

Chapter 2

Methodology for Studying Nematophagous Fungi

Juan Li, KD Hyde and Ke-Qin Zhang

Contents

Introduction	14
Habitats of Nematophagous Fungi	14
Field Collection	15
Culture Medium	16
Baits for Nematophagous Fungi	16
Treatment of Samples	19
Soil Sprinkling Technique	19
Soil Dilution Method	20
Baermann Funnel Technique	20
Differential Centrifugation Technique (Barron 1969)	22
Observation and Identification	23
Observation	23
Electron Microcopy	24
Fluorescence Microscopy	25
Isolation of Pure Culture Species	27
Macerated Inocula Method (Heintz 1978)	28
Identification	28
Use of Molecular Techniques in Identifying Nematophagous Fungi	30
Preservation and Permanent Slides	31
Storage	31
Slides	32
Semi-permanents Mounts in Lactophenol Sealed with Nail Varnish	32
Double Cover-Glass Method	33
Erythrosin-Glycerine and Venetian Turpentine Methods	35
Conclusions	36
References.....	36

K.-Q. Zhang (✉) · J. Li

Laboratory for Conservation and Utilization of Bio-Resources, and Key Laboratory of Microbial Diversity in Southwest China, Ministry of Education, Yunnan University, 650091 Kunming, Yunnan, China

e-mail: kqzhang1@ynu.edu.cn

K. Hyde

Institute of Excellence for Fungal Research, Mae Fah Luang University, Chiang Rai, Thailand

School of Science, Mae Fah Luang University, Chiang Rai, Thailand

Abstract There has been a huge increase in our knowledge of nematophagous fungi, however, the methodology used to study these organisms are unique and have changed little over the years. Nematophagous fungi are easy to find and cultivate, but their peculiar mode of life makes it necessary to use certain techniques to bring them under observation. These methods are detailed in this chapter and include techniques for obtaining mixed cultures of the fungi from nature, for isolating taxa into pure culture, for observing living material and for making permanent microscope preparations.

Keywords Baermann funnel • Differential centrifugation • Field collection; Soil sprinkling • Soil dilution • Slide preparations

Introduction

Nematophagous fungi are classified as either nematode-trapping fungi, which catch nematodes by producing sophisticated mycelial trapping devices, or endoparasitic fungi, which infect nematodes by adhesive conidia or modified conidia, which germinate within the host after ingestion. The protocols for studying nematophagous fungi differ to those used to study most other fungi, because they do not form aggregated mycelial structures visible to the unaided eye. Therefore, certain techniques differ from orthodox mycological methods are used to isolate and characterize nematophagous fungi. The techniques related to the recovery, isolation and maintenance of nematophagous fungi involve a number of unusual techniques which are unique to the study of this group.

Habitats of Nematophagous Fungi

Ecological surveys of nematophagous fungi suggest that this group has extensive worldwide distribution, in all climates and habitats examined (Gray 1987). Therefore, it can be relatively easy to isolate nematophagous fungi, particularly from soils and organic matter.

Gray (1984b) investigated ten different terrestrial habitats for nematophagous fungi in Ireland and found that although nematophagous fungi were abundant in all the habitats examined, agricultural pasture, coniferous leaf litter and coastal vegetation had the greatest number of samples with nematophagous fungi. It was also established that nematophagous fungi in soil are associated with specific soil variables, including soil pH, depth, moisture, nitrogen, phosphorus and potassium amount, and the densities of soil bacteria, fungi and nematodes (Gray 1985, 1988). Studies on the vertical distribution of nematophagous fungi in deciduous woodland with various biotic and abiotic soil factors indicated that this group is restricted to the upper litter and humus layers (Gray and Bailey 1985). Mitsui (1985) also found similar results indicating that most of nematophagous fungi occur in the top 10–30 cm of soil.

Persmark and Jansson (1997) investigated the nematophagous fungi in rhizosphere soils of barley, pea and white mustard, and found that the densities of nematophagous fungi slightly increased in the rhizosphere as compared to root-free soil. They also found that the most common species of nematophagous fungi in these soils was *Arthrobotrys oligospora*. However, *Arthrobotrys musiformis*, *A. robusta* and *Dactylella lobata* are also significantly more abundant than most other species (Jaffee et al. 1996; Persmark and Jansson 1997; Jaffee and Strong 2005).

The best source for isolation of nematophagous fungi is rotting vegetable matter, preferably on or within soil (Duddington 1955). Nematophagous fungi are abundant in forest leaf-mould, and even more so in rotting bark and twigs and in decomposing leaf and stem litter from the forest floor or from a garden compost heaps (Duddington 1955). Therefore, it is preferable to collect soil samples from these habitats whenever possible. A high nematode population density is however, no indicator of the possible biodiversity, and so should not be used as the sole criterion for site selection (Gray 1984b).

Field Collection

The size and quantity of samples is dependent on the subsequent isolation procedure adopted in the laboratory. Sampling methodology and subsequent treatment before specialized isolation are important factors affecting the success of recovering the fungi. The commonest and most efficient sampling system is to use cork borers with different specifications (Shepherd 1955; Fowler 1970; Farrell et al. 2006). Shepherd (1955) used a 20 mm diameter cork borer to take 225 mm long cores of soil, while Fowler (1970) used a 50 mm cork borer, removing 75 mm long cores. A 75 mm diameter bulb planter was used to remove large soil cores, some 125–150 mm long, along with its associated plant material.

Little or no change is likely to have occurred in the centre of the core if the samples are processed within 24 h of collection. After sample collecting, the cores are immediately placed in polythene bags and double-sealed to prevent evaporation and deterioration. Samples in polythene bags can be stored at room temperature for 21 days, or at 4 °C for nine weeks, with no reduction in the isolation efficiency (Fowler 1970). Samples are however, preferably processed on the same or subsequent days of collection to minimize any changes in biological or soil variables. Shepherd (1955) sterilized the cork borer, used to take the soil samples, in 70% alcohol in between sampling to avoid cross-contamination. This was not possible using the larger bulb-planter, but as cores were eventually split longitudinally and subsamples taken from the centre of each core, it was considered adequate to clean the bulb-planter between sampling with fresh moist tissue.

It is not known whether nematophagous fungi are evenly distributed throughout the soil or whether they are restricted to particular zones with the nematodes. It is better to take subsamples from the nematode layer whenever possible. Where no distinct nematode zones exists, then the material is gently mixed with a pestle and mortar and subsamples taken randomly from the homogenous sample.

Table 2.1 Media used for isolating nematodes

Media	Recipe	Methods	Reference
PDA	200 g peeled potato, 20 g agar; 20 g glucose; 1,000 ml water	Boil potatoes for 30 min, filter through cheesecloth	
CMA	20 g cornmeal; 20 g agar; 1,000 ml water	Boil cook cornmeal for 30 min, filter through cheesecloth	
Water agar (WA)	20 g agar; 1,000 ml water		
Oat meal medium	20 g oatmeal; 20 g agar; 1,000 ml water	Boil oatmeal for 30 min, filter through cheesecloth	
Maize meal agar	20 g maize meal (or crushed maize grains), 20 g agar; 1,000 ml water	The maize and water are warmed to about 70 °C, for 1 h	Duddington (1955)
Difco CMA	8.5 g Difco cornmeal; 10 g agar, 1,000 ml water		Rubner (1996)
Rabbit-dung agar ^a	Rabbit pellets; 20 g agar; 1,000 ml water	Soak rabbit pellets in tap water for 2 or 3 days, and pour off the supernatant fluid, filter and dilute with tap water until a pale straw colour	Duddington (1955)
Selective media	1 % water agar or 1 % tryptone glucose agar; 1,000 mg/l triton, 50 mg/l streptomycin sulfate, 50 mg/l rose Bengal		Lopez-Llorca and Duncan (1986)

^a This media encourages the multiplication of nematodes, but does not support good vegetative growth even of predacious fungi, which makes it useful for the study of internal parasites of nematodes or for material where, as sometimes happens, mould contamination is severe. A disadvantage is that the agar often becomes rather soft, that the nematodes are plentiful and the surface may be badly disturbed

Culture Medium

There are several culture media (Table 2.1) that have been used for incubation and isolation of nematophagous fungi. However, as a general rule, low nutrient media should be used for isolation and high nutrient media should be used for incubation. Once isolated the most commonly used media for incubation of nematophagous fungi are PDA and CMA. However, it should be noted that for nematophagous fungi, if more nutrients are present then they will generally produce less conidiophores.

Baits for Nematophagous Fungi

The addition of nematodes as baits in isolation media can effectively stimulate the growth and trap formation of nematophagous fungi, facilitating their accurate qualitative and quantitative assessment (Wyborn et al. 1969). Several nematode species

have been used as the target organism in tests involving nematophagous fungi (e.g., Jansson 1994; Zuckerman et al. 1994; Stirling et al. 2005) or nematocidal compounds (e.g., Lambert et al. 1995; Sharma et al. 2003; Schwarz et al. 2004). These studies have helped elucidate pathogenicity factors and antagonistic agents of plant-parasitic nematodes (Dong et al. 2007).

The most common nematode used in studies of nematophagous fungi is a free-living soil nematode *Panagrellus redivivus* which is a well known commercially produced nematode used as a food source for fish larvae (Gray 1983) and can be bought online (<http://www.atcc.org/>). *Panagrellus redivivus* does not lay eggs, but the juveniles hatch internally. They have a short life cycle which has four larval stages before becoming adults. The first larval stage is intrauterine, but the remaining stages are free-living (Stock and Nadler 2006). This nematode is easy to rear in large quantities in culture and has a high fecundity. Several methods have been designed to culture *P. redivivus*, however, oatmeal is the most commonly used medium [10 g oat meal in 23 ml tap water, sterilized by autoclaving at 121 °C for 30 min] (Gray 1983; Dong et al. 2007). After 7–10 days of incubation at room temperature, *P. redivivus* can be separated from the oatmeal by using a Baermann-funnel which allows active nematodes to pass through a filter (Schindler 1961; Coolen 1979). Aliquots (0.01 ml) of the nematode suspension are placed on slides and gently heated to immobilize the nematodes which are then counted at 60× magnification to estimate the population density.

