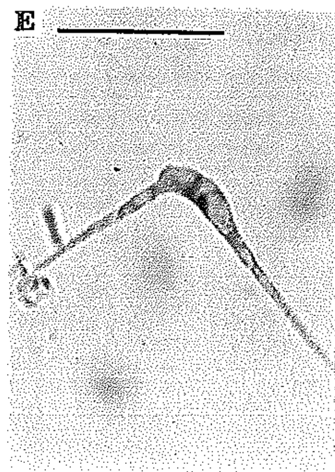
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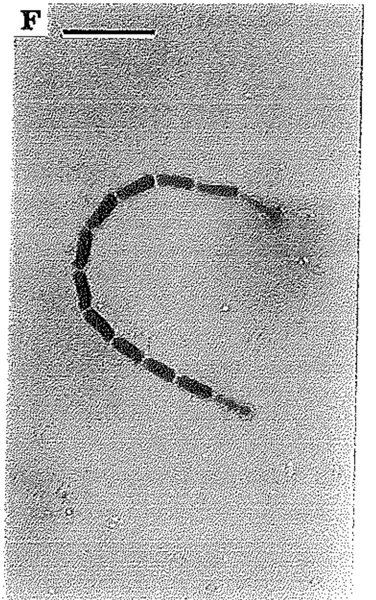
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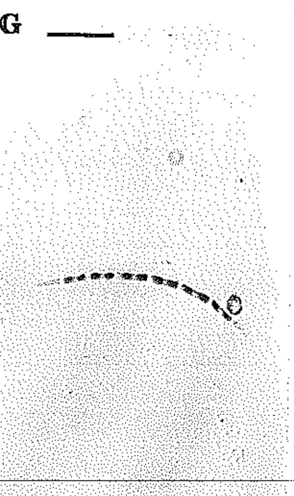
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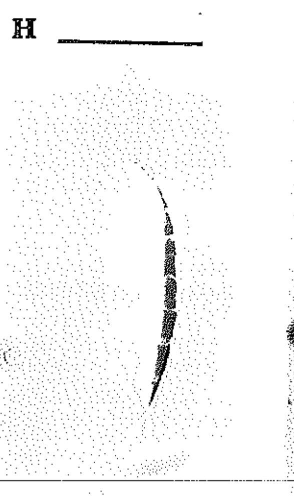
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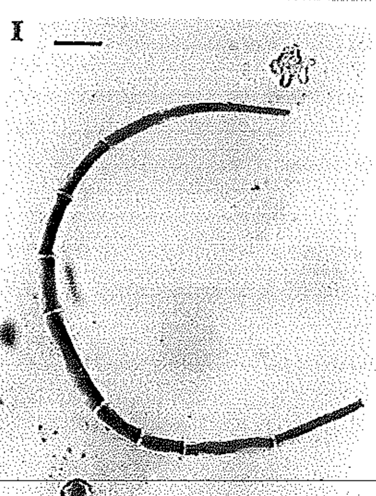
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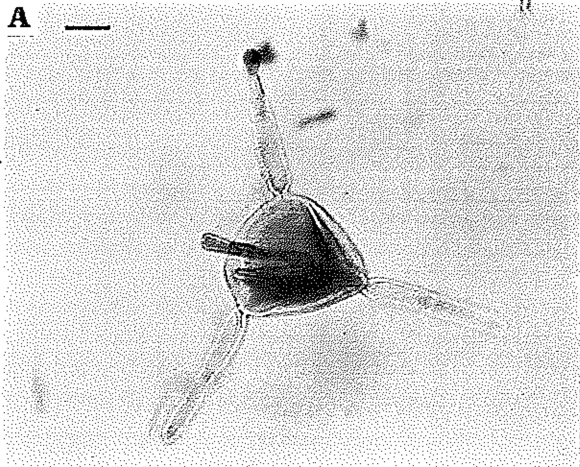
Fig. 15.2. Tetraradiate shape

A	*Brachiosphaera tropicalis*
B	*Clavariopsis* sp.
C	*Quadricladium*-like
D	*Tetrachaetum*-like
E	*Triscelophorus acuminatus*
F	unidentified JT013
G	unidentified JB003
H	unidentified JB003/1
I	unidentified J003/2

Scale bars = 20 μm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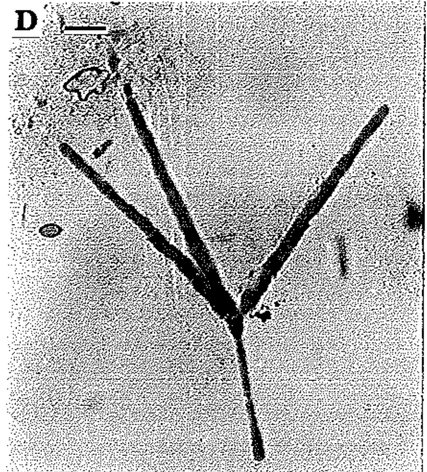
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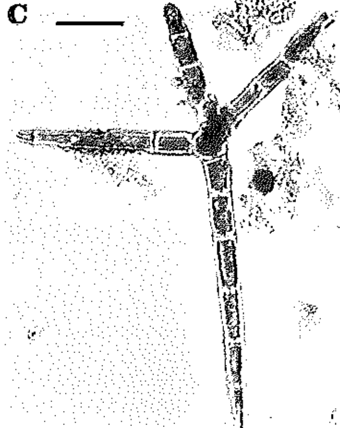
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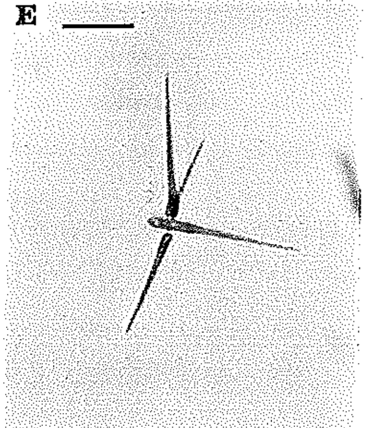
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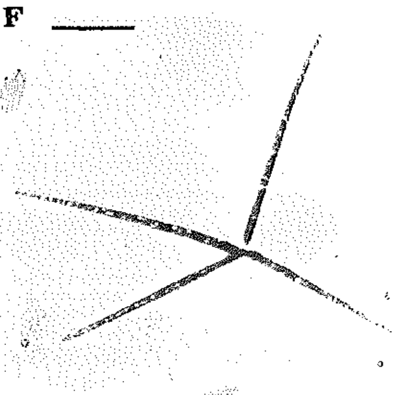
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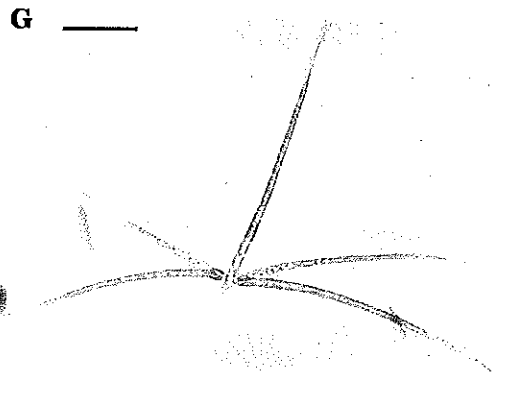
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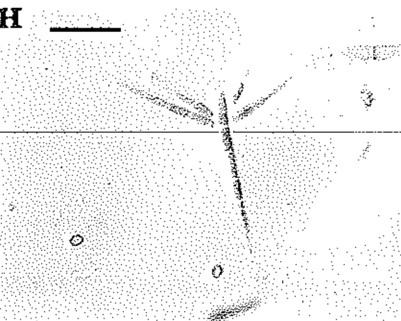
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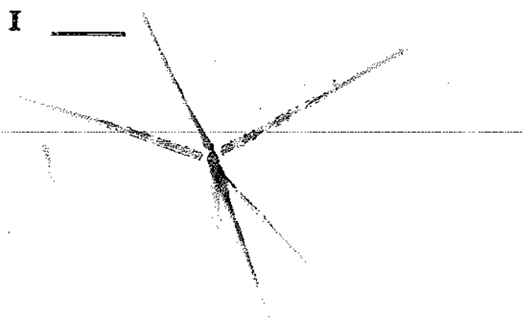
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Fig. 15.3. Branched shape

A *Campylospora chaetocladia*

B *C. filiformis*

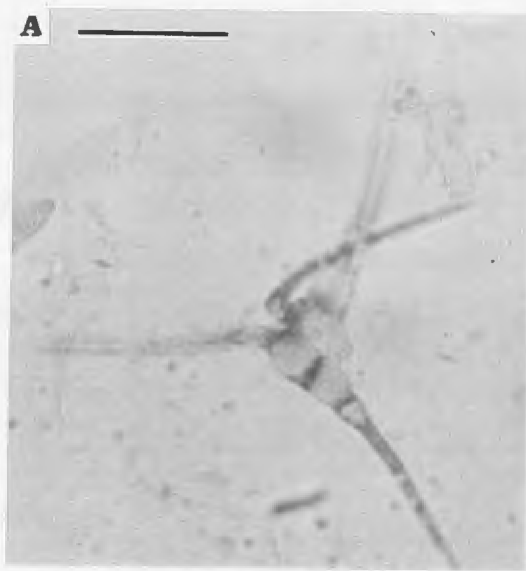
C *Dendrospora*-like

D *Dwayaangam* sp. 1

E *Dwayaangam* sp. 2

F,G *Flabellospora crassa*

Scale bars = 20 μm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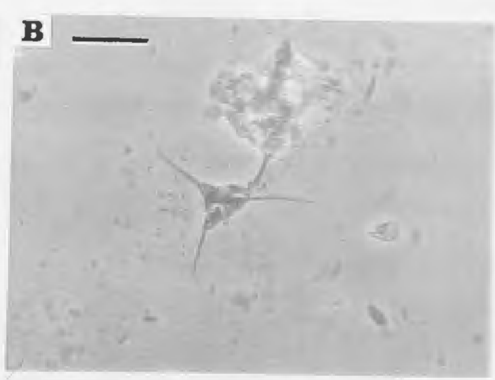
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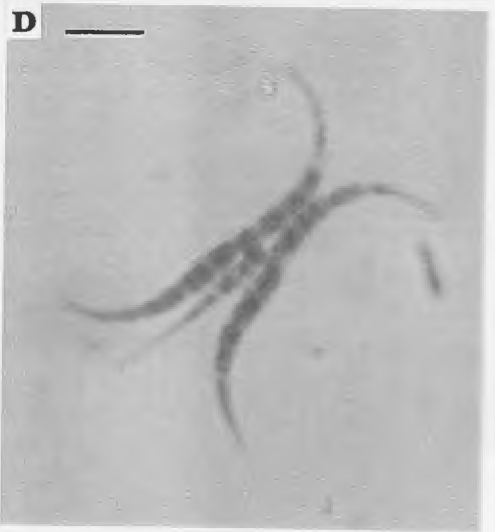
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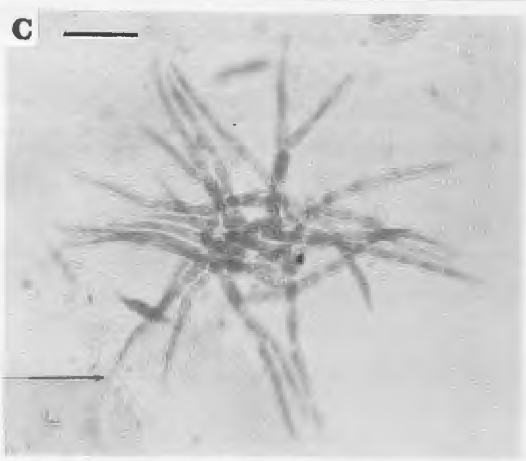
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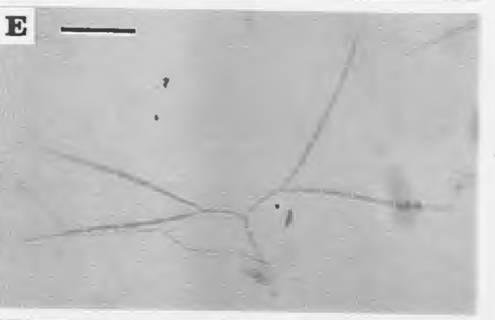
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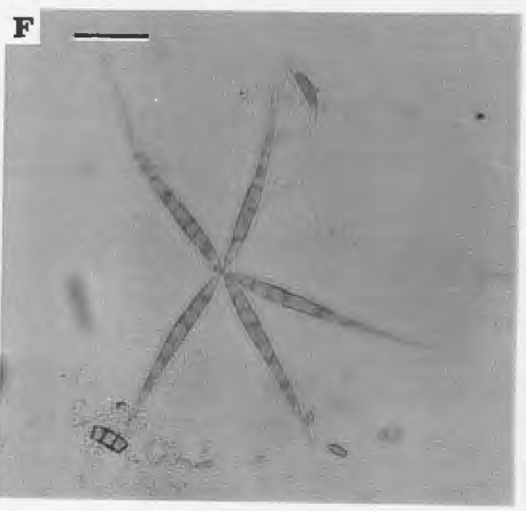
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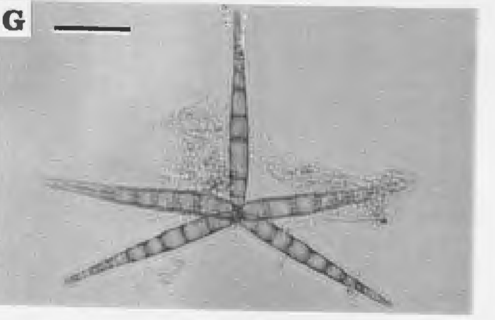
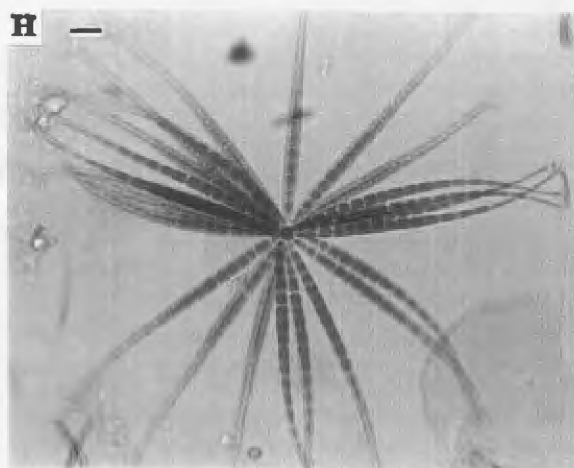

G

Fig. 15.3. Branched shape (continued)

H	*Flabellospora multiradiata*
I, J	*F. verticillata*
K, L	*Isthmotricladia gombakiensis*
M	*I. laeensis*-like
N	*Laridospora appendiculata*

Scale bars = 20 μm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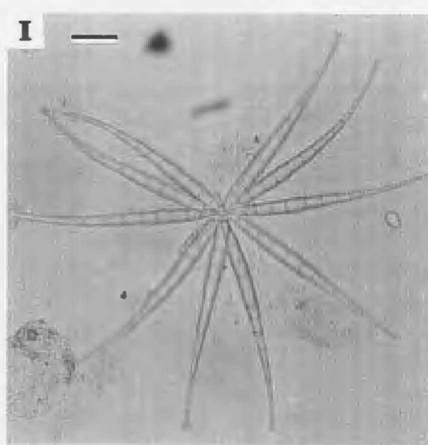
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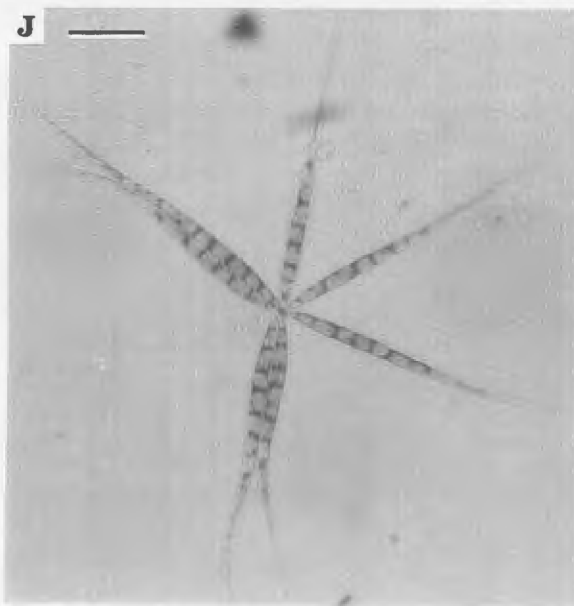
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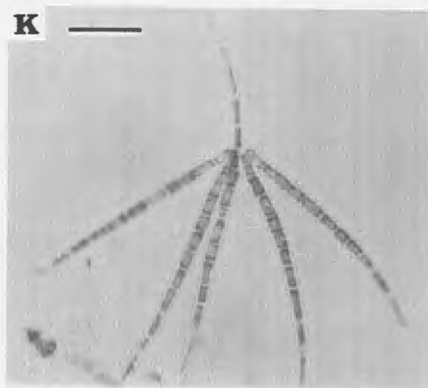
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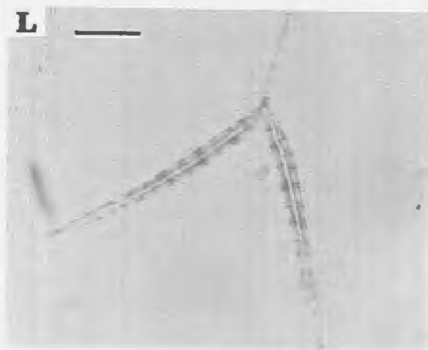
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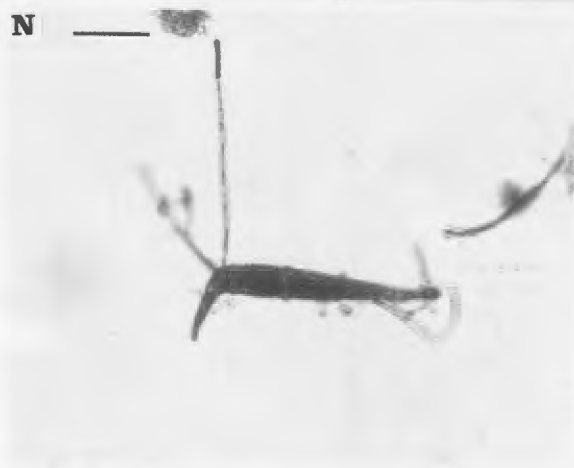

N

M

Fig. 15.3. Branched shape (continued)

O *Nawawia* sp. 1

P *Nawawia* sp. 2

Q *Nawawia* sp. 3

R *Phalangispora constricta*

S *Tricladium aciculum*

T *Trinacrium*-like

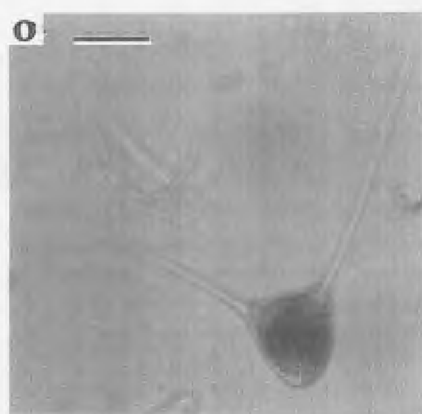
U *Varicosporium giganteum*

V *V. macrosporum*

W unidentified JB 020

X unidentified JB 034

Scale bars = 20 μm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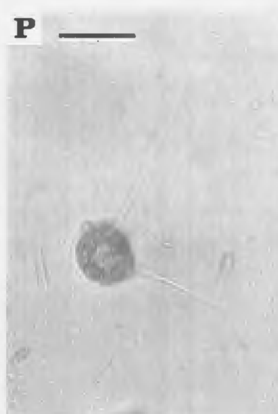
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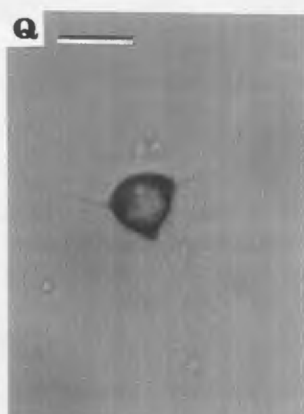
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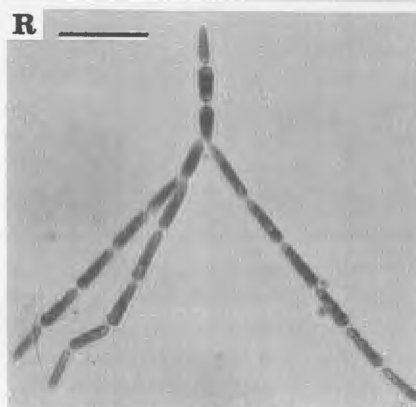
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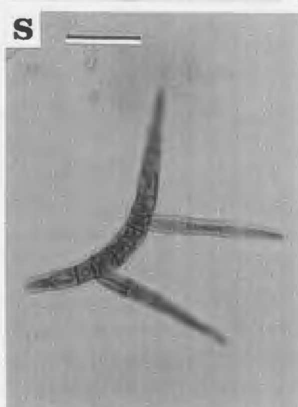
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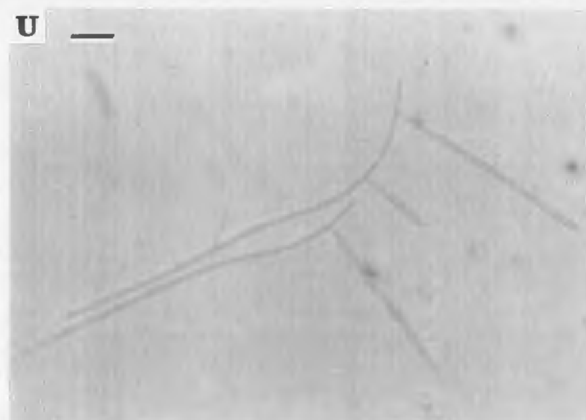
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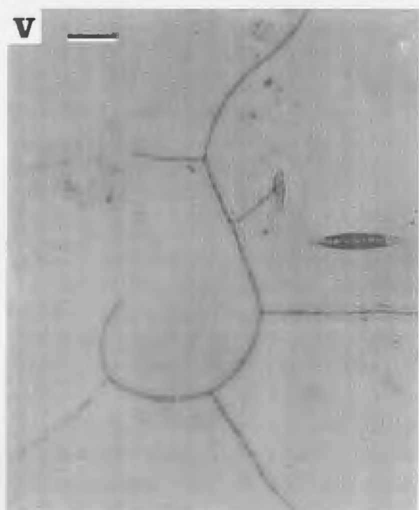

V

W

X

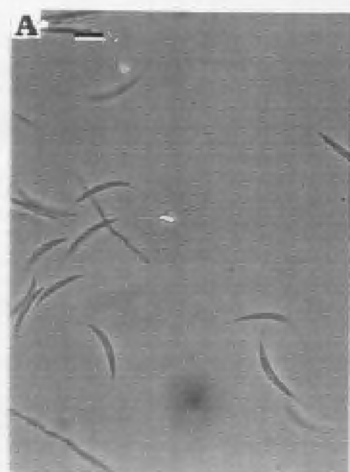
Fig. 15.4. Ovoid shape

A *Thozetella* sp.

