*Union Internationale des Sciences Biologiques*

ORGANISATION INTERNATIONALE DE LUTTE BIOLOGIQUE ET INTEGREE CONTRE LES ANIMAUX ET LES PLANTES NUISIBLES

SECTION REGIONALE OUEST PALEARCTIQUE

ISBN 92 9067 036 3

# WORKING GROUP "INTEGRATED CONTROL OF SOIL PESTS"

# GROUPE DE TRAVAIL "LUTTE INTEGREE CONTRE LES RAVAGEURS DU SOL"

# METHODS FOR STUDYING NEMATOPHAGOUS FUNGI

*EDITED BY B.R. KERRY & D.H. CRUMP*

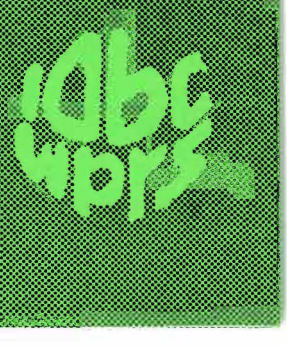
IOBC / WPRS BULLETIN
BULLETIN OILB / SROP

*1991 / XIV / 2*

*International Union of Biological Sciences*

INTERNATIONAL ORGANIZATION FOR BIOLOGICAL AND INTEGRATED CONTROL OF NOXIOUS ANIMALS AND PLANTS

WEST PALAEARCTIC REGIONAL SECTION



IOBC WPRS OILB SROP

WORKING GROUP

"INTEGRATED CONTROL OF SOIL PESTS"

GROUPE DE TRAVAIL

"LUTTE INTEGREE CONTRE LES RAVAGEURS DU SOL"

---

# METHODS FOR STUDYING NEMATOPHAGOUS FUNGI

CONTRIBUTIONS FROM MEMBERS OF THE SUBGROUP "PATHOGENS OF NEMATODES"

*EDITED BY B.R. KERRY & D.H. CRUMP*

IOBC / WPRS BULLETIN
BULLETIN OILB / SROP

*1991 / XIV / 2*

## PREFACE

The Pathogens of Nematodes subgroup was formed in 1976, as part of the IOBC/WPRS Working Group on the Integrated Control of Soil Pests, at a meeting in Cambridge, England. At about that time, soils that were suppressive to cyst or root-knot nematodes were described in England and California respectively, and in both places nematophagous fungi were considered the causal agents; the first demonstrations of the effective longterm biological control of plant parasitic nematodes. These developments coincided with the realisation that some nematicides were hazardous to health and the environment which resulted in their withdrawal from the market. Concerns remain about the safety of other compounds which has led some countries, such as The Netherlands, to legislate for substantial reductions in the amounts of pesticides applied to soil. As a consequence, interest has greatly increased in methods of nematode management, including biological control, that rely less on the use of nematicides. However, experience over the last decade suggests that practical biological control of any nematode pest will not be easily achieved. Biological agents are not a replacement for chemicals and will almost certainly require integration with other control measures.

Since 1976, members of the subgroup have identified many soils in N. Europe that suppress the multiplication of the cereal cyst nematode, *Heterodera avenae* Woll., in cereal monocultures and some soils that suppress *H. schachtii* Schmidt on sugar beet and *Globodera rostochiensis* Woll. on potatoes. Surveys of these soils revealed a fairly restricted range of parasitic fungi which infected the nematode females and/or their eggs. Methods were developed to estimate the levels of suppression in a range of soils. Applications of the partial soil sterilant, formalin (38% formaldehyde) or the fungicide, Captafol, applied at 3000 l/ha and 60Kg/ha respectively, often significantly reduced fungal parasitism and resulted in marked increases in nematode multiplication. In general, responses to these treatments were greater in cereal cyst nematode-infested soils than in those infested with the other cyst species. Applications of these chemicals could affect other soil organisms apart from the nematophagous fungi and results of experiments were often difficult to interpret even though neither chemical appeared to be significantly nematicidal in soil. However, as a result of these studies, members of the subgroup considered that soils suppressive to cyst nematodes were widespread in N. Europe where susceptible crops had been grown intensively in the presence of a nematode pest. Unfortunately, such natural control is slow to establish in soil and has proved difficult to manipulate.

Hence, the activities of the subgroup have tended to concentrate on the application of selected fungal agents to soil including those that have been developed commercially: *Verticillium chlamydosporium* Goddard and *Hirsutella heteroderae* Sturhan & Schneider were tested against *H. schachtii*, and *Paecilomyces lilacinus* (Thom.) Samson and *Arthrobotrys irregularis* Matruchot were tested against *Meloidogyne incognita* Kofoid and White. In all of these empirical tests the levels of control were variable and, in general, disappointing. It was clear that too little was known of the methods for handling these fungi, and of their ecology and biology in soil, to obtain predictable results.

Alongside these studies on the biological control of plant parasitic nematodes, other projects in Denmark and France have concentrated on the use of nematode trapping fungi for the control of animal parasitic nematodes that infect ruminants such as cattle and sheep. The fungi survive passage through the gut and infect nematodes in the faeces. Some fungal isolates have shown considerable potential as control agents.

Research on biological control of nematodes has begun to move from a predominantly observational to a more experimental science. It became clear at the meeting of the subgroup in Leuven in 1988 that for the successful development of a biological control agent fundamental knowledge was required on its ecology, epidemiology, and mode of action, and suitable methods for screening, culturing and applying the fungus needed to be developed. Because few nematophagous fungi have been studied in much detail there is a shortage of suitable techniques for research. However, several laboratories in at least six member countries are involved in the development of nematophagous fungi or their products for the control of nematodes. Members of the subgroup within these laboratories have developed a range of methods which are reviewed and brought together in this Bulletin. It is anticipated that the reader should be able to obtain ready access to methods for the isolation, selection and testing of nematophagous fungi. Although members of the subgroup have considerable experience in handling these biological control agents, it is inevitable in a subject so young that there are likely to be many improvements and developments in the methods described. However, it was felt important to try to standardise existing techniques, and it is hoped that the methods described in this Bulletin will provide a useful baseline against which others can be compared.

Brian Kerry

## LIST OF CONTRIBUTORS


| | |
|---|---|
| BELDER, den, Eefje | I.P.O. (Research Institute for Plant Protection), PO Box 9060, NL-6700 GW Wageningen, The Netherlands. |
| BOOGERT, van den, Paul | Institute for Soil Fertility, PO Box 30003, NL-9750 RA Haren, The Netherlands. |
| COOSEMANS, Josef | Katholieke Universiteit Leuven, Laboratory of Phytopathology & Plant Protection, Kardinaal Mercierlaan 92, B-3030 Leuven, Belgium. |
| CRUMP, David | Entomology & Nematology Dept., AFRC/IACR, Rothamsted Experimental Station, Harpenden, Herts. AL5 2JQ, England. |
| HAAN, de, Jasper | H.L. Hilbrands Laboratory for Soilborne Diseases, PO Box 323, 9400 AH Assen, The Netherlands. |
| KERRY, Brian | Entomology & Nematology Dept., AFRC/IACR, Rothamsted Experimental Station, Harpenden, Herts. AL5 2JQ, England. |
| LOHMANN, Ulrich | Institut für Pflanzenkrankheiten der Rheinischen Friedrich-Wilhelms Universität, Nussallee 9, 5300 Bonn 1, Germany. |
| NICOLAY, Rainer | Institut für Pflanzenkrankheiten der Rheinischen Friedrich-Wilhelms Universität, Nussallee 9, 5300 Bonn 1, Germany. |
| PELOILLE, Michele | Laboratoire Ecologie de Parasitologie, Institut National de la Recherche Agronomique, CR de Tours-Nouzilly, 37380 Monnaie, France. |
| SIKORA, Richard | Institut für Pflanzenkrankheiten der Rheinischen Friedrich-Wilhelms Universität, Nussallee 9, 5300 Bonn 1, Germany. |
| THIJSSEN, Leon | H L Hilbrands Laboratory for Soilborne Diseases, PO Box 323, 9400 AH Assen, The Netherlands. |

## CONTENTS

# Isolation and determination of the antagonistic potential of fungal endoparasites of nematodes

**U. Lohmann[1], R. A. Sikora[1] and P van den Boogert[2]**

**[1]Institute für Pflanzenkrankheiten der
Rheinischen Friedrich-Wilhelms Universität
Nussallee 9
5300 Bonn 1
FRG**

**[2]Institute for Soil Fertility
PO Box 30003
9750 RA Haren (Groningen)
The Netherlands**

## Introduction

Interest in fungal endoparasites of nematodes has increased greatly since 1964 (11). Most endoparasites are considered obligate parasites having no vegetative hyphal development outside the body of their host. Thalli inside the host, or conidiophores which break out through the host cuticle, produce infective spores. These become established in nematodes after encysting on, or adhering to, the cuticle or by germination in the buccal cavity or oesophagus following ingestion (5,6).

With the exception of a few glasshouse and microplot experiments (24,32,34,43) endoparasitic fungi have not been investigated thoroughly as potential biological control organisms. This is partly due to a lack of adequate techniques for precise determination of the number and distribution of infective spores or conidia in the soil. In this chapter, the techniques presently available for isolation and estimation of the antagonistic potential of endoparasitic fungi in soil, will be outlined and evaluated.

## Materials and Methods

### Nematode cultures

A constant supply of nematodes is necessary either as a source of bait for isolation of the endoparasites or for maintenance and purification after isolation. Dixenic laboratory cultures of nematodes are ideal for most routine studies such as the recovery of endoparasites or to study infection cycles (6). Microphagous nematodes such as *Panagrellus redivivus* (Linn.) Goodey and *Caenorhabditis elegans* (Maupas) Dougherty multiply well on bacteria cultures grown on Nigon's medium (13), 0.3% Miluvit (Milupa) or 1.5% Bacto-Agar (Difco) in Petri dishes and can be used as bait. Species of *Rhabditis* and *Acrobeloides* growing on corn meal or seremil agar (20) also have been used as a bait and for maintenance of axenic fungal cultures (3,4).

The mycophagous species *Aphelenchus avenae* Bastian or *Ditylenchus triformis* Hirschmann & Sasser cultivated on nutrient agars with fungi can also be used for testing. The use of axenic nematode cultures (27) is particularly useful for obtaining pure cultures of the slow growing endoparasitic fungi.

**Isolation of fungi**

Environments which contain numerous and diverse species of nematodes are most likely to contain a wide range of endoparasitic fungi (3,20,26). Aged compost and old pasture soils (25), rotting wood (44), leaf mould (16) and agricultural soils (18) are particularly good sources of nematodes and their parasites. Soil from under Bryophytes is also a good source of endoparasites (17). Isolation procedures vary greatly, therefore, their usefulness for a particular ecological niche or fungal species will be emphasized.

The number of replicates needed will vary greatly depending on whether qualitative or quantitative data are desired for any one soil sample. Replicate number can only be determined by experimentation. The number required also will vary with fungal species, time available, number of treatments, and availability of technical support etc. Statistical analysis should be used to determine replication following the accumulation of sufficient data.

**Soil sprinkling**

A pinch of leaf mould or other organic debris is sprinkled over a plate of dilute corn meal agar (15). After the initial development of bacteria and saprophytic fungi on the plates, the natural population of nematodes gradually increases. The nematodes are attacked by nematode-trapping fungi and, in a later stage of succession, also by endoparasitic fungi. Frequently mites or helminthic worms introduced with the organic particles cause problems. The plates need to be examined frequently over a long period of time making this method time consuming.

The technique has been slightly modified by pouring dilute corn meal or rabbit dung agar over 2 g of soil scattered in the bottom of a sterile Petri dish (19). This minimizes growth of saprophytic fungi and increases detection of nematophagous fungi. *P. redivivus* can be added to 2% water agar or 0.25 - 0.5% corn meal agar plates prior to the addition of 0.5 to 1.0 g of soil or organic debris (46). The plates are inspected at 1-3 day intervals for several weeks. The presence of numerous nematodes on baited plates exerts a fungistatic effect preventing profuse development of saprophytic fungi. Often the only fungi found are nematode-trapping or endoparasitic fungi. Slow-developing endoparasites, however, are often overlooked due to suppression by fast growing predacious fungi.

The soil sprinkling technique clearly has some disadvantages when compared to other methods (Table 1). The technique, however, closely simulates a natural ecosystem since it encourages the development of a variety of nematodes as well as other soil microorganisms and allows the isolation of a wide range of parasites and predators.

### The Baermann funnel

The use of the Baermann funnel for studying nematode-destroying fungi is based on the assumption that in any natural population of nematodes, a small percentage will be infected by fungal parasites which are still sufficiently mobile following infection for passage through the filter system (23). This method is selective for endoparasites as nematode-trapping fungi are excluded because they immobilize their prey.

After 24 to 48 h the nematodes extracted from the soil sample are concentrated in a centrifuge at 1000 rpm for 3 min, resuspended in distilled water and incubated at room temperature (23). Examination should be made after 4 days since suspensions immediately after centrifugation exhibit only low levels of fungal attack. An alternative method (3) is to remove the supernatant after centrifugation and to spread the nematode containing pellet onto water agar. The use of plates with fresh or dried agar allows a selection between flagellate endoparasites such as species of *Catenaria, Myzocytium, Nematoctonus* and *Harposporium* and non-flagellated Hyphomycetes.

### Sugar-flotation technique

The extraction of living and parasitized vermiform nematodes can be accomplished by the sugar-flotation technique (8,20,45). A 150 g soil sample is suspended and centrifuged with a sugar solution of the same specific gravity as that of nematodes (20). The supernatant containing the nematodes is spread on 1% water agar plates containing 300 ppm streptomycin sulphate. Nematode suspensions from laboratory cultures can be added as additional prey to increase isolation efficiency.

### Differential centrifugation technique

The differential centrifugation was modified (2) to separate the relatively small-spored endoparasites from large-spored nematode-trapping species. A 200 g well mixed soil sample is measured out in a beaker, added to 250 ml water in a Mason jar and blended for 30 to 60 s (6). The soil suspension is passed through various sieves, down to 50 $\mu$m aperture (20). 100 ml of the screened suspension are poured into two 50 ml centrifuge tubes and centrifuged at 750 G for 2 min to remove the heavier soil particles and large spores of predatory fungi. The supernatant is decanted, retained and centrifuged again at 2500 G for 30 min (20) or 1 hr (2,4,40). The supernatant is discarded and the residue saved. A few drops of water are added to the residue which is then stirred with a glass rod. The mixture is poured onto 2% (w/v) water agar and spread over half the plate or a portion of the residue is applied in a cross like manner (20,27). The plates are always baited with microphagous nematodes.

The method has advantages in that 1) only species capable of attacking the bait nematodes are recovered 2) saprophytic fungi do not grow on the plate and 3) the plate is relatively free from soil, improving detection and isolation (Table 1).

### Comparison of methods

The most common isolation method used has been soil sprinkling, even though, few endoparasites are recovered with this technique. Soil sprinkling, Baermann funnel and

**Table 1.** Comparison of different methods for qualitative and quantitative isolation of endoparasitic fungi and estimation of their activity

| Method/ Authors | Soil Volume (g) | Bait Nematode | Time to prepare/ examine | Selectivity for endoparasite | Contamination/ Chance of isolation | Biological Control Significance |
|---|---|---|---|---|---|---|
| Qualitative Methods: | | | | | | |
| Soil sprinkling: (4,15,19) | < 2 | yes/no | low/high | very low | high/low | low |
| Baermann Funnel: (3,6,23) | 10-100 | no | low/medium | medium | low/high | low |
| Sugar flotation: (20,44) | 10-100 | no | medium/medium | low | low/high | low |
| Differential Centrifugation: (2,6,20,27,40) | 100-200 | yes | high/medium | very high | low/high | low |
| Quantitative Methods: | | | | | | |
| Most Probable Number | | | | | | |
| + soil sprinkling: (12,21) | | yes | high/high | very low | high/low | low |
| + diff. centrifug. (12) | | yes | high/very high | very high | low/high | medium |
| Predation event (PE) | | | | | | |
| + soil filtration: (38) | 10-25 | no | medium/high | low | medium/low | low |
| + diff. centrifug. + host specific bait (36) | | yes | high/high | very high | low/high | *V.balanoides* *H. anguillulae* |
| + diff. centrifug.: + target nematode (41) | | yes | high/high | very high | low/high | Target nematode |
| Antibodies and Immunofluorescence microscopy | | | | | | |
| + diff. centrifug.: | | no | high/low | low | low/low | *V. balanoides* *D. coniospora* |

differential centrifugation techniques have been compared for their effectiveness in recovery of endoparasites (4). From 40 recorded species, 32 were detected with the Baermann funnel, 21 with soil sprinkling and 19 with the centrifugation technique. A limitation in the technique was the fact that lower fungi of the Chytridiomycetes, Oomycetes, and Zygomycetes were not detected. Species of *Myzocytium, Catenaria anguillulae* Sorokin and two *Haptoglossa* species were isolated using centrifugation, by storing the plates under humid conditions (5,20).

The centrifugation technique is effective and selective for endoparasitic Hyphomycetes. *Harposporium anguillulae* Lohde was recovered in 2% of 700 samples with soil sprinkling (22) and 6.2% of 161 samples with the Baermann funnel (25) as compared to 94% of 74 samples with differential centrifugation (20).

Both the differential centrifugation and the Baermann funnel technique should be employed in order to obtain a broad spectrum of endoparasitic fungi. The methods are reasonably selective for endoparasites and produce results within 4 weeks. Conversely, the soil sprinkling method is time consuming and rather unspecific.

**Estimation of activity of endoparasitic fungi**

The effectiveness of endoparasitic fungi as biological control agents can be assessed either by estimation of parasitism of all fungi in an ecosystem against one target organism or by counts of conidia or propagules of a single fungus. There are a few methods for measuring fungal density which can be used as an estimate of antagonistic potential. Techniques for estimation of conidial density for a specific fungal species have not been developed.

**Enumeration of fungal density**

The most-probable-number (MPN) technique for determining viable numbers of microorganisms employs a statistical approach in which successive dilutions are performed to reach an extinction point (1,9,10,39). Usually 3-10 replicates of each dilution are made and the pattern of positive and negative scores are recorded. A statistical table based on a Poisson distribution is used to determine the most probable number of viable microorganisms present in the original sample.

The soil sprinkling method was expanded by applying the MPN concept to nematophagous fungi (21). A positive score is the presence of parasitized nematodes which are added to the plates as a bait (35). Enumeration is made by using probability tables for the determination of *Rhizobium* bacteria in soil (7). This method has been modified by differentiating between nematode-trapping fungi and endoparasites with the centrifugation technique (2,12). In all cases, the methods are time consuming in both preparation and examination of the numerous replicates over long time periods.

The concept of counting predation has been used for quantifying the nematophagous potential in the soil (38). Samples of 10 to 25 g are washed through various sized sieves and the soil residue together with propagules of nematophagous fungi are then collected on a filter paper and placed on one-quarter strength corn meal agar.

The antagonistic potential has been determined for endoparasitic Chytridiomycetes, Oomycetes and Zygomycetes with the Baermann-funnel (4). Differential centrifugation (1) has been used for nematode-trapping fungi in the soil fraction after the first centrifugation and the activity of endoparasitic hyphomycetes determined after the second centrifugation. This method is quick and efficient for determination of the spectrum of nematophagous fungi present in the field, but it is not a good measure of the antagonistic potential.

None of the described techniques allows a good assessment of the potential for control of nematophagous fungi against a specific plant parasitic nematode in soil. A target nematode species can be added to a sample to determine the antagonistic potential of a soil (41). Quantitative estimations are also possible by counting predation or the degree of parasitism of the nematode population after specific time intervals (38).