Rhabditis terricola has also extensively been used as a target organism to stimulate trap formation in nematode-trapping fungi (Barron 1977). The population density of these nematodes, however, never reaches the levels as can be achieved with *Panagrellus redivivus*. Barron (1977) developed an improved method for culturing this species which was to centrally inoculate a water agar plate with 1 ml of the nematode suspension, and then sprinkled the area with 0.5 g of autoclaved Lipton's green pea dried soup (probably any green pea or other dried soup can be tested). Plates are inoculated at either 20 or 30 °C to obtain large populations of nematodes within a few days (Barron 1977).

Caenorhabditis elegans has extensively been used as a model organism in biological research (Brenner 1974), and is also extensively used to an invertebrate infection model to screen for virulence factors involving nematophagous fungi (Jansson 1994; Zuckerman et al. 1994; Stirling et al. 2005; Vaitkevicius et al. 2006). In general, juveniles and adults are used. Dong et al. (2007) has designed a simple, but reproducible, cost-effective and efficient way to obtain quantities of eggs in bulk.

This method is follows:

- Cultivate *Caenorhabditis elegans* at room temperature on oat meal medium (10 g oat meal in 23 ml tap water, sterilized by autoclaving at 121 °C for 30 min) and seeded with *Escherichia coli* OP50 in an Erlenmeyer flask. Nematode cultures are transferred to fresh medium on a fortnightly basis.
- Nematode inoculum is prepared by scraping those individuals wriggling on the interface of the medium and the sides of the flask and suspending them in deionised water (DW). A 400 µl suspension containing ca 5,000 worms is inoculated onto the Luria-Bertani (LB) medium (tryptone 10 g, yeast extract 5 g, NaCl 5 g,

tap water to 1,000 ml, pH adjusted to 7.0 with 5 M NaOH, sterilized by autoclaving at 121 °C for 30 min) in a Petri-dish (6 cm diam.) and spread evenly by repeated rocking.

- Following inoculation, the culture is left at room temperature. Cultures are observed microscopically daily.
- From the third day (72 h post inoculation), a large quantity of eggs could be seen on the surface, whilst the juveniles and adults wriggle through the superficial softened medium.
- The number of eggs laid, peaks on days 3–4. After day 4 the eggs on the culture begin to hatch. For the removal of non-egg developmental stages from the medium, the medium is repeatedly flooded with the M9 buffer ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 6 g, KH_2PO_4 3 g, NaCl 0.5 g, NH_4Cl 1 g, DW to 1,000 ml, sterilized by autoclaving at 121 °C for 30 min) for three cycles of 1 min, until all the juveniles or adults are suspended and removed.
- These worms are then transferred to fresh LB medium where they continued to lay eggs. The eggs from the original flooded medium can be scraped from the surface and suspended in the M9 buffer. The eggs are surface sterilized by suspending in freshly prepared sodium hypochlorite (0.5% available chlorine) for 5 min and then rinsed with sterile DW for five cycles. A total of $ca (1.20-1.40) \times 10^5$ eggs could be obtained from one Petri-dish culture, on day 3.
- To test the hatch rate of these harvested eggs, a 400 μl suspension of prepared eggs is inoculated onto the LB medium. The cultures are placed at room temperature and microscopically inspected on a daily basis.
- Five days later, juveniles from 80–90% of the eggs hatch. The methods reported herein were conducted three times with four replicate plates per trial. The results presented are from the final trial. The method provides a reproducible, cost-effective and efficient way to obtain quantities of eggs in bulk. This method could also be used to obtain synchronized juvenile stages. The surface-bound eggs are in various stages of development. However, juveniles of the same age could be obtained by serially flooding the culture whilst leaving the unhatched eggs intact.

Wyborn et al. (1969) found that 1,000 nematodes per plate was below the threshold for stimulation of the fungal species studied, whereas at the 10,000 nematodes per plate the fungi attained epidemic proportions and swamped the nematode population. A level of 5,000 nematodes per plate was demonstrated to be the most effective level, and this is recommended. Larger inocula of nematodes have been found to cause excessive damage to the surface of the agar, making subsequent microscopic observation difficult (Gray 1984c). Therefore, it is more effective to centrally inoculate plates if the soil samples are either very wet or clay-based, otherwise this material will be spread thinly over the surface of the medium by the nematodes, leaving no clear areas of agar for direct microscopic observation (Gray 1984c).

Although trap formation of nematode-trapping fungi can be stimulated by the addition of living nematodes (Cooke 1962), a number of other substances, such as

nemin, sodium cyanide, sodium azide, colchicine, cytochalasin B, abscisic acid, nitric oxide, yeast extract, and valine are also capable of stimulating trap formation (Higgins and Pramer 1967; Chen et al. 2001; Wang and Higgins 2005; Herrera-Medina et al. 2007; Xu et al. 2011). Nemin, which is a metabolic product of nematodes rapidly induces trap formation in culture without the problem of nematode contamination (Pramer and Stoll 1959; Feder et al. 1963; Nordbring-Hertz 1972; Barron 1977).

Treatment of Samples

Several techniques have been devised to isolate nematophagous fungi, such as the soil sprinkling method, the Baermann funnel technique and the differential centrifugation technique (Duddington 1955; Cooke 1961; Barron 1969). These methods are efficient and time saving and give an overview of the spectrum of nematophagous fungi infecting living nematodes in soils. Quantitative examinations are also possible. The soil sprinkling technique and the soil dilution method are good for quantifying nematode-trapping fungi (Dackman et al. 1987; Persmark and Jansson 1997), while the differential centrifugation technique are good for quantifying endoparasitic fungi. The soil sprinkling technique and the soil dilution method employ nematodes as baits, enabling microscopic observation of the fungi. Limitations are that they do not allow determination of whether the fungi are present in soil as spores or hyphae, and that they are time-consuming and labour-intensive (Persson et al. 2000). The Baermann funnel technique and differential centrifugation technique are mainly used to isolate endoparasitic species.

Soil Sprinkling Technique

The soil sprinkling technique was originally devised by Drechsler (1941), and developed by Duddington (1955), and then further by Eren and Pramer (1965). Duddington (1955) first isolated nematode-trapping fungi by plating out small amounts of soil onto nutrient agar. However, the slow growing nematode-trapping fungi were overgrown by the faster growing saprobic fungi that rapidly colonized plates. Eren and Pramer (1965) changed the nutrient agar to 2% water agar, with adding nematodes to be selective for nematode-trapping fungi. The low nutrient status of this agar reduces saprobic fungal growth, and by providing a separate nutrient source in the form of nematodes, the method becomes highly selective for nematode-trapping fungi. In a later experiment, CMA nutrient adjusted to 15%, was found to be a more effective isolation media (Fowler 1970).

A soil dilution technique combined with the sprinkling plate method gave a higher probability of isolating nematode-trapping fungi rather than the sprinkling plate method mentioned above, when similar soils were compared (Dackman et al. 1987). The soil dilution method is described below.

The soil sprinkling technique is widely used for the recovery of nematode-trapping fungi (Persson et al. 2000; Park et al. 2002; Farrell et al. 2006). The limitations of the soil sprinkling method are that they do not allow determination of whether the fungi are present in soil as spores or hyphae, and that they are time-consuming and labour-intensive (Persson et al. 2000).

Soil Dilution Method

A similar technique to isolate nematode-trapping fungi is the soil dilution method. This method were first proposed by Dackman et al. (1987) who found that the soil dilution technique can give approximately 10 times as many fungal species as the sprinkle plate method, when similar soils are compared. At present, the soil dilution method which was a development of the earlier sprinkling technique have also been used to isolate nematode-trapping fungi (Persmark and Jansson 1997).

The soil dilution method is performed in the following manner:

- 15 g soil is shaken in 15 ml 0.01 % (w/v) sodium hexametaphosphate in a 100-ml Erlenmeyer flask for 10 min.
- Heavy material is allowed to settle before the soil suspension is decanted into a graduated cylinder and the volume adjusted to 15 ml with water.
- A dilution series is prepared giving 1, 0.1, 0.01, and 0.001 g soil ml⁻¹.
- From each dilution a sample of 0.5 ml is spread on a water agar plate (1.5 % w/v) in such a way that a star is formed (Barron 1977), and the plates are left with the lid half open to allow excess water to evaporate.
- Five replicates are made from each dilution.
- On the following day, the plates are baited with approximately 500 nematodes per plate.
- The plates are incubated in the dark at approximately 22 °C and examined for growth of predatory fungi after 2 and 6 weeks.
- More frequent examination were found unnecessary. Plates showing growth of predatory fungi, manifested in the formation of trapping organs, are recorded as positive. The proportions of positive plates in each dilution is used to calculate the most probable numbers from an appropriate MPN table.

Baermann Funnel Technique

Living nematodes in soils will normally be colonized by fungal parasites. Therefore, nematophagous fungi can be isolated by extracting nematodes from soil and plating them onto low nutrient agar (Capstick et al. 1957; Giuma and Cooke 1972). Although many methods have been described for separating nematodes from the soil (Whitehead and Hemming 1965; Southey 1986), the Baermann funnel technique (Staniland 1954; Giuma and Cooke 1972) has been most extensively used, especially for extracting nematodes infected by nematophagous fungi.