B *Volutella* sp.

C unidentified JSP 01

Scale bars = 10 μm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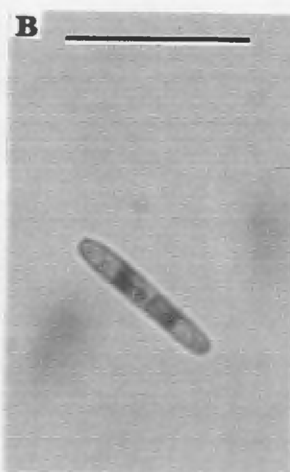
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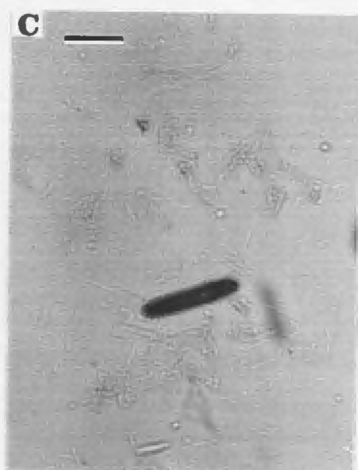
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Fig. 15.5. Helicoid shape

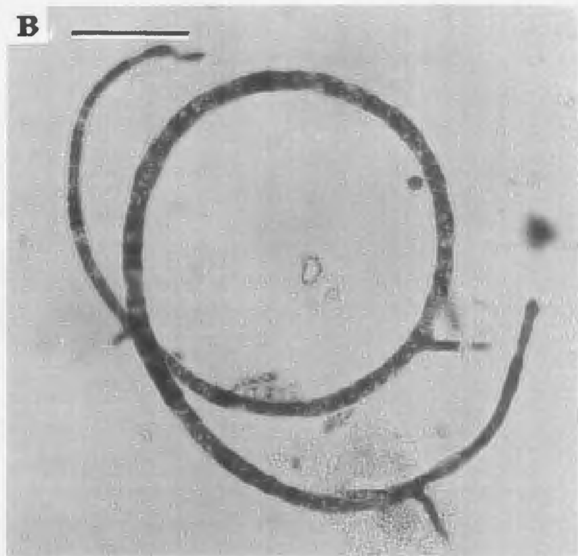
A, B *Helicomyces* sp.

C *Helicosporium* sp. 1

D *Helicosporium* sp. 2

Scale bars = 20 μm

A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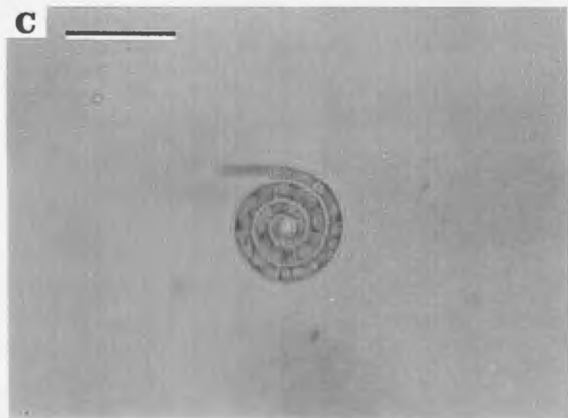
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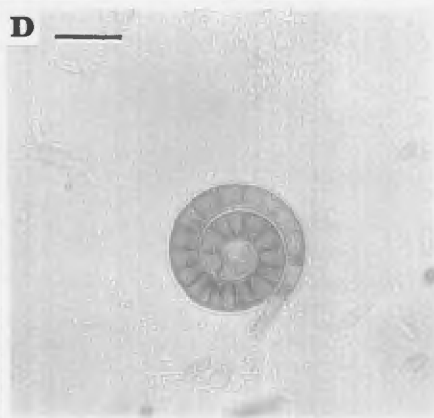

D

Fig. 15.6. Dematiaceous hyphomycetes

A *Beltrania rhombica*

B *Camposporium*-like sp. 1

C *Camposporium*-like sp. 2

D *Diplocladiella appendiculata*

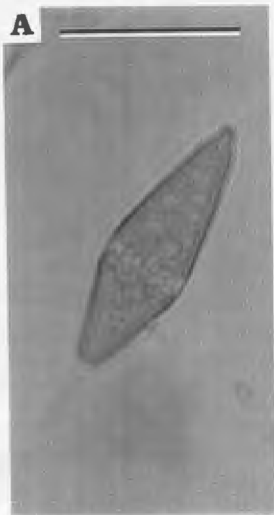
E *D. scalaroides*

F *Pseudobeltrania* sp.

G *Scutisporus* sp.

H *Sporidesmium tropicalis*

Scale bars = 20 µm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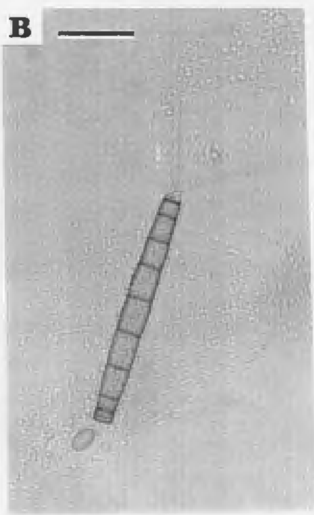
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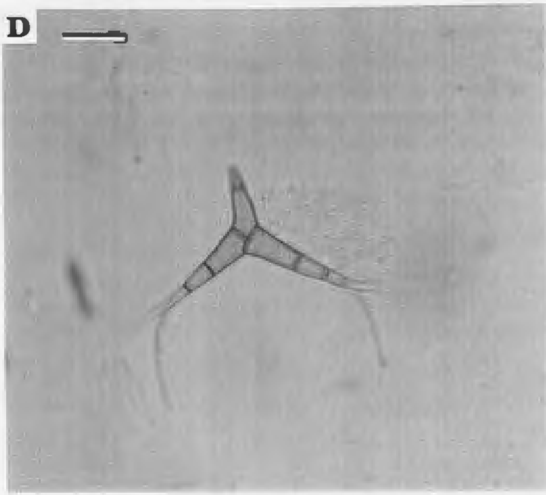
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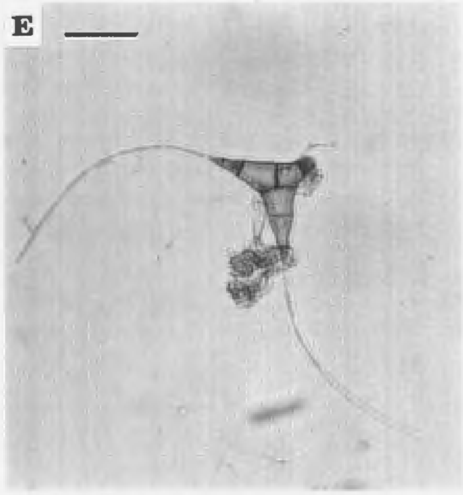
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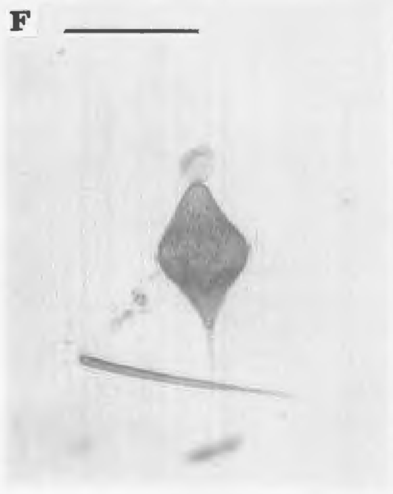
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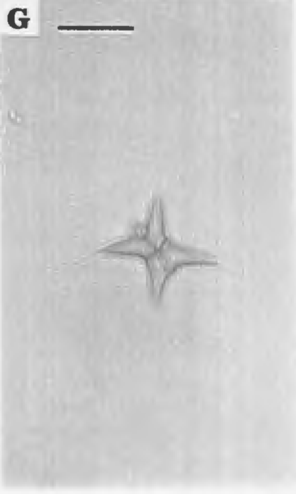
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H

Fig. 15.6. Dematiaceous hyphomycetes (continued)

I unidentified JT 007

J unidentified JBR 04

K unidentified JBR 05

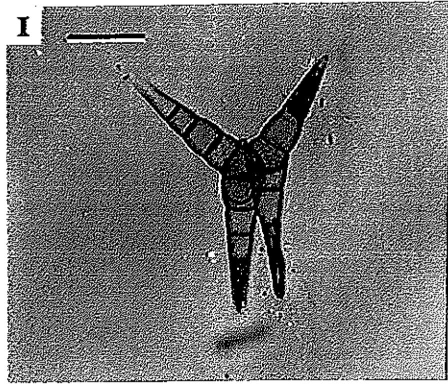
L unidentified JBR 06

M unidentified JBR 07

N unidentified JBR 011

O unidentified JB 036/1

Scale bars = 20 μm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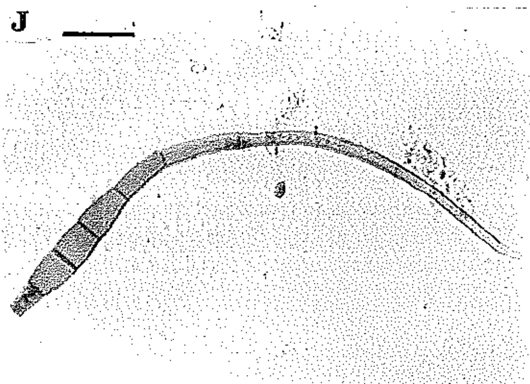
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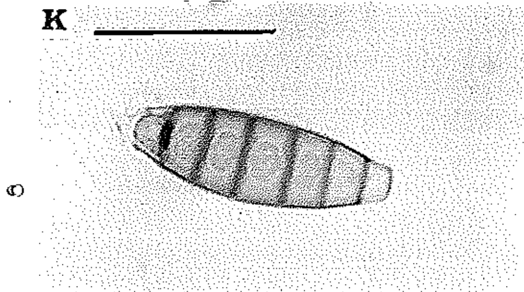
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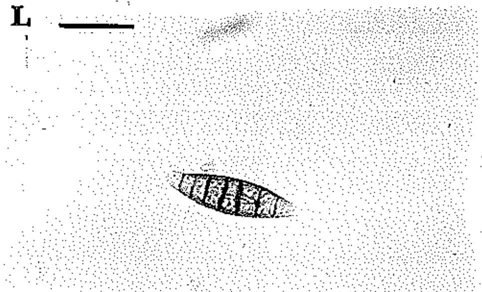
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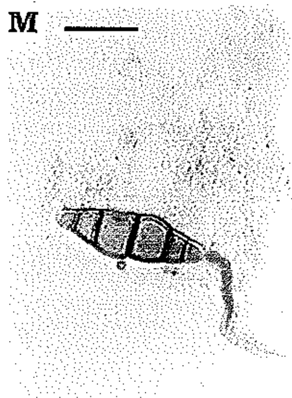
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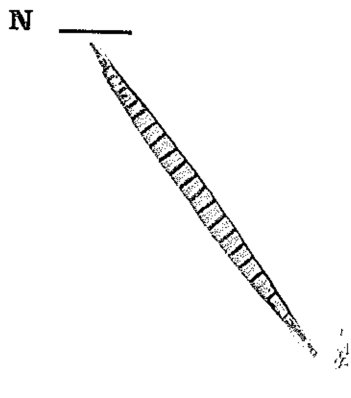
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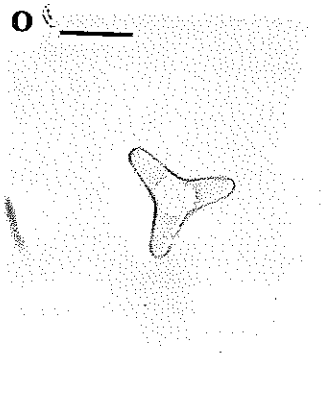

O

Fig. 15.7. Non-hyphomycetes

Basidiomycetes

A, B *Ingoldiella hamata*

Coelomycetes

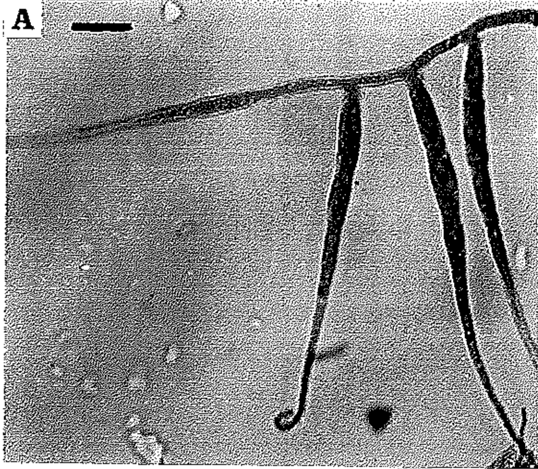
C *Chaetospermum camelliae*

D *Chaetospermum*-like

E *Pestalotia* sp.

F *Robillarda* sp.

Scale bars = 20 μm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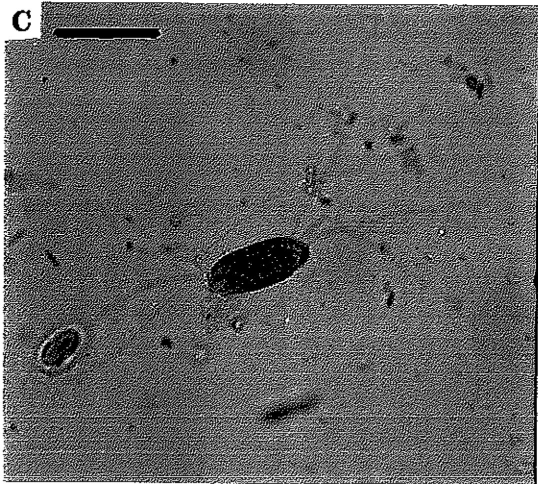
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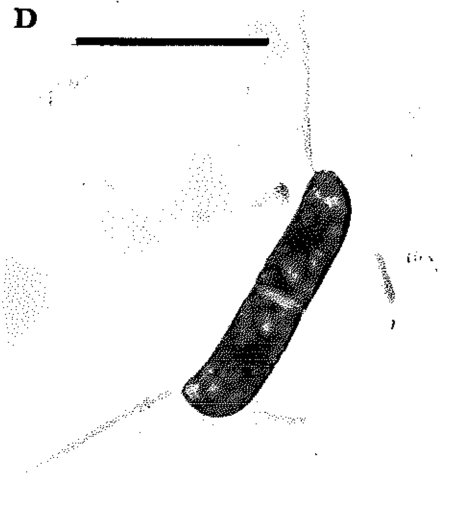
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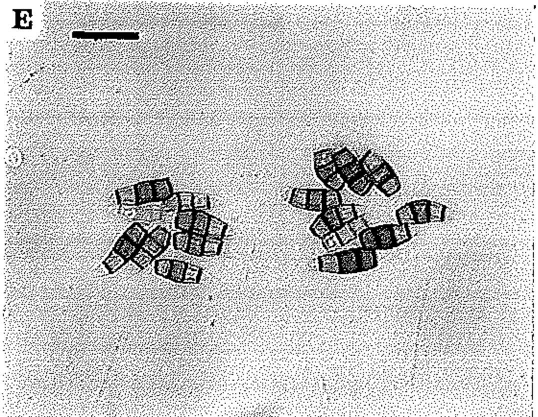
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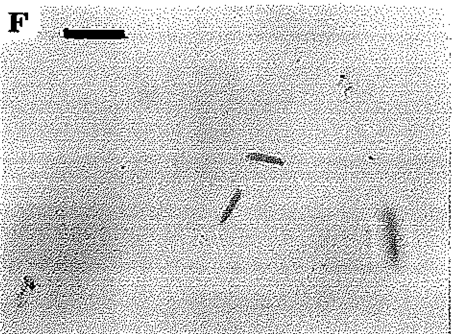

F

Numerous branched spores, particularly tetraradiate spores, were trapped and accumulated in higher number of species than other, more regular types of conidia such as ovoid or sigmoid shape. According to Iqbal & Webster (1973a), tetraradiate and branched spores were more easily trapped due to their high efficiency in being brought to the surface of water by air bubbles and caught up in foam.

Some of the terrestrial or non-aquatic hyphomycetes were also found in foam samples, such as *Beltrania rhombica, Pestalotia* sp., *Volutella* sp., *Thozetella* sp. and other dematiaceous hyphomycetes. It was possible these were washed into the river from leaf detritus or from soil on the bank during or after heavy rain (Ingold, 1968; Tan & Koh, 1995). Tan & Koh (1995) also found the non-aquatic hyphomycetes e.g. *B. rhombica*, commonly in foam. They concluded that rainfall may wash some terrestrial fungi into the streams, and it was probable that terrestrial fungi can adapt and survive in a submerged aquatic environment.

The most abundant species in foam in my study were the genera *Anguillospora* sp. and *Triscelophorus* sp., which were found consistently at all collecting sites, and throughout the period of observation from June-September. The fungal spores were common and similar with those found in the north and central part of Thailand (Hywel-Jones, unpubl. obs., Tubaki *et al.*, 1983). Examples of hyphomycete genera commonly found in the Northern and central

regions, and also in my study, include: *Anguillospora* sp., *Campylospora, Clavariopsis, Flabellospora, Isthmotricladia, Lunulospora, Phalangispora* and *Triscelophorus*.

Sivichai (2000) studied the aquatic fungi on wooden test blocks submerged in streams. Most of the fungi colonizing the test blocks were lignicolous ascomycetes and their anamorphs, although few Ingoldian fungi reported. I conclude that most Ingoldian fungi grow on leaves, with only some colonizing wood, and produce large numbers of conidia which can be trapped in foam.

In my study, 48 species of fungi were found in foam samples. It was observed that during the period of study, there was the richness of species compare with the other studies in Thailand. Tubaki *et al.* (1983) and Hywel-Jones (unpubl. obs.) reported only 20 species and 10 species of Ingoldian fungi from the North and central part of Thailand, respectively.

Additionally, some species found in my study commonly occurred in Malaysia and Singapore, such as, *Anguillospora* sp., *Beltrania rhombica, Dwayaangam* sp., *Flabellospora multiradiata, F. verticillata, Ingoldiella hamata, Isthmotricladia* spp., *Tricladium aciculum, Triscelophorus acuminatus* and *Varicosporium macrosporum* (Nawawi, 1974a, b; 1975a, b; 1976 a, b; 1985a, b; Tan & Koh, 1995). It may be possible that because of the small variations of

environment factors such as the geographic characteristics, climate and riparian vegetation structure between the south of Thailand and these two countries, therefore freshwater hyphomycetes occurred similarly in these regions.

4.3.2 Stream water

Fungal conidia in stream waters were observed from week 8 to week 14 (August to September) in order to compare the fungi with those found in foam samples. Table 13 shows the number of conidia found in filtered stream water at different collecting sites. In weeks 8 and 10, there was a slight trend showing an increase in conidia at sites downstream. It is possible that the conidia in stream water were accumulating from upstream to downstream from week 8 to week 10. The spore accumulation may be the result of heavy rain and the water current which can wash the spores in foam and accumulated in a downstream direction. However, this trend could not be supported for week 12 and 14. Importantly, there seemed to be a strong trend with time. Weeks 8 and 10 produced higher numbers of conidia in samples than week 12 and 14. It may be because of the availability of substrata or the result of low rate of leaf colonization by aquatic

Table 13. Total conidia in stream water at the four collecting sites.

Collections / Sites	Wk 8 Aug	Wk 10 Aug	Wk 12 Sept	Wk 14 Sept
Site I	460	444	320	164
Site II	458	729	137	134
Site III	826	648	183	149
Site IV	740	828	413	145
Total (conidia /l)	**2484**	**2649**	**1053**	**592**
Total (conidia /ml)	**2.48**	**2.64**	**1.05**	**0.59**

fungi in the stream. Additionally, the build up of the conidia may take time for a week, thus the conidia in water which were detected in this period became rare and lower numbers than weeks 8 and 10.

The dominant species found in stream waters were mainly *Anguillospora* sp. and dematiaceous hyphomycetes. Table 12 shows only a few species of hyphomycetes which were found in stream water compared with those found in foam. This present observation was similar to the study of Iqbal & Webster (1973a) who found fewer branched spores but more sigmoid and ovoid spores in river water. It is concluded that mostly branched and tetraradiate spores were trapped in foam, thus those which were left in stream water were regular shapes, such as ovoid and sigmoid shapes.

4.4 Pure culture collections

Sixty-five pure isolates were obtained from foam samples by the single spore and hyphal tip isolation method. Twenty-four isolates produced conidia on corn meal and potato dextrose agar. Only 15 isolates were identified as listed in Table 14. The others were not sporulating on the agar, but produced dark brown or black chlamydospore packed firmly in the mycelium (Table 15).

Only 36.9% of the isolates sporulated in culture and only 23% were identified. The first possible reason is that because of heavy bacterial contamination of the natural foam samples. The addition of a high concentration of antibiotics in the agar may have caused effect to the germination of some conidia. The second reason is probably because the isolation media which was not enriched, thus some conidia could not germinate. Additionally, the other possibility is that while examining the foam samples, only one conidium was found once on the slide, thus the pure culture could not obtained because there were not enough conidia for isolation.

Table 14. Sporulating cultures.