**Enumeration of *H. anguillulae* and *V. balanoides* conidia in soil**

Quantitative enumeration of a single fungus species allows important insights into its biology, ecology and significance for biological control. Two characteristics are essential for the development of a quantitative method: 1) conidia of a single fungus species should be selectively detected without interference from other parasites e.g. by using a bait nematode which is parasitized by a specific species and 2) the dynamics of parasitism should be dependent on conidia density in the soil fraction.

The following method was developed for detection of *H. anguillulae* and *V. balanoides* using *C. elegans* and *Aphelenchus avenae* Bastian as bait nematodes (36,37). The conidia are extracted by modifying the differential centrifugation technique in that a) the weight of the residue in each tube is determined after the second centrifugation b) the amount of residue on each water agar plate is measured by weighing the tubes again after inoculation of each plate with the residue.

*Caenorhabditis elegans* or *A. avenae* are added to each of 10 replicate plates to ensure selective infection by either *H. anguillulae* or *Verticillium balanoides* (Drechsler) Dowsett *et al* respectively. For calibration, autoclaved residue was incubated with 1 to 10,000 conidia/plate and the levels of nematode infection assessed. Significant differences ($P \leq 5\%$, $n=10$) in the rate of parasitism by *H. anguillulae* were obtained when parasitism was measured after specific time intervals (Fig. 1b) or when the dynamics of parasitism (Fig. 1a) were compared by non-linear regression analysis (14) for logistic growth (42). Thus either the mean degree of parasitism or the coefficient of increase of parasitism determined by regression analysis could be used to distinguish between different conidial densities on the detection plate. Similar results were obtained for *V. balanoides* (36,37).

The method allowed comparisons of fungal densities in soil samples on a logarithmic scale. Variances were high at low densities, because not every detection plate contained conidia. This may be due to the fact that only 10% of the residue was spread on all detection plates. Variation may be reduced by filling the grooves in the centre of detection plates with the entire soil fraction obtained after the second centrifugation (41).

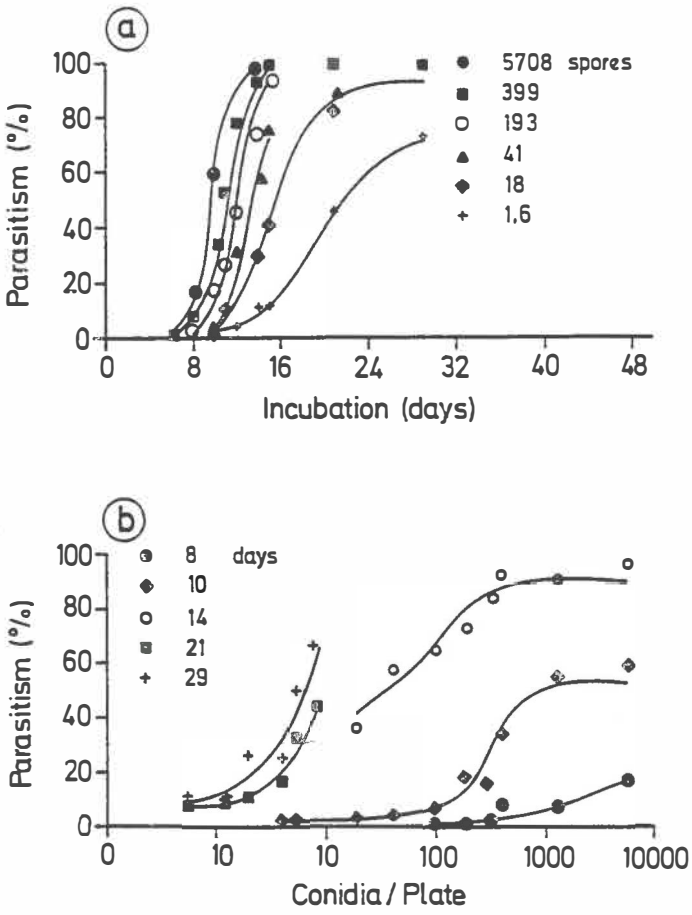



Fig.1. Degree of parasitism of a *Caenorhabditis elegans* population by various *Harposporium anguillulae* conidial densities on water agar with time of incubation (a). The same data are shown using the conidial density (on a logarithmic scale) as the independent variable after specific time intervals of inoculation of the fungus (b).

## Enumeration of fungi using antibodies

*Verticillium balanoides* can be detected using antibodies adhering to the conidial surface, which are then observed by fluorescence microscopy (Dürschner pers. comm.). Unfortunately, the specificity of the antibodies is not sufficient to exclude other soil fungal propagules. This problem also arose when immunofluorescence labelled antibodies (van den Boogert, unpublished) were used to detect *Drechmeria coniospora* (Drechsler) Gams & Jannson conidia in soil (Table 2). In addition, various fungal strains used for antibody production might yield different antibodies. With *D. coniospora*, this problem has been avoided by using a mixture of conidia from different origins for antibody production.

**Table 2.** Cross-reaction of antibodies from three different mice to two strains of *Drechmeria coniospora* as compared with conidia from 11 other soil fungi detected by immunofluorescence. (van den Boogert, unpublished)

| Fungus | Immuno-fluorescence | | |
|---|---|---|---|
| | Antibodies from mice strain | | |
| | 128 | 129 | 130 |
| *Drechmeria coniospora (Lund)* | + | + | + |
| *Drechmeria coniospora (Rolde)* | + | + | + |
| *Verticillium biguttatum* | ± | ± | ± |
| *Verticillium lecanii* | ± | + | + |
| *Verticillium albo-altrum* | + | + | + |
| *Gliocladium nigrovirens* | ± | ± | ± |
| *Gliocladium roseum* | ± | ± | ± |
| *Fusarium asparagi* | ± | + | + |
| *Fusarium acuminatum* | - | - | - |
| *Hormiactis fimicola* | + | + | + |
| *Trichoderma hamatum* | - | - | - |
| *Cylindrocarpon destructans* | - | - | - |
| *Botrytis aclada* | - | - | - |

a + = yes; ± = variable; - = no

## Further prospects and conclusion

With the methods described, either the natural population of nematodes in the soil or bait nematodes are used for the detection of endoparasitic fungi. Since only the infected nematodes indicate the presence of the fungus, all methods are clearly limited to the detection of virulent spores or conidia of the fungi. This may cause problems since different isolates of *V. balanoides* or *D. coniospora* showed different levels of virulence to the same nematode (20,27,33). In addition, the infection process includes the attraction of the host nematode (28,29), adhesion to its cuticle (30,31) and the formation of infection tubes (27), factors which may be influenced by environmental and chemical conditions on the detection plate. Therefore, the determination of the exact number of all viable conidia present in the soil probably cannot be reached using the baiting methods described here.

Methods for direct counting of conidia using antibodies are not yet available. For effective biological control, the antagonistic potential of a fungal population towards a target nematode is of major importance, not the total number of conidia present in the soil. The number of infected nematodes under the experimental conditions used is related to the inoculum density. At least for *V. balanoides* the number of infective propagules can be estimated using the calibrated baiting method outlined. Whether the introduction or enhancement of endoparasitic fungi meets the challenge of effective and economic control

under field conditions still needs investigation. The methods outlined here with further refinement should help us answer some of these basic questions.

## References


1. ALEXANDER, M. 1965. Most-probable-number method for microbial populations. In: *Methods of Soil Analysis. Part 2*, Chemical and Microbiological Properties (C.A. Black, ed.), American Society of Agronomy, Madison, Wisconsin, pp. 1467-1472.

2. BARRON, G.L. 1969. Isolation and maintenance of endoparasitic nematophagous hyphomycetes. *Canadian Journal of Botany* **47**:1899-1902.

3. BARRON, G.L. 1977. The nematode-destroying fungi. *Canadian Biological Publications Ltd.*, Guelph, Ontario, Canada.

4. BARRON, G.L. 1978. Nematophagous fungi: Endoparasites of *Rhabditis terricola*. *Microbial Ecology* **4**:157-163.

5. BARRON, G.L. 1981. Predators and parasites of microscopic animals. In: *Biology of conidial fungi,* **Vol. 2**. (Cole, G.T., B. Kendrick, eds.), London U.K., Acad. Press; pp. 167-200.

6. BARRON, G.L. 1982. Nematode-destroying fungi. In: *Experimental microbial ecology*. (Burns, R.H. and J.H. Slater, eds.). Blackwell, Oxford. pp. 533-552.

7. BROCKWELL, J. 1963. Accuracy of a plate-infection technique for counting *Rhizobium trifolium*. *Applied Microbiology* **11**:377-383.

8. CAVENESS, F.E. & JENSEN, H.J. 1955. Modification of the centrifugal flotation technique for the isolation and concentration of nematodes and their eggs from soil and plant tissue. *Proceedings of the Helminthological Society* Washington *22, 87-89.*

9. COCHRAN, W.G. 1950. Estimation of bacterial densities by means of the "most probable number". *Biometrics* **2**:105-106.

10. COLWELL, R.R. 1979. Enumeration of specific populations by the most-probable-number (MPN) method. In: *Native Aquatic Bacteria: Enumeration, Activity and Ecology* (Costerton, J.W. and R.R. Colwell, eds.), ASTM Special Technical Publication No. 695, American Society for Testing and Materials, Philadelphia, pp. 56-64.

11. COOKE, R.C. & GODFREY, B.E.S. 1964. A key to the nematode destroying fungi. *Transactions of the British Mycological Society* **47**:61-74.

12. DACKMAN, C., OLSSON, S., JANSSON, H.-B., LUNDGREN, B. & NORDBRING-HERTZ, B. 1987. Quantification of predatory and endoparasitic nematophagous fungi. *Microbial Ecology* **13**:89-93.

13. DOUGHERTY, E.C. 1960. Cultivation of Aschelminths, especially Rhabditid nematodes. In: *Nematology*. (J.N. Sasser & W.R. Jenkins, eds.), University of North Carolina press, Chapel Hill., pp. 297-318.

14. DRAPER, N.R. & SMITH, H. 1966. Applied regression analysis. Wiley & Sons, Inc., New York.

15. DRECHSLER, C. 1941. Some hyphomycetes parasitic on free-living terricolous nematodes. *Phytopathology* **21**:773-802.

16. DRECHSLER, C. 1963. A new nematode-destroying hyphomycete of the genus *Harposporium. American Journal Botany* **50**:839-842.

17. DUDDINGTON, C.L. 1951. The ecology of predacious fungi. I. Preliminary survey. *Transactions of the British Mycological Society* **34**:322-331.

18. DUDDINGTON, C.L. 1954. Nematode-destroying fungi in agricultural soils. *Nature* **173**:500-501.

19. DUDDINGTON, C.L. 1955. Notes on the technique of handling predacious fungi. *Transactions of the British Mycological Society* **38**:97-103.

20. DÜRSCHNER, U. 1983. Pilzliche Endoparasiten an beweglichen Nematodenstadien. *Mitteilungen der Biologischen Bundesanstalt für Land- und Forstwirtschaft* **217**:83 pp.

21. EREN, J. & PRAMER, D. 1965. The most probable number of nematode-trapping fungi in soil. *Soil Science* **99**:295.

22. FOWLER, M. 1970. New Zealand predacious fungi. *New Zealand Journal of Botany* **8**:283-302.

23. GIUMA, A.Y. & COOKE, R.C. 1972. Some endozoic fungi parasitic on soil nematodes. *Transactions of the British Mycological Society* **59**:213-218.

24. GIUMA, A.Y. & COOKE, R.C. 1974. Potential of *Nematoctonus* conidia for biological control of soil-borne phyotonematodes. *Soil Biology & Biochemistry* **6**:217-220.

25. GRAY, N.F. 1983. Ecology of nematophagous fungi: distribution and habitat. *Annals of Applied Biology* **102**,501-509.

26. GRAY, N.F. 1985. Ecology of nematophagous fungi: effect of soil moisture, organic matter, pH and nematode density on distribution. *Soil & Biology Biochemistry* **17**:499-507.

27. HASHEM, M.H. 1988. Untersuchungen zur Wirtsspezifität und zum Entwicklungszyklus endoparasitärer Nematodenpilze an beweglichen Nematoden. *Dissertation, Kiel*.

28. JANSSON, H.-B. 1982. Attraction of nematodes to endoparasitic nematophagous fungi. *Transactions of the British Mycological Society* 79:25-29.

29. JANSSON, H.-B. & NORDBRING-HERTZ, B. 1980. Interactions between nematophagous fungi and plant-parasitic nematodes: attraction, induction of trap formation and capture. *Nematologica* **26**:383-389.

30. JANSSON, H.-B. & NORDBRING-HERTZ, B. 1983. The endoparasitic nematophagous fungus *Meria coniospora* infects nematodes specifically at the chemosensory organs. *Journal of General Microbiology* **129**:1121-1126.

31. JANSSON, H.-B. & NORDBRING-HERTZ B. 1984. Involvement of sialic acid in nematode chemotaxis and infection by an endoparasitic fungus. *Journal of General Microbiology* **130**:39-43.

32. JANSSON, H.-B & JEYAPRAKASH, A. & ZUCKERMAN, B.M. 1985. Control of root-knot nematodes on tomato by endoparasitic fungus *Drechmeria coniospora. Journal of Nematology* **17**:327-329.

33. JANSSON, H.-B., DACKMAN, C. & ZUCKERMAN, B.M. 1987. Adhesion and infection of plant parasitic nematodes by the fungus *Drechmeria coniospora. Nematologica* **33**, 480-487.

34. JUHL, M. 1985. The effect of *Hirsutella heteroderae* on the multiplication of the beet cyst nematode on sugar beet. *Tidsskrivt Planteavl.* **89**:475-480.

35. KLEMMER, H.W. & NAKOMO, R.Y. 1964. A semiquantitative method of counting nematode-trapping fungi in soil. *Nature* **203**:1085.

36. LOHMANN, U. 1989. Untersuchungen zur Biologie und Anwendung der endoparasitären Pilze *Verticillium balanoides* Dowsett und *Drechmeria coniospora* Gams und Jansson als biologische Bekämpfungsmittelmittel gegen pflanzenparasitäre Nematoden. *Dissertation, Bonn.*

37. LOHMANN, U. & SIKORA, R.A. 1990. Estimation of *Verticillium balanoides* and *Harposporium anguillulae* conidial density in soil. In preparation *(Revue de Nematologie)*.

38. MANKAU, R. 1975. A semiquantitative method of enumerating and observing parasites and predators of soil nematodes. *Journal of Nematology* **7**:119-122.

39. MELCHIORRI-SANTOLINI, U. 1972. Enumeration of microbial concentration of dilution series (MPN). In: *Techniques for Assessment of Production and Decomposition in Fresh Waters* (Sorokin, Y.I. and H. Kadota, eds.), IBP Handbook No. 23, Blackwell Scientific Publications, Oxford, pp. 64-70.

40. MONOSON, H.L., CONWAY, T.D. & NELSON, R.E. 1975. Four endoparasitic nematode destroying fungi isolated from sand ridge state forest soil. *Mycopathologica* **57**:59-62.

41. NICOLAY, R. & SIKORA, R.A. 1988. Improved techniques for the detection of nematophagous fungi and their activity against target nematodes. *Révue de Nematologie* **11**:115-116.

42. RICHTER, O. 1985. Simulation des Verhaltens ökologischer Systeme: mathematische Methoden und Modelle. Weinheim, Deerfield Beach, pp. 219.

43. SAYRE, R.M. & KEELEY, L.S. 1969. Factors influencing *Catenaria anguillulae* infections in a free-living and a plant-parasitic nematode. *Nematologica* **15**:492-502.

44. SHEPHERD, A.M. 1959. *Harposporium crassum* sp. nov. *Transactions of the British Mycological Society* **38**:47-48.

45. STURHAN, D. & SCHNEIDER, R. 1980. *Hirsutella heteroderae* ein neuer nematodenparasitärer Pilz. *Phytopathologische Zeitung* **99**:105-115.

46. WYBORN, C.H.E., PRIEST, D. & DUDDINGTON, C.L. 1969. Selective technique for the determination of nematophagous fungi in soils. *Soil Biology Biochemistry* **1**:101-102.

# Selection of nematode-trapping fungi for use in biological control

**M. Peloille**

**Station de Pathologie Aviaire et de Parasitologie**
**37380 Monnaie, France**

## Introduction

Since the discovery of nematode-trapping fungi, about one hundred years ago, several attempts at using these organisms as agents of biological control against parasitic nematodes causing diseases either in plants or animals have been performed. *Arthrobotrys oligospora* Fres. has been most often used for that purpose in natural conditions, or *in vitro*, (2,4,8). This species is one of the most commonly found in nature (3,6,7) but it is not necessarily the most efficient (1). We have studied different criteria which need to be considered in the selection of an efficient nematode trapping fungus: Growth rate at various temperatures, predatory activity, ability to survive the intestinal transit of animals and ability to produce chlamydospores which can be used for the dispersal of the fungi.

## Materials and Methods

**Radial growth** was assessed by measuring two diameters, at right angles, of a 5-day old colony grown on C.M.A. (corn meal agar, Difco), incubated at 5, 10, 15, 20, 25, 30 and 37°C in the dark. Three replicates were made for each temperature. The inoculum is a disc of 1cm diameter, cut off the mother culture. The growth curve was established with the mean of the measures recorded.

**Predatory activity** has been studied *in vitro* at different temperatures : 10, 15, and 22°C (9). The plates containing CMA already covered with the fungus were inoculated with third stage larvae (L3) of a parasitic nematode at doses of 100, 200, 400 and 800 larvae per plate and incubated for 24, 72 or 168 hours in the dark. The remaining free larvae were then collected using Baermann's method and counted.

**Ability to survive intestinal transit** has been studied by looking for the presence of the fungus in the faeces of sheep already excreting eggs of parasites. Four sheep were fed, once each, with 500 g of a culture of the fungus on millet seeds and their faeces were collected during 5 days and maintained at 20°C for two weeks. The larvae produced were extracted by Baermann's method and counted. Faeces of four untreated sheep were used as controls. The time interval of two weeks allows the eggs to develop into third stage larvae (L3) and the fungus to grow and develop traps.

Samples of faeces were spread on CMA and put in culture during this period for the reisolation of the fungus applied. The plates were examined microscopically and any conidia removed and used to inoculate new plates of CMA to establish pure cultures for identification.

The *chlamydospores* were studied to test their ability to germinate. They were isolated individually using a binocular microscope and incubated at different temperatures. Twenty four spores were observed at each temperature and the number germinated was recorded daily and the percentage germinated calculated (5).

## Results

One strain of *Duddingtonia flagrans* (Dudd.), Cooke 1969, has been studied using the above methods to partly determine its potential as a biological control agent for *Teladorsagia circumcincta*.

Development occurred between 5 and 35°C (Fig. 1). The optimal growth rate was obtained at 30°C where the colony reached 85mm in diameter in 5 days. No development was observed at 37°C but the fungus was not killed at this temperature and was able to grow again if the temperature was subsequently decreased to 30°C.

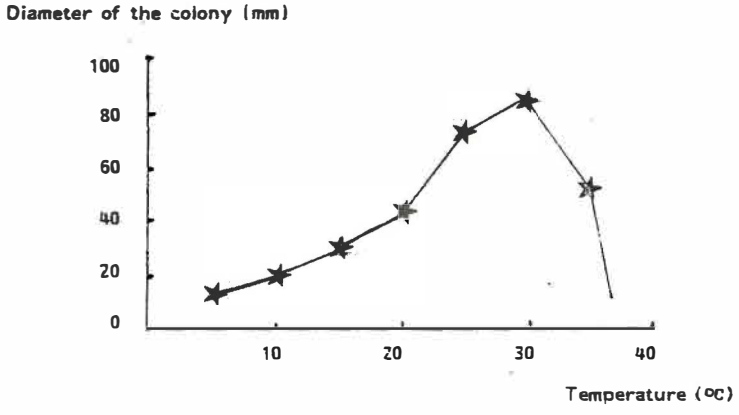



Fig. 1. Growth curve of *Duddingtonia flagrans* after 5 days incubation

**Predatory activity** *in vivo* has been tested against L3 of *T. circumcincta*. The plates were incubated at 18°C during the contact period. Results are presented in Figure 2.