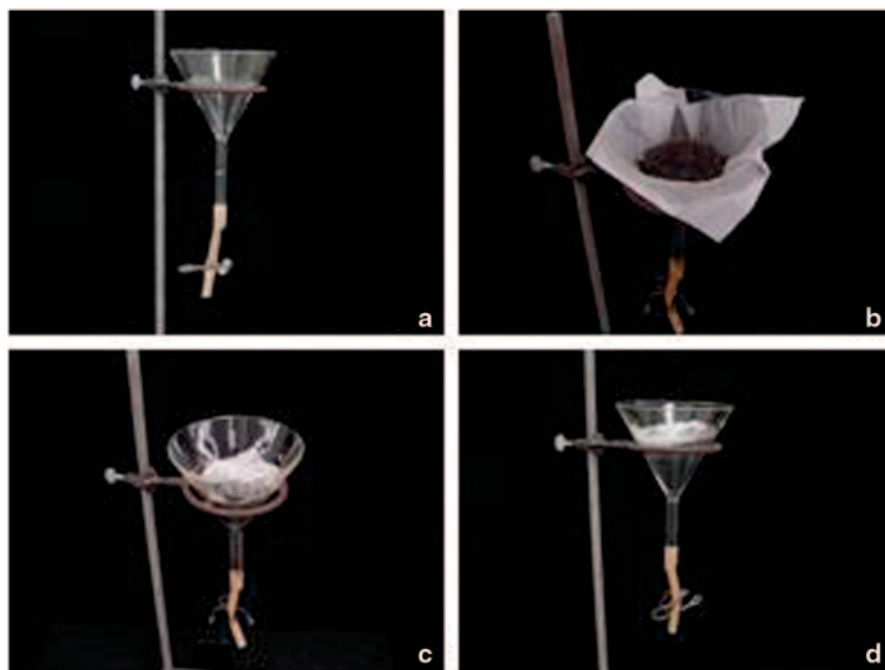


Fig. 2.1 Procedure for isolating living nematodes in soil by Baermann funnel technique

- The standard Baermann funnel apparatus is shown in Fig. 2.1a.
- Wrap approximately 30 g of fresh soil sample in a double layer of soft tissue (Fig. 2.1b).
- Placed this in the standard Baermann funnel apparatus with distilled water (Fig. 2.1c, d), the water level can be readjusted until it is in contact with the soil.
- The nematodes wriggle through the tissue and pass down through the funnel into a small collecting tube.
- After 24 h the collecting tube is removed and the nematode suspension concentrated by centrifugation at 1,000 rpm/min for 3 min.
- Supernatants are discarded and the nematode mass is resuspended in 3 ml of distilled water and incubated at room temperature (18–22 °C).
- Microscopic examination of suspensions should be carried out immediately after centrifugation and this may show that very few nematodes have obvious symptoms of fungal attack.
- After 4 days or more of incubation in distilled water, place samples into glass cavity blocks and re-examine. The nematodes which become sluggish or immobile may contain fungi.
- Remove the nematodes to a sterile 10 ml centrifuge tube containing 5 ml of sterile distilled water by means of a fine Pasteur pipette and concentrated as before.
- Discard the supernatant and resuspend the nematodes in a further 5 ml of sterile distilled water and centrifuge again.

- Transfer the wash nematodes to glass cavity blocks in a small amount of distilled water.
- Transfer those nematodes with emerging hyphae to 2% cornmeal agar plates, and leave the remaining nematodes containing thalli in the cavity blocks
- Examine at daily intervals in order to observe the development of the parasites. Fertile hyphae usually grow rapidly from the nematodes and can be subsequently identified.
- When conidia form, transfer them aseptically to fresh media, where they should usually germinate. In this way pure cultures of a number of species of nematophagous fungi can be obtained (Giurma and Cooke 1972).

Although some active nematodes may be caught on the sides of the funnel or may die from a lack of oxygen in the funnel system, this technique allows reasonably rapid isolation of endozoic parasites without the necessity for their maintenance on nematode populations or the use of antibiotics in the agar medium employed. A comparative investigation has shown the Baermann funnel technique to be significantly more effective in isolating endoparasitic nematophagous fungi than the soil sprinkling method (Gray 1984a).

However, only nematodes in early stages of infection seem mobile enough to do this. The chance of parasites with very rapid rates of development within their hosts being isolated using this method might therefore be small (Giurma and Cooke 1972), and the Baermann funnel technique is influenced by the indigenous nematode population density (Gray 1984a).

Differential Centrifugation Technique (Barron 1969)

Differential centrifugation is a common procedure in microbiology and cytology used to separate certain organelles from whole cells for further analysis of specific parts of cells (Wilchek and Bayer 1990). This technique has also been modified to investigate nematophagous fungi. The principle of differential centrifugation technique is to spin down the large predaceous type conidia at low speeds leaving the smaller conidia of the endoparasitic fungi in the supernatant. Therefore, this method can be used to separate the heavier spores of nematode-trapping fungi from the lighter spores of endoparasites. The supernatant is then centrifuged at a higher speed and the pellet containing the spores spread on plain agar baited with nematodes which subsequently become infected (Barron 1969).

The procedure for this technique is as follows:

- Approximately 200 cm³ of the soil sample is added to 250 cm³ of water and blended for 30–60 s.
- The resultant mixture is passed through a series of screens down to a No. 35 screen to remove any coarse material.
- The soil suspension is poured into 50-ml centrifuge tubes and spun down for 2 min at 1,000–2,000 rpm to remove heavy soil particles and large spores.

- The supernatant is decanted and retained to be centrifuged again at 4,500 rpm for 1 h.
- The supernatant is then discarded and the residue saved.
- This is broken up and mixed with a few drops of sterile water using a glass rod and then spread onto a water agar plate.

The residue is spread over half of the plate and then seeded with a few drops of concentrated nematode solution containing 2,000–3,000 nematodes. The centrifugation method results in lower efficiency and diversity of nematophagous fungi than the Baermann funnel technique. However, this technique can isolate conidia as well as the Baermann funnel, making this isolation technique especially useful for studies in specificity of infection and on the conidia (Gray 1984a).

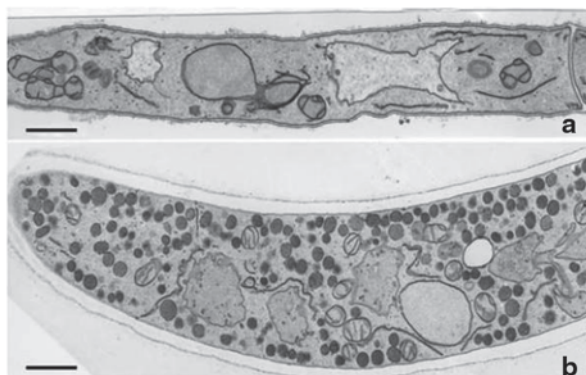
Observation and Identification

Observation

The identification of nematophagous fungi is normally established by direct observation. Nematophagous fungi are observed in culture and conidiophores and trap structure characteristics are determined. Conidiophores are often observed on top of crumbs of soil or on organic debris, where they are only visible at lower magnifications. Emergence times for nematophagous fungi vary considerably, and in order to recover the majority of species in a particular sample, the soil plates should be scanned frequently. Fowler (1970) compared 32 samples of garden soil at weekly intervals for 14 weeks, finding that most of nematophagous fungi present had been recorded within six weeks. Therefore Barron (1978) advised that plates should be examined daily for up to ten days to ensure that all the possible nematophagous species were observed. Presently, plates with small amounts of soil on nutrient agar are examined as frequently as possible, and at least at weekly intervals, for up to ten weeks. In this way even the most slowly developing species are recovered. Less frequent observations will result in fewer species being recorded.

Identification of the fungi by direct observation at $\times 400$ magnification using an objective lens with a long working distance is highly successful and significantly reduces the number of mounted preparations. When fungi are immature, the bottom of the Petri-dish can be marked using an indelible marker pen so that the specimen can be relocated and examined at a later date. A thin section of the agar (clear water agar) is better as it allows the researcher to focus on infected nematodes. In general, plates should be scanned at $\times 100$ magnification for clear observation of nematophagous fungi. Lower magnifications such as $\times 40$ or $\times 60$ magnification should only be used by experienced workers. Subsequent identification is carried out directly at $\times 100$ magnification using an objective with a long working distance. The entire surface of each plate is scanned in this way using a template fixed to a movable stage.

Fig. 2.2 Transmission electron micrograph (TEM) of vegetative hypha (upper panel) and a trap cell. Bar, 1 μ m. Note dense bodies only in the trap cell. Figure reproduced from Nordbring-Hertz (1984) with kind permission from Cambridge University Press



Electron Microscopy

Electron microscopy is a method which uses a beam of electrons to illuminate the specimen and produce a magnified image. This method has a greater resolving power than a light-powered optical microscope, because electrons have wavelengths about 100,000 times shorter than visible light (photons), and can achieve better than 50 pm resolution (Erni et al. 2009) and magnifications of up to about 10,000,000 $\times$, whereas ordinary, non-confocal light microscopes are limited by diffraction to about 200 nm resolution and useful magnifications below 2,000 $\times$. There are two forms of electron microscopy which are commonly used to provide surface information: Secondary Electron Microscopy (SEM), which provides a direct image of the topographical nature of the surface from all the emitted secondary electrons, and Scanning Auger Microscopy (SAM), which provides compositional maps of a surface by forming an image from the Auger electrons emitted by a particular element. Both of these techniques employ focusing of the probe beam (a beam of high energy electrons, typically 10–50 keV in energy) to obtain spatial localization.

Electron microscopy can provide valuable morphological information, as well as some remarkable views of nematophagous fungi and have been extensively used to observe species (Nordbring-Hertz 1972; Barron 1979; Saikawa and Morikawa 1985; Tunlid et al. 1991; Bordallo et al. 2002). Many workers, such as Heintz and Pramer (1972), Nordbring-Hertz (1972) and Janssona (Janssona 1982) have provided useful details of the techniques of transmission and scanning electron microscopy of nematophagous fungi. For example, Lysek and Nordbring-Hertz (1983) used SEM to obtain a clear figure of an adhesive network trap of *Arthrobotrys oligospora*, while Nordbring-Hertz (1984) used TEM to reveal a common feature observed in all traps of trap-forming fungi (Fig. 2.2). The presence of numerous cytosolic organelles (dense bodies) are formed directly on initiation of the trap. Normal vegetative hyphae invariably lack dense bodies (Fig. 2.2). The dense bodies develop from specialized regions of the endoplasmic reticulum and exhibit catalase and amino acid oxidase activity and thus

are peroxisomal in nature. They are supposed to be involved in penetration and digestion of the nematode.