Culture code	**Conidial production on agar**	**Identifications**	**Remarks**
T001/1	CMA	*Wiesneriomyces* sp.	
T002/4	PDA	UI	Black, ovoid shape conidia, 10 μm long
T005/2	PDA	UI	Black, ovoid shape conidia, 10 μm long, formed in a black acervuli
T005/5	CMA, PDA	*Pestalotia* sp.	
T007	CMA, PDA	*Pestalotia* sp.	
T013/1	CMA, PDA	*Pestalotia* sp.	
T015	CMA	*Robillarda* sp.	
T016/1	PDA	UI	Black, ovoid shape conidia, 10 μm long, formed in a mucous pink acervuli
T024	PDA	UI	Sigmoid shape conidia
T026	PDA	UI	Ovoid shape conidia containing in acervuli

Table 14. (continued)

Culture code	**Conidia production on agar**	**Identifications**	**Remarks**
T027	CMA, PDA	UI	Cylindrical shape conidia, 20 µm long
T028	CMA	UI	Round shape conidia containing in packed hyphae
T031	CMA	*Beltrania rhombica*	
T037	CMA	UI	Black, round shape conidia
T038	CMA	UI	Black, round shape conidia contained in black acervuli
T041	CMA	*Helicomyces* sp.	
T042	CMA	*Varicosporium* sp.	
J10	CMA,PDA	*Thozetella* sp.	
JL001	CMA,PDA	*Anguillospora* sp.	
B009	CMA,PDA	*Volutella* sp.	
V3B1	CMA,PDA	*Volutella* sp.	
V02	CMA,PDA	*Volutella* sp.	

Table 14. (continued)

Culture code	**Conidia production on agar**	**Identifications**	**Remarks**
V04	CMA,PDA	*Volutella* sp.	
V06	CMA,PDA	*Volutella* sp.	

CMA = Corn meal agar

PDA = Potato dextrose agar

UI = unidentified

Table 15. Non-sporulating cultures.

Culture code	Sporulation	Remarks
T001	Chlamydospore	
T001/2	Chlamydospore	
T001/3	Chlamydospore	
T001/4	Chlamydospore	
T002	Chlamydospore	
T004	-	No sporulation
T005	Chlamydospore	
T005/1	-	Yeast like colony
T005/3	-	No sporulation
T005/4	Chlamydospore	
T006	-	No sporulation
T008	-	No sporulation
T008/1	-	No sporulation
T009	-	No sporulation
T010	-	Yeast like colony
T012	Chlamydospore	
T013	-	Yeast like colony
T014	Chlamydospore	

Table 15. (continued)

Culture code	Sporulation	Remarks
T016	Chlamydospore	
T018	Chlamydospore	
T019	Chlamydospore	
T020	Chlamydospore	
T021	Chlamydospore	
T022	-	No sporulation
T023	-	No sporulation
T025	Chlamydospore	
T030	Chlamydospore	
T032	Chlamydospore	
T033	-	No sporulation
T035	Chlamydospore	
T036	-	No sporulation
T043	-	No sporulation
T044	-	No sporulation
T045	-	No sporulation
T046	Chlamydospore	
T047	Chlamydospore	

4.5 Identifications

Descriptions of hyphomycetes, coelomycetes and basidiomycetes in foam samples are listed alphabetically.

Hyphomycetes

1. Long, sigmoid shape

Fig. 16. *Anguillospora* sp. (Descals, 1997; Marvanová, 1997)

Conidial description: Conidia unbranched, scolecoid or filiform, eventually with a percurrent basal extension or excentric basal extension, 200-280 μm long, 2-3 μm wide. (scale bar = 20 μm)

Culture description: Conidia production on corn meal agar. Colony dark gray, margin entire, aerial mycelium very sparse. Thread-like conidial shape. Sporulation superficial on partly submerged mycelium in water.

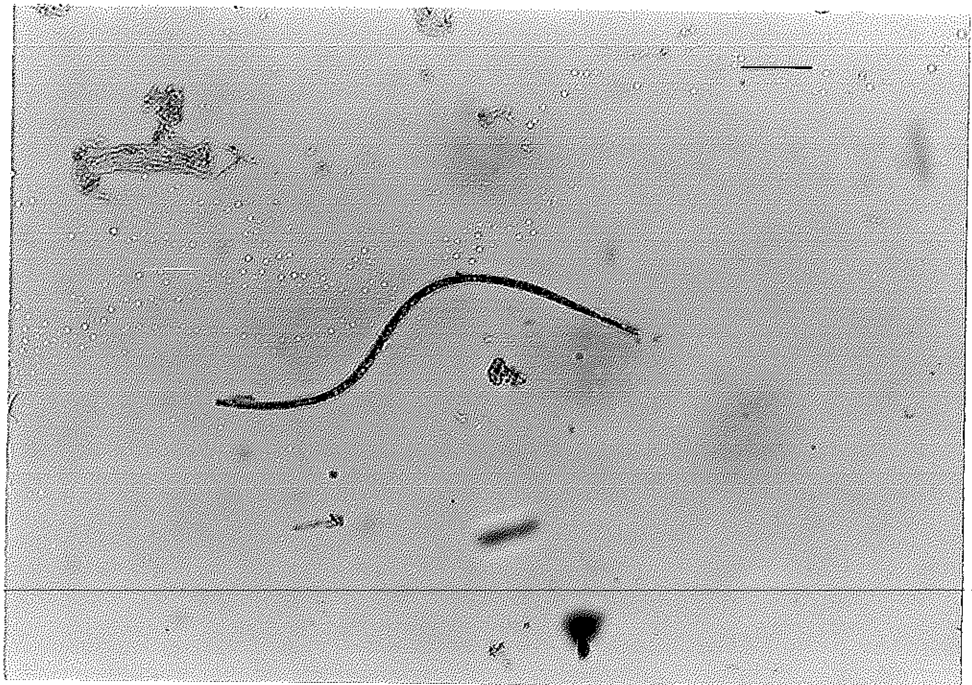


Fig. 17. *Anguillospora*-like (Marvanová, 1997)

Conidial description: Conidia unbranched, filiform or thread-like, conidia bent like knee, approximately 100-150 μm long, 2-3 μm wide. (scale bar = 20 μm)

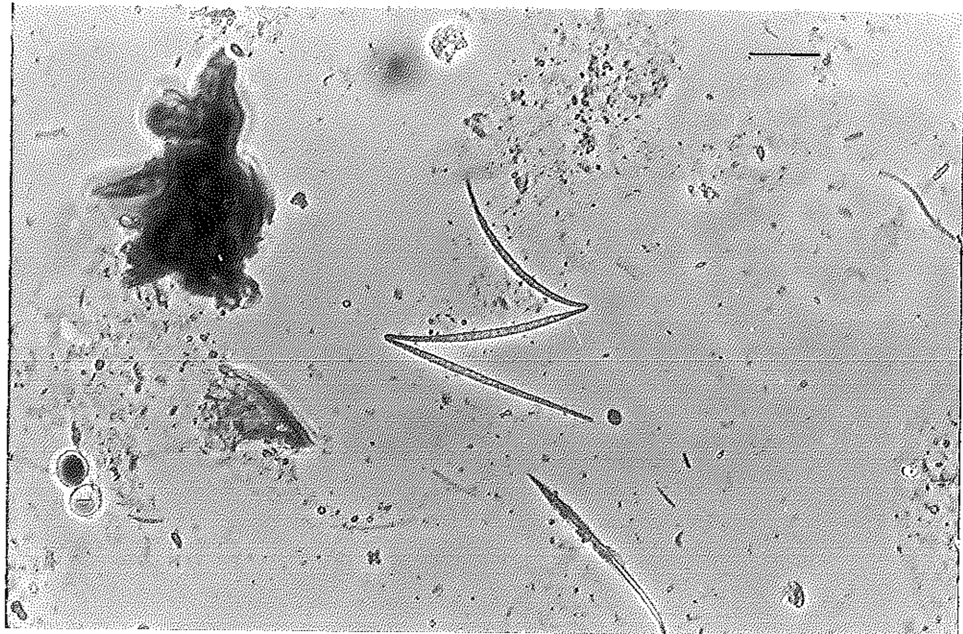


Fig. 18. *Condylospora spumigena* (Marvanová, 1997)

Conidial description: Conidia unbranched, filiform, septate with a single "kink", 72-104 μm long, 2-3 μm wide. (scale bar = 20 μm)

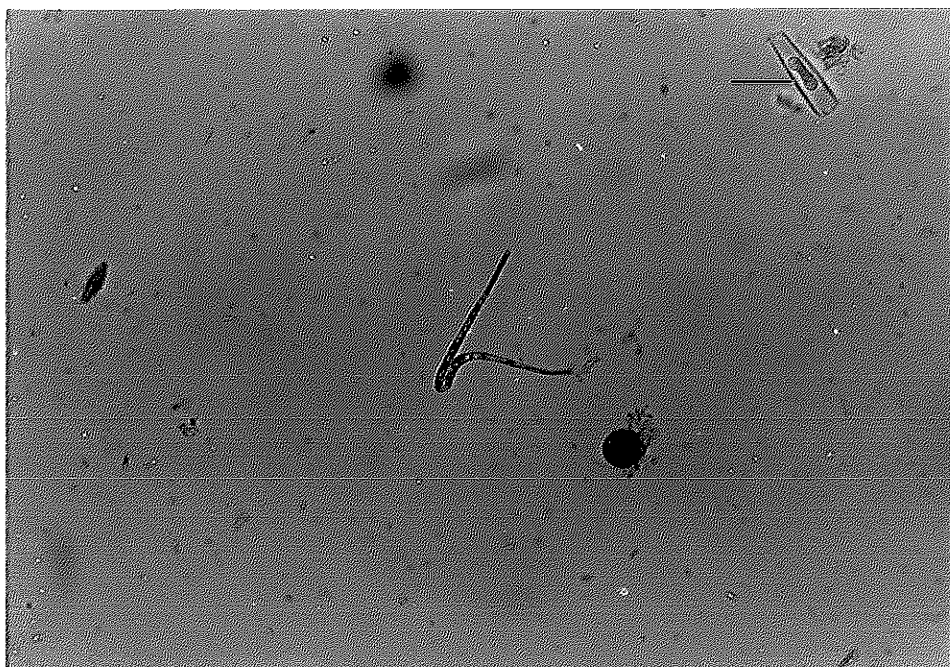


Fig. 19. *Lunulospora cymbiformis* (Marvanová, 1997)

Conidial description: Conidia unbranched, scolecoid or filiform with lateral detachment scar at a third of the way from one end of conidium. Lunate or L-shaped conidia, often inflated in the lower third, detachment scar prominent, ends subulate. (scale bar = 20 μm)

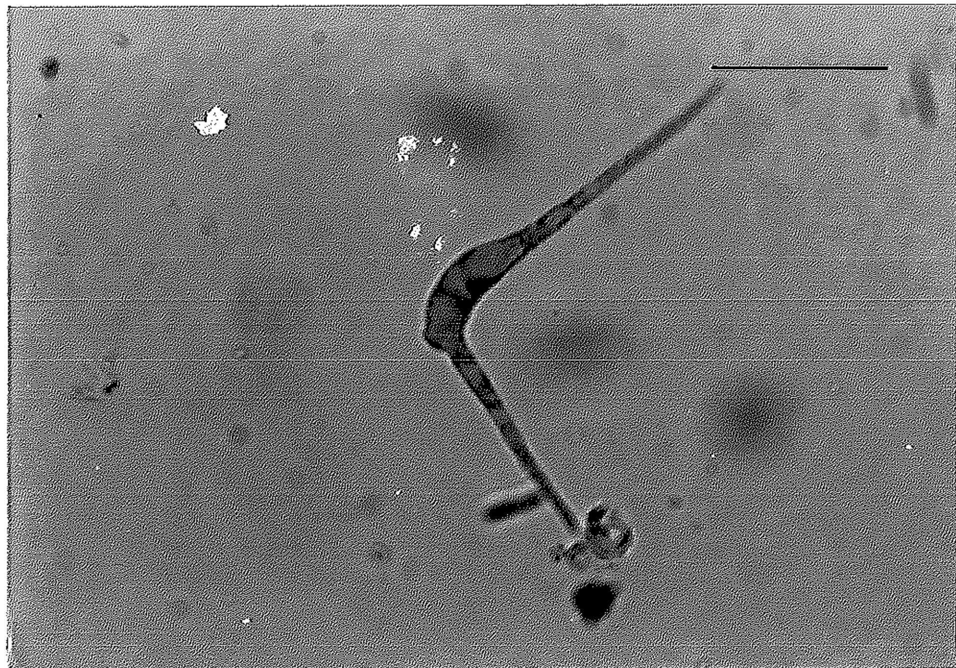


Fig. 20. ***Wiesneriomyces*** **sp. 1** (Kuthubutheen & Nawawi, 1988)

Conidial description: Phragmospore break up into 1 more-celled bits, 8-12 cells produced into the conidial chain which are connected by thin hyphal bridges. Conidial chains hyaline, tapering at both ends, falcate, 70-100 x 3-4 μm (scale bar = 20 μm)

Culture description: Colony with dark gray, margin entire, aerial mycelium very sparse. Phragmospore shape. Sporulation occurs superficial on partly submerged mycelium in water.

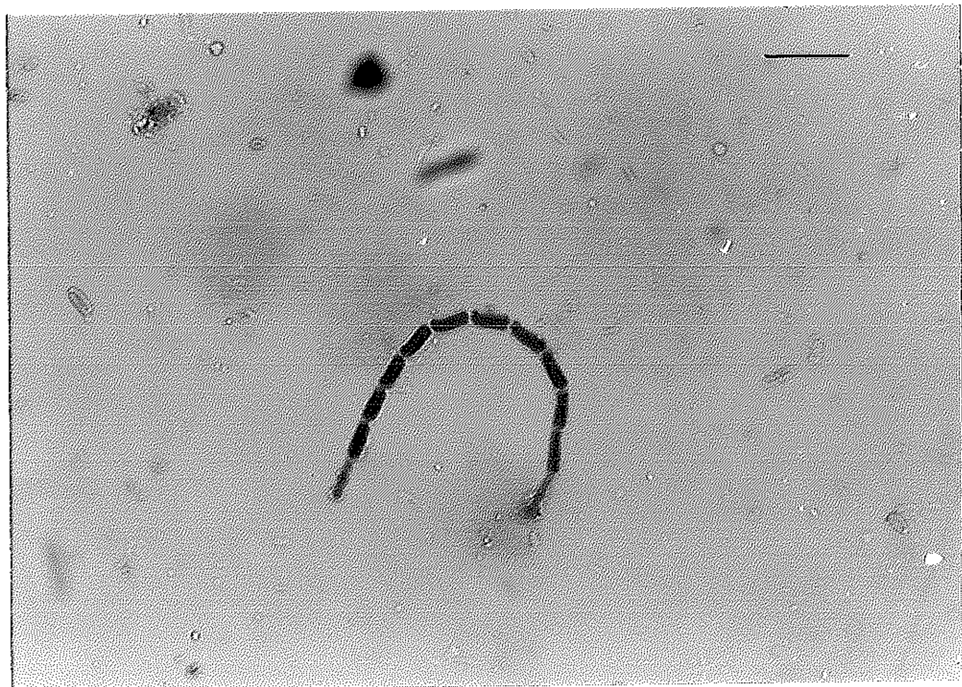
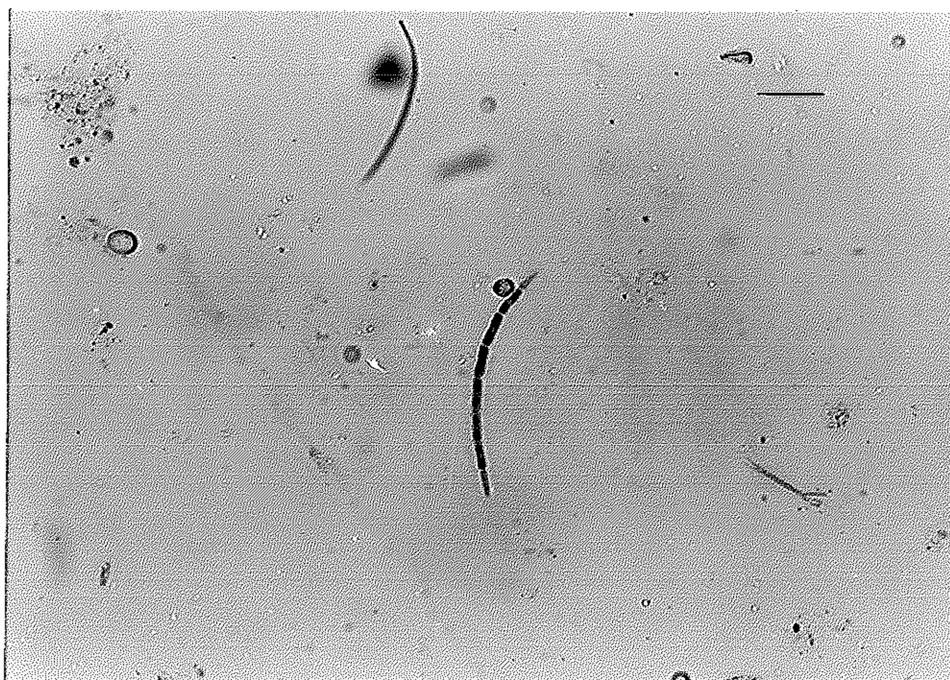


Fig. 21. *Wiesneriomyces* **sp. 2** (Kuthubutheen & Nawawi, 1988)

Conidial description: Phragmospore with break up into 1 more celled bits, 6–8 cells produced into the conidial chain which are connected by thin hyphal bridges. Conidial chains hyaline, tapering at both ends, falcate, 58–73 x 2–3 μ m, with individual. (scale bar = 20 μm)



2. Tetraradiate shape

Fig. 22. *Brachiosphaera tropicalis* (Marvanová, 1997)

Conidial description: Tetraradiate conidia, conidial body globose, turbinate or short clavate (length/ width ratio ca. 1:3 or less). The central sphere 40-60 μ m diameter. Branches inserting distinctly constricted with 4-5 branches, 90-190 μm long x 9-11 μm wide, 3-5 septate. (scale bar = 50 μm)

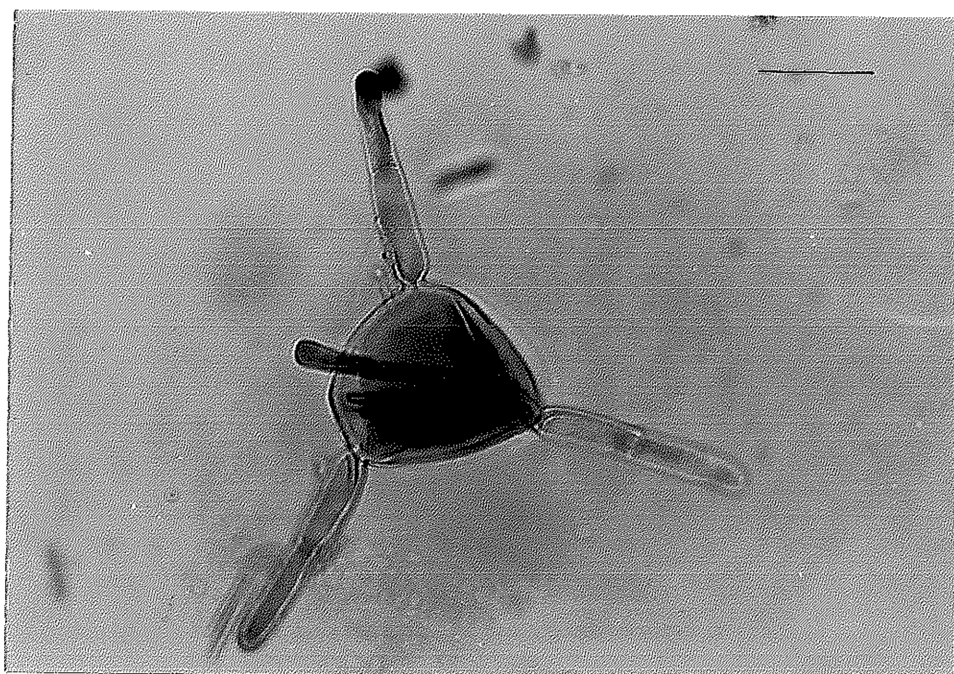


Fig. 23. *Clavariopsis* sp. (Ingold, 1975)

Conidial description: Conidium with clavate conidial body. Each conidium typically consists of 4 arms, septate, 30-50 x 10-15 μm, length-width ratio 2-3. (scale bar = 20 μm)

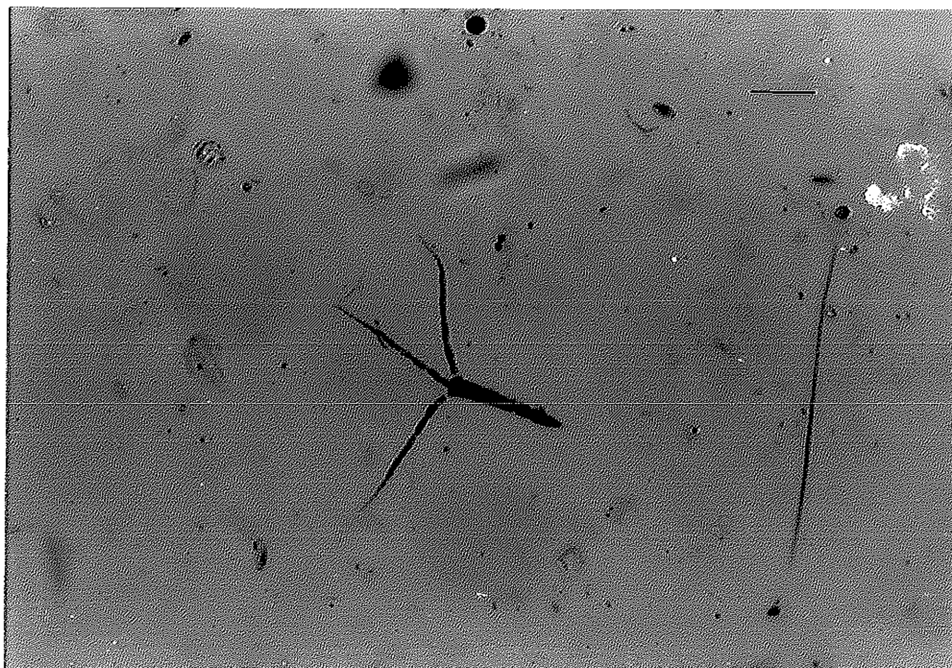


Fig. 24. *Quadricladium*-like (Ingold, 1975; Nawawi & Kuthubutheen, 1989)

Conidial description: Tetraradiate conidia, typically consisting of 4 broardly diverging arms, multiseptate. Conidial elements cylindrically, slightly, tapering distally 40-70 x 6.5-7.5 µm. (scale bar = 20 µm)