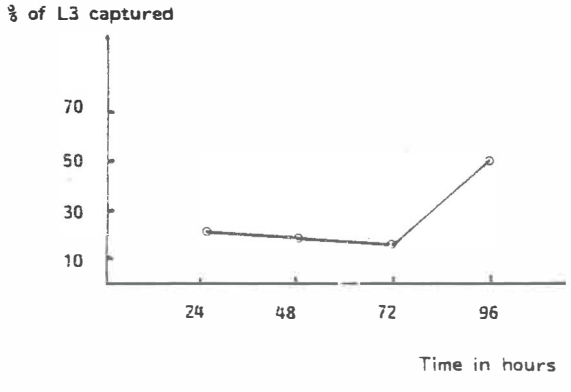



Fig. 2. Percentage of captured larvae

Within the first three days the larvae were trapped by the devices already present on the mycelium and the decreasing number of trapped L3, even if not significant, can be explained by the saturation of the existing traps. Between, 72 and 96 hours the percentage of trapped larvae increased up to 50%. The proportion of trapped larvae was still increasing after 96 hours. It is known that the addition of larvae to a culture of fungus stimulates the proliferation of traps and the increase in the proportion of trapped larvae is probably due to this secondary production of trapping devices.

**Ability to survive intestinal transit of sheep.** We followed the development of *D. flagrans* in the faeces of the sheep previously fed with a culture of the fungus on millet seeds. The faeces were collected during 5 days and maintained at room temperature. *D. flagrans* was found in 77.5% of the plates containing faeces excreted during the 24 hours following the ingestion of the fungus, and in 50% of the plates with faeces excreted between 24 and 48 hours but not in the faeces excreted afterwards until the end of the experiment.

**The predatory activity** of the fungus recovered in the faeces has been evaluated by counting the number of living L3 after two weeks in culture. The results are shown in table 1. The number of larvae is the mean of 4 independent countings in the experimental and in the control plots.

**Table 1.** Number of L3 of *T. circumcincta* in faeces, calculated from the total amount of faeces excreted daily

| Hours after ingestion | 24 | 48 | 72 | 96 | 180 |
|---|---|---|---|---|---|
| Experimental plot : a | 58 | 4392 | 9009 | 8515 | 19641 |
| Control plot : b | 93497 | 41899 | 47043 | 22349 | 22349* |
| a/b x 100 | 0,06 | 10,5 | 19,2 | 38,1 | 87,9* |

* For technical reasons, an accurate determination of the number of larvae could not be obtained in the control plot at 180 h. and the data from the previous determination were used for b.

These results are in agreement with the previous ones; when the fungus could be isolated from a majority of samples during the first 24 hours, the number of free L3 was very small, but this number increased after 48 hours concomitantly with a decrease in the fungal reisolations.

**The germination of chlamydospores** incubated at three temperatures: 25, 30 and 35°C was assessed over 8 days. The results are presented in figure 3.

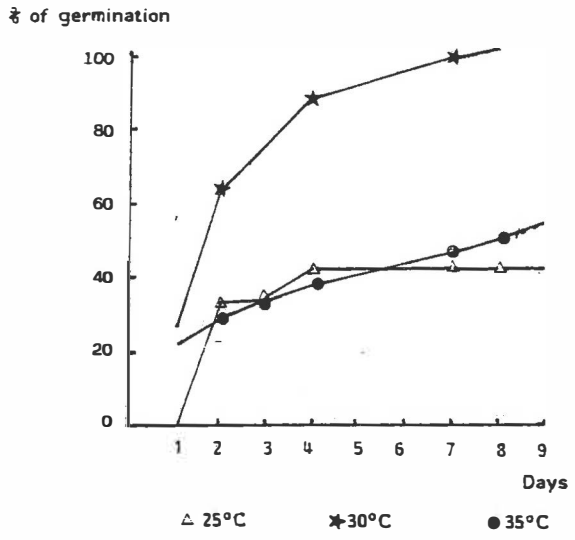



Fig. 3. Percentage of germinated chlamydospores of *D. flagrans at different temperatures*

The greater percentage of germination is obtained after 8 days of incubation at 30°C. It is noticeable that the optimal growth of the fungus also occurred at this temperature. The results obtained at 25 and 35°C are very similar but after 7 days of incubation the number of germinating chlamydospores was still increasing at 35°C whereas at 25°C no increase in germination was recorded after 4 days.

## Discussion and conclusion

For the purpose of use in biological control against zooparasitic nematodes, we consider that nematode-trapping fungi should:

- be able to grow between 15 and 35°C;
- survive intestinal transit of sheep;
- be able to reduce significantly the number of larvae in the environment (faeces, grass);
- produce chlamydospores easy to isolate and germinate.

*Arthrobotrys oligospora, A. tortor* and *Candelabrella musiformis* fulfill at least the first three conditions but their chlamydospores are difficult to isolate and are fragile (especially those of *A. tortor*). Moreover, the chlamydospores of *C. musiformis* are unable to germinate at temperatures above 25°C.

*Duddingtonia flagrans* fulfills all of the conditions above. It produces chlamydospores in abundance and they germinate easily up to 30°C.

Thus, this species appeared to be a good candidate for biological control experiments and our experimental results using sheep as a means of dispersal confirm its efficiency. Other species of nematode-trapping fungi could be considered for this purpose. The use

of chlamydospore-producing fungi would present the advantage of allowing a precise determination of the fungal inoculum.

**Acknowledgement**

I would like to express my thanks to Dr J.P. Lafont for reading this manuscript.

**References**

1. COOKE, R.C. 1963. The predacious activity of nematode-trapping fungi added to soil. *Annals of Applied Biology* **51**:295-299.

2. DESCHIENS, R., LAMY, L. and VAUTRIN, E. 1943. Essai de pratique de prophylaxie de l'anguillulore des végétaux par l'emploi d'Hyphomycètes prédateurs. *C.R. Academy of Science Paris* **216**:539-541.

3. DUDDINGTON, C.L. 1951. The ecology of predacious fungi. I. Preliminary survey. *Transactions of the British Mycological Society* **34**:322-331.

4. DUDDINGTON, C.L., EVERARD, C.O.R. and DUTHOIT, C.M.G. 1961. The effect of green manuring and a predacious fungus on cereal root eelworms *Plant Pathology* **10**:108-109.

5. GRUNER, L., PELOILLE, M., SAUVE, C. and CORTET, J. 1985. Survie et conservation d l'activité prédatrice vis-à-vis de nématodes trichstrongylidés après ingestion par des ovins de trois Hyphomycètes prédateurs. *C.R. Acadamy of Science Paris t.300* Serie III no. **14**:525-528.

6. MEKHTIEVA, N.A. 1971. Preliminary data on the composition of predatory fungi in Azerbaidjan) (in Russian). *Dokl. Akad. Nauk. Azerb. SSSR* **27**:72-74.

7. PELOILLE, M. 1981. Hyphomycètes prédateurs de nématodes dans une prairie du Limousin. *Entomophaga* **26**:(1):91-98.

8. PRIADKO, E.I. and DROBICHENKO, N.I. 1965. (Essais de mise en évidence de l'activité d'une préparation du champignon prédateur *Arthrobotrys oligospora* envers les larves de strongles intestinaux du mouton et de nématodes libres du sous-ordre des Rhabditata) (in Russian). *Mater Nauchn. Konf.* **1**:181-183.

9. VIRAT, M. and PELOILLE, M. 1977. Pouvoir prédateur *in vitro* d'une souche d'*Arthrobotrys oligospora* Fres. vis-à-vis d'un nématodes zooparasite. *Ann. Rech. Vét.* **8**:51-58.

# Estimation of suppressiveness and isolation of fungal parasites of cyst nematodes

**D H CRUMP**

**AFRC Institute of Arable Crops Research**
**Rothamsted Experimental Station**
**Harpenden, Herts., AL5 2JQ, UK**

## Introduction

Much of the work on fungal parasites of cyst nematodes has involved isolating fungi from eggs, hence many of these fungi have been termed egg parasites. However, Crump and Kerry (4) found fungi such as *Cylindrocarpon destructans* (Zins.) Scholten, *Verticillium chlamydosporium* Goddard, and *Fusarium spp.*, traditionally termed egg parasites, to be more effective as parasites of females; these were able to parasitise females soon after they emerged on the roots, before egg production had occurred. Indeed, parasitism of eggs during a growing season can only be achieved by first infecting the female. However, when immature females are infected they usually shrivel and are difficult to extract; also, the species of fungi isolated from females varies with the age of the female and at different times in the growing season (5,1). Estimates of levels of infection of females on a single sampling occasion are not accurate since the natural control of a nematode population occurs during many weeks in the soil. Crump (2) described an observation chamber method of monitoring the changes in numbers of females of the beet cyst nematode (*Heterodera schachtii* Schmidt) developing on sugar beet roots as a way of assessing the amount of natural control operating in a field soil, i.e. the suppressiveness of the soil to the nematode. This method is summarised here, together with a method for isolating fungi from individual female nematodes.

The fungi *Nematophthora gynophila* Kerry and Crump and *V. chlamydosporium* are the main two involved in the natural decline of cereal cyst nematode (*H. avenae* Woll.) populations (7), and *V. chlamydosporium* is one of the main parasites of the beet cyst nematode (1,2,6). Resting spores of both of these fungi can be quantitatively extracted from soil (3). However, the technique has now been modified to make it simpler and quicker without loss in efficiency, and this modified technique is described below.

## Materials and Methods

### Isolation of Fungi from Female Nematodes

The first stage in isolating fungi from female nematodes is to examine the females under a dissecting microscope and select those showing signs of infection. There are three fungi that are obligate parasites of females: *Catenaria auxiliaris* (Kuhn) Tribe, *N. gynophila* and an undescribed Lagenidiaceous fungus. Although these cannot be cultured easily on agar they all have characteristic hyphae and resting spores (9,8), and cause

recognisable symptoms in the female, i.e. disruption of the cuticle and softening of the body. Hence dissection of the female should ascertain the presence of any of these fungi.

Infection of the female by other fungi does not usually cause the cuticle to break down, but does cause it to tan prematurely. Occasionally, fungal spores are formed in or around the infected female; this has been seen with *V. chlamydosporium, C. destructans* and *Fusarium spp*. However, for the majority of infected females the fungus has to be cultured on agar to induce it to sporulate and hence be identified. The quickest way of doing this is to place a sterile glass coverslip (24 mm square) onto nutrient agar in a Petri dish; antibiotics can be incorporated into the agar to reduce the chance of bacterial contamination. Females are then placed in a drop of water on the coverslip about 0.5 mm in from each edge, four females per coverslip. In general, only fungi that can feed on the female will grow away from it, across the coverslip and onto the agar where they grow more rapidly. The fungi that produce spores and can therefore be identified usually do so before the colonies merge. In fact some fungi produce spores on the coverslip close to the female, presumably as nutrients in the female become depleted, thus allowing rapid identification.

This method lessens the need to surface sterilise the female, which is necessary if they are plated directly onto nutrient agar (surface sterilisation may kill fungi inside the female), and it is quicker than plating females onto water agar followed by sub-culturing onto nutrient agar. Also, fewer Petri dishes are used since each can accommodate four females.

**Measurement of Suppressiveness**

Triangular observation chambers were described by Crump (2) that are large enough to support a sugar beet plant while the nematode goes through two generations. These chambers have three sides that are easily removed so that the surface roots can be examined under a microscope (x15 magnification) and females either counted or removed for isolation of fungi. This method assumes that most of the control is operating on the female stage of the nematode. In a non-suppressive soil the numbers of females increase through the growing season, and most of them survive to form cysts full of healthy eggs. In a suppressive soil there is a decrease in numbers of healthy females. If this loss of females is quantified it gives a comparative measure of the suppressiveness of the soil to a particular nematode species.

The standard method adopted is to fill these observation chambers with the test soil and plant with a suitable host plant (sugar beet and potatoes have been successfully grown). Once females begin to appear on the surface roots regular observations (weekly or 2 weekly) are made. On two sides of the chamber females are left intact on the roots and counted on each sampling occasion. On the third side all tanned females or cysts are removed, cleaned of pieces of sub-crystalline layer and soil debris, and placed onto coverslips on nutrient agar as described above to assess the numbers infected and to identify the fungal species present. On the final sampling occasion all females and new cysts are removed from sides 1 and 2 of the chambers and dissected individually at x50 magnification; those that contain eggs and have no visible signs of infection are considered healthy. The number of healthy females and cysts at the end of the experimental period are expressed as a percentage of the maximum number observed during the period. This percentage survival can then be used as a comparative measure

of suppressiveness; the lower the survival rate the greater the suppressiveness of the soil. Further information on the control operating in the soil is obtained from the proportions of the females infected, and the fungal species isolated, on each sampling occasion.

When several soils containing the beet cyst nematode were tested with this method (2) the percentage survival of females was least (16%) in soil from a plot at Broom's Barn Experimental Station which had been monocultured with sugar beet for 20 years and where fungal parasites were known to be suppressing the nematode, and greatest (60%) in a field soil from Great Barton where multiplication of the nematode was known to be high.

Since the measure of suppressiveness obtained with this method is only comparative, it is only useful for comparing different soils or different treatments of one soil. However, it also gives information on the fungal parasites present in a soil and the time in the growing season at which infection occurs. For example. *V. chlamydosporium* was found to be most active on the first generation of the beet cyst nematode, whereas *C. destructans* was more active on the second. As well as investigating the natural control of the beet and other cyst nematode populations in field soils, this method could be used to investigate the factors that might increase it. Using the same soil in all chambers, other conditions could be altered, such as the addition of soil amendments or of artificially cultured fungal inoculum, to assess their effect on nematode multiplication.

**Extraction of *N. gynophila* and *V. chlamydosporium* spores from soil**

The original method for extracting resting spores of *N. gynophila* and *V. chlamydosporium* from soil (3) was designed to provide a direct method of quantifying these important nematophagous fungi that did not depend on the assessment of infection levels in nematode females that developed over a long period of time. Although the method was quicker and more accurate than methods in which the fungi were isolated from infected females, it was subsequently realised that a further saving of time could be made so that larger numbers of samples could be processed without loss of extraction efficiency.

The modified method is fundamentally the same as the original, i.e. wet sieving the soil followed by centrifugation in a high specific gravity (magnesium sulphate) solution to separate the fungal spores from soil. However, savings have been made by introducing an extra centrifugation stage to separate the fine soil sediment from the water instead of collecting the sediment on a 10 $\mu$m aperture sieve, which took a long time for the water to pass through. This also enables the sediment to be mixed directly with magnesium sulphate rather than floated in a separate layer on top of the magnesium sulphate in aqueous suspension. The latter method meant great care was needed not to shake the bottle and mix the two layers.

The modified spore extraction procedure is as follows:-

1. A 25 g soil sample is washed through 1,000, 150, 53 and 10 $\mu$m aperture sieves with a fine spray.

2. The sediment on the 10 μm sieve is washed into a 380 ml centrifuge bottle and centrifuged at 630 relative centrifugal force (R.C.F.) for 4 minutes. The supernatant (water) is discarded.

3. 250 ml of magnesium sulphate solution (S.G. 1.3) are added to the bottle, which is shaken to disperse the pellet, and centrifuged at 39 R.C.F. for 4 minutes. The fungal spores stay in suspension while most of the soil particles settle in the pellet.

4. The supernatant is poured onto a 10 μm aperture sieve and the pellet is discarded. The spore suspension is washed on this sieve to remove the magnesium sulphate.

5. The spore suspension is then washed into a 10 ml graduated centrifuge tube and concentrated into 0.5 ml by centrifuging at 850 R.C.F. for 4 minutes and the supernatant is discarded.

6. A 0.01 ml aliquot is pipetted onto a counting slide, and the spores counted under a high powered microscope, as before.

The method relies on identification of the fungal spores amongst other soil particles on the counting slide, but the spores are quite characteristic. Spores of *C. auxiliaris*, which are also easily identified, have occasionally been observed in these suspensions, but the efficiency of the technique in extracting these spores has not been measured.

**Table 1.** Numbers of *N. gynophila* and *V. chlamydosporium* spores/g soil extracted from four soils by two extraction methods.

(Means of 6 replicates)

| Method | *N. gynophila* | | | | | *V. chlamydosprium* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | A† | B | C | D | Mean ± $SE_D$ | A | B | C | D | Mean ± $SE_D$ |
| Revised | 16 | 1 | 31 | 3 | 13 | 13 | 33 | 175 | 114 | 84 |
| | | | | | 1.9 N.S. | | | | | 8.8 N.S. |
| Original | 23 | 2 | 33 | 2 | 15 | 13 | 45 | 191 | 91 | 85 |
| Mean | 20 | 2 | 32 | 3 | | 13 | 39 | 183 | 103 | |
| ± $SE_D$ | | | 2.7 *** | | | | | 12.5 *** | | |

† Soil A, clay loam; B and C silty loam; D peat loam

In the original method it was recommended that the pellet be suspended twice in magnesium sulphate since some spores were being dragged down with the soil particles when centrifuged. With the modified method only one centrifugation in magnesium sulphate is necessary to achieve the same efficiency of extraction, hence greatly reducing overall time. The modifications have therefore made the technique quicker and easier to use without loss in efficiency. The technique is regularly used to quantify the amount of *N. gynophila* and *V. chlamydosporium* in soils, and to monitor changes in the numbers of these spores in field trials.

A comparison of the original and new procedures was made on four soils of different textures containing spores of both fungi. In general, fewer spores were extracted by the new method but the differences were small and not statistically significant (Table 1).

## References


1. CRUMP, D.H. (1987). Effect of time of sampling, method of isolation and age of nematode on the species of fungi isolated from females of *Heterodera schachtii* and *H. avenae*. *Revue de Nematologie* **10**:369-373.

2. CRUMP, D.H. (1987). A method for assessing the natural control of cyst nematode populations. *Nematologica* **33**:232-243.

3. CRUMP, D.H. & KERRY, B.R. (1981). A quantitative method for extracting resting spores of two nematode parasitic fungi, *Nematophthora gynophila* and *Verticillium chlamydosporium* from soil. *Nematologica* **27**:330-338.

4. CRUMP, D.H. & KERRY, B.R. (1987). Studies on the population dynamics and fungal parasitism of *Heterodera schachtii* in soil from a sugar beet monoculture. *Crop Protection* **6**:49-55.

5. GINTIS, B.O., MORGAN-JONES, G. & RODRIGUEZ-KABANA, R. (1983). Fungi associated with several developmental stages of *Heterodera glycines* from an Alabama field soil. *Nematropica* **13**:181-200.

6. HEIJBROEK, W. (1983). Some effects of fungal parasites on the population development of the beet cyst nematode (*Heterodera schachtii* Schm.). *Mededelingen van de Faculteit Landbouwwetenschappen Rijksuniversiteit Gent* **48**:433-439.

7. KERRY, B.R. & CRUMP, D.H. (1977). Observations on fungal parasites of females and eggs of the cereal cyst nematode, *Heterodera avenae*, and other cyst nematodes. *Nematologica* **23**:193-201.

8. KERRY, B.R. & CRUMP, D.H. (1980). Two fungi parasitic on females of cyst nematodes *(Heterodera spp.)*. *Transactions of the British Mycological Society* **74**:119-125.

9. TRIBE, H.T. (1977). A parasite of white cysts of *Heterodera: Catenaria auxiliaris*. *Transactions of the British Mycological Society* **69**:367-376.

# Estimation of the activity of fungi parasitic on nematode eggs

R. Nicolay and R. A. Sikora

Institut fur Pflanzenkrankheiten der
Rheinischen Friedrich-Wilhelms-Universitat
Nussallee 9
5300 Bonn 1
FRG

## Introduction

Egg parasitic fungi infect a stage in the life-cycle of plant parasitic nematodes that is exposed to attack over extended time periods between host crops. Hence, they may have considerable potential in the biological control of *Heterodera, Globodera* and *Meloidogyne* species.