Fluorescence Microscopy

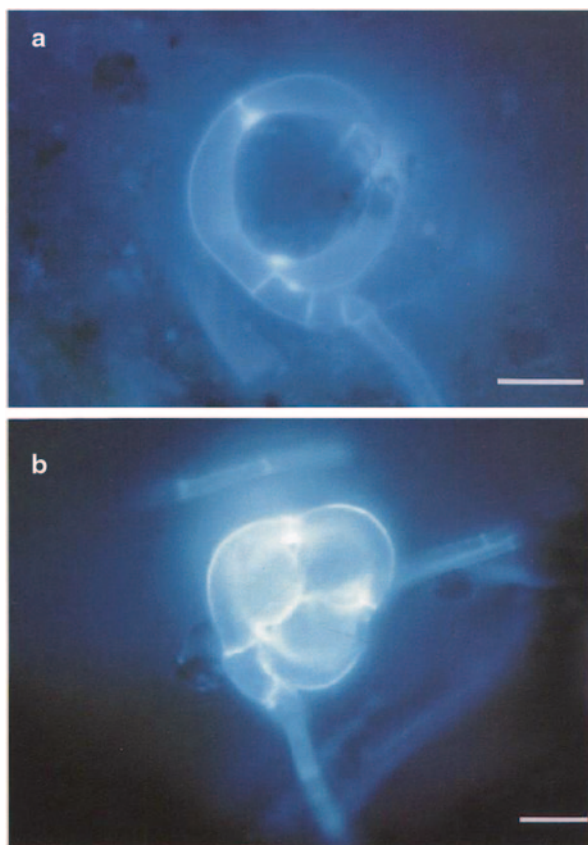
With sophisticated equipment and an increasing number of fluorochromes, fluorescence techniques have become an effective tool in microscopy to directly observe soil dwelling fungi *in situ* (Jensen and Lysek 1991; Saxena and Lysek 1993; Jensen 1994). This has been made possible by the use of specific fluorochromes such as fluorescein-di-acetate (FDA) and calcofluor white. Fluorescein-di-acetate (FDA) is a substance which does not give any fluorescence by itself; but it can be taken up by actively metabolizing cells or hyphae, and hydrolyzed enzymatically to yield free fluorescein, a true fluorochrome. Thus, only living and actively metabolizing microorganisms become visible when stained with FDA (Correa et al. 1986; Soderstrom and Erland 1986; Jensen 1994). Calcofluor white (correct name Calcofluor white M2R new) is an optical brightener with a high affinity to β -1, 4-glucans such as cellulose or chitin. It is thus specific for hyphal walls, and does not differentiate between living and dead tissue. Calcofluor stains many types of fungal structures, for example spores or fruiting body initials (Von Sengbusch et al. 1983).

Investigation of fungi in the soil by fluorescence microscopy is now possible. Staining of key taxonomic features by fluorochromes, for example developing conidiophores and spores of *A. oligospora*, make it an ideal tool for the rapid identification of species of fungi that grow actively in soil, and which would be extremely difficult to observe by conventional microscopy. The technique can also be used to study fungal growth in soils over time, which exhibit periodic effects and even allows some considerations concerning their ecology (Jensen et al. 1997; 1998). It also has been used to study the colonization of soils contaminated with urban pollutants and to study the occurrence of fungi in the ventilation systems of buildings (Neumeister et al. 1996). Here, we give examples of using fluorescence microscopy to examine the trap formation of nematophagous fungi. Figure 2.3 shows the constricting rings of *Arthrobotrys dactyloides* which was stained with calcofluor white before (a) and after contraction (b) (Jensen et al. 1998). These inflation acts very rapidly, triggered when a nematode touches the inside of the ring. The three cells enlarge their volume such that the ring is completely filled. The nematode is quickly ensnared and cannot escape. Figure 2.3 also shows a recently captured nematode, but due to the selectiveness of calcofluor white for glucans, it is stained poorly and hence is only visible as a shadow (Jensen et al. 1998).

Figure 2.4 shows an example of double staining of *A. oligospora* with FDA and calcofluor white (Jensen et al. 1998). If the filters are chosen adequately, the stains highlight different cellular components: FDA (Fig. 2.4a) stains actively metabolizing areas such as capture organs, while calcofluor white makes the entire complex visible (Fig. 2.4b; Jensen et al. 1998).

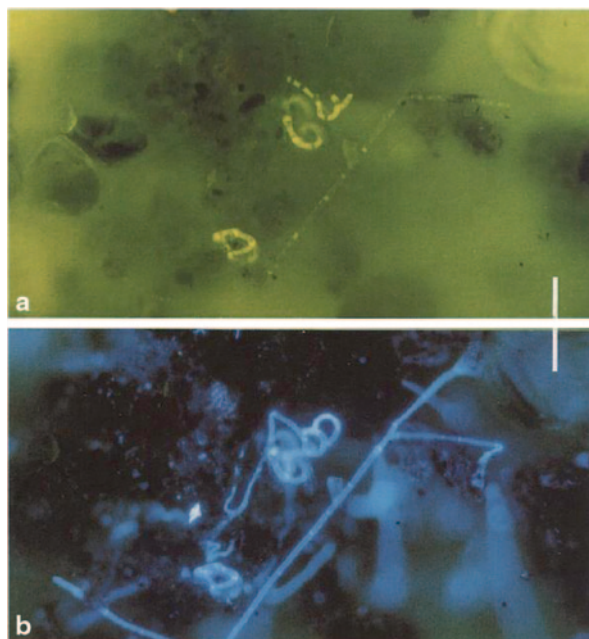
When using fluorescence microscopy to investigate nematophagous fungi in soil we recommend the use of sterilized (autoclaved) compost, to avoid any effect of soil

Fig. 2.3 Constricting rings of *Arthrobotrys dactyloides* before (a) and after contraction (b). In (b) the captured nematode captured is just visible. Staining with calcofluor white. Bar = 10 μ m. (Figure reproduced from Jensen et al. (1998) with kind permission from Elsevier)



fungistasis and inoculation of the fungi from pre-cultures on malt extract agar plates. Incubation should be done in Petri-dishes at room temperature. In order to preserve the exact localization of hyphae, traps, or other specific structures and surrounding soil particles, the soil samples need to be treated very carefully. Soil samples are cut vertically from the surface to the bottom of the Petri-dish with cover slips to obtain a number of soil slides to an average depth of 2.5 mm. Each soil slide is placed on one cover slip. Staining and manipulation should be done with much care to avoid distortion of the fungal structures. Observation is best through a cover slip using the hanging drop technique (Jensen 1994; Jensen and Lysek 1995). When staining by fluorochromes, if stain by FDA, a stock solution should be prepared from 500 mg FDA dissolved in 100 ml acetone and stored at -18°C . A working solution should be prepared by diluting 0.1 ml of the stock in 5 ml 60 mM phosphate buffer pH 6.88. Since this degrades very rapidly, the working solution should be freshly prepared for every observation. It is mixed into the soil and microscopic observation started immediately; images are visible 1 min after preparation. Due to the rapid photofading of fluorescein, staining only lasts 20 min. If stain by Calcofluor white, a stock solution

Fig. 2.4 Hyphae with sticky networks of *A. oligospora* between soil particles stained with FDA (**a**) and calcofluor white (**b**). Due to the triggering light and filtering the FDA (**a**) and calcofluor white (**b**) give different images of the same structure. Bar = 100 μm . (Figure reproduced from Jensen et al. (1998) with kind permission from Elsevier).



should be prepared from 50 mg calcofluor dissolved in 5 ml buffer (Tris pH 9.0). The working solution consists of 0.1 ml of stock solution in 9.9 ml buffer. Both solutions are stable and should be stored in the dark at room temperature. For microscopic observations, the working solution should be simply mixed with the soil to be investigated immediately prior to observation. The fluorescence is very stable; for example, mounts kept in a wet chamber could be studied for at least 24 h after staining.

Isolation of Pure Culture Species

In order to identify and characterize the morphology of nematophagous fungi, it is necessary to obtain pure cultures of species (Gray 1983). The method used to obtain pure subcultures of nematophagous fungi is to transfer a small piece of agar which contains numerous fungal conidia to a new fresh plate with nutrition medium. This method has the disadvantage of picking up surface contamination from the agar, which causes unwanted bacterial and mould growth on the plates. Heintz (1978) used two techniques for transferring nematophagous fungi from pure cultures onto fresh plates, a standard method uses plug inocula and macerated inocula.

Standard method

- 3 mm diameter disks of agar are cut from the periphery of an actively growing culture on nutrient agar using a sterile cork borer
- The disks are transferred to new plates.

Macerated Inocula Method (Heintz 1978)

- A 50–60 mm diameter colony of the fungus is removed from an agar plate with a sterile scalpel, removing as little agar as possible and placed in 100 ml of sterile distilled water.
- The colony is macerated until a homogenous suspension is produced.
- 0.5 ml aliquots are then spread uniformly over the entire surface of each agar plate.

The second technique uses macerated inocula, which allows a more uniform growth to be produced on the new plate. Heintz (1978) noted that this method resulted in the development of a typical fungal thallus within three days.

Numerous methods have been used to transfer conidia to obtain pure cultures of nematophagous species. Shepherd (1955) used a sterile platinum wire while Barron (1977) used a sterile dissecting needle to remove the conidia. However, Gray (1984b) used a single haired sable paint brush to pick off individual conidia. He believed that the single hair is extremely fine, short and flexible, and can be easily directed to particular conidia under $60\times$ or $100\times$ magnification. A popular method to transfer conidia is to use a sterile toothpick (Hao et al. 2005; Zhang et al. 2005; Li et al. 2006). The single hair is extremely fine, short and flexible, and can be easily directed to particular conidia under $60\times$ or $100\times$ magnification. The collected conidia are streaked onto nutrient agar.