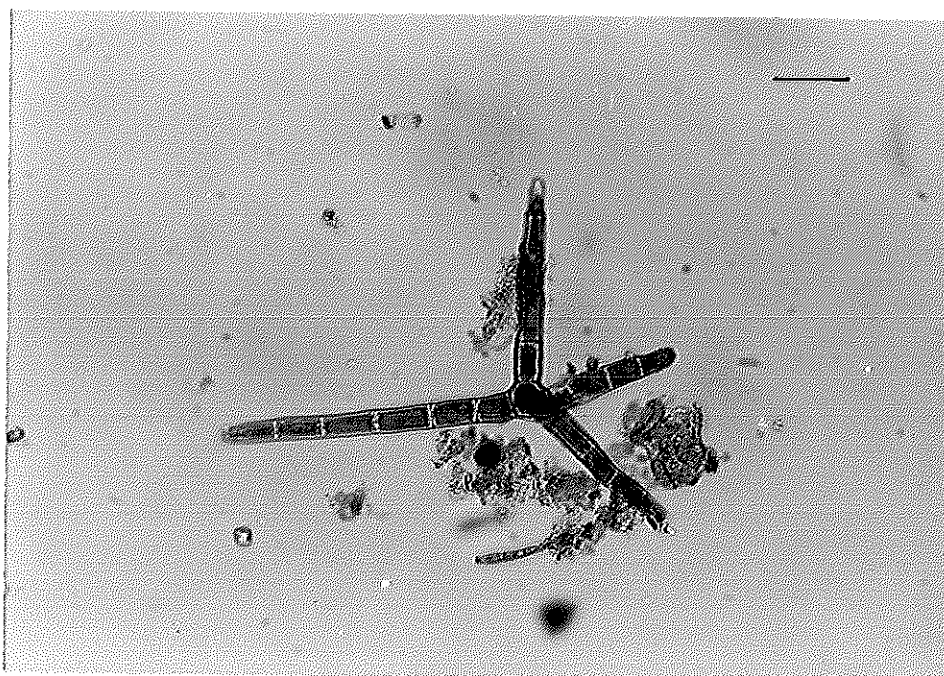


Fig. 25. *Tetrachaetum*-like (Ingold, 1975)

Conidial description: Tetraradiate conidium consisting of 4 broardly diverging arms, multiseptate. Branches are 190 µm long, main axis is 140 x 5 µm. (scale bar = 20 µm)

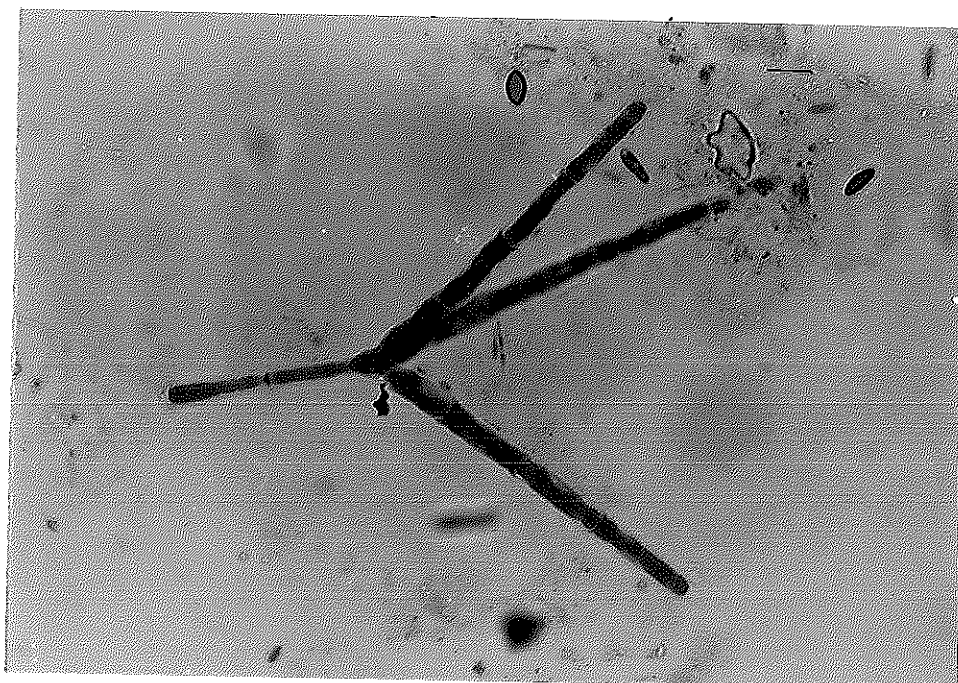
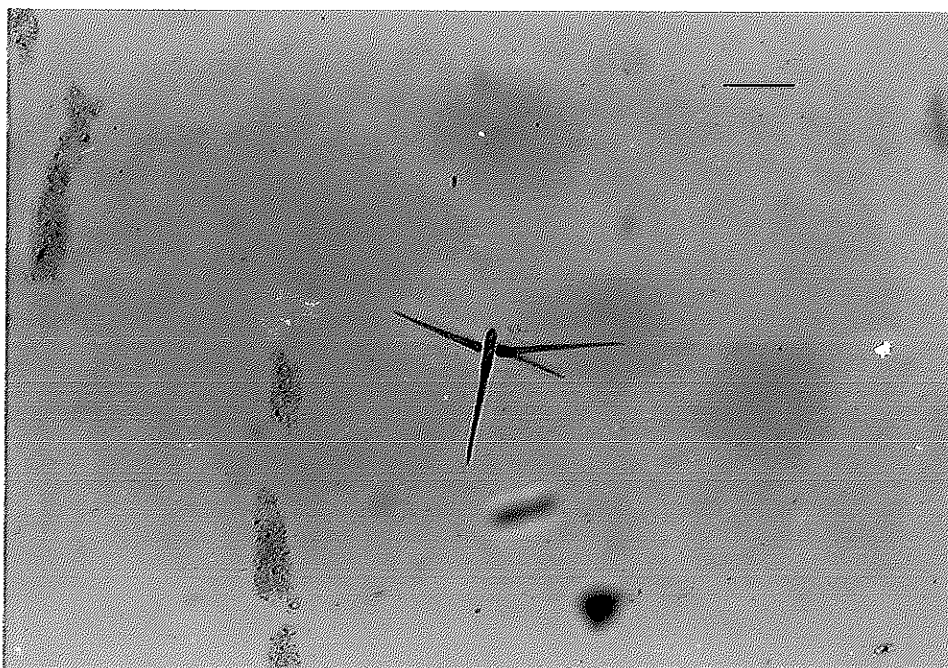


Fig. 26. ***Triscelophorus acuminatus*** (Marvanová, 1997)

Conidial description: The tetraradiate conidia, typically consists of four broadly diverging arms, axis obclavate, multiseptate, branches cylindrical, axis 40-70 x 3 µm long, branches 30-50 µm. (scale bar = 20 µm)



3. Branched shape

Fig. 27. *Campylospora chaetocladia* (Ingold, 1975; Marvanová, 1997)

Conidial description: The conidial body comprise of 2 parts: a triangular and a fusoid one, large with branches spanning 70-100 μm, branches longer than the conidial body, integrated and straight. (scale bar = 20 μm)

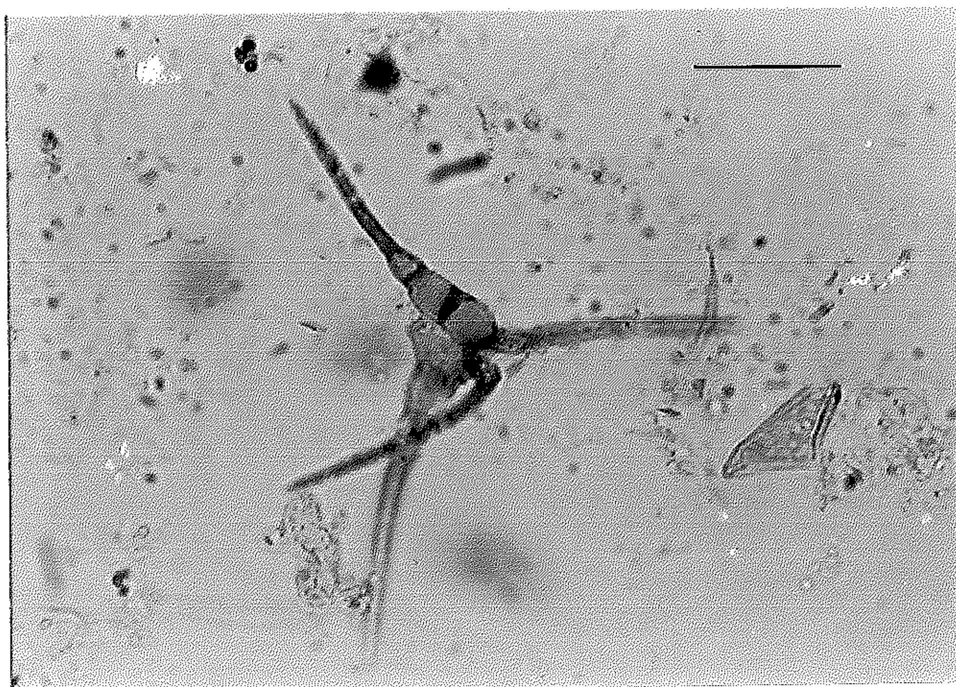


Fig. 28. *Campylospora filicladia* (Ingold, 1975; Nawawi, 1974b)

Conidial description: Septate mycelium, hyaline, comprise of 2 halves. Conidial body less than 20 µm. The proximal half is triangular, 4-celled measuring 6-7.5 µm high and 10-12 µm wide. The distal half is allantoid, 4-celled and measures 9-13 µm long, 3-4.5 µm wide. (scale bar = 20 µm)

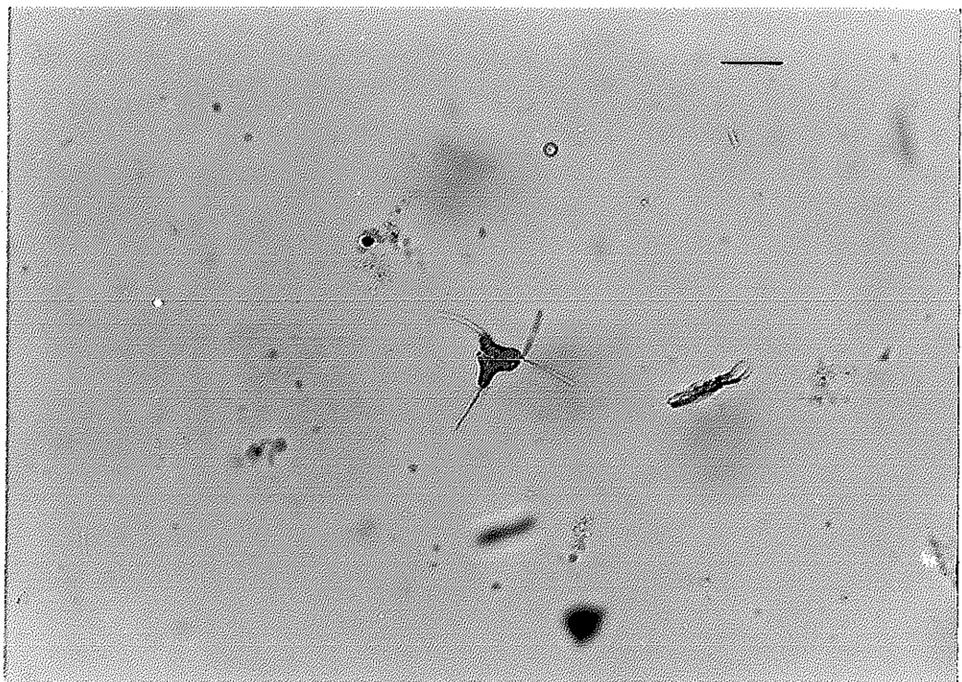


Fig. 29. ***Dendrospora*-like** (Ingold, 1975)

Conidial description: The terminal thalloconidium consists of a straight main axis, from near the base of which whorls of laterals arise at more than one level and some of these laterals may show themselves basal branching.

(scale bar = 20 μm)

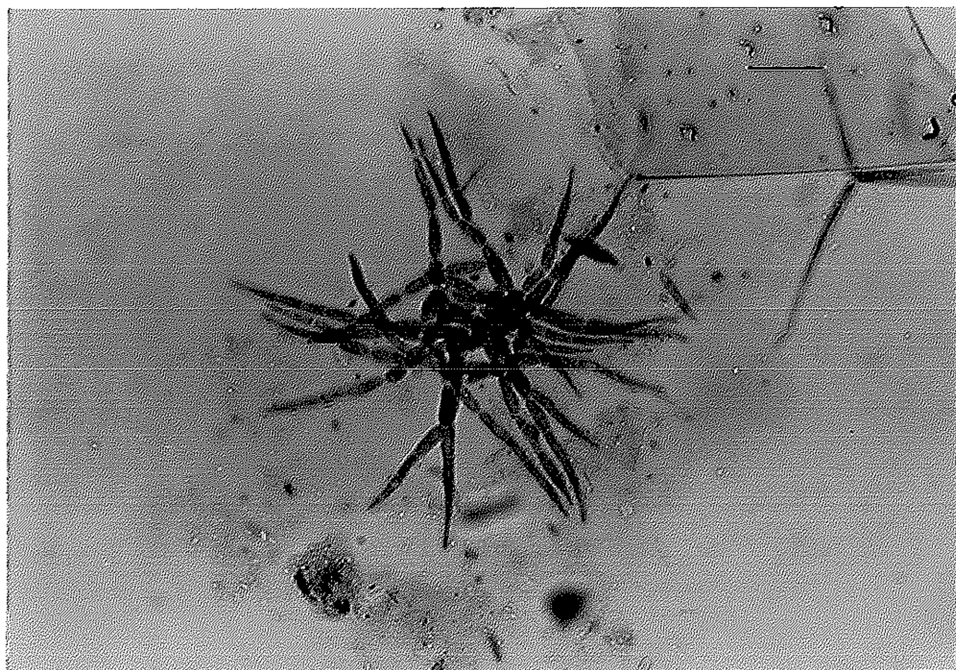


Fig. 30. ***Dwayaangam* sp. 1** (Descals & Webster, 1982)

Conidial description: Conidia multicellular, 50-80 μm long, 50-60 μm wide. Resemble bovine horns, consisting of 2 halves and a main axis.

(scale bar = 20 μm)

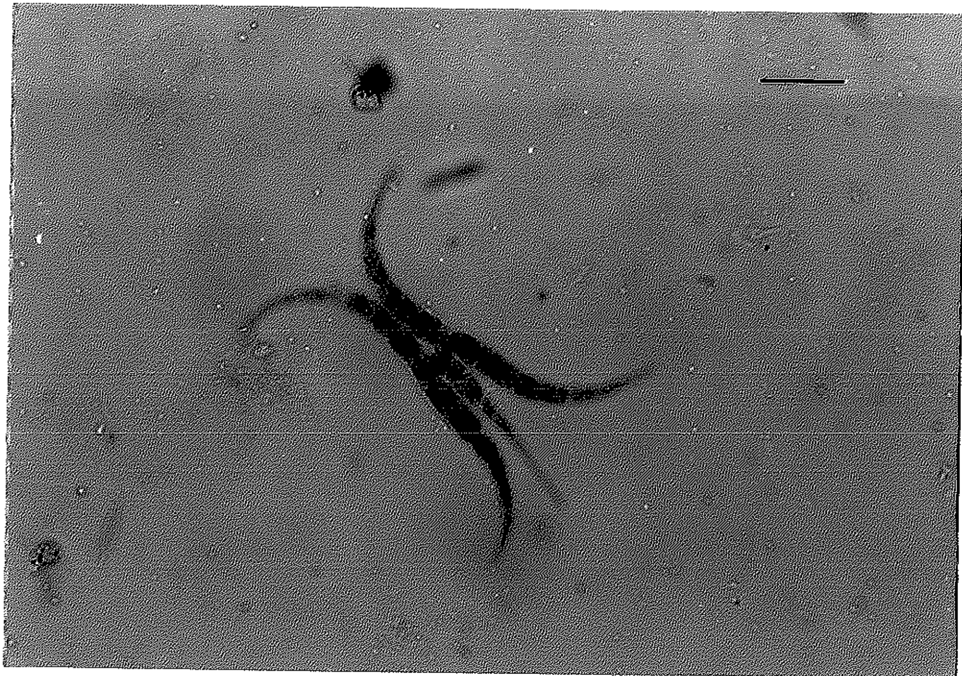


Fig. 31. ***Dwayaangam* sp. 2** (Descals & Webster, 1982)

Conidial description: Conidia multicellular, main axis compose of 2 branches which 50-60 μm long, 2-3 μm wide. Each branch composed of 1 lateral branch. (scale bar = 20 μm)

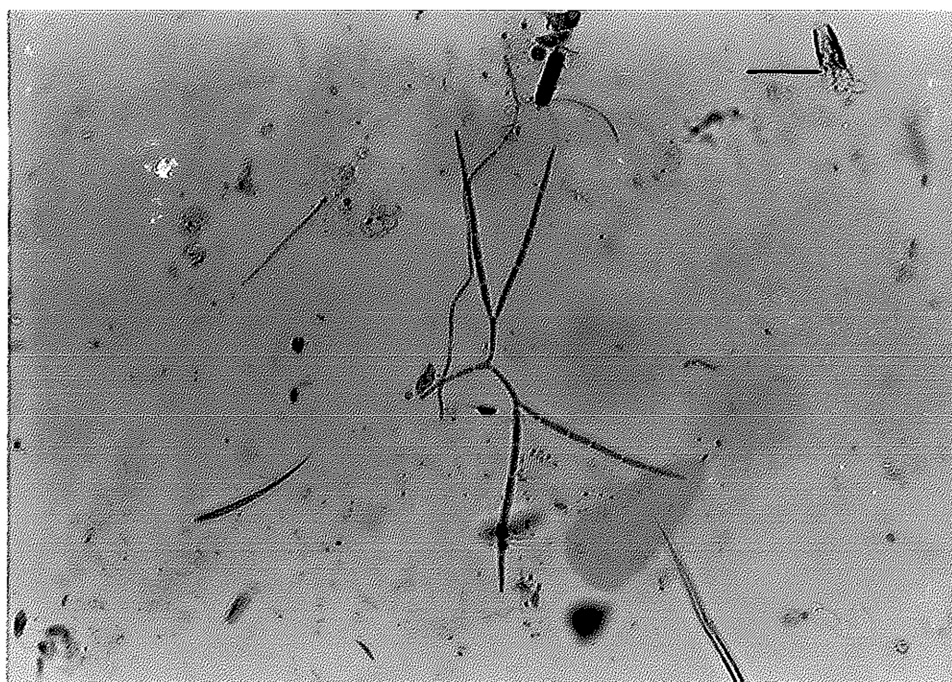
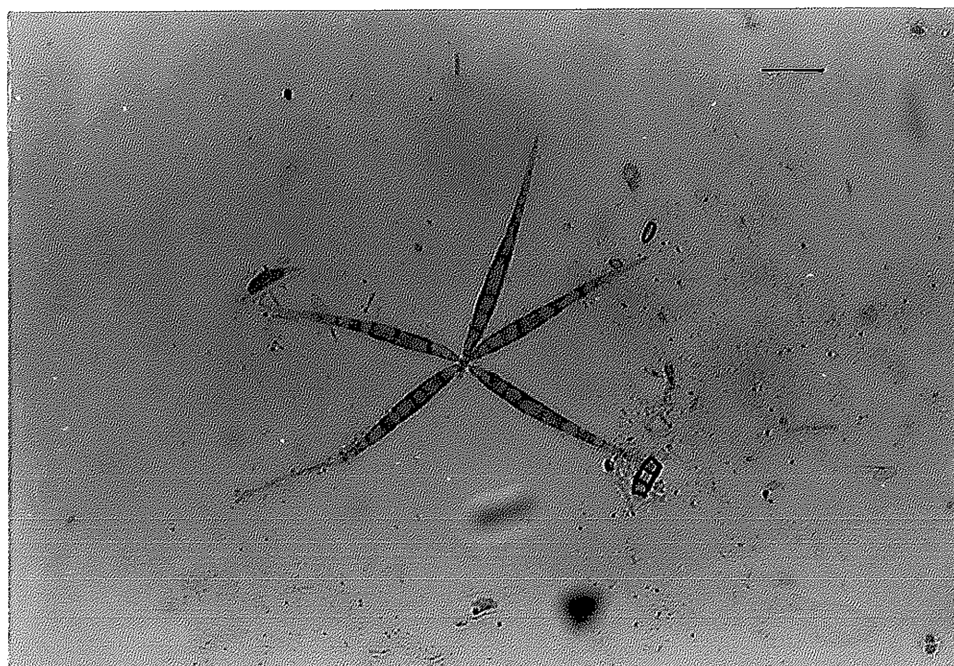


Fig. 32. *Flabellospora crasssa* (Marvanová, 1997)

Conidial description: The terminal thalloconidium has a very short stalk ending in a small spherical head, clavate or capitate as wide as branches or thinner, with radiating branches typically 4-5, 50-90 μm long. (scale bar = 20 μm)



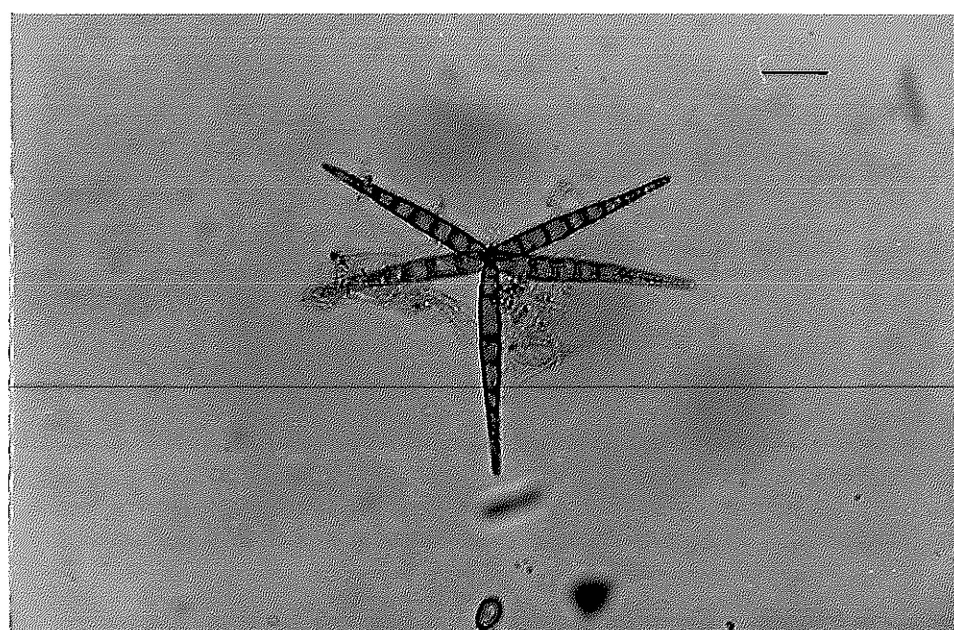


Fig. 33. *Flabellospora multiradiata* (Ingold, 1975; Marvanová, 1997)

Conidial description: The terminal thalloconidium has a stalk ending in a small spherical head, with typically 18-19 branches, 90-140 μm long.