Because they often attack young females and reduce female fecundity these fungi could also be considered female parasites. Difficulties in distinguishing between true egg parasitism and female parasitism will affect the selection of methods for estimation of egg parasitism and can influence the interpretation of results.

All known egg parasitic fungi are able to grow saprophytically in the soil and therefore are facultative parasites that are not dependent on nematode eggs for their nutrition. Active mycelial growth through soil is essential for their establishment and infection of sedentary hosts (7). However the measurement of fungal growth in soil samples as an estimation of fungal egg parasitic potential is unrealistic because the parasitic activity depends on true parasitism and not simple contact with the egg. For this reason methods devised to investigate the role of egg parasites in regulating plant parasitic nematode densities should be based on the determination of rates of parasitism in viable eggs.

The methods used to determine fungal egg parasitism in soil samples and their advantages and disadvantages will be discussed in this chapter.

## Materials and Methods

### Determination of parasitism in field populations

Early reports of attempts to examine the extent of fungal disease in cyst nematode populations usually refer to the rate of "infected cysts". Vitality classes were set up for cysts (5, 12). The pathology of cyst nematodes has been reviewed (11).

For quantitative investigations, however, the rate of parasitism of the eggs is decisive, not fungal colonization of the cyst, since any parasitic or saprophytic fungus could enter this entity. The advantage of this procedure is that it is simple and does not require

greenhouse bioassays. On the other hand, rates of parasitism in field populations including several generations of the nematode are difficult to interpret, because they show no more than a temporal phase of the development of egg parasitism within the cysts.

Where many juveniles have hatched, assessments of fungal parasitism based only on the proportion of infected eggs may lead to an overestimation of the significance of parasitism (6). Empty egg-shells resulting from infection by fungi are hard to distinguish from those egg-shells remaining after juvenile emergence or those produced by physiological or abiotic causes of death (1). This method does not differentiate between parasitism of viable eggs and saprophytic growth in dead eggs. In most cases, however, fungi that have invaded newly produced eggs are probably parasitic rather than saprophytic (10).

For the reasons outlined, techniques are required that allow determination of parasitism in newly formed eggs which ultimately give a reliable estimation of parasitic activity in a particular soil sample.

**Addition of egg masses to field soil**

Several methods were developed for detecting *Dactylella oviparasitica* Stirling & Mankau and evaluating its significance in field soils (10). One technique requires mixing root pieces containing egg masses from *Meloidogyne*-infected plants grown in the greenhouse with soil collected from the field. The soil and roots are placed in containers, and the egg masses are examined 15-25 days later. Fungal activity under field conditions can be evaluated by burying soil and *Meloidogyne*-infected root pieces in porous ceramic tubes, bags of fine nylon screening, or other materials which allow free movement of water and gases. Although the presence of *D. oviparasitica* could usually be determined by these methods, they were considered unsatisfactory if counts of parasitized eggs were used to estimate the seasonal variation in the activity of the parasite, or to compare levels of parasitism in different fields. Many eggs escape parasitism because they readily hatch after incorporation into soil. The degree of hatch varies with egg masses source and with environmental factors such as moisture (10).

**Investigation of newly formed eggs in a bioassay**

A greenhouse bioassay was developed to assess both the presence of parasitic fungi and the percentage of infected eggs in soil where *Heterodera schachtii* was reproducing (8, 9).

Two 1500 g subsamples were taken from soil samples from the field and placed in 15cm diameter clay pots. A three week old sugarbeet seedling was planted in each pot and placed in a greenhouse where air temperatures were in the range of 24-30°C. Ninety days later, the cysts were extracted from the pots. Females and cysts were washed from the roots and collected on a sieve. The cysts from the soil and roots were combined and a 100-cyst subsample taken from each pot. Eggs were released from the cysts, suspended in water, and fungal parasites assessed. Egg suspensions were passed through a 250 $\mu$m sieve to remove cyst wall fragments and debris. The eggs were collected on a 25 $\mu$m sieve, washed several times with sterile distilled water and transferred to a graduated cylinder. A 5, 10 or 20 ml suspension was prepared with sterile distilled

water containing 1 ml of 0.05% streptomycin sulphate and 1 ml 0.1% penicillin solutions/total 10ml water. The suspension volume selected depends on egg density. After the suspensions were agitated, a sample was removed for counting, and a 0.5 ml sample pipetted onto five 5 cm diameter Petri dishes containing 0.8% distilled-water agar. The dishes were incubated at 25°C for 24-48 hrs and 100 eggs/dish examined at random for growth of fungal hyphae.

Although the majority of eggs examined were from the newly produced generation of the nematode this method did not consistently exclude cysts of earlier generations and in addition, after 90 days at 24-30°C an appreciable amount of newly formed juveniles could have hatched. Thus the problems described above cannot be totally excluded and a certain amount of experimental error can be expected with this method.

A similar technique was described for assessment of parasitism of *Meloidogyne* eggs by *D. oviparasitica* (10). Tomato seedlings were planted into field soil in pots, second-stage *Meloidogyne* juveniles were added if the soil was lightly infested. Plants were grown at 25-27°C for about 40 days, after which the egg masses were removed and eggs examined for fungal parasitism. It was assumed that the number of egg masses found to contain *D. oviparasitica* by this method is related to the level of parasitism occuring naturally in that field soil.

Methods to estimate the fungal egg parasitic potential of soil samples have to fulfil two conditions: 1) numbers of empty egg-shells must be at a minimum and 2) cysts previously present in the soil must be excluded from the examination process. The techniques described below were developed for examination of *Heterodera schachtii* Schmidt eggs (7), but could be modified for any cyst nematode.

**Observation chambers**

Observation chambers were constructed from 9 cm diameter Petri dishes (2) and filled with 70 g soil/chamber (≥ 5 replicates). Six seeds of rape were sown per chamber and tne chambers placed next to each other at a 45° angle with the lid facing downward in plastic trays filled to a 3cm depth with moistened sand. The trays with the sand were incubated at 21°C with 16 hrs artificial lighting (4000 Lux) in a growth chamber and watered by moistening the sand with deionized water. Each chamber was inoculated with 1500 *H. schachtii* juveniles ten days after seeding. Forty days after inoculation 20 newly formed cysts were removed from the roots in each chamber.

The cysts were crushed in a tissue homogenizer and the rate of egg parasitism determined using the following modification of the technique described earlier (4). The egg suspension is shaken in a test tube to break up clumps of eggs and then poured onto a 20 μm sieve. The residue on the sieve was washed with deionized water, placed in graduated 15 ml centrifuge tubes and centrifuged for 5 mins at 2500 rpm. The supernatant was removed with a pipette until 0.3 ml suspension remained. A 0.3 ml solution containing 100 mg/1 streptomycin sulphate and 200 mg/1 penicillin G was added to the 0.3 ml sediment in each tube. The resulting 0.6 ml suspension was spread over a 1.5% water agar Petri dish (9 cm diameter). Large cyst wall fragments were left in the tube. Approximately 1000 eggs and juveniles were applied per plate and the plates incubated at room temperature for 48 hrs before examining the eggs.

The observation chambers originally used to observe the degradation of newly formed females by parasitic fungi (2) are used here to simplify separation of newly produced cysts from older cysts already present in the soil to be examined. The build-up of a muddy silt film on the chamber surface may, however, prevent unobstructed examination of the root surface through the lid after prolonged incubation. Dislodging of cysts from the roots can further hamper the efficiency of this technique.

**Soil-fractioning technique**

A 70 g soil sample was suspended in 100 ml water, poured onto a 250 $\mu$m sieve and washed with water. The fungi and eggs were freed from the cysts by gently crushing the cysts on the sieve with a rubber stopper. The cyst contents released were then washed through the sieve with water. The filtrate containing the eggs as well as fungi was added to 800 ml beakers and centrifuged at 5600G for 20 mins. The sediment was mixed 1 : 1.5 (w/w) with sterilised sand to improve its texture and the resulting mixture added to a 7cm diameter pot.

The pots were placed in plastic trays on a 3 cm deep layer of moist sand and sown with ten rape seeds/pot. In addition to the natural nematode infestation in the filtrate, each pot was inoculated with 100 *H. schachtii* juveniles 10 days after seeding. The pots were incubated for 40 days after inoculation at 21°C in a growth chamber with 16 hrs artificial lighting (4000 Lux) and watered with deionized water. The shoots were then removed at the soil surface in order to prevent nematode development and hatching of the second generation. The pots were stored for an additional 14 days at 17°C to allow further egg parasitism. The newly formed cysts were extracted and the rate of parasitism determined as described above.

The study of only newly formed eggs with this technique has the advantage that empty eggs caused by hatching remain at a minimum. The technique allows the use of all the cysts that developed on the roots for quantitative collection of data on the parasitic potential in a field soil and not just a sub-sample. It is based on the assumption that high levels of hyphae or spores of egg parasitic fungi in field soil will be reflected in high rates of egg parasitism when the fungi are challenged with newly forming eggs in young females. Highly significant correlations between the percentage of unsterile field soil in a mixture with sterilised soil and the percentage of infected eggs was detected (7) demonstrating that the soil-fractioning technique is efficient in detecting different densities as well as in determining relative activity of parasitic fungi attacking eggs. In addition, this technique exposes all development stages, from immature females to second-stage juveniles in the egg to fungal attack. Although an accurate quantitative prediction of the influence of fungal egg parasitism on the population dynamics of cyst nematodes in the field is not possible, comparisons can be made between different fields or different treatments. It allows studies of the influence of agronomic and environmental factors on the activity of egg parasites. The applicability of this technique is, however, restricted to soils with similar texture, since the addition of sand (w/w) may distort fungal densities. Because it is time-consuming the technique is more applicable for research rather than for routine examinations.

## Discussion

It is impossible to observe the parasitic activity on eggs in cysts over extended periods of time, e.g. years, efficiently without exertion of large amounts of time and manpower. Therefore, techniques that estimate the activity of fungal egg parasites in soil at a predetermined development stage of the nematode are more useful and efficient. The overlapping of several nematode generations in the field as well as hatching of eggs makes any method that disregards these factors inappropriate. All of the methods described have advantages and disadvantages (Table 1).

**Table 1.** Comparison of different techniques to estimate the activity of egg parasitic fungi.

| Technique | Advantages | Disadvantages |
|---|---|---|
| Investigation of field populations | simple, time-saving | difficult to interpret |
| Addition of egg masses to soil (10) | simple, detection of true egg parasites | hatch of juveniles, dependent on environmental factors |
| Bioassay (8, 10) | examination of young eggs | no exclusion of old cysts |
| Observation chambers (7) | examination of young eggs (exclusion of old cysts) | possible problems in distinguishing younger from older cysts |
| Soil-fractioning (7) | examination of young cysts, exclusion of old cysts, quantitative cyst counts | dependent on soil texture, high expenditure of time and equipment |

In general, simple techniques usually give inadequate results. However, even the most time-consuming soil-fractioning technique produces results limited to the events surrounding egg parasitism on the root surface.

The nematologist or invertebrate pathologist should select methods that fit definite research goals before initiating his programme! The recent trend in research on fungal egg parasitism has been channelled towards the detection of isolates to be used for commercial products for nematode control. Methodology has not kept pace with this trend. This has often resulted in the worldwide promotion of isolates with low levels of activity and a significant loss in research funding, manpower, and progress. In many

instances, the techniques used favour isolation of saprophytic fungi and not parasitic species. Improved techniques for working with true fungal parasites should increase detection of active isolates and lead to effective biological control systems.

## References


1. BURSNALL, L.A. & TRIBE, H.T., 1974. Fungal parasitism in cysts of *Heterodera*. II. Egg parasites of *H. schachtii*. *Transactions of the British Mycological Society* **62**:595-601.

2. CRUMP, D.H. & KERRY, B.R., 1977. Maturation of females of the cereal cyst-nematode on oat roots and infection by an *Entomophthora*-like fungus in observation chambers. *Nematologica* **23**:398-402.

3. KERRY, B.R., 1987. Biological control. In: Brown, R.H. & Kerry, B.R. (eds.): *Principles and practice of nematode control in crops*. Academic press, Sydney.

4. KERRY, B.R. & CRUMP, D.H., 1977. Observations on fungal parasites of females and eggs of the cereal cyst-nematode, *Heterodera avenae*, and other cyst nematodes. *Nematologica* **23**:193-201.

5. KORAB, J.J., 1929. Results of a study of the beet nematode *Heterodera schachtii* at the nematode laboratory of the Belaya Tserkov Research Station. (Russian with English summary.) *Sbornik Sortocovo Semenovodcheskogo Upravleniya* **8**:29-67.

6. LOPEZ-LLORCA, L.V. & DUNCAN, J.M., 1986. New media for the estimation of fungal infection in eggs of the cereal cyst nematode, *Heterodera avenae* Woll. *Nematologica* **32**:486-490.

7. NICOLAY, R. & SIKORA, R.A., 1989. Techniques to determine the activity of fungal egg parasites of *Heterodera schachtii* in field soil. *Revue de Nematologie* **12**:97-102.

8. NIGH, E.A., 1979. *Acremonium strictum* and *Fusarium oxysporum*, two fungal egg parasites of *Heterodera schachtii*, the sugarbeet cyst nematode. *Dissertation, University of California, Riverside*.

9. NIGH, E.A., THOMASON, I.J. & VAN GUNDY, S.D., 1980. Identification and distribution of fungal parasites of *Heterodera schachtii* eggs in California. *Phytopathology* **70**:884-889.

10. STIRLING, G.R., 1979. Techniques for detecting *Dactylella oviparasitica* and evaluating its significance in field soils. *Journal of Nematology* **11**:99-100.

11. TRIBE, H.T., 1977. Pathology of cyst-nematodes. *Biological Review* **52**: 477-507.

12. WILCOX, J. & TRIBE, H.T., 1974. Fungal parasitism in cysts of *Heterodera*. I. Preliminary investigations. *Transactions of the British Mycological Society* **62**:585-594.

# A bioassay for assessing the biological control potential of facultative parasitic fungi infecting the potato cyst nematode

**Jasper de Haan and Leon Thijssen**
**H.L. Hilbrands Laboratorium voor Bodemziekten, Mr. P.J. Troelstralaan 1A, 9402 BA Assen, The Netherlands**

## Introduction

Many organisms are known as predators and parasites of nematodes, but not all of these organisms are able to suppress nematode multiplication significantly. Fungi are probably the most promising organisms for the biological control of nematodes. For biocontrol of the potato cyst nematode (PCN) two groups of fungi are interesting: fungi which parasitise eggs or young females, and fungi which influence nematode populations in other ways, e.g. by metabolic products or by blocking the natural openings of the cysts, thus preventing the hatched juveniles escaping from cysts. A number of these fungi are facultative parasites of nematodes which can also live saprophytically.

Various methods are described to isolate fungi from eggs and cysts, but there is a great need for screening methods to evaluate nematophagous fungi for their abilities to control plant parasitic nematodes. A bioassay to test facultative parasitic fungi on PCN is described.

## Materials and Methods

### Preparation of fungal inoculum

Three fungi, *Cylindrocarpon destructans* (Zinssm.) Scholten, *Fusarium oxysporum* Schlecht and *Plectosphaerella cucumerina* (Lindf.) Gams, which previously had been isolated from PCN, were grown in pure culture on 2% malt extract agar in Petri dishes.

Conical flasks were filled with 50 g of potting compost (66% organic matter, pH 5.6), which was sieved (5 mm apertures) to remove coarse organic material. Subsequently the flasks were closed with cotton plugs, and their contents were autoclaved (20 minutes at 120°C). Five discs (5 mm diam.) from a fungal colony on agar were transferred aseptically to the potting compost in each flask. After incubation for 14 days at 20°C, the fungal inocula were ready to be used in the bioassay.

### Bioassay

The contents of each flask (50 g of fungal inoculum) were added in a plastic bag to 200 g of a sandy field soil (2.6% organic matter; pH 4.5. While the bag was kept closed, the fungal inoculum was mixed thoroughly with the soil.

A clay pot (300 ml) was three quarters filled with this mixture. Then *Globodera pallida*

Stone pathotype Pa 2, was added as cysts: 10 cysts per pot, which resulted in an initial population density of 6 hatchable eggs/g soil. A small potato tuber of the variety 'Mentor' was planted and the remaining quarter of soil put in the pot. There were two control series: one in which only autoclaved potting compost (without fungus) was added (PC) and one in which only field soil was put in the clay pots (Untreated). Pots were placed in a glasshouse which was heated in winter and spring. In this bioassay each treatment consisted of 5 replications.

After 12 weeks, the plant tops were cut off, the soil was air dried and cysts were separated from the soil using a Schuiling centrifuge (Fa. A. Volkers & Zn, The Netherlands). Debris retained on the sieve was air dried and the cysts separated from organic matter by flotation in acetone (8). To determine the effect of the facultative parasites on the multiplication of PCN, the cyst samples were counted and the fecundity, and hatching rate of the eggs were determined.

The hatching rate was assessed after 5 months when the newly formed cysts had passed their period of dormancy. From each replicate, 25 cysts were taken at random and put in tap water for 5 days and afterwards in potato root diffusate. The hatched juveniles were counted at 7 day intervals. At the end of the hatching experiment, after 15 wk, the remaining cyst content was determined and the fecundity and the hatching rate calculated. Finally, the multiplication rate of PCN was expressed as a Pf/Pi-value, based on the total number of hatchable eggs added per pot (Pi) and the numbers of newly formed cysts, their contents and the hatching rate of the juveniles (Pf).

## Results

Two of the fungi tested, *P. cucumerina* and *F. oxysporum*, reduced the numbers of newly formed cysts, by 53 and 46% respectively, compared with the substrate control, i.e. treatment PC (Table 1). The addition of uncolonised potting compost to the field soil resulted in a small, but not statistically significant, increase in the number of cysts compared to untreated.

Differences in the fecundity of the females were found between PC and the treatments with *P. cucumerina* and *C. destructans*. The fecundity of the nematode was reduced by 58 and 22% in soil treated with *P. cucumerina* or *C. destructans* respectively (Table 2). Hatching of eggs was not significantly reduced by any of the treatments.

The multiplication of PCN was considerably affected by the fungi but the nematode population increased considerably in all pots. The $P_f/P_i$-values varied from 14 for the treatment with *P. cucumerina* and 30 for the treatment with *F. oxysporum* to 63 for PC (Table 2). Compared with PC, *P. cucumerina* and *F. oxysporum* reduced the multiplication of PCN by 78 and 52% respectively. There was no statistical difference between PC and untreated soil in terms of $P_f/P_i$-values.

**Table 1.** Effects of three fungi and potting compost on the numbers of newly formed cysts of PCN.

| Treatment | Cysts | Reduction (%)* |
|---|---|---|
| *P. cucumerina* | 121 A** | 53 |
| *F. oxysporum* | 137 A | 46 |
| *C. destructans* | 253 B | 1 |
| Untreated | 199 AB | 22 |
| Potting compost (PC) | 255 B | 0 |

* Compared with potting compost
** Data having different letters are statistically different at $P < 0.10$

**Table 2.** Effects of three fungi and potting compost on fecundity, hatching and multiplication of PCN

| Treatment | Eggs/cyst | Hatch (%) | Pf/Pi |
|---|---|---|---|
| *P. cucumerina* | 174 A* | 87 | 14 a* |
| *F. oxysporum* | 360 BC | 91 | 30 ab |
| *C. destructans* | 324 B | 79 | 45 bc |
| Untreated | 345 BC | 88 | 42 bc |
| Potting compost (PC) | 418 C | 89 | 63 c |

*Data having different letters are statistically different at P=O.10.