In addition, pure cultures of some endoparasites can also be obtained by transferring nematodes, which are infected but with the fungus not yet sporulating, onto nutrient agar supplemented with aureomycin (30 g/l) to reduce bacterial contamination. Endoparasites are more difficult to transfer and grow in culture. The simplest method is to remove the infected nematode with a needle and wash it several times in saline to remove any contamination. This is best done by using a series of cavity slides and transferring the nematode from slide to slide. The infected nematode is then placed on a fresh water agar plate and inoculated with nematodes.

Identification

Identification of nematophagous fungi depends on the morphology and morphometry of conidia (shape, septa and size) and conidiophore branching, and modifications at the apex (Drechsler 1937; Cooke and Godfrey 1964), but also the trapping structure, and presence or absence of chlamydospores. Five kinds of trapping devices have been recognized, including adhesive networks, adhesive hyphae, constricting rings, adhesive knobs and nonconstricting rings (Fig. 2.5). The adhesive hypha is a short erect branch of a few swollen cells produced on a hypha with an adhesive layer covering its surfaces (Figs. 2.5, 1–4). The adhesive knob is a globose or subglobose

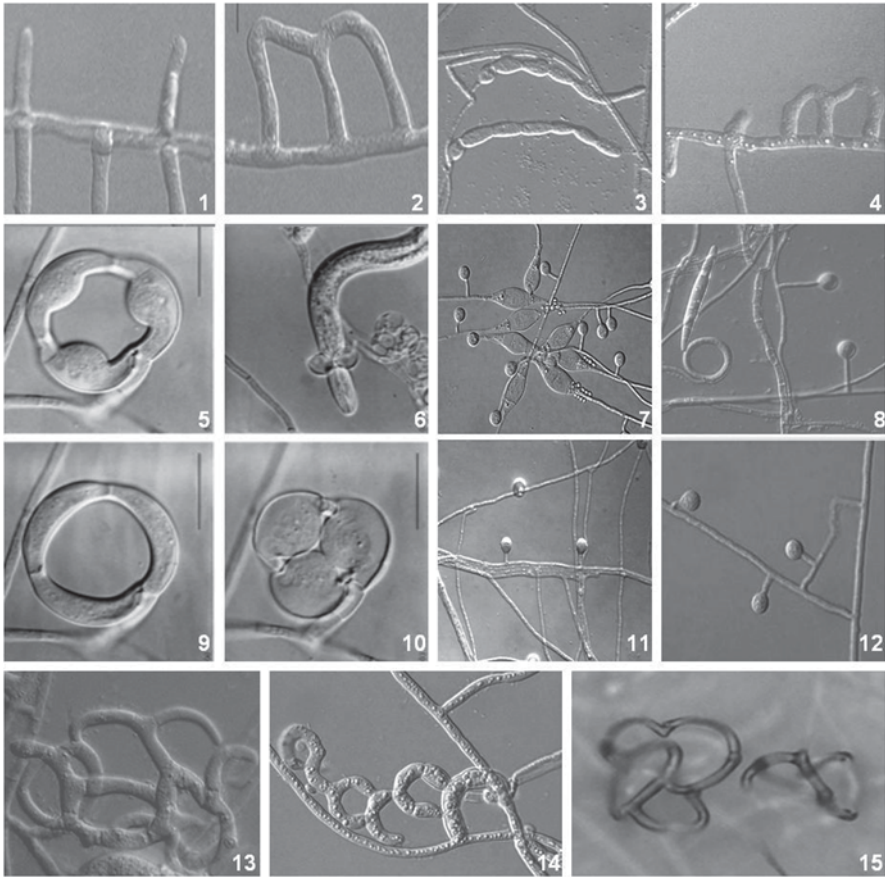


Fig. 2.5 Morphology on traps of nematode-trapping fungi. 1–4. Adhesive hypha. 5–6, 9–10. Constricting rings. 7, 11–12. Adhesive knobs. 8. Adhesive knobs and nonconstricting rings. 13–15. Adhesive networks

cell that is either sessile on the hypha or with an erect stalk (Figs. 2.5, 7–8, 11–12). Nonconstricting rings always occur alongside adhesive knobs, and are produced when erect lateral branches from vegetative hypha thicken and curve to form a generally three-celled ring that then fuses to the supporting stalk (Figs. 2.5, 8). The constricting ring contains three ring cells. When a nematode enters a constricting ring, the three ring cells are triggered to swell rapidly within 1–2 s (Figs. 2.5, 5–6, 9–10). The adhesive network is formed by an erect lateral branch growing from a vegetative hypha, curving to fuse with the parent hypha and developing more loops exterior to the original loop or on the parent hypha (Figs. 2.5, 13–15; Barron 1979; Yang et al. 2007).

Use of Molecular Techniques in Identifying Nematophagous Fungi

Rapidly developing molecular techniques such as the polymerase chain reaction (PCR) have revolutionized identification and classification of fungi (White et al. 1990; Foster et al. 1993). Application of such techniques for identification of nematophagous fungi can be important tools for rapid and accurate identification. For example, Hu et al. (2006) has identified *Arthrobotrys multisecondaria* (YMF1.01821) as a new nematode-trapping fungus based on the production of unicellular secondary conidia from both distal and basal ends. However, the extracellular serine protease genes, ITS and β -tubulin fragments which were cloned from *Arthrobotrys multisecondaria* show a high degree of similarity to those from *Monacrosporium microscaphoides*. Therefore, although these two fungi exhibited obvious differences in secondary conidia morphology, Li et al. (2008) has considered *Arthrobotrys multisecondaria* to be a spontaneous mutant of *Monacrosporium microscaphoides*. This example suggests that there may be several mistakes regarding the identification of nematode-trapping fungi when based only on the morphological observations.

Another example indicating the importance of molecular technology concerns the taxonomic revisions of nematode-trapping fungi. With molecular technology, the systemic classification of nematode-trapping fungi has been redefined because there is multitude inconsistencies in the evolutionary relationships as compared to the established taxonomy, which was traditionally based on morphology of conidia and conidiophores. Since the pioneering work by Drechsler (Drechsler 1937), nematode-trapping fungi have been classified in a number of genera based on morphology of conidia and conidiophores. Based on the phylogenetic analyses of the 5.8 S rDNA, ITS1 and ITS2 of 28 nematode-trapping fungi, Liou and Tzean (1997) found that the 28 nematode-trapping fungi can be separated into four clades, each with a unique trapping device, and were first to propose that trapping organs may reflect evolutionary relationships, and appear more significant than conidiogenous cells and conidia for genus and species delimitation. Pfister (1997) also found that trapping devices are more informative than other morphological characters in delimiting genera. Subsequently, based on the small subunit (SSU) ribosomal DNA (18 S rDNA) from 15 species of nematode-trapping fungi, Ahren et al. (1998) found that nematode-trapping fungi clustered into three lineages: species with constricting rings, species with various adhesive structures (net, hyphae, knobs and nonconstricting rings) and species have no trapping devices. Based on results obtained from morphological and molecular characters, Hagedorn and Scholler (1999) and Scholler et al. (1999) classified nematode-trapping fungi into four genera: *Dactylellina* characterized by stalked adhesive knobs including species characterized by nonconstricting rings and stalked adhesive knobs; *Gamsylella* characterized by adhesive branches and unstalked knobs; *Arthrobotrys* characterized by adhesive networks; and *Drechslerella* characterized by constricting rings. Based on sequence analyses of 28 S and 5.8 S rDNA and β -tubulin, Li et al. (2005) proposed an emended generic concept for nematode-trapping fungi. *Arthrobotrys* is characterized by

adhesive networks, *Dactylellina* by adhesive knobs, and *Drechslerella* by constricting-rings. Phylogenetic placement of taxa characterized by stalked adhesive knobs and nonconstricting rings also is confirmed in *Dactylellina*. Species that produce unstalked adhesive knobs that grow out to form loops were transferred from *Gamsylella* to *Dactylellina*, and those that produce unstalked adhesive knobs that grow out to form networks were transferred from *Gamsylella* to *Arthrobotrys*. *Gamsylella* as currently circumscribed cannot be treated as a valid genus.

The use of molecular techniques is widespread and has helped to establish relationships between different organisms. However, these techniques only came into use in the previous 15 years and thus much can be learnt from these techniques. They can be used not only for the investigation of phylogenetic, taxonomic, and diagnostic problems in nematophagous fungi, but also provide the basis for gaining deeper insights in their biological research. However, we should not use molecular data in isolation to other data such as morphological data because researchers may infer wrongly perceived ideas from incorrectly generated phylogenetic trees due to the unwise selection of ingroups and outgroups.

In 2012, the International Code of Nomenclature for Algae, Fungi and Plants was revised and no longer allows a single fungal species (or genus) to have two names, with the older name taking priority except in exceptional circumstances (Hawksworth 2012). The asexual genera of nematode-trapping fungi are linked with a sexual *Orbilbia* morphs and may need to take the *Orbilbia* name, or alternatively their names may be maintained. Discussions are presently underway by the *Orbilomyces working group* of the *International Commission on the Taxonomy of Fungi* (<http://www.fungaltaxonomy.org/subcommissions>) to establish which is the best name (or names) to adopt for this group(s) of fungi.

Preservation and Permanent Slides

Storage

In general, nematophagous fungi lose their viability if maintained for long periods in pure culture. The storage methods most suited to each fungal species or genera needs to be determined (Ryan et al. 2000). The maintenance of predatory activity of the fungal isolates is one of the basic prerequisites to ensure predatory activity. Several methods have therefore, been devised for preserving fungal cultures for prolonged periods and thus retaining the original characteristics of the isolates. Methods include preservation in mineral oil, cryopreservation, cold storage at 4 °C and preservation of species in silica gel (Duddington 1955). However, all these methods can more or less reduce the predatory activity and sporulation capacity.