(scale bar = 20 μm)

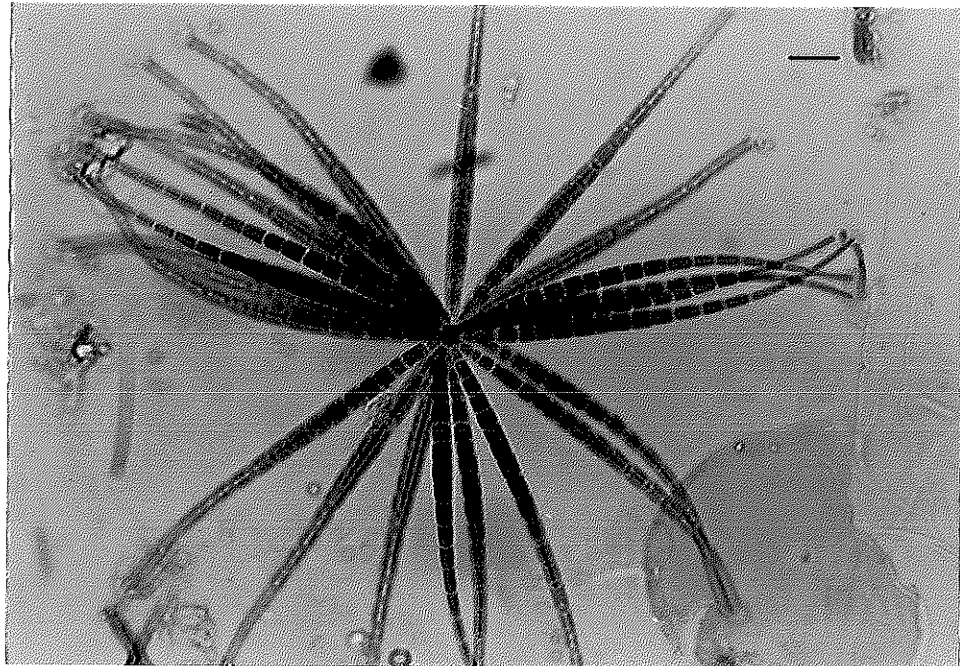
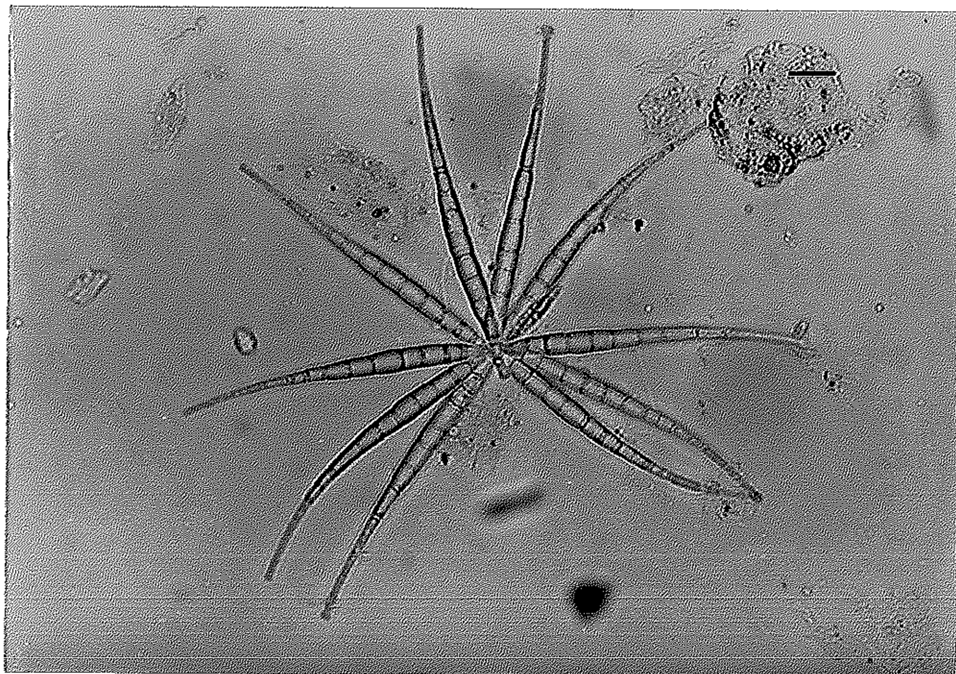


Fig. 34. ***Flabellospora verticillata*** (Ingold, 1975; Marvanová, 1997)

Conidial description: The terminal thalloconidium has a stalk ending in a small spherical head, with typically 5-7 branches, 50-90 μm long. (scale bar = 20 μm)



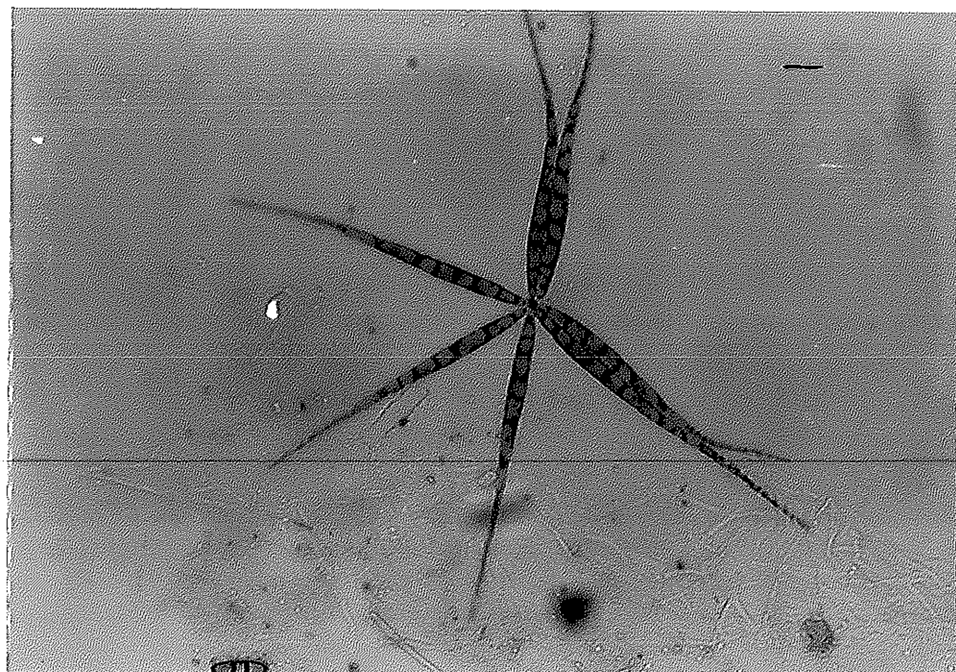


Fig. 35. *Isthmotricladia gombakiensis* (Ingold, 1975; Marvanová, 1997)

Conidial description: Conidial stalk clavate or capitate, as wide as branches or thinner. The single, terminal, septate, hyaline thalloconidium consists of a longish thin stalk with 20-27μm long, with 3-5 branches 70-100 μm long. (scale bar = 20 μm)

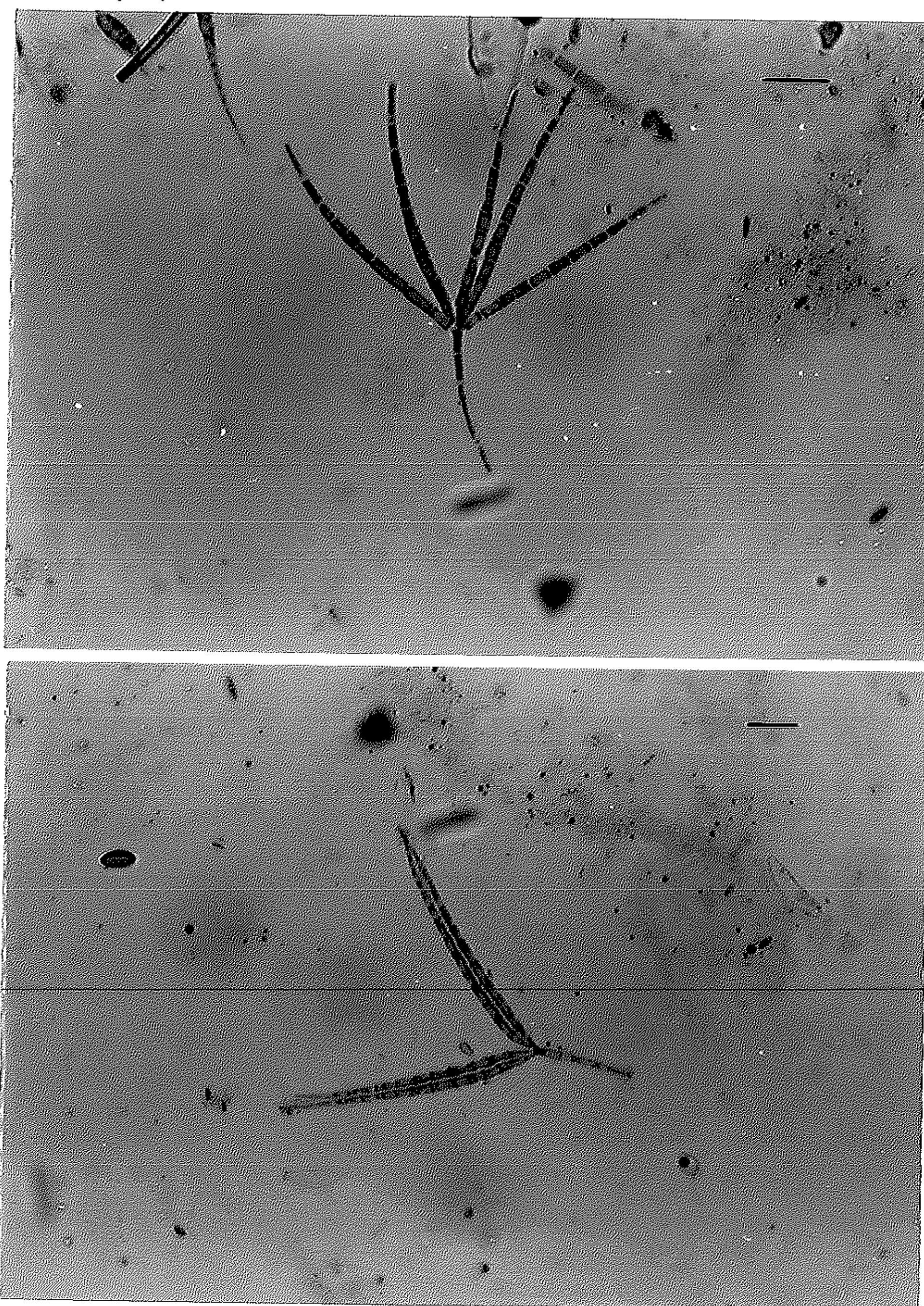


Fig. 36. ***Isthmotricladia laeensis*** (Ingold, 1975; Marvanová,1997)

Conidial description: The single, terminal, septate, hyaline thalloconidium consists of a longish thin stalk, 14-20 μm long, with 3 branches 35-52 μm long. (scale bar = 20 μm)

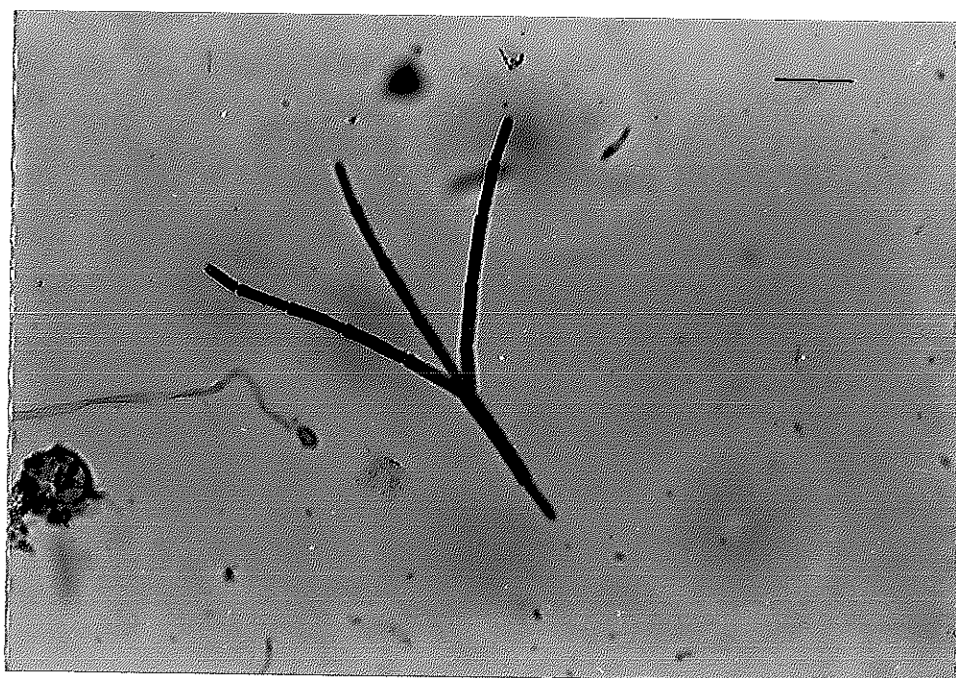


Fig. 37. *Laridospora appendiculata* (Nawawi, 1976a)

Conidial description: Conidia consisting of a fusoid conidial body distinctly wider than the branches, hyaline, septate conidia, with 60-100 μm long with a bend in the sub-basal cell, 5-6 celled main axis. (scale bar = 20 μm)

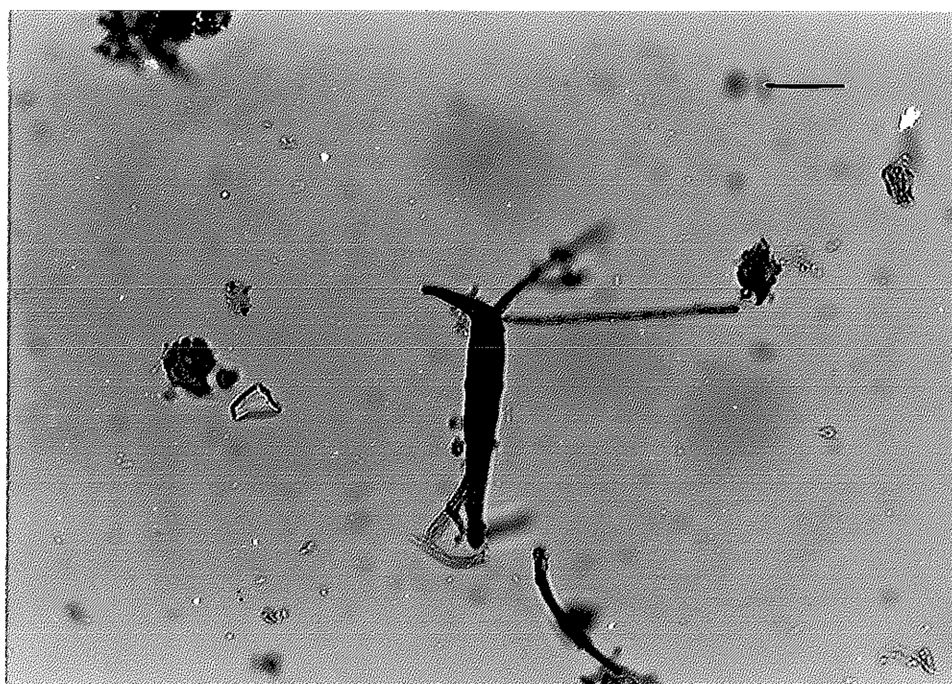


Fig. 38. *Nawawia* **sp. 1** (Hyde *et al.*, 1996; Marvanová, 1980)

Conidial description: Conidial body globose or short clavate or round-tetrahedral, 20-25 μm long or more (from base to apex) with 2 hyaline appendages, 80-120 μm long, arising from the apical corners of the conidium. (scale bar = 20 μm)

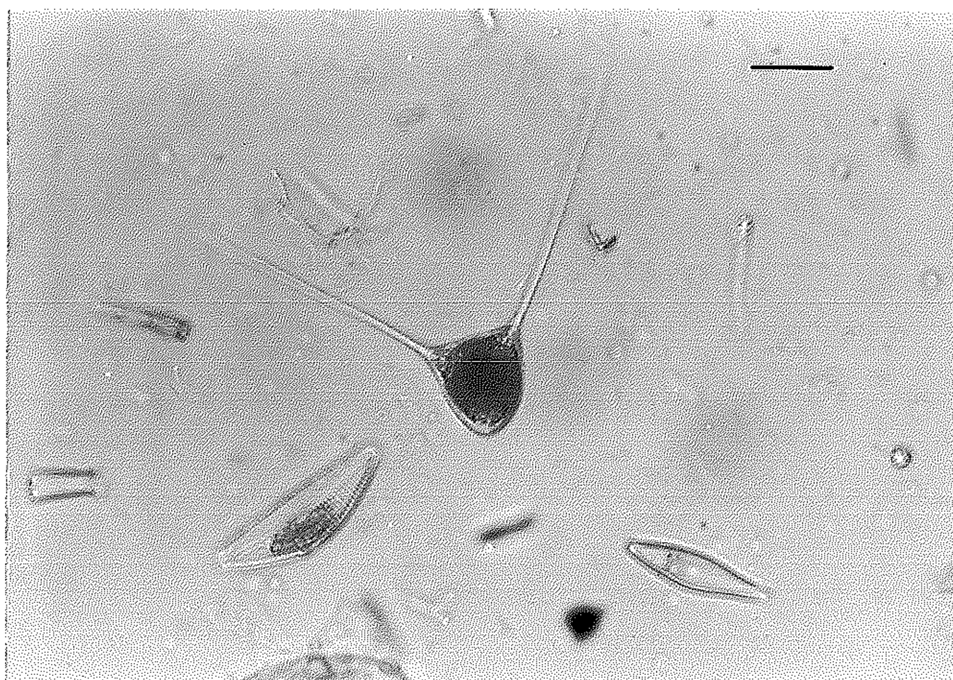


Fig. 39. ***Nawawia*** **sp. 2** (Hyde *et al.*, 1996; Marvanová, 1980)

Conidial description: Conidial body globose or short clavate or round-tetrahedral, 12-20 µm long or more (from base to apex) with 2 hyaline appendages, 60-80 µm long, arising from the apical corners of the conidium. (scale bar = 20 µm)

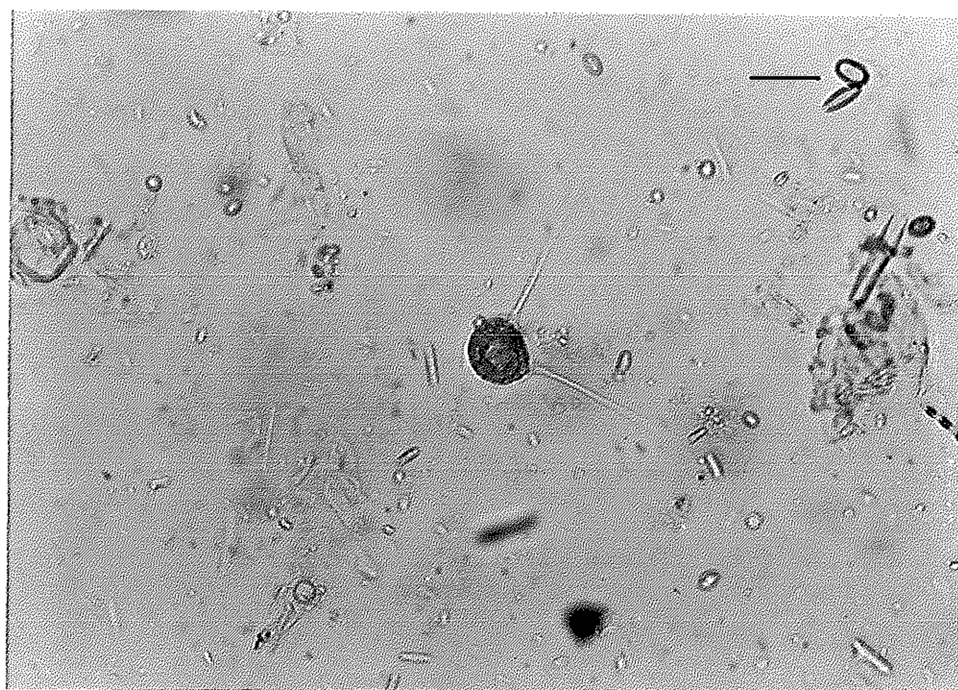


Fig. 40. *Nawawia* **sp. 3** (Hyde *et al.*, 1996; Marvanová, 1980)

Conidial description: Conidial body globose or short clavate or round-tetrahedral, 12-20 μm long or more (from base to apex) with 2-3 hyaline appendages, 60-80 μm long, arising from the apical corners of the conidium. (scale bar = 20 μm)

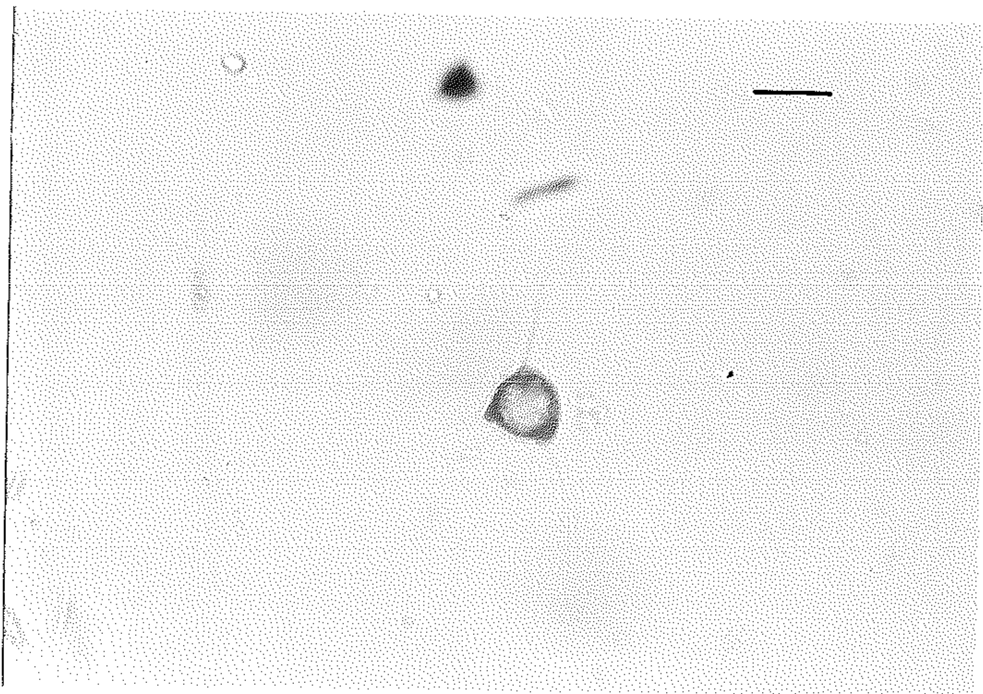


Fig. 41. *Phalangispora constricta* (Marvanová, 1997; Kuthubutheen, 1987)