## Discussion

In the bioassay described, potting compost was chosen as substrate for the multiplication of fungi, because it is more comparable to field soil than artificial media, like agar. It is likely that fungi introduced on this medium establish themselves more quickly in soil than fungi introduced on artificial media. Although the multiplication of PCN tends to be greater when potting compost was added, no statistical differences were observed between PC and untreated, nor in the numbers of newly formed cysts, nor in the fecundity, nor in the multiplication rate of PCN.

Treatments with fungi were compared with the amendment of potting compost, because the fungi were grown on this substrate. Potting compost contains a lot of organic matter, which by itself may have an impact on the multiplication of PCN. Kerry *et al.* (6) found that media, which they used for the preparation of fungal inocula, had a varying impact on the multiplication of the cereal cyst nematode, *Heterodera avenae* Woll. The effect of a fungal treatment on PCN multiplication may partly be due to the amendment of organic matter. Differences between treatments with fungi and substrate control have to be determined, rather than differences between treatments with fungi and field soil.

In addition to the parameters estimated, eggs and cysts can be plated on agar to assess the percentages of parasitised eggs and colonized cysts. This was not done in these experiments. Probably not many new eggs were parasitised by the fungi tested, as the proportion of eggs that hatched was large (79-89%) in all treatments. This implies that the mode of action of these fungi probably results from the colonisation of young females and/or a reduction in their fecundity; *P. cucumerina* reduced the fecundity in this bioassay significantly. But it is also possible that fungi cause a decrease in the numbers of second-stage juveniles that invade the roots. More detailed studies are needed to elucidate the modes of action of facultative parasitic fungi.

Using the bioassay described, it is possible to assess the potential of facultative parasites of cyst nematodes in the glasshouse. However, results obtained in the glasshouse cannot be translated directly to field situations and fungi, which are able to reduce PCN multiplication in the glasshouse, have to be tested under field conditions afterwards. This bioassay resembles the field situation more than the method described by La Mondia and Brodie (7), which is a modification of the one described by Foot (4). They used closed canisters, in which root growth occurs, but shoot growth is prevented. It is likely that the plant shoot has an impact on the rhizosphere through the release of metabolites to the roots. These substances which leach from roots may attract fungi towards the rhizosphere where the young females of cyst nematodes are located and probably easily found by the fungi. It is therefore better to use whole plants instead of only root systems. For laboratory experiments plants grown in Petri dishes filled with soil and inoculated with fungi can be used to examine parasitism of females and eggs (3,5).

It is not possible in this bioassay in pots to make observations on the rhizosphere. To visualize the effect of fungi on cyst nematodes in the rhizosphere, the triangular observation chamber technique can be used which was developed and described by Crump (1). This method makes it possible to determine the effect of fungi on nematodes at various time intervals. Sampling at different times may be important, as usually non-sterile soil is used in a bioassay and the amount of parasitism by a fungus may vary with time (2).

In this bioassay the fungi are grown on potting compost. This substrate is suitable for laboratory and glasshouse experiments. but not for field applications. To introduce facultative parasites of nematodes into fields other methods have to be developed. First of all the ability of these fungi to suppress nematode multiplication has to be demonstrated, for which the described bioassay may be helpful.

**Acknowledgement**

We thank Geraldien van der Woude for carrying out the glasshouse experiment.

**References**

1. CRUMP, D.H. 1987. A method for assessing the natural control of cyst nematode populations. *Nematologica* **33**:232-243.

2. CRUMP, D.H. 1987. Effect of time sampling, method of isolation and age of nematode on the species of fungi isolated from females of *Heterodera schachtii* and *H. avenae*. *Revue Nematol.* **10**:369-373.

3. CRUMP, D.H. & KERRY, B.R. 1977. Maturation of females of the cereal cyst-nematode on oat roots and infection by an *Entomophthora*-like fungus in observation chambers. *Nematologica* **23**:398-402.

4. FOOT, M.A. 1977. Laboratory rearing of potato cyst nematode: a method suitable for pathotyping and biological studies. *New Zealand Journal of Zoology* **4**:183-186.

5. JUHL, M 1977. An easy and space-saving Petri dish method for testing fungi on *Heterodera avenae*. *Nematologica* **23**:122-123.

6. KERRY, B.R., SIMON, A. & ROVIRA, A.D. 1984. Observations on the introduction of *Verticillium chlamydosporium* and other parasitic fungi into soil for control of the cereal cyst-nematode *Heterodera avenae*. *Ann. appl. Biol.* **105**:509-516.

7. LA MONDIA, J.A. & BRODIE, B.B. 1984. An observation chamber technique for evaluating potential biocontrol agents of *Globodera rostochiensis*. *Journal of Nematology* **16**:112-115.

8. OOSTENBRINK, M 1950. Het aardappelaaltje (*Heterodera rostochiensis* Wollenweber) een gevaarlijke parasiet voor de eenzijdige aardappelcultuur. Versl. Meded. Plziektenk. Dienst, Wageningen 115: 230pp.

# Methods for studying the growth and survival of the nematophagous fungus, *Verticillium chlamydosporium* Goddard, in soil.

**Brian Kerry**
**AFRC, IACR, Rothamsted Experimental Station,**
**Harpenden, Herts., AL5 2JQ, England.**

## Introduction

*Verticillium chlamydosporium* Goddard has been extensively studied as a potential biological control agent (5) since it was identified as an important parasite causing the decline of *Heterodera avenae* Woll. populations in many soils in N. Europe (3). The fungus has been isolated from the eggs and females of several species of cyst and root-knot nematodes in soils of different textures around the world (5). *Verticillium chlamydosporium* is a facultative parasite which can also proliferate in soil, particularly in the rhizosphere, where it feeds saprophytically. Most attention has been paid to the parasitic phase and the selection of isolates that are virulent against particular pest species. Little is known about the saprophytic development of *V. chlamydosporium* in soil. However, a knowledge of the extent of colonisation of the soil and survival therein is essential for an understanding of the epidemiology of this fungus, the development of application methods and the selection of suitable isolates as biological control agents.

A method primarily used to extract spores of the obligate parasite, *Nematophthora gynophila* Kerry & Crump, has also been used for estimating the numbers of chlamydospores of *V. chlamydosporium* in soil (see chapter 3). However, the fungus can grow actively in soil and on roots so that assessments of abundance based only on chlamydospores may underestimate the number of propagules, particularly when the fungus is growing actively. Few attempts have been made to develop selective media for the isolation and estimation of nematophagous fungi in soil. Semi-selective media have been developed for *Paecilomyces lilacinus* (Thom.) Samson (2,7) and *V. chlamydosporium* (2) but the medium for the latter supported the development of many contaminants which made estimates of population density difficult in UK soils. This paper describes the use of another medium which has proved effective for the isolation of *V. chlamydosporium* from a range of soil types and plant rhizospheres. A simple method for estimating hyphal growth in soil is also described.

## Materials and Methods

The semi-selective medium has been used in a number of experiments to estimate the density of colony forming units (cfu) in soils of different textures and on root surfaces at different times after application of selected isolates to soil, or in soils naturally infested by the fungus. The growth of all isolates of *V. chlamydosporium* is partly suppressed by the fungicides incorporated in the medium; isolates differ in their sensitivity and it is important to test that the medium is suited to the isolate used. This can be done most simply by adding, in sterile conditions, a known number of propagules to the medium and comparing

the numbers of colonies that develop with those growing on a non-selective medium such as corn meal agar. In a test of 25 isolates at Rothamsted, the growth of some was not reduced on the selective medium but that of about 20 was reduced by, on average, 47% ± 4.8.

**Selective medium.** The medium contains: 37.5 mg carbendazim, 37.5 mg thiabendazole, 75 mg rose bengal, 17.5 g NaCl, 50 mg each of streptomycin sulphate, aureomycin and chloramphenicol, 3ml Triton X 100, and 17 g corn meal agar (Oxoid) in a litre of distilled water. The fungicides and the antibiotics are added to the cooled agar after autoclaving.

**Estimation of the numbers of colony forming units.** Soil samples should be screened through a 1 mm aperture sieve and thoroughly mixed. A representative 2 g subsample is taken and added to 18 ml of 0.05% water agar solution. The suspension is vigorously shaken for 15 secs before a 2 ml subsample is transferred to a similar volume of agar solution as part of a dilution series; this process is repeated for each dilution. For $10^{-2}$ and $10^{-3}$ dilutions of each sample, three Petri dishes (9 cm diam.) are each inoculated with 0.2 ml of the suspension which is aseptically spread over the surface of the selective medium. The numbers of colony forming units are usually counted after incubation at 18°C in the dark for two weeks, but the time and temperature used depends on the isolate; some isolates grow faster at 25°C. Colonies usually produce conidiophores with conidia on the selective medium. To confirm identification individual colonies can be subcultured onto corn meal agar and incubated for 3-4 wks to allow the characteristic chlamydospores to develop.

**Measurement of the extent of colonisation in soil.** A method developed by Lumsden (6) was modified to examine hyphal growth and chlamydospore production in soil. Petri dishes (5 cm diam.) are filled with sieved soil adjusted to 30% moisture. The soil surface is smoothed with a spatula and nylon fabric (50 μm apertures) is held in close contact with it by means of a plastic ring; the ring is made by cutting out the base of a second Petri dish with a hot scalpel. A source of inoculum, e.g. colonised cereal grain or alginate granule, is placed in the centre of the fabric and soil carefully added to the depth of the ring which is then covered with a Petri dish lid. The dishes should be placed in a moist chamber and incubated at 18°C for 4 wks. The soil and original inoculum source are then removed from the nylon mesh which is lifted and any remaining soil agregates carefully shaken off. The mesh can be pressed onto the selective medium and removed. Colonisation is estimated from the diameter of the colonised area that develops after incubation at 18°C for 3-7 days; Petri dishes should be checked regularly so that growth on the agar does not affect the estimate of colonisation. The mesh may also be stained with methylene blue lactophenol and examined microscopically; *V. chlamydosporium* hyphae could not be recognised, so growth was therefore assessed in terms of the distribution of the characteristic chlamydospores.

## Results

Data from experiments examining the importance of the rhizosphere in the survival of *V. chlamydosporium* and the need for an energy source in the inoculum for successful establishment of the fungus in soil have been published elsewhere (4,5). The number of cfu in four soils from microplots in which spring barley cv. Triumph had been grown for 10 years and in which *H. avenae* multiplication was suppressed, are presented below and

compared with counts for the chlamydospores extracted using the method described by Crump in this volume. Soil samples (500 g) were collected in spring before drilling.

**Table 1.** Numbers ($log_{10}$) of cfu and chlamydospores of *V. chlamydosporium* in four soils suppressive to *H. avenae*. (Means of 2 replicates; figures in parentheses are the untransformed counts)

| Soil Texture | *V. chlamydosporium* density ($log_{10}$ numbers/g soil) cfu | chlamydospores |
|---|---|---|
| A. Silty loam | 3.322 (2242) | 2.349 (247) |
| B. Calcareous silty loam | 3.122 (1504) | 2.901 (979) |
| C. Calcareous silty loam | 3.707 (5496) | 3.041 (1208) |
| D. organic loam | 3.163 (1583) | 3.149 (1620) |
| | SED ± 0.068 | |

There are significant differences ($P < 0.05$) in the abundance of *V. chlamydosporium* in the four soils. Also, there was a significant interaction ($P < 0.001$) between soil and the type of propagule; in soil D there was no significant difference between the numbers of cfu and chlamydospores whereas in soil A, chlamydospores represented only 11% of the total number of cfu.

Hyphal growth on the meshes in soil was very limited in tests comparing different isolates of *V. chlamydosporium* originally derived from chlamydospores or infected eggs of cyst nematodes in soil. After 4 wks no isolate had colonised more than 1 cm from the point source of inoculum and the origin of the fungus did not significantly affect that growth (Table 2). The isolates were placed on the meshes in alginate granules containing wheat bran (5).

**Table 2.** Extent of hyphal growth in soil of *V. chlamydosporium* isolated from soil or nematode eggs. (Means of 4 replicates for each isolate)

| Origin of isolate | Number of isolates tested | Growth (mm) Mean ± S.E. | |
|---|---|---|---|
| Soil | 6 | 6.7 | 0.57 |
| Nematode eggs | 8 | 5.6 | 0.81 |

Estimating the extent of colonisation by direct staining of chlamydospores underestimated growth by about 50% ; presumably because chlamydospores are only formed on the older sections of hyphae.

## Discussion and Conclusions

The semi-selective medium has proved useful for the estimation of numbers of cfu of *V. chlamydosporium* in soil. Approximately 1.5 x $10^3$ to 7.5 x $10^3$ cfu/g were present in soils suppressive to *H. avenae* whereas fewer than 350 cfu/g were found in soils where the nematode was causing damage (Kerry unpublished data). Growth of some isolates may be slow and their numbers are likely to be underestimated if any contaminants eventually grow over the agar. However, if known numbers of chlamydospores of selected isolates are added to soil, similar cfu counts are obtained from samples taken immediately afterwards.

It is not possible to distinguish reliably whether a cfu has developed from a hyphal fragment, conidium or chlamydospore; all these propagules occur in soil. Hence it is difficult to assess whether the fungus is growing vegetatively in soil or is surviving as resting spores, unless a series of samples are taken at different times to assess whether cfu numbers are increasing or not. In general, rapidly increasing cfu counts are associated with hyphal growth or conidiation; large numbers of cfu in soils counted on a single occasion do not necessarily mean that the fungus is active and care must be taken in the interpretation of data from dilution plates.

Although *V. chlamydosporium* can proliferate and survive in soil (4,5), the extent of colonisation on the meshes is limited even when an energy source is incorporated with the inoculum. Hyphal growth is likely to be affected by many factors including isolate and inoculum type, method of application, and soil conditions. However, the rate of growth of *V. chlamydosporium* appears slow in comparison with some other fungi, such as *Rhizoctonia solani* Kühn, that may extend 5 cm in only 12 days (1). Colonisation in the rhizosphere may be more extensive (4). Successful establishment at practical application rates of a fungus with such limited powers of colonisation in soil is likely to depend on the type of inoculum used and its thorough incorporation. The methods described are useful for testing different types of inoculum and formulations of *V. chlamydosporium* in a range of conditions for the development of selected isolates as biological control agents.

## Acknowledgements

I am grateful to Iain Kirkwood, supported on MAFF Grant CSA 958, and Frans de Leij, supported by the Agricultural Genetics Company, for the use of some of their data.

## References

1. BLAIR, I.D., 1942. Studies on the growth in soil and the parasitic action of certain *Rhizoctonia solani* isolates from wheat. *Canadian Journal of Research* **20**:174-185.

2. GASPARD, J.T., JAFFEE, B.A. & FERRIS, H., 1990. *Meloidogyne incognita* survival in soil infested with *Paecilomyces lilacinus* and *Verticillium chlamydosporium*. *Journal of Nematology* **22**:176-181.

3. KERRY, B.R., 1982. The decline of *Heterodera avenae* populations. *EPPO Bulletin* **12**:491-496.

4. KERRY. B.R., 1988. Two microorganisms for the biological control of plant parasitic nematodes. *Proceedings of the Brighton Crop Protection Conference -Pests and Diseases 1988*, 606-607.

5. KERRY, B.R., 1989. Fungi as biological control agents for plant parasitic nematodes. In: Biotechnology of fungi for improving plant growth. Whipps, J.M. & Lumsden, R.D. (Eds.), Cambridge University Press, Cambridge, 153-170.

6. LUMSDEN, R.D., 1981. A nylon fabric technique for studying the ecology of *Pythium aphanidermatum* and other fungi in soil. *Phytopathology* **71**:282-285.

7. MITCHELL, D.J., KANNWISCHER-MITCHELL, M.E. & DICKSON, D.W., 1987. A semi-selective medium for the isolation of *Paecilomyces lilacinus* from soil. *Journal of Nematology* **19**:255-256.

# Methods for introducing *Verticillium chlamydosporium* into soil

**J. Coosemans**

**Laboratory fo Phytopathology and Plant Protection**
**University of Leuven**
**Kardinaal Mercierlaan 92, B-3030 Leuven, Belgium**

## Introduction

Chemical control is becoming more and more expensive, because of increased costs in the synthesis of new compounds with new modes of action and is increasingly undesirable because of environmental hazards associated with their application. Undoubtedly, biological control will play a more important role in integrated control of nematodes in the future. However, there is a big gap between results of small scale experiments and the practical use of biological control; although research has been done over more than 30 years, the use of nematophagous organisms is still rather limited.

Research is needed to study the optimal environmental conditions (temperature, soil pH, humidity and soil fertility) before antagonists can be introduced under practical circumstances. At present, biological agents tend to be mainly treated as chemical products; a certain quantity of spores (active ingredient) is introduced in the soil to control a specific pest. A more profound knowledge of the survival of antagonistic bacteria and fungi is urgently needed if predictable control is to be achieved. Therefore, observations on the natural occurrence of some nematophagous fungi and research on their optimal growth conditions have begun in order to collect data on sporulation, germination, mycelial growth and survival in dried formulations.

This contribution will deal with the possible use of the fungal parasite, *Verticillium chlamydosporium* Goddard against the beet cyst nematode, *Heterodera schachtii* Schmidt, and will discuss two basic methods for introducing *V. chlamydosporium* into the host plant's rhizosphere to avoid the necessity of a broadcast application in the field. Antagonistic activity was achieved either by using the paperpot (chainpot) technique or by seed pellet inoculation as a means of introduction of the antagonist.

In natural conditions, fungi continuously destroy nematodes in virtually all soils. There are, however, great technical problems in observing and assessing the importance of these organisms. Among these problems should be mentioned: the complexities of the soil habitat, the specialized techniques required to extract, count and isolate the fungal antagonists and the fact that many of these organisms are obligate parasites or do not readily sporulate.

In the literature, quite a lot of data can be found discussing the introduction of trapping fungi into the soil, e.g. *Arthrobotrys* spp., *Dactylaria* spp.. Most experiments with these fungi involved adding inoculum to the soil which had been amended with organic matter

to stimulate fungal growth. This amendment resulted in better growth of the fungus but predacious activity was not usually sufficient to affect nematode populations.

The apparent non-specific nature of nematode predation by most trapping fungi appears to be a serious disadvantage in their potential as commercial biological agents against second-stage juveniles of nematodes. Also, the following problems remain: (1) the inoculation rate is usually high (fungus on granules of cereal seed medium at a rate of 140 g/m$^2$) and has to be incorporated one month in advance of the crop; (2) colonisation of the soil by the fungus is often unsatisfactory and survival is short; (3) the lack of ring and network traps, as they have to be induced by living nematodes; (4) only nematodes of an appropriate body size are captured.

Tribe (7) reviewed the relationship of fungi to cyst pathology and listed *V. chlamydosporium*, *Catenaria auxiliaris* (Kuhn) Tribe and *Nematophthora gynophila* Kerry & Crump as the major pathogens within *H. schachtii* and *H. avenae* Woll. cysts. He recognised two phases of fungal attack on females and eggs but both occur whilst the females and young cysts are still attached on the roots of the host.

The biological control agents of endoparasitic root-knot and cyst nematodes have been studied because of the economic importance of these pests on a range of crops and the relative ease with which they can be extracted from the soil compared to some other nematodes. The fungi attacking these nematodes may be specific and do not attack freeliving species. By killing the females or their contents they have good potential for being effective biological control agents. Their effect is indirect: second-stage juveniles attack the roots and females are formed, then the females are parasitised so that the nematode population decreases by reducing the number of viable eggs and larvae remaining in soil. Hence, fewer nematodes are present to infect subsequent crops.

Among the endoparasites *V. chlamydosporium* is a promising species because it can easilv be cultured *in vitro*. In the soil it produces mycelium and colonizes the rhizosphere. The chlamydospores allow survival under adverse conditions (1). Kerry (6) described some species of endoparasitic fungi that prevented the population increase of *H. avenae* and *H. schachtii* and reduced the damage caused by root-knot nematodes. This was reported for *H. schachtii* on sugar beets in the presence of *V. chlamydosporium* (4). Heijbroek (5) also found the parasite, *V. chlamydosporium*, to be pathogenic to beet cyst nematodes.