Barron (1977) developed a soil storage method which can preserve nematophagous fungi for much longer periods without loss of viability. In this method 30-ml screw top vials are half filled with moist greenhouse soil and sterilized. Fresh

nematodes are added to a plate containing infected nematodes, and softly mixed together. The resulting suspension, including bacteria and other contaminants, is then used to inoculate the vials. Approximately 0.5 ml of the suspension is added to each vial, which is allowed to stand at room temperature for several days to ensure infection of the healthy nematodes and is then stored at 5 °C. When required the vial is shaken to loosen the soil, and the material plated out as normal using the soil sprinkling technique.

Slides

In order to identify the nematode-trapping fungi that grow on the culture medium, microscopic slides must be prepared. Fowler (1970) initially used a dissecting microscope to locate fungi by examining the plates at 60× magnification. Alternatively where immediate identification was not possible subcultures were prepared. Shepherd (1955) used high-power microscopy to identify fungi directly by adding a drop of water to the agar, lowering a cover slip over the specimen, and examining it under oil immersion. Wood (1973) examined his isolates in temporary water mounts or in lactophenol acid fuchsin, while live nematodes were fixed in 5% formalin containing acid fuchsin. It is important to retain the hyphae *in situ* for subsequent examination and so permanent preparations were made using the method originally described by Dixon and Duddington (1951).

Lactophenol cotton blue (LPCB) wet mounts are the most widely used method for staining and observing fungi (Leck 1999). A thin section of the agar containing the fungus is removed using a scalpel and transferred onto the slide. The agar thickness is reduced by gently warming the slide, and the specimen is stained using cotton blue in lactophenol. A piece of clear adhesive tape can be gently touched on the colony and then placed on a drop of stain on the slide to observed intact specimens with string of spores or other structures that break up when making agar mounts.

It is difficult to make good permanent preparations of nematophagous fungi and these are less satisfactory to study than water mounts of living material. Several techniques for making permanent mounts of nematophagous and other fungi have been described, including lactophenol mounts with nail varnish sealant (Hawkswell 2001), Kohlmeyer's permanent mount method (Volkmann-Kohlmeyer and Kohlmeyer 1996) and Venetian turpentine method (Duddington and Dixon 1951, 1953).

Semi-permanents Mounts in Lactophenol Sealed with Nail Varnish

When examining spores of *Russulaceae* in Melzer's reagent under the microscope and subsequently mounting them in lactophenol, Hawkswell (2001) found that the iodine staining faded, rendering details of the spore ornamentation indistinct.

However, by adding a quantity of iodine to lactophenol, he found that the staining intensity was enhanced and it did not fade and the details of spore ornamentation remained clear (Hawkswell 2001). The routines for staining and mounting are as follows:

- Take a small piece of fungal tissue or infected nematodes, place it on a microscope slide and add a drop of iodine.
- Place a cover glass over the specimen, cover with a strip of filter paper and exert firm pressure with a finger to compress the sample.
- Pour a few drops of distilled water over the slide, gently remove the cover slip and carefully pour off the surplus water.
- Give the specimen a second rinse with distilled water, pour off the surplus water and blot dry with tissue.
- Add a drop of iodine-lactophenol and place a cover slip over the specimen, carefully avoiding air bubbles.
- Remove the surplus iodine-lactophenol with tissue and seal the cover glass with nail varnish.

All specimens mounted in lactophenol must be sealed with nail varnish or a similar sealant if it is intended to keep the slide on a permanent basis. Permanent slides prepared by this method will be good for at least 18 months Hawkswell (2001) and the specimens retain the stain and ornamentations details clearly.

Double Cover-Glass Method

The “double cover-glass method” was introduced by Diehl (1929) who described the mounting of the specimen between two cover glasses of different sizes. It was revised by Kohlmeyer and Kohlmeyer (1972) and also adapted for fungal cultures by Connell and Padgett (1988).

- A large square cover glass (25×25 mm, No. 1) is attached on a clean slide (76×26 mm) by means of two droplets of distilled water (Figs. 2.6, 1).
- A larger drop of distilled water is placed in the centre of the cover glass, to which the specimen is added in the form of whole mounts, squash mounts or sections (Figs. 2.6, 2).
- The object is then covered with a smaller cover glass (18×18 mm, No. 1, Figs. 2.6, 3) allowing immediate investigation of the living material with bright-field, phase contrast or Nomarski interference contrast. The use of immersion oil is also possible, although easier in the later stages, since special care must be taken when wiping off the oil.
- After the necessary observations, measurements, drawings and/or photographs have been made, a droplet of concentrated glycerin is added to the water at one or two sides of the small cover slip (Figs. 2.6, 4).

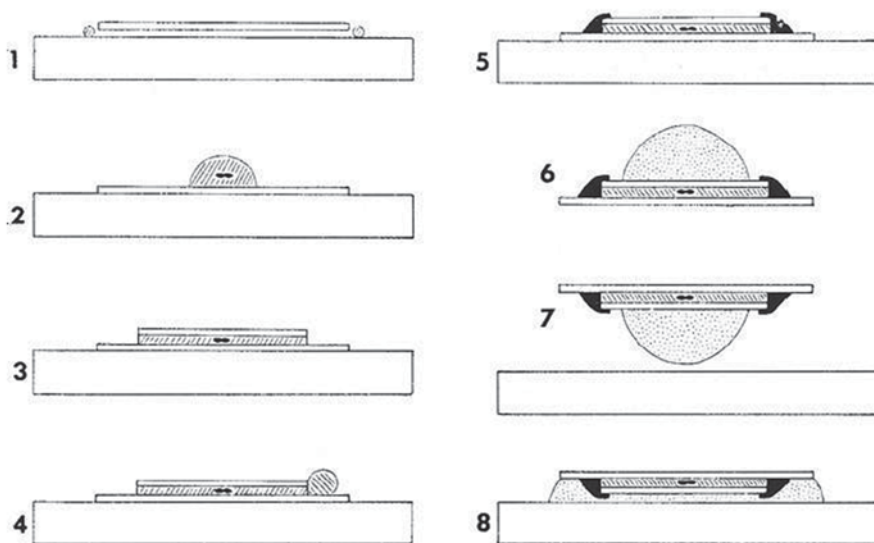


Fig. 2.6 Double cover glass method for permanent microscopic mounts. (From Kohlmeyer and Kohlmeyer 1972)

- The slide is stored horizontally in a dustproof container for a few days to allow the water to evaporate. Excessive glycerin is removed from the edges of the larger cover glass easily done with filter paper—or if needed, more glycerin is added.
- The mount is then sealed with a thin ring of clear fingernail polish; it is best to repeat this step after an hour to be sure the glycerin is perfectly sealed in (Figs. 2.6, 5).
- When the nail polish is fully dried, the large cover glass is removed from the slide with the aid of a needle and a drop of mounting medium is placed on the small cover glass (Figs. 2.6, 6).
- Then the preparation is turned over (Figs. 2.6, 7) and placed on the slide. The drop of mounting medium flattens out (more effectively if one puts a small weight e.g., old AA battery on top for a few minutes), surrounding the edges of the small cover glass (Figs. 2.6, 8), thereby permanently sealing in the small cover glass and nail polish.
- Any excess medium is squeezed out at the sides and can be taken off with a needle. The preparation is now stored horizontally until the medium is hardened, but it can be used after a day, should further inspection at the microscope be necessary.
- The sealing procedures are best done under a hood to avoid breathing the toxic fumes of the media. Nearly as important as the sealing technique is the labelling.

For this method, paper labels are not always permanent especially if written in pencil, neither with pencil written on frosted glass. Kohlmeyer and Kohlmeyer (1972) recommend to use slides with one frosted end, and the labelling being done with

waterproof ink. Kohlmeier and Kohlmeier have slides as old as 30 years that are in perfect condition; the fungi being permanently sealed, there is no drying out or cracking and the writing is as clear as on the first day. Again, this method is more time-consuming than just using fingernail polish, but the results are so much more satisfying for anybody who needs to prepare voucher or holotype specimens, and it should always be used for preserving type material.

Erythrosin-Glycerine and Venetian Turpentine Methods

Duddington and Dixon (1951) have introduced methods for making rapid preparations for nematophagous fungi from agar culture. They believed that formalin-acetic acid fixative is excellent for preservation of material. For endoparasitic nematophagous fungi, the Erythrosin-glycerine method is most useful. The procedure of this method is as follows:

- A small piece of agar containing the specimen is fixed in the following mixture: commercial formalin, 10 ml; glacial acetic acid, 5 ml; distilled water, 85 ml for not less than 24 h.
- The specimen is washed in water until no smell of fixative is detectable.
- The material is placed in a saturated aqueous solution of erythrosin for 24 h.
- The specimen is transfer to 10% glycerine and allowed to concentrate until most of the water has evaporated.
- Addition of 0.5% of glacial acetic acid is added to improve staining.
- The dehydration is completed in a desiccator, and the material can then be mounted in glycerine or in glycerine jelly.
- Duddington (1955), however, believed that nematode-trapping fungi can be mounted satisfactorily using the Venetian turpentine method. The procedures of this method are as follows:
 - Fix agar slices in the formalin-acetic acid for not less than 24 h.
 - Washed with water until no smell of acetic acid.
 - Stain in haematoxylin for about 5 min.
 - 'blue' in tap water or ammonia vapour.
 - Place in 10% glycerine.
 - When the concentration of the glycerine is saturated, wash in industrial methylated spirit until all glycerine is removed.
 - Dehydrate in absolute alcohol.
 - Place in a 10% solution of turpentine in absolute alcohol.
 - When the dish containing the material is left in the desiccator. Cleared with xylol and mounted in Canada balsam.

Please note that the dehydration and clearing must be carried out very gradually. For the preservation of material, the formalin-acetic acid fixative is excellent. The method causes no noticeable distortion, and the fungi can be left in the gelatin indefinitely without harm.

Conclusions

Although many standard experimental techniques, as presented above, have been developed for studying nematophagous fungi, they may not be suitable for all studies. Future studies should develop and adopt more efficient and better methods and researchers are encouraged to improve or design novel techniques to facilitate the investigation of nematophagous fungi.