Conidial description: Conidia fuscous with a short stalk and several primary as well as secondary branches, with deep constrictions between cells. Conidial cells predominantly cylindrical branching scopiform or diverging. The conidial chain from basal to tip measuring at 120-140 µm long and the lateral branches are 80-95 µm long. (scale bar = 20 µm)

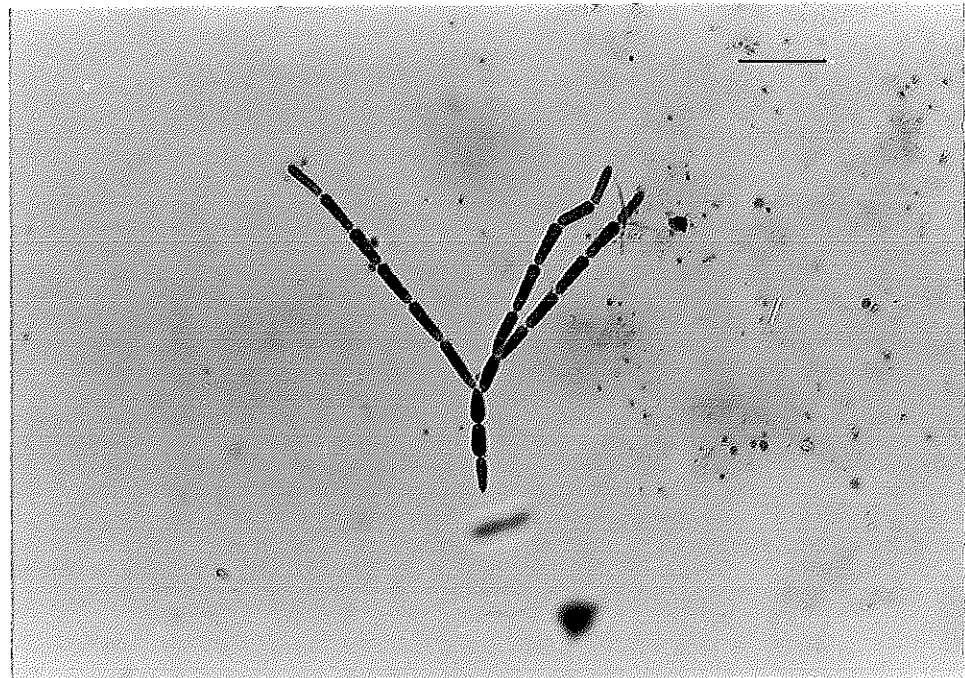


Fig. 42. ***Tricladium aciculum*** (Ingold, 1975; Nawawi, 1985a, b)

Conidial description: Thalloconidium consisting of long main axis and two lateral arising at the different levels, hyaline, content granular with minute globules. Main axis a smooth curve 75-90 x 4-5 μm. Arms 1-2 in number, 7-12 μm long diverging at the narrow angles. (scale bar = 10 μm)

Fig. 43. *Trinacrium*-**like** (Ingold, 1975)

Conidial description: Conidia with multicells, 3 branches with 3-4 septates, 35-45 μm long 2-3 μm wide. (scale bar = 20 μm)

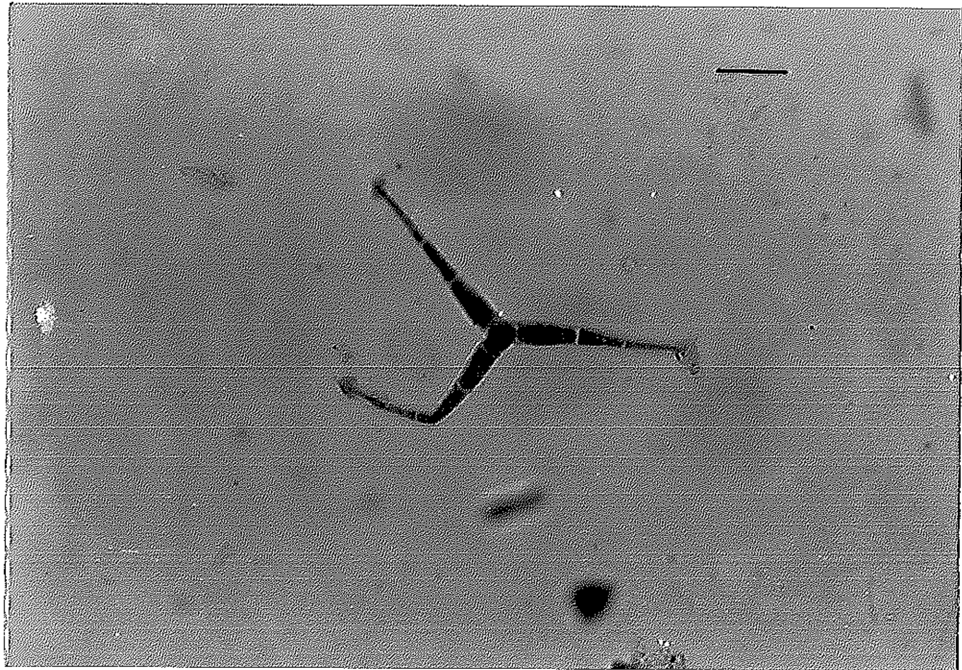


Fig. 44. *Varicosporium giganteum* (Ingold, 1975)

Conidial description: Conidia with narrow-fusoid or cylindrical axis, primary branches are broardly inserted on the main axis, main axis measuring at more than 300 μm long. (scale bar = 20 μm)

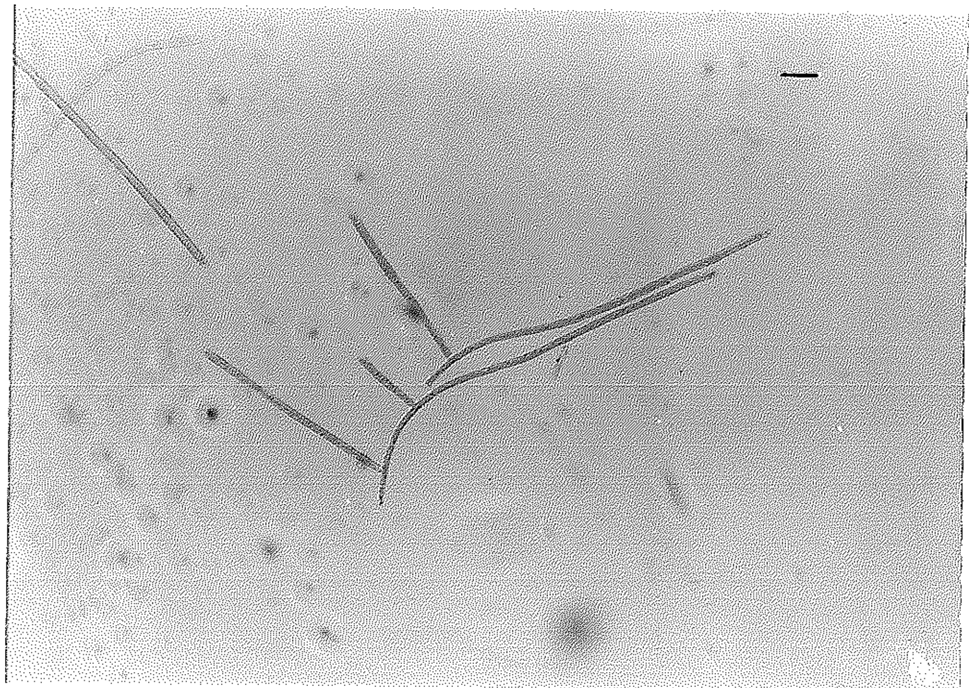
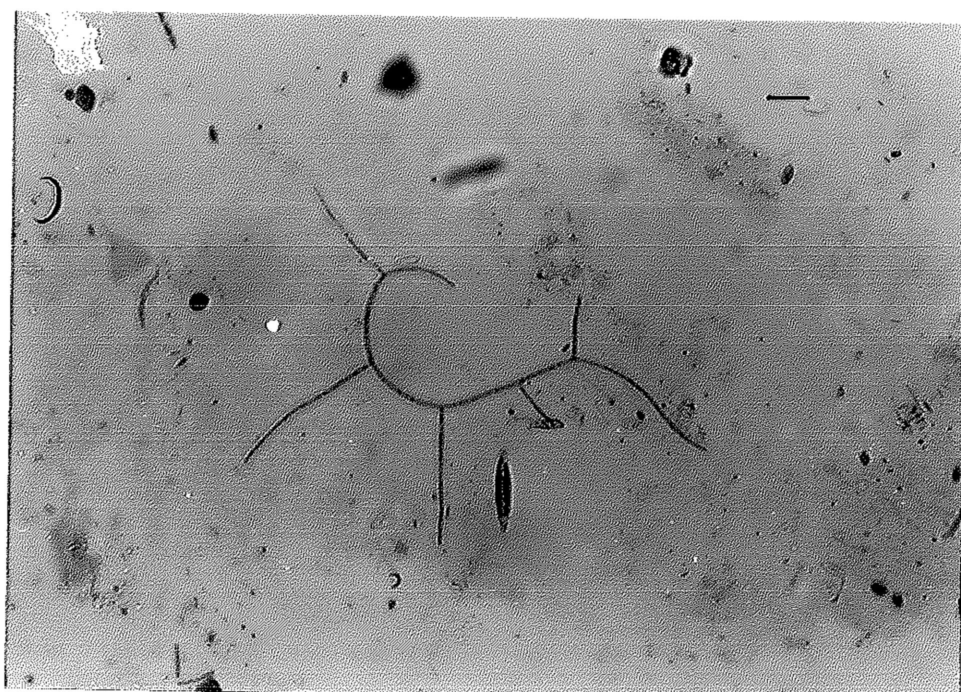


Fig. 45. *Varicosporium macrosporum* (Ingold, 1975; Nawawi, 1974a)

Conidial description: Conidia with narrow-fusoid or cylindrical axis, primary branches are broardly inserted on the main axis. The main axis measuring at 197-322 μm long, 2-2.5 μm wide, usually bent backward, 9-16 septates.

(scale bar = 20 μm)

Colony description: Conidial production on corn meal agar. A compact, circular, white colony is produced, with scantily aerial mycelium. Four-week colonies turn pinkish and the surface become powdery.



4. Ovoid shape

Fig. 46. ***Thozetella* sp.** (Pirozynski & Hodges, 1973)

Conidial description: Irregularly sigmoid or sickle-shaped twisted in two planes, 40-60 µm long, 2.5-3 µm wide in the middle, tapering to 1 µm at each end, thick-walled, with filiform setula 4-6 µm long. (scale bar = 20 µm)
Colony description: Sporulation on corn meal and potato dextrose agar. Dark gray slimy, glistening white mass of conidia are produced within a few days.

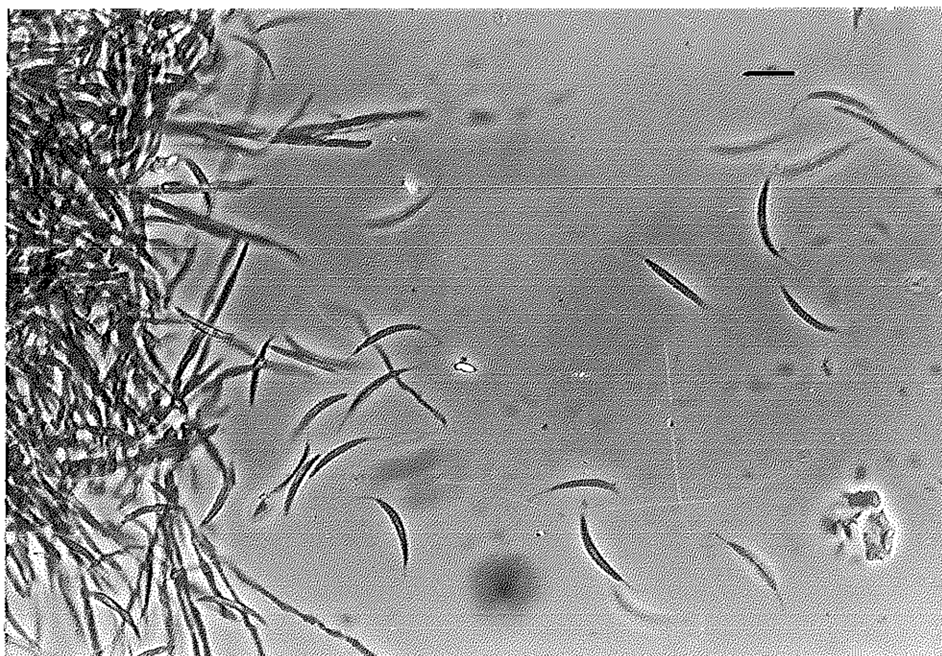
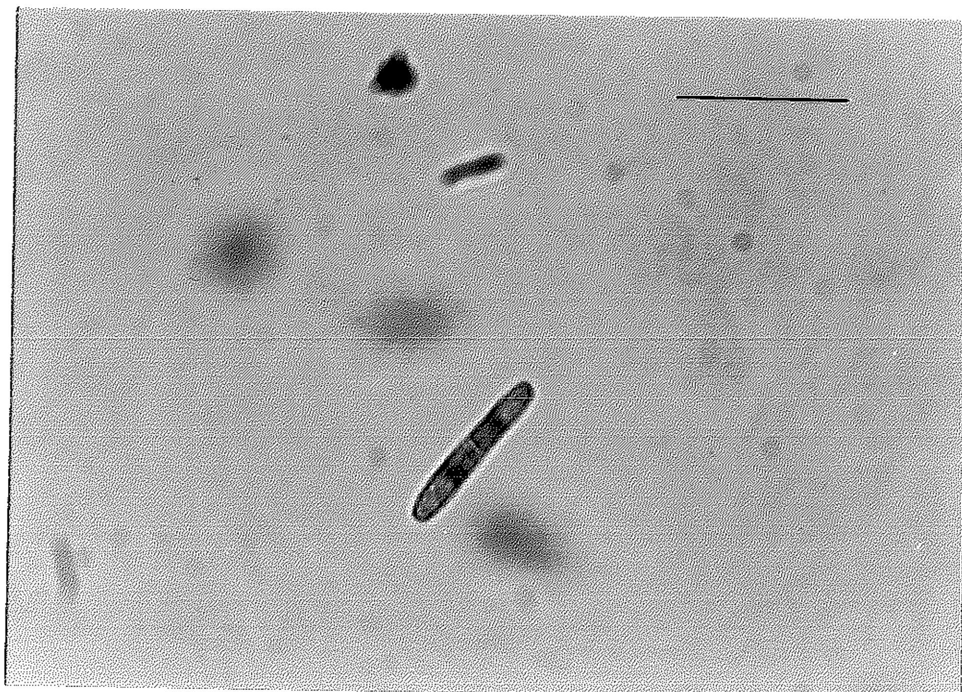


Fig. 47. ***Volutella* sp.** (Chilton, 1954)

Conidial description: Ovoid or cylindrical shape conidia with hyaline, single-celled, 5-10 μm long. (scale bar = 10 μm)

Colony description: Sporulation on corn meal and potato dextrose agar. Fast growing.. Sporodochium development with setae, pink colour mass of conidia produced in a mucus embedded mass.



5. Helicoid shape

Fig. 48. *Helicomyces* **sp.** (Goos, 1987; 1989)

Conidial description: Helicoid shape, coiled 3-5 times, usually in one plane, sometimes 3 dimensions, diameter of coils measuring of 32-65 μm, each cell containing one or two large vacuole. (scale bar = 20 μm)

Colony description: Conidial production on corn meal agar. Dark brown colony with aerial mycelium. Sporulation occurs superficial on partly submerged mycelium in water.

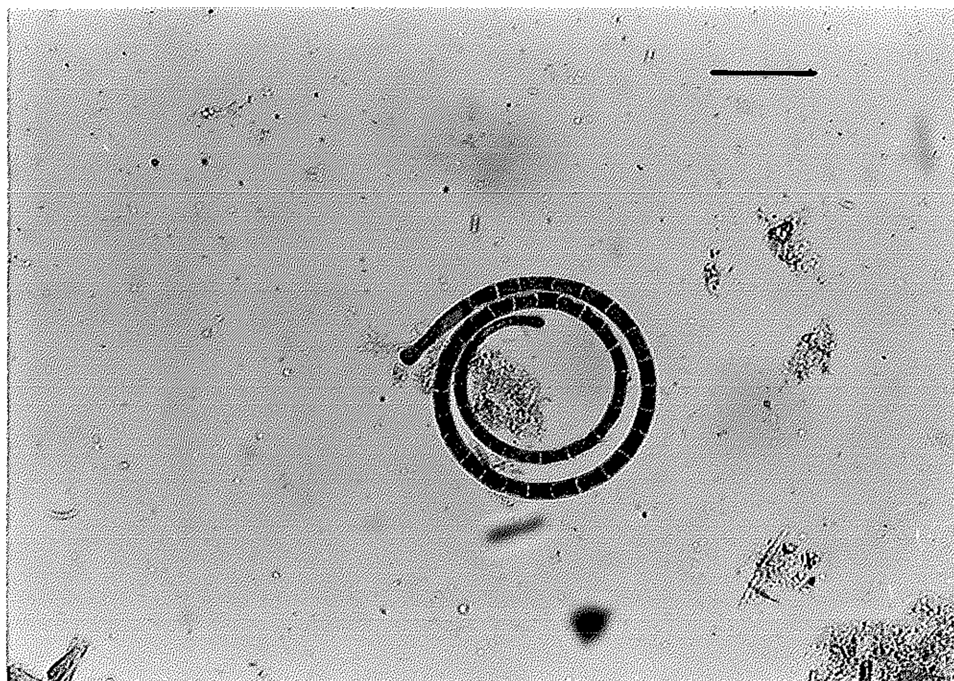


Fig. 49. ***Helicosporium* sp. 1** (Goos, 1987; 1989)

Conidial description: Helicoid conidium, usually coiled in one plane, hyaline, multiseptate, smooth, thin walled, diameter of coils measuring 50 μm, coiled 3-5 times. (scale bar = 50 μm)

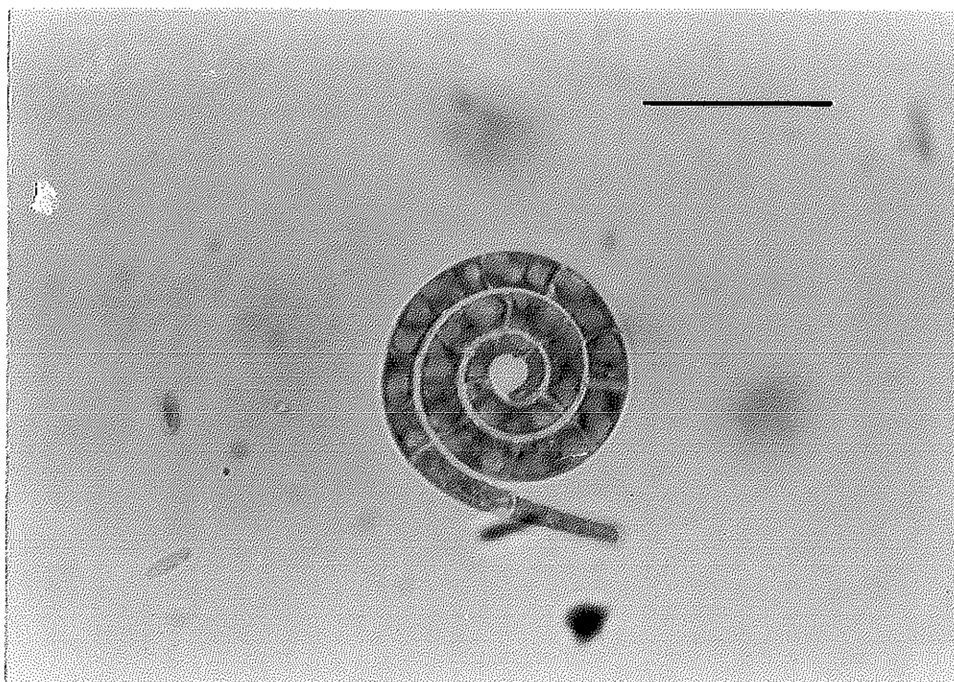
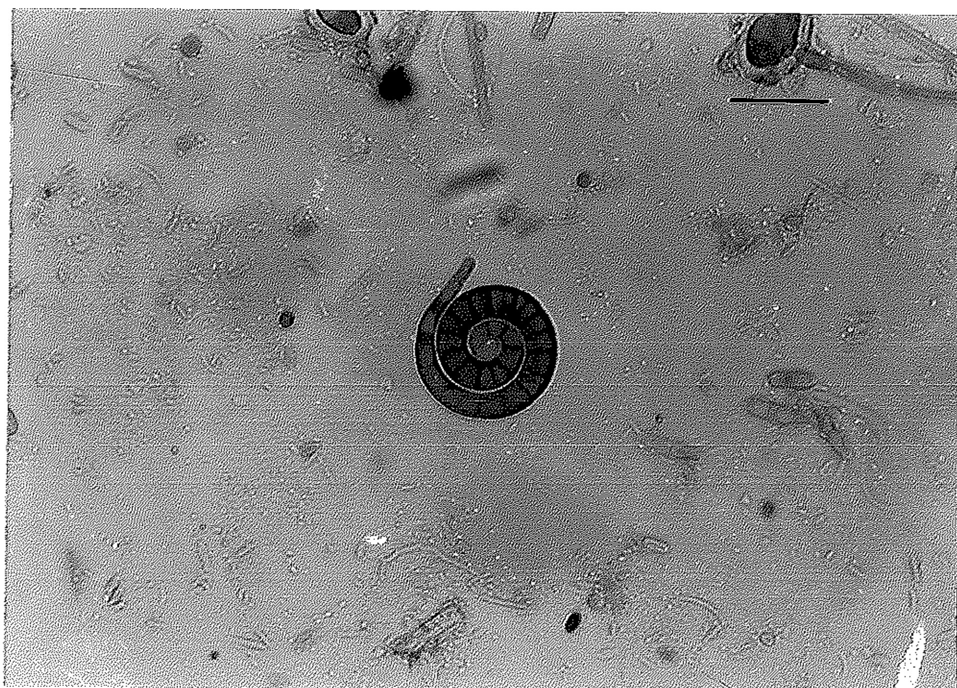


Fig. 50. *Helicosporium* **sp. 2** (Goos, 1987; 1989)

Conidial description: Helicoid conidium, usually coiled in one plane, hyaline, multiseptate, smooth, thin walled, diameter of coils measuring 20-50 μm, coiled 2-3 times. (scale bar = 20 μm)



6. Dematiaceous hyphomycetes

Fig. 51. *Beltrania rhombica* (Ingold, 1975)

Conidial description: Dematiaceous hyphomycetes, 1-celled conidia, brown colour with a paler middle band. The conidial body widest at 10 μm, with 1 hyaline seta. (scale bar = 20 μm)

Colony description: Conidia production on corn meal agar and potato dextrose agar. Dark brown colony with gray aerial mycelium.