## Materials and Methods

*Verticillium chlamydosporium* was originally isolated from an infested cyst of *H. schachtii* and screened and compared to other *V. chlamydosporium* isolates for its colonisation capacity, persistence, virulence, ease of production and storage survival in the absence of a host. *V. chlamydosporium* has always been grown on potato dextrose agar (PDA) and the inoculum consisted of the resting structures (chlamydospores) as they were best adapted for storage and application. However, after successive cultures, the original strain of *V. chlamydosporium* lost the ability to produce chlamydospores, so that it was necessary to test other production procedures. Two inoculation techniques were used, i.e. the paperpot (chainpot) technique and the seed pelleting technique.

*V. chlamydosporium* was cultured in Petri dishes on PDA and incubated at a temperature of 29°C. As the fungal growth is known to be very slow, it took 20 days to colonise the complete Petri dish (d 9 cm); approximately 2 x $10^6$ chlamydospores were formed two months after inoculation on each dish. The spores from 30 Petri dishes were washed into 30 litres of sandy loam soil containing large amounts of organic matter (5%C) and amended with oatmeal (5% w/v). This substrate was used to fill 1400 chain pots; each pot held 20 ml of the mixture which contained 2 x $10^3$ chlamydospores/ml. Three weeks after inoculation sugarbeets were sown in the paperpots in a glasshouse covered with plastic foil at an ambient air temperature of 7°C for 4 weeks. Later on, the paperpots, with the young beets, were transplanted in a soil which was infested with *H. schachtii* (inoculum density 8 eggs and larvae per g of soil). In the same circumstances, paperpots were filled with the same soil substrate containing Temik 10G (aldicarb) at a dose rate of 2 kg and 4 kg Temik/ha. A similar test was done in the field in plots (6 m x 6 m) each treatment being replicated 4 times. The plots were harvested and yield and nematode infestations recorded.

Seed inoculation of pelleted seeds was also used to introduce *V. chlamydosporium* into soil. In this method chlamydospores were absolutely necessary, as they were more resistant than conidia or hyphae and had to be mixed with the pelleting material. In order to solve the problem of poor chlamydospore production, other propagules produced in liquid culture were either ground and incorporated in alginate pellets or used as air dried cultures. These techniques posed practical problems and gave poor survival, so it seemed very interesting to look for new, better methods. Freeze-drying of shake cultures seemed to be very promising, but the question arose whether the fungus would still germinate after treatment. *V. chlamydosporium* was grown in a shake culture (rotation: 40 rpm), containing diluted molasses (saccharose 30 g/1; pH = 6). Each Erlenmeyer flask contained 125 ml of sterilized medium, and was inoculated with 5 pieces (diameter 5 mm) originating from the outermost zone of a Petri dish containing actively growing mycelium. When all the saccharose was consumed after 20 days, the cultures were transferred to a butt, frozen at -18°C during 24 hours and eventually freeze-dried. Afterwards, the fungus was ground to a powder.

The viability was studied immediately after seed pellet inoculation (7 g powder/150 g seeds), and also every 2 weeks for 16 weeks after seed inoculation. The observations were made by plating the treated seed pellets on a water-agar medium at 20°C, followed by microscopical observation (400 seeds for each treatment). The seeds were also sown in a heated glasshouse in sterilised sandy loam soil, steamed pot soil and white sand. Five, ten and fifteen weeks after sowing, 20 representative seedlings were examined for root colonisation: the roots were placed in a Petri dish in a wet chamber. Microscopical observation was used to evaluate the length of *V. chlamydosporium* colonized roots.

Fifteen weeks after sowing, soil samples were taken with a small core (ø 5 mm) at a radial distance of 1.5 and 3 cm to the seedlings at a depth of 0-5 and 5-10 cm. After mixing the 3 subsamples per seedling, 1.5 g soil was sprayed over a PDA nutrient medium in Petri dishes. Rhizosphere colonisation was evaluated after an incubation period of 10 days at 20°C.

**Table 1a.** Influence of *Verticillium chlamydosporium*, on cyst formation of *Heterodera schachtii* on sugar beets, in paperpots treated with fungus or Temik before transplanting into soil heavily infested with the nematode (Each figure is the mean of 6 samples).

| Treatment | Number of cysts per 100 gram soil at harvest (Samples taken 20 cm from beet) | |
|---|---|---|
| | Total number | Cysts with eggs and larvae |
| Control paperpot | 65 A* | 39 A* |
| paperpot + 2 kg/ha Temik | 15 B | 9 B |
| paperpot + 4 kg/ha Temik | 14 B | 4 C |
| paperpot *V. chlamydosporium* | 14 B | 11 B |

**Table 1b.** Influence on sugar beet crop

| Treatment | Net weight t/ha | | extractable sugar t/ha | |
|---|---|---|---|---|
| without paperpot | 100 = 59.8 | A* | 100 = 9.2 | A* |
| control paperpot | 98 | A | 96 | A |
| paperpot 2 kg Temik | 141 | B | 142 | B |
| paperpot 4 kg Temik | 143 | B | 148 | B |
| paperpot *V. chlamydosporium* | 146 | B | 146 | B |

* Means followed by the same letter are not significantly different (SAS; GLM procedure) according to Duncan's Multiple Range Test ($P < 0.05$).

## Results and Discussion

In earlier experiments, a mycelium/conidiospore mixture of *V. chlamydosporium* had been used for the inoculation of paperpots. The advantage of this approach was an immediate colonization of the substrate in the paperpot by the fungus, while the disadvantage was the inconvenience of the liquid inoculum.

Inoculation with chlamydospores was quite promising, especially under controlled conditions. In a glasshouse experiment, no viable cysts could be detected in the inoculated soil immediately around the sugarbeet roots. In this situation, the humidity was very well regulated during the cropping period. In the field, newly formed cysts were counted and examined for their viability and for the presence of chlamydospores at the end of the growing season of the sugar beets. Although an immediate reduction of the cyst population was not expected, the cyst population was significantly reduced even after the first crop. This confirms data from the literature which describe fungal attack of

females and young cysts on the roots of the hosts. The colonization of remaining cysts with chlamydospores was much greater under controlled glasshouse conditions than in the field.

There was an apparent decrease of viable cysts after Temik application, as well as after the *V. chlamydosporium* treatment. There was also an influence on the sugarbeet crop. In both the treatments, the net weight of the sugar beets and the extractable sugar content increased to a significant level.

A first problem using the seed pelleting method was the compatibility of the fungal inoculum in the seed pellet with pesticides normally present in pelleted sugar beet seeds. For these purposes, *V. chlamydosporium* was screened *in vitro* against fungicides and insecticides commonly used in seed pellets (2).

For each unit of sugarbeet seeds (= 100,000 seeds) in practice 2.3 kg coating powder was used; from these data the concentration of active products in the seed was:

- thiram : 2800 ppm
- hymexazole : 2400 ppm
- mercaptodimethur : 2600 ppm

The comparison of concentrations resulting in 100% growth inhibition with concentrations used in practice (Table 2) made clear that the use of hymexazole was quite impossible in seed coating (Table 3); even for thiram and for mercaptodimethur, the growth inhibition was about 65% at concentrations of 2500 ppm. This made it difficult to combine the normally used pesticides with the incorporation of *V. chlamydosporium*.

**Table 2.** In vitro-screening of seed disinfectants against *Verticillium chlamydosporium* (products in g per unit seeds = 1000,000 seeds = ± 1.4 kg seeds).

| Products | Dose (product g or ml/unit) | Active ingredient | Dose (a.i. g/unit) |
|---|---|---|---|
| Fungicides | | | |
| TMTD 80% | 8 g | thiram | 6.4 |
| Tachigaren 70% | 8 g | hymexazole | 5.6 |
| Rovral 50% | 3 g | iprodione | 1.5 |
| Insecticides | | | |
| Mesurol 50% | 12 g | mercaptodimethur | 6 |
| Curater 334 g/l | 9 ml | carbofuran | 3 |

**Table 3.** Mycelium growth of *Verticillium chlamydosporium* in mm ø at day 14. Growth inhibition (in % of control treatment) by increasing concentrations of thiram, hymexazole and mercaptodimethur (n = 5).

| inoculation | conc. (ppm) | thiram ø | thiram % | hymexazole ø | hymexazole % | mercaptodimethur ø | mercaptodimethur % |
|---|---|---|---|---|---|---|---|
| mycelium | 0 | 43 | 0 | 43 | 0 | 43 | 0 |
| | 0.1 | 40 | 7 | 37 | 14 | 43 | 0 |
| | 1 | 35 | 19 | 29 | 33 | 43 | 0 |
| | 10 | 29 | 33 | 20 | 54 | 43 | 0 |
| | 100 | 22 | 44 | 11 | 74 | 42 | 2 |
| | 500 | 20 | 54 | - | 100 | 40 | 7 |
| | 1000 | 17 | 61 | | | 36 | 16 |
| | 1500 | 16 | 63 | | | 30 | 30 |
| | 2000 | 14 | 67 | | | 23 | 47 |
| | 2500 | 14 | 67 | | | 15 | 65 |
| | 3000 | 13 | 70 | | | 8 | 81 |
| | 3500 | 12 | 72 | | | - | 100 |
| | 4000 | 11 | 74 | | | | |
| spores | 0 | 34 | 0 | 34 | 0 | 34 | 0 |
| | 0.1 | 31 | 9 | 30 | 12 | 34 | 0 |
| | 1 | 27 | 21 | 24 | 29 | 33 | 3 |
| | 10 | 21 | 38 | 17 | 50 | 32 | 6 |
| | 100 | 14 | 59 | 10 | 71 | 29 | 15 |
| | 500 | 11 | 68 | - | 100 | 27 | 21 |
| | 1000 | 8 | 77 | | | 23 | 32 |
| | 1500 | 8 | 77 | | | 11 | 68 |
| | 2000 | 7 | 79 | | | - | 100 |
| | 2500 | 7 | 79 | | | | |
| | 3000 | 7 | 79 | | | | |
| | 3500 | 7 | 79 | | | | |
| | 4000 | 7 | 79 | | | | |

Five days after placing sugarbeet seeds, inoculated with freeze-dried *V. chlamydosporium* inoculum on PDA nutrient medium the fungus could be detected on all pellets. *Verticillium chlamydosporium* remained viable for at least 16 weeks after treatment.

Root colonization by *V. chlamydosporium* inoculated pelleted seeds was higher in steamed pot soil than in a white sand. This confirms the positive influence of organic material on the mycelium development, as was illustrated earlier in the paperpot technique (Table 4).

**Table 4.** Mean root colonisation (mm) by *V. chlamydosporium* after seed pellet inoculation and effect of saccharose (molasses) 5, 10 and 15 weeks after sowing in steamed pot soil and white sand.

| Soil type | steamed pot soil | | | white sand | | |
|---|---|---|---|---|---|---|
| | type of seeds | mean | (mm) | type of seeds | mean | (mm) |
| 5 weeks | Z4* | 21.7 | A | Z4 | 19.8 | A |
| | Z2 | 20.4 | A | Z2 | 17.9 | A |
| | ZO | 18.4 | B | Z0 | 7.3 | B |
| 10 weeks | Z2 | 65.5 | A | Z4 | 58.3 | A |
| | Z4 | 64.8 | A | Z2 | 51.9 | B |
| | Z0 | 59.1 | B | Z0 | 35.0 | C |
| 15 weeks | Z4 | 124.7 | A | -** | | |
| | Z2 | 123.0 | A | - | | |
| | Z0 | 122.1 | A | - | | |

Means followed by the same letter are not significantly different (SAS; GLM procedure) according to Duncan's Multiple Range Test ($\alpha$ = 0,05).

Legend: *: ZO, Z2 and Z4: 0 g, 6.9 g and 13.8 g molasses-Aerosil 200 per 150 g seed
(3.45 g molasses-Aerosil 200 = 1 g saccharose)
**: not analysed (substrate had a depth of only 65 mm).

Studies on rhizosphere colonization showed that in 70% of soil samples, *V. chlamydosporium* could be detected at 3cm radial distance from the roots and to a depth of 10 cm (Table 5).

**Table 5.** Rhizosphere colonisation by *V. chlamydosporium* after seed pellet inoculation as influenced by molasses addition, 15 weeks after sowing in steamed pot soil (n = 20)

| Distance to the seedling (cm) | Depth (cm) | Seed type * | | |
|---|---|---|---|---|
| | | Z0 | Z2 | Z4 |
| 1.5 | 0 - 5 | 18** | 18 | 17 |
| 1.5 | 5 - 10 | 16 | 17 | 16 |
| 3.0 | 0 - 5 | 16 | 18 | 17 |
| 3.0 | 5 - 10 | 14 | 16 | 14 |

Legend: *: ZO, Z2 and Z4: 0 g, 6.9 g and 13.8 g molasses-Aerosil 100 per 150 g seed.
(3.45 g molasses-Aerosil 200 = 1 g saccharose)
**: max. = 20

## Conclusion

It can be concluded that the results of the paperpot/chainpot technique are promising. As it has to be admitted that this application is rather expensive, the target crops probably will be those with high value, which are normally transplanted.

Seed inoculation is also possible: *V. chlamydosporium* colonizes the root system as well as the rhizosphere. A higher organic matter content in the soil is favourable for both the mycelial growth and the parasitic effect.

## Acknowledgement

Planting, harvesting and sugar content determinations were done with the technical help of IRBAB, Tienen, Belgium.

## References

1. BARRON, G.L., 1981. Predators and parasites of microscopic animals. In: *Biology of conidial fungi*, Vol. 1. Cole, G.T., Kendrick, B. (Eds). Academic Press, New York, 167-200.

2. COOSEMANS, J., 1990. Seed pellet inoculation of sugar beet seeds with freeze dried *Verticillium chlamydosporium* culture against *Heterodera schachtii*. *Med. Fac. Landbouww. Rijksuniv. Gent* **55**:713-718.

3. CRUMP, D.H., & KERRY, B.R., 1983. Possibilities for biological control of beet cyst nematodes with parasitic fungi. Aspects of Applied Biology 2: *Pests, diseases, weeds and weed beet in sugar beet*, 59-64.

4. HEIJBROEK, W., 1983. Sugar beet pests and crop rotation. Instituut voor Rationele Suikerproduktie, Bergen Op Zooms, Nederland, 223-235.

5. KERRY, B.R., 1984. Nematophagous fungi and the relation of nematode populations in soil. *Helminthological Abstracts*, *Series B* 53: 1-14.

6. TRIBE, H.T., 1979. Extent of disease in populations of *Heterodera*, with especial reference to *H. schachtii*. *Annals of Applied Biology* **92**:61-72.

# Screening for the activity of fungal metabolites

**Eefje den Belder**

**Research Institute for Plant Protection**
**P.O.B. 9060 6700 GW Wageningen**
**The Netherlands**

## introduction

In the last decades nematophagous fungi have been studied as biological control agents of plant-parasitic nematodes. Their general biology is very well documented (20,33,34,48). It is therefore surprising, that knowledge about the physiological and biochemical mechanisms underlying pathogenicity as well as about the production of toxic metabolites produced by fungi, is very limited (19,20). Results from tests on the production of nematicidal compounds by nematophagous fungi in culture filtrates have been recently summarized (19). They show much variation, even within the same fungal species and it seems that the toxic substances obtained from culture filtrates depend on culture conditions and the media used (19). The role of chemical substances in the infection process has been clearly demonstrated for the pre-penetration stages, such as attraction and adhesion (3,17,18,36,54). However, this role is not yet clear in the later stages such as penetration, subsequent toxification/inactivation and digestion of the nematodes.

Increased knowledge of fungal metabolites may not only help to understand the nature of these organisms but also can contribute to:

1) the evaluation of the usefulness of these organisms or their products.
2) the development of procedures for comparing and standardizing the potency of antagonists.
3) the testing of their safety and the optimalization of their use as biological control agents either of the organisms or of their metabolites.

With the rapidly developing technologies of genetic engineering and biotechnology, the identification of fungal nematoxic metabolites will be even more important because it will be possible to transfer genes which regulate the production of nematoxic metabolites to plants or to bacteria, which normally colonize the rhizosphere or the nematode cuticle.

In this chapter, various screening methods of fungal metabolites will be discussed in relation to their role during the following events of nematode infection: attraction, adhesion, penetration, toxification and digestion.

## Materials and Methods

### Attraction

Three quantitative screening methods have been developed for nematode-attracting substances.

Method 1

Small sand-filled metal cylinders like those used in antibiotic assays, (diameter about 7mm, height about 12mm), are saturated with an excess of culture filtrates or sterile medium (control). Petri dishes are filled with a mixture of 20g sand and 7ml of sterile medium, and an even distribution of the sand-medium slurry is achieved by gently swirling of the dishes. Two ml of a suspension of nematodes (10,000/ml) are pipetted into the dish and evenly distributed by further swirling. The cylinders with treatment and controls are placed alternately within a circle into the Petri dish (2 replications/treatment). Attraction or repulsion is determined by counting the number of nematodes in the cylinders present after one or more hours (3,4).

Method 2

A piece of filter paper cut in the shape of a Y (7.5cm long) is used. Solutions of test and control substances (0.1ml) are pipetted on the arms of the Y. After evaporation of the solutions, the filter paper is moistened with a water mist and placed into a Petri dish. A nematode suspension is pipetted onto the base of the Y and the Petri dish is placed in the dark for 24 hrs at room temperature and almost 100% RH. The nematodes are extracted by removing the two short arms from the Y and placing them into water for 2 hrs. The nematodes recovered are counted (41).

Method 3

Discs (1 cm diameter) are cut with a cork borer from the outer edge of a fungus colony growing on solid medium (18) or from a mixture of attractant-agar in different concentrations (2). The discs are placed into two opposite quadrants of a fresh agar medium dish, 1cm from the edge of the dish (18) or against each other (2,5). Two control discs of the same agar medium without fungus or attractant are placed into the remaining two quadrants. The dishes are used immediately or incubated for 24 hrs (in the dark, at room temperature). A gradient of substances diffusing into the agar medium is established. A drop of nematode suspension is placed in the middle of the dish and the position of nematodes is checked at different times, e.g. after 10, 30, and 60 mins, using a microscope. Variations on this method could be either to replace agar discs by sterile filter paper discs (37), or to allow the fungal filtrate to diffuse into the agar of the Petri dish for twenty minutes after which the remaining liquid is removed. Another simple variation is to pipette fungal material on agar in a Petri dish opposite a 1-ml droplet containing nematodes (distance fungal material-nematodes is about 3cm); to determine nematode migration the dishes can be observed at 3 hrs intervals (36).

Method 2 and 3 are less laborious and more precise than method 1.

## Adhesion

For the fungus *Arthrobotrys oligospora* Fres. and some other species binding has been reported between a protein (lectin) located on the fungal trapping structure and a carbohydrate on the surface of the nematode (39,40,43,45). These protein-carbohydrate interactions can be studied in inhibition experiments using commercial carbohydrates to saturate the fungal trap surface so that nematode capture will be inhibited. The fungus must be grown on strips of dialysis membrane, to be placed on the surface of a low nutrient medium or on an agar surface. Trap formation of the fungus is induced by the addition of a small amount of nematodes to 3-4 days-old fungal colonies (38). The strips or agar pieces with the fungus traps are then transferred to empty Petri dishes and flooded for at least 20-24 hrs with test carbohydrate solutions (e.g. 20 or 200 mM solution of *N*-acetyl-D-galactosamine, α-D-galactose, in distilled water or phosphate buffered saline, PBS). After the removal of excess carbohydrate, a nematode suspension is added to test the capture ability of the fungus. Different concentrations of the commercial carbohydrates as well as other chemicals, e.g. glutaraldehyde may be tested (39). The colony is examined microscopically over a 6-24 hrs-period for nematode capture (45). Control solutions consist of PBS only.