References

- Ahrén, D., Ursing, B. M., & Tunlid, A. (1998). Phylogeny of nematode-trapping fungi based on 18 S rDNA sequences. *FEMS Microbiology Letters*, 158, 179–184.
- Barron, G. L. (1969). Isolation and maintenance of endoparasitic nematophagous hyphomycetes. *Canadian Journal of Botany*, 47, 1899–1902.
- Barron, G. L. (1977). *The nematode-destroying fungi*. Canadian Biological Publications Ltd.
- Barron, G. L. (1978). Nematophagous fungi: Endoparasites of *Rhabditis terricola*. *Microbial Ecology*, 4, 157–163.
- Barron, G. L. (1979). Observations on predatory fungi. *Canadian Journal of Botany*, 57, 187–193.
- Bordallo, J., Lopez-Llorca, L., Jansson, H. B., Salinas, J., Persmark, L., & Asensio, L. (2002). Colonization of plant roots by egg-parasitic and nematode-trapping fungi. *New Phytologist*, 154, 491–499.
- Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics*, 77, 71–94.
- Capstick, C., Twinn, D., & Waid, J. (1957). Predation of natural populations of free-living nematodes by fungi 1). *Nematologica*, 2, 193–201.
- Chen, T. H., Hsu, C. S., Tsai, P. J., Ho, Y. F., & Lin, N. S. (2001). Heterotrimeric G-protein and signal transduction in the nematode-trapping fungus *Arthrobotrys dactyloides*. *Planta*, 212, 858–863.
- Connell, S. L., & Padgett, D. E. (1988). An improved technique for making permanent slide cultures of fungi. *Mycopathologia*, 101, 165–166.
- Cooke, R. C. (1961). Agar disk method for the direct observation of nematode-trapping fungi in the soil. *Nature*, 191, 1411–1412.
- Cooke, R. C. (1962). The ecology of nematode-trapping fungi in the soil. *Annals of Applied Biology*, 50, 507–513.
- Cooke, R. C., & Godfrey, B. (1964). A key to the nematode-destroying fungi. *Transactions of the British Mycological Society*, 47, 61–74.
- Coolen, W. (1979). Methods for the extraction of *Meloidogyne* spp. and other nematodes from roots and soil. In F. Lamberti & C. E. Taylor (Eds.), *Root-knot nematodes (Meloidogyne species)*. *Systematics, biology and control* (pp. 317–329). New York: Academic Press.
- Correa, B., Purchio, A., Paula, C., & Gambale, W. (1986). Evaluation of a fluorescent method (fluorescein diacetate and ethidium bromide solution) in the study of the viability *Cryptococcus neoformans* strains. *Mycopathologia*, 96, 91–96.
- Dackman, C., Olsson, S., Jansson, H. B., Lundgren, B., & Nordbring-Hertz, B. (1987). Quantification of predatory and endoparasitic nematophagous fungi in soil. *Microbial Ecology*, 13, 89–93.
- Diehl, W. W. (1929). An improved method for sealing microscopic mounts. *Science*, 69, 276–277.
- Dixon, S. M., & Duddington, C. L. (1951). Permanent preparation of fungi growing on agar. *Nature*, 168, 38–39.
- Dong, L. Q., Mo, M. H., Yang, J. K., & Zhang, K. Q. (2007). A method for obtaining quantities of *Caenorhabditis elegans* eggs. *Nematology*, 9, 743–744.

- Drechsler, C. (1937). Some hyphomycetes that prey on free-living terricolous nematodes. *Mycologia*, 29, 447–552.
- Drechsler, C. (1941). Predacious fungi. *Biological Reviews of the Cambridge Philosophical Society*, 16, 265–290.
- Duddington, C. (1955). Notes on the technique of handling predacious fungi. *Transactions of the British Mycological Society*, 38, 97–103.
- Duddington, C., & Dixon, S. M. (1951). Permanent preparations of fungi growing on agar. *Nature*, 168, 38–39.
- Duddington, C., & Dixon, S. M. (1953). Some methods for making permanent preparations of micro-fungi. *The Journal of the Quekett Microscopical Club*, 3, 305.
- Eren, J., & Pramer, D. (1965). The most probable number of nematode-trapping fungi in soil. *Soil Science*, 99, 285.
- Erni, R., Rossell, M. D., Kisielowski, C., & Dahmen, U. (2009). Atomic-resolution imaging with a sub-50-pm electron probe. *Physical Review Letters*, 102, 96101.
- Farrell, F., Jaffee, B., & Strong, D. (2006). The nematode-trapping fungus *Arthrobotrys oligospora* in soil of the Bodega marine reserve: Distribution and dependence on nematode-parasitized moth larvae. *Soil Biology and Biochemistry*, 38, 1422–1429.
- Feder, W. A., Everard, C., & Wootton, L. (1963). Sensitivity of several species of the nematophagous fungus *Dactylella* to a morphogenic substance derived from free-living nematodes. *Nematologica*, 9, 49–54.
- Foster, L. M., Kozak, K. R., Loftus, M. G., Stevens, J. J., & Ross, I. K. (1993). The polymerase chain reaction and its application to filamentous fungi. *Mycological Research*, 97, 769–781.
- Fowler, M. (1970). New Zealand predaceous fungi. *New Zealand Journal of Botany*, 8, 283–302.
- Giuma, A., & Cooke, R. (1972). Some endozoic fungi parasitic on soil nematodes. *Transactions of the British Mycological Society*, 59, 213–218.
- Gray, N. (1983). Ecology of nematophagous fungi: *Panagrellus redivivus* as the target organism. *Plant and Soil*, 73, 293–297.
- Gray, N. (1984a). Ecology of nematophagous fungi: Comparison of the soil sprinkling method with the Baermann funnel technique in the isolation of endoparasites. *Soil Biology and Biochemistry*, 16, 81–83.
- Gray, N. (1984b). Ecology of nematophagous fungi: Methods of collection, isolation and maintenance of predatory and endoparasitic fungi. *Mycopathologia*, 86, 143–153.
- Gray, N. (1984c). The effect of fungal parasitism and predation on the population dynamics of nematodes in the activated sludge process. *Annals of Applied Biology*, 104, 143–149.
- Gray, N. (1985). Ecology of nematophagous fungi: Effect of soil moisture, organic matter, pH and nematode density on distribution. *Soil Biology and Biochemistry*, 17, 499–507.
- Gray, N. (1987). Nematophagous fungi with particular reference to their ecology. *Biological Reviews*, 62, 245–304.
- Gray, N. (1988). Ecology of nematophagous fungi: Effect of the soil nutrients N, P and K, and seven major metals on distribution. *Plant and Soil*, 108, 286–290.
- Gray, N., & Bailey, F. (1985). Ecology of nematophagous fungi: Vertical distribution in a deciduous woodland. *Plant and Soil*, 86, 217–223.
- Hagedorn, G., & Scholler, M. (1999). A reevaluation of predatory orbiiliaceous fungi. I. Phylogenetic analysis using rDNA sequence data. *Sydowia*, 51, 27–48.
- Hao, Y., Mo, M., Su, H., & Zhang, K. Q. (2005). Ecology of aquatic nematode-trapping hyphomycetes in southwestern China. *Aquatic Microbial Ecology*, 40, 175–181.
- Hawkswell, A. (2001). Iodine-lactophenol as a mycological mounting medium. *Field Mycology*, 2, 12.
- Hawksworth, D. L. (2012). Managing and coping with names of pleomorphic fungi in a period of transition. *IMA Fungus*, 3, 15–24.
- Heintz, C. E. (1978). Assessing the predacity of nematode-trapping fungi *in vitro*. *Mycologia*, 70, 1086–1100.
- Heintz, C., & Pramer, D. (1972). Ultrastructure of nematode-trapping fungi. *Journal of Bacteriology*, 110, 1163–1170.