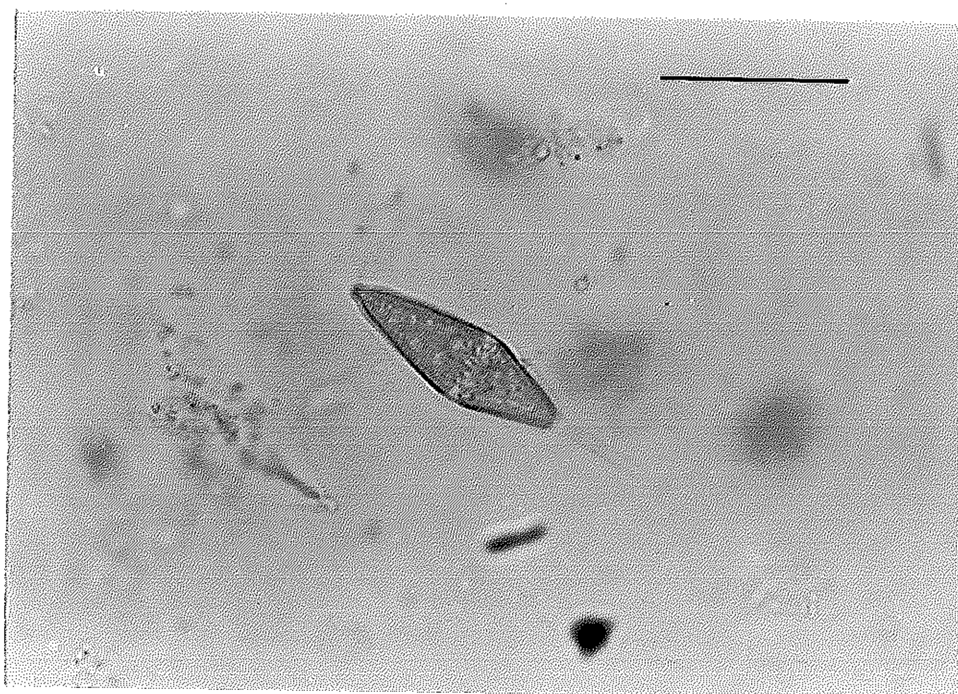


Fig. 52. ***Camposporium*****-like sp. 1** (Ingold, 1975)

Conidial description: Dematiaceous hyphomycetes with brown colour, thick walled, rod-like (70-100x8-12 μm). Main body of the conidium is usually prolonged apically into a narrow and nearly hyaline projection, often slightly curved, which may be up to 200 μm, with 2-3 setae. (scale bar = 20 μm)

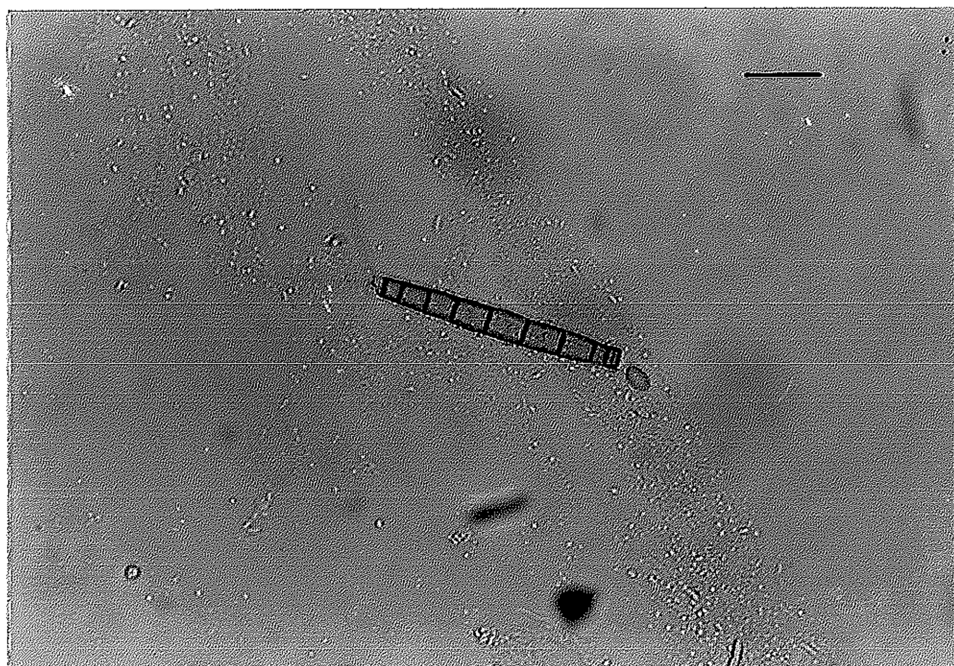


Fig. 53. ***Camposporium*** **-like sp. 2** (Ingold, 1975)

Conidial description: Dematiaceous hyphomycetes with brown colour, thick walled, rod-like (100-120 x 8-12 μm). Main body of the conidium is usually prolonged apically into a narrow and nearly hyaline projection, often slightly curved, which may be up to 200 μm, with 2-3 setae. (scale bar = 20 μm)

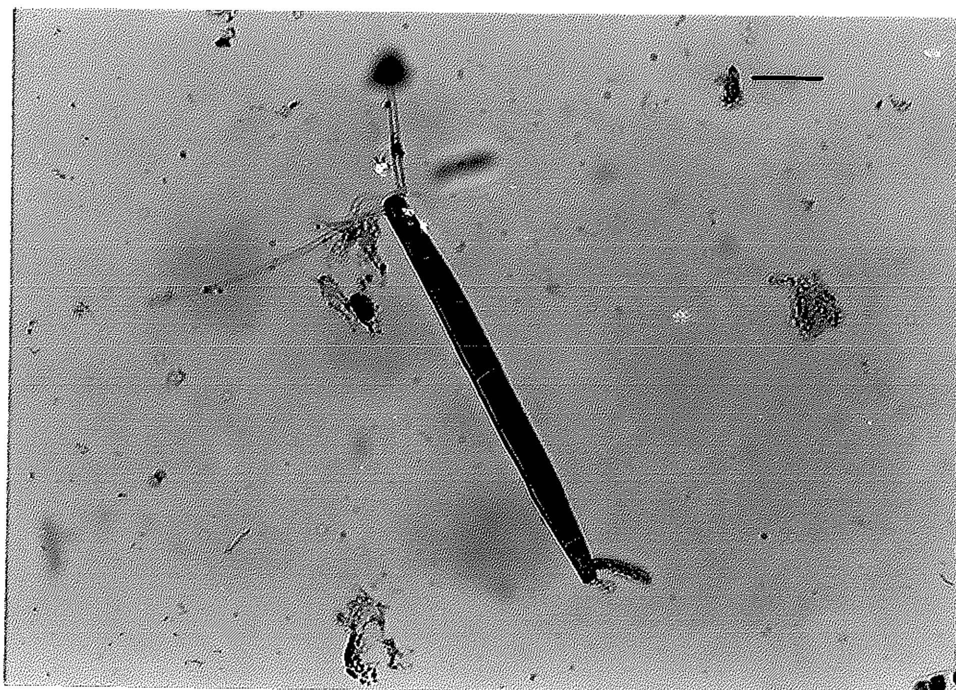


Fig. 54. ***Diplocladiella appendiculata*** (Ellis, 1976; Marvanová, 1997; Nawawi, 1987)

Conidial description: Conidial body triangular or Y-shaped, 8-celled distoseptate, with 2 hyaline filiform branches at each distal ends, measuring 48-60 μm across. (scale bar = 20 μm)

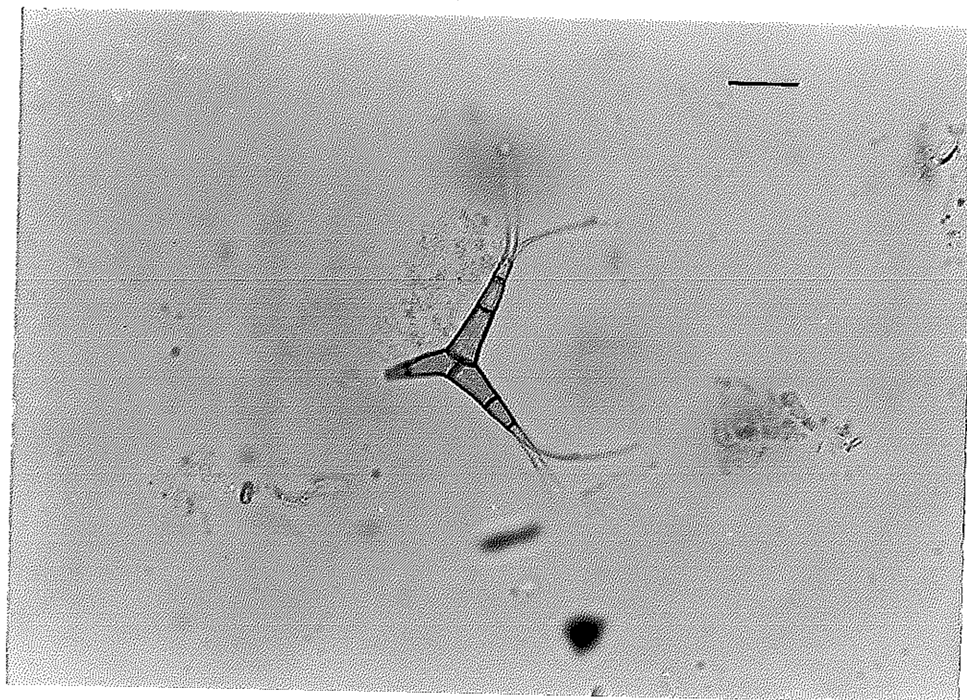


Fig. 55. ***Diplocladiella scalaroides*** (Ellis, 1976; Marvanová, 1997; Nawawi, 1987)

Conidial description: The conidium consists of 2 arms , five of its eight cells are brown, but the basal cell is colourless and each arm is terminated by an unpigmented hair-like cell, 15-30 μm wide or up to 40 μm , 3-4 μm thick.

(scale bar = 20 μm)

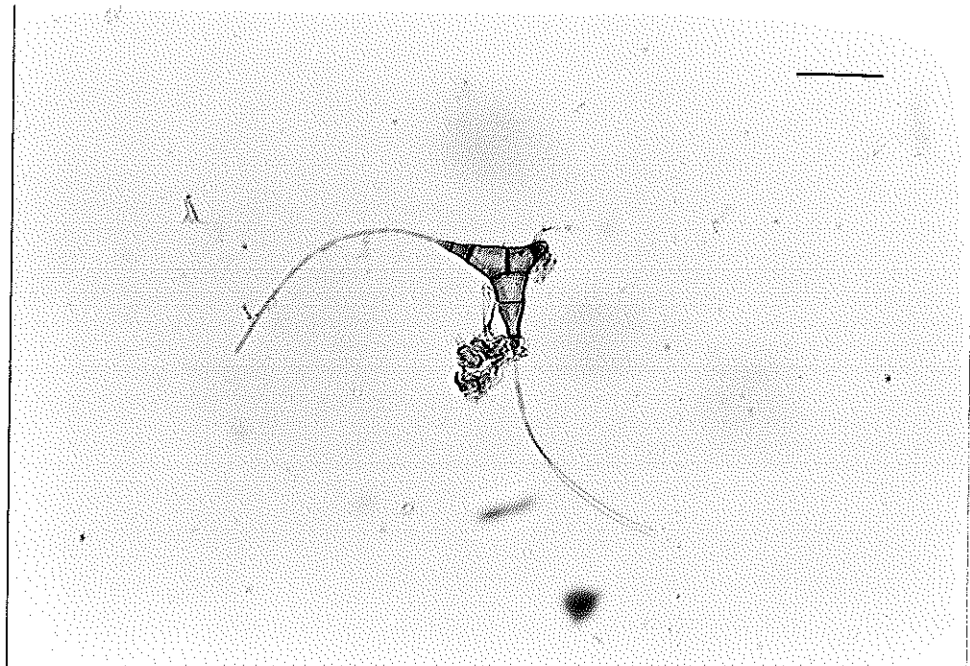


Fig. 56. ***Pseudobeltrania*** **sp.** (Ingold, 1975)

Conidial description: Dematiaceous hyphomycete with brown colour. 1-celled conidium widest at 15 µm, with 1 hyaline setae, total length measuring at 40 µm. (scale bar = 30 µm)

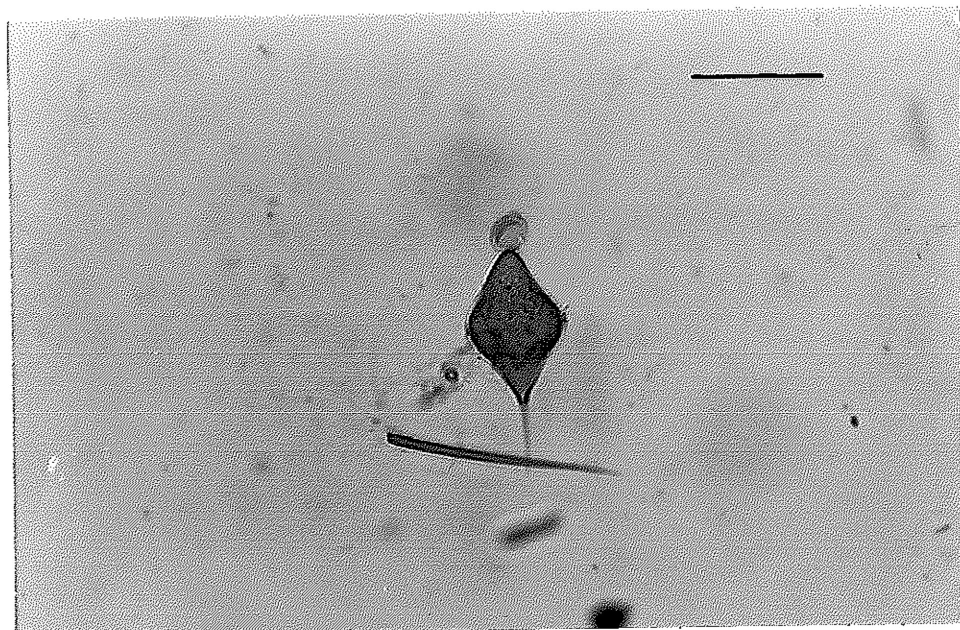
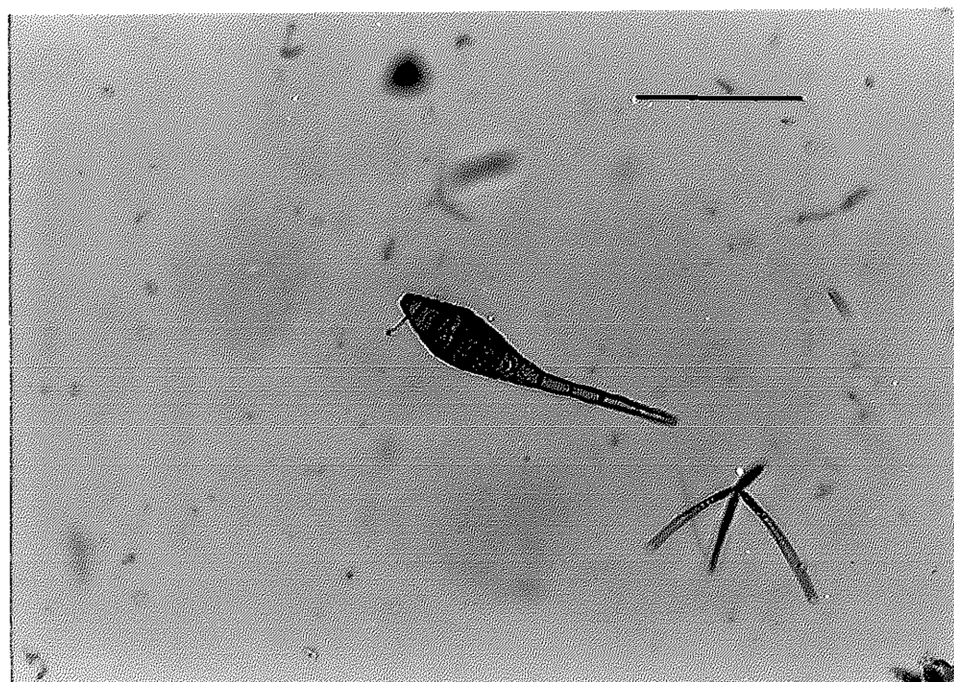


Fig. 57. ***Scutisporus* sp.** (Ando & Tubaki, 1985)

Conidial description: Conidia pale brown, butterfly-shaped measuring at 20-25 μm at the longest point, with 4 slender hyaline, ciliate appendages to each end cell. (scale bar = 30 μm)

Fig. 58. *Sporidesmium tropicalis* (McKenzie, 1995)

Conidial description: Conidia solitary, acrogenous, brown, basal and apical cells are smooth or slightly curved, apex rounded, 40-60 μm long, 5 μm at the widest point. (scale bar = 20 μm)



Coelomycetes

Fig. 59. *Chaetospermum camelliae* (Ingold, 1975; Jones, pers. comm.)

Conidial description: Ovoid conidia with 20 μm long x 10 μm wide , with 3-5 setae at both ends of conidium. (scale bar = 20 μm)

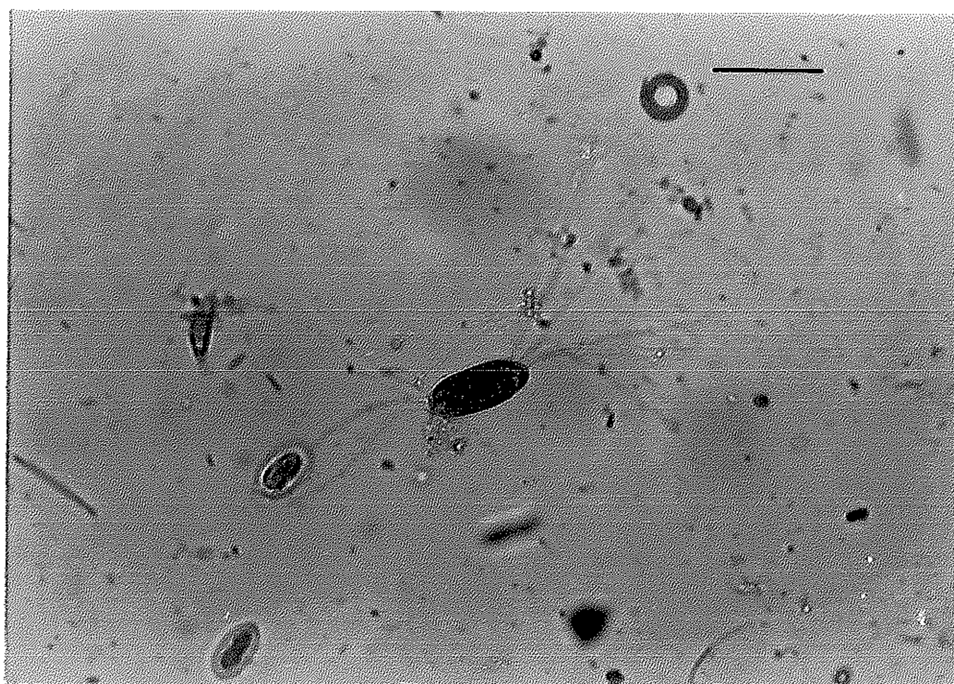


Fig. 60. ***Chaetospermum*-like** (Ingold, 1975)

Conidial description: Coelomycetes with ovoid shaped conidium with 3-5 hyaline setae at two poles of conidium. The conidium body is 20-25 μm long, 3-5 μm wide. (scale bar = 20 μm)

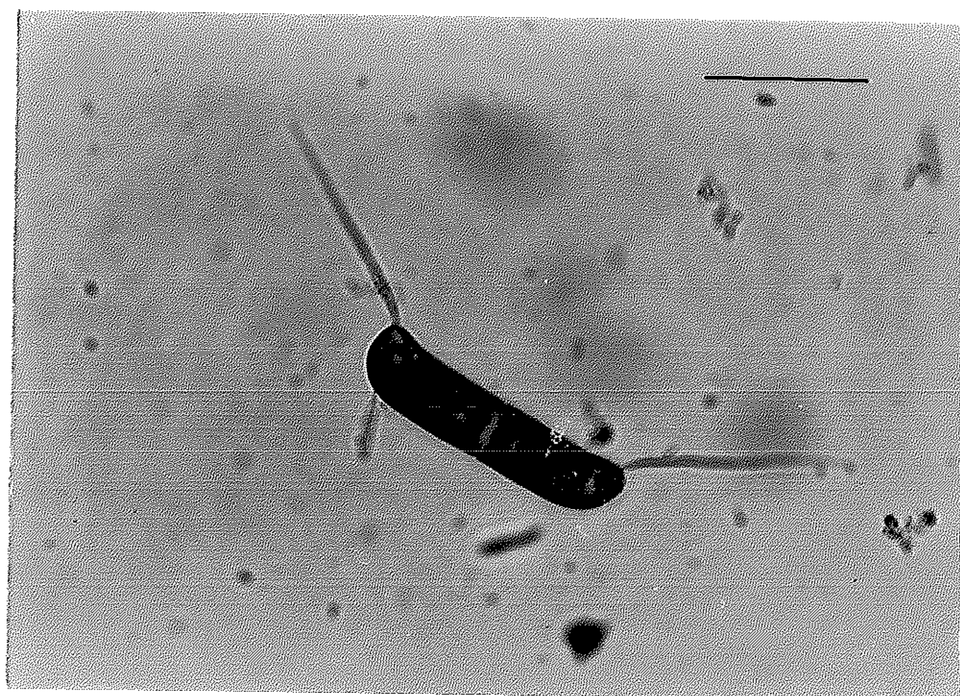


Fig. 61. ***Pestalotia*** **sp.** (Sutton, 1969)

Conidial description: Coelomycetes with 5-6 celled conidium. Fusiform shaped, dark brown colour, straight or slightly curved, barely constricted at the septa, 29-35 x 6-9 μm, with 3-9 cylindrical appendages. (scale bar = 20 μm)

Colony description: Conidial production on corn meal agar and potato dextrose agar. Conidial masses are formed in acervulus gelatinously, black colour, white-gray aerial mycelium, better mycelium development on potato dextrose agar.