It is also possible to treat nematodes with a purified fungal protein obtained from a mycelial homogenate of *A. oligospora*. Entrapment is then also observed microscopically and compared with entrapment of untreated nematodes (43).

## Penetration

The role of fungal metabolites during penetration of the nematode cuticle or egg shell has only partly been elucidated by light and electron microscope studies (10,11,29,49,50,52).

In cytochemical staining experiments phosphatase activity has been illustrated to be important at different stages of fungal penetration of the nematode cuticle and of nematode digestion (see digestion for method used (14)). Also catalase and D-amino acid oxidase activity in the dense bodies in capture structures of *A. oligospora* has been shown (52). Location of catalase activity is tested with a conventional staining method. Glutaraldehyde-fixed fungal trap cells are incubated with 3,3'-Diaminobenzidine (DAB) and $H_2O_2$ (0.06% $H_2O_2$ and 10mg DAB in 0.1 M bicarbonate buffer pH 10.5 for 4 hrs at 37°C). Catalase-active regions will show intensive staining. Control experiments are performed by pre-incubation for 30 min with aminotriazole to inhibit catalase activity. Oxidase activity is demonstrated with the cerium method. With this technique glutaraldehyde-fixed fungal cells are incubated with $CeCl_3$ (0.1 M Tris-maleate buffer pH 7.5, containing 50 mM amino triazole and 5 mM $CeCl_3$) at 37°C for 30 min, followed by incubation in the same medium supplemented with 50 mM methanol, 50 mM D-alanine or 50 mM sodium glycollate, for 3 hrs at 37°C under continuous aeration. Controls were performed by bubbling oxygen-free nitrogen through the solutions during the incubation.

## Toxification

*In vitro* evaluation of culture filtrates

To demonstrate fungal production of nematicidal substances, the effects of culture filtrates of fungi are tested on the mobility of second-stage juveniles and on egg hatch (19). In general, culture filtrates are produced by growing fungal mycelium in liquid medium (potato dextrose, Czapek's Dox), in Erlemeyer flasks for 7-259 days, at 23-30°C after inoculation with a spore suspension (11) or after inoculation with 5-10 mm agar blocks containing mycelium (21,41). Mycelial fragments are removed by centrifugation (32), or filtration through filter paper (30). Different concentrations of filtrates are prepared with sterile water to which suspensions of nematode juveniles or egg masses are added, Sometimes extracts obtained from the mycelial fragments (13) or conidia (14) are used in the evaluation.

The nematicidal fungal effects can be evaluated after times varying from 4 to 48 hrs of incubation exposure by:

- direct counting of the number of immobilized juveniles (real immobilization is checked from time to time by putting the immobilized juveniles in distilled water (32,42,51) or by gently touching the nematodes with a fine needle (31)

- counting the dead juveniles or collecting live nematodes by means of a Bearmann funnel (9)

- staining the nematodes with acridine orange (16) (heat-killed specimens show immediate fluorescence but staining of specimens killed by chemicals can take much longer and it is not always clearly visible)

- recording egg-hatch during 3-30 days (31,46).

As a control a similar series in distilled water is often used (1). Other controls include medium (13,30), medium plus agar discs without fungus (41), solutions of individual medium components, such as salts and sugars (30), neutralized culture filtrate (to evaluate pH dependence), and autoclaved or boiled fractions of the culture filtrate (20 min at 121°C) (30,32).

In many studies changes in pH and medium compositions have been insufficiently considered and in that case it is doubtful whether the inactivation of nematodes is due to substances released into the liquid medium by the fungi or due to other factors, e.g. medium salts. Frequently it is impossible to separate effects of the fungus from those of the substrate in which the fungus is grown. Therefore, the effect of the fungus alone, the substrate alone and of the autoclaved colonized substrate should be included in the tests (48). Another useful alternative approach could be screening of purified fungal enzymes separately, in combination, or in sequence, as already described for fungus-insect interactions (26,27).

*In vivo* evaluation of culture filtrates: soil tests

The effects of culture filtrates can be evaluated by the measurement of the inhibition of penetration of the roots by nematode larvae. For this purpose roots must be exposed to the fungal culture filtrate for 0-180 min. After that they are rinsed with water (41), transferred to sterilized soil, and inoculated with freshly hatched larvae to determine the effects on penetration. Controls are untreated-inoculated and non-inoculated plants (22) or plants treated with medium not containing fungus (41). Each treatment must be replicated at least five times. Larval penetration is checked from 12 hrs up to 5 days after inoculation. The entire root system of each plant is stained in cotton blue in lactophenol or in warm acid fuchsin lactophenol, then cleared in glycerol and the juveniles within the roots are counted. Sixty days after inoculation the length and fresh weight of roots and shoot is measured and the final population of the nematode larvae in the soil and in roots is determined (22).

In a second method tomato plants are planted in pots which are filled with 1,500 g sterilized soil, with sterilized soil to which culture filtrate is added, with blended mycelial fragments, or with a mixture of culture filtrate and blended mycelial fragments. Sixty days after inoculation with larvae, the length and fresh weight of roots and shoots are measured (9). Both soil tests have the same limitations as the *in vitro* evaluation of the culture filtrates: it is uncertain which factor(s) cause the lower juvenile penetration.

Screening for specific enzymatic activity

Extra cellular enzyme activity is assayed by incubating the fungus on solid media with enzyme specific substrates during a fixed period. Enzyme activity is shown as a clear zone (halo) around the fungal colony. Chitinase presence is tested (8) by including colloidal chitin (0.2%) or other chitin substrates (23) as a sole carbon source in a liquid salt synthetic medium solidified with 1.5% agar. Fungi are incubated on this solid chitin medium in test tubes at room temperature for 5-10 days. The presence of chitinase activity is shown as a clear zone under or around the colony (8,15). Protease activity is tested on skim-milk (0.8 and 1.5%) agar dishes. Species that show a clear zone around the colony are regarded as protease positive (8). Casein, gelatin, dyed elastic and erythrocyte concentrate are also used as enzyme specific substrates (6,25).

It is also possible to grow fungi in liquid medium supplemented with different carbon and nitrogen sources such as glucose, laminarin, chitin, N-acetylglucosamine, peptone and fungal cell wall (12). After a growing period of 3-5 days the mycelium is collected by centrifugation at 27000 x g for 20 min at 4°C. The supernatant is filtered through a Whatman filter and lyophilized. Enzyme activity is assayed by incubating the lyophilized culture, with enzyme-specific substrates during a fixed period and measuring the release of free glucose from laminarin when glucanase is present, or N-acetylglucosamine from chitin when chitinase is active. Enzyme activity can be measured at different pHs, temperatures and substrate concentrations. It is also possible to test different combinations of substrates in the reaction mixture or to test different stages of the fungi such as trap development (52).

These studies will contribute to our understanding of enzyme synthesis. However, the relevance of these enzymes *in vivo* is not clear: the composition of the culture medium

can markedly affect the extracellular protein pattern. For instance two isolates of *Paecilomyces lilacinus* (Thom) Samson show chitinase activity as well as protease activity but the nematode eggs are not parasitized (8). There is not necessarily a direct relation between enzyme activity and infection ability.

The role of fungal metabolites could be clarified by examining the effects of purified fungal enzymes on nematodes, nematode cuticles, eggs and egg shells. This has only been done with semi-pure commercial enzyme preparations (28,35). The effects respectively on the egg shell or juvenile cuticle were evaluated. Unfortunately methods to obtain purified fungal enzymes from nematophagous fungi have hardly been described although some have been developed for entomopathogenic fungi (27).

**Digestion**

How nematodes are digested by fungi is not yet fully clear. Cytochemical staining of digestive enzymes has been performed especially for acid phosphatase. Cultures of the fungus ***A. oligospora*** are grown on agar and nematode trapping structures are induced by adding nematodes. The nematodes are fully digested after one week. A second sample of nematodes is then added. After five minutes and after 1, 3 and 6 hrs, these nematodes are fixed by flooding with 4% formaldehyde. After fixation (12 hrs), enzyme activity is demonstrated by incubating with enzyme-specific incubation media, hexazonium-parasoanilin and $\alpha$-naphthyl phosphate (49) $\beta$-glycerophosphate or glucose-6-phosphate (53). Controls are performed in the absence of substrate or in the presence of 10 mM NaF, an inhibitor of acid phosphatase activity. The material is observed with the light microscope or the transmission electron microscope to check acid phosphatase activity.

## Conclusions

In the field of biological control of nematodes research is largely devoted to the discovery and characterization of species of nematode-destroying fungi (47). The role of fungal metabolites in nematode pathology has rarely been a subject of study. Techniques to be applied in studies of fungal metabolites vary according to the different infection stages. Fungus-nematode adhesion is verified in inhibition tests at the molecular level in which fungal lectin-nematode saccharide binding is reduced by pretreatment of the fungus with different saccharides (39,40,45) or pretreatment of nematodes with proteins (43).

The later infection stages such as penetration and digestion are mainly clarified in histochemical staining experiments in which enzyme activity is detected after incubation with enzyme-specific substrates. The results obtained are sometimes ambiguous (49). A more effective approach would be immunohistochemical techniques which have now become available and produce more precise results (19).

Nematode-attracting or toxic substances are mainly screened by testing culture filtrates *in vitro* or in soil. Contradictory results are often obtained. The effects of fungal metabolites can hardly be separated from those of the substrate in which the fungus grows and it is difficult to find the causal factors of lower nematode vitality/higher mortality.

A precise detection method for extracellular enzymes is also known. Dackman (8) and Chet (12) use specific substrates to assay enzyme activity but unfortunately the results

obtained thus far do not show a specific connection between production of an enzyme and pathogenesis (8,44).

Possibly the screening of purified metabolites, as described for entomopathogenic fungi (7), will throw more light on their role in nematode pathology and in general, the techniques developed in studies on fungus-fungus or fungus-insect interaction may also be applicable in fungus-nematode interactions. In this manner we can achieve better understanding in the fungus-nematode system, and make a better judgement of the possibilities and limitations of the use of fungi and their metabolites in biological control of nematodes.

## References


1. ALAM, M.M., KHAN, M.W. & SAXENA, S.K. 1973. Inhibitory effect of culture filtrates of some rhizosphere fungi of okra on the mortality and larval hatch of certain plant-parasitic nematodes. *Indian J. Nematol.* **3**:94-98.

2. BALAN, J. 1985. Measuring minimal concentrations of attractants detected by the nematode *Panagrellus redivivus*. *Nematologica* **30**:105-111.

3. BALAN, J. & GERBER, N.N. 1972. Attraction and killing of the nematode *Panagrellus redivivus* by the predacious fungus *Arthrobotrys dactyloides*. *Nematologica* **18**:163-173.

4. BALAN, J., KRIZKOVÁ, L., NEMEC, P. & VOLLEK, V. 1974. Production of nematode-attracting and nematicidal substances by predacious fungi. *Folia Microbiol*, **19**:512-519.

5. BALAN, J., KRIZKOVÁ, L., NEMEC, P. & KILOZSVÁRY, A. 1976. A qualitative method for detection of nematode attracting substances and proof of production of three different attractants by the fungus *Monacrosporium rutgeriensis*. *Nematologica* **22**:306-311.

6. CHALUPOVÁ, V. & LENHART, K. 1984. The relation between proteolytic activity and ovicidal activity of mutants of *Verticillium chlamydosporium*. *Acta Univ. Palaki. Olomuc*. **106**:79-87.

7. CHARNLEY, A.K. 1982. Physiological aspects of destructive pathogenesis in insects by fungi: a speculative review. In: Invertebrate microbial interactions. eds. Anderson, J.M., Rayner, A.D.M. & Walton, D.W.H., Univ. Press, Cambridge, 229-270.

8. DACKMAN, C., CHET, I. & NORDBRING-HERTZ, B. 1989. Fungal parasitation of the cyst nematode *Heterodera schachtii:* infection and enzymatic activity. *FEMS Microbiol. Ecol*. **62**:201-208.

9. DESAI, M.V., SHAH, H.M. & PILLAI, S.N. 1972. Effect of *Aspergillus niger* on root knot nematode *Meloidogyne incognita*. *Indian J. Nematol*. **2**:210-214.

10. DOWSETT, J.A. & REID, J. 1977a. Transmission and scanning electron microscope observations on the trapping of nematodes by *Dactylaria candida*. *Can. J. Bot.* **55**:2963-2970.

11. DOWSETT, J.A. & REID, J. 1977b. Light microscope observations on the trapping of nematodes by *Dactylaria candida*. *Can. J. Bot.* **55**:2956-2962.

12. ELAD, Y., CHET, I. & HENIS, Y. 1982. Degradation of plant pathogenic fungi by *Trichoderma harzianum*. *Can. J. Microbiol.* **28**:719-725.

13. GIUMA, A.Y. & COOKE, R.C. 1971. Nematoxin production by *Nematoctonus haptocladus* and *N. concerrens*. *Trans. Br. Mycol. Soc.* **56**:89-94.

14. GIUMA, A.Y., HACKETT, A.M. & COOKE, R.C. 1973. Thermostable nematotoxins produced by germinating conidia of some endozoic fungi. *Trans. Br. Mycol. Soc.* **60**:49-56.

15. GODOY, G., RODRÍQUEZ-KÁBANA, R. & MORGAN-JONES, G. 1982. Parasitism of eggs of *Heterodera glycines* and *Meloidogyne arenaria* by fungi isolated from cysts of *H. glycines*. *Nematropica* **12**:111-119.

16. IIZUKA, H. *et al*. 1962. Nematicidal action of microorganisms. *Agr. Biol. Chem.* **26**:199-200.

17. JANSSON, H-B., JEYAPRAKASH, A. & ZUCKERMAN, B.M. 1985. Differential adhesion and infection of nematodes by the endoparasitic fungus *Meria coniospora* (Deuteromycetes). *Appl. Environ. Microbiol.* **49**:552-555.

18. JANSSON, H-B. & NORDBRING-HERTZ, B. 1979. Attraction of nematodes to living mycelium of nematophagous fungi. *J. Gen. Microbiol.* **112**:89-93.

19. JANSSON, H-B. & NORDBRING-HERTZ, B. 1989. Infection events in the fungus-nematode interaction. In: *Diseases of nematodes*, Vol. 2, Eds. Poinar, G.O., Jansson, H-B, CRC Press, Florida, 59-71.

20. JATALA, P. 1986. Biological control of plant-parasitic nematodes. *Ann. Rev. Phytopathol.* **24**:453-489.

21. KENNEDY, N. & TAMPION, J. 1978. A nematoxin from *Nematoctonus robustus*. *Trans. Brit. Mycol. Soc.* **70**:140-141.

22. KHAN, T.A., AZAM, M.F. & HUSAIN, S.I. 1984. Effect of fungal filtrates of *Aspergillus niger* and *Rhizoctonia solani* on penetration and development of root-knot nematodes and the plant growth of tomato var. *Marglobe*. *Indian J. Nematol.* **14**:106-109.

23. KUNERT, J., ZEMEK, J., AUGUSTÍN, L., KUNIAK, L. & CHALUPOVÁ, V. 1985. Chitinolytic activity of ovicidal soil fungi. *Biologia (Bratislava)* **40**:1157-1165.

24. KUNERT, J., ZEMEK, J., AUGUSTÍN, J. & KUNIAK, L. 1987. Proteolytic activity of ovicidal soil fungi. *Biologia (Bratislava)* **42**:695-705.

25. KUNERT, J., ZEMEK, J., AUGUST'IN, J.L. & LÝSEK, H. 1982. Polysaccharide-hydrolyzing activity of ovicidal fungi. *Biológia (Bratislava)* **27**:291-299.

26. LEGER, R.J.S., CHARNLEY, A.K. & COOPER, R.M. 1986. Cuticle-degrading enzymes of entomopathogenic fungi: synthesis in culture on cuticle. *J. Invertebr. Pathol.* **48**:85-95.

27. LEGER, R.J.S., COOPER, R.M. & CHARNLEY, A.K. 1986. Cuticle-degrading enzymes of entomopathogenic fungi: cuticle degradation *in vitro* by enzymes from entomopathogens. *J. Invertebr. Pathol.* **47**:167-177.

28. LOPEZ-LLORCA, L.V. & DUNCAN, G.H. 1987. A study of fungal endoparasitism of the cereal nematode *(Heterodera avenae)* by scanning electron microscopy. *Can. J. Microbiol.* **34**:613-619.

29. LYSEK, H. & KRAJCI, D. 1987. Penetration of ovicidal fungus *Verticillium chlamydosporium* through the *Ascaris lumbricoides* egg shells. *Folia Parasitol.*, **34**:57-60.

30. MANI, A. & SETHI, C.L. 1984a. Some characteristics of culture filtrate of *Fusarium solani* toxic to *Meloidogyne incognita*. *Nematropica* **14**:121-129.

31. MANI, A. & SETHI, C.L. 1984b. Effect of culture filtrates of *Fusarium oxysporum* f. sp. *ciceri* and *Fusarium solani* on hatching and juvenile mobility of *Meloidogyne incognita*. *Nematropica* **14**:139-144.

32. MANKAU, R. 1969. Nematicidal activity of *Aspergillus niger* culture filtrates. *Phytopathology* **59**:1170.

33. MANKAU, R. 1972. Utilization of parasites and predators in nematode pest management ecology. *Proc. Tall Timbers Conf. Ecol. Anim. Control Habitat Management* **4**:129-143.

34. MANKAU, R. 1980. Biological control of nematode pests by natural enemies. *Ann. Rev. Phytopathol.* **18**:415-440.

35. MILLER, P.M. & SANDS, D.C. 1977. Effects of hydrolytic enzymes on plant-parasitic nematodes. *J. of Nematol.* **9**:192-197.

36. MONOSON, H.L., GALSKY, A.G., GRIFFIN, J.A. & McGRATH, E.J. 1973. Evidence for and partial characterization of a nematode attraction substance. *Mycologia* **65**:78-86.

37. MONOSON, H.L. & RAINIERI, G.M. 1972. Nematode attraction by an extract of a predacious fungus. *Mycologia* **64**:628-631.

38. NORDBRING-HERTZ, B. 1983. Dialysis membrane technique for studying microbial interaction. *Appl. Environ. Microbiol.* **45**:290-293.

39. NORDBRING-HERTZ, B. FRIMAN, E. & MATTIASSON, B. 1982. A recognition mechanism in the adhesion of nematode-trapping fungi. In: Lectins, Biology, Biochemistry and Clinical Biochemistry, Vol. II, Eds., de Gruyter & Co., New York, 83-90.

40. NORDBRING-HERTZ, B. & MATTIASSON, B. 1979. Action of a nematode-trapping fungus shows lectin-mediated host-microorganism interaction. *Nature* **281**:477-479.

41. NORDMEYER, D. & SIKORA, R.A. 1983. Effect of a culture fitrate from *Fusarium avenaceum* on the penetration of *Heterodera daverti* into roots of *Trifolium subterraneum*. *Nematologica* **29**:88-94.

42. OLTHOF, Th. H.A. & ESTEY, R.H. 1963. A nematotoxin produced by the nematophagous fungus *Arthrobotrys oligospora* Fresenius, *Nature* **197**:514-515.

43. PREMACHANDRAN, D. & PRAMER, D. 1984. Role of N-acetylgalactosamine-specific protein in trapping of nematodes by *Arthrobotrys oligospora*. Appl. and *Environ. Microbiol.* **47**:1358-1359.

44. ROSATO, Y.B., MESSIAS, C.L. & AZEVEDO, J.L. 1981. Production of extracellular enzymes by isolates of *Metharizium anisopliae*. *J. Invertebr. Pathol.* **38**:1-3.

45. ROSENZWEIG, W.D. & ACKROYD, D. 1983. Binding characteristics of lectins involved in the trapping of nematodes by fungi. *Appl. Environ. Microbiol.* **46**:1093-1096.