- Herrera-Medina, M. J., Steinkellner, S., Vierheilig, H., Ocampo Bote, J. A., & Garrido, J. M. G. (2007). Absciscic acid determines arbuscule development and functionality in the tomato arbuscular mycorrhiza. *New Phytologist*, 175, 554–564.
- Higgins, M., & Pramer, D. (1967). Fungal morphogenesis: Ring formation and closure by *Arthrobotrys dactyloides*. *Science*, 155, 345–346.
- Hu, W., Li, Y., Mo, M., & Zhang, K. Q. (2006). A new nematode-trapping hyphomycete of *Arthrobotrys*. *Mycotaxon*, 95, 181–184.
- Jaffee, B., & Strong, D. (2005). Strong bottom-up and weak top-down effects in soil: Nematode-parasitized insects and nematode-trapping fungi. *Soil Biology and Biochemistry*, 37, 1011–1021.
- Jaffee, B., Strong, D., & Muldoon, A. (1996). Nematode-trapping fungi of a natural shrubland: Tests for food chain involvement. *Mycologia*, 88, 554–564.
- Jansson, H. B. (1994). Adhesion of conidia of *Drechmeria coniospora* to *Caenorhabditis elegans* wild type and mutants. *Journal of Nematology*, 26, 430–435.
- Jansson, H. B. (1982). Attraction of nematodes to endoparasitic nematophagous fungi. *Transactions of the British Mycological Society*, 79, 25–29.
- Jensen, C. (1994). *Fluoreszenzmikroskopische Möglichkeiten zur in situ-Beobachtung nematophager Pilze im Boden. Dissertationes Botanicae*, 217. Berlin: Cramer.
- Jensen, C., & Lysek, G. (1991). Direct observation of trapping activities of nematode-destroying fungi in the soil using fluorescence microscopy. *FEMS Microbiology Letters*, 85, 207–210.
- Jensen, C., & Lysek, G. (1995). Fluorescence microscopy of fungi in native soil-improvement by additional substances. *Microscopy and Analysis*, 49, 7–9.
- Jensen, C. H., Neumeister, H., & Lysek, G. (1997). Nematophagous fungi-study by fluorescence microscopy and edx-technique of the periodicity of trap formation in soil. *Biological Rhythm Research*, 28, 365–373.
- Jensen, C., Neumeister-Kemp, H., & Lysek, G. (1998). Fluorescence microscopy for the observation of nematophagous fungi inside soil. *Mycologist*, 12, 107–111.
- Kohlmeyer, J., & Kohlmeyer, E. (1972). Permanent microscopic mounts. *Mycologia*, 64, 666–669.
- Lambert, M., Kremer, S., & Anke, H. (1995). Antimicrobial, phytotoxic, nematocidal, cytotoxic, and mutagenic activities of 1-hydroxypyrene, the initial metabolite in pyrene metabolism by the basidiomycete *Crinipellis stipitaria*. *Bulletin of Environmental Contamination and Toxicology*, 55, 251–257.
- Leck, A. (1999). Preparation of lactophenol cotton blue slide mounts. *Community Eye Health*, 12, 24.
- Li, Y., Hyde, K. D., Jeewon, R., Cai, L., Vijaykrishna, D., & Zhang, K. Q. (2005). Phylogenetics and evolution of nematode-trapping fungi (*Orbiliaceae*) estimated from nuclear and protein coding genes. *Mycologia*, 97, 1034–1046.
- Li, Y., Jeewon, R., Hyde, K. D., Mo, M. H., & Zhang, K. Q. (2006). Two new species of nematode-trapping fungi: Relationships inferred from morphology, rDNA and protein gene sequence analyses. *Mycological Research*, 110, 790–800.
- Li, J., Yang, J., Liang, L., & Zhang, K. Q. (2008). Taxonomic revision of the nematode-trapping fungus *Arthrobotrys multisecondaria*. *Journal of Microbiology*, 46, 513–518.
- Liou, G. Y., & Tzean, S. S. (1997). Phylogeny of the genus *Arthrobotrys* and allied nematode-trapping fungi based on rDNA sequences. *Mycologia*, 89, 876–884.
- Lopez-Llorca, L., & Duncan, J. (1986). New media for the estimation of fungal infection in eggs of the cereal cyst nematode. *Nematologica (Netherlands)*, 32, 486–490.
- Lysek, G., & Nordbring-Hertz, B. (1983). Die Biologie nematodenfangender Pilze. *Forum Mikrobiologie. Darmstadt*, 6, 201–208.
- Mitsui, Y. (1985). Distribution and ecology of nematode-trapping fungi in Japan. *JARQ*, 18, 182–193.
- Neumeister, H., Moritz, M., Schleibinger, H., & Martiny, H. (1996). Investigation of allergic potential induced by fungi on air filters of HVAC systems. *Proceedings of Indoor Air '96*, Nagoya, Japan.
- Nordbring-Hertz, B. (1972). Scanning electron microscopy of the nematode-trapping organs in *Arthrobotrys oligospora*. *Physiologia Plantarum*, 26, 279–284.

- Nordbring-Hertz, B. (1984). Carbohydrate interactions in nematode-trapping fungi. In DH Jennings & ADM Rayner (Eds.), *The ecology and physiology of the fungal mycelium* (pp. 419–432). Cambridge: Cambridge University Press.
- Park, J., Gams, W., Scholler, M., Ghisalberti, E., & Sivasithamparam, K. (2002). Orbiliaceous nematode-trapping fungi and related species in Western Australia and their biological activities. *Australasian Mycologist*, 21, 45–52.
- Persmark, L., & Jansson, H. B. (1997). Nematophagous fungi in the rhizosphere of agricultural crops. *FEMS Microbiology Ecology*, 22, 303–312.
- Persson, C., Olsson, S., & Jansson, H. B. (2000). Growth of *Arthrobotrys superba* from a birch wood resource base into soil determined by radioactive tracing. *FEMS Microbiology Ecology*, 31, 47–51.
- Pfister, D. H., (1997). Castor, Pollux and life histories of fungi. *Mycologia*, 89(1), 1–23.
- Pramer, D., & Stoll, N. R. (1959). Nemin: A morphogenic substance causing trap formation by predaceous fungi. *Science*, 129, 966–967.
- Rubner, A. (1996). Revision of predacious hyphomycetes in the *Dactylella-Monacrosporium* complex. *Studies in Mycology*, 39, 1–134.
- Ryan, M., Smith, D., & Jeffries, P. (2000). A decision-based key to determine the most appropriate protocol for the preservation of fungi. *World Journal of Microbiology and Biotechnology*, 16, 183–186.
- Saikawa, M., & Morikawa, C. (1985). Electron microscopy on a nematode-trapping fungus, *Acaulopage pectospora*. *Canadian Journal of Botany*, 63, 1386–1390.
- Saxena, G., & Lysek, G. (1993). Observation of nematophagous fungi in natural soils by fluorescence microscopy and their correlation with isolation. *Mycological Research*, 97, 1005–1011.
- Schindler, A. (1961). A simple substitute for a Baermann funnel. *Plant Disease Reporter*, 45, 747–748.
- Scholler, M., Hagedorn, G., & Rubner, A. (1999). A reevaluation of predatory orbiliaceous fungi. II. A new generic concept. *Sydowia*, 51, 89–113.
- Schwarz, M., Köpcke, B., Weber, R. W. S., Sterner, O., & Anke, H. (2004). 3-Hydroxypropionic acid as a nematocidal principle in endophytic fungi. *Phytochemistry*, 65, 2239–2245.
- Sharma, V., Walia, S., Kumar, J., Nair, M. G., & Parmar, B. S. (2003). An efficient method for the purification and characterization of nematocidal azadirachtins A, B, and H, using MPLC and ESIMS. *Journal of Agricultural and Food Chemistry*, 51, 3966–3972.
- Shepherd, A. M. (1955). Some observations on the distribution and biology of fungi predaceous on nematodes. Ph. D. Thesis, University of London.
- Soderstrom, B., & Erland, S. (1986). Isolation of fluorescein diacetate stained hyphae from soil by micromanipulation. *Transactions of the British Mycological Society*, 86, 465–468.
- Southey, J. F. (1986). *Laboratory methods for work with plant and soil nematodes*. HMSO Books.
- Staniland, L. (1954). A modification of the Baermann funnel technique for the collection of nematodes from plant material. *Journal of Helminthology*, 28, 115–118.
- Stirling, G., Wilson, E., Stirling, A., Pankhurst, C., Moody, P., Bell, M., & Halpin, N. (2005). Amendments of sugarcane trash induce suppressiveness to plant-parasitic nematodes in a sugarcane soil. *Australasian Plant Pathology*, 34, 203–211.
- Stock, S. P., & Nadler, S. A. (2006). Morphological and molecular characterisation of *Panagrellus* spp. (Cephalobina: Panagrolaimidae): Taxonomic status and phylogenetic relationships. *Nematology*, 8, 921–938.
- Tunlid, A., Johansson, T., & Nordbring-Hertz, B. (1991). Surface polymers of the nematode-trapping fungus *Arthrobotrys oligospora*. *Journal of General Microbiology*, 137, 1231–1240.
- Vaitkevicius, K., Lindmark, B., Ou, G., Song, T., Toma, C., Iwanaga, M., Zhu, J., Andersson, A., Hammarström, M. L., Wai, S. N., Tuck, S. (2006). A *Vibrio cholerae* protease needed for killing of *Caenorhabditis elegans* has a role in protection from natural predator grazing. *Proceedings of the National Academy of Sciences*, 103, 9280–9285.
- Volkman-Kohlmeier, B., & Kohlmeier, J. (1996). How to prepare truly permanent microscope slides. *Mycologist*, 10, 107–108.

- Von Sengbusch, P., Hechler, J., & Müller, U. (1983). Molecular architecture of fungal cell walls. An approach by use of fluorescent markers. *European Journal of Cell Biology*, 30, 305–312.
- Wang, J., & Higgins, V. J. (2005). Nitric oxide has a regulatory effect in the germination of conidia of *Colletotrichum coccodes*. *Fungal Genetics and Biology*, 42, 284–292.
- White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: a guide to methods and applications*. (Innis, M. A., Gelfand, D. H., Sninsky, J. J., White, T. J., eds). Academic Press, New York, USA: 315–322.
- Whitehead, A., & Hemming, J. (1965). A comparison of some quantitative methods of extracting small vermiform nematodes from soil. *Annals of Applied Biology*, 55, 25–38.
- Wilchek, M., & Bayer, E. (1990). *Methods in enzymology*. San Diego: Academic Press Inc.
- Wood, F. (1973). Nematode-trapping fungi from a tussock grassland soil in New Zealand. *New Zealand Journal of Botany*, 11, 231–240.
- Wyborn, C., Priest, D., & Duddington, C. (1969). Selective technique for the determination of nematophagous fungi in soils. *Soil Biology and Biochemistry*, 1, 101–102.
- Xu, L. L., Lai, Y. L., Wang, L., & Liu, X. Z. (2011). Effects of abscisic acid and nitric oxide on trap formation and trapping of nematodes by the fungus *Drechslerella stenobrocha* AS6.1 *Fungal Biology*, 2(114), 97–101.
- Yang, Y., Yang, E., An, Z., & Liu, X. (2007) Evolution of nematode-trapping cells of predatory fungi of the Orbiliaceae based on evidence from rRNA-encoding DNA and multiprotein sequences. *Proceedings of the National Academy of Sciences, USA*, 104, 83–79.
- Zhang, J., Mo, M., Deng, J., Liu, X. F., Bi, T. J., & Zhang, K. Q. (2005). *Dactylella zhongdianensis* sp. nov, a new predacious antagonist of nematodes. *Mycotaxon*, 92, 289–294.
- Zuckerman, B., Matheny, M., & Acosta, N. (1994). Control of plant-parasitic nematodes by a nematocidal strain of *Aspergillus niger*. *Journal of Chemical Ecology*, 20, 33–43.

Nematode-Trapping Fungi

Zhang, K.-Q.; Hyde, K.D. (Eds.)

2014, XI, 392 p. 137 illus., 14 illus. in color., Hardcover

ISBN: 978-94-017-8729-1