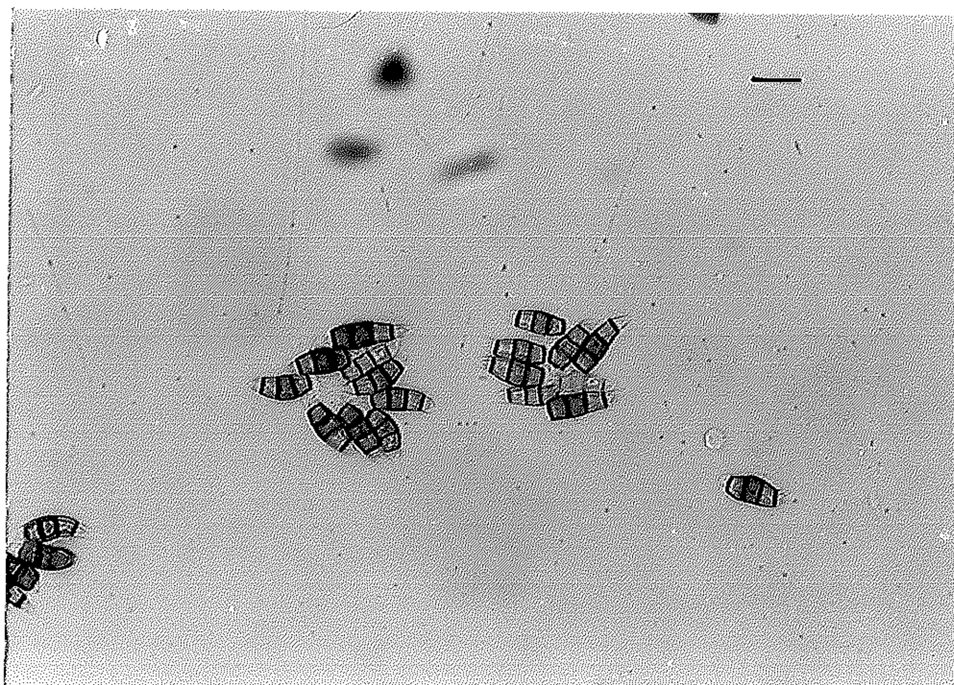
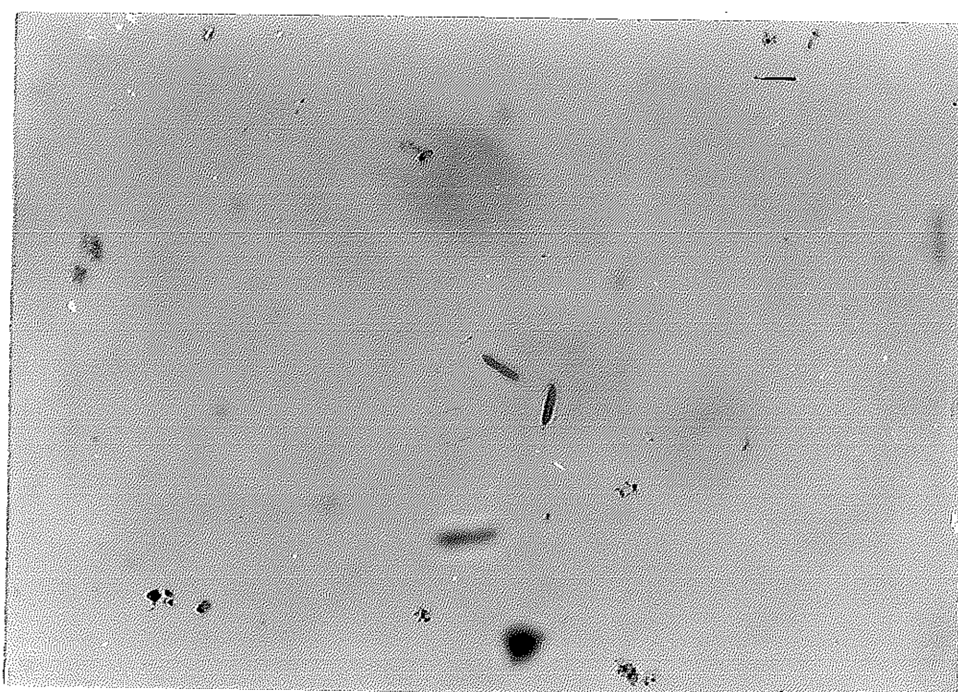


Fig. 62. ***Robillarda*** **sp.** (Cunnell, 1958)

Conidial description: Conidia with fusiform shape, 20-40 μm long, straight or rarely slightly curve. Bearing 2-3 setae, 15-23 μm at one end of the conidia. (scale bar = 20 μm)

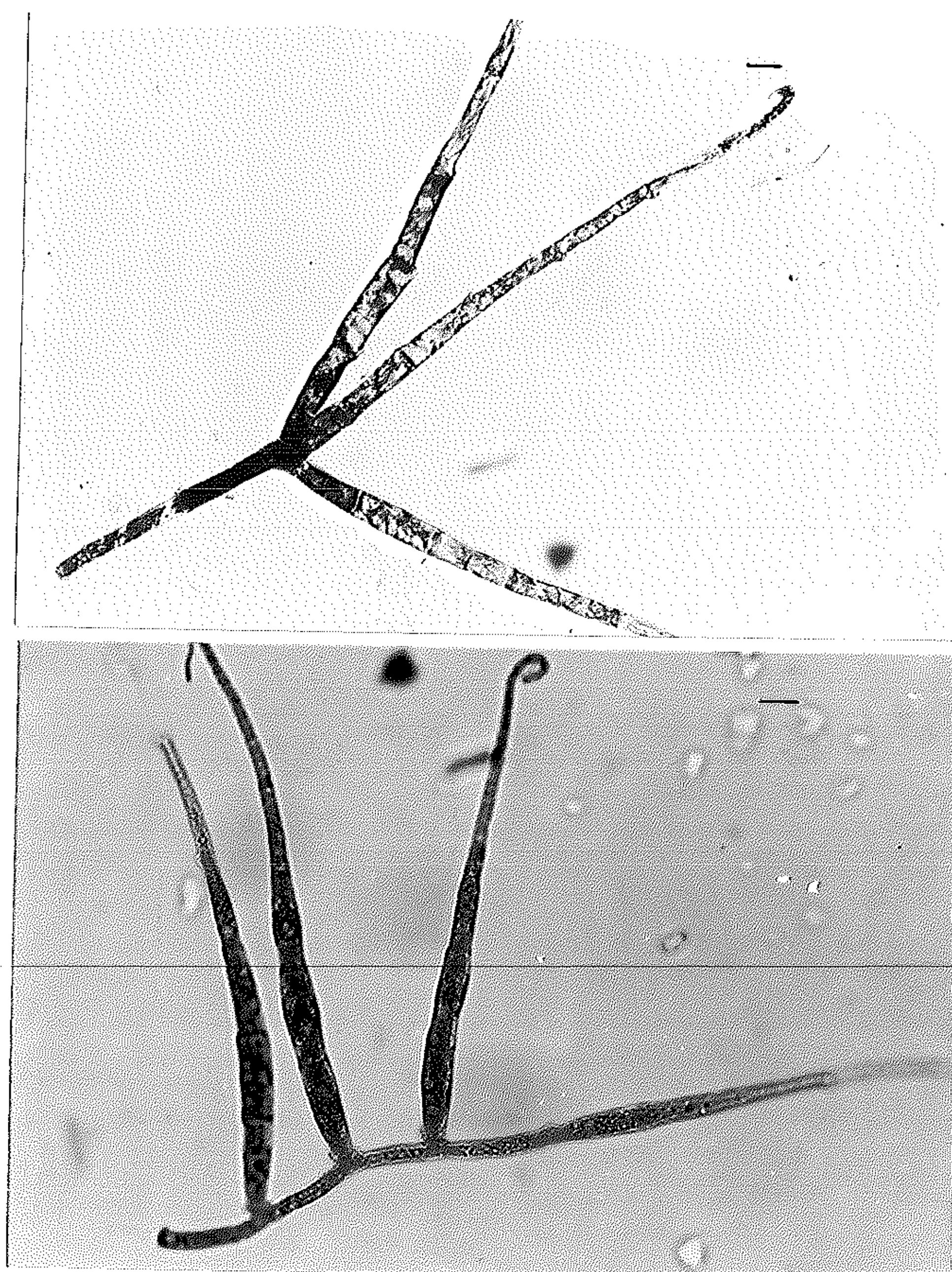
Colony description: Sporulation on corn meal agar, slow growing, whitish aerial mycelium is thin and the older part is pale.



Basidiomycetes

Fig. 63. ***Ingoldiella hamata*** (Bandoni & Marvanová, 1989; Nawawi, 1973)

Conidial description: Basidiomycetes with tetraradiate conidia, consisting of main axis and two lateral arms which arise at the same level in succession, 117-196 μm long, 7 μm at their widest point, clamp connections are formed. (scale bar = 50 μm)



Chapter 5

Conclusions

1. Estimation of the viability of conidia in foam samples

The total number of fungi trapped in foam, estimated by total viable plate counts method, varied from 2.3×10^3 to 3.4×10^5 CFU/ml in fresh foam and from 5×10^3 to 3.2×10^5 CFU/ml in old foam. These values were not significantly different ($P>0.05$). The highest numbers of total fungi in both types of foam were in week 10 (late August). Rainfall may be the main factor affecting the total number of fungi. It may increase water current in the stream, which may cause the release of conidia from leaf litter into the stream. Additionally, the water flowing system may enhance sporulation and spore release of some species. This leads to higher numbers of fungi in foam samples in this period.

Three vital stains were used in this study: tetrazolium bromide (MTT), acridine orange and DAPI. Acridine orange and DAPI showed higher percentages of conidia viability than MTT staining, but were not significantly different ($P>0.05$). However, MTT staining is more practical than those two vital stainings. MTT was used to estimate the viability of fungi by the presence of a purple-colored formazan. The conidial viability in fresh and old foam was in the range of 44-77% and 42-69%, respectively. There was

variation in the viability of conidia in fresh and old foam for every collecting time, but not significantly different at $P>0.05$. The viability in fresh and old foam was similar, because the conversion from fresh to old foam might be in a week. Therefore, the younger conidia were probably continuously being trapped in old foam resulting in the viability of conidia in fresh and old foam begin in a similar range.

2. Estimation of viability of laboratory-produced conidia under different conditions

2.1 Fungal viability under aeration

The viability for all four species showed little variation, the viability decreased to 83-88% over an aeration period of seven days. The air bubbles under laboratory conditions provide a good source of oxygen. Thus, the survival of the laboratory-produced conidia for all four species was still high (83-88%), compare with the viability in natural fresh and old foam which varied from 42-77%. Therefore, it may possible that the survival of laboratory-produced conidia under aerated conditions could be a predictor of fungal viability in foam.

2.2 Fungal viability under drying conditions

The same four species of laboratory-produced conidia were used to test the viability under drying conditions. After 10 hours drying, the viability of all four species decreased to 3-45 %. *Anguillospora* sp. and *Helicomyces* sp. showed higher percentages of viability than *Thozetella* sp. and *Volutella* sp. The reasons was because the difference in their conidial size, shape and amount of food reserves.

3. Diversity of freshwater fungi in foam samples

Thirty-five genera, 48 species of freshwater fungi in foam samples were identified based on their conidial shape. The identified fungi were dominated by hyphomycetes with three genera of coelomycetes and one genus of basidiomycetes. The predominate species found in foam were *Anguillospora* sp. and *Triscelophorus* sp.

Most of the conidia in stream waters were less branched spores but more dematiaceous hyphomycetes with ovoid conidial shape. The common species found in water samples were *Anguillospora* sp. and some dematiaceous hyphomycetes It may be possible that the conidia in stream waters were accumulated from upstream to downstream from week 8 and 10, because of the heavy rainfall.

4. Culture collections

Sixty-five pure isolates were obtained from foam samples. Twenty-four isolates produced conidia on corn meal and potato dextrose agar. Only 15 isolates were fully identified.

Suggestions for future research

My work has been the first study of aquatic hyphomycetes in the South of Thailand. However, this study was restricted to one river in one wildlife sanctuary. The South of Thailand has many wildlife sanctuaries and national parks; such as Hala Bala (Narithawat Province), Khao Sok (Surat Thani Province), and Thaleban (Sathun Province). To make a more complete study of the aquatic hyphomycetes of the South of Thailand, I would recommend that future work is extended to survey other streams and rivers in other national parks and wildlife sanctuaries.

Importantly, my work was confined to one time of the year (June to September, 1998). This was in the middle of the rainy season. However, it is possible that different species of aquatic fungi may be present at different times of the year. For the Central and Northern parts of Thailand Hywel-Jones (pers. comm.) noted that the cool season produced species that were not found at other times of the year. However, in the South, I do not think this will be so

important since there is not a marked cool season. The end of the rainy season could be a time when different species are active as the water level in rivers and streams becomes lower.

Many of the fungi found in this survey could only be identified to genus level. In Thailand there is a problem with access to literature, especially old literature. And in taxonomy the old literature does not go out of date and is just as important as new literature. It is important to try and build a large library of this taxonomic literature so that identifications can be done more easily. With access to this literature it is possible that many new species could be recognized and described.

For the laboratory studies on aeration and drying it is clear that examination over a longer period of time may have provided further information on the effects of drying and aeration on the longevity of the species examined. In my study I had to confine myself to a few species that produced spores easily and in large numbers. Unfortunately it was not possible to include a branched-spored species in this study. If possible it would be good to look for other species to be included in a further, more extensive laboratory study.

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Appendix A

Culture media

1. Potato dextrose agar (Difco, Detroit, USA)

Potato , infusion form	200 g
Bacto dextrose	20 g
Bacto agar	15 g
Distilled water	1 l

2. Corn meal agar (Difco, Detroit, USA)

Difco cornmeal agar	17.0 g
Distilled water	1 l

All media were autoclaved at 121 °C for 15 minutes at 15 lb/inch^2

Antibiotics; Kanamycin, Streptomycin and Tetracycline concentration 1 g/l were added into the media.

Appendix B

Statistical analysis

Table A1. Total viable plate count in foam (CFU/ml x 10^4).

Collection times	Fresh foam	Old foam
wk0	0.23 ±0.5a	0.6 ±0.8b
wk2	3.97 ± 0.9a	7.5 ± 1.5b
wk4	0.67 ± 0.9a	1.05 ± 0.6b
wk6	2.79±0.06a	4.0 ± 1.2b
wk8	10.6 ± 1.0a	14.8 ± 1.1b
wk10	34.4 ± 1.2a	32.9 ± 1.1b
wk12	9.17 ± 1.5a	2.08 ± 0.7b
wk14	4.2 ±1.0a	2.39 ± 0.7b

a, b = Means in each column followed by the same letter,

non significant difference at P>0.05

Table A2. Percentage viability of conidia in fresh foam using three different stains.

	wk 8	wk 10	wk 12	wk 14
MTT	44.612± 6.88a	62.611 ± 9.135b	64.517 ± 10.163c	60.015 ± 10.142d
AO	45.727±12.09a	89.798 ± 18.84b	77.495 ± 14.65c	66.068 ± 8.25d
DAPI	ND	83.008 ±17.842b	69.723 ±11.426c	65.349 ± 9.895d

MTT = tetrazolium bromide

AO = acridine orange

DAPI = 4′-6 diamidino-2-phenylindole

ND = Non detected

a, b, c, d = Means in each column followed by the same letter, non significant difference at P>0.05

Table A3. Percentage viability of conidia in old foam using three different stains.

	wk 8	wk 10	wk 12	wk 14
MTT	42.192 ± 6.88a	64.706 ±11.87b	55.475 ± 8.5c	51.368 ± 8.29d
AO	35.91± 9.026a	86.63 ± 20.73b	69.816 ± 12.75c	55.398 ± 7.36d
DAPI	ND	77.68 ± 19.48b	55.114 ± 9.66c	55.006 ± 7.64d

MTT = tetrazolium bromide

AO = acridine orange

DAPI = 4′-6 diamidino-2-phenylindole

ND = Non detected

a, b, c, d = Means in each column followed by the same letter, non significant difference at $P>0.05$

Table A4. Percentage viability of conidia in fresh and old foam (MTT staining).

Collection times	Fresh foam	Old foam
wk0	77.554 ± 5.301a	69.659± 3.44a
wk2	67.12 ± 6.247a	52.68 ± 4.804a
wk4	58.13 ± 9.629a	60.694 ± 3.224a
wk6	53.347 ± 9.60a	56.156 ± 14.344a
wk8	44.612 ± 6.88a	42.192 ±6.88a
wk10	62.611 ± 9.135a	64.706 ±11.847a
wk12	64.517 ± 10.163a	55.475 ± 8.5a
wk14	60.015 ± 10.142a	51.368 ± 8.259a

a = Means in each column and row followed by the same letter, non significant difference at P>0.05

Table A5. The summary of percentages of variance of different curves fitted by non-linear regression analysis of the laboratory experiment under aerated conditions.

% variance	Exponential decay curve	Logistic curve	Generalised logistic curve	Gompertz curve
Single curve	86.4	86.74	86.31	86.7
Vary a	87.3	87.64	87.2	86.5
Vary a and c	88.0	88.24	87.9	100
Vary a, b, c and m	88.0	88.14	86.3	88.0

Table A6. Percentage viability of four species after aeration for seven days.

Species \ Time (days)	2	3	5	7
Anguillospora	99.99 ± 0.009	95.94 ± 0.98	90.27 ± 1.22	83.09 ± 2.02
Helicomyces	99.23 ± 0.82	97.88 ± 1.69	91.17 ± 2.01	88.2 ± 1.42
Thozetella	99.4 ± 0.51	95.83 ± 1.43	87.9 ± 1.53	86.42 ± 3.36
Volutella	99.22 ± 0.77	98 ± 1.87	86.4 ± 1.43	84 ± 1.57

Exponential decay curve fitted to spore viability under aerated conditions

***** Nonlinear regression analysis *****

Response variate:	ALIVE
Explanatory:	TIME
Grouping factor:	SPECIES, all parameters separate
Fitted Curve:	A + B*R**X
Constraints:	R < 1

*** Summary of analysis ***

	d.f.	s.s.	m.s.	v.r.
Regression	11	5629.6	511.78	29.72
Residual	32	551.1	17.22	
Total	43	6180.7	143.74	
Change	3	-52.2	-17.39	-1.01

Percentage variance accounted for 88.0

Standard error of observations is estimated to be 4.15

* MESSAGE: The following units have large standardized residuals:

43 2.97

*** Estimates of parameters ***

	estimate	s.e.
R SPECIES *Anguillospora*	1.015	0.142
B SPECIES *Anguillospora*	-425	4370
A SPECIES *Anguillospora*	636	4378
R SPECIES *Helicomyces*	0.914	0.180
B SPECIES *Helicomyces*	78	104
A SPECIES *Helicomyces*	134	114
R SPECIES *Thozetella*	0.712	0.133
B SPECIES *Thozetella*	67.1	12.9
A SPECIES *Thozetella*	165.53	8.77
R SPECIES *Volutella*	0.969	0.169
B SPECIES *Volutella*	234	1097
A SPECIES *Volutella*	-20	1107

Table A7. The summary of percentages of variance of different curves fitted by non-linear regression analysis of the laboratory experiment under drying conditions.

% variance	Exponential decay curve	Logistic curve	Hyperbolic curve
Single curve	59.3	10	59.1
Vary a	79.2	78.8	79.0
Vary a and c	87.9	89.1	86.5
Vary a, b, c and m	94.9	75.8	92.0

Table A8. Percentage viability of four species after drying for 10 hours.

Time / Species	0 min	1 min	5 min	15 min	30 min	1 hr	5 hrs	10 hrs
Anguillospora	96.88 ± 0.67	96.93 ± 1.67	93.62 ± 1.16	91.51 ± 0.71	90.11 ± 0.72	88.32 ± 1.49	48.93 ± 3.68	45.22 ± 0.87
Helicomyces	100 ± 1.73	100 ± 1.65	100 ± 0.93	98.23 ± 1.2	92.8 ± 2.45	89.33 ± 0.33	53.11 ± 2.24	37.22 ± 0.88
Thozetella	98.5 ± 2.5	98.7 ±1.5	83.24 ± 1.53	82.94 ± 1.38	10.22 ± 0.77	8.22 ± 0.11	8.23 ± 0.23	7.97 ± 0.61
Volutella	97.8 ± 0.89	96.79 ± 0.33	96.28 ± 1.26	64.77 ± 0.81	21.72 ± 0.81	5.82 ± 0.01	5.80 ± 0.01	3.66 ± 0.28

Hyperbolic curve fitted for spore viability under drying conditions data

***** Nonlinear regression analysis *****

Response variate:	ALIVE
Explanatory:	TIME (minutes)
Grouping factor:	SPECIES, all parameters separate
Fitted Curve:	A + B/(1 + D*X)

*** Summary of analysis ***

	d.f.	s.s.	m.s.	v.r.
Regression	11	327911	29810.1	66.72
Residual	52	23233	446.8	
Total	63	351144	5573.7	
Change	3	-18006.	-6001.9	-13.43

Percentage variance accounted for 92.0

Standard error of observations is estimated to be 21.1

*** Estimates of parameters ***

	estimate	s.e.
D SPECIES *Anguillospora*	0.00379	0.00350
B SPECIES *Anguillospora*	158.5	53.2
A SPECIES *Anguillospora*	35.9	56.2
D SPECIES *Helicomyces*	0.00255	0.00234
B SPECIES *Helicomyces*	213.3	87.0
A SPECIES *Helicomyces*	-10.7	90.0
D SPECIES *Thozetella*	0.0590	0.0159

B SPECIES *Thozetella*	214.5	14.7
A SPECIES *Thozetella*	-5.3	11.7
D SPECIES *Volutella*	0.0564	0.0147
B SPECIES *Volutella*	221.9	14.7
A SPECIES *Volutella*	-11.4	11.8

Vitae

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