46. SAKHUJA, P.K., SINGH, I. & SHARMA, S.K. 1978. Effect of some fungal filtrates on the hatching of *Meloidogyne incognita*. *Indian Phytopathol.* **31**:376-377.

47. SAYRE, R.M. & STARR, M.P. 1989. In: Diseases of nematodes, Vol. 1., eds. Poilar, G.O., Jansson, H-B, CRC Press. Florida, 69-102.

48. STIRLING, G.R. 1989. Biological control of plant-parasitic nematodes. In: *Diseases of nematodes*, Vol. 2, Eds. Poinar, G.O., Jansson, H-B., CRC Press, Florida, 93-139.

49. TOTH, R., TOTH, D. & NORDBRING-HERTZ, B. 1980. Cytochemical localization of acid phosphatase in the nematode-trapping fungus. *Nematologica* **72**:813-817.

50. TZEAN, S.S. & ESTEY, R.H. 1979. Transmission electron microscopy of fungal nematode-trapping devices. *Can. J. Plant Sci.*, **59**:785-795.

51. VAISHNAV, M.U., PATEL, H.R. & DHRUJ, I.U. 1985. Effect of culture filtrates of *Aspergillus spp.* on *Meloidogyne arenaria*. *Indian J. Nematol.* **15**:116-117.

52. VEENHUIS, M., NORDBRING-HERTZ, B. & HARDER, W. 1984. Occurrence, characterization and development of different types of microbodies in the nematophagous fungus *Arthrobotrys oligospora*. *Fems Microbiol. Lett.* **24**:31-38.

53. VEENHUIS, M., NORDBRING-HERTZ, B. & HARDER, W. 1985. An electron microscopical analysis of capture and initial stages of penetration of nematodes by *Arthrobotrys oligospora*. *Antonie van Leeuwenhoek* **51**:385-398.

54. ZUCKERMAN, B.M. & JANSSON, B-H. 1984. Nematode chemotaxis and possible mechanisms of hosts/prey recognition. *Ann. Rev. Phytopathol*. **22**:95-113.

APPENDIX

**Fungal species isolated from beet (BCN), cereal (CCN) and potato (PCN) cyst nematodes**

| Fungus | Reference | | |
|---|---|---|---|
| | BCN | CCN | PCN |
| *Acremonium crotocinigenum* | 48 | | |
| *Acremonium schlerotigenum* | 37 | | |
| *Acremonium* sp. | 32 | 13 | 6 |
| *Acremonium strictum* | 29,32 | | |
| *Altenaria alternata* | 29 | | |
| *Amerosporium* sp. | 48 | | |
| *Aphanocladium* sp. | 48 | | |
| *Arthrobotrys oligospora* | 28 | | |
| *Ascodesmis sphaerospora* | | | 3 |
| *Aspergillus erythrocephalus* | 37 | | |
| *Aspergillus flavus* | 37 | | |
| *Aspergillus fumigatus* | 37 | | |
| *Aspergillus ochraceus* | 48 | | |
| *Aspergillus sydowii* | | | 30 |
| *Aspergillus tenuissima* | 29 | | |
| *Aspergillus versicolor* | 29,37,48 | | |
| *Black yeast* | 2,43,44,45 | | |
| *Byssochlamys nivea* | 48 | | |
| *Catenaria auxiliaris* | 1,8,9,28,41, 42,43,44,45 | 21,38,39 | |
| *Chrysosporium pannorum* | 48 | | |
| *Chrysosporium* sp. | | 26 | |
| *Cladosporium cladosporiodes* | | 4 | 11,30 |
| *Cladosporium herbarum* | 7,4,29 | 4 | 34 |
| *Cladosporium* sp. | | 26 | |
| *Cladosporium sphaerospermum* | 37 | | |
| *Colletotrichum coccodes* | | | 6,46,47 |
| *Coniothyrium fackelii* | | | 3 |
| *Cylindrocarpon destructans* | 2,4,5,8,9,14, 17,24,29,31, 43,44,45,48 | 4,8,24,26,45 | 6,27,30 |
| *Cylindrocarpon didymum* | | | 30 |
| *Cylindrocarpon gracile* | | | 30 |
| *Cylindrocarpon olidium* | 4,37 | 4 | |
| *Cylindrocarpon* sp. | | 13 | 3,11,15 |
| *Drechslera australiensis* | | | 30 |
| *Endogone* sp. | | 49 | |
| *Exophiala jeanselmei* | | | 46,47 |

| Fungus | Reference BCN | CCN | PCN |
|---|---|---|---|
| *Exophiala mansonii* | 2,4 | | |
| *Exophiala pisciphila* | 43,45 | 13 | 30 |
| *Exophiala* sp. | | | 11,15 |
| *Fusarium avenaceum* | 48 | 4 | |
| *Fusarium culmorum* | 4,37 | 4 | 6 |
| *Fusarium equiseti* | 37 | 4 | 3 |
| *Fusarium flocciferum* | 4,37 | | |
| *Fusarium moniliforme* | | | 30 |
| *Fusarium oxysporum* | 4,29,32,37 | | 3,6,15,27, 30,34,35 |
| *Fusarium redolens* | 48 | | |
| *Fusarium semitectum* | | | 30 |
| *Fusarium solani* | 29,37,48 | | 15,30 |
| *Fusarium* sp. | 5,9 | 13,26,39 | 6 |
| *Fusarium tabacinum* | 2 | | |
| *Geomyces pannorum* | | | 3 |
| *Gliocladium catenulatum* | | | 6,30 |
| *Gliocladium roseum* | 37 | 26 | 30 |
| *Helminthosporium* sp. | | 39 | |
| *Hirsutella heteroderae* | 31 | | |
| *Hirsutella rhossiliensis* | 19 | | |
| *Humicola fuscoatra* | 48 | | |
| *Humicola grisea* | 48 | | 3,11,30, 46,47 |
| *Isaria* sp. | 28 | | |
| *Lagenidiaceous fungus* | | 25 | |
| *Margarinomyces heteromorpha* | | | 46,47 |
| *Metarrhizium anisopliae* | 18,33 | | |
| *Microdochium bolleyi* | | 13,26 | |
| *Microcladium* sp. | | 39 | |
| *Mortierella elongata* | | 4,26 | |
| *Mortierella hyalina* | 37 | | |
| *Mortierella polycephala* | 37 | | |
| *Mortierella* sp. | 48 | 13,26 | 3 |
| *Mucor circinelloides* | | | 3 |
| *Mucor plumbeus* | | | 3 |
| *Mucor racemosus* | 48 | | |
| *Mucor* sp. | 48 | | |
| *Nematophthora gynophila* | 4,8,9,21,45 | 4,13,20,21,22, 26,40,45 | 10 |
| *Oidiodendron flavum* | | | 3 |
| *Paecilomyces lilacinus* | 4 | 4,13 | 30 |
| *Paecilomyces* sp. | | 26 | |
| *Paecilomyces variotii* | | | 30 |
| *Penicillium aurantiogriseum* | 37 | | |

| Fungus | Reference | | |
|---|---|---|---|
| | BCN | CCN | PCN |
| *Penicillium charlesii* | 48 | | |
| *Penicillium chrysogenum* | 48 | | 30 |
| *Penicillium decumbens* | 29,48 | | |
| *Penicillium fellutatum* | | | 30 |
| *Penicillium ganthinellum* | 48 | | |
| *Penicillium meleagrinum* | 48 | | |
| *Penicillium purpurescens* | | | 3 |
| *Penicillium restrictum* | | | 30 |
| *Penicillium roseopurpureum* | 48 | | |
| *Penicillium rubrum* | | | 30 |
| *Penicillium* sp. | 48,49 | 26 | |
| *Penicillium variobile* | 29,48 | | |
| *Penicillium waksmanii* | | | 3 |
| *Phialophora malorum* | 2,28 | | |
| *Phialophora* sp. | | 26 | |
| *Phoma americana* | | | 30 |
| *Phoma exigua* | | | 46,47 |
| *Phoma herbarum* | | | 3 |
| *Phoma medicaginis* | 4 | | |
| *Phoma* sp. | 2,48 | 26 | |
| *Pleospora* sp. | 37 | | |
| *Preussia* sp. | 37 | | 34,35 |
| *Pseudeurotium ovale* | | | 46,47 |
| *Pyrenochaeta terrestris* | 4 | 4 | |
| *Pythium elongatum* | 48 | | |
| *Pythium sp.* | 28 | | |
| *Pythium ultimum* | 29,48 | 39 | |
| *Ramichloridium schulzeri* | | | 30 |
| *Rhizopus nigricans* | 48 | | |
| *Rhizopus stolonifer* | 29 | | |
| *Scolecobasidium tschawytschae* | | | 30 |
| *Scopulariopsis* sp. | | | 46,47 |
| *Septofusidium herbarum* | 48 | | |
| *Stachybotrys chartarum* | | | 30 |
| *Sterile mycelium* | 2,37,48 | 13 | 11 |
| *Trichocladium aspermum* | | | 3,30 |
| *Trichocladium opacum* | | | 11 |
| *Trichoderma harzianum* | | | 30 |
| *Trichoderma longibrachiatum* | | | 30 |
| *Trichoderma* sp. | 37 | | 3 |
| *Trichosporium populneum* | 36 | | |
| *Ulocladium atrum* | | | 3,30 |
| *Ulocladium consortiale* | 37 | | |
| *Varicosporium* sp. | | | 11 |
| *Verticillium bulbillosum* | | 13 | |

| Fungus | Reference | | |
|---|---|---|---|
| | BCN | CCN | PCN |
| *Verticillium chlamydosporium* | 2,4,5,8,9,17, 23,24,29,31, 37,43,44,45, 48,49 | 4,13,16,20,21, 23,24,26,39, 44,45 | 6 |
| *Verticillium falcatum* | 48 | | |
| *Verticillium* sp. | | 13 | 11 |
| *Verticillium suchlasporium* | | 12 | |
| *Volutella ciliata* | 4 | 4 | |
| *Xanthothecium peruvianum* | | | 46,47 |

## References


1. BAUNACKE, W. 1922. Untersuchungen zur Biologie und Bekämpfung des Rübennematoden *Heterodera schachtii* Schmidt. *Arb. biol. Abt. (Anst.-Reichsanst.), Berl* **11**:185-288.

2. BURSNALL, L.A. & TRIBE, H.T. 1974. Fungal parasitism in cysts of *Heterodera*: II Egg parasites of *H. schachtii. Trans. Br. Mycol. Soc.* **62**: 595-601.

3. CLOVIS, C.J. & NOLAN, R.A. 1983. Fungi associated with cysts, eggs and juveniles of the golden nematode (*Globodera rostochiensis*) in Newfoundland. *Nematologica* **29**:346-356.

4. CRUMP. D.H. 1987. Effect of time of sampling, method of isolation and age of nematode on the species of fungi isolated from females of *Heterodera schachtii* and *H. avenae. Revue Nematol.* **10**:369-373.

5. CRUMP, D.H. 1987. A method for assessing the natural control of cyst nematode populations. *Nematologica* **33**:232-243.

6. CRUMP. D.H. 1989. Interaction of cyst nematodes with their natural antagonists. *Aspects of Applied Biology*, **22**:135-140.

7. CRUMP, D.H. & KERRY, B.R. 1977. Maturation of females of the cereal cyst nematode on oat roots and infection by an *Entomophthora* like fungus in observation chambers. *Nematologica* **23**:398-402.

8. CRUMP, D.H. & KERRY, B.R. 1983. Possibilities for biological control of beet cyst nematode with parasitic fungi. *Aspects of Applied Biology*, **2**:59-64.

9. CRUMP, D.H. & KERRY, B.R. 1987. Studies on the population dynamics and fungal parasitism of *Heterodera schachtii* in soil from a sugar beet monoculture. *Crop Protection* **6**:49-55.

10. CRUMP, D.H. & MOORE, H. 1990. Infection of the potato cyst nematode by the nematophagous fungus, *Nematophthora gynophila*. *Nematologica* **36**:324-326.

11. DACKMAN, C. 1988. Fungal parasitism of the potato cyst nematode *Globodera rostochiensis.* XIXth Int. Symposium, ESON, Uppsala, Sweden, 7-13 August 1988 (Abst).

12. DACKMAN, C. & BAATH, E. 1989. Effect of temperature and time on growth of *Verticillium chlamydosporium* and *V. suchlasporium* and infection of *Heterodera avenae* eggs. *Mycologia* **81**:161-163.

13. DACKMAN, C. & NORDBRING-HERTZ 1985. Fungal parasites of the cereal cyst nematode *Heterodera avenae* in southern Sweden. *J. Nematol.* **17**:50-55.

14. GOFFART, H. 1932. Untersuchungen am Hafernematoden *Heterodera schachtii* Schm. unter besonderer Berucksichtigung der schleswig-holsteinischen verhätnisse. *III. Arbeiten aus der biologischen Reichsanstalt für Land-und Fortwirtschaft, Berlin-Dahlem* **20**:1-28.

15. GOSWAMI, B.K. & RUMPENHORST, J.H. 1978. Association of an unknown fungus with potato cyst nematodes, *Globodera rostochiensis* and *G. pallida*. *Nematologica* **24**:251-256.

16. GRAHAM, C.W. & STONE, L.E.W. 1975. Field experiments on the cereal cyst nematode *(Heterodera avenae)* in south-east England 1967-72. *Ann. appl. Biol.* **80**:61-73.

17. HEIJBROEK, W. 1983. Some effects of fungal parasites on the population development of the beet cyst nematode (*Heterodera schachtii*). *Med. Fac. Landbouw. Rijksuniv. Gent.* **48**:433-439.

18. HOLLRUNG, D. 1890. Die Hallesche versuchs-station für Nematoden-vertilgung. Mitteilung über die Arbeiten im Jarhe 1889. *Zeitschrift des vereins für die Rübenzucker-Industrie des Deutschen Reich.* **40**:140-149.

19. JAFFEE, B.A. & MULDOON, A.E. 1989. Suppression of cyst nematode by natural infestation of a nematophagous fungus. *J. Nematol.* **21**:505-510.

20. JUHL, M. 1982. Cereal cyst nematode, soil heating and fungal parasitism. *EPPO Bull.* **12**:505-511.

21. KERRY, B.R. 1975. Fungi and the decrease of cereal cyst nematode populations in cereal monoculture. *EPPO Bull.* **5**:353-361.

22. KERRY, B.R. 1980. Biocontrol: Fungal parasites of female cyst nematodes. *J. Nematol.* **12**:253-259.

23. KERRY, B.R. 1984. Report of a meeting of the IOBC/WPRS, Integrated control of soil pests working group. *Pedobiologia* **27**:151-157.

24. KERRY, B.R. & CRUMP, D.H. 1977. Observations on fungal parasites of females and eggs of the cereal cyst nematode, *Heterodera avenae* and other cyst nematodes. *Nematologica* **23**:193-201.

25. KERRY, B.R. & CRUMP, D.H. 1980. Two fungi parasitic on females of cyst nematodes *(Heterodera* spp.). *Trans. Br. Mycol. Soc.* **72**:119-125.

26. KERRY, B.R., CRUMP, D.H. & MULLEN, L.A. 1982. Natural control of the cereal cyst nematode *Heterodera avenae* Woll. by soil fungi at three sites. *Crop Protection* **1**:99-109.

27. KONDAKOVA, E.I. & BELOKURSKAYA, V.I. 1973. Rate of infection of *Heterodera rostochiensis* with fungi. *Vses. Inst. gel'mint., K.I. Skryabine* 11:43-46.

28. KORAB, J.J. 1929. Results of a study of the beet nematode *Heterodera schachtii* at the nematode laboratory of the Belaya Tserkov Research Station. *Sb. Sort. Semenov. Upravl.* **8**:29-67.

29. LOPEZ, D.J. & ROMERO, M.D. 1988. Fungal parasite of cysts and eggs of *Heterodera schachtii* in the Duero Valley. *Nematol. Medit.* **16**:63-65.

30. MORGAN-JONES, G., RODRIGUEZ-KABANA, R. & JATALA, P. 1986. Fungi associated with cysts of potato cyst nematodes in Peru. *Nematropica* **16**:21-31.

31. MULLER, J. 1982. The influence of fungal parasites on the population dynamics of *Heterodera schachtii* on oil radish. *Nematologica* **28**:161.

32. NIGH, E.A., THOMASON, I.J. & VAN GUNDY, S.D. 1980. Identification and distribution of fungal parasites of *Heterodera schachtii* eggs in California. *Phytopathology* **70**:884-889.

33. RADEMACHER, B. & SCHMIDT, O. 1933. Die bisherigen Erfahrungen in der Bekämfung des Rübennematoden *(Heterodera schachtii* Schm.) auf dem Wege der Reizbeeinflussung. *Archiv. für Pflanzenbau, Berlin* **10**:237-296.

34. ROESSNER, J. 1987. Fungi as antagonists of *Globodera rostochiensis*. *Nematologica* **33**:106-118.

35. ROESSNER, J. 1988. Effect of moisture on fungal parasitism of *Globodera rostochiensis. XIXth Int. Symposium, ESON., Uppsala, Sweden, 7-13 August, 1988 (abst.).*

36. ROZYPAL, J. 1934. Houby na hád'átku repném *Heterodera schachtii* Schmidt v Moravských pudách. *Vestnik Ceskoslovenské Akademie Zemedelské* **10**:413-422.

37. SALEH, H. & QUADRI, A.N. 1989. Fungi associated with *Hederodera schachtii* in Jordan. II. Identity and incidence. *Nematol. medit.* **17**:109-112.

38. STIRLING, G.R. 1981. Parasites and predators of cereal cyst nematode *(Heterodera avenae)* in South Australia. *Helm. Abs. B.* 50: Entry 434.

39. STIRLING, G.R. & KERRY, B.R. 1983. Antagonists of the cereal cyst nematode *Heterodera avenae* in Australian soils. *Aus. J. Exp. Agric. Anim. Husb.* **23**:318-324.

40. STOYANOV, D. 1982. Cyst forming nematodes on cereals in Bulgaria. *EPPO Bull.* **12**:341-344.

41. THOMAS, E. 1982. On the occurrence of parasitic fungi and other pathogens and their influence on the development of populations of the beet nematode *(Heterodera schachtii)* in the Northern Rhineland. *Gesunde Pfl.* **34**:162-168.

42. TRIBE, H.T. 1977. A parasite of white cysts of *Heterodera*: *Catenaria auxiliaris. Trans Br. mycol. Soc.* **69**:367-376.

43. TRIBE, H.T. 1977. Pathology of cyst nematodes. *Biological Reviews.* **52**:477-507.

44. TRIBE, H.T. 1979. Extent of disease in populations of *Heterodera*, with especial reference to *H. schachtii*. *Ann. appl. Biol.* **92**:61-72.

45. TRIBE, H.T. 1980. Prospects for the biological control of plant parasitic nematodes. *Parasitology* **81**:619-639.

46. VAN DER LAAN, P.A. 1953. Een Schimmel asl parasiet van de cyste-inhoud van de aardappelcyste-naaltje *(Hederodera rostochiensis* Wollenw.). *Tijdschrift over Plantenziekten* **59**:101-103.

47. VAN DER LAAN, P.A. 1956. Onderzoekingen over schimmels, die parasiteren op de cyste-inhoud van het aardappelcystenaaltje (*Heterodera rostochiensis* Wollenw.). *Tijdschrift over Plantenziekten* **62**:305-321.

48. VINDUSKA, L. 1982. Fungi parasitising on the cysts of beet eelworm *Heterodera schachtii. Rostlinna Vyroba* **28**:257-262.

49. WILLCOX, J. & TRIBE, H.T. 1974. Fungal parasitism in cysts of *Heterodera*. I. Preliminary investigations. *Trans. Br. mycol. Soc.* **62**:585-594.

David H. Crump